Towards waste valorization: Recovery of valuable compounds from animal by-products and opportunities of application

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Universitat de Lleida

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Towards waste valorization: Recovery of valuable compounds from animal by-products and opportunities of application

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"Progress is made by trial and failure; the failures are generally a hundred times more numerous than the successes; yet they are usually left unchronicled. The reason is that the investigator feels that even though he has failed in achieving an expected result, some other more fortunate experimenter may succeed, and it is unwise to discourage his attempts."

William Ramsay

"The reward of the young scientist is the emotional thrill of being the first person in the history of the world to see something or to understand something. Nothing can compare with that experience."

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Abstract

Meat industry generates vast amounts of waste, which must be properly treated and managed to avoid environmental and health concerns. The recycling of animal co- and by-products into added-value products could help turn this problem around. Hence, diverse science and technology-driven approaches have been coupled with market, legislative, and consumer knowledge for the successful valorization of these residues in the frame of Circular Economy.

This Ph.D. thesis addresses an underestimated and therefore unexploited waste produced during the rendering process, called *fines*. Given its high content in lipids and protein, of around 37 and 46%, respectively, the focus was placed on the recovery of these components for further applications. Thus, three main phases were established: 1st fat recovery, 2nd protein recovery as protein hydrolysates, and 3rd potential applications of protein hydrolysates.

Firstly, *fines* were submitted to five different methods to extract the lipidic fraction: Soxhlet method, Folch method, extraction with cyclopentyl methyl ether, aqueous extraction, and aqueous enzymatic extraction. After evaluating the employed methodologies in terms of yield and assigning penalty points based on the Eco-Scale approach, the aqueous extraction was chosen as the greenest method. It allows overcoming the main drawbacks of classical methods, such as the generation of a high amount of waste and organic vapor emissions. Since water is a non-hazardous solvent, the fat was successfully extracted while preparing partially defatted *fines* (PDF) for the next phase. The consistency of the extracted fat was optimized, obtaining a semi-solid product ready for application as an ingredient in animal feed.

Secondly, two commercially available proteases, Alcalase 2.4L and Neutrase 0.8L were used to recover the protein from PDF. Hydrolysis conditions were optimized aiming to maximize the degree of hydrolysis (DH), monitored using the *o*-phthaldialdehyde (OPA) assay. The maximum DH achieved with Alcalase 2.4L was 21.4% under optimal conditions: E/S ratio 5%, pH 8, temperature 55 °C, and time 24 h. Neutrase 0.8L exhibited lower efficiency in hydrolyzing the protein present in PDF and therefore, lower protein recovery. After optimizing four variables (E/S ratio, initial pH, temperature, and time) through Response Surface Methodology (RSM), the maximum DH achieved was 7.2% with the variables set at: E/S ratio 15%, initial pH 8, temperature 40 °C, and time 10.5 h. Peptides profiles were displayed using electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC), indicating that most of the peptides in the hydrolysates were < 5 kDa. Enzymatic hydrolysis had a significant impact on the volatiles profile and functional groups. However, the amino acid profiles of the hydrolysates and PDF were very similar. In addition, nitrogen-to-protein conversion factors (NPCF) were determined for the different fractions obtained.

Lastly, the process was scaled-up (10-fold) from shake-flask to a reactor successfully, demonstrating the feasibility for future scale-up.

Finally, the usefulness of the obtained protein hydrolysates as a low-cost nitrogen source was assessed. The protein hydrolysates were incorporated as protein ingredients in microbiological growth media replacing peptones or as biostimulants for plants. The capacity of protein hydrolysates to support bacterial growth was excellent, even outperforming commercial ones in some cases. In the case of yeast, the effects were strain-dependent: one of the tested strains displayed good performance while the other one exhibited lower growth and fermenting capacity in the presence of the low-cost protein hydrolysates. Several fungi strains took advantage of the low-cost protein hydrolysates, improving their capability to transform 5-hydroxymethylfurfural when used as whole-cell biocatalysts. Finally, the protein hydrolysates did not improve tomato and radish plants growth.

In conclusion, the present Doctoral Thesis provides an insight into the opportunities for extracting compounds with added-value from *fines* through sustainable practices.

Resumen

La industria cárnica genera enormes cantidades de desechos, que deben tratarse y gestionarse adecuadamente para evitar problemas ambientales y de salud. El reciclaje de residuos y subproductos animales en productos de valor añadido podría ayudar a revertir este problema. Por lo tanto, diversos enfoques han sido adaptados por la ciencia y la tecnología, acoplándolos con los conocimientos del mercado, la legislación y los consumidores para la valorización exitosa de estos residuos en el marco de la Economía Circular.

Esta Tesis Doctoral se centra en un residuo subestimado y sin explotar que se produce durante el proceso de tratamiento de subproductos cárnicos, llamado *finos*. Dado su alto contenido en lípidos y proteínas, aproximadamente del 37 y 46% respectivamente, la investigación se focalizó en la recuperación de estos componentes para futuras aplicaciones. Se establecieron tres etapas principales: 1.ª recuperación de la fracción lipídica, 2.ª recuperación de proteínas en forma de hidrolizados de proteínas y 3.ª aplicaciones potenciales de los hidrolizados de proteínas.

En primer lugar, los *finos* se sometieron a cinco métodos diferentes para extraer la fracción lipídica: método de Soxhlet, método de Folch, extracción con ciclopentil metil éter, extracción acuosa y extracción enzimática acuosa. Después de la evaluación de las metodologías empleadas en términos de rendimiento y la asignación de puntos de penalización en base al Eco-Scale, se eligió la extracción acuosa como el método más ecológico. Este método permite superar los principales inconvenientes de los métodos clásicos, como la generación de gran cantidad de residuos y las emisiones de vapores orgánicos. Dado que el agua no es un solvente peligroso, la grasa se extrajo con éxito mientras se obtuvieron *finos* parcialmente desgrasados (PDF) para la siguiente fase. Por último, la consistencia de la grasa extraída se optimizó, obteniendo un producto semisólido, listo para su aplicación como ingrediente en la alimentación animal.

En segundo lugar, se utilizaron dos proteasas disponibles comercialmente, Alcalasa 2.4L y Neutrasa 0.8L, para recuperar la proteína presente en los PDF. Se optimizaron las condiciones de hidrólisis con el objetivo de maximizar el grado de hidrólisis (DH) y el proceso se monitorizó mediante el reactivo *o*-ftalaldehído (OPA). El DH máximo alcanzado con la enzima Alcalasa 2.4L fue 21,4 % en las siguientes condiciones óptimas: ratio E/S 5%, pH 8, temperatura 55 °C y tiempo 24 h. La enzima Neutrasa 0.8L exhibió una menor eficiencia en la hidrólisis de las proteínas presentes en los PDF y, por consiguiente, una menor recuperación de proteína. Después de la optimización de cuatro variables (ratio E/S, pH inicial, temperatura y tiempo) a través de la metodología de superficie de respuesta (RSM), el DH máximo alcanzado fue 7,2% en las siguientes condiciones: ratio E/S 15%, pH inicial 8, temperatura 40 °C y tiempo

10,5 h. Los perfiles peptídicos de las hidrólisis se determinaron mediante electroforesis (SDS-PAGE) y cromatografía de exclusión molecular (SEC), mostrando el predominio de los péptidos < 5 kDa en los hidrolizados obtenidos. La hidrólisis enzimática tuvo un gran impacto en el perfil de los compuestos volátiles y los grupos funcionales. Sin embargo, se observaron similitudes entre los perfiles aminoacídicos de los hidrolizados y los PDF. Además, se determinaron los factores de conversión de nitrógeno a proteína (NPCF) para las diferentes fracciones obtenidas. Por último, el proceso se escaló (10 veces), realizando el paso de matraz a reactor exitosamente, demostrando la viabilidad del escalado.

Finalmente, se evaluó la utilidad de los hidrolizados de proteínas producidos como fuente de nitrógeno de bajo coste. Los hidrolizados de proteínas se incorporaron como fuente de proteína en medios de cultivos para microbiología como substitutos de las peptonas, o como bioestimulantes para plantas. La capacidad de los hidrolizados de proteína para sustentar el crecimiento bacteriano fue excelente, incluso superaron la capacidad de las fuentes de nitrógeno comerciales en algunos casos. En el caso de la levadura, los efectos fueron dependientes de la cepa: una de las cepas probadas mostró un buen rendimiento mientras que la otra exhibió menor crecimiento y capacidad de fermentación en presencia de los hidrolizados de proteína de bajo coste. La suplementación con los hidrolizados de proteína obtenidos tuvo un efecto positivo para varias cepas de hongos, mejorando su capacidad para transformar el 5-hidroximetilfurfural cuando estos microorganismos se emplearon como biocatalizadores. Finalmente, los hidrolizados de proteína no mejoraron el crecimiento de las plantas de tomate y rábano.

En conclusión, la presente Tesis Doctoral proporciona una visión de las oportunidades para extraer compuestos de valor añadido de los *finos* a través de prácticas sostenibles.

Resum

La indústria càrnia genera enormes quantitats de deixalles, que s'han de tractar i gestionar adequadament per evitar problemes ambientals i de salut. El reciclatge de residus i subproductes animals en productes de valor afegit podria ajudar a revertir aquest problema. Per tant, diversos enfocaments han estat adaptats per la ciència i la tecnologia, acoblant-los amb els coneixements del mercat, la legislació i els consumidors per a la valorització amb èxit d'aquests residus en el marc de l'Economia Circular.

Aquesta Tesi Doctoral s'enfoca en un residu subestimat i sense explotar produït durant el procés de tractament de subproductes carnis, anomenat *fins*. Donat el seu alt contingut en lípids i proteïnes, aproximadament del 37 i 46% respectivament, la investigació es va centrar en la recuperació d'aquests components per a futures aplicacions. Es van establir tres etapes principals: 1.ª recuperació de la fracció lipídica, 2.ª recuperació de proteïnes en forma de hidrolitzats de proteïnes i 3.ª aplicacions potencials dels hidrolitzats de proteïnes.

En primer lloc, els *fins* es van sotmetre a cinc mètodes diferents per extreure la fracció lipídica: mètode de Soxhlet, mètode de Folch, extracció amb ciclopentil metil èter, extracció aquosa i extracció enzimàtica aquosa. Després de l'avaluació de les metodologies emprades en termes de rendiment i l'assignació de punts de penalització en base a l'Eco-Scale, es va triar l'extracció aquosa com el mètode més ecològic. Aquest mètode permet superar els principals inconvenients dels mètodes clàssics, com la generació de gran quantitat de residus i les emissions de vapors orgànics. Atès que l'aigua no és un solvent perillós, el greix es va extreure amb èxit i alhora es van obtenir *fins* parcialment desgreixats (PDF) per a la següent fase. Finalment, la consistència del greix extret es va optimitzar, obtenint un producte semi-sòlid, a punt per a la seva aplicació com a ingredient en l'alimentació animal.

En segon lloc, es van utilitzar dues proteases disponibles comercialment, Alcalasa 2.4L i Neutrasa 0.8L, per recuperar la fracció proteica present en els PDF. Es van optimitzar les condicions d'hidròlisi amb l'objectiu de maximitzar el grau d'hidròlisi (DH) i el procés es va monitoritzar mitjançant el reactiu *o*-ftalaldehid (OPA). El DH màxim assolit amb l'enzim Alcalasa 2.4L va ser 21,4 % en les següents condicions òptimes: ràtio E/S 5%, pH 8, temperatura 55 °C y temps 24 h. L'enzim Neutrasa 0.8L va exhibir una menor eficiència en la hidròlisi de les proteïnes presents en els PDF i, per tant, una menor recuperació de proteïna. Després de l'optimització de quatre variables (ràtio E/S, pH inicial, temperatura i temps) a través de la metodologia de superfície de resposta (RSM), el DH màxim aconseguit va ser 7,2% en les següent condicions: ràtio E/S 15%, pH inicial 8, temperatura 40 °C i temps 10,5 h. Els perfils peptídics de les hidròlisi es van determinar mitjançant electroforesi (SDS-PAGE) i cromatografia d'exclusió molecular (SEC), mostrant el predomini

dels pèptids < 5 kDa en els hidrolitzats obtinguts. La hidròlisi enzimàtica va tenir un gran impacte en el perfil dels compostos volàtils i els grups funcionals. No obstant això, es van observar similituds entre els perfils aminoacídics dels hidrolitzats i els PDF. A més, es van determinar els factors de conversió de nitrogen a proteïna (NPCF) per a les diferents fraccions obtingudes. Finalment, el procés es va escalar (10 vegades), realitzant el pas de matràs a reactor amb èxit, demostrant la viabilitat de l'escalat.

Finalment, es va avaluar la utilitat dels hidrolitzats de proteïnes produïts com a font de nitrogen de baix cost. Els hidrolitzats de proteïnes es van incorporar com a font de proteïna en medis de cultius per a microbiologia com a substituts de les peptones, o com a bioestimulants per a plantes. La capacitat dels hidrolitzats de proteïna per sustentar el creixement bacterià va ser excel·lent, fins i tot van superar la capacitat de les fonts de nitrogen comercials en alguns casos. En el cas dels llevats, els efectes van ser dependents de la soca: una de les soques va mostrar un bon rendiment mentre que l'altra va exhibir menor creixement i capacitat de fermentació en presència dels hidrolitzats de proteïna de baix cost. La suplementació amb els hidrolitzats de proteïna obtinguts va tenir un efecte positiu per diverses soques de fongs, millorant la seva capacitat per transformar el 5-hidroximetilfurfural quan aquests microorganismes es van emprar com a biocatalitzadors. Finalment, els hidrolitzats de proteïna no van millorar el creixement de les plantes de tomàquet i rave.

En conclusió, la present Tesi Doctoral proporciona una visió de les oportunitats per extreure compostos de valor afegit dels *fins* a través de pràctiques sostenibles.

List of contributions

Manuscripts

Cosovanu, D., Llovera, M., Villorbina, G., Canela-Garayoa, R., and Eras, J. (2021). A simple and fast method for metabolomic analysis by gas liquid chromatography-mass spectrometry. *Metabolomics*, *17*(2), 22. https://doi.org/10.1007/s11306-021-01771-w

Millán Acosta, A., Cuesta Turull, C., **Cosovanu, D.**, Sala Martí, N., Canela-Garayoa, R. (2021). Novel and Efficient Biotechnological Approach to Produce 2,5-Diformylfuran from Biomass-Derived 5-Hydroxymethylfurfural. *ACS Sustainable Chem. Eng., 9*(43), 14550-14558. https://doi.org/10.1021/acssuschemeng.1c05308

Millán Acosta, A., **Cosovanu, D.**, Cabañeros López, P., Tjalfe Thomsen, S., Gernaey, K. V., Canela-Garayoa, R. (2021). Co-cultivation of a novel *Fusarium striatum* strain and a xylose consuming *Saccharomyces cerevisiae* yields an efficient process for simultaneous detoxification and fermentation of lignocellulosic hydrolysates. *Chemical Engineering Journal, 426,* 131575. https://doi.org/10.1016/j.cej.2021.131575

Delpino-Rius, A., **Cosovanu, D.**, Eras, J., Vilaró, F., Balcells, M., Canela-Garayoa, R. (2018). A fast and reliable ultrahigh-performance liquid chromatography method to assess the fate of chlorophylls in teas and processed vegetable foodstuff. *Journal of Chromatography A, 1568*, 69-79. https://doi.org/10.1016/j.chroma.2018.07.016

Conference participations

Cosovanu, D.G., Eras Joli, J., Villorbina Noguera, G., Millán Acosta, A., and Balcells Fluvià, M. (2018). From meat by-products to peptones by enzymatic hydrolysis. Application in microbiological growth media. Poster contribution at European Congress on Biotechnology. Geneve, Switzerland.

Cosovanu, D.G., Villorbina, G., Balcells, M. and Eras, J. (2017). Aqueous enzymatic extraction of lipids and production of peptides from non-edible animal by-products. Poster contribution at IV Forum SCICC (Sustainable Chemistry, Innovative and Competitive Companies). Zaragoza, Spain.

Table of contents

List of abbreviations

ABPs	Animal by-products
ACE	Angiotensin-converting enzyme
AE	Atom Economy
AHP	Analytic hierarchy process
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
AU	Anson unit
BSE	Bovine spongiform encephalopathy
CCD	Central composite design
CP	Crude protein
Da	Dalton
DFF	2,5-Diformylfuran
DH	Degree of hydrolysis
DHMF	2,5-Di(hydroxymethyl)furan
DM	Dry matter
DMI	Dry matter intake
E/S	Enzyme/substrate
EC	European Commission
EU	European Union
FAMEs	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FID	Flame ionization detector

Fines	Fine solid particles
FPH	Fish protein hydrolysate
FTIR	Fourier transform infrared
GC	Gas chromatography
HMF	5-Hydroxymethylfurfural
HPLC	High performance liquid chromatography
IS	Internal standard
ISP	Isoelectric solubilization/precipitation
LAB	Lactic acid bacteria
MF	2-Methylfuran
MUFA	Monounsaturated fatty acids
MW	Molecular weight
NR	Nitrogen recovery
OD_{600}	Optical density at 600 nm
OPA	*o*-Phthaldialdehyde
PDF	Partially defatted *fines*
PP	Penalty point
PR	Protein recovery
PUFA	Polyunsaturated fatty acid
R_i	Response factor
RPF	Rumen protected fats
RSM	Response Surface Methodology
RT	Room temperature
S/L	Solid/liquid

SAFA	Saturated fatty acids
SCP	Specific crude protein
SDS	Safety Data Sheet
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
T	Temperature
t	Time
TP	True protein
U	Unit of activity
UHPLC	Ultra-high pressure liquid chromatography
US	United States
UV-Vis	Ultraviolet-visible
WSH	Wheat straw hydrolysate

List of acronyms for culture media

HA	Alcalase hydrolysate
HN	Neutrase hydrolysate
LB	Luria-Bertani medium
LB-C	Luria-Bertani control medium
LB-HA	Luria-Bertani medium containing Alcalase 2.4L hydrolysate
LB-HN	Luria-Bertani medium containing Neutrase 0.8L hydrolysate
ME	Malt Extract medium
ME-50HA	Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate
ME-50HN	Malt Extract medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate
ME-50Meat	Malt Extract medium containing meat peptone
ME-50WP	Malt Extract medium without peptone and 50% of malt extract
MEA	Malt extract agar medium
MEA-50HA	Malt Extract Agar medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate
MEA-50HN	Malt Extract Agar medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate
MEA-50WP	Malt Extract Agar medium without peptone and 50% of malt extract
MEA-HA	Malt Extract Agar medium containing Alcalase 2.4L hydrolysate
MEA-HN	Malt Extract Agar medium containing Neutrase 0.8L hydrolysate
MEA-WP	Malt Extract Agar medium without peptone
ME-HA	Malt Extract medium containing Alcalase 2.4L hydrolysate
ME-HN	Malt Extract medium containing Neutrase 0.8L hydrolysate

ME-WP	Malt Extract medium without peptone
RHA	Alcalase 2.4L hydrolysate produced in the reactor
RHN	Neutrase 0.8L hydrolysate produced in the reactor
MS	Murashige and Skoog medium
YD	Yeast Dextrose medium
YD-RHA	Yeast Dextrose medium containing Alcalase 2.4L hydrolysate produced in the reactor
YD-RHN	Yeast Dextrose medium containing Neutrase0.8L hydrolysate produced in the reactor
YDX	Yeast Dextrose Xylose medium
YDX-RHA	Yeast Dextrose Xylose medium containing Alcalase 2.4L hydrolysate produced in the reactor
YDX-RHN	Yeast Dextrose Xylose medium containing Neutrase 0.8L hydrolysate produced in the reactor
YP	Yeast Peptone medium
YPD	Yeast Peptone Dextrose medium
YPDX	Yeast Peptone Dextrose Xylose medium

Chapter 1. Introduction

1. From Linear to Circular Economy

Throughout its evolution and diversification, our industrial economy has been dominated by Linear Economy, established in the early days of industrialization. This model relies on the pattern of "take-make-use-dispose", a unidirectional model where raw materials are extracted, goods are manufactured, sold to customers, used, and finally discarded as waste at their end-life, without other purposes (Figure 1.1) (Borrello et al., 2017; Esposito et al., 2018).

Figure 1.1. The Linear Economy model characterized by the "take-make-consume-throw away" pattern. Figure adapted from Wautelet, (2018).

The Food and Agriculture Organization of the United Nations (FAO) predicted that the world's population will reach 9.1 billion by 2050, almost 17% higher than today (FAO, 2011). Moreover, currently, resources are consumed 50% faster than can be replaced, and by 2050 our demand will require three planets worth of natural resources (Esposito et al., 2018). Therefore, this system is testing the limits of the planet and threatening the resilience of the Earth system, making it unsustainable (Esposito et al., 2018; Stahel, 2016). Vast amounts of wastes have been disposed to landfill or through incineration, leading to environmental problems, increasingly jeopardizing the earth's life-support systems (European Commission, 2020; Geissdoerfer et al., 2017). For instance, four of the nine Planetary Boundaries have already been crossed: climate change, biosphere integrity, biogeochemical flows, and land-system change (Steffen et al., 2015). In consequence, many companies have noticed higher resources prices and supply disruptions while several global crises, such as climate change, diminished biodiversity, as well as food, water, and energy shortages have emerged, and their effects are dangerous for our future (Ellen MacArthur Foundation, 2013; Keijer et al., 2019). In the last decades, the concern about the finite nature of many resources as well as the limited environmental tolerance has grown tremendously (Keijer et al., 2019), but, for instance, still one-third of plastic waste globally is not collected or managed (Stahel, 2016). This awareness has led to the transition from a Linear Economy, based on inefficient use of natural resources as if they were limitless, to a more sustainable economic model, known as Circular Economy (Figure 1.2).

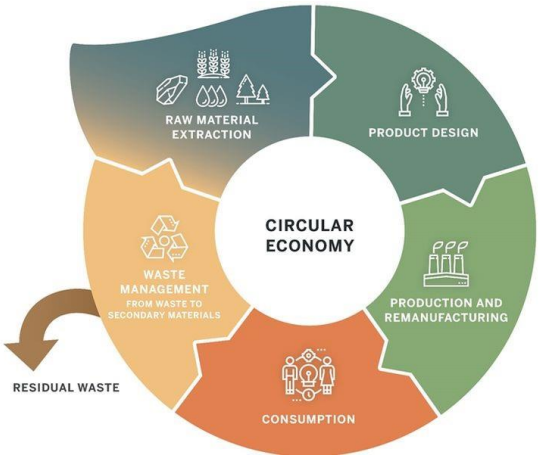

Figure 1.2. The Circular Economy model: extension of products life cycle, waste reduction, and creation of value.

The Circular Economy concept is associated with Pearce and Turner back to 1990, grounded in the study of non-linear systems, particularly living ones (Ellen MacArthur Foundation, 2013). As disseminated by Ellen MacArthur, the Circular Economy is driven by four principles: i) waste is equal food; meaning that restorative loops are the central idea, ii) building resilience through diversity, iii) creating energy from renewable resources, and iv) thinking in systems (Homrich et al., 2018), and this approach has emerged as a way to achieve sustainability (Araujo Galvão et al., 2018). Despite a lack of consensus for the definition of Circular Economy from an academic perspective, it can be defined as "restorative and regenerative by design, and aims to keep products, components and material at their highest utility and value at all times". Due to its potential as a disruptive and innovative model and because it forces a rethinking of many aspects, it is of academic interest (Esposito et al., 2018).

The Circular Economy is a model for efficient utilization, and it would turn goods that are at the end of their service life into resources for others, taking advantage of the scientific approach of the industrial ecology, aiming at closing loops (Figure 1.3) in industrial ecosystems and minimizing waste (Sariatli, 2017; Stahel, 2016), a win-win approach from an economic and value perspective (Homrich et al., 2018).

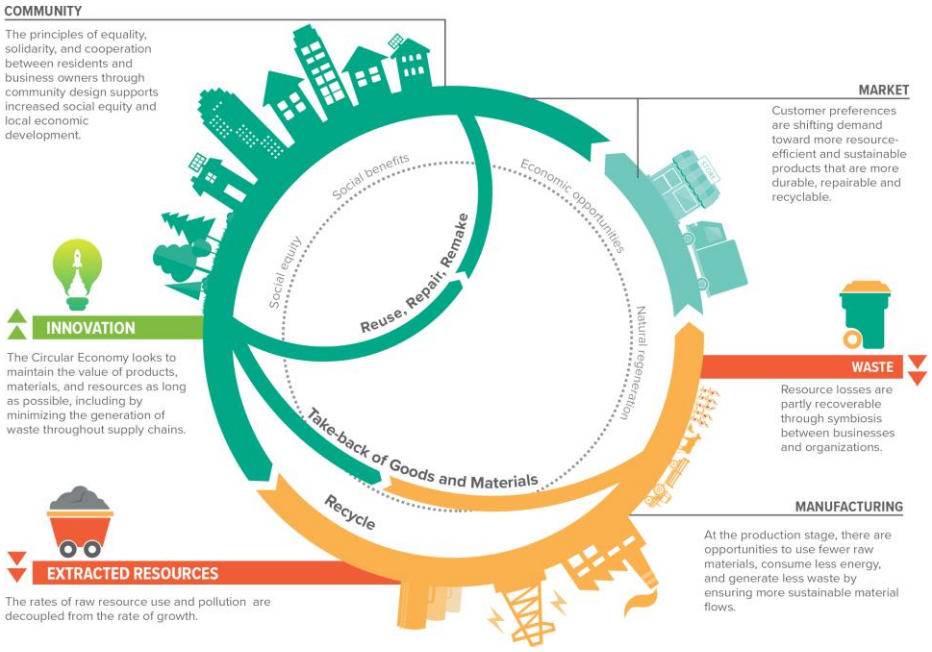

Figure 1.3. Loop system resulting in the circulation of materials through sequential product-life extension activities (reuse, repair, remanufacture, and recycling) (Brennan et al., 2015). Figure from the City of Richmond, (2019).

With Waste Management Hierarchy (Figure 1.4) introduced in European Union (EU) through the Directive 2008/98/EC, the effort must go through the whole value chain and a key process will be waste prevention or avoidance and re-use (Girotto et al., 2015), being the disposal the last option in waste management. The new system goes from “waste as a problem” to “waste as a resource”. If these concepts are carried out extensively, new materials consumption could be reduced by 53% by 2050 (Esposito et al., 2018).

Figure 1.4. Waste Management Hierarchy defined by EU Directive to be applied by the EU Member States in the waste management. Figure adapted from Waste Framework Directive (European Commission). Created with BioRender.com.

Despite the different schools of thoughts of Circular Economy (Homrich et al., 2018), all the organizations and communities are pursuing the same goal, optimum utilization of materials, culminating in no waste or properly handling of by-products to produce value-added substances or by-products (Toldrá et al., 2016), delivering economic, social, and environmental benefits (European Commission, 2020).

Given the role of the Circular Economy, Europe has made substantial progress in turning waste a resource and promoting ways of waste management, such as recycling (European Commission, 2014). This approach allows Europe to grow resource productivity by up to 3% annually (Ellen MacArthur Foundation, 2015). Five main waste streams have been identified to play a role in the transition to a Circular Economy: municipal waste, packaging waste, food waste, bio-waste and residues, and critical raw materials (CRMs) (European Commission, 2020).

2. Meat industry: meat products and by-products

To meet the food demand of the increasing human population, the food industry has become increasingly market-driven and must increase by 70% to ensure food security (European Commission, 2020; Lee et al., 2017). In consequence, it will lead to a vast amount of waste and by-products. However, by enforcing best waste management practices, a higher amount of nutrients and other resources can be recovered.

As stated by FAO, most of this population increase will occur in developing countries, coupled with rapid urbanization and rising of the living standards. In developing countries, a shift from a traditional diet based on cereals and other staples to a diet containing more animal protein is expected (Boland et al., 2013; Lynch et al., 2018b). Besides this, in Asia, for example, the consumption of animal protein per capita increased by 225% between 1961 and 2011, the latter date accounting near to 40% of total protein consumption. On the other hand, in the same continent and time frame, the consumption of crop-based protein increased by only 22% (Boland et al., 2013). A faster increase in the rate consumption of meat was observed in Sub-Saharan countries (Lynch et al., 2018b). Thus, the global demand for protein is expected to double by 2050 (Aspevik et al., 2017; Lafarga & Hayes, 2017). This change is due not only to the rise of income but also to the recognition of the important role of protein in a healthy diet to fulfill the nutritional needs (Alao et al., 2017; Aspevik et al., 2017; Drummond et al., 2019; Hall & Schönfeldt, 2013).

The predicted increased meat consumption will bring a concomitant increase in the amounts of generated by-products (Hejnfelt & Angelidaki, 2009; Lynch et al., 2018b; Mullen et al., 2017; Zhang et al., 2013). However, the use of co-products and by-products for human consumption has decreased; thus, increasing the production of animal-based products will result in more by-products and food waste (Darine et al., 2008, 2010; Nollet & Toldrá, 2011). Currently, 1.3 billion metric tons/year of food waste are lost or wasted

globally, which could be avoidable (Tonini et al., 2018). Nevertheless, around 60 million metric tons/year of animal by-products are produced, which are considered unavoidable, also known as product-specific waste. Therefore, this type of waste cannot be prevented, just managed considering both legal regulations and the best ecological and economical solutions (Hicks & Verbeek, 2016b).

Considering the global pressure for increasing the supply of foods with high protein content coupled to the finite resources and the environmental impact, it is extremely important the minimization, recovery, and utilization of food by-products and wastes (Lemes et al., 2016; Selmane et al., 2008).

2.1. Classification of meat by-products

The increasing demand for meat results in tons of solid and liquid by-products and wastes that need to be properly managed (Callegaro et al., 2019; Lapeña et al., 2018; Toldrá et al., 2012). The main sources of by-products are dead animals (on-farm deaths), slaughterhouse waste, and meat processing for human consumption (Pandey et al., 2020). In the EU, all the non-meat parts of a dressed carcass that arise during the slaughter of animals for human consumption can be referred to as meat by-products or fifth quarter. This term comprises edible co-products, such as offal, and inedible parts, or animal by-products (Drummond et al., 2019; Mora et al., 2019; Mullen et al., 2015). However, the definition of the terms can vary depending on the country and legislation (Mullen et al., 2017). The different classification and nomenclature systems can lead to confusion. Here, we use the definitions given in European Commission (EC) regulations.

2.1.1. Animal co-products

In accordance with EC Regulation No. 853/2004, offal or edible animal by-products or co-products may be defined as “fresh meat other than that of the carcass, including viscera and blood” (European Parliament & Council of the European Union, 2004). Some edible by-products can be consumed directly as foods and others are unsuitable for human consumption when produced at the slaughterhouse but can be processed for usage in human food, also known as variety meats (Drummond et al., 2019). There are variations among the countries, depending on culture, tradition, and needs, but some are widely accepted, like blood, liver, lung, heart, tongue, kidney, brain, spleen, tail, ears, and mechanically recovered meat (Mora et al., 2019; Toldrá et al., 2012, 2016). The weights of a selection of bovine, ovine, and porcine co-products are shown in Table 1.1. These parts have high nutritional value and are integrated as part of the diet in different countries worldwide (Toldrá et al., 2012). Around 12% of the live weight of bovine and 14% of pigs render as offal or co-products. However, many edible co-products are re-directed

for purposes other than human consumption despite their potential as a high-quality protein source for nutraceutical ingredients and food (Drummond et al., 2019; Lynch et al., 2018a).

Table 1.1. Expected weight of co-products based on animal type (Lynch et al., 2018b).

	Porcine	Bovine				Sheep
		Yearling	Steers	Cows	Bulls	
Animal weight (kg)	60 – 75	200 – 300	300 – 500	300 – 600	400 – 450	55 – 65
Hide/skin (kg)	3.6 – 4-5	14 – 21	21 – 35	21 – 42	28 – 32	7.5 – 8.5
Bones (kg)	3.5 - 5.6	30 – 42	40 – 55	40 – 65	45 – 52	4.0 – 6.0
Head (kg)	3.6 – 4.5	16 – 24	24 – 40	24 – 48	32 – 35	5.5 – 6.5
Feet (kg)	1.0 – 1.5	4 – 6	6 – 10	6 – 12	8 – 9	1.1 – 1.3
Blood (L)	3.5 – 3.8	14 – 16	18 – 25	18 – 36	24 – 26	1.5 – 1.8
Heart (g)	180 – 245	1275 – 1470	1200 – 2000	1800 – 2400	1687 – 2062	300 – 1000
Kidney (g)	130 – 220	635 – 940	600 – 1200	580 – 1600	800 – 1200	300 – 600
Liver (g)	1150 – 1660	2700 – 4800	3500 – 6200	3000 – 8600	5180 – 6400	900 – 2200
Lung & trachea (g)	750 – 1100	2240 – 2570	3980 – 6640	6000 – 8600	3480 – 6710	700 – 2000
Tongue (g)	150 – 210	1500 – 1750	1400 – 1880	1380 – 1490	1570 – 1940	500 – 600
Rumen & reticulum (g)			6340 – 10600	6000 – 15500	8470 – 10350	2900 – 4600
Omasum (g)			1800 – 4860	5120 – 8700	4310 – 5270	1000 – 1200
Abomasum (g)			1000 – 3030	2140 – 4700	2230 – 2730	

2.1.2. Animal by-products

Following EC Regulation No 1069/2009, animal by-products (ABPs) may be defined as entire bodies or parts of animals, including blood, feather, glands, hair, heads, hides, bones, meat trimmings, skin, fatty tissues, horns, hoofs, feet, skull, and viscera; products of animal origin, or other products obtained from animals which are not intended for human consumption, including oocytes, embryos, and semen (Aspevik et al., 2017; European Parliament & Council of the European Union, 2009; Fu et al., 2019; Mora et al., 2019; Toldrá et al., 2016). In the EU, around 20 million metric tons of ABPs are produced annually (Fu et al., 2019). In the case of ABPs, the amount that is considered inedible to humans ranges between 25 and 42% of the total weight of animals (EFPRA, 2016). Depending on their origin and the potential risk for transmitting pathogens and toxic substances that can affect public health, animal health, and the environment, ABPs are divided into categories 1, 2, and 3. Category 1 corresponds to the material with high specific risk, for example, transmissible diseases such as bovine spongiform encephalopathy (BSE), as well as animal by-products containing some specifies substances and environmental contaminants.

ABPs from this category are expected to be buried or incinerated at designed sites and by an approved agency. Category 2 corresponds to the ABPs with high risk, infected or contaminated carcasses, as well as materials declared unfit for human consumption due to the presence of foreign bodies (died in farm animals, diseased animals, manure, and digestive tract content). These can be used as feedstock in composting, biogas generation, and energy production after sterilization (pre-treatment at 133 °C, 300 kPa for 20 min). Category 3 is the one with lower risk (catering residues, meat, precooked foods) and includes inedible and free from infection carcass materials, as well as animal materials that fit but are not intended for human consumption for commercial reasons and the only one that can be used for animal feed and pet food. Moreover, due to the risk of transmissible spongiform encephalopathy, category 3 by-products are classified according to their origin, ruminants or non-ruminants (Aspevik et al., 2017; Cascarosa et al., 2012; Drummond et al., 2019; Hejnfelt & Angelidaki, 2009; Lasekan et al., 2013; Mora et al., 2019; Mullen et al., 2017). However, in the United States (US), the term animal by-product is reserved for everything produced by or from the animal, except dressed meat. Animal by-products can be divided into edible and inedible (Jayathilakan et al., 2012).

2.2. Disposal and valorization of meat by-products

Waste valorization is the process of converting the organic components of waste into more valuable products, including chemicals, materials, and fuels (Arancon et al., 2013). Meat by-products have great potential as a source of added value substances or products. If handled properly, they constitute an excellent valorization opportunity, and therefore it is no longer practical to discard (Lynch et al., 2018b; Mullen et al., 2017; Toldrá et al., 2012, 2016; Zhang et al., 2013). The raw materials include about 60% water, 20% protein and minerals, and 20% fat (Pandey et al., 2016).

Different industrial processes and markets have been secured in order to treat and reduce by-product generation. However, in general, these are low-value markets, carrying a neutral (no disposal cost) or negative (costs money to dispose of) value (Lafarga & Hayes, 2014; Mullen et al., 2017). Furthermore, one of the issues that are facing the industries is the disposal of waste in an economically and environmentally acceptable manner (Tesfaye et al., 2017). Such practice may increase the costs and is difficult to follow where adequate disposal systems are unavailable. The improper use of certain animal by-products can drive to environmental problems (Arvanitoyannis & Ladas, 2008) or raise catastrophic and outbreak events such as the spread of BSE or the occurrence of dioxins in feeding stuffs (European Parliament & Council of the European Union, 2009; Toldrá et al., 2016).

The main routes for the valorization of meat co – and by-products that are followed currently are shown in Figure 1.5.

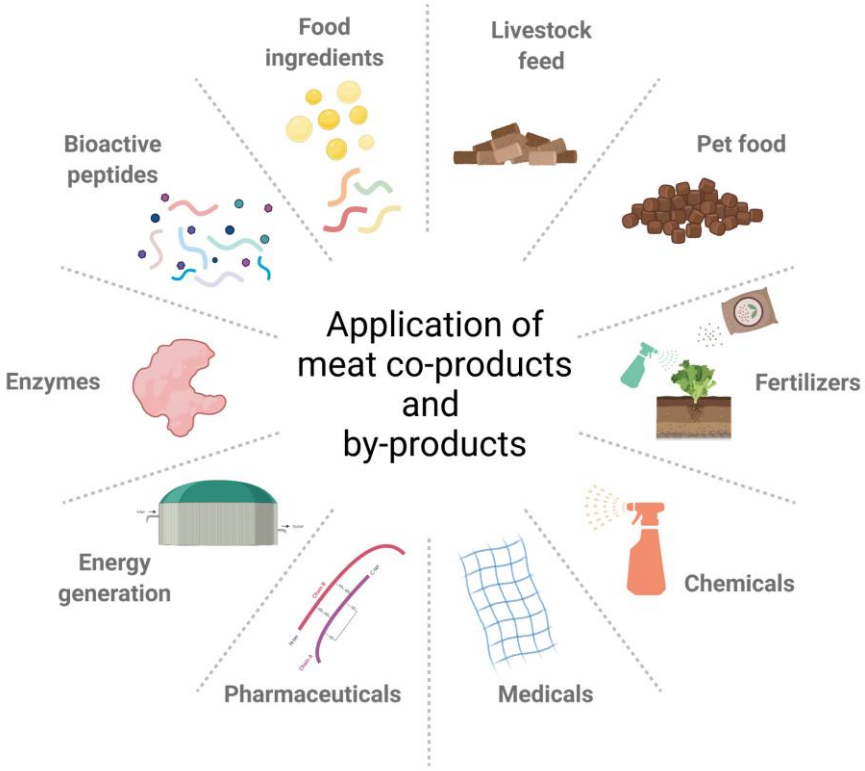

Figure 1.5. Main routes of meat co- and by-products application. Figure adapted from Toldrá et al., (2016). Created with BioRender.com

A large variety of applications have been described for meat by-products based on classical or new and improved technologies. However, the development of techniques to dispose or repurpose it cleanly and efficiently are increasingly being sought. Furthermore, considerable interest has gained in the last years, industries showing great efforts (Darine et al., 2010; Lafarga & Hayes, 2014; Mora et al., 2014).

The edible co-products have been consumed directly in typical dishes, although the use depends on market demand (Toldrá et al., 2012). However, great efforts on the recovery of protein have been ongoing since 1970 (Gault & Lawrie, 1980; Webster et al., 1982; Young & Lawrie, 1974). Among the different proteins that can be recovered, collagen is of great interest due to its many health-promoting properties and functional peptides, with applications in health, cosmetics, and personal care areas (Lafarga & Hayes, 2014; Mora et al., 2014; Mullen et al., 2017). The awareness of the obtention of higher value products by extraction of separate components has led to emerging technologies (Mullen et al., 2017). Enzymatic hydrolysis has surged as a good method to convert the by-products into a broad spectrum of valuable

products, such as functional ingredients (flavor enhancers, emulsifiers, enhancers of water bonding capacity or nutrients) or bioactive peptides (Bah et al., 2015; Mora et al., 2014; Ryder et al., 2016).

The most common practices for inedible by-products include processes as rendering, composting, and anaerobic digestion, obtaining fertilizers, energy, and ingredients for pet food and livestock feed (Hejnfelt & Angelidaki, 2009; Lasekan et al., 2013; Swisher, 2014; Toldrá et al., 2016). As in the case of edible by-products, protein hydrolysates production from inedible by-products is one of the fields currently ongoing to utilize better animal by-products (Bhaskar et al., 2007), representing an exciting alternative and a growing market exists (Bhaskar et al., 2007). By-products such as hair, nails, or feathers can be profitable after hydrolysis (Toldrá et al., 2016). For instance, protein hydrolysates have demonstrated efficient improvement in the fertility of soils (da Silva, 2018). On the other hand, there has also been a growing interest in the use of protein in non-food applications. Such applications include media ingredients for industrial fermentation (Arancon et al., 2013; Liang et al., 2011; Pasupuleti et al., 2010; Solaiman et al., 2011; Tzimorotas et al., 2018), renewable flocculants (Piazza & Garcia, 2010), wood adhesives (Park et al., 2000), biopesticides, commodity organic compounds, bio-plastics, cosmetics, enzymes, and hormones (Hicks & Verbeek, 2016a, 2016b; Lynch et al., 2018b; Piazza & Garcia, 2014).

It is evident that exist different available options to manage the by-products generated by the meat industry. Moreover, the recovery of compounds of interest represents an area of opportunity to enhance economic performance and to improve the environmental impact of the meat industry, making an important contribution to the world's supply in the future (Mullen et al., 2015; Vilg & Undeland, 2017). Nevertheless, the recycling and the potential use of meat by-products to produce value-added products rely on a compromise among public perception, legal regulations, available technology, and the cost, but also sustainability concerns to avoid depletion of water and land reserves (Hicks & Verbeek, 2016b; Rahman et al., 2014; Vilg & Undeland, 2017).

2.2.1. Conventional methodologies

According to the legislation and depending on the category of ABPs, there are several ways to manage animal carcasses and meat by-products, including using them in their current form, disposing of them through incineration or landfill, or adding value through bioprocessing or valorization technologies. The choice of the method depends on the cost, customs, and legislation (Hejnfelt & Angelidaki, 2009; Hicks & Verbeek, 2016b).

As previously mentioned, edible by-products are rich in nutrients and there are markets for them. However, the acceptance of these by-products depends on several factors, such as tradition, culture,

religion, and regulatory requirements. Furthermore, in the last years, the edible by-products markets have been vanishing due to several health-related threats, and the use of by-products has diverted to their integration in processes and applications for non-human consumption (Mora et al., 2019; Rahman et al., 2014). Those by-products not intended for human consumption have been disposed of in other ways, such as incineration or landfill.

2.2.1.1. Incineration

Incineration is the simplest means of waste disposal and can be used as means of energy; however, high energy is required for most of ABPs due to the high moisture content. This method is favored when the destruction of pathogens is deemed necessary to protect public health. The main advantages of this method are the significant reduction in the volume of waste; however, it causes environmental problems (Girotto et al., 2015).

2.2.1.2. Landfilling

Landfilling consists of burying the by-products, generating methane (CH_4) and carbon dioxide (CO_2). This process can lead to serious environmental problems, such as the proliferation in the soil and the transmission of pathogenic microorganisms to animals that gaze on the land, the risk of entering toxic substances into the soils, or the groundwater contamination via leaching. Therefore, in the last years, landfilling has been discouraged (Gooding & Meeker, 2016; Hicks & Verbeek, 2016b; Lasekan et al., 2013; Staroń et al., 2016).

However, the management of the waste is focused on the efficient utilization of biomass by reuse or recycling into other product lines instead of simple reduction or disposal. Other methods have been used to handle meat by-products, such as composting, anaerobic digestion, and rendering.

2.2.1.3. Composting

Composting involves a combination of chemical and microbiological processes (involves thermophilic and mesophilic microorganisms) occurring through three stages, producing a soil-like beneficial product called “compost”. The soil-like stable form can be mixed with soil or directly used as medium for plants (Lasekan et al., 2013). The process requires between 4 and 12 months, temperatures within the range 60 – 70 °C, aerobic conditions, and optimization through regular adjustments to ensure that carbon is converted in CO_2, rather than CH_4 and good quality compost.

2.2.1.4. Anaerobic digestion

Anaerobic digestion is carried out in enclosed vessels, where a complex series of biochemical reactions take place. Microorganisms break down organic-rich material in the absence of oxygen and the main products are CH_4 and CO_2, with traces of hydrogen sulfide (SH_2). The CH_4 constitutes a source of energy, while the digestion effluents can be made into compost via aerobic digestion and used as fertilizer (Hejnfelt & Angelidaki, 2009; Hicks & Verbeek, 2016b; Lasekan et al., 2013; Mora et al., 2019).

2.2.1.5. Rendering

Rendering is one of the oldest methods to process ABPs (Zagklis et al., 2020). This process is relatively complex, but the two major steps are: i) mechanical fragmentation and ii) heating (Pandey et al., 2020). These steps convert rendering in the safest and most economical method of inactivating disease-causing microbes. The primary products resulting from the meat rendering plants are the constituents components of the meat by-products: solid protein (called meal), melted fats, and water (Hicks & Verbeek, 2016a; Meeker, 2006).

Among the primary methods to manage ABPs, rendering is the most common method, well environmentally regulated, and suitable method for the collection and profitable utilization of meat by-products with a low carbon footprint. The economic value of the rendered products is up to 10 times the value of products from composting and up to 6 times the value of products from anaerobic digestion (Table 1.2) (Gooding & Meeker, 2016; Meeker, 2006). Besides this, based on an Analytic Hierarchy Process (AHP) analysis, Zagklis et al., (2020) concluded that the process of rendering is the preferred method compared to anaerobic digestion, composting, incineration, and rendering followed by incineration.

Table 1.2. Gate-to-gate comparison of composting, digestion, and rendering 1000 kg of meat by-products in terms of amounts and economic value of the products. Table adapted from Gooding and Meeker, (2016).

Basis: 1000 kg of meat by-products processed			
Process	**Composting**	**Anaerobic digestion**	**Rendering**
Product 1	N in compost	CH_4 in biogas	Fat
amount (kg)	10 to 20	100 to 180	200
value ($)	10 to 20	20 to 40	170
Product 2	P in compost	N and P in digestate	Protein
amount (kg)	~ 10	30 to 40	210
value ($)	up to 40	40 to 60	130
Total product value ($)	**30 to 60**	**50 to 100**	**290 to 310**

In the EU, around 15 million metric tons of ABPs are processed annually by rendering (Toldrá et al., 2016). The primary purposes of rendering are: i) sterilization of by-products for safety, ii) removal of fat to prevent oxidation during storage, iii) drying to inhibit bacterial growth and facilitate transportation and storage, and iv) separation of ABPs into their main components, which are proteins along with inorganic substances such as calcium and phosphorus and fat (Hicks & Verbeek, 2016a; Tena et al., 2014).

- The rendering process (dry rendering)

To achieve the purposes mentioned above, the basic principles of rendering are followed. All the rendering processes involve the application of heat, the separation of fat, and the extraction of moisture to obtain a protein-rich residue. The quality and the nutritive value of the finished products depend on several conditions and parameters and will determine the ability for a given purpose (Moutinho et al., 2017). The indicators of evaluation are sensory attributes, such as color, texture, flavor, odor, and physical qualities, as solubility and particle size distribution. Before rendering, the material should be collected and handled as soon as possible to minimize microbial degradation of protein and fats. During the rendering, the critical parameters are the steam pressure, the agitation speed, the temperature, and the length of the cooking process. Finally, rendering products can be edible or inedible, depending on the raw material (Hicks & Verbeek, 2016a).

All rendering systems can be classified as wet or dry rendering and both can be carried out in batch, semi-continuous, or continuous (Hicks & Verbeek, 2016a; Meeker, 2006). The most used system nowadays is the dry rendering and a diagram of a dry batch rendering process is shown in Figure 1.6.

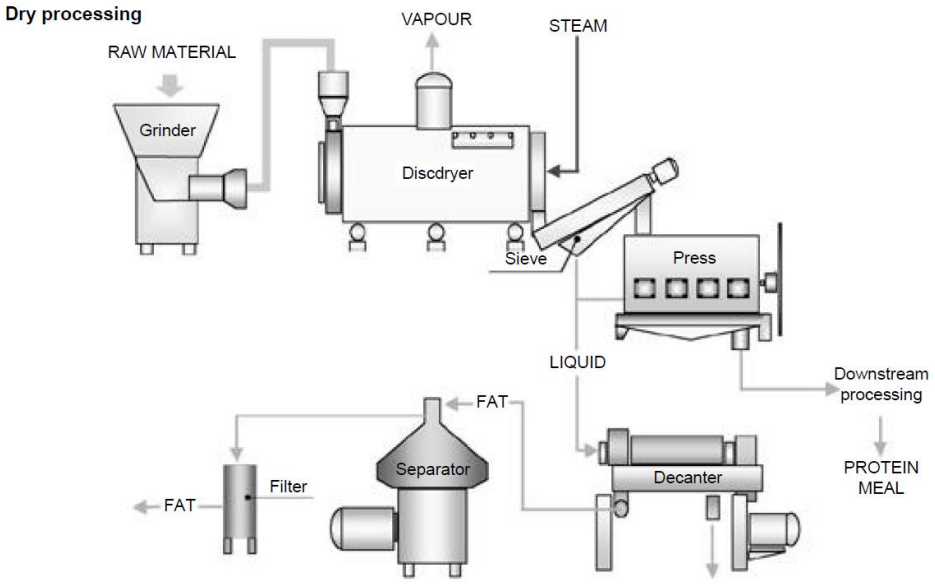

Figure 1.6. Schematic diagram of the dry rendering process (Woodgate & van der Veen, 2004).

The rendering process begins with introducing the solid and semi-solid raw material in a crusher where it is crushed, grounded into consistent particle size, and blended. The reduction of the size improves cooking efficiency. Next, the grounded material is conveyed to a cooking vessel, a steam-jacketed cylindrical vessel equipped with an agitator, where is cooked. In the US, the temperature ranges from 115 to 145 °C and the time required varies from 40 to 90 min while in the EU, the typical parameters are temperature over 133 °C, pressure 3 bar by steam for 20 min, although there are several approved methods (Aspevik et al., 2017). Adjustments on time and temperature are made depending on the type of raw material and the system. During the thermal treatment, water is evaporated, fat is melted, and pathogens are killed. Once the material is ready, the content of the cooker is discharged to the percolator drain pan, which contains a perforated screen. The perforated screens allow the draining of the fat, separating it from the protein solids. After this step, which lasts for 1 or 2 hours, the protein solids still contain about 25% fat. This fraction is conveyed to the screw press, where the separation of fat from protein solids is completed. The solids, known as cracklings, contain protein, minerals, and some residual fat, around 10%. Finally, it is grounded with a hammer mill and sifted (12-mesh screen) to produce the protein meal, then transferred for storage or shipment. On the other hand, the fat discharged from the screw contains water and fine solid particles, also called *fines*, and is sent to a centrifuge to "polish" the fat by removing the *fines* and the water. Finally, the animal fat is stored in the fat storage tank, while a part of the *fines* can be recycled to join the feed entering the drainer conveyer. However, depending on the conditions and the type of raw material, the geographic location of the plants, and other parameters, large amounts of *fines* are recovered and only a part can be recycled, thus generating a waste that needs to be properly managed. Therefore, after the rendering, stable, safe-to-use, and valuable products are obtained, the meal high in protein and the purified fat, and in some cases a waste, the *fines* (Aspevik et al., 2017; Bradford, 1967; Garcia et al., 2006; Gooding & Meeker, 2016; Hicks & Verbeek, 2016a; Meeker, 2006, 2009; Pandey et al., 2016, 2020; Prokop, 1985; Woodard & Curran, 2006).

This process relies mainly on the application of heat. The heat is transferred into the crushed raw material, causing the evaporation of water and water-based substances present in the by-products. This provokes an increased and sustained partial pressure within the cells, causing the lysis of the cells' walls. In consequence, three main effects are observed. First, the water and water-based substances present in the solids are released. Second, due to the binomial temperature/time, protein structure in the material is denatured, causing changes in carboxyl and carbonyl groups. These alterations allow more moisture to be driven off, leaving an altered structure, shorter amino acid chains in the protein structure. And third, fats and lipids molecules are emulsified and separated from solid materials. Besides this, due to their hydrophobicity, they are also separate from the released water (Meeker, 2006; Yarem, 2006).

Over the years, the processes and technologies have changed and improved. Modern rendering facilities can monitor the process via computer technology, allowing better control of time and temperature, achieving appropriate levels to kill specific microorganisms (Meeker, 2006).

- Rendered products

The products of the rendering process are safe and provide economic value (Pandey et al., 2020). The meal is one of the primary products obtained after rendering. Meals are protein-rich-solids and comprise meat and bone meal, meat meal, poultry by-product meal, hydrolyzed poultry feather meal, blood meal, hydrolyzed hair, hydrolyzed leather, and leather meal, etc. (Hicks & Verbeek, 2016a; Meeker, 2006). The annual global production of meals from ABPs is estimated at 13 million metric tons, with the United States, South America, and the European Union accounting for around 90% of the total (Figure 1.7) (Hicks & Verbeek, 2016b).

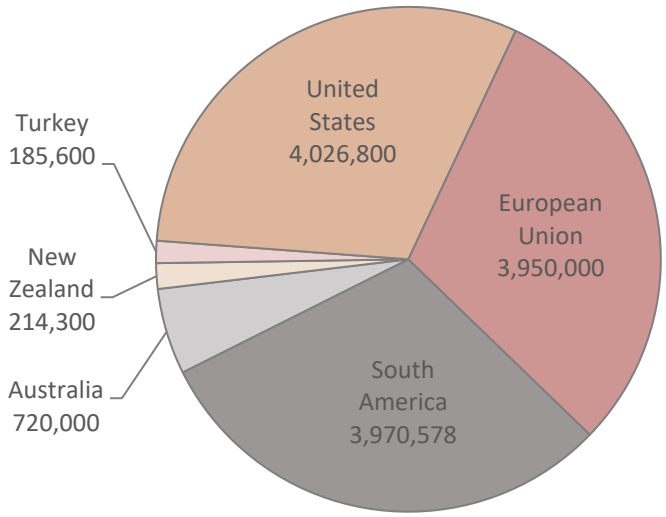

Figure 1.7. Global production estimated for animal by-product protein meals expressed in metric tons. Figure adapted from Hicks and Verbeek, (2016b).

In the US, the major use of the high protein by-products from the rendering industry is as an ingredient in the animal feed industry (animal pets, poultry, fish, and pigs), which is considered a major contributor to the growth of the pet food industry (Mullen et al., 2017). Therefore, animal feeds play an important role in the global food industry, as the products arising from meat processing and rendering influence greatly the ability to produce animals in an economical way (Jayathilakan et al., 2012). In fact, according to the Global Feed Survey, worldwide feed production increased to 963 million metric tons in 2013, being China the largest feed market in the world (Swisher, 2014). In the US, the rendered products used by the animal feed industry represent about 85% of the total (Pandey et al., 2016).

Meat and bone meal (MBM) is one of the most used meals manufactured worldwide with a steady availability, averaging production of 3.5 million metric tons per year in the EU (Moutinho et al., 2017). It is considered an excellent source of nutrients, such as proteins, amino acids, vitamins, calcium, and phosphorus and it has good digestibility (Pandey et al., 2016, 2020; Toldrá et al., 2012). In addition to the use of MBM as a protein source and considering that one of the most abundant proteins in MBM is collagen, it has been flagged as a source of bioactive peptides, observing positive effects when they are fed to young broiler chicks (Muir et al., 2013). Besides this, the absence of known anti-nutritional factors appears to be a better alternative compared to plant ingredients (Moutinho et al., 2017).

MBM is a heterogeneous mixture of particles derived from bones and soft matter, conferring a dark red-brown color, low protein solubility, high mineral, and low fat content. However, large variations in the chemical composition exist among rendering plants and within the same rendering facilities, due to several factors, as the rendering method and the composition of the raw material (Pérez-Calvo et al., 2010). The blending of raw material from different sources is a common practice to ensure better consistency (Garcia et al., 2006; Hicks & Verbeek, 2016a). Once MBM is produced, the duration and method of storage also affect the product quality due to the autooxidation of fatty acids and the increase in moisture content (Hendriks et al., 2006).

Due to progressive restriction of the use of MBM in animal feed as a consequence of the appearance of transmissible spongiform encephalopathies, the overproduction of MBM should be eliminated or used in alternative environmentally friendly processes (Garcia & Phillips, 2009). Considering the characteristics of MBM in terms of heating value and nutritional content, other industrial applications, such as solid fertilizer and fuel, have been proposed, being the thermal degradation treatment (pyrolysis, combustion, or gasification) in a thermal plant one of the most chosen methods to solve the problem. The most investigated is co-combustion with coal and pyrolysis (Toldrá et al., 2016). The use of MBM as a secondary fuel in thermochemical processes could result in a noticeable saving in Europe. Moreover, apart from energy recovery, proteins such as prions are destroyed. However, one of the aspects to keep in mind is the fact that ashes produced from MBM represent up to 30% of the original weight, leading to large amounts of ashes whose fate is a major environmental concern. However, the high phosphate content (around 56%) converts the ashes into a good fertilizer, a suitable low-cost phosphate source for industry, agricultural soil enrichment, or heavy metal immobilization in soil or water (Cascarosa et al., 2012; Coutand et al., 2008; Deydier et al., 2005; Toldrá et al., 2016).

As mentioned above, soil application is a promising strategy for sustainable recycling due to large amounts of nutritive elements, such as nitrogen (N), phosphor (P), and calcium (Ca). Several studies have reported the potential of MBM as an effective fertilizer (Jeng et al., 2006, 2004; Mondini et al., 2008). Mondini et al. (2008) reported a significant increase in available mineral N and an enhancement of the size and activity of soil microbial biomass, indicating an increase in the soil quality. On the other hand, Jeng et at. (2004, 2006) reported significantly higher yields for cereals and ryegrass crops. Thus, the effectiveness of MBM as N and P fertilizer has been positively evaluated.

At laboratory or demonstration-scale, potential applications of meals have been proposed, such as alternative nitrogen sources to replace yeast extract during the fermentation of potato starch to produce bioethanol (Izmirlioglu & Demirci, 2010), control of plant pathogens (Lazarovits, 2001), renewable flocculants (Piazza & Garcia, 2010), as the primary ingredient in an adhesive (Park et al., 2000), culture media for microbial production of eicosapentaenoic acid by *Pythium irregulare* (Liang et al., 2011), production of plastic material (Garcia et al., 2004, 2006), etc.

Animal fats

Fat is the other main product obtained during rendering and includes several types of animal fat, such as tallow, lard, yellow grease, white grease, and poultry fat (Meeker, 2006). As well as meals, rendered fats have been extensively used as a supplement in the animal feed of both ruminant and non-ruminant animals because of their good content in fatty acids and some fat-soluble vitamins (Freeman, 1983; Mora et al., 2019; Partridge et al., 1986). Fats are the most caloric-density feed ingredient available and it has been used for years to increase the energy density of animals' diet. Depending on the species being fed, the energy contributions of fat range from 2.6 to 3.8 times the energy content of corn (Hess et al., 2008; Meeker, 2006). However, it was observed that fats decrease the dry matter intake (DMI), depress ruminal digestion, and are likely to cause milk fat depression (Manriquez et al., 2019). Later, it was demonstrated that the supplementation of fats in the form of calcium soaps, also known as rumen-protected fats (RPF) when supplemented to ruminants, had other positive effects, such as improvement of the digestibility of dry matter, organic matter, crude protein, ether extract, and fiber fractions (Behan et al., 2019; Jenkins & Palmquist, 1984). Tyagi et al., (2010) reported an increase in milk production and its persistency and improved reproductive performance of lactating crossbred cows when RPF were supplemented at 2.5% of DMI. Recently, Manriquez et al., (2019) reported similar effects on dairy cows. Other positive effects attributed to added fat are higher conception rate, increased pregnancy rate, and reduced service periods (Handojo et al., 2019; Manriquez et al., 2019; McNamara et al., 2003; Tyagi et al., 2010). On the other

hand, Behan et al., (2019) did not observe any effect on the body weight, nutrient intake, digestibility, and rumen fermentation parameters in Dorper sheep when RPF were supplemented.

Calcium soaps or calcium salts of fatty acids are obtained through saponification with an alkali. These compounds are protected against rumen degradation circumvent rumen fermentation (Manriquez et al., 2019; Proaño et al., 2015). Consequently, they are dissociated in the abomasum, where free fatty acids and calcium ions are released, molecules that can be digested and absorbed in the intestine (Proaño et al., 2015; Schneider et al., 1988). Besides this, the physical requirements of many fats used in feeds are different from those of natural fats or oils (Sharma et al., 2013). Rendered fats can be unsatisfactory soft, causing problems during the fabrication of the final products. Saponification has been used to improve fat consistency (Proaño et al., 2015). Thus, this chemical reaction between a fatty acid and a base can solve the consistency problem and simultaneously benefit animals when fed in controlled amounts (Handojo et al., 2019).

However, in the last years, bioenergy production acted as a strong demander for rendered fats and used cooking oil as feedstocks (Swisher, 2014). The drastic rise in the oil prices and the uncertainty of day-to-day supplies have led many countries to look to alternatives and ecologically accepted substitutes, considering biodiesel as a solution (Meeker, 2006). Currently, the EU is producing about 11 million metric tons of biodiesel annually, and about 920,000 metric tons are generated from categories 1, 2, and 3 animal fats. Animal fat as raw material instead of common edible vegetable oils offers economic, environmental, and food security advantages, being the cheapest option for biodiesel production when cheap waste cooking oils or waste animal fats are used (Adewale et al., 2015). Moreover, diesel fuel replacement has several advantages, such as renewability, portability, lower sulfur and aromatic content, higher efficiency, higher cetane number, better emission profile, and safer handling (Banković-Ilić et al., 2014). Besides this, the production and use of biodiesel are considered environmentally friendly because it is biodegradable, non-toxic, and has a good combustion emission profile, leading to reductions in carbon dioxide, carbon monoxide, particulate matter, and unburned hydrocarbons. However, the replacement of diesel by biodiesel will require nonrelevant modifications in engines. The most common way to produce biodiesel is by transesterification with a low molecular weight alcohol to yield a mixture of fatty acid methyl esters and glycerol as a side product, but some limitations have been observed due to the protein content, phosphoacylglycerols, and water in substrates. In consequence, the resulting biodiesel requires some refinement, as degumming for protein and phosphoacylglycerols removal, vacuum drying for water reduction, and winterization for the reduction in saturated fatty acids (Mora et al., 2019; Toldrá et al., 2012, 2016).

Besides this, fatty acids are used in chemical processes like rubber and plastic polymerization, softeners, lubricants, and plasticizers. Furthermore, rendered fats also have expanded their use for various cosmetics applications like hands and body lotions, creams, and bath products (Toldrá et al., 2012).

2.2.2. Alternative and emerging processing technologies

Efforts to recover and to improve the value of animal coproducts and by-products are not new (Drummond et al., 2019). In the case of animal-originated waste, the target ingredients are proteins, lipids, and biomolecules (Mullen et al., 2017). These materials are a source of too valuable compounds to be discharged in the environment (Galanakis, 2012). The recovery of protein-rich products has been extensively studied and is still under active investigation (de Queiroz et al., 2017; Piazza & Garcia, 2014). Although the use of these by-products in the feeding of animals or as fertilizers, employing technological processes like rendering is technologically and economically viable, a paradigm shift might be necessary to utilize better animal co- and by-products (Lasekan et al., 2013).

Consequently, there is a growing interest in the recovery and recycling of co-products and ABPs through sustainable and environmentally-friendly methodologies employing the most up-to-date and effective tools (Anzani et al., 2019; Mullen et al., 2015; Toldrá et al., 2012), using some principles in the field of chemistry, biochemistry, and microbiology (Lasekan et al., 2013). The recovery of target compounds from any raw material usually follows five distinct stages, going from macroscopic to the molecular level with the ultimate goal of i) maximizing the yield of the target compounds, ii) suiting the demands of industrial processing, iii) clarifying the high added-value ingredients from impurities and toxic compounds, iv) avoiding deterioration and loss of functionality during processing, and v) ensuring the food-grade nature of the final product, when required (Galanakis, 2012). Specifically, the recovery of proteins from meat co-products and by-products, as well as other secondary streams, can be divided into three main steps: pretreatment, extraction, and downstream treatments (Drummond et al., 2019). Several techniques have been developed based on the raw material and the final use of the targeted compound, recovering many meat proteins, peptides, or amino acids with a wide range of applications (Table 1.3).

Table 1.3. Current technologies employed for protein recovery from meat co- and by-products. Table adapted from Drummond et al., (2019); Mullen et al., (2015).

Source	Pre-treatment	Extraction technology	Downstream processing	High added-value product	Reference
Liver	Homogenization + phosphate buffer + centrifugation	Enzymatic hydrolysis	Ultrafiltration Reverse-phase high-performance liquid chromatography (RP-HPLC) purification	Antioxidant peptides	(Di Bernardini et al., 2011)
	Homogenization	Salt extraction	Centrifugation	Peptides with emulsifying properties	(Steen et al., 2016)
Heart	Homogenization	Surimi	Centrifugation	Surimi like-material for use in frankfurters	(Desmond & Kenny, 1998)
	Homogenization + distilled water + centrifugation	Isoelectric solubilization/precipitation (ISP)	None	Functional protein concentrate, with low ash, fat, and cholesterol	(Dewitt et al., 2002)
Skin	Cutting + washing	Microbial degradation Autoclave/microwave alkaline hydrolysis	Chromatographic purification	Protein hydrolysates	(Gousterova et al., 2005)
	None	Enzymatic hydrolysis	Chromatographic purification	Antihypertensive angiotensin-converting enzyme (ACE)-inhibitory peptides	(Ichimura et al., 2009)
Hair	None	Sub-critical water hydrolysis	Centrifugation	Amino acids	(Esteban et al., 2008)
	Rinsing	Chemical solubilization with Mistolin HTG 50	Centrifugation + ultrafiltration	A fairly pure extract of keratin	(Cassoni et al., 2018)
	Chopping + thermal treatment + drying + crushing + sieving	Keratinolytic bacteria fermentation and enzymatic hydrolysis (*Amycolatopsis keratiniphila* D2)	Centrifugation + vacuum filtration + freeze-drying	Protein supplements for animal feed with improved nutritional value	(Falco et al., 2019)
	Washing + drying	Thermal hydrolysis process (THP)	Ultrafiltration + reverse osmosis	Keratin hydrolysates	(Tasaki, 2020)

Source	Pre-treatment	Extraction technology	Downstream processing	High added-value product	Reference
Feathers	Washing + autoclave + drying + grinding	Fermentation and enzymatic hydrolysis (genus *Thermoactinomyces*)	Centrifugation, filtration, and drying	Soluble proteins, amino acids, enzymes, and hydrolysates as soil amendment and biological control agent	(Gousterova et al., 2011; Vasileva-Tonkova et al., 2009)
	None	Keratinolytic bacteria fermentation and enzymatic hydrolysis (*Meiothernus ruber* H328)	Centrifugation	Chiral separation membrane	(Sueyoshi et al., 2011)
	None	Keratinolytic bacteria fermentation and enzymatic hydrolysis (*Pseudomonas stutzeri* strain K4)	Centrifugation	Protease production, culture media	(Chaturvedi et al., 2014)
	Washing + soaking with ethanol + washing + drying	Thermal alkaline treatment followed by enzymatic hydrolysis	Centrifugation	Protein-rich hydrolysate	(Cheong et al., 2018)
Bone	Homogenization	Alkaline extraction	Acid precipitation or dialysis	Food additive in sausages	(Boles et al., 2000)
	Boiling + filtration + centrifugation	Enzymatic hydrolysis	Ultrafiltration	Bioactive peptides with antihypertensive effect	(Saiga et al., 2008)
	Washing with water and acetone + drying + grinding	Sub-critical water hydrolysis Alkaline hydrothermal hydrolysis Thermal decomposition	Filtration	Hydroxyapatite and collagen	(Barakat et al., 2009)
	Drying + grounded	Enzymatic hydrolysis	Acid precipitation + freeze-drying	Bone hydrolysate	(Pagán et al., 2013)
	Boiling + high pressure (HP)	Enzymatic hydrolysis	Filtration	ACE-inhibitory peptides	(Zhang et al., 2013)

Source	Pre-treatment	Extraction technology	Downstream processing	High added-value product	Reference
Blood	Bovine serum albumin (BSA) purification	Sub-critical water hydrolysis	None	Amino acids	(Rogalinski et al., 2005)
	Centrifugation + acid precipitation + freeze drying	Enzymatic hydrolysis	Centrifugation + membrane-filtration + freeze drying	Iron-binding peptides	(Lee & Song, 2009)
	Centrifugation	Ethanol precipitation	Filtration and drying	Functional peptides (food additives in human foodstuffs)	(Álvarez et al., 2009)
	Centrifugation + water + freeze-drying	Enzymatic hydrolysis	Ultrafiltration	Antioxidant peptides with applications in food, cosmetics, and medicine industries	(Sun et al., 2011)
	Centrifugation + hemolyzation	Enzymatic hydrolysis assisted by high hydrostatic pressure (HHP)	Centrifugation	Peptides and biopreserved blood	(Toldrà et al., 2011)
	Centrifugation + osmotic shock + chloroform + freeze drying	Medium pressure and moderate temperature hydrolysis	Chromatographic purification	Antioxidant peptides	(Álvarez et al., 2012b)
	Centrifugation + osmotic shock + chloroform + freeze drying	Acid hydrolysis	Precipitation and centrifugation	Amino acids and peptides for animal and pet food	(Álvarez et al., 2012a)
Lung	Homogenization	Mild condition of pH (4, 7, and 9, temperature (20, 30, and 40 °C) and time (30 and 60 min)	Microfiltration and ultrafiltration	Concentrated protein extracts with excellent functional properties	(Darine et al., 2008)
	Homogenization	Alkaline solubilization	Acid precipitation	Protein concentrates with good functional properties	(Darine et al., 2010)
	Mincing	pH-shift or ISP	Centrifugation	Soluble proteins	(Lynch et al., 2018a)

Animal co-products and by-products processing usually begin with the macroscopic pre-treatment (washing, mincing, thermal treatment, precipitation, homogenization, freeze-drying, etc.), aiming the stabilization and the preparation of the raw material matrix to facilitate the next step, the extraction of the compounds of interest, thus increasing the efficacy or the yield of the process. Several methodologies have been described and are well-documented towards the recovery of target compounds or molecules and their physicochemical properties. Chemical hydrolysis, sub-critical water hydrolysis, enzymatic hydrolysis, thermal and microwave treatments, etc. have been widely investigated over the last years. However, each technique presents its advantages and disadvantages (Table 1.4). Finally, the downstream processing consists of the isolation of the clarification of the target compounds from co-extracted impurities or the non-target compounds. Centrifugation, membrane filtration, and chromatographic separation are the most employed methodologies. However, some of them, such as chromatographic methods, are worth only when the target compounds are very valuable. These methods are laboratory-intensive and time-consuming (Drummond et al., 2019; Galanakis, 2012).

Table 1.4. Main advantages and disadvantages of the current technologies employed for the protein recovery from meat by-products. Table adapted from Drummond et al., (2019) and Lynch et al., (2018b).

Technology	Advantages	Drawback
pH shift or ISP	Inexpensive reagents; aqueous solvents; low processing time; easy scale-up; good emulsification properties	Medium-high water consumption; low yields depending on the raw material
Salt extraction	Inexpensive reagents; aqueous solvents	Protein functionality negatively affected
Enzymatic hydrolysis	High yield; predictable products; easy to scale up; aqueous solvents	Expensive reagents
Sub-critical water hydrolysis	Low processing time; green technology; batch or continuous operation; no reagents required; aqueous solvents	High cost of equipment; loss of product functionality, amino acid degradation
Surimi processing	Low-cost reagents, aqueous solvents	Low yields for some raw materials
Chemical hydrolysis	High yields; inexpensive reagents	Techno-functional properties lost due to hydrolysis; amino acid degradation

One of the most studied and promising lines is the production of protein hydrolysates (Bhaskar et al., 2007; Lasekan et al., 2013; Mora et al., 2014). The method of choice for the hydrolysis of proteins depends on their sources, the target compounds to be obtained, and the desired functionality and application (Drummond et al., 2019; Hou et al., 2017). Acidic, alkaline, sub-critical water, microbial, and enzymatic hydrolysis are widely used to recover and generate protein hydrolysates, peptides, and amino acids (Drummond et al., 2019; Kadam et al., 2015; Kristinsson & Rasco, 2000). A general procedure of the manufacturing of protein hydrolysates is shown in Figure 1.8.

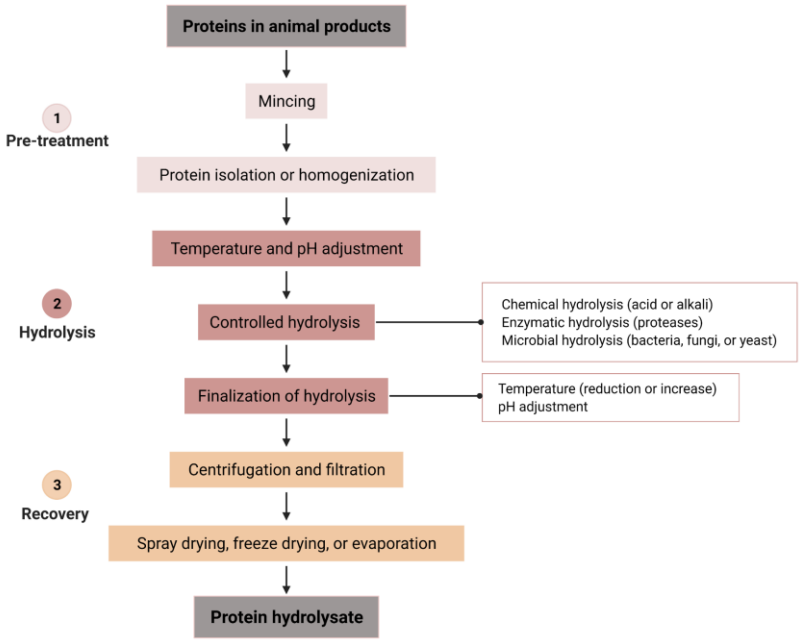

Figure 1.8. General procedure for the production of protein hydrolysates from animal co- and by-products. Figure adapted from Hou et al., (2017) and Villamil et al., (2017). Created with BioRender.com.

However, enzymatic hydrolysis is preferred over chemical hydrolysis for several reasons, mainly related to the size of the peptides and amino acids availability (da Silva, 2018; Etemadian et al., 2021). The main differences are presented in Table 1.5.

Table 1.5. Differences between enzymatic and chemical hydrolysis of proteins (Aspevik et al., 2017; de Queiroz et al., 2017; Drummond et al., 2019; Kristinsson and Rasco, 2000; Tavano, 2013; Villamil et al., 2017).

Enzymatic hydrolysis	**Chemical hydrolysis**
Precise in the cleavage of peptide bonds: peptides produced are predictable and homogenous	New peptides can be produced and new functions can be discovered Yield products with modified amino acids
No desalting step is needed	A salt is produced after neutralization and must be removed
Mild conditions, there is no loss of amino acids or side reactions, and good amino acid balance	Destructive to particular amino acids and can yield products with modified amino acids Acid hydrolysis destroys tryptophan, methionine, and cysteine and converts glutamine and aspartame into glutamic and aspartic acids Alkaline hydrolysis destroys serine and threonine
Free amino acids are poorly produced	Appreciable amount of free amino acids is produced
Proteases are expensive	Low-cost and quick
Require a carefully controlled environment (pH, temperature, agitation, and time)	Simple process
Small amount of protein can be hydrolyzed (5 – 10 mg/mL)	Large amount of protein can be processed (50 – 500 mg/mL)

2.2.2.1. Enzymatic hydrolysis

Enzymatic hydrolysis is an efficient process to solubilize and extract protein from meat by-products and improve its biological and nutritional value, obtaining high value-added ingredients with commercial interest (Fu et al., 2018; Villamil et al., 2017). It is certainly one of the most applied methods in the obtention of protein hydrolysates due to enzyme specificity, absence of toxic/chemical/microbial contaminants, performance, high nutritional quality, ease to control, and relatively mild conditions (Aspevik et al., 2017; da Silva, 2018; Etemadian et al., 2021; Pasupuleti & Braun, 2010; Villamil et al., 2017). All these advantages have led to the employment of this method on various meat co-products and by-products, as shown in Table 1.6. Enzymatic hydrolysis consists mainly of the enzymatic reaction itself and a final heating step to inactivate the enzyme. However, sample pre-treatment and recovery of protein hydrolysates play an important role (Drummond et al., 2019; Villamil et al., 2017). During the hydrolysis, the molecular weight of intrinsic proteins and peptides is reduced, while the number of ionizable and polar groups on the protein surface is increased, which influences the improvement of their ability to form hydrogen bonds with water. As a result, a mixture of low molecular weight polypeptides and peptides are obtained and more water-soluble than the intact protein (Aspevik et al., 2017; Villamil et al., 2017).

Table 1.6. Sources and conditions used in the enzymatic hydrolysis of animal by-products.

Source	Enzymes	Optimum conditions and parameters	DH (%)	Outcome	Reference
Bovine lung and rumen	Pepsin Papain Neutrase Alcalase	E/S ratio = 2%; t = 4 h; pH = 3.0, T = 50 °C E/S ratio = 2%; t = 4 h; pH = 5.5, T = 50 °C E/S ratio = 2%; t = 4 h; pH = 7.0, T = 50 °C E/S ratio = 2%; t = 4 h; pH = 8.5, T = 50 °C	4 – 15 5 – 20 2 – 12 5 – 15	Protein hydrolysates	(Webster et al., 1982)
Leather waste	Protoderm 100 T	E/S ratio = 500 – 15,000 U/g, t = 120 min; pH = 9 – 10, T = 56 °C		Powder leather flour, gelatin products, and protein hydrolysate	(Bajza & Vrček, 2001)
Sheep visceral mass (stomach, large, and small intestine)	P “Amano” 6	E/S ratio = 1%; t = 45 min; pH = 7; T = 43 °C	34.7	Protein hydrolysates rich in some essential amino acids and bioactive peptides	(Bhaskar et al., 2007)
Calf skin and blood	Alkaline proteinase containing subtilisin DY (APB 72)	E/S ratio = 4%; t = 5 – 6 h; pH = 8 – 8.3; T = 50 – 55 °C	65 – 68	Peptone for bacterial growth media	(Vasileva-Tonkova et al., 2007)
Chicken meat	Alcalase® 2.4L	E/S ratio = 4.2%, t = 6 h, pH = 8.0, T = 52.5 °C	31	Protein hydrolysates as protein supplementation in food systems	(Kurozawa et al., 2008)
Poultry viscera (intestine, spleen, gall bladder, and connective tissue)	Endogen enzymes	t = 6 h; pH = 2.8; T = 55 °C	55	Protein hydrolysate suitable as nutrient source for rats, pets, aquaculture, bacteria, and other commercially grown microorganisms	(Jamdar & Harikumar, 2008)
Chicken leg bone	Alcalase® 2.4L	E/S ratio = 2%; t = 4 h; pH = 8; T = 50 °C		ACE-inhibitory peptides	(Cheng et al., 2009)

Source	Enzymes	Optimum conditions and parameters	DH (%)	Outcome	Reference
Pig bones	Neutrase® 0.8L	E/S ratio = 2.5%; t = 120 min; pH = 6.0 – 7.0, T = 55 °C	12.14	Protein hydrolysate with potential use as emulsifier and dispersant for preparing sauces and soups	(Pagán et al., 2013)
Bovine collagen from achilled tendon	Alcalase® 2.4L Collagenase Thermolysin Endopeptidase K Pepsin Trypsin	E/S ratio = 1%; t = 1 – 240 min; pH = 8.0; T = 55 °C E/S ratio = 1%; t = 1 – 240 min; pH = 7.5; T = 37 °C E/S ratio = 1%; t = 1 – 240 min; pH = 8.0; T = 37 °C E/S ratio = 1%; t = 1 – 240 min; pH = 7.5; T = 37 °C E/S ratio = 1%; t = 1 – 240 min; pH = 2.0; T = 37 °C E/S ratio = 1%; t = 1 – 240 min; pH = 8.0, T = 37 °C	4 – 15 1 – 18 0.5 – 7 1.7 – 6 0.5 – 3 1 – 7	Potent ACE-inhibitory peptides	(Zhang et al., 2013)
Deer, sheep, and pig blood and plasma	Papain Bromelain FP400 FPII	E/S ratio = 10%; t = 1 – 24 h; pH = 6.5, T = 55 °C E/S ratio = 10%; t = 1 – 24 h; pH = 6.5, T = 50 °C E/S ratio = 10%; t = 1 – 24 h; pH = 6.5, T = 50 °C E/S ratio = 10%; t = 1 – 24 h; pH = 6.5, T = 50 °C		Hydrolysates with varying degrees of in vitro antioxidant activities	(Bah et al., 2015)
Bovine *Musculus semimembraneous* meat myofibrils and connective tissue collagen	Acidic fungal protease Fungal protease II Fungal protease 31,000 Fungal protease 60,000 HT proteolytic	E/S ratio = 0.4 – 10%; t = 24 h; pH = 6.0, T = 50 °C E/S ratio = 0.4 – 3%; t = 24 h; pH = 6.0, T = 50 °C E/S ratio = 0.4 – 2%; t = 24 h; pH = 6.0, T = 50 °C E/S ratio = 0.4 – 1%; t = 24 h; pH = 6.0, T = 50 °C E/S ratio = 0.2 – 0.4%; t = 24 h; pH = 6.0, T = 50 °C		Bioactive peptides containing significant bioactivity with potential application in the production of health-promoting products	(Ryder et al., 2016)
Goat by-products (liver, lungs, and heart)	Alcalase® 2.4L Brauzyn®	E/S ratio = 0.8%; t = 180 min; pH = 7.0; T = 60 °C E/S ratio = 1%; t = 120 min; pH = 6.5; T = 70 °C	17.6 14.4	Protein hydrolysates with potential for application as functional ingredient in various products of food industry	(de Queiroz et al., 2017)
Pig skin	Pepsin Alcalase	E/S ratio = 2400 U/g; pH = 7.0, T = 50 °C E/S ratio = 3000 U/g; pH = 8.5, T = 60 °C	10 – 20 10 – 20	Protein hydrolysate with good functionality and structural properties	(Zhang et al., 2017)

Source	Enzymes	Optimum conditions and parameters	DH (%)	Outcome	Reference
Pork (intestines, spleen, liver, and tails), beef (intestines), and chicken (heart, liver, and digestive tract)	Alcalase® 2.4L Papain	E/S ratio = 0 – 0.5%; t =0 – 2 h; pH = no adjustment or 8.0, T = 60 °C E/S ratio = 0 – 0.5%; t = 0 – 2 h; pH = no adjustment or 8.0, T = 60 °C		Protein hydrolysates as nitrogen source for growth media	(Lapeña et al., 2018)
Fleshing meat	Pepsin Trypsin Dispase Alcalase® 2.4L Pancreatin Papain	E/S ratio = 1%; t = 16 h; pH = 3, T = 37 °C E/S ratio = 1%; t = 16 h; pH = 7.8, T = 37 °C E/S ratio = 1%; t = 16 h; pH = 7.3, T = 37 °C E/S ratio = 1%; t = 16 h; pH = 6.5, T = 60 °C E/S ratio = 1%; t = 16 h; pH = 7.8, T = 37 °C E/S ratio = 1%; t = 16 h; pH = 7.3, T = 37 °C	1.2 5.0 7.1 30.9 6.4 6.5	Collagen hydrolysates for gelatin production, thickening agents, texturizers, and others	(Anzani et al., 2018)
Chicken feathers	Savinase Ultra 16L Autoclave-NaOH + Savinase Ultra 16L Microwave-NaOH + Savinase Ultra 16L	E/S ratio = 2.6%, t = 4 h, pH = 9, T = 55 °C E/S ratio = 2.6%, t = 4 h, pH = 9, T = 55 °C E/S ratio = 2.6%, t = 4 h, pH = 9, T = 55 °C		Protein hydrolysate	(Cheong et al., 2018)
Porcine plasma and minced beef	Alcalase® 2.4L FG Flavourzyme® Protamex® Neutrase® 0.8L ProteAX Protease P 6SD Protease A 2SD Papain P1 Bromelain Sumizyme BNP-L	E/S ratio = 0.5%; t = 5 h; pH = 7.5, T = 50 °C E/S ratio = 0.5%; t = 5 h; pH = 7.0, T = 50 °C E/S ratio = 0.5%; t = 5 h; pH = 7.5, T = 55 °C E/S ratio = 0.5%; t = 5 h; pH = 7.0, T = 50 °C E/S ratio = 0.5%; t = 5 h; pH = 7.0, T = 50 °C E/S ratio = 0.5%; t = 5 h; pH = 7.0, T = 40 °C E/S ratio = 0.5%; t = 5 h; pH = 7.0, T = 50 °C E/S ratio = 0.5%; t = 5 h; pH = 7.5, T = 55 °C E/S ratio = 0.5%; t = 5 h; pH = 7.0, T = 40 °C E/S ratio = 0.5%; t = 5 h; pH = 7.0, T = 50 °C	16 – 20 15 – 17 11 – 16 8 – 9 15 – 22 17 – 26 20 – 25 14 – 15 10 – 13 12 – 14	Low bitter and high umami protein hydrolysates with potential to be used as protein ingredients in food products	(Fu et al., 2018)
Duck meat	Flavourzyme	E/S ratio = 3%, t = 1 h, pH = 5.45, T = 50 °C		Antioxidant peptides	(Wang et al., 2018)
Bovine hides	Alcalase from *Bacillus licheniformis*	E/S ratio = 4.25%; t = 6 h; pH = 7 – 7.5; T = 60 °C	17.3 – 19.2	Hydrolysates with potential application as protein source for feed, food, or other high-quality application	(Anzani et al., 2019)

- Pre-treatment

The first stage in the recovery of proteins depends on the by-product. Applied methods for samples pre-treatment for enzymatic hydrolysis do not differ very much from those mentioned previously. Usually, the by-products require a stabilization step, unless immediately processing. The next step consists of the homogenization, size reduction, and adjustment of the moisture content, depending on the raw material. Some liquid streams may have very low protein contents (1 – 3%) and moisture reduction is carried out. Several approaches, such as membrane filtration (microfiltration, ultrafiltration, nanofiltration) and reverse and forward osmosis can be used to increase protein concentration. On the other hand, homogeneous mixtures of water are prepared when by-products with very low moisture content must be hydrolyzed. Additionally, fat and other undesirable compounds should be reduced at the lowest possible percentage to avoid oxidation, coloration, and the formation of unpleasant smells and tastes and highly toxic compounds (Drummond et al., 2019; Villamil et al., 2017). Solvent-based methods and heat treatment are standard practices to remove and to ensure reduced fat content in animal co- and by-products (Bhaskar et al., 2008, 2007; Boyaci et al., 2014; Garcia et al., 2011; Klompong et al., 2007; Kristinsson & Rasco, 2000; Liang et al., 2011; Nebel & Mittelbach, 2006; Pérez-Palacios et al., 2008; Rahman et al., 2014). Thermal treatment is a conventional method to reduce fat content and sterilize the material and inactivate endogenous enzymes (Guérard et al., 2001). However, using organic solvents is often necessary to dry the sample previously, to obtain an efficient extraction (Hewavitharana et al., 2020). Besides this, Klompong et al., (2007) reported lower protein solubility after solvent fat extraction due to exposure of hydrophobic domains and protein aggregations, thus affecting hydrolysis efficiency and the properties of protein hydrolysates negatively (Villamil et al., 2017).

- Hydrolysis

Protein hydrolysis consists of the cleavage of peptide bonds to obtain free amino acids, oligopeptides (less than 20 amino acid residues), and polypeptides (more than 21 amino acid residues without 3-dimensional structure), consuming a molecule of water for each broken bond (Hou et al., 2017; Villamil et al., 2017). A simple mechanism associated with the enzyme-catalyzed formation of the product (no inhibition) is depicted in Figure 1.9. The mechanism of enzymatic hydrolysis by proteases can be supposed mainly in three consecutive stages: first, the formation of the enzyme-substrate complex between the peptide chain and the enzyme, second, the cleavage of the peptide bond between the carbon and the amino group of the peptide linkage to liberate one of the two peptides (hydrolysis), and third, a nucleophylic attack by a deprotonated water molecule on the remains of the complex to dissociate the second product from the active site and to regenerate the free enzyme. This is possible since the active site of an enzyme is highly

specific for certain substrates, allowing to discriminate between a substrate and a competing molecule (Kristinsson & Rasco, 2000; Nelson & Cox, 2013; Pagán et al., 2013).

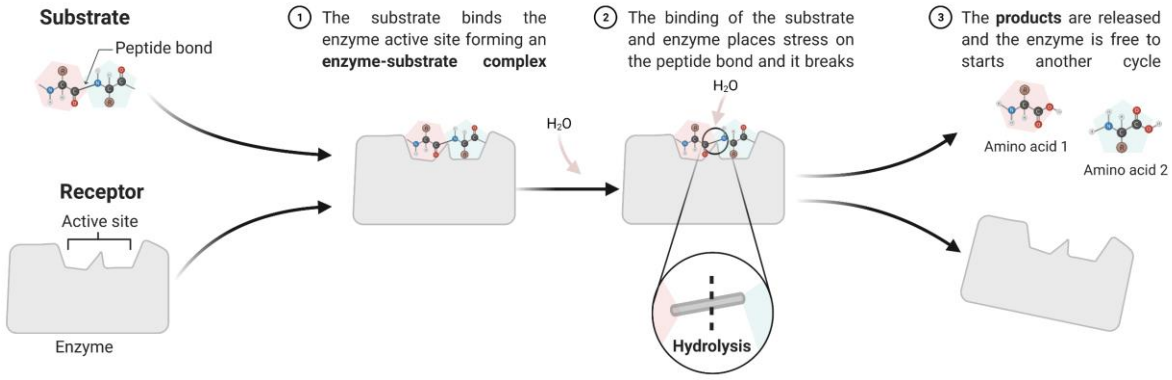

Figure 1.9. Enzymatic hydrolysis mechanism. The overall mechanism can be presented as: E+S↔ES↔EP→E+P; where E, S, and P represent the enzyme, substrate, and product; ES and EP are transient complexes of the enzyme with the substrate and with the product. Created with BioRender.com.

The determining factor for the generation of peptide sequences with the desired composition and properties is the specificity of the enzyme or protease. Proteases can be classified as exopeptidases or endopeptidases according to their mode of action. Exopeptidases act near the end of polypeptide chains while endopeptidases within the internal region of the protein molecule (Hou et al., 2017; Kristinsson & Rasco, 2000; Tavano, 2013). The most common enzymes for the synthesis of protein hydrolysates are digestive enzymes like pepsin, trypsin, and chymotrypsin and bacterial and fungal peptidases like alcalase, neutrase, and flavourzyme (da Silva, 2018). Although the use of endogenous enzymes (autolysis) is also an efficient strategy, reproducibility and controllability of the hydrolysis process are limited (Lapeña et al., 2018). The characteristics of the protein source to be hydrolyzed (the amino acid composition, the structure, the protein solubility, and the denaturation degree) will affect the choice of the enzyme and the subsequent products (Drummond et al., 2019; Pasupuleti & Braun, 2010). For instance, to obtain a hydrolysate from a protein source with a high content in hydrophobic amino acids, an enzyme that breaks down hydrophobic amino acids would be chosen (Hou et al., 2017; Pasupuleti & Braun, 2010). Besides making the correct choice, enzyme efficiency is highly dependent on reaction temperature (T), pH, and enzyme/substrate ratio (E/S ratio). Moreover, the achievement of an intended degree of hydrolysis and a determined molecular weight distribution of the peptides are based on the processing time (Drummond et al., 2019; Ozuna et al., 2015).

The hydrolysis starts when a determined amount of enzyme is added. Previously to enzyme addition, the temperature and the pH of the homogeneous mixture obtained from pre-treatment are adjusted according to the ideal working conditions of the enzyme. It is worth mentioning that these parameters

(E/S ratio, temperature, and pH) are generally selected to optimize the kinetics of the selected enzyme for a specific substrate (Kristinsson & Rasco, 2000). Moreover, constant stirring is necessary to ensure that the mixture is well mixed. Once the reaction between enzyme and substrate is initiated, changes in the pH of the solution are observed due to the release of protons in the media. Several methods have been proposed to deal with the above problem, like the addition of buffer solutions to moderate pH changes (Anzani et al., 2019, 2018; Bah et al., 2015; Cheong et al., 2018; Lapeña et al., 2018; Ryder et al., 2016; Zhang et al., 2013) or the maintenance of the optimal pH by the constant addition of neutralizing solutions such as sodium hydroxide (NaOH) and hydrochloric acid (HCl) (de Queiroz et al., 2017; Klompong et al., 2007; Kurozawa et al., 2008; Pagán et al., 2013; Vasileva-Tonkova et al., 2007; Webster et al., 1982; Zhang et al., 2017). However, the presence of buffer salts might affect the functional properties of the hydrolysates, such as foaming or emulsifying properties and sodium chloride (NaCl) can affect the hydrolysis rate, thus increasing reaction time (Kristinsson & Rasco, 2000). Finally, once the desired degree of hydrolysis is achieved, the termination of the enzymatic activity reaction can be done by changes in temperature, usually temperature ranging from 75 to 100 °C for 5 to 30 min (Lapeña et al., 2018) or either lower or raise the pH (Benítez et al., 2008; Kristinsson & Rasco, 2000).

The key parameter to determine the efficiency of a protease in hydrolyzing a protein source is the degree of hydrolysis (DH). DH is defined as the percentage of cleaved bonds out of the total amount of peptide bonds in the native protein, assuming that a DH of 100% represents that the protein is fully transformed into free amino acids (Hou et al., 2017; Lynch et al., 2018b; Nielsen et al., 2001; Villamil et al., 2017). There are several methods for the measurement of DH, UV spectrophotometric methods like *o*-phthaldialdehyde (OPA) or trinitrobenzenesulfonic acid (TNBS), electrochemicals like pH-stat, or formol titration, and gravimetric like trichloroacetic acid soluble nitrogen (SN-TCA). There is no consensus on the best method to determine the DH (Bah et al., 2015; Silvestre, 1997).

- Recovery

Protein hydrolysates downstream processing is essential, as dictates the end-use of the product. Generated hydrolysates need to be concentrated, isolated, and finally stabilized (Drummond et al., 2019). Commonly, once the inactivation is finished, the slurry is desludged by centrifugation, which results in several fractions: an oil layer in the top, a lipid-protein fraction between aqueous and oil layers, an aqueous layer, and the sludge in the bottom. Through this step, most of the lipids and non-soluble fractions are eliminated from the aqueous layer, although a second centrifugation step might be performed on the soluble fraction. If the raw material was defatted before enzymatic hydrolysis, centrifugation can be performed to separate the soluble fraction from the non-soluble one. Increasingly,

depending on the end-use of the hydrolysates, ultrafiltration with 10,000 or 50,000 Da cut-off membranes have been introduced. Using 10,000 Da cut-off membranes, endotoxins are removed, although endotoxins specifications may not be critical. On the other hand, chromatographic techniques have been employed on the laboratory scale for the separation of different peptides according to their molecular weight. However, there are difficulties for industrial scaling currently (Villamil et al., 2017). Finally, after filtration or chromatographic separation, the final soluble fraction is generally spray-dried or freeze-dried to reduce water content while converting the hydrolysate to a powder form or a concentrated hydrolysate. This final step also stabilizes the product, increasing the shelf life and providing greater ease in handling, transporting, and storage (Drummond et al., 2019; Kristinsson & Rasco, 2000; Pasupuleti & Braun, 2010; Villamil et al., 2017).

- Characterization

The DH is the parameter employed to follow reaction kinetics and measure the extent of the enzymatic hydrolysis. However, a partial characterization is achieved by DH evaluation, and other characteristics have to be studied to obtain better knowledge about the functional properties and the composition, these influencing protein hydrolysates performance (Silvestre, 1997).

The protein content is an important indicator of nutritional quality. Therefore, reliable quantitative methods to determine total protein content are essential (Moore et al., 2010). The general nitrogen-to-protein conversion factor (NPCF) of 6.25 (based on the assumptions that all the proteins contained 16% nitrogen and all the nitrogen was derived from protein) is extensively used to determine total (crude) protein from total N (Hall and Schönfeldt, 2013; Krul, 2019; Mariotti et al., 2008; Sriperm et al., 2011). However, it has been known for some time that it is not true that all proteins contain 16% N and all the N is from protein (Figure 1.10) (Krul, 2019; Lyman et al., 1956). The most recent NPCF values (*k*) for meat range from 5.38 to 5.74 (averaging 5.6) (Mariotti et al., 2008). Besides this, as Kjeldahl and Dumas methods do not distinguish protein-based nitrogen from non-protein nitrogen, total N can be increased by adding non-protein nitrogen-containing compounds, thus increasing apparent protein content (Moore et al., 2010). In consequence, this factor tends to inaccurately overestimates total protein. Several emerging methods (HPLC, electrophoresis and lab-on-a-chip, mass spectroscopy, etc.) with a high degree of selectivity have been proposed for protein quantification or authentication of food protein ingredients. However, these methods are not yet sufficiently developed or practical to replace current total nitrogen techniques used in routine protein measurements (Moore et al., 2010). Therefore, the accurate determination of nitrogen-to-protein conversion factors, depending on the protein source, is an acceptable alternative (Hall and Schönfeldt, 2013; Krul, 2019; Moore et al., 2010).

Mossé, (1990) reported the determination of several ingredient-specific NPCF, such as k_A, k_P, k_N, and k, while demonstrating that the utilization of an ingredient-specific factor is an appropriate approach (i.e., k_A is suitable for use in purified protein fractions).

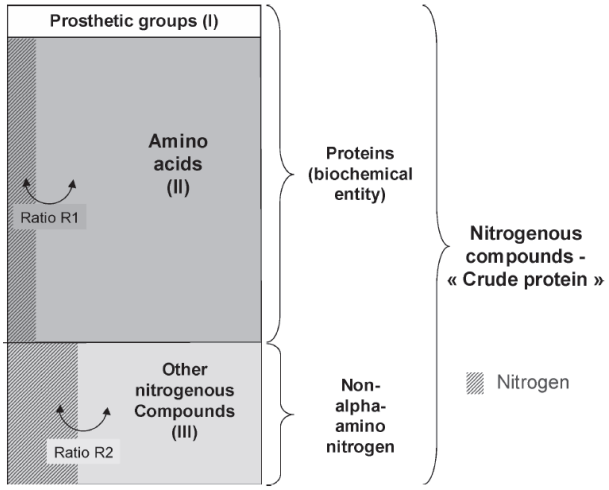

Figure 1.10. Nitrogenous material in foodstuffs consists in i) protein and free amino acids, the former being protein-bound amino acids (which contain the nitrogen) and non-nitrogenous groups in protein (the prosthetic groups) and ii) various nitrogenous compounds other than amino acids (Mariotti et al., 2008).

Nutritive value, taste properties, hydrodynamic properties, such as solubility, viscosity, gelling capacity, and water absorption, and surface-active properties such as emulsifying capacity, foaming, and film formation, are of interest if protein hydrolysates are used as an ingredient in food and animal feed products (Kristinsson & Rasco, 2000; Šližyte et al., 2005; Villamil et al., 2017). The protein value depends mainly on its capacity to satisfy the need for nitrogen and essential amino acids. In addition, the taste properties are considered to avoid the bitter and unpalatable taste given by peptides with high average hydrophobicity. Among functional properties, solubility is the most important one, affecting other functional properties such as emulsification and foaming, and it is an excellent indicator of protein hydrolysate potential applications. Thus, the functional properties, most of them correlated to a low DH, are relevant to the food industry because they affect the texture, the nutritional value, and the sensory properties of the final product (Chalamaiah et al., 2010; Fu et al., 2018; Šližyte et al., 2005; Villamil et al., 2017). However, if the final application of protein hydrolysates is as a nitrogen source in microbiological media, a higher DH is desired. Additionally, amino acid profile, free amino acids content, and molecular weight distribution are usually determined. Amino acid content is a major determinant of the functionality and value of the hydrolysates when used as fermentation ingredients (Lapeña et al., 2018).

3. Protein hydrolysates in biotechnology

Protein hydrolysates, also known as peptones, are the soluble product produced by partial or extensive hydrolysis and encompasses a complex mixture of oligopeptides, peptides, free amino acids, and other compounds, such as, minerals, carbohydrates, and lipids that varies depending on the raw material (Nasri, 2017; Pasupuleti et al., 2010). The main application of the protein hydrolysates is as nitrogen and nutritional source, and the functionality of the products obtained may be due to specific peptides, or the combination of peptides, or due to non-protein components in the product. The first application of protein hydrolysates dates back to 1880 when Nägeli recorded better bacteria performance in culture media containing partially digested meat proteins (Sandle, 2011). Nevertheless, culture media and applications of peptones have evolved over the years (Figure 1.11). Nowadays, protein hydrolysates are used in several areas of fermentation and biotechnology. Such areas include protein production in microbial cells, mammalian cells, and insect cells cultures, and production of vaccines, plant cell culture, and primary metabolites (Pasupuleti et al., 2010). It is worth noting that peptones are the most expensive ingredients of culture media, making up a large fraction of the total expenses of a biotechnological process (Callegaro et al., 2019). Therefore, to further develop bioeconomy, it is crucial the substitution of cost-intensive with low-cost nitrogen sources from cheap raw materials. The utilization of protein-rich residues and unexploited waste represents an alternative for developing low-cost protein hydrolysates and value-adding processes (Pleissner & Venus, 2016).

Figure 1.11. Evolution of culture media (Bonnet et al., 2019; Sandle, 2011; Siemensma et al., 2010).

3.1. Use of protein hydrolysate in growth media

A culture medium or a growth medium is a liquid, semi-solid, or solid designed to support the growth of a population of microorganisms, cells, or small plants. A significant factor in growing an organism successfully is the choice of the nutritional components and growth regulators (Gamborg & Phillips, 1995). All organisms require water, an energy source, a carbon source, a nitrogen source, some mineral elements, and possibly growth factors plus oxygen if aerobic (Bonnet et al., 2019; Stanbury et al., 2017). In microbiology, two types of media are used: defined medium and complex medium. A defined medium is composed of pure chemicals in precisely known proportions. A complex medium includes ingredients of natural origin, such as peptones, compositions of which are not entirely known (Zhang & Greasham, 1999). However, the specific nutritional requirements of a microorganism used are as complex and varied as the species and the strains and the process in question. For instance, only two amino acids are essential for *Leuconostoc mesenteroides*, glutamic acid and valine, while for *Lactobacillus brevis*, no less than 15 amino acids were necessary for its growth. Nowadays, supplementation is not usually performed with pure amino acids, but with protein hydrolysates (Bonnet et al., 2019).

Nitrogen is an essential element for the anabolic synthesis of nitrogen-containing cellular substances, such as amino acids, purines, DNA, RNA, and substances required for growth. The supply of nitrogen sources strongly influences growth and metabolism (Kampen, 2014; Zhang et al., 2020). Few organisms, such as algae and fungi, are able to use ammonium nitrate (NH_4NO_3) and sodium nitrate ($NaNO_3$) as nitrogen sources; however, yeast and bacteria may have some limitations utilizing nitrogen in this form. Complex organic nitrogen sources are the alternative, providing many other nutrients. Moreover, in many instances, growth will be faster and more efficient with a supply of organic nitrogen, considering that these compounds reduce the number of compounds that the cells would otherwise have to synthesize *de novo*. However, nitrogen availability must be considered in each case (Kampen, 2014; Stanbury et al., 2017).

Protein hydrolysates have demonstrated to be the preferred nitrogen source of a wide variety of microorganisms, such as *Escherichia coli* (*E. coli*), *Pichia pastoris* (*P. pastoris*), *Saccharomyces cerevisiae* (*S. cerevisiae*), *Aspergillus awamori* (*A. awamori*), etc. Their complexity may stimulate and improve the metabolic activity and growth of the organisms (Pleissner & Venus, 2016). Lysogeny broth (LB) and LB agar[1] are the most referenced bacterial media for the growth and maintenance of *E. coli* strains, the most popular bacterium for the production of recombinant proteins. These culture media include tryptone as

[1] LB broth contains 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl in distilled water. LB agar contains 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl and 20 g/L agar in distilled water.

a nitrogen source. It is worth mentioning that the growth performance can vary changing the nitrogen source.

Ranganathan et al., (2010) replaced tryptone in LB medium with six different protein hydrolysates, three from animal sources and three from plant sources, observing differences in growth curves of *E. coli* (Figure 1.12). It was observed that the peptones tested were equal or better in supporting growth. Whey hydrolysate followed by soy hydrolysate and wheat hydrolysate were better than tryptone while the rest were more or less identical.

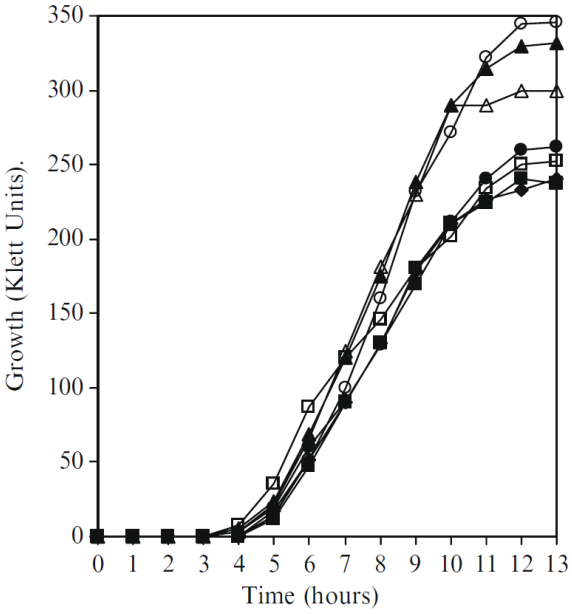

Figure 1.12. Growth curve of *E. coli* in various culture media. LB medium (◆) and media where tryptone was replaced by: enzymatic hydrolysate of casein (bovine) (■); Enhancetone – enzymatic digest of gelatin from porcine and yeast (□); soy protein – enzymatically hydrolyzed grits (▲); wheat protein - enzymatically hydrolyzed wheat gluten (△); pea protein - enzymatic hydrolysate(●); and enzymatic hydrolysate of whey protein and casein (bovine) (○).

Therefore, higher biomass concentrations can be obtained through the optimization of the medium, and protein hydrolysates from cheap materials have demonstrated their potential. However, one of the developments that has to take place is the partnership between protein hydrolysates manufacturers and the end-users, this enabling a better understanding of the capabilities of manufacturers and the requirements of the end-users (Pasupuleti et al., 2010).

3.2. Use of protein hydrolysates in fermentations

Fermentation technology is widely used to produce various economically important compounds, which have applications in the energy production, pharmaceutical, chemical, and food industries (Singh et al., 2017). As a result, fermentation specialists are constantly looking for a variety of ways to produce higher biomass concentrations (Ummadi & Curic-Bawden, 2010). There are four groups of commercially important fermentation: production of biomass or microbial cells as the intended products, production of microbial enzymes, production of primary and secondary metabolites, and biotransformation (Sitanggang et al., 2010). Although fermentation processes have been used for generations, the need for sustainable production of products, meeting the market requirements in a cost-effective manner has put forward a challenging demand (Singh et al., 2017).

Strains improvement by genetic engineering and the optimization of process parameters are the critical factors for successful fermentation. Modification of metabolic pathways is out of the scope of this study; therefore, the focus will be on the medium composition. The specific nutritional requirements of microorganisms used in fermentations are complex. A detailed investigation is needed to establish the most suitable conditions and appropriate nutritional compounds for an individual fermentation process in order to maximize the metabolite yields and/or productivity (Sitanggang et al., 2010). High productivity is a prerequisite for industrial production of any type of metabolite, this reducing the overall cost of the products, as well as the production costs (Singh et al., 2017). Therefore, a crucial aspect is to supply the fermentation medium with the right ingredients, especially carbon and nitrogen sources. The limitation of essential nutrients can restrict the growth of microbial cells and/or product formation (Singh et al., 2017).

Industrial fermentations are usually performed in semi-defined or complex media (Ummadi & Curic-Bawden, 2010). The carbon source is the most important medium component, followed by the nitrogen source. Different nitrogen sources will result in different yields and effects, depending on the strain and the process. Although protein hydrolysates can be added to the fermentation media to provide a broad spectrum of adequate nutrients for growth and viability, they are usually the most expensive ingredients in the fermentation media (Li et al., 2017; Ummadi & Curic-Bawden, 2010; Vázquez et al., 2020). Protein hydrolysates from various protein-rich materials and wastes have been used as an alternative to cost-intensive nitrogen sources in several biotechnological processes (Table 1.7), demonstrating their potential to increase productivity.

Table 1.7. Biotechnological production processes based on protein hydrolysates from various materials rich in protein. Adapted from Pleissner & Venus, (2016) with modifications.

Protein source	Carbon source	Strain	Product	Reference
0.088% (w/w) nitrogen supplied by whey protein hydrolysate[a]	50 g/L lactose supplied by whey protein permeate and hydrolysate	*Lactobacillus helveticus*	99.5% lactose converted into lactic acid, lactic acid yield 96%	(Fitzpatrick & O'Keeffe, 2001)
5.1 g/L nitrogen supplied by fish waste[b]	100 g/L glucose	*Lactobacillus rhamnosus*	79 g/L lactic acid	(Gao et al., 2006)
1% (w/w) excess sludge hydrolysate [c]	100 g/L glucose from tapioca starch	*Bacillus coagulans*	79 g/L lactic acid, productivity 2.8 g/(L h)	(Ma et al., 2014)
5 g/L meat and bone meal hydrolysate[d]	13 g/L glycerol	Recombinant *E. coli*	0.04 g/L cyanophycin	(Solaiman et al., 2011)
5 g/L meat and bone meal hydrolysate[e]	13 g/L glycerol	Recombinant *E. coli*	0.11 g/L cyanophycin	(Solaiman et al., 2011)
Food waste hydrolysate containing 0.28 g/L free amino nitrogen[f]	Food waste hydrolysate containing 31.9 g/L glucose[f]	Recombinant *E. coli*	29.9 g/L succinic acid	(Sun et al., 2014)
Food waste hydrolysate containing 0.28 g/L free amino nitrogen[f]	Food waste hydrolysate containing 31.9 g/L glucose[f]	*Actinobacillus succinogenes*	24.1 g/L succinic acid, 13.7 g/L by-products	(Sun et al., 2014)
Food waste hydrolysate containing 0.1 g/L free amino nitrogen[f]	Food waste hydrolysate containing 17.9 g/L glucose[f]	*Chlorella vulgaris*	1.1 g/L lipids containing among other 0.2 g/L α-linolenic acid	(Lau et al., 2014)
Food waste hydrolysate containing 1.9 g/L free amino nitrogen[f]	Food waste hydrolysate containing 106.9 g/L glucose[f]	*Chlorella pyrenoidosa*	9 g/L lipids containing among other 2.7 g/L α-linolenic acid	(Pleissner et al., 2015)
30 g/L spent yeast (autolyzed)	30 g/L spent yeast untreated and glycerol	*Cryptococcus curvatus*	19 g/L lipids for biodiesel production	(Ryu et al., 2013)
10 ml of chicken intestines auto-hydrolysate[g]	100 mL enzymatically hydrolyzed potato[h]	*Mucor circinelloides*	49 lipid content mg/g biomass	(Tzimorotas et al., 2018)
4 g/L chicken feather peptone[i]	150 g/L sugar beet molasses	*Aspergillus niger*	68.8 g/L citric acid, yield 48%	(Ozdal & Kurbanoglu, 2019)

[a] Obtained by enzymatic hydrolysis
[b] Pretreatment of fish waste at 121 °C for 20 min, afterward acid hydrolysis of residues at 121 °C for 20 min
[c] Excess sludge formed during the aerobic biological treatment process of municipal sewage hydrolyzed at 121 °C for 20 min
[d] Enzymatic hydrolysis was carried out using Alcalase 2.4 L for 4 h at pH 8.5 and 50 °C, followed by the addition of Flavourzyme and incubation for 4 h at pH 7 and 50 °C
[e] Alkaline hydrolysis carried out using 0.1 g $Ca(OH)_2$ at 85 °C for 16 h
[f] Fungal hydrolysis was carried out in submerged fermentation using extracellular enzymes secreted by *Aspergillus awamori* and *Aspergillus oryzae*
[g] Hydrolyzed at 60 °C for 3 h by autolytic endogenous enzymes activated using acetic acid buffer at pH 2.5
[h] Hydrolyzed using thermostable α-amylase from Bacillus licheniformis at 80 °C for 3 h, followed by hydrolysis using a glucoamylase at 40 °C for 24 h
[i] Obtained by chemical hydrolysis

3.2.1. Production of biomass

A large number of processes described in the literature deal with lactic acid bacteria (LAB). LAB have been used as starter cultures for fermenting food for a long time, even before the importance of microorganisms was recognized. A well-designed and control process of culture production is required to meet a high yield of vigorous biomass and specific metabolic activities. LAB specifies are so diverse, thus involving optimization of nutritional requirements for each culture/strain. Due to the limited capacity to synthesize amino acids, the availability of nitrogen sources is essential for the growth of LAB. Some LAB can secrete proteases and break proteins in peptides and amino acids, while those LAB that lack proteolytic systems rely on basic building blocks such as amino acids and ammonia. Moreover, it was observed that small peptides or oligopeptides are of superior nutritional value because of their ability to transport more than one amino acid into the cell at the expense of one adenosine triphosphate (ATP). Hence, considering the mixture of oligopeptides, small peptides, and amino acids present in protein hydrolysates, they represent a suitable nitrogen source for LAB and have been successfully used for fermentation (Ummadi & Curic-Bawden, 2010). A wide range of protein hydrolysates are available in the market; however, the production of new protein hydrolysates from cheap raw material will increase the options for formulating an optimal growth medium.

3.2.2. Production of primary and secondary metabolites

Increased demand for fuel ethanol as a sustainable energy source with the growing debate about the use of food sources has led to the development of various biotechnological processes to produce bio-based compounds from residues of low value, such as lignocellulose-based ethanol plants (Pleissner & Venus, 2016). Nevertheless, this process is still limited by several factors, such as price and performance of the enzymes, efficient fermentation of all sugars, pretreatment costs, and ability to handle lignocellulosic materials at high solid concentrations. One of the factors that have proven to be successful for improving fermentation performance in lignocellulosic materials is the adaptation of the fermenting microorganism. *S. cerevisiae* is used universally for the industrial production of fuel ethanol (Jørgensen, 2009). *S. cerevisiae* is able to use a wide variety of nitrogen sources, such as ammonium ion, urea, peptone, yeast extract, corn steep liquor, free amino acids, and others. However, not all the nitrogen sources support the growth and the ethanol yield equally well (Beltran et al., 2004; Li et al., 2017), being the assimilable nitrogen the most important component in the fermentation medium (Laopaiboon et al., 2009).

A comparative study on the performance of four often-used nitrogen sources (ammonium sulfate ($(NH_4)_2SO_4$), urea, peptone, and yeast extract) in a very high gravity (VHG) fermentation of corn starch by

S. cerevisiae was performed (Li et al., 2017). The authors observed that by adding $(NH_4)_2SO_4$ or urea as nitrogen sources, the maximum ethanol yields were 6 and 8% (v/v), respectively. Whereas adding complex nitrogen sources (peptone or yeast extract), the ethanol yield increased up to 18% (v/v) with peptone and 21% (v/v) with yeast extract. These results suggest that organic complex nitrogen sources might be more suitable for ethanol production by *S. cerevisiae* in VHG fermentations.

In another comparative study, Jørgensen, (2009) tested six different nutrients/nitrogen sources (magnesium sulfate ($MgSO_4$), $(NH_4)_2SO_4$, urea, corn steep liquor, yeast extract, and peptone), alone or in combination to improve fermentation of hydrolyzed wheat straw by *S. cerevisiae*. Significant variations were observed in the performance of the microorganism as a result of the nitrogen source. The addition of only inorganic nitrogen sources to the media did not improve the fermentation rate compared to the control. The fermentation was prolonged, and it was not completed within 73 h. However, when complex nitrogen sources were added, alone or in combination, a shorter lag phase and higher fermentation rate were observed. After 25 h, the fermentation was almost completed.

These results are in agreement with other studies, where the presence of free amino acids in the media can substantially reduce fermentation time by alleviating stress or lack of nutrients (Arrizon & Gschaedler, 2002; Jones & Ingledew, 1994; Pereira et al., 2010; Wang et al., 2007) and reduce cultivation time (Albers et al., 1996). The preference of *S. cerevisiae* for a given nitrogen source can be explained by a mechanism called Nitrogen Catabolite Repression (NCR).

3.2.3. Enzyme production

Enzymes have been extensively used in food, textiles, paper industries, fermentations, renewable energy, chemicals, etc. (Sitanggang et al., 2010). Microorganisms are the most important source for enzyme production, with proteases accounting for about 60% of the total industry enzymes (Hajji et al., 2008). *Aspergillus* species are frequently employed in biotechnological processes for enzyme production. However, the carbon and nitrogen source play an important role in the enzyme properties (Maiorano et al., 2008).

A comparative study on alkaline protease production by *Aspergillus clavatus* ES1 in culture media containing different nitrogen sources (casein peptone, urea, casein, $NaNO_3$, yeast extract, *Sardinella* heads and viscera flour (SHVF), and $(NH_4)_2SO_4$)) was performed (Hajji et al., 2008). The addition of organic nitrogen sources, such as peptones and yeast extract, resulted in higher protease production levels, whereas the presence of inorganic nitrogen sources showed weak production levels. Similar results were observed in the biotechnological production of fructosyltransferase (FTase) by fungi and bacteria.

Complex nitrogen sources enhanced enzyme production, while inorganic nitrogen sources can limit enzyme production (Maiorano et al., 2008).

Microorganisms such as fungi *Aspergillus fumigatus* and *Aspergillus flavus* and bacteria *Bacillus* sp., *Streptomyces* sp., and *Amycolatopsis keratiniphila* have been cultured in keratinous substrates (hair, feather, wool), observing secretion of keratinases (Callegaro et al., 2019; Falco et al., 2019). These microorganisms secrete keratinolytic enzymes in order to make the organic nitrogen source available. This biotechnological approach dies not only result in the production of enzymes but also simultaneously keratin protein hydrolysates are produced to be used in other applications, such as nitrogen source (Pleissner & Venus, 2016).

3.2.4. Biotransformation

Biotransformation can be defined as the enzyme-catalyzed conversion of one chemical into another that may be more or less toxic to produce goods of social and/or economic value. The most potent markets that fueled biotransformations are fine chemicals and pharmaceuticals. Over the last 50 years, fungi use for the production of commercial products has increased rapidly (Pera et al., 2008). Fungi represent one of the most diverse groups of microorganisms, playing key roles in nature. These microorganisms possess several strategies to counteract a myriad of toxic compounds, including bioadsorption, hydrophobin production, metal interaction, extracellular enzymatic system, and intracellular enzymatic system. Through these systems other products or by-products are produced, representing an important tool for various purposes (Olicón-Hernández et al., 2017). A direct relation was observed between morphology and fungal product formation. Therefore, factors affecting morphology, such as the carbon source, type and levels of nitrogen source, trace minerals, pH, temperature, etc., and physical factors can be modulated and optimized to achieve maximum performance (Papagianni, 2004; Rodríguez Porcel et al., 2005).

Considering that media components play an essential role in controlling fungal morphology, yield, and product formation (Papagianni, 2004), optimizing carbon and nitrogen sources can be an effective method to achieve high productivity. One-factor-at-a-time (OFAT) is a common experimental method to study the effect of one variable or factor at a time while keeping all the other variables as constants (Singh et al., 2017; Zhang et al., 2016). For instance, this approach has been applied by Prasad et al., (2011) in the biotransformation of meloxicam into new metabolites (e.g. 5'-hydroxymethyl meloxicam, 5'-carboxy meloxicam) by a filamentous fungus, *Cunninghamella blakesleeana*. The influence of carbon and nitrogen sources was studied, replacing glucose with other carbon sources and peptone by thiourea or inorganic nitrogen sources. The commonly used carbon source, glucose, favored the transformation of most of the

meloxicam added. On the other hand, the inorganic nitrogen source, NH_4NO_3 was the one favoring the major transformation of meloxicam. Moreover, nitrogen sources significantly influenced the transformation of meloxicam, observing significant differences among the metabolites produced, thus indicating the importance of media optimization.

A wide variety of products can be biotransformed, such as 5-hydroxymethylfurfural (HMF) by *Scheffersomyces stipitis* (Ra et al., 2013) and *Pseudomonas aeruginosa* (Pan et al., 2020); amoxapine, mirtazapine, and methdilazine by *Cunninghamella elegans* (Moody et al., 2002; Shanmugam et al., 2003; Zhang et al., 1996); naproxen by *Trametes vesicolor* (Marco-Urrea et al., 2010); aflatoxin B_1 by *Lactobacillus helviticus* (Zhang et al., 2021); etc. Protein hydrolysates are the most common nitrogen source used in culture media for biotransformations.

3.3. Use of protein hydrolysates in plant culture

The availability of mineral nutrients in the soil represents one of the most critical limiting factors for crop productivity, being nitrogen the main limiting nutrient for plant growth (Callegaro et al., 2019; Santi et al., 2017). Commercial fertilizers have been used for years to ensure good production and avoid low fertility of soils (da Silva, 2018). The burial of animal by-products and other waste also played an important role as fertilizer to ensure good harvests. Nonetheless, nowadays, the burial and the frequent use of chemical agents are discouraged to avoid severe environmental problems, such as soil and water contamination (da Silva, 2018; Gooding & Meeker, 2016). Therefore, efficient N capture and utilization and maximization of crop productivity is worldwide an important goal (Santi et al., 2017). Recently, a promising tool known as biostimulants has been introduced to improve the sustainability of production systems for horticultural crops (Colla et al., 2015). Biostimulants can be defined as “materials which contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to benefit nutrient uptake, nutrient use efficiency, tolerance to abiotic stress, and/or crop quality, independently of its nutrient content” (Calvo et al., 2014; du Jardin, 2015; Santi et al., 2017). Biostimulants are generally classified into three major groups, including humic substances (HS), hormone containing products (HCP), and amino acid containing products (AACP) (du Jardin, 2015).

Protein hydrolysates represent an emerging class of crop management products and have gained attention as natural plant biostimulants in vegetable cropping systems (Colla et al., 2017; da Silva, 2018; Santi et al., 2017; Sestili et al., 2018). Currently, more than 90% of the protein hydrolysates available in the horticulture market are produced through chemical hydrolysis of animal protein-rich residues; however, plant and biomass of dedicated legume crops are also considered sources of protein

hydrolysates. Plant-derived protein hydrolysates are mainly produced enzymatically, but they are less common because they have been recently introduced to the biostimulants market (Colla et al., 2015; Sestili et al., 2018). Application of protein hydrolysates on leaves or roots may activate several molecular and physiological mechanisms and have been reported to improve the performance of a wide range of horticultural and agronomic crops (Callegaro et al., 2019; Colla et al., 2017, 2015; da Silva, 2018; Sestili et al., 2018).

For instance, Santi et al., (2017) assessed the effect of animal-based protein hydrolysates on maize root, comparing an equal amount of N supplied by free amino acids mixture mimicking the protein hydrolysate composition or an inorganic nitrogen source, ammonium dihydrogen phosphate ($NH_4H_2PO_4$). Significant differences were observed among the tested nitrogen source. Protein hydrolysates were more efficient in stimulating root growth and micronutrient accumulation, followed by free amino acids and inorganic N, suggesting a specific role for small peptides in controlling root growth. Moreover, genome-wide transcriptional analysis shown changes in the expression of a high number of genes, allowing to hypothesize the role of peptides present in the protein hydrolysate.

Another comparative study was performed by Sestili et al., (2018), where tomato (*Solanum lycopersicum* L. cv. Console F1) received six different treatments. A plant-based protein hydrolysate was used as foliar spray or substrate drench at two N levels of concentration. The results showed that the application of protein hydrolysis induces higher growth, root length, and dry weight compared to the control. Moreover, tomato performance parameters and N content were enhanced when protein hydrolysates were applied as a substrate drench. It seems that application of the protein hydrolysates may have generated a signal transduction pathway through modulation of endogenous phytohormone biosynthesis, deriving to the stimulation of plant growth and yield. On the other hand, the increase in plant biomass was associated with the stimulation of the root architecture, thus inducing “nutrient acquisition mode” that favors N use efficiency. Besides this, protein hydrolysates differentially regulated in a N-dependent manner the expression of genes involved in nitrate (NO_3^-), ammonium (NH_4^+), and amino acid transporters as well as the key genes involved in N metabolism.

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Hypothesis and objectives

The hypothesis underlying the current research was that *fines*, *a priori* valueless meat by-product generated during the processing of animal by-products (ABPs) into pet food, contain valuable resources with a strong economic potential if treated in the correct manner. Currently, these by-products are discarded as waste or under-utilized; and in some cases, incur considerable cost to processors.

The main objective of this Doctoral Thesis was the recovery of fat and protein fractions from *fines* through the application of mild conditions and their processing into non-edible products beyond their usual profitability. To address the main goal, the following specific objectives were proposed:

I. To determine the amount of valuable compounds to be extracted from the *fines* (Chapter 2).
II. To ascertain the optimal fat extraction methodology to promote a cleaner alternative based on the Eco-Scale approach and improve fat consistency (Chapter 2).
    - Evaluation of fat extraction efficiency of several techniques.
    - Evaluation of fat extraction methodology by assigning penalty points (Eco-Scale approach).
    - Determination of optimal conditions to obtain a semi-solid fat to be used as a precursor in animal feed.
III. To optimize a methodology for the enzymatic hydrolysis of the protein fraction from *fines* and perform the characterization of the protein hydrolysates (Chapter 3).
    - Determination of the optimal hydrolysis conditions with two proteases, Alcalase 2.4L and Neutrase 0.8L.
    - Recovery of the protein hydrolysates to be used for further applications.
    - Characterization of the hydrolysates measuring the nitrogen content, amino acid composition, molecular weight distribution, volatiles, and changes in functional groups.
    - Scale-up of the hydrolysis process.
IV. To prove the feasibility of using the produced protein hydrolysates as substitutes of commercial protein hydrolysates in microbiological growth media or other nitrogen sources.
    - Use of protein hydrolysates in Luria-Bertani medium for bacteria growth (Chapter 4).
    - Determine the suitability of the protein hydrolysates in yeast growth and fermentation (Chapter 5).
    - Study of the effect of protein hydrolysates on the capability of different fungi strains to biotransform 5-hydroxymethylfurfural (Chapter 6).
    - Assess the effect of the protein hydrolysates in germination and *in vitro* culture of tomato and radish plants (Chapter 7).

Chapter 2. Extraction of lipidic fraction from *fines* and methodologies comparison based on the Eco-Scale approach

1. Introduction

Animal protein consumption has increased worldwide (Lynch et al., 2018). This surging demand has contributed to generate larger volumes of animal by-products (ABPs) (Toldrá et al., 2016). In the European Union (EU), about 18 million metric tons of ABPs are produced annually (Drummond et al., 2019; Mora et al., 2019). The amount of ABPs considered inedible to humans ranges between 25 and 42% of the total weight of animals (EFPRA, 2016).

Rendering is the most common, well environmentally regulated, safest, and suitable method for the collection and profitable utilization of meat by-products (Pandey et al., 2020). The rendering process is relatively complex, but the two major steps are i) mechanical fragmentation and ii) heat/pressure treatment (Pandey et al., 2020). The primary products resulting from meat rendering plants are protein-rich solids called meal and animal fats (Meeker, 2006). However, in the rendering process, other intermediate by-products are generated, such as solids that bypass screw presses and solid impurities discarded with fat, also known as *fines*. These solids can be recovered using settle tanks or centrifuges and recycled back to the rendering system under certain circumstances. Depending on several factors, such as the type of raw material, preservation, the geographic location of the plant, changes in climatic conditions, etc., high amounts are produced. In order to meet the quality of the finished products, only a part can be recycled, thus generating a waste that must be managed. The method to handle *fines* is of the choice of the renderer, but it will carry a negative value (costs money to dispose of). Other solutions should be considered for both economic and environmental reasons when by-products or wastes cannot be avoided (Meeker, 2006; Prokop, 1985). Apart from recycling, no applications have been described for this surplus of *fines* to the best of our knowledge.

Rendered animal proteins and fats derived from category 3 are attractive ingredients for pet food, animal nutrition, and aquaculture (Mora et al., 2019; Partridge et al., 1986). In fact, in the EU in 2016 about 1.9 million metric tons of food-grade proteins and about 1 million metric tons of fats were used in pet foods (Mora et al., 2019). On the one hand, protein serves as a concentrated source of protein and amino acids, and, on the other hand, fats are the most caloric-density feed ingredient available, and it has been used for years to increase the energy density of animal's diet without increasing the dry matter intake (DMI). Depending on the species being fed, the energy contributions of fat range from 2.6 to 3.8 times the energy content of corn (Meeker, 2006). In addition, later on, it was demonstrated that the supplementation of fats in the form of soaps had other positive effects, as the improvement of the digestibility of dry matter (DM) and organic matter (Behan et al., 2019), higher conception rate, increased pregnancy rate, etc. (Tyagi et al., 2010).

Despite being a well-known and common procedure, the production of rendered fats and their integration in animal feed sometimes need adjustments. The physical characteristics of these fats sometimes do not meet the standards in terms of firmness; they can be unsatisfactory soft, causing problems during the fabrication of final products. These lipids can be summited to different treatments to improve consistency, to obtain solid or semi-solid fats (Salimon et al., 2011). Among them, the mixing of natural fats or oils with alkalis has been proposed as a good method to obtain saponified fats, and at the same time, a firmer product (Handojo et al., 2019; Proaño et al., 2015).

Considering the origin of *fines*, the extraction of the lipidic fraction is of interest. As lipids are present in tissues in different physical forms, there are several ways to extract lipids depending on sample characteristics, being organic solvents widely used due to their high efficiency (Amusan, 2008; Azadmard-Damirchi et al., 2010; Señoráns & Luna, 2012; Shin et al., 2013). However, there is little scientific information about the most convenient method for lipid extraction in each type of meat product or by-product (Pérez-Palacios et al., 2008). Besides this, due to environmental, public health, and economic concerns, organic solvents should be avoided and alternatives must be considered (Hewavitharana et al., 2020; Jiao et al., 2014; Long et al., 2011; Servaes et al., 2015). Aqueous-based solvent systems have been explored in the last years to replace traditional organic solvent, although low extraction yields have been reported. This fact can be overcome by aqueous enzymatic extraction, which usually results in greater yields (Gai et al., 2013; Jiao et al., 2014; Rosenthal et al., 1996, 2001; Tzompa-Sosa et al., 2014). With the introduction of the 12 Principles of Green Chemistry, much effort has already been made to replace or reduce toxic and hazardous substances and research is aiming at cleaner processes (Poliakoff et al., 2002; Reinhardt et al., 2008). However, quantitative tools are required to evaluate the greenness of a process. In the last years, several green metrics have been proposed to make possible comparisons and to select the greenest alternative, such as Eco-Scale (Armenta et al., 2017; Gałuszka et al., 2012; Saleh et al., 2020; Van Aken et al., 2006).

Given the above considerations, this study aimed to find new ways to revalorize *fines*. The lack of information led us to conduct our research on their chemical characterization. This present study takes advantage of the composition of *fines* to extract the fat, being this one of the products with a wide range of applications and highly demanded as a precursor in animal feed. To this end, different extraction methods were tested and evaluated, and the Eco-Scale approach was applied to define the greenest technique. Finally, the extracted fat was summited to several assays to improve its consistency.

2. Materials and methods

2.1. Materials and reagents

The *fines* were kindly provided by the local company Subcarn Echevarria S.L. (Cervera, Lleida, Spain). The sample was sent in plastic bags and kept at 4 °C until further use.

The enzymes used were purchased from Sigma-Aldrich (St. Louis MO, USA). Alcalase® 2.4L (EC 3.4.21.62) is an endo-protease from *Bacillus licheniformis* of the serine type with an enzymatic activity of 2.58 AU/g. The working conditions range between 50 and 70 °C and between 6 and 9.5 of pH. Neutrase® 0.8L (EC 3.4.24.28) is also an endo-protease from *Bacillus amyloliquefaciens* with an enzymatic activity of 0.95 AU/g. The working conditions range between 45 and 55 °C and between 5.5 and 8 of pH. Protamex® (3.4.21.14) is a protease from *Bacillus sp.* with an enzymatic activity of 1.5 AU/g at pH values between 5.0 and 11.0 and the temperature near 60 °C. Flavourzyme® (3.4.11.1) is a fungal protease/peptidase complex from *Aspergillus oryzae* with both endoprotease and exopeptidase activities of ≥ 500 U/g. The working conditions range between 30 and 65 °C and pH values between 4 and 8.

Methanol (CH_3OH), chloroform ($CHCl_3$), cyclopenthyl methyl ether (CPME), and sulfuric acid (H_2SO_4) were from Thermo Fisher Scientific (Waltham, MA, USA). *n*-Hexane was from Honeywell (Charlotte, EU). Sodium chloride (NaCl), calcium oxide (CaO), and 2,6-di-*t*-butyl-4-methylphenol (BHT) were from Sigma-Aldrich (St. Louis, MO, USA). Calcium chloride ($CaCl_2$) and anhydrous sodium sulfate (Na_2SO_4) were from Panreac (Barcelona, Spain). Fatty acid methyl esters (FAMEs) reference standard was from Supelco (Sigma-Aldrich, Madrid, Spain). Triundecanoin was from Fluka (Sigma-Aldrich). Sodium hydrogen carbonate ($NaHCO_3$) was from Prolabo (Barcelona, Spain).

2.2. Sample characterization

An initial characterization *of fines* was made in terms of moisture, lipids, ash, and protein (N x 6.25) following standard methods (AOAC, 2000). The moisture content was measured by gravimetric heating in an oven-drying at 95 – 100 °C until constant weight (934.01). The ash content was determined by gravimetric heating of the samples in a muffle furnace at 550 °C for 6 h using quartz crucibles (942.05). The total nitrogen (N) was analyzed by Dumas method. Lipid content was obtained by Soxhlet extraction with *n*-hexane. Unless stated, the measurements were performed in triplicate.

2.3. General procedures for fat extraction

The fat from fines was extracted from the dried *fines* using different extraction methods (Figure 2.1). *Fines* were dried at 40 °C for 48 h in an oven with forced air circulation (JP Selecta, Barcelona, Spain).

a) Automated Soxhlet extraction

In a typical experiment, a conventional *n*-hexane Soxhlet extraction was performed with 1 g of dried sample. Dried *fines* were placed in a cellulose extraction thimble (25 mmx80 mm, i.d.; Scharlab, Barcelona, Spain) containing cotton wool. The thimbles were fitted with the metal adaptors, which were then loaded into an automated Soxhlet extractor system (JP Selecta Det-Gas, Barcelona, Spain). Beakers, previously dried at 105 °C and cooled down, were filled with 50 mL of *n*-hexane and placed in the extraction system under each extraction thimble. The extraction was performed in three steps: I) immersion of the sample in *n*-hexane for 60 minutes, II) rinsing of the sample with *n*-hexane for 2 h, and III) collection of the sample in the beakers and recovery of the clean *n*-hexane. After the extraction, the oil fraction was dried in a drying oven at 105 °C to a constant mass and was cooled in a desiccator. The total lipid content was determined gravimetrically. Being Soxhlet extraction a conventional method, the value determined was set at 100% extraction yield and was used to compare performances with the other methods. The recovered crude fat material was derivatized and analyzed. The remaining oil was stored at 4 °C.

b) Folch extraction

In a typical experiment, the fat extraction was carried out according to the method reported by Folch et al. (Folch et al., 1957) with slight modifications. A known amount of dried sample (2 g) was mixed with 20 mL of $CHCl_3$/CH_3OH (2:1, v/v). Due to the toughness of the tissue, 30 min of incubation were required at room temperature (RT) in a rotatory shaker at 60 rpm. Then, the mixture was centrifuged and filtered under vacuum through paper filters using a Büchner funnel. The remaining solid was re-homogenized twice with 5 mL of the $CHCl_3$:CH_3OH mixture, centrifuged, filtered, and collected together. The crude extract containing the fat was mixed thoroughly with 5 mL of 1% NaCl solution. The two phases were separated by centrifugation at 5000 rpm for 5 min. Finally, the organic phase was collected and evaporated under nitrogen steam or using a rotary evaporator. The extracted crude fat was determined gravimetrically, then derivatized and analyzed. The remaining extracted oil sample was stored at 4 °C.

c) Fat extraction by a green solvent – CPME

In a typical experiment, 2 g of a dried sample were extracted with 20 mL of CPME. The mixture was placed in a rotatory shaker at 60 rpm for 30 min at 25 °C or 60 °C. After the extraction, the samples were

centrifuged for 5 min at 5000 rpm and the solvent was separated. To determine the total fat extracted, the solvent was removed using a rotary evaporator. The fat was determined gravimetrically. The remaining extracted fat was stored at 4 °C.

d) Aqueous extraction at high temperature

In a typical experiment, 20 g of starting material were mixed with 30 mL of tap water or tap water containing 0.033 g $CaCl_2$/mL. The mixture was heated at 80 °C and 180 rpm in an orbital shaker (211DS Labnet International) for 4 h. Then, the samples were cooled down and centrifuged for 15 min at 5000 rpm at RT. Three fractions were observed: a sludge, a gelatinous phase, and an oily phase. Subsequently, the oil released was withdrawn using a Pasteur micropipette and transferred to a 15 mL falcon® tub containing hot water. After 15 min of mixing and centrifugation, the crude fat was recovered. The fat was determined gravimetrically, and the fatty acid profile was determined. The remaining extracted fat was stored at 4 °C.

e) Aqueous enzymatic extraction (AEE)

In a typical experiment, 20 g of sample and 30 mL of distilled water were added into the reaction flask (50 mL). The mixture was stirred and previous to enzyme addition, pH and temperature were adjusted to the optimum values (recommended by the manufacturers), as indicated in Table 2.1. Once the optimum temperature was reached, a known amount of fresh enzyme was added (1 or 5%). The mixture was incubated in a shaking incubator at 180 rpm for 2 h. Then, the enzymes were inactivated in a water bath at 95 °C for 10 min. The samples were centrifuged at 5000 rpm for 15 min and three fractions were observed: a sludge, an aqueous phase, and an oily layer. Subsequently, the oil released was withdrawn using a Pasteur micropipette and determined gravimetrically.

Table 2.1. Conditions during enzymatic hydrolysis.

Commercial enzyme	Working conditions		Protease activity (AU/g)[a]
	pH	Temperature (°C)	
Alcalase 2.4L	8	55	2.4
Neutrase 0.8	6.5	40	0.8
Protamex	7	55	1.5
Flavourzyme	6	55	500[b]

[a]AU: One Anson unit is defined as the amount of enzyme which, under specified conditions, digests urea-denatured hemoglobin at an initial rate such that there is liberated an amount of TCA-soluble product per minute which gives the same color with Folin-Ciocalteu Phenol reagent as one milliequivalent of tyrosine at 25 °C at pH 7.5

[b]U: One unit is the amount of enzyme which hydrolyzes 1 mmol of L-leucine-p-nitroanilide per minute

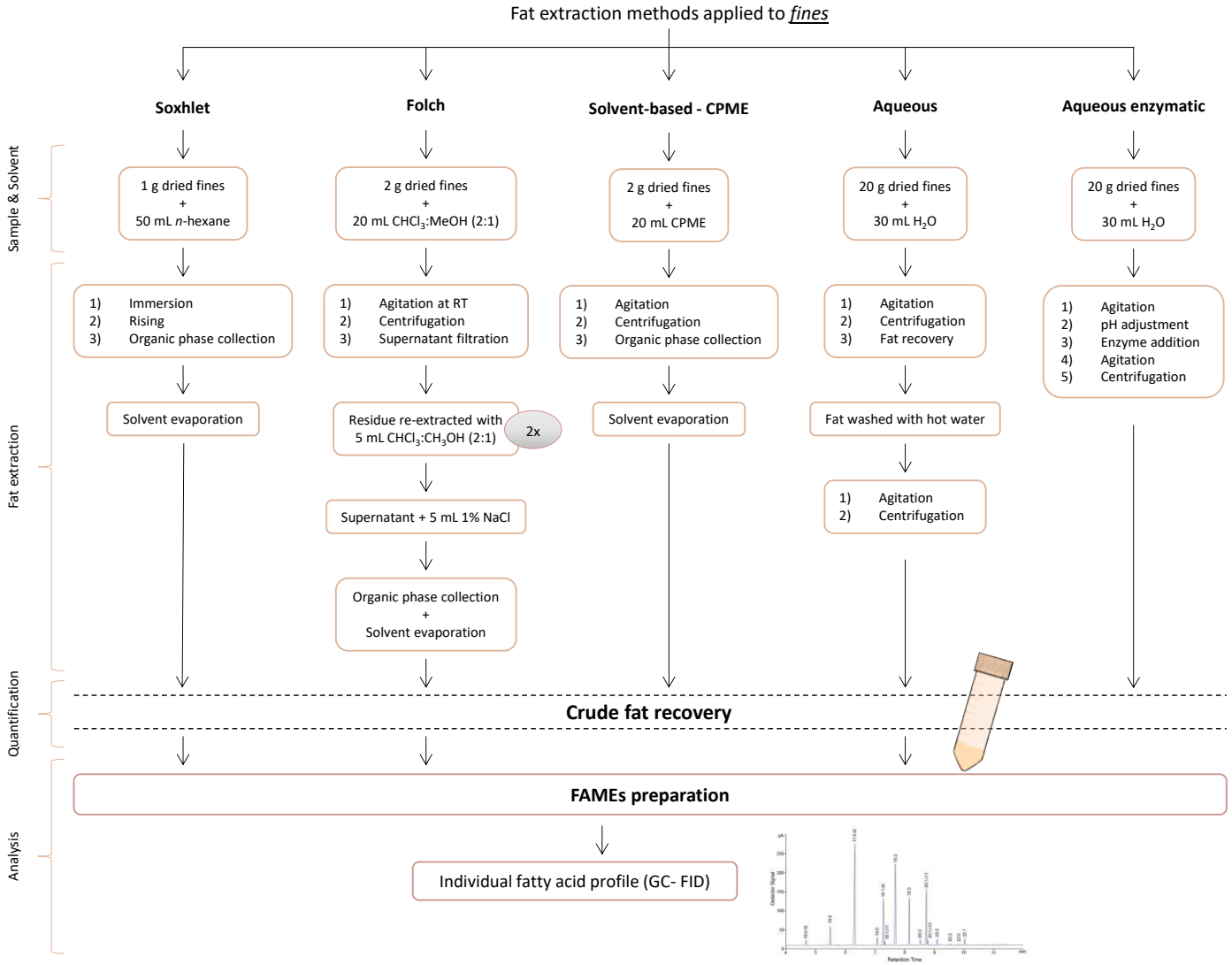

Figure 2.1. Experimental procedure of the fat extraction methods and FAMEs analysis.

2.3.1. Fat recovery

The efficiency of the extraction method was expressed as extraction yield and was calculated as the percentage of the ratio between the amount of fat extracted with the method assayed and the amount of fat extracted using the Soxhlet method, used as reference method (Equation 2.1).

$$Yield\ (\%) = \frac{weight\ of\ extracted\ fat\ (g)}{weight\ of\ theoretical\ total\ fat\ in\ the\ sample\ (g)}\ x\ 100$$

Equation 2.1

2.4. Fatty acid profile analysis

2.4.1. Preparation of fatty acid methyl esters (FAMEs)

Between 5 – 10 mg of fat were added into a 10 mL reaction flask with a polytetrafluoroethylene (PTFE) cap. A magnetic stirrer, 10 mg of BHT, 100 µL of a *n*-hexane solution containing the glyceryl triundecanoate (IS) at 40 mg/mL, and 2 mL of 2% H_2SO_4 in CH_3OH (v/v) were added. The mixture was stirred at RT for 10 min. Then, the flask was placed in a heating block at 80 °C for 30 min 800 rpm. After, the mixture was brought to RT and treated with 2 mL of *n*-hexane, vigorously shacked for 60 seconds, and 2 mL of a saturated NaCl solution, and again shacked for 60 seconds. Then, between 0.5 and 0.7 mL of a saturated solution of $NaHCO_3$ (until the neutralization of the acid was achieved) were added and the mixture was shacked for 60 seconds. The sample was centrifuged, and the organic phase was separated. The extraction was repeated twice. The organic phase recovered was dried with anhydrous Na_2SO_4, and then the FAMEs analyzed by GC-FID.

2.4.2. Gas chromatography (GC) analysis

Gas chromatography – flame ionization detector (GC-FID) was used to determine the fatty acid composition of the animal fat extracted with the different methodologies. The fatty acid profile was analyzed after conversion into their FAMEs. GC–FID analysis was performed in an Agilent 7890A (Palo Alto, CA, USA) gas chromatograph coupled to an Agilent 5973N flame ionization detector equipped with a DB-23 capillary column (30 m × 0.25 mm i.d.; film thickness 0.25 µm) (Agilent J&W). The oven temperature was programmed from 150 °C (held for 1 min) to 180 °C at 25 °C/min and finally increased to 230 °C at 10 °C/min (held for 5 min). The samples were injected with a split ratio of 1:20 and an injection volume of 1 µL. Hydrogen was used as the carrier gas and the flow was 1 mL/min. The acquisition was done using Agilent ChemStation software (Agilent Technologies, Palo Alto, CA, USA).

The fat components were identified based on the comparison of their retention time with a FAMEs mix standard containing 37 compounds (CRM47885). Quantification of the identified fatty acids was performed based on the response factors (R_i).

2.4.3. Response factor (R_i) calculation

The response factor (R_i) for each fatty acid identified was determined following standard methods (AOAC 996.06, 1998). The R_i for each fatty acid was calculated as follows (Equation 2.2):

$$R_i = \frac{Ps_i}{Ps_{C11:0}} \, x \, \frac{W_{C11:0}}{W_i}$$

Equation 2.2

where Ps_i = peak area of individual fatty acid in mixed FAMEs standard solution; $Ps_{C11:0}$ = peak area of C11:0 fatty acid in mixed FAMEs standard solution; $W_{C11:0}$ = weight of internal standard in mixed FAMEs standard solution; and W_i = weight of individual FAME in mixed FAMEs standard solution.

The percentage of saturated (Equation 2.3) and unsaturated (Equation 2.4) fatty acids in the samples were calculated as follows:

$$\%\, saturated\ fat = \frac{\sum saturated\ W_i}{W_{sample}} \, x\ 100$$

Equation 2.3

$$\%\, unsaturated\ fat = \frac{\sum unsaturated\ W_i}{W_{sample}} \, x\ 100$$

Equation 2.4

2.5. Environmental metrics – Eco-Scale

The analytical Eco-Scale tool described by Gałuszka et al., (2012) with slight modifications was used to compare and assess the environmental feasibility of the tested extraction methods. It can act as a potential tool to correlate several production methods of a single product with respect to environmental friendliness (Katakojwala and Mohan, 2020). Determination of the greenest process was made by assigning penalty points to six crucial parameters: price of the components, amount, safety, energy use,

occupational hazard, and waste generated. The penalty points were cumulative for all components of the preparation. Parameters included and their corresponding penalty points are shown in Table 2.2.

Table 2.2. The penalty point to calculate the Eco-Scale.

Parameter	Conditions	Penalty points (PP)	
	Reagents		
Price of the extraction (per sample)	Inexpensive (< 1€)	1	
	Expensive (> 1€ and < 5€)	3	
	Very expensive (> 5€)	5	
Amount (per sample)	< 10 mL	1	Amount PP x Hazard PP
	> 10 mL and < 100 mL	2	
	> 100 mL	3	
Safety (Hazard)[a]	None	0	
	Less severe hazard	1	
	More severe hazard	2	
	Instruments		
Energy use (per sample)	≤ 0.1 kWh	1	
	≤ 1.5 kWh	2	
	> 1.5 kWh	3	
Occupational hazard	Process hermetization	0	
	Emissions of vapors and gases to the air	3	
	Waste		
Waste (per sample)	None	0	
	< 1 mL	1	
	> 1 mL< and < 10 mL	3	
	> 10 mL	5	

[a] PPs were assigned to each of the hazard categories posed by a reagent. Each reagent can have more than one hazard category, thus the sub-total PP value for a single reagent may be greater than 2

Penalty points for the price of components were assigned according to the values proposed by Van Aken et al., (2006) with slight modifications. The extraction price was calculated by summing up the solvent price and the rest of materials required per sample. Safety Data Sheets (SDS) were consulted to determine the hazard of solvents and reagents. Penalty points for energy consumption were assigned according to the values proposed by Raynie & Driver, (2009). The recovered solvent was considered as waste.

The Eco-Scale represents a scale from 0 to 100, wherein 100 corresponds to an ideal process. Finally, the Eco-Scale score for a particular process was calculated by deducing the sum of all the penalty points from 100 (Equation 2.5).

$$\text{Eco-Scale} = 100 - \sum \text{penalty points}$$

Equation 2.5

Moreover, the Eco-Scale values can be categorized as an excellent green process if > 75, acceptable green process if < 75 and > 50, and inadequate green process if < 50.

2.6. General procedure to improve fat consistency

The fat extracted from *fines* was summited to thermal and chemical processes to improve consistency (Figure 2.2).

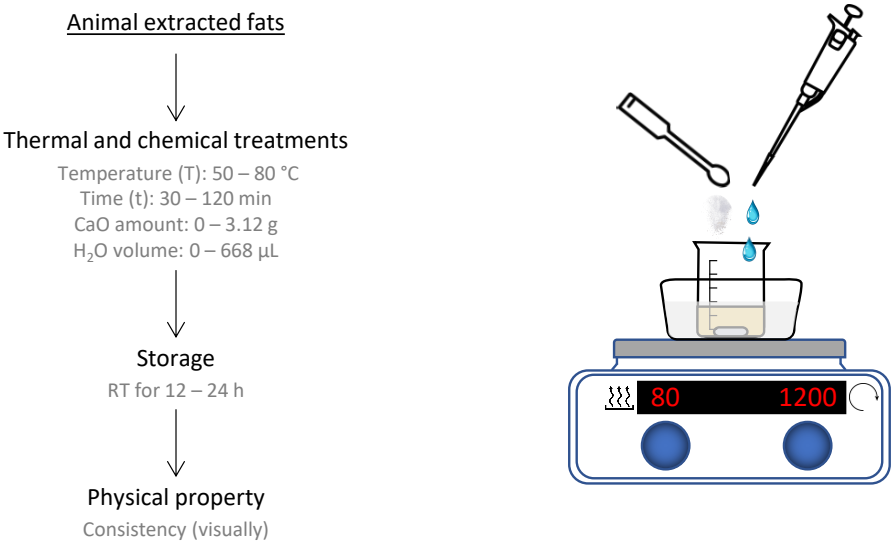

Figure 2.2. Work plan to enhance the consistency of rendered fat to supply to the local livestock industry.

A known amount of animal fat (15 g) was placed in a 100 mL beaker. Subsequently, different ratios of CaO and distilled H_2O were slowly added. The mixture was stirred continuously at 800 rpm using a magnet to ensure uniform dispersion of the components. The beaker was transferred into a silicone bath and several temperatures and times were tested. The reaction was stopped by removing the beaker from the silicone bath and the reaction mass was allowed to cool down. After 12 – 24 h at RT, the texture was visually evaluated.

2.7. Statistical analysis

All the experiments and analyses were performed in triplicate unless indicated otherwise. Results were expressed as means ± standard deviations (SD). Differences between conditions were determined with Tukey honestly significant difference (HSD) test on a level of significance at $p < 0.05$ using JMP Pro 14 (Statistical Discovery™ from SAS, Cary, NC, USA).

3. Results and discussion

3.1. *Fines* characterization

The *fines* were obtained during the rendering of category 3 non-ruminant animal by-products to produce fat and meat and bone meal (MBM) following the standard processing methods established in the European Regulations EC 1069/09 and 142/11 (temperature, over 133 °C; pressure, 3 bar by steam for 20 min; maximum particle size, 50 mm). *Fines* chemical characterization was performed before their use for other applications. The characterization is essential to understanding their composition and their possible applications.

The raw material was received as a heterogeneous powder with particles of variable size and brown colored, as shown in Figure 2.3.

Figure 2.3. Raw sample as received.

The general characteristics of the dried *fines* are displayed in Table 2.3. The proximate analysis showed that the main components in the sample were fat and protein, representing around 83% of the total dry weight. The total fat content includes true fat (triglycerides) as well as alcohol, waxes, terpenes, steroids, pigments, esters, aldehydes, and other lipids. On the other hand, crude protein is an indicator of protein content. It was calculated using the 6.25 nitrogen-to-protein conversion factor (NPCF) and comprises true

protein (depending on amino acid composition) and non-protein nitrogen. The high amount of fat and protein confirms that this by-product can be beneficiated as a source of fats and protein. The moisture content was very low, as it did not exceed 3%. In terms of processing and transportation, low moisture content is preferred. Moreover, this implies that the material can be safely stored for long periods with no concerns of deterioration due to microbial growth. The remaining 17% corresponds to ashes (12%) and other non-identified compounds.

Table 2.3. Total chemical composition (%) (based on dry matter content).

	Moisture (*n* = 3)	**Fat (*n* = 4)**	**Protein (*n* = 3)**	**Ash (*n* = 3)**
Fines	2.8 ± 0.1	37.2 ± 0.3	45.8 ± 0.5	12.2 ± 0.2

As no available information about *fines* composition was found after extensive research on literature and patents, MBM was selected for comparison. Table 2.4 shows the chemical composition of a wide range of MBM samples. As expected, the composition of dried *fines* is somehow different compared to the one reported in the literature for MBM. Even though, considerable variations were observed for some characteristics of MBM, as they tend to vary in composition among different rendering facilities and within a rendering plant (Hicks & Verbeek, 2016). As can be observed in Table 2.4, the percentages of moisture and crude protein for MBM ranged from 1.9 to 5.7 and 44.6 to 62.8%, respectively. Although higher percentages of protein were observed in MBM in some cases, the protein content of *fines* is within the range. Due to the high temperature applied during rendering, the proteins present in MBM have very low solubility. As *fines* were exposed to the same treatment, similar solubility was expected, around 5.35% of the total protein (Garcia et al., 2006). Solubility can be a useful indicator of how denatured a protein source has become during rendering and drying (Hicks & Verbeek, 2016).

The percentages of fat and ash in MBM varied from 8.9 to 16.0 and 20.7 to 52.9 %, respectively. Fat content in our sample was around 37%, which is considerably higher than fat content reported in MBM, almost doubling the maximum value observed in MBM. In contrast, the ash content is slightly lower in *fines* compared to MBM. The high ash content in MBM is considered to be one of the major drawbacks due to its negative effect on protein quality, energy, concentration of all essential amino acids, and amino acids balance, limiting MBM use in several animal diets (Moutinho et al., 2017; Shirley & Parsons, 2001). Arguably, the low ash content in *fines* indicates the presence of high-quality protein and higher gross energy compared to MBM. This characteristic might increase the possibilities of utilizations of this waste.

Table 2.4. MBM characteristics reported in the literature.

Reference	(Johnson et al., 1998)	(Karakas et al., 2001)			(Ravindran et al., 2002)	(Jeng et al., 2004)	(Adedokun & Adeola, 2005)	(Garcia et al., 2006)	(Hendriks et al., 2006)	(Sriperm et al., 2011)	(Li et al., 2011)	(Muir et al., 2013)	(Hicks & Verbeek, 2016)	(Moutinho et al., 2017)	*Fines* from this work
Animal	Swine						Swine	Cattle, swine, and poultry					Mixed species	70% Swine	Swine
Country	US	Denmark			New Zealand	Norway	US	US and Canada			China		US and Canada	Spain	Spain
	% (DM basis)														
CP (N x 6.25)	55.0	66.9	58.6	44.4	55.6	49.3	53.8	44.6 – 62.8[a]	58.4	54.8	52.0	50.3	49.5 – 59.4	53.1	45.8
Fat	10.0	11.2	11.2	10.7	10.6		11.6	8.9 – 16.0	10.8			6.0	8.9 – 16.0	15.3	37.2
DM	97.0	97.8	98.4	98.3	95.35	97.0	99.1	94.3 – 98.9	95.8	95.1	96.1	93.4	92.9 – 100	97.0	97.2
Ash	24.1	20.9	29.3	43.0	27.8	33.6	26.1	20.7 – 39.9	29.4			36.1	20.7 – 52.9	26.9	12.2

[a]CP = N x 6.45

3.2. Assessment of the method performance

The performance of five different methods of fat extraction from *fines* was investigated and compared. The extraction yields of the tested methods are summarized in Figure 2.4. Extraction yields were calculated based on the crude fat content obtained with the Soxhlet method, assuming that the fat extracted represents the total amount of fat in the sample. The results show that not significant differences were observed between Soxhlet and Folch methods ($p > 0.05$). The obtained results concur with previously published data (Pérez-Palacios et al., 2008; Tzompa-Sosa et al., 2014). Pérez-Palacios et al., (2008) have reported that Soxhlet (without previous acid hydrolysis) and Folch extractions did not differ in total lipid extraction for a product with very high fat content, around 48%. It also has been shown that performing acid hydrolysis previous to Soxhlet extraction has a significant effect for this type of product, as lower content of lipids was extracted (Pérez-Palacios et al., 2008). Given the role of the polarity of organic solvents in solubilizing fat and their problematic aspects, a greener solvent was tested, CPME. Independently of the extraction temperature, a significantly lower extraction yield was observed ($p < 0.05$), approximately 75%. Thus, due to the nature of lipids present in *fines*, *n*-hexane and the mixture of $CHCl_3$ and CH_3OH show a higher capacity and affinity to dissolve the fat. Moreover, Nebel & Mittelbach, (2006) also reported the high efficiency of *n*-hexane in extracting the fat from a similar substrate, MBM.

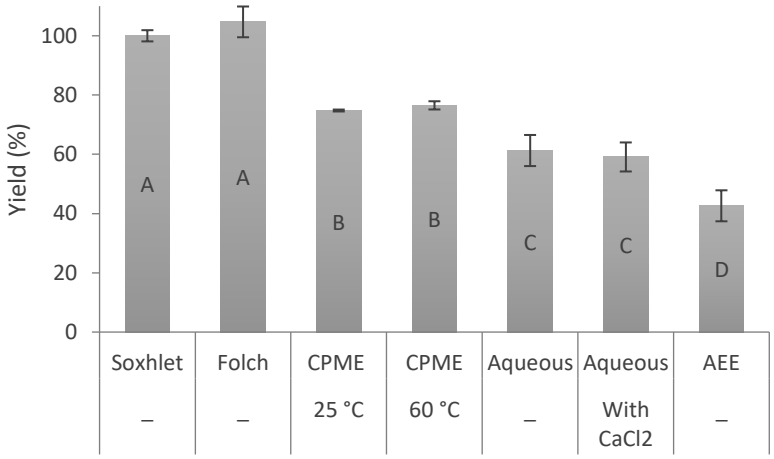

Figure 2.4. Performance of the different tested methods on fat extraction from dried *fines*. Values are represented as mean ± standard deviation of three independent experiments (Folch extraction $n = 6$ and aqueous enzymatic extraction $n = 36$). Identical letters indicate non-significant differences ($p > 0.05$).

In contrast, aqueous-based methods extracted lower amounts of lipids. Adding hot water with or without $CaCl_2$ released around 60% of the lipids compared to the Soxhlet method. Considering that the extraction of fat using water is based on the insolubility of fat in water and subsequent recovery of fat on the upper

layer of the mixture, these results were expected, and they are in accordance with previous reports. However, aqueous enzymatic extractions have been reported to overcome the low extraction yields of aqueous processes by using enzymes that hydrolyze structural proteins (Dumay et al., 2006; Rosenthal et al., 1996). Considering the high amount of protein in *fines*, commercial enzymes were selected to break down the large proteins into a range of smaller peptides and amino acids and to increase lipid liberation.

Nevertheless, regarding the solvent, other parameters than extraction yield are important. Aqueous processes are considered non-hazardous alternatives to organic solvents used in the traditional methods, which are hazardous and flammable liquids with potential toxic emissions during extraction and costly (Servaes et al., 2015). Conversely, water is environmental-friendly and safe. Besides this, water cost is much lower compared to the organic solvents, facts that have led water and aqueous-based solvent systems to represent an increased crucial choice for the placement of conventional solvent extraction methods (Gai et al., 2013).

3.2.1. Effect of enzyme in on aqueous fat extraction yield

Four commercial enzymes were chosen for comparison based on previously reported data (Gai et al., 2013; Jiao et al., 2014; Liang et al., 2012; Rosenthal et al., 2001; Šližyte et al., 2005a; Šližyte et al., 2005b; Wang et al., 2016). Alcalase 2.4L, Neutrase 0.8L, Flavourzyme, and Protamex were added at 2 levels of concentration, aiming to recover a higher amount of fat, resulting in 36 independent experiments. The recovery of fat after different enzymatic treatments is presented in Figure 2.5. High variability was observed among the controls. This variability might be due to the lack of homogeneity of the sample, the influence of the withdrawal method, or the completeness of the centrifugation-aided separation applied before withdrawal (Hall et al., 2017). Because of that, a comparison among the different concentrations of enzyme within the data set with the same enzyme was performed. No significant differences were observed among the control and the experiments with different amounts of enzymes, except for the enzyme Flavourzyme. However, no clear relation between commercial protease, protease concentration, and the lipids extracted was observed.

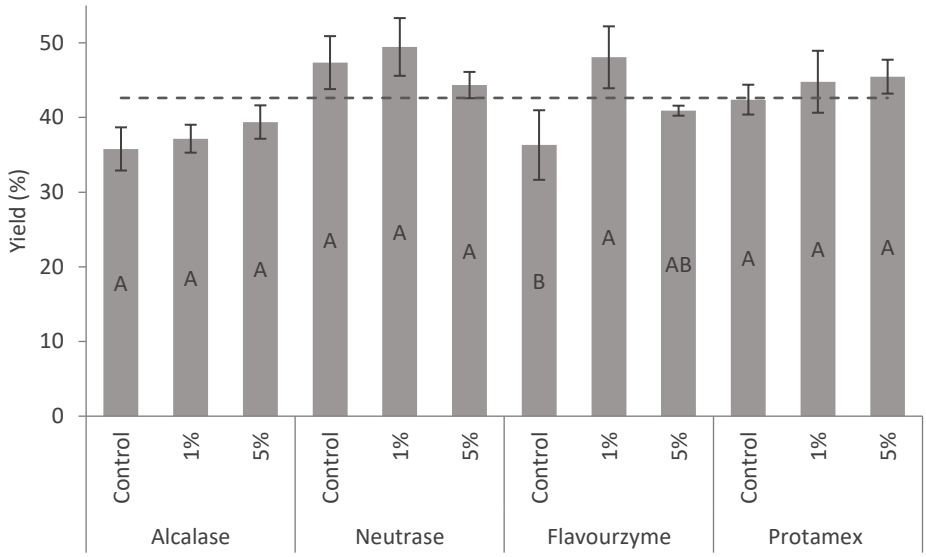

Figure 2.5. Effect of enzyme and enzyme percentage on oil yield from dried *fines*. Yields are represented as mean ± standard deviation of three independent experiments. The dashed line represents the mean of all the experiments. Identical letters indicate non-significant differences ($p > 0.05$).

Surprisingly, aqueous enzymatic extraction resulted in the lowest yield among the methods compared, about 40%. The low extraction yields might be due to i) protein present in the raw material, ii) the state of the raw material, and iii)) the amount of water added during the extraction. Šližyte et al., (2005a, 2005b) pointed out that for fish by-products the production of oil fraction can be prevented when the amount of proteins in the raw material is more than 17% of raw material. In addition, during the process of rendering, *fines* are summited to high temperature and pressure. In consequence, the interactions between lipids and proteins are stronger, forming a protein gel network and insoluble aggregates, making more resistant the denatured proteins, thus hindering their enzymatic breakdown and lipid release (Pérez-Palacios et al., 2008; Šližyte et al., 2005a). Besides this, the addition of water during the extraction process also plays an important role (Daukšas et al., 2005; Šližyte et al., 2005c). Daukšas et al., (2005) reported that water is the a crucial factor in oil extraction from cod by-products, obtaining higher yields in the control experiments without water addition compared to enzymatic hydrolysis with added water. In analogy to the results obtained in our study, Daukšas et al., (2005) reported that the amount of oil fraction obtained after treatment by adding only water was also higher than the amount of oil fraction obtained after hydrolysis with enzymes and added water. In contrast, Wang et al., (2016) reported the improvement in the oil extraction rate from pig fatback by using the same enzymes as in this study. The effect of the proteases on oil yield was in the following order: alcalase > protamex > neutrase > flavourzyme, while the rate of oil extraction was increased up to 97%, depending on the enzyme and the enzyme amount (Wang et al., 2016).

3.2.2. Influence of extraction method on lipid composition

The fatty acid composition was determined by GC-FID as methyl ester of fatty acid. Prior to the analysis by GC-FID, a derivatization step was performed in order to reduce polarity and make more amenable fatty acids analysis (Hewavitharana et al., 2020). As shown in Table 2.5, six saturated fatty acids (SAFA), five monounsaturated fatty acids (MUFA), and one polyunsaturated fatty acid (PUFA) were identified. The main fatty acids in the extracted fractions were palmitic acid C16:0 (36.90 – 37.32%), stearic acid C18:0 (19.72 – 20.93%), and oleic acid *c*9-C18:1 (31.99 – 33.48%), accounting for approximately 90% of the total acids identified. These values match with those reported by Nebel & Mittelbach, (2006), who tested several solvents to extract fat out of MBM for biodiesel applications. The main fatty acids in the fat extracted from MBM were palmitic (27.9%), stearic (20.3%), and oleic acids (38.6%), accounting for 86% of the fat extracted using *n*-hexane. Considering that the fatty acid profile of the fat extracted from *fines* is similar to the fat from MBM, the production of biodiesel could be an appropriate alternative. Additionally, it is worth mentioning that this alternative is not competing with the first-generation feedstocks, as edible oil stocks from rapeseed, soybean, or maize (Breil et al., 2016).

Table 2.5. Fatty acid composition (expressed in mg fatty acid/g fat extracted ± standard deviation).

Fatty acid	**t_R (min)**	**R_i**	**Concentration (mg fatty acid/g fat extracted)**			
			Soxhlet	**Folch**	**Aqueous**	**Aqueous (with $CaCl_2$)**
C12:0	2.84	1.04	1.5 ± 0.0^{ab}	1.3 ± 0.1^{b}	1.4 ± 0.1^{ab}	1.7 ± 0.2^{a}
C14:0	3.62	1.07	12.8 ± 0.1^{ab}	11.4 ± 0.9^{b}	12.4 ± 0.3^{ab}	13.4 ± 0.8^{a}
C16:0	4.57	1.10	209.0 ± 2.0^{ab}	188.0 ± 15.0^{b}	207.1 ± 5.0^{ab}	217.2 ± 7.2^{a}
*c*9-C16:1	4.74	1.05	12.1 ± 0.1^{a}	10.6 ± 0.8^{a}	11.5 ± 0.3^{a}	11.8 ± 0.3^{a}
C17:0	5.08	1.09	3.3 ± 0.0^{ab}	2.9 ± 0.2^{b}	3.2 ± 0.0^{ab}	3.6 ± 0.4^{a}
*c*9-C17:1	5.25	1.10	2.1 ± 0.0^{a}	1.9 ± 0.2^{a}	2.0 ± 0.0^{a}	2.1 ± 0.1^{a}
C18:0	5.63	1.12	112 ± 1^{ab}	101.7 ± 8^{b}	114.0 ± 2.5^{ab}	121.8 ± 5.6^{a}
*c*9-C18:1	5.80	1.13	189.6 ± 1.3^{a}	166.8 ± 13.5^{a}	181.4 ± 4.0^{a}	186.2 ± 4.2^{a}
*c*11-C18:1	5.83	1.14	15.5 ± 0.1^{a}	13.8 ± 1.1^{a}	14.8 ± 0.4^{a}	15.2 ± 0.3^{a}
*c*9,12-C18:2	6.08	1.14	1.5 ± 0.0^{b}	1.6 ± 0.1^{ab}	1.8 ± 0.0^{a}	1.8 ± 0.0^{a}
C20:0	6.74	1.15	2.0 ± 0.0^{ab}	1.7 ± 0.1^{b}	2.0 ± 0.1^{ab}	2.4 ± 0.3^{a}
*c*11-C20:1	6.92	1.14	5.0 ± 0.0^{a}	4.4 ± 0.3^{a}	4.8 ± 0.2^{a}	4.9 ± 0.1^{a}
Total			566.4 ± 4.6	506.2 ± 39.9	556.6 ± 4.63	582.0 ± 18.9

Means in a row with different letters indicate significant differences ($p < 0.05$)

Although different fat extraction methods result in significantly different yields, differences in the concentrations of fatty acids among the methods tested were statistically insignificant. However, there were some exceptions for the Folch method and the aqueous extraction with $CaCl_2$, where significantly higher concentrations of some fatty acids were determined in the latter one ($p < 0.05$). These differences were observed for lauric acid (C12:0), myristic acid (C14:0), palmitic (C16:0), heptadecanoic acid (C17:0), stearic acid (C18:0), and arachidic acid (C20:0).

Saturated and unsaturated percentages were calculated, and the results are shown in Figure 2.6. The extracted crude fat was more abundant in SAFA, accounting for 60% of the total fatty acids. No significant differences were observed in the proportions for the methods tested. However, the saturated to unsaturated ratio was higher than the standard value reported by FEDNA (values from November 2015) for animal blended fats, being this ratio 0.69. Pérez-Calvo et al., (2010) reported an increase in the saturated fatty acid content after the rendering process, compared to the content in raw material. This fact may be related to a higher sensibility to oxidation of the polyunsaturated fatty acids at high temperatures and a lower melting point of the polyunsaturated fatty acids compared to saturated fatty acids. Therefore, the raw material and the conditions of the specific rendering methods applied (type of rendering, temperature, and time) can affect the fatty acid profile.

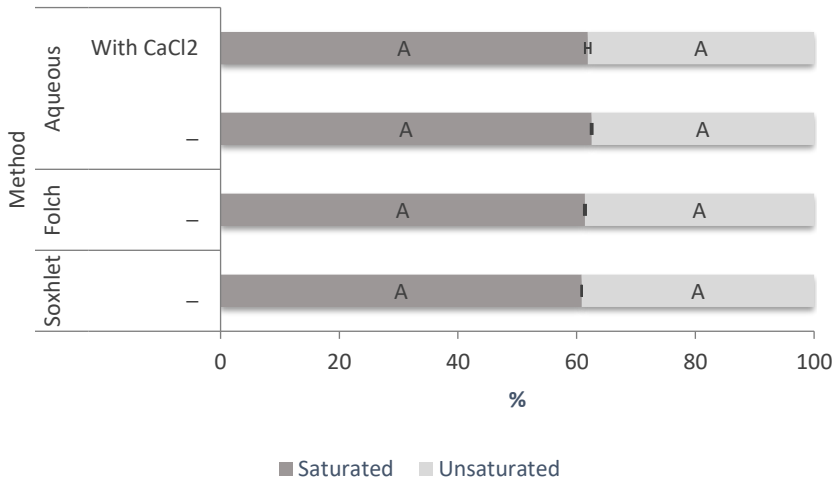

Figure 2.6. Saturated and unsaturated fatty acids (%). Different letters indicate significant differences ($p < 0.05$).

3.3. Assessment of the ecological and economic performance

The tested methods can easily be evaluated based on the efficiency in extracting the fat and a large number of studies are focused on this kind of comparison (Fiori et al., 2013; Hewavitharana et al., 2020; Pérez-Palacios et al., 2008; Shin et al., 2013; Shin & Park, 2015). However, considering that worldwide research aimed at cleaner processes (Poliakoff et al., 2002), the greenness of the methods needs to be considered. The principal question is: "How to evaluate different procedures in order to choose the greenest one?" (Gałuszka et al., 2012) . Several assessment methods have been proposed, each of them having its advantages and disadvantages (Tobiszewski & Orłowski, 2015). The environmental impact or the greenness of a reaction or process can be assessed by tools, such as Atom Economy (AE), E-factor (Sheldon, 2012), Life Cycle Assessment (LCA) (Kralisch et al., 2015), National Environmental Method Index (NEMI) (https://www.nemi.gov/home/) (Keith et al., 2007), Green Analytical Procedure Index (GAPI) (Płotka-Wasylka, 2018), or Eco-Scale (Clarke et al., 2018; Gałuszka et al., 2012; Mohamed et al., 2020; Saleh et al., 2020; Van Aken et al., 2006). Environmental metrics such E-factor and AE were introduced as new tools for efficiency in organic synthesis due to the growing concern regarding the generation of waste and the use of toxic and hazardous materials in fine chemical and allied industries (Sheldon, 2018).

The Eco-Scale is a semi-quantitative tool that relies on the comparison of several parameters that affect a process by assigning PP. This comprehensive tool allows the selection of the greenest alternative and enables the modification of proposed methods based on their weakest points. A mix of Eco-Scale parameters previously introduced by Van Aken et al., (2006) for the purposes of organic synthesis and the analogous approach proposed by Gałuszka et al., (2012) for analytical procedures were adopted in this study to evaluate the greenness of fat extraction methods. Penalty points were assigned to the cost of the components, the safety for the operator and the environment, the energy required, and the waste generated. Each step contributes to pollution and has a different potential for being made greener (Gałuszka et al., 2012). Moreover, generally, a higher number of steps is directly correlated to a less green procedure.

A green analytical evaluation has been made for each tested method by assigning penalty points and the final scores are shown in Table 2.6, Table 2.7, Table 2.8, Table 2.9, and Table 2.10. The most relevant issues that affect the greenness of fat extraction are discussed in this section.

Table 2.6. The penalty points and final Eco-Scale score for fat extraction following the Soxhlet method.

Parameter	Experimental conditions	PP
Price of the components	> 5 €	5
Reagents (amount x hazard)	*n*-hexane (50 mL) (4 pictograms)	2 x 8 = 16
Energy	Soxhlet (> 1.5 kWh) hot plate (≤ 0.1 kWh)	3 1
Occupational hazard	emission of vapors and gases to the air	3
Waste	*n*-hexane (50 mL)	5
	∑ PP = 33 **Eco-Scale total score = 67**	

Table 2.7. The penalty points and final Eco-Scale score for fat extraction following the Folch method.

Parameter	Experimental conditions	PP
Price of the components	> 1€ and < 5€	3
Reagents (amount x hazard)	$CHCl_3$ (20 mL) (2 pictograms) CH_3OH (10 mL) (3 pictograms) saline solution (2.5 mL) (0 pictograms)	2 x 4 = 8 2 x 6 = 12 0
Energy	shaker (≤ 0.1 kWh) centrifuge (≤ 1.5 kWh) rotatory evaporator (> 1.5 kWh)	1 2 3
Occupational hazard	emission of vapors and gases to the air	3
Waste	$CHCl_3$ and CH_3OH (30 mL)	5
	∑ PP = 37 **Eco-Scale total score = 63**	

Table 2.8. The penalty points and final Eco-Scale score for fat extraction using CPME.

Parameter	Experimental conditions	PP
Price of the components	> 1€ and < 5€	3
Reagents (amount x hazard)	CPME (20 mL) (2pictograms)	2 x 4 = 8
Energy	incubator (≤ 1.5 kWh) centrifuge (≤ 1.5 kWh) rotatory evaporator (> 1.5 kWh)	2 2 3
Occupational hazard	emission of vapors and gases to the air	3
Waste	CPME (20 mL)	5
	∑ PP = 26 **Eco-Scale total score = 74**	

Table 2.9. The penalty points and final Eco-Scale score for fat extraction using hot water.

Parameter	Experimental conditions	PP
Price of the components	< 1€	1
Reagents (amount x hazard)	tap water (30 mL) (0 pictograms)	0
Energy	hot plate (≤ 1.5 kWh) incubator (> 1.5 kWh)	2 3
Occupational hazard	None	0
Waste	Water (1 – 10 mL)	3
	∑ PP = 9 **Eco-Scale total score = 91**	

Table 2.10. The penalty points and final Eco-Scale score for fat extraction using aqueous enzymatic extraction.

Parameter	Experimental conditions	PP
Price of the components	> 1€ and < 5€	3
Reagents (amount x hazard)	distilled water (30 mL) (0 pictograms) enzymes (<10 mL) (4 pictograms) HCl (<10 mL) (2 pictograms) NaOH (< 10 mL) (1 pictogram)	0 1 x 8 = 8 1 x 4 = 4 1 x 2 = 2
Energy	hot plate (≤ 1.5 kWh) incubator (> 1.5 kWh)	2 3
Occupational hazard	None	0
Waste	None	0
	∑ PP = 22 **Eco-Scale total score = 78**	

Eco-Scale scores for the extraction of fat from *fines* ranged from 63 to 91. Soxhlet and Folch methods were undoubtedly the best methods to extract the higher amount of crude fat from *fines*. However, applying the Eco-Scale approach, the above methods showed the poorest Eco-Scale scores, 67 and 63, respectively. According to the ranking, these punctuations fall under acceptable green process (< 75 and > 50). Extraction with CPME was also ranked as an acceptable green analysis. The remaining two methods, aqueous extraction at high temperature and aqueous enzymatic extraction, received 91 and 78 points, respectively. Although both techniques can be ranked as excellent green processes (> 75), the difference between them is high. Parameters regarding safety (reagents and occupational hazard), followed by energy requirements had the greatest incidence in the final score.

3.3.1. Considerations regarding safety

One of the most problematic aspects of fat extraction is the use of organic solvents (Jiao et al., 2014). The solvent has a great influence on i) fat recovery and ii) hazard parameters. Considering the volume required for each extraction and the safety labels of each solvent the penalty points assigned to *n*-hexane, $CHCl_3$/CH_3OH, CPME, and water were 16, 20, 8, and 0, respectively. The organic solvents used are hazardous and flammable liquids with potential toxic emissions during extraction, that can cause harm to the environment and human health (López et al., 2020; Señoráns & Luna, 2012; Servaes et al., 2015). As safety is of paramount importance, reduction or replacement with greener environmental alternatives is crucial. In the last years, water and aqueous-based solvent systems have represented an increasingly crucial choice for the placement of the conventional solvents, such as *n*-hexane and CH_3OH in the food industry (Gai et al., 2013). This fact is not surprising, given that water is an environmental-friendly, cheap, and safe solvent, resulting in less operation danger and is considered as the green solvent par excellence (Armenta et al., 2017; Rosenthal et al., 1996). However, the amount of solvent required and the possibilities of using mixed solvents are additional aspects to be considered (Armenta et al., 2017). Rosenthal et al., (1996) reported that the highest possible solid:solvent ratio is desirable in the extraction step to obtain fewer stable emulsions and generate less effluents. The highest solid:solvent ratio was 1:2 for water-based extractions, whereas ratios ranging from 1:10 to 1:50 were necessary for organic solvent-based extractions.

Although aqueous processes result in a lower yield, requirements regarding health and environment are satisfied. Aqueous extraction eliminates the problem of solvent safety, less volume is necessary, and the cost is much lower compared to organic solvents.

3.3.2. Considerations regarding energy

Extraction methodologies usually require electricity to work, consuming considerable amounts of energy. Energy production involves the consumption of raw materials and the production of wastes and emissions to air, water, and soil. Moreover, nuclear and fossil fuel-based sources of electricity are not supported for green approaches. Therefore, an evaluation of energy consumption must be done (Armenta et al., 2017).

In general, the energy penalty points sum up the cumulative energy demand for sample storage, extraction, and purification. Proper sampling, preservation, and adequate preparation of samples are important to obtain valid results. However, sample preservation usually requires energy, contributing to a less green process. The sample preparation depends on the type of sample, the nature of the lipids, and the type of analytical procedure used (Señoráns & Luna, 2012). In the preparation of a sample for solvent

extraction, it is often necessary to dry the sample prior to fat extraction when organic solvents are used. Many organic solvents are immiscible with water and it can interfere with the efficiency of the extraction. The next step in the preparation is the grinding of the sample to i) increase the surface area of the lipids exposed to the solvent and ii) enhance the diffusion of water-soluble components (Rosenthal et al., 1996). Besides this, acid or alkaline hydrolysis can be performed prior to the extraction to enhance the extraction efficiency. In this step, the bonds that keep lipids attached to protein and carbohydrates are broken (Hewavitharana et al., 2020; Shin et al., 2013). All these steps increase energy supply and contribute to pollution, leading to a less green process. Although samples were stored at 4 °C until fat extraction was performed, this parameter was not considered in the Eco-Scale approach.

Soxhlet method is known to be a time-consuming procedure, but it resulted to be greenest in terms of energy consumption. This method received 4 penalty points while the rest received between 5 and 7 penalty points. Most of the energy consumption is related to the heat supply, although processes related to the recovery or concentration of lipids, involving the combined use of heat sources, vacuum pumps, and rotating motors are also important demanders. Aqueous extraction at high temperature was the most affected method by this factor, contributing to 5 out of 9 total penalty points, although the work-up procedure did not involve any additional solvent evaporation step.

3.3.3. Considerations regarding waste

Solvent and energy requirements are significant variables to establish the greenness of an extraction method, but produced waste and potential emissions must also be considered (Armenta et al., 2017). After the extraction process, three fractions were obtained: crude fat, recovered solvent, and a protein-rich fraction. Depending on fat recovered, crude protein was concentrated up to 70%. The concentrated protein fraction was not added to waste calculations, as it was considered a new material with significant value for another process (Chapter 3). The recovered solvent was treated as waste and was disposed of following good disposal practices.

Soxhlet and Folch methods and extraction with CPME conduce to solubilization of lipids in the organic phase. Since there is necessary one additional step to obtain fat free of solvent, the solvents were eliminated and, in some cases, recovered. Among 10 and 50 mL of solvent per g of sample treated were considered as waste, although solvent recycling might be investigated. Moreover, efficient extraction hoods were employed to reduce the occupational risk of the operators. However, a significant amount of solvent is likewise emitted to the atmosphere; thus the emission of solvent to air is punished in Eco-Scale (Armenta et al., 2017). On the other hand, the insolubility of lipids in water results in the formation of an upper layer containing the fats, allowing the fats and protein separation in one step. Hence, avoiding

waste of energy in solvent evaporation, volatile organic compounds emissions, and the use of hazardous reagents, results in a decrease in waste.

3.3.4. The ideal green analysis

The Eco-Scale approach provided a wide range of Eco-Scale scores. Taking into account the above consideration, the ideal green process can be characterized by cost-effectiveness, elimination or minimal use of reagents, low energy use, and no waste generation (Gałuszka et al., 2012). Although both Soxhlet and aqueous lipid extraction are of industrial relevance (Tzompa-Sosa et al., 2014), the highest Eco-Scale score was obtained by aqueous extraction at high temperatures. This technique met the criteria, especially regarding safety and environmental issues. Moreover, as the fat extracted is considered as a low added value compound, the price of the extraction components was taken into account to select a process economically profitable. Apart from the above considerations, the aqueous extraction method was the cheapest. Besides this, aqueous extraction resulted in simultaneous production of i) crude fat and ii) protein concentrate, being this process less damaging for protein. Therefore, aqueous extraction at high temperature was selected as the most adequate method and the Eco-Scale approach was a handy tool in selecting the greenest methodology to perform fat extraction from meat by-products.

3.4. Evaluation of animal fat consistency. A precursor for animal feed

The animal fat extracted by aqueous extraction at high temperature consisted of 60% of saturated and 40% of unsaturated fatty acids. The most abundant unsaturated fatty acid was oleic acid, while the predominant saturated fatty acid was palmitic acid. The consistency was liquid at RT.

Animal fat can be used as an ingredient in feed for the livestock sector and pet food. To integrate it into animal feed, a harder fat consistency is necessary. A total of 15 combinations of 3 variables (temperature, time, and molar ratio fat/CaO/H_2O) were tested, aiming at selecting the optimal conditions to obtain a semi-solid fat. All the conditions are summarized in Table 2.11. The efficiency of each experimental condition was determined visually. A harder fat consistency after the cooling period means an improvement in the consistency of the product.

Table 2.11. Conditions tested to improve the texture of animal fat and the consistency visually observed.

Assay	Temperature (°C)	Time (min)	Molar ratio fat/CaO/H_2O	Consistency (at RT)
1	50	30	1/1/0	Liquid
2	60	105	1/1/0	Liquid
3	50	30	2/1/0	Liquid
4	60	120	2/1/0	Liquid
5	80	60	3/1/0	2 phases
6	50	30	3/1/0	Liquid
7	50	30	5/1/0	Semi-liquid
8	50	60	5/1/0	Liquid
9	50	30	10/1/0	Liquid
10	50	60	10/1/0	Liquid
11	50	30	3/1/2	Semi-liquid
12	50	30	3/1/1	Unctuous solid
13	50	30	5/1/1	Unctuous solid
14	50	30	5/0/1	Semi-liquid
15	50	30	10/1/1	Semi-liquid

Among the investigated parameters, the results in the study showed that an increase of incubation temperature to 80 °C resulted in a non-homogeneous product where phase separation was observed (2 layers). Besides this, when fat was incubated for more than 30 min, independently of the temperature, no differences in fat consistency were observed when compared to the initial sample. On the other hand, the mixing of the fat with calcium oxide and water was found to affect solidification positively. However, in the absence of additional water, the consistency of the mixture was liquid. Only in one of ten assays performed without extra water (assay 7) an effect on fat consistency was observed. This finding should not be considered as a surprise since the reaction that allows consistency modification is saponification. After mixing water, CaO, and fat, calcium salts of fatty acids from acylglycerides were formed as a soap.

After depicting the importance of water in this reaction, concentrations of calcium oxide and H_2O ranging from 2 to 7% and from 0 to 5%, respectively, were tested at 50 °C and 30 min of incubation (assays 11 – 15). Two of the tested conditions gave as a product an unctuous solid, considered as an improvement. The stoichiometric molar ratios were 3:1:1 and 5:1:1 (Table 2.11). It has been found that the amount of calcium oxide can influence palatability. Thereby, a greater amount of calcium oxide increases the alkalinity of feed blocks and thus tends to reduce consumption. Besides this, an excessive calcium content can affect the correct utilization of other minerals such as magnesium in cattle digestion (Jenkins & Palmquist, 1984). Palatability is an important consideration in feed formulation which will have

a direct impact on product acceptability (Mullen et al., 2017). Therefore, it is important to obtain a product that meets the nutritional requirements of the animals. Because of this, the conditions of assay 13 were selected as optimal and the consistency is shown in Figure 2.7. The amount of CaO added implies around 4% of the weight of the final partially solidified product. The product obtained consists of a homogeneous dispersion of fats, calcium fatty acids soaps, and glycerol. This change in consistency provides wider utility for management; it can be integrated more easily as an ingredient for feedstock than traditional fats. Moreover, it is an economical ingredient in animal feed.

Figure 2.7. Fat consistency obtained under conditions of assay 13 after cooling down at RT.

Achieving the modification of the consistency with CaO to be used as an ingredient in animal feed is not merely a physical improvement. This fact could benefit animal feed in terms of micelles formation that facilitate the digestion and assimilation of fats and other fat-soluble vitamins (Pablos Pérez, 2008). Moreover, a fraction of the final product obtained in this work consists of calcium fatty acids soaps, also known as rumen-protected fats (RPF) or rumen bypass fats when used in ruminant diets. Supplementation of the ruminant diet with RPF overcomes the drawbacks of adding fats, which is a well-known practice to increase the energy value of the diet. Extensive research proved mixed results of supplementing diets with fats and rumen bypass fats. Even at low levels of supplementation, fats show to decrease the dry matter intake (DMI) in some reports, while no changes were observed in others (Moallem et al., 2007). Moreover, fats show to depress fiber digestibility and milk fat depression (Manriquez et al., 2019). Besides this, a high level of fats could be toxic to rumen microbes (Behan et al., 2019), while RPF allows greater energy consumption, and it has been associated with positive effects on milk production, higher conception rate, increased pregnancy rate, and reduced service period (Handojo et al., 2018; Schneider et al., 1988; Tyagi et al., 2010). This improvement is due to the protection of the nutrient from microbial fermentation and biohydrogenation in rumen. The RPF remain insoluble at normal rumen pH and they can escape rumen fermentation and be utilized as a source of energy, after being converted into free fatty acids and calcium in the abomasum and absorbed from the small intestine (Schneider et al., 1988).

The utilization of fat by monogastric animals compared to ruminant species differ. Freeman, (1983) reported the factors which influence the utilization of the dietary fat by monogastric animals and included the extent of saturation of the constituent fatty acids, the proportion of glycerides, the chain length of fatty acids, the level of inclusion, and the age of the animal. Besides this, Pablos Pérez, (2008) reported that results obtained with soaps are worse than those obtained with triglycerides in monogastric animals. The lack of glycerol plays an important role, which is considered vital for the formation of micelles, which are necessary for the good digestibility of fats by monogastric animals. The new product obtained after partial saponification was composed mainly by fats, glycerol, and calcium fatty acids soaps. Based on the previous considerations, it will be of great use in monogastric animal feed.

Therefore, this simple procedure satisfied the renderer problem and, at the same time, the addition of partially saponified fat as an ingredient in diet may have potential benefits for animals.

4. Conclusions

In this study, an under-utilized waste from the meat rendering industry was characterized in terms of moisture, fat, protein, and ash contents (2.8% moisture, 37.2% fat, 45.8% crude protein, and 12.2% ash). As the fat is a fraction of interest, several extraction methodologies were tested. The performance of the methods was evaluated based on the extraction yield (%), using Soxhlet as the reference method. The Folch method revealed the exact yield as the Soxhlet method, while the methods with CPME, water, and enzymes exhibited lower yields. However, the aqueous extraction method was proposed as the greenest technique based on the Eco-Scale approach. The fat extracted with this method was summited to different treatments to improve the consistency. Finally, an efficient procedure was optimized to achieve a modification on the consistency of rendered fats. A semi-solid fat was obtained when 15 g of liquid fat was mixed at 50 °C with 0.62 g of CaO and 199 µL of water for 30 min.

5. References

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Chapter 3. Towards generation of protein hydrolysates from *fines* through enzymatic hydrolysis: optimization and characterization

The results described in **Section 3.4.2.2. Size exclusion chromatography (SEC)** analyses have been obtained in collaboration with the Department of Food Science (UCPH FOOD) (University of Copenhagen).

The results described in **Section 3.5. Scale-up of enzymatic hydrolysis** have been obtained at PROSYS Research Centre - DTU Chemical Engineering (Technical University of Denmark).

1. Introduction

The meat industry generates considerable amounts of solid and liquid waste (Arancon et al., 2013; Aspevik et al., 2017; Thoresen et al., 2020). With the continuous growth of meat consumption, mainly in developing countries, a concomitant increase in animal co- and by-products is expected (Boland et al., 2013; Etemadian et al., 2021; Lynch et al., 2018). The adequate disposal following strict regulations is primordial to avoid environmental threats and health problems, such as the spread of bovine spongiform encephalopathy (BSE) (Toldrá et al., 2016). Cleaner and effective management ways are increasingly being sought (Lafarga & Hayes, 2014). Besides this, it has been estimated that the efficient use of cattle and pig by-products might generate about 11.4% and 7.5% of the gross income of the productive sector, respectively (Jayathilakan et al., 2012). Specific rules apply for the handling of animal parts intended for human consumption (European Parliament & Council of the European Union, 2004) or animal by-products (ABPs) (European Parliament & Council of the European Union, 2009; Lynch et al., 2018), which determine their potential utilization. The applications for edible meat by-products based on improved or new technologies consist mainly of ingredients for food, feed, and pet food industries. The main applications addressed towards inedible by-products are substances of interest for chemical or pharmaceutical industries, fertilizers, or energy generation (Toldrá et al., 2016). Although these applications are technologically and economically viable (Bhaskar et al., 2007), in frame with the objectives of Circular Economy, enzymatic hydrolysis is well-accepted and one of the most promising valorization opportunities for the meat industry (da Silva, 2018; Vázquez et al., 2020). For instance, from the protein fraction can be obtained, through enzymatic proteolysis, valuable molecules, such as extracts with functional properties, bioactive peptides, and protein hydrolysates (Toldrá et al., 2016) with potential application in the formulation of food, animal nutrition (Hou et al., 2017), nutraceutical industries (Etemadian et al., 2021), and biotechnology (Pasupuleti & Demain, 2010).

The nature of substrate, enzyme specificity, and hydrolysis conditions are crucial parameters in the enzymatic hydrolysis, as they affect nutritional, bioactive, and functional properties of peptides and protein hydrolysates (Anzani et al., 2019; Bhaskar et al., 2007; de Queiroz et al., 2017). Factors like pH, temperature, enzyme/substrate ratio, and time can be modulated to produce hydrolysates with composition and properties of interest (Pagán et al., 2013). Most of the studies reported in the literature concerning enzymatic hydrolysis of animal co- and by-products refer to raw materials (native proteins) or heat-treated materials at 90 °C for short periods (partially denatured proteins), such as goat viscera (de Queiroz et al., 2017), deer, sheep, and pig plasma (Bah et al., 2015), pig skin (Zhang et al., 2017), pork, beef, and chicken by-products, and salmon viscera (Lapeña et al., 2018), etc., focused primarily on the

research of bioactive peptides and food ingredients (Hou et al., 2017; Lafarga & Hayes, 2014; Lemes et al., 2016; Martínez-Alvarez et al., 2015; Mora et al., 2014; Ryan et al., 2011; Toldrá et al., 2012; Vercruysse et al., 2005). Sterilized waste materials according to the conditions established in European Union (EU) (Aspevik et al., 2017) or other heat-treated materials have received little attention, and the studies are limited to meat and bone meal (MBM), which feed use has become increasingly restricted (Deydier et al., 2005). Garcia et al., (2011) and Piazza & Garcia, (2014) reported the enzymatic solubilization of MBM proteins with flavourzyme, versazyme, subtilisin, and trypsin. Nevertheless, no information has been found about the hydrolysis of *fines*, a thermally treated fraction generated during MBM production. The amount produced of *fines* is dependent on several parameters, such as the quality and the type of raw material, the geographic location of the plants, etc. Although a part of the *fines* has potential for recycling into the rendering system or the primary treatment system for wastewater (Meeker, 2006), large amounts of *fines* are generated and must be handled in a proper way. This surplus does not have market value, representing a waste that needs to be managed appropriately to avoid pollution and major aesthetic and health problems.

This fraction, *fines*, contains a similar protein content to MBM but a higher amount of fat. These types of materials are generally regarded as difficult substrates for enzymatic hydrolysis due to low water content, high fat content, and insolubility in water. However, because of their composition, they could constitute a cheap and valuable protein source for value-added products if handled properly, resulting in better economic and environmental sustainability.

Hence, the objective of this study was to evaluate the enzymatic hydrolysis of the proteinaceous fraction of *fines*. Considering the demand for reliable sources of quality protein hydrolysates for non-feed applications, the research was first focused on the obtention of protein hydrolysates with the higher degree of hydrolysis (DH) possible. Secondly, hydrolysates were characterized, and finally, the feasibility of scaling up the process was evaluated. Given their characteristics, potential applications have been proposed, thus indicating a successful transformation of this unexploited waste.

2. Materials and methods

2.1. Materials

Inedible by-products, called *fines*, were a kind gift provided by the local company Subcarn Echevarria S.L. (Cervera, Lleida, Spain). *Fines* were stored at 4 °C.

The enzymes used were purchased from Sigma-Aldrich (St. Louis MO, USA). Alcalase 2.4L (EC 3.4.21.62) is an endo-protease from *Bacillus licheniformis* of the serine-type and with an enzymatic activity of 2.58 AU/g. Neutrase 0.8L (EC 3.4.24.28) is also an endo-protease from *Bacillus amyloliquefaciens* and with an enzymatic activity of 0.95 AU/g.

Dithiothreitol, hydrochloric acid (HCl), serine standard, glycine, tryptone, acrylamide/bis-acrylamide, peptone from soybean, peptone Primatone® RLT, and β-mercaptoethanol were from Sigma-Aldrich (St. Louis MO, USA. Sodium hydroxide, (NaOH), sodium-dodecyl-sulfate (SDS), Coomassie Blue G250, bromophenol blue, acetonitrile, methanol, and formic acid were from Fisher (Madrid, Spain). *o*-Phthaldialdehyde was from Across Organics (Geel, Belgium). Di-sodium-tetraborate decahydrate and acetic acid were from Panreac (Barcelona, Spain). Bacteriological meat extract (A1710HA) was purchased from Biokar Diagnostics (Allonne, France). Glycerol was from Vidrafoc (Barcelona, Spain).

All analytical reagents including amino acid mixture (AAS18), hydroxyproline (Hyp), diglycine (Gly-Gly), reduced glutathione (GSH), glutathione disulfide (GSSG), bacitracin, insulin from bovine pancreas, and myoglobin from equine skeletal muscle were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Sample pre-treatment

Prior to enzymatic hydrolysis, the *fines* were dried at 40 °C for 48 h and sieved through a 2 mm screen (10 mesh). The homogenized material was partially defatted following the procedure previously selected as optimal (Chapter 2 Section 3.3). Briefly, *fines* were mixed with water and the mixture was incubated at 80 °C in an orbital shaker (211DS Labnet International) for 4 h at 180 rpm, then cooled down to room temperature (RT) and centrifuged (Beckman Coulter Avanti® J-26 XP). After centrifugation, the fat and the interphase were easily removed while the remaining partially defatted *fines* (PDF) containing proteinaceous non-soluble solids were characterized and used for further hydrolysis experiments. All the fractions obtained were divided in plastic bags and kept in the fridge at 4 °C or in the freezer at -20 °C until further use.

2.3. Enzymatic hydrolysis optimization for Alcalase 2.4L

To optimize the hydrolysis conditions for Alcalase 2.4L, the effects of the variables enzyme/substrate (E/S) ratio (%), pH, and time were tested. The PDF fraction was mixed with deionized water or sodium phosphate buffer (50 mM, pH 8) with a solid/liquid (S/L) ratio of 1:4 (w/v). Solutions containing 250 g/L of PDF, resulting in a dry matter (DM) content concentration in a range of 9 – 12.5%, were prepared in Nalgene™ polypropylene centrifuge bottles with sealing caps. Mixture solutions were shacked vigorously

and conditioned at 55 °C and 200 rpm for 30 minutes in a shaking incubator (211DS Labnet International). The pH of water solutions was adjusted at 8.0 - 8.3 by adding 6 N NaOH before enzyme addition. Then, the reaction was started by adding 0.026, 0.129, and 0.258 AU/g substrate (corresponding to 1, 5, and 10% of Alcalase 2.4L (w/w)), and the reaction was conducted at 55 °C and 200 rpm. The reactions were carried out under controlled conditions of pH or not controlled. Under controlled conditions, the pH was manually adjusted every 2 h until 12 h at 8.0 – 8.3 by adding 1 N NaOH. Next, at 24 h it was adjusted if necessary. The process lasted for 24 – 48 h and aliquots were taken during the hydrolysis at specific times and inactivated at 95 °C for 15 min. Then, cooled hydrolysates were centrifuged at 13000 rpm at RT for 10 min (Eppendorf® Centrifuge 5424R). Finally, the DH of the hydrolysates was determined. All the experiments were performed in triplicate.

2.4. Enzymatic hydrolysis optimization for Neutrase 0.8L

To optimize hydrolysis conditions for Neutrase 0.8L, Response Surface Methodology (RSM) was employed with a Central Composite Design (CCD). According to the literature (Dey & Dora, 2014; Yolmeh & Jafari, 2017; Zhang et al., 2016) four different variables, E/S ratio, hydrolysis time, initial pH value, and hydrolysis temperature were selected to perform one-factor-at-a-time (OFAT) experiments in order to choose their reasonable ranges. Fixed levels of factors were E/S ratio 5%, hydrolysis time 4 h, pH 7, and temperature 50 °C. Based on the data obtained performing OFAT experiments, the experimental ranges and the center point values of the independent variables were selected and are shown in Table 3.1. Therefore, a four-factor, five-level orthogonal CCD was developed, consisting of 29 treatments including 2^4 = 16 factorial points, eight axial points (α = 1.48), and five replicates of the central point. DH was selected as the response to evaluate the OFAT and the combination of the independent variables. Several parameters have been estimated for the quadratic model and the behavior of the model was explained by a second-order polynomial equation (Equation 3.1) (Montgomery, 2012):

$$Y = b_0 + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} b_{ij} X_i X_j$$

Equation 3.1

where Y represents the parameter to be modeled (DH); b_0 is a constant coefficient; b_i represents the regression coefficients for linear effects; b_{ii} represents the quadratic coefficients for quadratic effects, and b_{ij} represents the regression coefficients for interaction effects. X_i and X_j are the independent coded variables. The design was validated through the combination of parameters to obtain a higher response.

Table 3.1. Enzymatic hydrolysis variables and respective levels for CCD with Neutrase 0.8L.

Factors	Levels				
	α (-1.48)	**-1**	**0**	**1**	**α (1.48)**
X_1 - E/S (%)	2.59	5.0	10	15	17.41
X_2- Time (h)	0.57	2.5	6.5	10.5	12.43
X_3- Initial pH	5.52	6	7	8	8.48
X_4 - T (°C)	37.59	40	45	50	52.41

Each assay was composed of 500 mg of PDF in 50 mL of deionized water. The pH of the samples was adjusted with 1N NaOH. Then, the samples were placed in a shaking incubator at 200 rpm in the specified conditions. The hydrolysis was terminated by inactivating the enzyme at 95 °C for 15 min. DH of the supernatants was determined after cooling down and centrifugation at 13,000 rpm for 10 min (Eppendorf® Centrifuge 5424R).

2.5. Protein hydrolysates production in shake flasks

Once optimal conditions were determined, peptones for further experiments were prepared with Alcalase 2.4L and Neutrase 0.8L. Solutions containing 250 g/L of PDF were incubated in the optimal conditions of temperature and agitation. After 30 min, pH was adjusted and the enzyme was added. After the specific time required for each reaction, samples were incubated at 95 °C for 15 min in a water bath. After enzyme inactivation, the samples were centrifuged (Beckman Coulter Avanti® J-26 XP) for 30 min at 7000 rpm at RT and two fractions were collected: the sludge on the bottom and the supernatant containing the soluble protein hydrolysate. The supernatant was filtered using a Büchner funnel with filter paper and then it was freeze-dried under vacuum (Telstar LyoQuest) to obtain a dry powder. Final dry materials were termed HA – hydrolysate obtained with Alcalase 2.4L and HN – hydrolysate obtained with Neutrase 0.8L. The samples were kept in the fridge at 4 °C until further use.

2.6. Protein hydrolysates production in a bench-scale reactor

After complete characterization of protein hydrolysates produced in shake flasks and assessment of their potential for several applications, the process was scaled-up (Table 3.2). Enzymatic hydrolysis experiments of PDF with Alcalase 2.4L and Neutrase 0.8L were carried out in duplicate in a 3-L bench-scale reactor (Applikon, Delft, The Netherlands) with a 1.25 L working volume and equipped with a heating shell and two 6-bladed Rushton impellers. Temperature, pH, and stirring speed were controlled automatically. Process parameters were previously optimized for each enzyme, although several conditions were slightly

modified. Enzymatic hydrolysis with Alcalase 2.4L was performed for 24 h at 55 °C, the stirring rate was fixed at 600 rpm, the S/L ratio was 1:4 (w/v), the E/S ratio was 5%, and the pH was kept around a set point value of 8.0 ± 0.1 by automatic addition of 2N NaOH. The pH-stat method was used to determine the DH. Enzymatic hydrolysis with Neutrase 0.8L was carried out for 10.5 h at 40 °C, the stirring rate was fixed at 600 rpm, the pH was initially adjusted to 8 with 2N NaOH, the S/L ratio was 1:4 (w/v), and the E/S ratio was 15%. At the end of the enzymatic process, the reactor was autoclaved and the enzyme was inactivated. Then, protein hydrolysate was separated from the residual non-solubilized sample by centrifugation at 4500 rpm for 60 min (Heraeus™ Multifuge X3R, Thermo Fisher Scientific). In addition, ultrafiltration (Vivaflow® 200, Sartorius, Germany) with a cut-off membrane of 100 kDa was employed to further remove insoluble particles. Finally, ultrafiltrated hydrolysates were freeze-dried (CoolSafe™ 4L, LaboGene, Denmark). Final dry materials were termed as RHA – hydrolysate obtained with Alcalase 2.4L and RHN - hydrolysate obtained with Neutrase 0.8L. The samples were kept in the fridge at 4 °C until further use.

Table 3.2. Amount of sample and water used in the different stages of enzymatic hydrolysis.

Experimental phase	**PDF (g)**	**H_2O (mL)**	**Protein hydrolysates code**	
			Alcalase 2.4L	**Neutrase 0.8L**
1st Optimization	0.5 - 25[a]	100	-	-
2nd Production in shake flasks	25	100	HA	HN
3rd Production in a reactor	250	1000	RHA	RHN

[a] depending on the experimental design

2.7. Analyses

2.7.1. Macrocomponents and elementary analysis

The percentage of moisture, fat, ash, and nitrogen of the *fines* fractions obtained were determined following standard methods (AOAC 2000). The moisture content was measured using an oven (934.01). The ash content was determined using a muffle furnace (942.05). The total protein was analyzed by Dumas method with a nitrogen-to-protein conversion factor of 6.25 (968.06). Lipid content was obtained by Soxhlet extraction with hexane. Measurements were performed, at least, in triplicate.

Elementary analysis (Na, Mg, P, S, K, Ca, Mn, Fe, Cu, and Zn) was performed by inductively coupled plasma hyphenated with mass spectrometry (ICP-MS) using an Agilent 7700 Series (Agilent Technologies, USA, Santa Clara). The samples were analyzed by Scientific and Technical Services (SCT) from the University of Lleida (Lleida, Spain).

2.7.2. Degree of hydrolysis (DH)

The degree of hydrolysis (DH) was measured spectrophotometrically using *o*-phthaldialdehyde (OPA) as reactant following the method described by Nielsen et al., (2001) with slight modifications or, alternatively, by the pH-stat method (Adler-Nissen, 1986).

OPA reagent was prepared by dissolving 7.62 g of di-Na-tetraborate decahydrate and 200 mg of SDS in 150 mL of deionized water. Once reagents were completely dissolved, 160 mg of OPA previously dissolved in 4 mL of ethanol, were transferred quantitatively to the solution mentioned above. Finally, 176 mg of dithiothreitol were added and the solution was made up to 200 mL with deionized water. Serine standard was prepared at a concentration of 0.1 mg/mL. For the measurements, the volumes were reduced to 120 µL of sample and 900 µL of OPA reagent. The method is based on the reaction of alfa amino (α-NH) groups with OPA, which gives a compound detectable at λ=340 nm in a UV-Vis spectrophotometer. The reaction can be conducted at RT in a matter of minutes (Rutherfurd, 2010).

To calculate the DH following formula was employed (Equation 3.2):

$$DH\ (\%) = \frac{h}{h_{tot}} \times 100$$

Equation 3.2

where h is the number of hydrolyzed peptide bonds and h_{tot} is the total number of peptide bonds per protein equivalent (estimation of 7.6 meqv/g). The value of h_{tot} was not calculated for PDF. An established value by Adler-Nissen, (1986) for meat samples was used.

DH according to pH-stat method was calculated as follows (Equation 3.3):

$$DH\ (\%) = \frac{h}{h_{tot}} \times 100 = \frac{B\ \times\ N_b}{M_P\ \times\ \alpha\ \times\ h_{tot}} \times 100$$

Equation 3.3

where B is the added base volume (mL), N_b is the normality of the base, M_P is the mass (g) of initial protein (N x 6.25), α is the average degree of dissociation of α-NH groups in the protein substrate (0.897 at pH 8 and 55 °C), and h_{tot} is the total number of peptide bonds in the substrate (estimation of 7.6 meqv/g).

2.7.3. Protein recovery (PR)

Protein recovery (PR) was determined based on the weight of sample in each fraction after hydrolysis and nitrogen determined using Dumas method, following Equation 3.4:

$$PR\ (\%) = \frac{mass\ of\ protein\ in\ each\ fraction\ (g)}{total\ mass\ of\ protein\ in\ the\ substrate\ \ (g)} \times 100$$

Equation 3.4

where the total protein in the substrate was the sum of the protein quantified in the supernatant and the remaining non-hydrolyzed solid precipitate.

2.7.4. Estimation of molecular weight – SDS-PAGE

Molecular weight distribution was obtained under denaturing conditions by electrophoresis with an SDS - polyacrylamide gel (1D SDS-PAGE) using handcast 4% polyacrylamide stacking gel and 15% polyacrylamide resolving gel, as described by Laemmli, (1970). Gel preparation and vertical electrophoresis were carried out using Bio-Rad Mini Protean II system (Bio-Rad Laboratories, Inc., California, US). Between 10 and 15 µL of sample containing 50 µg of protein were mixed with 3.5 µL of Laemmli loading buffer (62.5 mM Tris-HCl pH 6.8, 2% (v/v) SDS, 25% (w/v) glycerol, and 0.01% (w/v) bromophenol blue), and 2 µL of β-mercaptoethanol, heat denatured at 99 °C for 10 min then cooled and centrifuged at 13,000 rpm for 15 seconds. Then, the denatured samples were loaded in the gel and run for 1 h and 120 V in 1X Tris/Glycine/SDS running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) at RT in a Bio-Rad equipment. The SDS-peptide complexes were resolved in the gel based on their size, and to evaluate protein molecular weights 5 µL of two standards protein were used: a low molecular weight marker containing six recombinant proteins from 1.7 to 42 kDa (Thermo Scientific, Waltham, Massachusetts, US) and a broad molecular weight marker containing ten proteins ranging from 10 to 180 kDa (Nippongenetics, Düren, Germany). After electrophoresis, gels were stained with methanol:acetic acid:distilled water (5:1:4) containing 0.1% of Coomassie Brillant Blue R250 for 30 min and destained with methanol:acetic acid:distilled water (1:1:8) three times.

2.7.5. Estimation of molecular weight distribution – SEC

Peptide molecular weight (MW) distribution of hydrolysates and commercial protein hydrolysates was analyzed according to the previous method reported by Fu et al., (2018). Briefly, size exclusion chromatography was carried out using an ultra-high performance liquid chromatography (UHPLC) system

(Thermo Scientific Dionex Ultimate 3000, Denmark) with a Phenomenex BioSep™ SEC-S2000 column (300 mm × 4.6 mm) (Torrance, CA, USA). Ten µL of each diluted (1 mg/mL) and filtered hydrolysate were injected. The separation was under isocratic elution using 30% acetonitrile with 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.2 mL/min and detection at 214 nm. MW standard curves were plotted using the standards Gly-Gly (132 Da), GSH (307 Da), GSSG (613 Da), bacitracin (1423 Da), insulin (5733 Da), and myoglobin (17600 Da). Data were processed and acquired via Chromeleon 7.0 Chromatography Data System software.

2.7.6. Amino acid composition

The amino acid content of samples was determined on freeze-dried and pulverized tissue. Hydrolysis of the samples (50 mg) was carried out by acidic hydrolysis using 5 mL of 6N HCl (110 °C, overnight, under N_2) (Colgrave et al., 2008; Dai et al., 2014). Hydrolysis tubes were cooled and centrifuged at 3,000 *g* for 30 min to remove particulate matter. Aliquots of 25 µL of hydrolysate were evaporated using SpeedVac™ SPD131DDA (Thermo Electron Corporation) and reconstituted in 500 µL of water:acetonitrile (20:80, v/v). Samples were filtered through a 0.22 µm hydrophilic polytetrafluoroethylene (PTFE) membrane prior to injection. The injection volume was 5 µL.

Quantitation of individual amino acids was performed using a method described by Guo et al., (2013) with modifications. UHPLC was performed using a Waters Acquity system (Waters, Milford, MA) equipped with a BEH Amide column (2.1 x 150 mm; 1.7 µm) (Waters, Manchester, UK). The mobile phase consisted of solvent A (10 mM ammonium formate in water with 0.15% formic acid) and solvent B (ammonium formate - saturated acetonitrile with 0.15% formic acid). The gradient elution followed was 15% A and 85% B maintained for 3 min at 0.5 mL/min. Then, from 15% to 20% A in 3 min; from 20% to 24% A in 1.5 min; from 24% to 60% A at 0.6 mL/min in 1.5 min and maintained for 3 min. Finally, initial conditions were regained in 2 min. The flow rate of the phase was 0.5 mL/min, and the column temperature was maintained at 30 °C. The column was cleaned with weak (20% acetonitrile) and strong (80% acetonitrile) washing solvents between injections.

Detection and quantitation of amino acids in the hydrolysate were performed using a Multiple Reaction Monitoring method (MRM) in a Waters Triple Quadrupole Detector (TQD) mass spectrometer (Micromass MS Technologies, Manchester, UK). The system was equipped with an electrospray ionization (ESI) source operated in positive ion mode. Parameters in the source were set as described in the bibliography by Guo et al., (2013). MRM transitions were tested successfully and optimized in our conditions for phenylalanine (Phe), leucine (Leu), isoleucine (Ile), methionine (Met), valine (Val), proline (Pro), hydroxyproline (Hyp), tyrosine (Tyr), alanine (Ala), threonine (Thr), glycine (Gly), glutamic acid (Gln/Glx), serine (Ser),

aspartic acid (Asp/Asx), histidine (His), arginine (Arg), lysine (Lys), and cysteine (Cys). Tryptophan (Trp) was not determined as it was totally degraded during the hydrolysis conditions. Cone voltage and collision energy were optimized for each individual amino acid. A stock solution containing a commercial amino acid standard mixture and Hyp were serially diluted in water:acetonitrile (20:80, v/v) to prepare a 7-point standard curve. Data were processed using QuanLynx software. Amino acids were quantified from absolute response without internal standard and results were expressed as mg amino acid/g sample.

2.7.7. Calculation of nitrogen-to-protein conversion factors (NPCF)

Nitrogen-to-protein conversion factors (NPCF), *k*, were calculated following the methodology described in the literature (Mossé, 1990; Sosulski & Imafidon, 1990; Sriperm et al., 2011) with slight modifications. Based on the total nitrogen determined by Dumas method and the amino acids quantification (Hyp was not included in the calculation), k_A, k_P, and *k* were calculated from $\sum E_i$, $\sum D_i$, and N_L, while *k* is an average of k_A and k_P.

A first definition was established for k_A (Equation 3.5):

$$k_A = \frac{\sum_{i=1}^{17} E_i}{\sum_{i=1}^{17} D_i}$$

Equation 3.5

where $\sum E_i$ is the sum of anhydrous amino acids residues and E_i is the mass of anhydrous amino acid residue (g) of the *i*th amino acid kg^{-1} DM (Equation 3.6). Mass of an anhydrous amino acid residue was calculated by reducing the assay weight to account for the loss of a water molecule that occurs during polymerization. $\sum D_i$ is the sum of the total N content from each of the amino acids reported and D_i is the mass of N (g) of the *i*th amino acid kg^{-1} DM (Equation 3.7). Amide N ($NH_{3(N\ atom)}$) from Asn and Gln residues was not included.

$$\sum_{i=1}^{17} E_i = \sum \left[AA_{i,g\ kg^{-1}}\ x\ \left(\frac{AA_{i(MW)} - H_2O_{(MW)}}{AA_{i(MW)}} \right) \right]$$

Equation 3.6

$$\sum_{i=1}^{17} D_i = \sum \left[AA_{i,g\ kg^{-1}}\ x\ \left(\frac{AA_{i(N\ atom)}\ x\ NN_{(MW)}}{AA_{i(MW)}} \right) \right]$$

Equation 3.7

Another useful factor corresponds to k_P (Equation 3.8), which was defined as:

$$k_P = \frac{\sum_{i=1}^{17} E_i}{N_L}$$

Equation 3.8

where N_L is the total N determined by Dumas.

Finally, the factor k is an average of k_A and k_P (Equation 3.9):

$$k = \frac{k_A + k_P}{2}$$

Equation 3.9

Total (crude) protein content was estimated by multiplying N_L by NPCF of 6.25. True protein content was determined from the sum of all the amino acids. Nitrogen recovery (NR) was calculated as follows (Equation 3.10):

$$NR = \frac{\sum D_i}{N_L}$$

Equation 3.10

2.7.8. Fourier transform infrared (FTIR) spectroscopy

FTIR analysis was performed to characterize the functional groups present in the initial by-product and the further fractions obtained. The analyses were carried out using a Jasco FT/IR-6300 spectrometer. A total of 16 scans were completed from 650 to 4000 cm^{-1} at a resolution of 4 cm^{-1} with a diamond ATR sampler.

2.7.9. Volatile compounds analysis

The volatile compounds were analyzed following the methodology described by Tan et al., (2018) with slight modifications. Gas chromatography coupled to mass spectrometry (GC-MS) (6890N GC system interfaced with a 5973 MSD, Agilent Technologies, Palo Alto, CA, USA) together with manual injection headspace-solid phase microextraction (HS-SPME) system was used to detect the compounds in the different samples. One gram of freeze-dried sample was transferred to a 10 mL glass vial (previously maintained at 70 °C). After 30 min the SPME syringe with a polydimethylsiloxane (PDMS) fiber was

exposed in the upper space of the vial at 70 °C to absorb the volatile compounds. After 30 min of exposition, the fiber was withdrawn and transferred immediately to the gas chromatograph injector port at 250 °C for 10 min for thermal desorption. The temperature ramp was set as follow: initial temperature at 40 °C with a 3 min hold, ramped to 70 °C at 2.5 °C/min, then increased to 150 °C at 8 °C/min and finally increased by a ramp of 20 °C/min to 260 °C and held for 10 min. A DB-5MS UI capillary column (5% phenylmethylsiloxane, 30m x 0.25 mm, film thickness 0.25 µm, Agilent, USA) was used for the separation. The instrument detector was operated in electron ionization (EI) mode with an ionization voltage of 70 eV. Data were recorded in full scan mode (35 - 500 amu). The carrier gas used was helium at a constant flow rate of 1.5 mL/min. The front inlet was kept at 250 °C in splitless mode. Software Enhanced Data Analysis was used for data acquisition. Peak areas and mass spectra were extracted from the chromatograms using the Data Analysis software (Agilent ChemStation) followed by identification of mass spectra employing NIST11 database. The Retention Index (RI) of each compound detected was calculated following the methods of van Den Dool and Kratz (van Den Dool & Dec. Kratz, 1963). Comparison with retention indices reported by NIST (NIST Chemistry WebBook, 2018) was used for confirmation of the volatile compound identifications.

2.8. Statistical analysis

Statistical calculations were carried out with JMP Pro 14 statistical software (Statistical Discovery™ from SAS, Cary, NC, USA). The values were expressed as means ± standard deviations (SD) of at least triplicate samples. Normality of the data was evaluated by Shapiro-Wilk normality test. The results were subjected to analysis of variance (ANOVA) to compare the mean values. Statistical significance was assessed with the *p*-value with a 95% confidence level ($p < 0.05$) and Tukey's honestly significant difference (HSD) test.

For the RSM, analysis of the experimental data and calculation of predicted data were carried out using JMP Pro 14 to evaluate the effect of each independent variable to a response. The Student *t*-test ($\alpha = 0.05$) was employed to determine the statistical significance of the coefficients.

3. Results and discussion

3.1. Pre-treatment and partially defatted *fines* (PDF) characterization

Homogenized *fines* were treated with hot water based on our previous results (Chapter 2). This pre-treatment of the material allows the partial removal of fat from the *fines* (which can be used for other purposes) using the greenest and less costly methodology. Furthermore, warm-water collagen can be

recovered. In consequence, the proteinaceous solids containing non-soluble protein, from now on called partially defatted *fines* (PDF) were obtained from *fines*, separating them from fat that can interfere in the enzymatic process. Figure 3.1 shows the raw material and different fractions recovered after the thermal treatment and centrifugation. The chemical analysis of physically defatted dry material indicates the loss of close to half of total fat in the original material. The protein content experienced an increase from 45.8 ± 0.5% to 57.7 ± 1.7% based on DM. The ash content, moisture, and C/N ratio were 16.9 ± 1.0%, 56.7 ± 5.2%, and 5.1 ± 0.1%, respectively.

Fines PDF Warm-water collagen Fat

Figure 3.1. *Fines* and the fractions recovered after thermal treatment: PDF, warm-water collagen, and fat.

As previous investigations pointed out (Bhaskar et al., 2007; Šližyte et al., 2005a), the amount of lipids in the material has a significant impact on the hydrolysis process. Partial removal of fat results in a sharp reduction of viscosity, which allows a higher DH (Bhaskar et al., 2007). Šližyte et al., (2005a, 2005b) pointed out that the highest amount of lipids in the raw material gave the lowest percentage of solubilized proteins. Besides this, the amount of emulsion, which is not a desired fraction after hydrolysis, is negatively linearly correlated to the amount of protein (Šližyte et al., 2005b). Furthermore, hydrolysates with low lipid content are desired to enhance product stability (Nilsang et al., 2005).

3.2. Optimization of hydrolysis with Alcalase 2.4L

Process optimization is a topic of central importance. The focus in this section was to find the optimal conditions to maximize the DH, since a high DH usually corresponds to very low MW peptides, high content in free amino acids, increased recovery yield of protein-nitrogen, and improved solubility of the substrate (Lynch et al., 2018). Many factors, such as temperature, time, E/S ratio, and pH affect the course of the proteolysis and are essential for optimizing the activity of the enzymes (Etemadian et al., 2021; Gbogouri et al., 2004; Lasekan et al., 2013). Hydrolysis reactions were performed in the different conditions previously established in Material and methods (Section 2.3) by mixing the proteinaceous material with water or phosphate buffer. This step was necessary in order to allow more surface area for

the action of the enzyme and to enhance enzyme homogeneity, promote tissue swelling, and reduce the localized concentration of hydrolysis products (Benjakul & Morrissey, 1997). Moreover, pH is a relevant point in the enzymatic hydrolysis with several enzymes, such as alcalase, as the proteolysis changes the pH of the medium, affecting the activity of the enzyme (Sousa et al., 2004). The enzymatic reactions were followed by measuring the DH. DH is an indicator of broken peptide bonds during hydrolysis, and it is a primary response in determining the optimal conditions and obtaining protein hydrolysates with different functionalities (de Queiroz et al., 2017). In this study, the effects of pH, time, and E/S ratio were evaluated.

3.2.1. Effect of pH on DH

Hydrolysis reactions were performed over 24 h to evaluate the impact of pH conditions: uncontrolled pH (no adjustment of pH during the process but initially set up at 8), controlled pH (adjustment of pH at specific times), and buffer. The hydrolysis curves under the different pH conditions with 1 and 5% E/S ratios were compared and results are shown in Figure 3.2. It is clear that pH influenced and had a significant effect on DH, regardless of the E/S ratio. The DH increased when augmenting the incubation time and the higher DH was obtained when the pH was kept at the optimal pH (pH 8) and a higher amount of enzyme was added. Meanwhile, during the hydrolysis without pH control, under both E/S ratios, the pH dropped from 8.0 – 8.5 to 6.5 – 6.7 until the end of the process; therefore, the reaction changed the pH of the reaction medium. As there is a significant relation between pH and DH (Benjakul & Morrissey, 1997; Ovissipour et al., 2013), it is not surprising that the pH stabilization is correlated to DH stationary phase. The activity of Alcalase 2.4L is sensitive to pH and although Carvalho et al., (2017) mentioned an optimal pH range of 6.5 – 8.5, the pH instability of the medium (Figure S3.1 in Supplementary data) might affect enzyme stability by causing irreversible denaturation on its conformational structure, resulting in a continuous loss of enzyme activity. Hence, this could be one of the reasons for the lower DH obtained. This decrease in pH is due to the release of H^+ owing to the cleavage of peptide bonds. When the pH of the system is close to 7, the α-carboxyl groups (pKa 3.1) from peptides are fully deprotonated, while α-amino groups (pKa 7.3) are partially protonated, resulting in a buffering effect and maintaining pH constant for a while (Carvalho et al., 2017; Sousa et al., 2004). Although the use of buffer solutions has been reported for the enzymatic hydrolysis with alcalase (Anzani et al., 2018; Benjakul & Morrissey, 1997; Nam et al., 2008; Valencia et al., 2014), the obtained results indicate a lower activity of the enzyme in this medium. This can be due to the lack of buffering capacity for the reaction.

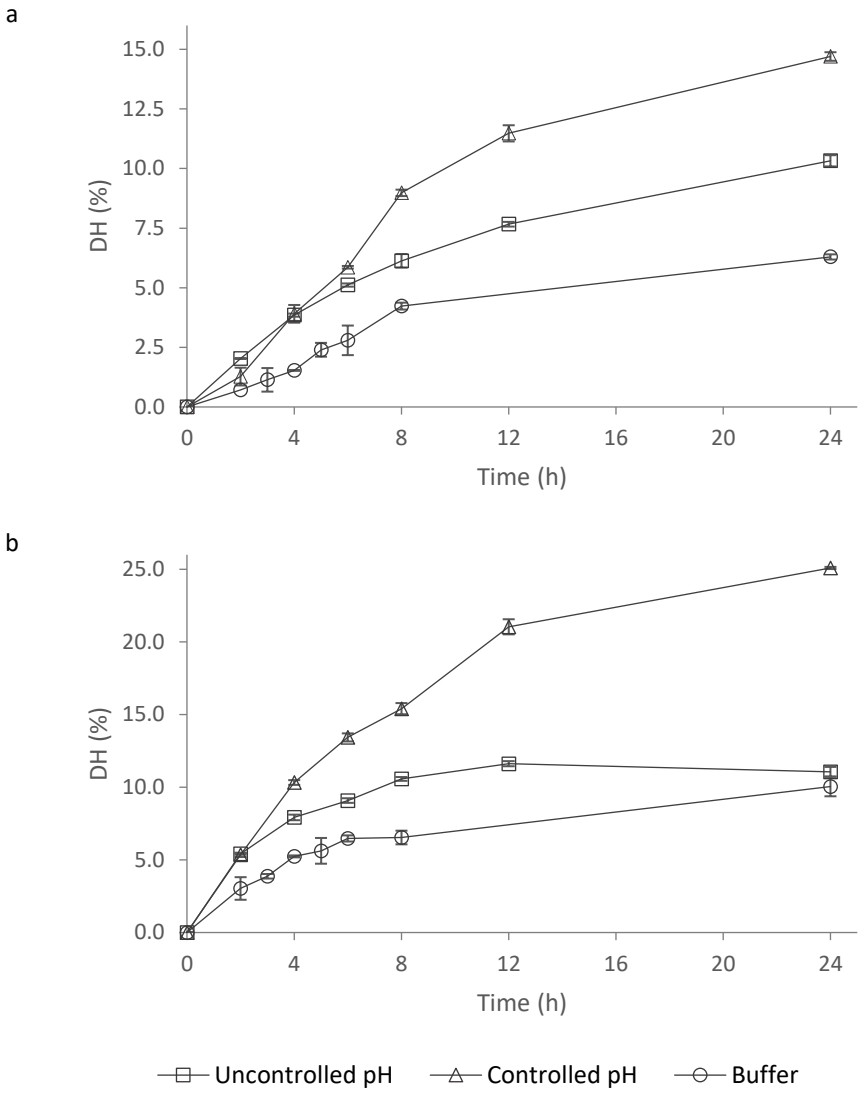

Figure 3.2. Hydrolysis curves (DH *vs.* time) of PDF with a) 1% E/S ratio and b) 5% E/S ratio under different pH conditions. The symbols correspond to the values obtained experimentally while the lines are drawn for illustration purposes.

Experimental results show that medium pH is a key parameter in the enzymatic hydrolysis of PDF from *fines*. Controlling the pH during the process gave the highest DH and it was selected as optimal condition for further experiments. These results are in agreement with other studies (Abdul-Hamid et al., 2002; Chalamaiah et al., 2010; Fallah et al., 2015; Garcia et al., 2011; Guérard et al., 2001; F. G. Hall et al., 2017; Klompong et al., 2007; Shahidi et al., 1995; Vázquez et al., 2020), where no optimization of pH was performed but it was kept constantly around 8 – 8.5 to succeed in the enzymatic hydrolysis with serine proteases.

3.2.2. Effect of E/S ratio on DH

The effect of the E/S ratio was investigated once the optimal pH condition was selected. The hydrolysis curves obtained for different enzyme concentrations are displayed in Figure 3.3. As observed, the DH at E/S ratios of 5 and 10% were significantly higher than DH at 1% E/S ratio. Both 5 and 10% E/S ratios provided a similar rate of hydrolysis and prolonging the reaction beyond 24 h did not produce a remarkable improvement in the DH. A slightly higher final DH value (26%) was obtained with the relation E/S of 5% and a reaction time of 48 h. Nevertheless, the differences between 24 and 48 h and 5% and 10% were not very remarkable. Therefore, increasing enzyme concentration over 5% does not affect DH. This dose-response effect indicates that higher enzyme dosages did not further improve yields. A similar dose-response effect was described by Lapeña et al., (2018) in different substrates with alcalase. Noman et al., (2018) also observed a slight decrease in DH with E/S ratio beyond 3% with pepsin, attributing this decline to enzyme aggregation, which leads to an increase in substrate diffusion inhibition, causing the saturation of the reaction rate. Hence, an E/S ratio of 5% was selected as optimum for further experiments.

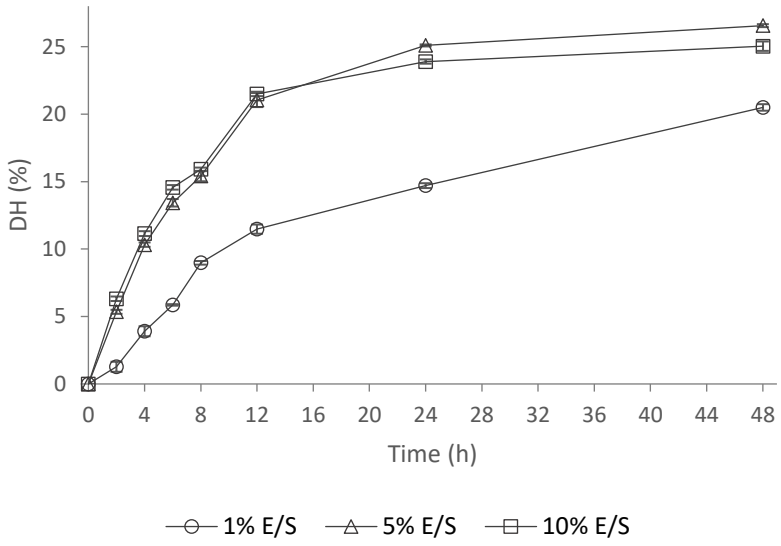

Figure 3.3. Hydrolysis curves (DH *vs*. time) of different E/S ratios.

No reports were found in the literature using the same enzyme and *fines* as a substrate to enable the DH comparison, although it is not surprising that the enzymatic hydrolysis of PDF takes about 24 h to achieve a DH of 25% compared with other raw meat by-products. For instance, Kurozawa et al., (2008) reported the hydrolysis of chicken meat up to a DH of 31% in less than 6 h under similar conditions. Whereas, Webster et al., (1982) found that heated lung samples were less solubilized and more challenging to defat

than their raw counterparts. The treatment at elevated temperatures promotes the formation of protein-fat interactions, thus hindering their separation and therefore, protein hydrolysis.

Given the nature of the substrate, which consisted mainly of insoluble peptides and proteins, the reaction obeys a sequence of two first-order processes (Kristinsson & Rasco, 2000a; Liaset et al., 2000). In the first stage, the loosely bond polypeptides chains are cleaved from insoluble protein peptides quickly, whereas, in the later stage, the more compacted core proteins were hydrolyzed more. Three main hypotheses have been proposed to explain the downward tendency of the hydrolysis curve: i) a decrease in the concentration of available peptide bonds, ii) enzyme inhibition by proteolysis products, and iii) enzyme inactivation (Guérard et al., 2001; Qi & He, 2006; Valencia et al., 2014). In this study, the same substrate concentration and different E/S ratios tend to a similar profile, although to different limit values. Hence, the concentration of peptide bonds is not clear to be a key factor. The inhibition by product was not determined; nevertheless, it is a relevant factor for the technological application of enzymatic hydrolysis. Withdrawal of products can improve the efficiency of the process when product inhibition is observed (Valencia et al., 2014). Enzyme inactivation could have a significant effect on the hydrolysis curve due to the long process and pH variations of the media, which may reduce enzyme activity. However, further work is needed to elucidate the underlying causes. Guérard et al., (2001) determined that for protein hydrolysis of cooked tuna, which consisted largely of insoluble proteins, the main factor controlling the hydrolysis rate was the concentration of hydrolyzable bonds. However, Valencia et al., (2014) pointed out that some experiments were not properly designed to test the hypothesis and in the case of fresh salmon, the responsible for the typical shape of the hydrolysis curve was the inhibition of the reaction by hydrolysis products.

Table 3.3 shows the relation between $\log_{10}$ (E/S) and DH after 4, 8, 12, and 24 h of reaction. A linear relation was observed for almost all the E/S ratios where the correlation coefficients R^2 showed values above 0.8. This relationship, previously reported in the literature (Benjakul & Morrissey, 1997; Khantaphant & Benjakul, 2008; Kristinsson & Rasco, 2000b; Pagán et al., 2013) allows the determination of the exact concentration of enzyme or E/S ratio needed to hydrolyze the substrate to a specific DH in different times under the optimized conditions. However, extrapolation out of the range of the experimental points can lead to inaccurate results.

Table 3.3. Coefficient values (a and b) at different times. Linear model DH = $a + b \cdot \log(E/S)$.

Time (h)	*a* (%)	*b* (%)	R^2
4	4.17	7.58	0.96
8	9.28	7.33	0.95
12	11.96	10.67	0.94
24	15.45	10.20	0.84

3.2.3. Effect of time on DH and PR

After considering different conditions of medium pH and E/S ratios, the effect of time over DH and PR was evaluated under the optimal conditions. DH and the corresponding PR of the hydrolysates after 4, 8, 12, and 24 h of enzymatic hydrolysis are presented in Figure 3.4a. Figure 3.4b shows the PR and C/N of the remaining non hydrolyzed solid. After 4 h of hydrolysis, about 50% of the initial nitrogen was solubilized and recovered in the supernatant and the DH was around 12%. After 8 h of hydrolysis, significant changes in the PR and DH were observed. After 12 h of incubation, no significant increase in PR occurred, while DH increased from 16 to 22%. Since the soluble protein/peptides increased only during the first 8 h of hydrolysis, the DH increase in the next 4 h indicates that the enzyme attacks already solubilized peptides, decreasing their molecular weight. This fact clearly indicated that this subtilisin is very effective in hydrolyzing the protein material present in PDF. As other authors already concluded, alcalase is often the best candidate due to its broad catalytic specifications and higher protein recoveries (Adamson & Reynolds, 1996; Dey & Dora, 2014; Lapeña et al., 2018; Piazza & Garcia, 2014). The values of DH are in accordance with reported values for alcalase from Novo Nordisk, 15 – 25% (Liaset et al., 2000). Piazza & Garcia, (2014) reported the recovery of 49.7% of the protein from MBM, while other studies with alcalase showed protein recoveries up to 70% from fish (Liaset et al., 2000; Ovissipour et al., 2013; Shahidi et al., 1995) and up to 90% from chicken meat (Kurozawa et al., 2008). Although no significant differences were observed for PR and DH between 12 and 24 h, the latter hydrolysis time was selected for protein hydrolysates preparation using Alcalase 2.4L for further experiments.

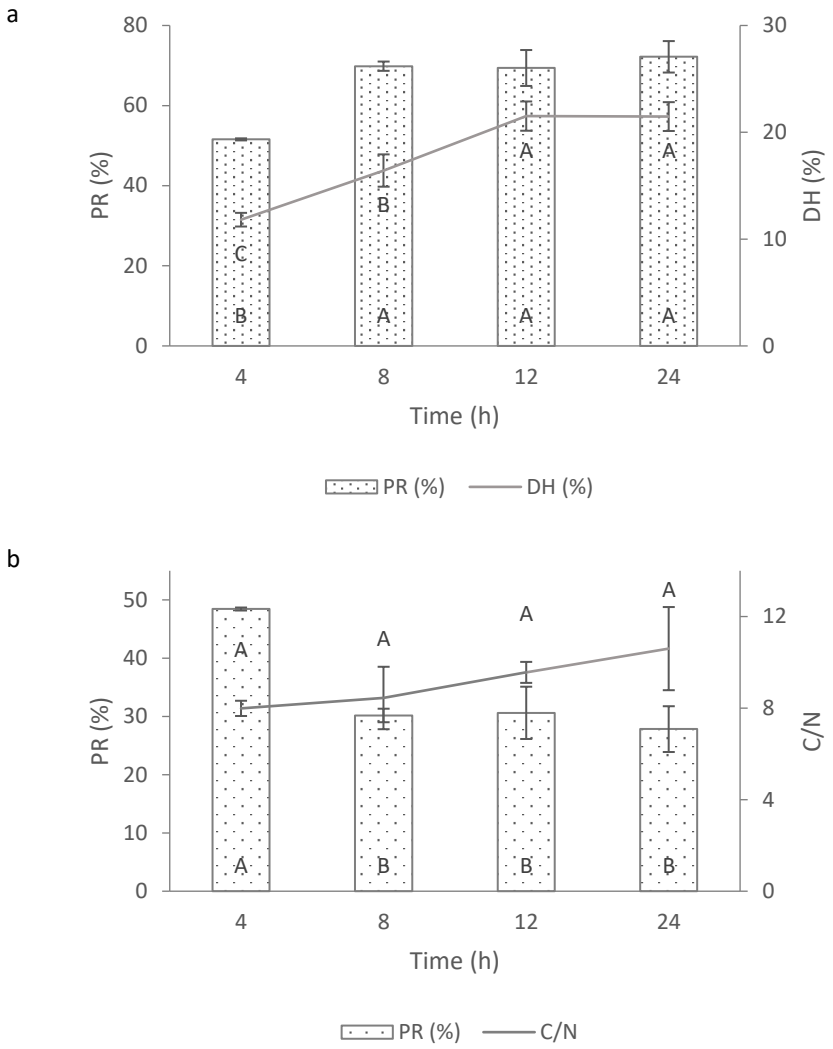

Figure 3.4. Protein recovery, degree of hydrolysis, and C/N ratios obtained with enzymeAlcalase 2.4L under optimal conditions of pH and E/S ratio in the fractions a) recovered supernatant (HA hydrolysate) and b) remaining non-hydrolyzed solid (precipitate). Different letters indicate significant differences ($p < 0.05$).

3.3. Optimization of hydrolysis with Neutrase 0.8L

RSM is a statistical technique that has been successfully used in the optimization of several processes (Bhaskar et al., 2008; Bhaskar & Mahendrakar, 2008; de Queiroz et al., 2017; Dey & Dora, 2014; Jiao et al., 2014; Yusoff et al., 2016; Zhang et al., 2016). The application of statistical experimental design techniques reduces the resources needed (Kampen, 2014; Puri et al., 2002). Given the high number of experiments required to determine the optimal E/S ratio and pH conditions to obtain the higher DH with Alcalase 2.4L, RSM was selected for further experiments with Neutrase 0.8L.

3.3.1. OFAT experiments

In this study, RSM has been used to optimize the enzymatic hydrolysis of PDF with Neutrase 0.8L. First, OFAT experiments were performed to select the working range for CCD experimental design. OFAT experiments data is shown in Figure 3.5.

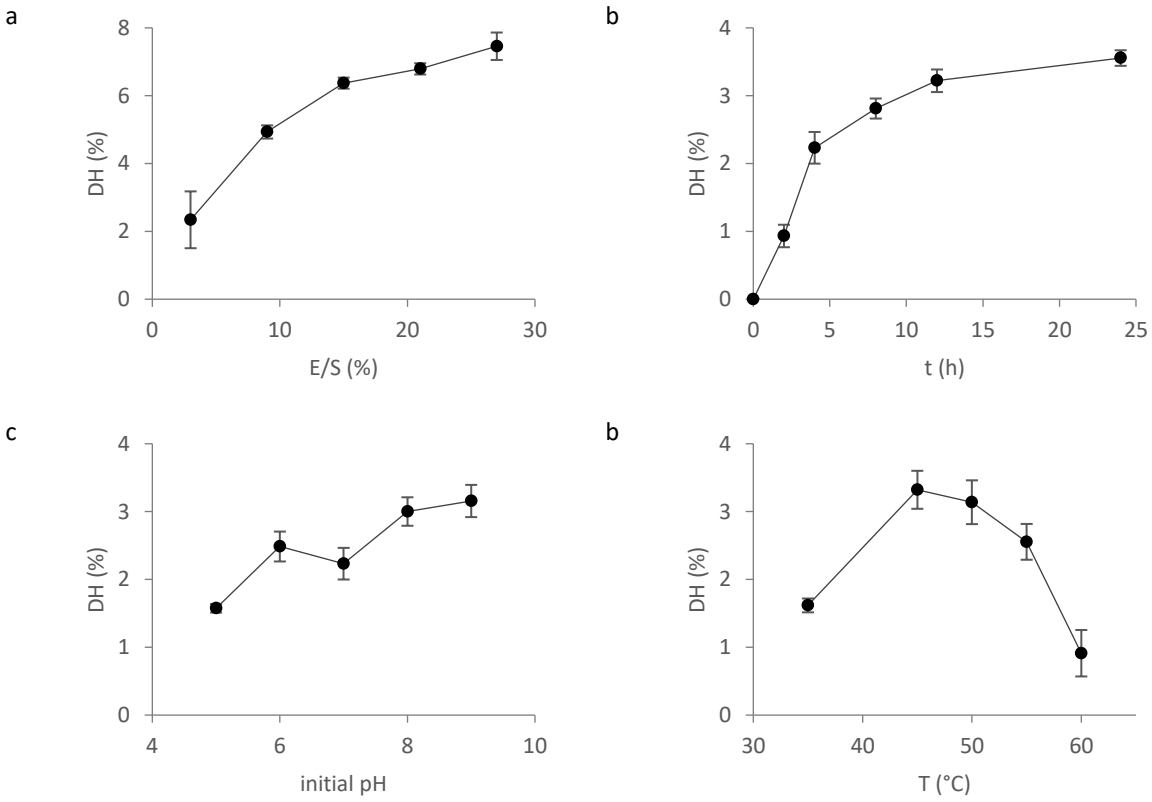

Figure 3.5. Main effects plots of OFAT experiments on a) DH *vs.* E/S ratio, b) DH *vs.* incubation time, c) DH *vs.* initial pH, and d) DH *vs.* hydrolysis temperature. Data are expressed as mean ± standard deviation of triplicate determinations.

As observed, at a low concentration of E/S ratio (3%) the DH was 2%. When increasing the E/S ratio, DH was gradually increased up to 8%. An E/S ratio of 10% was selected as the center point. As expected, the DH increased with the progress in the reaction time. However, incubation times longer than 12 h did not improve DH substantially. Thus, 6.5 h was selected as the center point. Higher DH values were obtained at pH values of 8 and 9. However, the differences were rather small and an initial pH value of 7 was selected as the center point. The reaction temperature had an important impact on the DH. Reaction temperature between 40 and 50 °C resulted in a higher DH, and 45 °C was selected as the center point.

3.3.2. RSM optimization of hydrolysis parameters

The influence of E/S ratio (X_1), incubation time (X_2), initial pH value (X_3), and temperature (X_4) on the hydrolysis by the neutral protease was determined using a CCD experimental design. The experimental and predicted values for DH at different combinations of the four independent variables are displayed in Table 3.4.

Table 3.4. CCD for the enzymatic hydrolysis of PDF fraction with enzyme Neutrase 0.8L.

	Factors				Response	
Run	**X_1**	**X_2**	**X_3**	**X_4**	**$DH_{experimental}$ (%)**	**$DH_{predicted}$ (%)**
1	-1	-1	-1	-1	0.00	-0.15
2	-1	-1	-1	1	0.49	0.47
3	-1	-1	1	-1	0.68	0.64
4	-1	-1	1	1	0.85	0.52
5	-1	1	-1	-1	3.92	3.86
6	-1	1	-1	1	2.14	2.43
7	-1	1	1	-1	4.00	4.34
8	-1	1	1	1	2.44	2.17
9	1	-1	-1	-1	1.89	2.08
10	1	-1	-1	1	3.24	2.80
11	1	-1	1	-1	3.95	3.57
12	1	-1	1	1	3.58	3.56
13	1	1	-1	-1	6.66	6.90
14	1	1	-1	1	5.63	5.58
15	1	1	1	-1	8.15	8.09
16	1	1	1	1	5.97	6.03
17	-1.48	0	0	0	1.72	1.80
18	1.48	0	0	0	6.07	6.31
19	0	-1.48	0	0	0.00	0.72
20	0	1.48	0	0	5.93	5.52
21	0	0	-1.48	0	4.41	4.33
22	0	0	1.48	0	4.85	5.25
23	0	0	0	-1.48	4.19	4.05
24	0	0	0	1.48	2.53	2.98
25	0	0	0	0	5.07	4.56
26	0	0	0	0	4.70	4.56
27	0	0	0	0	4.54	4.56
28	0	0	0	0	4.61	4.56
29	0	0	0	0	4.46	4.56

X_1 – E/S ratio; X_2 – time; X_3 – initial pH, and X_4 – temperature

The shapes of the 3D response surface plots representing the DH are illustrated in Figure 3.6, depicting the influence of two independent variables on the DH while the other two were fixed at their central levels. It is evident that with increasing E/S ratio (X_1) and the incubation time (X_2) the DH gradually increased (Figure 3.6a). However, regardless of E/S ratio (X_1), time (X_2), and pH (X_3) values, when the temperature (X_4) increased, the DH decreased (Figure 3.6b, c, and d). Hence, under the conditions studied, the enzymatic reaction with Neutrase 0.8L requires a temperature in the coded range between –1.48 and –0.5, corresponding to 37.6 and 42.5 °C, respectively. This might be due to the denaturation of the enzyme at higher temperatures. On the other hand, DH increased with an increase in the initial pH value (X_3) (Figure 3.6e and f).

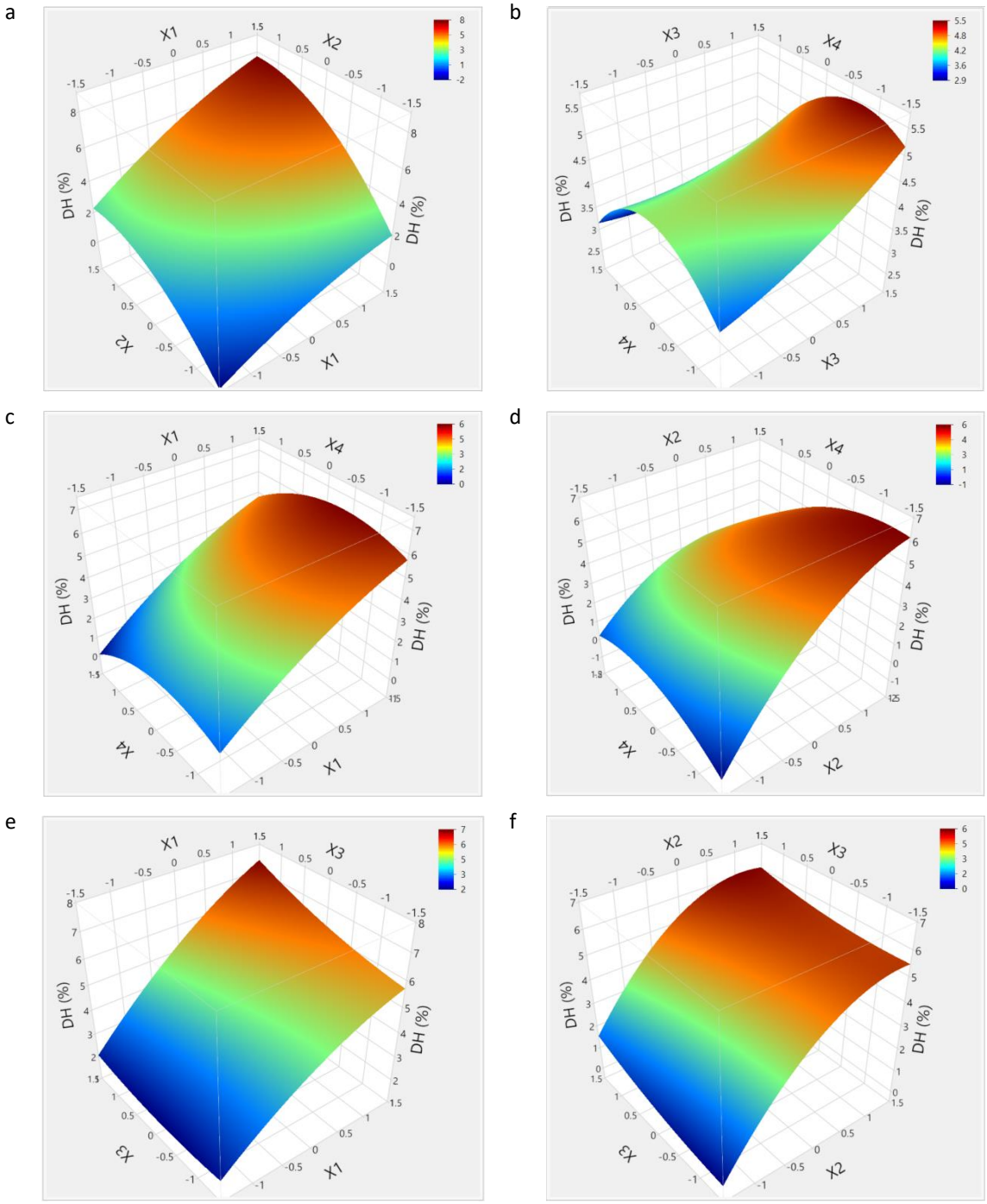

Figure 3.6. Response surface plots showing the effects of independent variables E/S ratio (X_1), time (X_2), initial pH (X_3), and temperature (X_4) on DH in enzymatic hydrolysis of PDF with enzyme Neutrase 0.8L: a) E/S ratio *vs.* time; b) temperature *vs.* initial pH; c) temperature *vs.* E/S ratio; d) temperature *vs.* time; e) initial pH *vs.* E/S ratio, and f) initial pH *vs.* time.

ANOVA is presented in Table 3.5. As previously mentioned, the statistical significance has a threshold of 0.05. A *p*-value < 0.05 indicates that the variable or model term is considered significant. The *p*-value for the model is smaller than 0.05 (*p*-value < 0.001) and the F value is several times higher than F tabulated ($F_{0.05}^{14,14} = 2.484$) for α = 0.05, indicating that the variation accounted by the model is significantly greater

than the unexplained variation (Dey & Dora, 2014) and the model is statistically significant. Moreover, the Lack of fit is used to test the fitness of the model (Zhang et al., 2016) and this value should be insignificant (Montgomery, 2012). As expected, the *p*-value for this parameter is higher than 0.05 (*p*-value = 0.1122); therefore, the model fitted the experimental data and suggests that the model is sufficiently accurate to predict the DH for any combination of experimental independent variables. The coefficient of determination ($R^2_{adjusted}$) of the model is 0.9630. It is satisfactory because it disclosed that about 96% of the variation in behavior within the range of values studied could be explained by the independent variables. Less than 4% of the variation is unexplained or due to experimental error.

Table 3.5. Analysis of variance (ANOVA) for the response surface quadratic model.

Source	DF	Sum of squares	Mean square	F value	*P*-value Prob>F
Model	14	120.73680	8.62406	53.0334	<0.001
Residual	14	2.27662	0.16262		
Lack of fit	10	2.0512963	0.205130	3.6416	0.1122
Pure error	4	0.2253200	0.056330		
C. Total	28	123.01341			
R^2=0.9815; $R^2_{adjusted}$=0.9630; $R^2_{predicted}$=0.9064					

The experimental data obtained were fitted to a second-order polynomial model as a function of the studied independent variables and their interactions to describe the behavior of the system. The equation was derived using the constant, linear, and quadratic regression coefficients and it is as follows (Equation 3.11):

$$y = 4.56 + 1.52X_1 + 1.62X_2 + 0.31X_3 - 0.36X_4 + 0.20X_1X_2 + 0.18X_1X_3 - 0.08X_2X_3 + 0.03X_1X_4 - 0.51X_2X_4 - 0.19X_3X_4 - 0.23{X_1}^2 - 0.65{X_2}^2 + 0.10{X_3}^2 - 0.47{X_4}^2$$

Equation 3.11

The magnitude of the coefficients is directly proportional to the importance of variables and their effects (Cheong et al., 2018). It is in concordance with Table 3.6, where the F value of each parameter and their interactions are described. The higher coefficient and the higher F value are observed for X_2, while the smaller values are observed for the interaction between X_2 and X_3. Negative signs of regression coefficients of quadratic terms (X_1, X_2, and X_4) emphasized the existence of local maximum of DH, as pointed out by Elmalimadi et al., (2017).

3.3.3. Effect of parameters

The significance of each factor on the dependent parameter is shown in Table 3.6. The same criterium was followed, *p*-value < 0.05 indicates that the variable or the interactions are significant. As observed, all the linear factors, the quadratic effects of X_2 and X_4 variables, and the interactions X_2X_4 and X_3X_4 were significant. All the other interactions were not significant. The most influential factors were the X_1 (E/S ratio) and X_2 (time), being F value an indicator of this. Compared to OFAT experiments, in this model, we can observe the significance of the interactions and the quadratic effects.

Table 3.6. Effect test of the independent variables and their interactions.

Source	DF	Sum of squares	F value	*p*-value Prob>F
X_1	1	47.11452	289.7297	<.0001
X_2	1	53.46283	328.7685	<.0001
X_3	1	1.947405	11.9755	0.0038
X_4	1	2.663886	16.3815	0.0012
X_1X_2	1	0.668306	4.1097	0.0621
X_1X_3	1	0.493506	3.0348	0.1034
X_2X_3	1	0.094556	0.5815	0.4584
X_1X_4	1	0.012656	0.0778	0.7843
X_2X_4	1	4.192256	25.7802	0.0002
X_3X_4	1	0.551306	3.3902	0.0869
X_1^2	1	0.573496	3.5267	0.0814
X_2^2	1	4.600718	28.292	0.0001
X_3^2	1	0.11518	0.7083	0.4142
X_4^2	1	2.419759	14.8803	0.0017

X_1 – E/S ratio; X_2 – time; X_3 – initial pH, and X_4 – temperature

3.3.4. Optimum conditions

The optimum conditions were extracted by employing the desirability profile with a maximum desirability level of 0.89 (a value of 1 indicates the highest desirability), as shown in Figure 3.7. Optimum DH or the highest DH possible, about 8.1 ± 0.7%, can be obtained with an E/S ratio (X_1) of 1, a hydrolysis time (X_2) of 1, initial pH (X_3) of 1, and a temperature (X_4) of -1. The real values were E/S ratio (X_1) of 15%, a hydrolysis time (X_2) of 10.5 h, initial pH (X_3) of the substrate 8, and a temperature (X_4) of 40 °C. The DH, as well as desirability level, were reduced significantly at an E/S ratio below 15% and a hydrolysis time of 10.5 h.

A higher E/S ratio could have a positive effect on the DH, but due to enzyme cost, 15% was selected to ensure economic feasibility if the process might be transferred to the industry. Meanwhile, DH was less affected by the variables initial pH and temperature, and it is in concordance with the F value displayed in Table 3.6. The DH ranged between 7 and 8% in the experimental pH levels, while temperatures higher than 45 °C decreased the response.

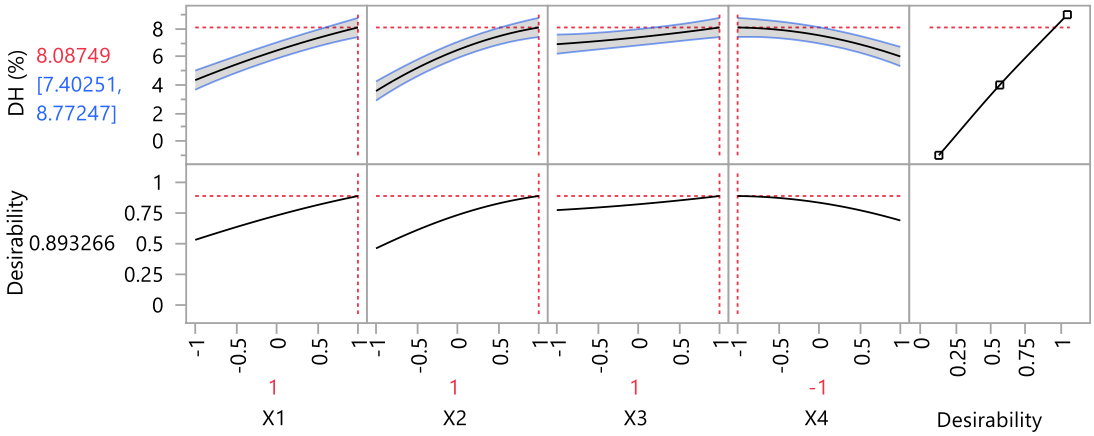

Figure 3.7. Profiles for the predicted DH and the desirability level for different factors (X_1 – E/S ratio; X_2 – time; X_3 – initial pH, and X_4 – temperature) for optimum DH for hydrolysis of PDF with Neutrase 0.8L.

To the best of our knowledge, this is the first time that RSM has been applied in the hydrolysis of this substrate. Several authors have previously reported the use of RSM in enzymatic hydrolysis, experimenting high correlation of experimental results with those predicted by RSM. Dey & Dora, (2014) and Zhang et al., (2016) used RSM to optimize shrimp waste hydrolysis using alcalase. Bhaskar et al., (2008) and Bhaskar & Mahendrakar, (2008) used RSM in the hydrolysis optimization of visceral waste proteins of catla (*Catla catla*) using alcalase and a neutral protease. Cheong et al., (2018) investigated chicken feather valorization by thermal alkaline pre-treatment followed by enzymatic hydrolysis optimization through RSM. Hence, is a very useful and widely used technique.

3.3.5. Validation of the predictive model

Upon statistical optimization, the JMP Pro 14 software predicted the highest DH using 15% of E/S ratio, an incubation time of 10.5 h, an initial pH value of 8, and the temperature of incubation 40 °C. The validity of the theoretical model was confirmed under the predicted optimal conditions. A DH value of 7.2 was observed from the experiments under the specified conditions. DH value was a good fit for the value forecasted (8.1%) by the regression model. This finding proved the validity of the prediction model and that the hydrolysis conditions achieved by RSM were reliable and practical.

3.4. Characterization of the protein hydrolysates

3.4.1. Macrocomponents and elementary analysis

The proximate and mineral element composition based on the dry weight of the substrate, the protein hydrolysates, and their corresponding solid precipitates is detailed in Table 3.7. As expected, a considerable increase in protein content was observed as a result of protein solubilization and the removal of insoluble non-protein substances in the freeze-dried hydrolysates when compared to *fines*, from 46% to 70%, regardless of the enzyme used. These hydrolysates could be used as a good source of protein. Protein recoveries were 70 and 40% for Alcalase 2.4L and Neutrase 0.8L, respectively. This undoubtedly indicates the higher efficiency of Alcalase 2.4L in hydrolyzing proteins. Lipid content was reduced compared to the substrate, showing that the centrifugation step was efficient in purifying the protein hydrolysate (Kurozawa et al., 2008). Moreover, reducing fat content is beneficial for the hydrolysates, as it might significantly increase the stability of the material towards lipid oxidation while contributing to enhance product stability (Dey & Dora, 2014; Ovissipour et al., 2009; Shahidi et al., 1995). A greater reduction of fat content was observed in the hydrolysis with Neutrase 0.8L, although the lipid content in HA can be considered satisfactory. The higher amount of fat in HA can be attributed to fat globules present in the supernatant after removing non-hydrolyzed material (Chalamaiah et al., 2010). As Neutrase 0.8L is less efficient in hydrolyzing the substrate, lipids might have been excluded with the insoluble protein fraction by centrifugation (Nilsang et al., 2005). Although a high ash content was expected in the hydrolysates most likely due to the addition of alkali during pH control (Gbogouri et al., 2004; Hall et al., 2017; Kristinsson & Rasco, 2000a), lower ash content was observed in the hydrolysates. In analogy to what was observed by Garcia et al., (2011) in the case of the ash content, the hydrolysates contained less than 10%. Very high ash content was observed in the sludge, indicating that a substantial fraction of these precipitates is composed of particles of insoluble bone. Ash content is slightly higher in HA than HN. This might be due to the higher amount of NaOH solution added to control the pH, although it is not considered inconvenient for most applications.

Table 3.7. Proximate elemental composition of *fines*, PDF, freeze-dried hydrolysates, and precipitates based on DM.

Based on DM		Samples					
		Fines	**PDF**	**HA**	**HA precipitate**	**HN**	**HN precipitate**
	Protein (%)	45.8 ± 0.5	57.7 ± 1.7	69.2 ± 5.1	23.7 ± 2.0	70.5 ± 1.4	46.3 ± 2.6
	Ash (%)	12.2 ± 0.2	16.9 ± 1.0	9.8 ± 0.2	42.2 ± 2.9	6.5 ± 0.2	25.2 ± 1.8
	Fat (%)	37.2 ± 0.3	18.9	12.9	26.3	2.6	21.1
	C/N	6.8 ± 0.1	5.1 ± 0.1	4.2 ± 0.1	10.6 ± 0.5	3.8 ± 0.1	5.8 ± 0.2
Mineral element	^{23}Na (%)	0.58	0.34	3.47	0.78	2.44	0.30
	^{24}Mg (%)	0.12	0.16	0.02	0.40	0.07	0.13
	^{31}P (%)	2.56	4.09	0.40	9.20	0.69	3.30
	^{34}S (%)	0.53	0.61	0.79	0.25	0.73	0.59
	^{39}K (%)	0.65	0.30	0.43	0.07	0.81	0.08
	^{44}Ca (%)	3.61	5.81	0.07	15.51	0.09	4.80
	^{55}Mn (ppm)	2.98	6.98	0.23	14.33	0.19	6.03
	^{56}Fe (ppm)	149.81	252.39	23.01	655.51	10.02	351.79
	^{63}Cu (ppm)	7.57	10.93	13.19	4.44	11.96	11.70
	^{66}Zn (ppm)	89.24	125.30	8.08	293.61	2.54	136.29

PDF: partially defatted *fines*, HA: Alcalase 2.4L hydrolysate, and HN: Neutrase 0.8L hydrolysate

As we can observe in Table 3.7, the substrate is especially rich in phosphorus and calcium. The high content of phosphorus and calcium observed in the precipitates confirm the presence of bone particles in this fraction, being these the two major constituents (Deydier et al., 2005). The amounts of mineral elements in the obtained freeze-dried protein hydrolysates were reduced, except for Na. The presence of these mineral elements in the protein hydrolysates is not a negative aspect. Some of them, such as Zn, Cu, and Mg act as cofactors, S is a component of some amino acids, P is involved in energy transfer, Ca is a component of membranes, K participate in protein biosynthesis, while Na is the major intracellular cation (Kampen, 2014; Klompong et al., 2009). Hence, these minerals are required for microbial growth and support the use of these hydrolysates in microbiological growth media (Lobo-Alfonso et al., 2010; Taskin, 2013).

3.4.2. Determination of peptide MWs

3.4.2.1. SDS-PAGE

Determination of the molecular weight of the proteins in *fines* and the PDF was not possible with conventional techniques due to the low solubility. However, Garcia & Phillips, (2009) achieved the solubilization of 24.0 ± 1.8% of protein from soft tissue and 10.9 ± 1.9% of protein from bone (MBM is mainly composed of these two general classes of particles) after extraction with a very aggressive solution. The molecular weight of most of the extracted protein and peptides was below 65 kDa, even though an intense band was observed at 116.3 kDa. This study revealed that soluble MBM protein was highly polydisperse, a factor that increases the difficulty of functional utilization. Given the origin of *fines*, a similar profile was assumed for the non-hydrolyzed PDF.

The electrophoretic profile of protein hydrolysates obtained with Alcalase 2.4L in the optimal pH and E/S ratio conditions after 4, 8, 12, and 24 h of hydrolysis compared with commercial hydrolysates is presented in Figure 3.8. Independently of the hydrolysis time, a polypeptide wide and diffuse band pattern below 17 kDa was observed in the hydrolysates. Considering the above assumption, larger peptide fragments were expected in the initial stage of the reaction (4 h). However, the obtained results are in agreement with Piazza & Garcia, (2014), who reported that after 4 h of hydrolysis of MBM with alcalase, about 90% of the proteins solubilized from MBM had a molecular weight smaller than 30 kDa, whereas after 24 h of incubation a decrease in the concentration of peptides > 10 kDa and an increase in the peptides < 5 kDa were observed. A similar pattern was given by a commercial meat hydrolysate (Primatone RLT), implying that HA could be used as a nitrogenous source in microbiological growth media. This peptone is the most similar regarding color, solubility, and composition to HA. No bands were observed in lanes 6 - 8, despite manufacturer characterization of the protein hydrolysates added in lane 6 as a peptone with a predominance of high molecular weight polypeptides. Hence, we suggest that the absence of bands in these lanes was because peptides were too small to be detected under the analytical electrophoresis conditions (Khantaphant & Benjakul, 2008).

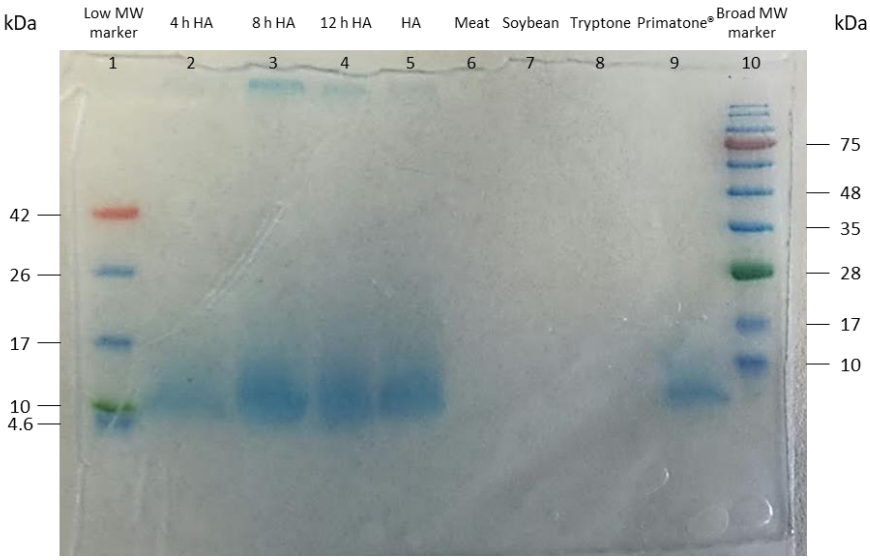

Figure 3.8. SDS-PAGE gel showing the fragments in hydrolyzed samples with Alcalase 2.4L (HA) in the optimal conditions after 4, 8, 12, and 24 h (lanes 2 – 5), meat peptone, soybean peptone, tryptone, and Primatone® RLT (meat tissue) (lanes 6 – 9), Low Range Protein Ladder from 2 to 42 kDa (lane 1) and Wide Range Protein Ladder from 10 to 180 kDa (lane 10).

Other techniques can be used to reveal the molecular weight. Replacing glycine by tricine in the Laemmli running buffer will improve the separation of peptides between 1 and 5 kDa (BIO-RAD, 2010). SEC, Matrix-Assisted Laser Desorption/Ionization - Time-Of-Flight (MALDI-TOF), multi-angle light-scattering, or MS also could be used in the determination of peptides more accurately (Garcia & Phillips, 2009; Mazotto et al., 2017).

3.4.2.2. Size exclusion chromatography (SEC)

For the purpose of a more complete characterization and to detect peptides in a molecular weight range not detected by SDS-PAGE, SEC analysis was carried out to determine the molecular weight distribution of the two prepared hydrolysates and commercial peptones for comparison, as displayed in Figure 3.9. MW distribution ranges include < 0.5 kDa, 0.5 – 1 kDa, 1 – 5 kDa, 5 – 10 kDa, and > 10 kDa. All the hydrolysates were characterized by presenting a very high relative percentage of peptides with small and medium molecular weights (< 5 kDa), which accounted for more than 92% of all the peptides. This fact indicates that Alcalase 2.4L and Neutrase 0.8L considerably degraded PDF and hydrolyzed proteins into small and medium peptides or free amino acids. However, significant differences were observed among the samples. The relative percentage of peptides with a medium molecular weight (> 1 kDa) was higher in HN than HA, thus corroborating well the lower DH obtained with Neutrase 0.8L. Furthermore, several authors correlated the release of small peptides with increasing DHs (Chalamaiah et al., 2010; Karoud et al., 2019; Khantaphant & Benjakul, 2008; Noman et al., 2018). Approximately 80% of all the peptides in

HA corresponds to small molecular weight peptides (< 1 kDa), and it is interesting to note that the molecular weight distribution of this was almost identical to meat peptone. Tryptone and soybean peptones showed greater differences compared to HA and HN. Commercial peptones were analyzed because we had a special interest in using the hydrolysates in microbiological growth media.

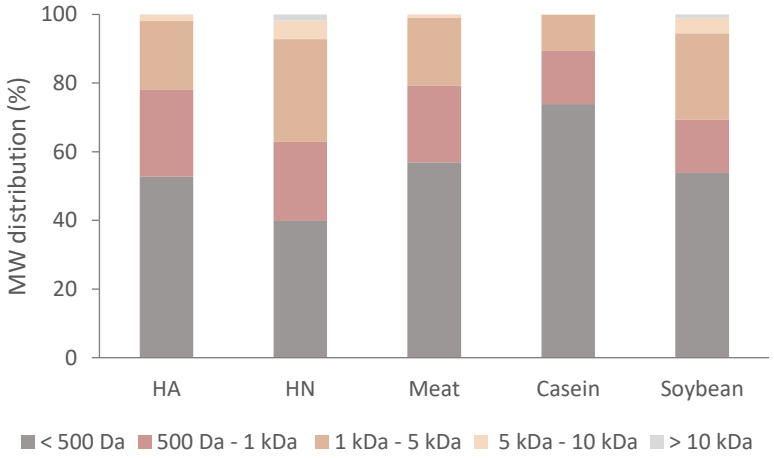

Figure 3.9. The MW distribution of the hydrolysates obtained with Alcalase 2.4L (HA) and Neutrase 0.8L (HN), and commercial peptones (meat tissue, tryptone from casein, and soybean).

Protein hydrolysates have different applications according to their DH, the molecular weight of the released peptides, and the amino acid sequence (de Queiroz et al., 2017). The size is one of the most important factors concerning desired functional properties used as functional materials (Wang et al., 2013). Hence, given the similarities of HA and HN with the commercial peptones, similar applications can be developed for them.

3.4.3. Amino acid composition

Amino acid composition of *fines*, PDF, protein hydrolysates, and precipitates are presented in Table 3.8. Total ion chromatograms (TIC) of standards are shown in Figure S3.2 (Supplementary data). The methodology employed was not optimal to determine tryptophan and glutamic acid/glutamine and aspartic acid/asparagine separately. During the hydrolysis with HCl, tryptophan is completely degraded while glutamine and asparagine are completely hydrolyzed to glutamic acid and aspartic acid, respectively (Fountoulakis & Lahm, 1998). Therefore, Glx includes glutamic acid and glutamine, and Asx includes aspartic acid and asparagine. Among the amino acids quantified herein, the content of Glx, Leu, Gly, and Asx tended to predominate in all the samples. Data presented in Table S3.1 (Supplementary data) also showed the predominance of these amino acids in MBM. Therefore, the amino acid composition of *fines* determined in this study and their comparison with several publications regarding MBM revealed that the

amino acid profile was very similar, even though the waste used is not considered MBM. After the thermal treatment, as consequence of fat and warm water-soluble collagen removal, the recovered fraction (PDF) used for hydrolysis showed a significantly higher concentration of some amino acids, specifically Ala, Asx, Cys, Glx, Ile, Leu, Phe, Ser, Tyr, and Val. In contrast, the enzymatic hydrolysis showed that on the whole, there were no significant differences between the substrate and the hydrolysates, with the exception of Cys and Val for HA and Ala, Arg, Asx, Cys, Hyp, Ile, and Pro for HN. Besides this, the hydrolysis gave similar relative percentages of all amino acid residues, as shown in Figure S3.3 (Supplementary data). These findings agreed with Cheng et al., (2009); Kurozawa et al., (2008); and Lapeña et al., (2018), who reported only slight changes in the proportion of all amino acids residues when compared the initial substrate and the hydrolysates obtained from protein-rich industrial by-products and animal co-products.

Table 3.8. Amino acid composition (mg amino acid/g sample) and total amino acid content expressed as mean ± SD.

Amino acid	Concentration (mg amino acid/g sample)					
	Fines	**PDF**	**HA**	**HA precipitate**	**HN**	**HN precipitate**
Ala	16.2 ± 0.3^{c}	19.8 ± 0.2^{b}	20.1 ± 0.6^{b}	9.2 ± 1.1^{d}	24.1 ± 1.4^{a}	16.2 ± 0.5^{c}
Arg	36.3 ± 2.9^{c}	41.7 ± 2.4^{c}	49.1 ± 2.7^{a}	20.6 ± 1.7^{c}	51.1 ± 3.3^{a}	34.6 ± 1.4^{c}
Asx	39.8 ± 2.4^{d}	49.7 ± 1.2^{bc}	54.4 ± 1.4^{ab}	22.4 ± 3.4^{e}	56.3 ± 1.4^{a}	43.9 ± 1.2^{cd}
Cys	1.5 ± 0.8^{cd}	5.2 ± 1.0^{ab}	2.7 ± 0.2^{bcd}	3.1 ± 1.1^{bc}	0.5 ± 0.2^{d}	7.4 ± 1.8^{a}
Glx	82.4 ± 2.7^{b}	90.5 ± 1.5^{ab}	96.0 ± 1.8^{a}	39.5 ± 2.3^{d}	94.3 ± 5.8^{a}	61.6 ± 0.1^{c}
Gly	50.8 ± 8.8^{ab}	52.4 ± 10.6^{ab}	51.7 ± 8.6^{ab}	16.7 ± 2.0^{c}	68.5 ± 13.8^{a}	30.2 ± 1.2^{bc}
Hyp	6.1 ± 0.4^{b}	5.6 ± 0.2^{b}	6.2 ± 0.3^{b}	2.3 ± 0.0^{c}	11.2 ± 0.3^{a}	2.4 ± 0.1^{c}
His	15.0 ± 1.4^{ab}	17.3 ± 0.9^{ab}	17.9 ± 0.2^{a}	7.3 ± 0.5^{c}	17.6 ± 1.8^{a}	14.2 ± 0.5^{b}
Ile	12.6 ± 0.7^{bc}	20.4 ± 1.1^{a}	16.8 ± 1.2^{ab}	8.7 ± 2.6^{c}	14.9 ± 2.7^{b}	22.0 ± 0.8^{a}
Leu	49.8 ± 2.2^{b}	65.8 ± 1.1^{a}	68.2 ± 0.6^{a}	24.9 ± 2.1^{c}	62.9 ± 5.8^{a}	46.2 ± 2.0^{b}
Lys	35.5 ± 6.1^{b}	42.3 ± 3.3^{ab}	47.0 ± 3.3^{a}	17.9 ± 3.0^{c}	46.4 ± 4.6^{ab}	38.7 ± 1.3^{ab}
Met	7.3 ± 0.4^{ab}	6.9 ± 4.6^{ab}	11.5 ± 3.2^{a}	2.6 ± 0.7^{b}	9.3 ± 1.6^{ab}	9.3 ± 1.8^{ab}
Phe	11.3 ± 0.7^{b}	15.5 ± 0.7^{a}	16.2 ± 1.2^{a}	6.6 ± 0.5^{c}	15.1 ± 1.3^{a}	12.0 ± 0.4^{b}
Pro	34.5 ± 0.0^{b}	39.1 ± 2.7^{b}	39.5 ± 1.4^{b}	13.8 ± 2.0^{d}	54.3 ± 4.0^{a}	22.9 ± 1.0^{c}
Ser	24.7 ± 1.3^{c}	30.7 ± 1.6^{b}	38.2 ± 0.3^{a}	12.2 ± 1.2^{d}	36.5 ± 2.4^{a}	22.6 ± 0.3^{c}
Thr	29.9 ± 1.0^{a}	31.3 ± 0.6^{a}	31.8 ± 1.2^{a}	14.9 ± 0.1^{c}	31.8 ± 1.4^{a}	19.1 ± 0.9^{b}
Tyr	14.8 ± 0.8^{c}	19.5 ± 1.0^{ab}	22.0 ± 1.2^{a}	9.5 ± 1.5^{d}	19.6 ± 1.4^{ab}	18.2 ± 0.0^{bc}
Val	29.7 ± 1.7^{c}	39.4 ± 1.0^{a}	34.1 ± 1.2^{b}	12.9 ± 1.1^{d}	36.1 ± 2.2^{ab}	26.6 ± 0.7^{c}
Total	498.3 ± 26.5	593.0 ± 23.4	623.5 ± 6.2	244.9 ± 25.2	650.6 ± 51.8	448.4 ± 3.6

Means in the same row not connected by the same letter differ significantly (Tukey HSD test, $\alpha = 0.05$)
PDF: partially defatted *fines*, HA: Alcalase 2.4L hydrolysate, and HN: Neutrase 0.8L hydrolysate
Asx, no separate analysis of Asp/Asn; Glx, no separate analysis of Glu/Gln

The amino acid compositions of HA and HN were relatively comparable, except for the content of Ala, Hyp, and Pro, observing significantly higher concentrations in HN. It is interesting to note that Pro and Hyp are amino acids abundant in collagen (Anzani et al., 2018; Cheung & Li-Chan, 2017; Chi et al., 2014; Nam et al., 2008; Sotelo et al., 2016). Although alcalase is able to degrade collagen (Gomez-Guillen et al., 2011; Zhang et al., 2013), this fact suggests that Neutrase 0.8L was more effective in hydrolyzing collagen structure than Alcalase 2.4L. These results are in agreement with stated by Webster et al., (1982), who reported that neutrase was the most effective enzyme hydrolyzing collagenous tissue and alcalase was by far the least effective compared to pepsin, papain, and neutrase. This can be explained by the specificity of each enzyme. Neutral proteases prefer substrates containing aromatic amino acids, Leu, Ile, Val, and Arg-Gly linkage (Hou et al., 2017; Webster et al., 1982; Xu et al., 2014), which appear quite frequently in collagen. Conversely, alcalase has a broad specificity (Zhang et al., 2013) for sites containing mainly hydrophobic residues in either P'_2 or P'_3 positions, Ala and Ser at P'_1, Glu in the P_1 position, Gly, Pro, Ala, Val, and Ser at various P_4–P'_4, and Leu-Tyr bond (Adamson & Reynolds, 1996; Cheung & Li-Chan, 2017; Xu et al., 2014), although not necessarily for those amino acids bonds predominant in the collagen or gelatin structures (Webster et al., 1982). Thus, the broader specificity of alcalase had yielded hydrolysates with higher DH.

According to Table 3.8, we can see that HA and HN precipitates amino acid profiles were significantly different. As a consequence of the broader specificity of alcalase, a lower amount of amino acids remained in the recovered HA precipitate, as shown in Figure 3.10. These results are in agreement with PR. Moreover, the high N content of the peptones obtained indicates that can be a good source of high-nutritional quality products for use in microbiological growth media as a nitrogen source or in fermentation media (Kampen, 2014; Stanbury et al., 2017).

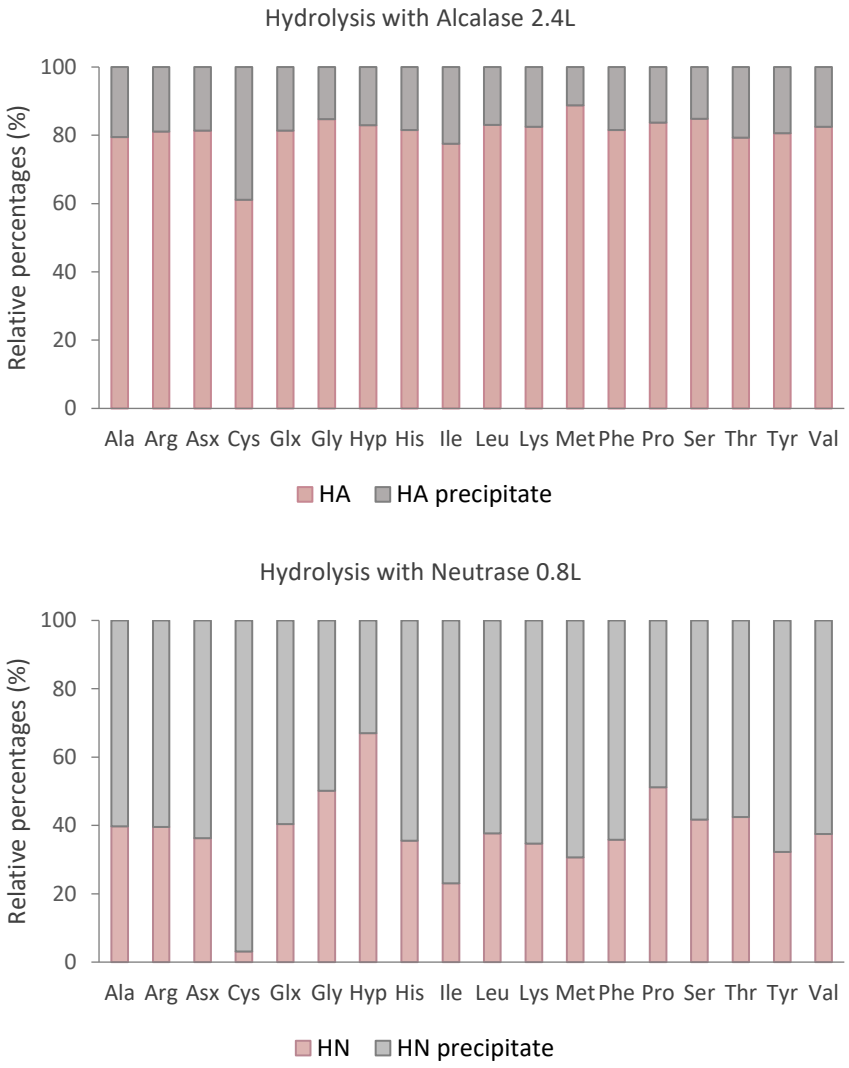

Figure 3.10. Relative amino acid percentages in the hydrolysates (HA and HN) and the recovered non-soluble fractions (HA and HN precipitates) obtained with Alcalase 2.4L and Neutrase 0.8L in the optimal conditions.

3.4.4. Nitrogen to protein conversion factors (NPCF)

Nitrogen-to-protein factors are empiric factors calculated from total nitrogen determined by Kjeldahl or Dumas and amino acid concentration determined by HPLC. Three types of NPCF (k_A, k_P, and k) have been determined: k_A, the ratio of protein to amino nitrogen, k_P, the ratio of protein to total nitrogen, and k, the average of k_A and k_P (Chen et al., 2017). The factor k_A assumes that all the nitrogen measured comes from protein, while factor k_P includes protein nitrogen and non-protein nitrogen found in the sample (Templeton & Laurens, 2015). Data for factors determination, N_L, $\sum E_i$, and $\sum D_i$, for *fines*, PDF, protein hydrolysates, and their recovered precipitates are described in Table 3.9. All these factors were calculated based on the basis of 17 amino acids analyses without Trp and NH_3. As expected, k_A values were higher than k_P values. The k_A values were very similar among the samples, while remarkable differences were

observed for k_P and *k* factors. A similar trend was observed by Templeton & Laurens, (2015) for k_A in algal biomass samples, who reported that these similarities lie in the amino acids profile. On the whole, no significant differences were observed in the relative composition in the studied samples (Figure S3.3 in Supplementary data). Conversely, k_P factor lies in the N_L and as a consequence of the enzymatic hydrolysis and the obtention of different fractions, this value varied. Mossé, (1990) and Templeton & Laurens, (2015) agreed that the most conservative and accurate choice is factor *k*. However, k_P is the factor that closely reflected the protein content of algal biomass in the absence of accurate measurements of non-protein nitrogen content and NH_3.

NR ranged from 80 to 92%, suggesting that N recovery from total amino acid analysis is relatively high despite not including N from NH_3. The values reported in the present work for NR agree with those reported by Sriperm et al., (2011).

Table 3.9. Nitrogen to protein conversion factors, including NL, $\sum E_i$, $\sum D_i$, and NR.

Sample	N_L (g/kg)	$\sum E_i$ (g/kg)	$\sum D_i$ (g/kg)	NR (%)	Specific NPCF		
					k_A	k_P	*k*
Fines	76.8	420.3	70.8	92.2	5.94	5.47	5.71
PDF	92.3	502.3	83.4	90.4	6.02	5.44	5.73
HA	110.8	528.5	88.6	79.9	5.97	4.77	5.37
HA precipitate	37.9	208.1	34.8	91.6	5.99	5.49	5.74
HN	112.1	545.3	93.1	83.1	5.85	4.87	5.36
HN precipitate	74.1	382.7	63.7	86.1	6.01	5.17	5.59

PDF: partially defatted fines, HA: Alcalase 2.4L hydrolysate, and HN: Neutrase 0.8L hydrolysate

The *k* values for *fines* and PDF were 5.71 and 5.73, which are within most recent values for meat, ranging from 5.38 to 5.74 (averaging 5.6) (Mariotti et al., 2008). Sriperm et al., (2011) recommended a *k* of 5.1 ± 0.1 for MBM while Piazza & Garcia, (2010) calculated a NPCF of 6.45 based on the amino acid analyses of 12 MBM samples reported by Adedokun & Adeola, (2005). Sosulski & Imafidon, (1990) reported a NPCF for meat products of 5.2 ± 0.2 although suggested a factor of 5.7 for animal and plant foods. Although the NPCF 6.25 is usually used and recommended by AOAC (King-Brink & Sebranek, 1993), from the published literature it seems that no consensus has been reached. Precipitates have somewhat similar *k* values to *fines* and PDF. Smaller *k* values were found for the hydrolysates, nevertheless, no data was found in the literature for comparison. The *k* values for HA and HN were very close, 5.37 and 5.36, respectively. A lower *k* can be attributed to a higher amount of nitrogen in the proteins. In particular, the presence of larger amounts of rich nitrogen-containing amino acids (Lys, Gln, Asn, Gly, His, and Arg) increase total protein as determined by total nitrogen content to inaccurate amounts. Hence, lower *k*

values are required to avoid overestimation in samples rich in high nitrogen-containing amino acids (Hall & Schönfeldt, 2013).

The choice of conversion factor can greatly affect the protein estimation, as shown in Table 3.10. Using the k_A, k_P, and k factors, on the whole, lower specific crude protein (SCP) values were obtained compared with crude protein (CP) and true protein (TP). As Sriperm et al., (2011) reported, this was because NPCF were calculated from anhydrous amino acid residues, while TP is the summation of the amino acid residues.

Table 3.10. Specific crude protein, crude protein, and true protein contents (g/kg).

Sample	Specific crude protein (SCP) (g/kg)			Crude protein (CP) (g/kg)	True protein (TP) (g/kg)
	SCP (k_A)	SCP (k_P)	SCP (k)		
Fines	455.8	420.3	438.1	479.8 ± 5.2	492.2 ± 26.1
PDF	555.4	502.3	528.8	576.7 ± 17.2	587.4 ± 23.2
HA	661.1	528.5	594.8	692.5 ± 50.8	617.2 ± 6.2
HA precipitate	227.1	208.1	217.6	237.1 ± 20.4	242.6 ± 25.2
HN	656.1	545.3	600.7	700.4 ± 13.8	639.4 ± 51.5
HN precipitate	444.7	382.7	413.7	462.9 ± 25.7	445.9 ± 3.5

PDF: partially defatted *fines*, HA: Alcalase 2.4L hydrolysate, and HN: Neutrase 0.8L hydrolysate

Comparing all the fractions analyzed, CP levels were similar to TP, except for the hydrolysates. Strikingly, for *fines*, PDF, and precipitates, the most accurate approximations were obtained using the NPCF 6.25, although several reports have demonstrated that exists a poor correlation coefficient between protein content based on amino acids and predicted protein content based on N x 6.25 (Chen et al., 2017; Hall & Schönfeldt, 2013; Sriperm et al., 2011; Templeton & Laurens, 2015). This might be due to very low amounts of non-protein nitrogen in these samples. On the other hand, for HA and HN the N x 6.25 resulted in an overestimation of the protein. This is due to the presence of larger amounts of rich nitrogen-containing amino acids. More realistic and accurate estimations were obtained with factor *k*. This suggests that depending on the type of sample, different factors should be employed. Therefore, the ideal method for determining TP is from amino acid composition (Zhang et al., 2020). However, there are several limitations in performing it in routine protein measurements, such as the price and time. Determination of specific factors for a given matrix can be used to improve total protein estimations. Therefore, in the present work, a *k* value of 5.37 is recommended for protein hydrolysates from meat by-products, while a k_A value of 6 is recommended for *fines*, PDF, and recovered precipitates containing non-soluble protein.

3.4.5. Functional groups analysis

Fourier Transform Infrared (FTIR) is considered a valuable tool with a growing interest in the characterization of enzymatic protein hydrolysates (Aspevik et al., 2017). FTIR analysis was performed on freeze-dried samples to study the main changes in the structure of the resulting protein hydrolysates. Aqueous solutions were not used to avoid the strong absorbance of water, near 3500 cm^{-1} and 1645 cm^{-1} bands, as the latter overlaps the amide I band of proteins and some side chains bands (Barth, 2007). The spectra after the different treatments were notably different, as shown in Figure 3.11. *Fines* showed some characteristic bands in the region 2800 – 2980 cm^{-1} which correspond to symmetric and asymmetric C-H stretching vibrations, typically of long-chain fatty acids and oils. The bands at ~1740 cm^{-1} and ~3000 cm^{-1} were reported to be associated with C=O stretching vibrations of esters or carboxylic acids and -C=C-H stretching vibrations of the *cis*-unsaturation, respectively, being important bonds in the chemistry of fats and oils (Tesfaye et al., 2017; Upadhyay et al., 2018). The peak in the region 1100 – 1250 cm^{-1} was reported to correspond to the stretching vibrations of C-O bond of esters composed of two asymmetric vibrations consisting C-C(=O)-O and O-C-C (Koca et al., 2010). After the thermal treatment, a decreasing trend of the defined bands related to fat was observed, reflecting lower levels of lipids. As expected, after the enzymatic hydrolysis, the most significant changes were observed in the region 1300 – 1650 cm^{-1}. Strong signals at 1230 cm^{-1}, 1550 cm^{-1}, and 1650 cm^{-1} were observed and these bands were assigned to amide III, amide II, and amide I, respectively (Güler et al., 2016; Tesfaye et al., 2017). These peaks are related to peptide bonds group vibration (CO-NH), therefore to the concentration of protein in the samples. Amide III was associated with mixed N-H in plane bending and C-H stretching vibrations of amide linkage and corresponds to the α-helix conformation (Dash & Ghosh, 2017; Tesfaye et al., 2017). Amide II is due to the mixed C-N stretching vibrations and N-H bending. Amide I was mainly related to C=O stretching vibrations ascribed to the typical α-helix conformation (Güler et al., 2016; Wang et al., 2013). Güler et al., (2016) reported that upon hydrolysis, the amide I band was reduced over time and shifted towards lower wavenumbers whereas a broad band around 1593 and 1588 cm^{-1} increased, which were attributed to the antisymmetric stretching of free carboxylates COO^-. Besides this, the band around 1402 cm^{-1}, attributable to symmetric stretching modes of free carboxylate anions, also increased in intensity during digestion. These facts confirm products released from hydrolysis. The bands at 1400 cm^{-1} and 1460 cm^{-1} also increased during the hydrolysis and these signals correspond to $-CH_2$ and $-CH_3$, thus conforming the disruption of a long C-C skeletal chain (Cheong et al., 2018). Moreover, Poulsen et al., (2016) reported that the band at 1400 cm^{-1} may be related to the increase in carboxyl groups at the C-terminus of generated peptides, and changes in the side chains of the residue becoming more exposed and thereby visible in the spectrum and/or a result of changed hydrogen bonding as hydrolysis proceeds. Additionally,

the bands in the region 3050 – 3400 cm^{-1} are characteristic of proteins, corresponding to Amide A and Amide B. FTIR spectra reflected not only higher protein content in HA and HN but also the lower levels of fat. In the precipitates obtained after the hydrolysis the strongest band was observed at 1020 cm^{-1}, and it corresponds to P-O stretching (Miculescu et al., 2012; Paschalis et al., 2011) indicating the presence of bone. Based on the bands at 1760 cm^{-1}, 2860 cm^{-1}, and 2910 cm^{-1} the precipitate mainly comprises bone, non-hydrolyzed protein, and fat. The differences between hydrolysates and precipitates spectra are in agreement with the protein recoveries.

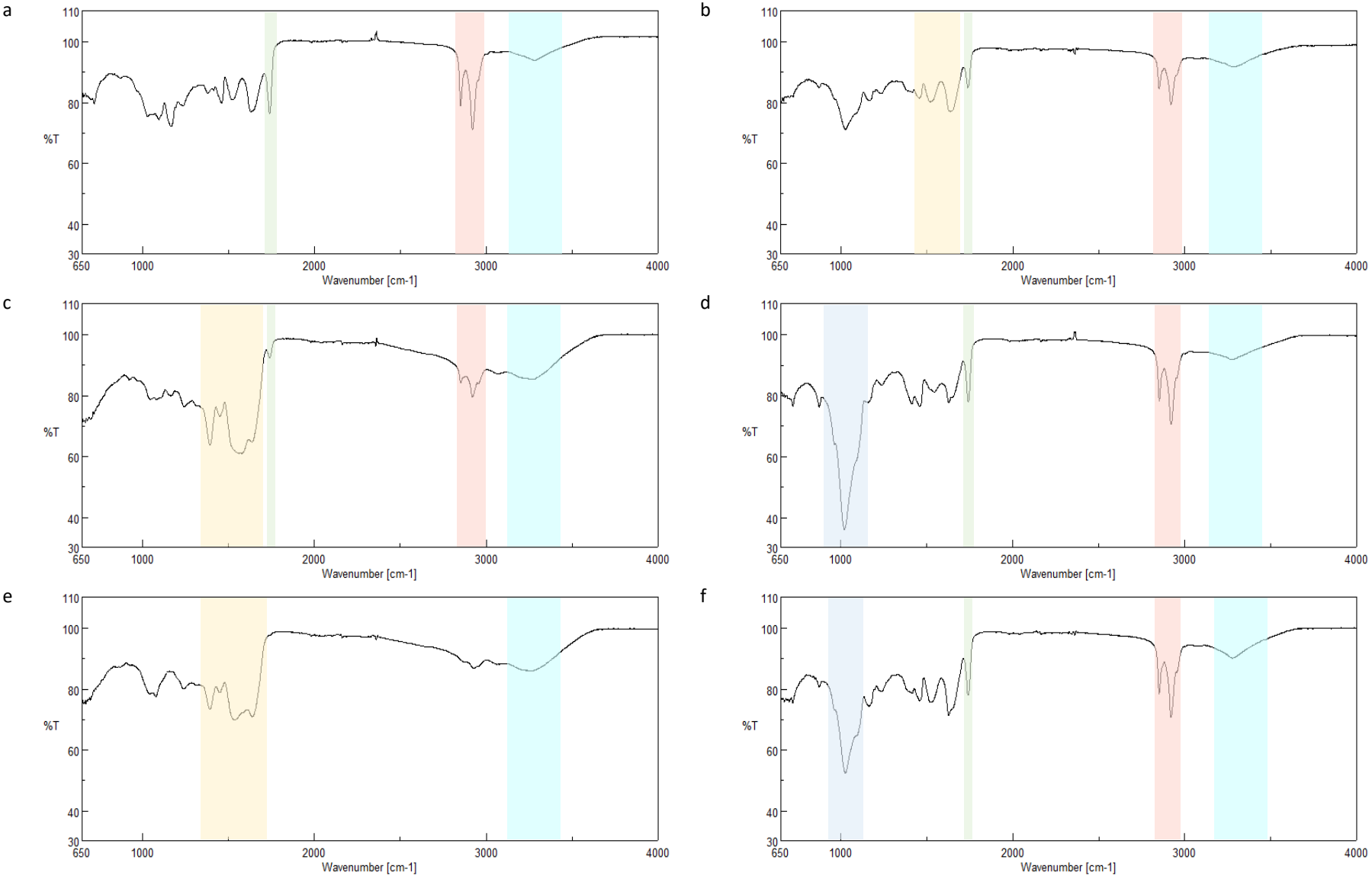

Figure 3.11. IR spectra of different fractions: a) *fines*, b) PDF, c) Alcalase 2.4L hydrolysate (HA), d) HA precipitate, e) Neutrase 0.8L hydrolysate (HN), and f) HN precipitate.

3.4.6. Volatile compounds

Volatile compounds detected in the raw material and the subsequent fraction obtained after heat treatment and enzymatic hydrolysis with Alcalase 2.4L and Neutrase 0.8L are listed in Table 3.11. GC-MS chromatograms of the analyzed samples are displayed in Figure S3.4 (Supplementary data). Although the produced protein hydrolysates were not intended for human consumption, the production of off-flavors is undesired if intended for pet food. HC-SPME-GC-MS was used to determine the development and disappearance of desirable and undesirable volatile compounds during heat treatment and hydrolysis. Around 78 different volatile compounds were detected, whereas only 70 were identified. Of the detected compounds, 42 were found in *fines* and aldehydes were the most abundant. These volatile compounds indicate lipid oxidation, and they are the major contributors to off-flavors and odors (Ross & Smith, 2006). Besides this, hexanal, the major component in *fines*, is often used as an indicator of oxidative stability (Shahidi, 1998). The volatiles profile of PDF changed as a result of the heat treatment. The predominant compounds were not identified. At the end of the enzymatic hydrolysis, 11 new compounds were detected when overlapped to PDF. This indicates that some compounds were affected by the enzymatic treatment. Proteolysis influences volatile compounds development due to the formation of free amino acids and other low molecular weight compounds. The main routes for the generation of volatile compounds are Maillard and Strecker reactions and lipid oxidation (Armenteros et al., 2012).

The major groups of volatile compounds found in the hydrolysates were aldehydes, carboxylic acids, alkanes, alcohols, and ketones. The same type of volatile compounds was found by Zhang et al., (2019) in microbone meal and Flavourzyme treated microbone meal and by Tan et al., (2018) in cod bone hydrolysates. HA volatiles profile was predominated by ketones and the second largest flavor compounds were alkanes. Three ketones, 2-heptanone, 2-nonanone, and 2-decanone accounted for 65% of the volatiles in HA. The odor and flavor of these ketones are perceived as cheesy, fruity and nice cheesy, and floral and fermented, respectively (The Good Scents Company Information System, 2021). 2-Heptanone was already present in PDF but at lower relative abundance. Hydrolysis conditions, such as salt addition might have influenced ketone formation (Armenteros et al., 2012). Due to their low odor threshold value, ketones would make an important contribution to aroma (Tan et al., 2018). On the other hand, acids prevailed in HN. Hexanoic, heptanoic, octanoic, and nonanoic acid accounted for 66% of the volatiles. Octanoic and nonanoic acids were not detected in PDF; therefore they were formed as a consequence of enzymatic hydrolysis. Acids are known to contribute with unpleasant and unwanted odor (cheesy and rancid odor descriptors) (Bak et al., 2018), although the rancid notes could be hidden by the presence of other volatile or even non-volatile compounds (Armenteros et al., 2012). As previously mentioned in the literature, enzyme specificity can influence the flavor of hydrolysates (Fu et al., 2019). However, a

particular class of compounds in a high concentration does not mean that it is an important contributor to the aroma, as this also depends on other factors such as odor threshold and detector sensitivity for different compounds (Fu et al., 2019). Besides this, the hydrolysates should be tested in a real product for a realistic evaluation (Bak et al., 2018).

Table 3.11. Relative peak area of volatile compounds detected in raw material and the obtained fractions.

Code	RT (min)	Volatile compound	Sample						Retention index	
			Fines	PDF	HA	HA precipitate	HN	HN precipitate	Exp.	Auth. Std
ald	2.50	Pentanal	1.92	0.82	ND	ND	ND	ND		
oth	3.65	Not identified	15.44	2.91	4.21	ND	1.42	ND		
alka	3.68	2-Methylheptane	ND	ND	ND	3.058	ND	ND	747	760
alka	3.71	4-Methylheptane	ND	ND	ND	3.74	ND	ND	749	765
alc	3.79	1-Pentanol	3.52	0.91	0.54	1.03	1.08	0.21	753	760
ket	4.31	2-Hexanone	ND	ND	0.47	ND	ND	ND	779	789
alka	4.60	Octane	ND	ND	0.37	1.40	ND	0.89	794	
ald	4.68	Hexanal	21.22	3.86	0.37	1.59	0.83	2.94	801	801
alka	5.25	2,4-Dimethylheptane	0.50	3.21	2.32	5.86	0.13	4.44	814	822
est	5.53	Pentyl formate	0.18	ND	ND	ND	ND	ND	821	826
alke	5.91	2,4-Dimethyl-1-heptene	0.29	1.47	1.37	3.29	0.13	1.38	832	
alka	6.81	4-Methyloctane	0.24	1.50	1.83	4.06	0.10	3.40	856	858
alc	7.16	1-Hexanol	0.28	0.25	ND	0.41	0.22	ND	865	865
ket	7.97	2-Heptanone	0.56	0.52	6.97	2.43	0.24	0.88	887	889
aci	8.36	Pentanoic acid	ND	ND	ND	ND	0.99	ND	898	925
ket	8.41	Nonanone	ND	0.14	0.12	0.26	ND	ND	889	
ald	8.54	Heptanal	2.93	1.09	0.14	0.55	0.20	1.01	902	902
pyr	9.00	2,5-Dimethylpyrazine	ND	ND	0.30	ND	ND	ND	911	911

Code	RT (min)	Volatile compound	Sample						Retention index	
			Fines	**PDF**	**HA**	**HA precipitate**	**HN**	**HN precipitate**	**Exp.**	**Auth. Std**
alke	9.84	(1*R*)-2,6,6-Trimethylbicyclo[3,1,1]hept-2-ene	1.28	ND	0.63	ND	ND	0.64	927	935
alka	11.44	4-Methylnonane	ND	0.323	0.270	0.54	ND	0.32	959	958
alka	11.63	2-Methylnonane	0.12	1.18	0.83	1.55	ND	0.94	963	962
alc	12.06	1-Heptanol	1.47	0.99	0.83	2.85	0.96	1.76	971	971
fur	12.96	2-Pentylfuran	0.37	0.25	ND	1.29	ND	2.07	989	989
ket	13.03	2-Octanone	0.17	ND	4.22	1.72	ND	ND	990	992
aci	13.57	Hexanoic acid	ND	3.22	ND	ND	27.57	ND	1000	1008
alka	13.79	Decane	ND	0.34	0.40	0.71	ND	0.55	1001	
ald	13.80	Octanal	6.89	2.34	0.38	1.25	0.36	2.70	1006	1005
ter	14.75	*o*-Cymene	1.37	ND	ND	ND	ND	ND	1027	1026
alka	14.77	4-Methyldecane	ND	2.47	1.57	2.62	ND	2.39	1028	1059
ter	14.99	*D*-Limonene	7.17	0.16	0.82	0.78	ND	1.06	1033	1032
ket	15.65	3-Octen-2-one	0.88	ND	0.39	ND	0.21	0.18	1047	1046
fur	16.18	5-ethyl-3H-furan-2-one	0.52	ND	ND	ND	0.607	ND	1059	1056
oth	16.37	Not identified	0.53	8.52	ND	5.84	ND	ND	1063	
est	16.73	Ethyl 4-oxopentanoate	ND	ND	ND	1.08	0.77	ND	1071	
alc	17.16	1-Octanol	1.93	1.44	1.13	5.44	0.82	3.32	1080	1080
ket	17.81	2-Nonanone	0.24	ND	24.05	5.86	ND	ND	1095	1095
aci	17.92	Heptanoic acid	ND	ND	ND	ND	5.992	ND	1097	1071

Code	RT (min)	Volatile compound	Sample						Retention index	
			Fines	PDF	HA	HA precipitate	HN	HN precipitate	Exp.	Auth. Std
alka	18.07	3,7-Dimethyldecane	ND	4.34	ND	4.40	ND	6.32	1101	
ald	18.29	Nonanal	7.53	3.63	ND	ND	ND	ND	1109	1106
fur	19.56	5-Propyloxolan-2-one	0.12	ND	ND	ND	0.29	ND	1155	
alka	19.95	2-Methylundecane	0.07	0.75	0.32	0.75	0.25	0.92	1169	1163
ket	20.40	2-Piperidinone	ND	ND	1.62	ND	ND	ND	1186	1174
aci	20.65	Octanoic acid	6.24	0.99	ND	ND	18.38	ND	1195	1192
ket	20.67	2-Decanone	ND	ND	34.41	7.74	ND	1.65	1195	1196
ald	21.01	Decanal	2.30	0.70	ND	ND	ND	0.83	1210	1209
alka	21.05	2,4-Dimethylundecane	ND	0.70	0.22	ND	ND	0.65	1212	
alka	21.14	2,6-Dimethylundecane	0.13	1.93	0.85	1.59	0.84	1.93	1216	1214
alka	21.33	4-Methyldodecane	ND	1.16	0.49	0.90	0.61	1.17	1225	1259
ter	21.83	Carvone	0.10	ND	ND	0.40	ND	0.29	1249	1246
alka	22.00	4,6-Dimethyldodecane	ND	2.70	0.96	1.78	1.05	2.59	1256	
fur	22.07	(5*R*)-5-butyloxolan-2-one	0.62	ND	0.63	0.59	1.75	0.70	1260	1260
oth	22.45	Not identified	ND	14.25	ND	4.89	5.01	12.51		
aci	22.57	Nonanoic acid	6.56	ND	ND	ND	13.739	ND	1284	1281
est	22.74	Hexanoic acid, pentyl ester	0.41	ND	ND	ND	ND	ND	1291	1287
oth	23.36	Not identified	ND	4.94	ND	2.93	ND	6.32		
oth	23.74	Not identified	2.22	ND	ND	ND	3.16	ND		

Code	RT (min)	Volatile compound	Sample						Retention index	
			Fines	**PDF**	**HA**	**HA precipitate**	**HN**	**HN precipitate**	**Exp.**	**Auth. Std**
fur	24.11	5-Pentyloxolan-2-one	0.94	0.60	0.93	1.33	1.98	1.22	1365	1363
ald	24.19	2-Undecenal	0.46	0.69	ND	ND	ND	ND	1369	1368
aci	24.23	*n*-Decanoic acid	0.25	ND	ND	ND	ND	ND	1371	1368
ald	24.29	(*Z*)-2-butyloct-2-enal	ND	1.18	ND	1.36	ND	3.46	1375	1372
alka	25.15	4-Methyltetradecane	ND	0.429	ND	ND	ND	ND	1428	1460
oth	26.07	Not identified	ND	9.73	ND	3.47	3.18	7.20	1491	
phe	26.31	3,5-di*tert*-butylphenoxy	ND	ND	ND	ND	1.55	ND	1512	1513
oth	26.58	Not identified	ND	4.28	ND	2.47	ND	4.36	1539	
oth	28.01	Not identified	0.73	3.17	2.46	3.21	4.47	4.84	1703	
est	29.08	bis(2-methylpropyl) benzene-1,2-dicarboxylate	ND	ND	ND	ND	0.677	ND	1871	1870.9
oth	29.29	Homosalate	0.07	0.25	0.22	ND	ND	0.71	1905	1904
ket	29.30	2-Heptadecanone	0.12	ND	0.65	0.71	ND	0.78	1908	1900
aci	29.61	Hexadecanoic acid	0.30	0.16	ND	ND	ND	ND	1964	1963
phe	30.80	4-[2-(4-hydroxyphenyl)propan-2-yl]phenol	0.20	0.37	0.80	0.84	0.46	1.30	2198	

PDF: partially defatted *fines*, HA: Alcalase 2.4L hydrolysate, and HN: Neutrase 0.8L hydrolysate
ND: not detected
alc = alcohol, ald = aldehyde, est = ester, fur = furan, ket = ketone, aci = acid, alka = alkane, alke = alkene, phe = phenol, ter = terpene, pyr = pyrazine, and oth = other

3.5. Scale-up of enzymatic hydrolysis

After the initial studies of optimization, protein hydrolysates production at the shake flask level, and characterization, the hydrolysis process was transferred to a bench-scale reactor (Figure 3.12) to investigate the scale-up and applicability of the process. Transferring a process is challenging due to substantial differences (Zaghloul et al., 2011). A 10-fold scale-up was carried out with Alcalase 2.4L and Neutrase 0.8L. E/S ratios and temperature were kept as previously optimized. Agitation speed was increased to ensure a good degree of mixing and complete homogenization. Besides this, pH was monitored constantly and controlled in the proteolysis with Alcalase 2.4L, while with Neutrase 0.8L it was initially adjusted at 8 and monitored during the process. To evaluate the performance of the enzymatic hydrolysis several factors were considered: DH, PR, protein content, and MW distribution.

Figure 3.12. Reactor set up for enzymatic hydrolysis of partially defatted *fines* (PDF).

The hydrolysis curves with both enzymes are shown in Figure 3.13. After the enzymatic hydrolysis of PDF with Alcalase 2.4L in the 3-L reactor, a DH of 41.2 ± 0.8% and a PR of 85.5 ± 0.8% were achieved after 24 h. Hydrolysis with Neutrase 0.8L also gave higher DH and PR after 10.5 h, 10.8 ± 0.1% and 46.1 ± 1.2%, respectively. These values are considerably higher than the ones obtained in shake flasks under similar conditions. Since substrate, E/S ratio, hydrolysis time, and temperature were unaltered, the reasons for the increased DH and PR values in the stirred bench reactor compared to shake flasks can be associated with the impeller presence and the agitation speed (Elmalimadi et al., 2017; Kadić et al., 2014; Zaghloul et al., 2011). Furthermore, the effect of pH control also must be considered in the hydrolysis with Alcalase 2.4L.

In the present study, the effect of different impeller geometries was not considered. However, with the reported observations in mind, comparing agitation type (orbital shaking *vs.* radial direction with a wide discharge steam suitable for mixing off-bottom suspension particles) and speed (200 rpm *vs.* 600 rpm) in shake flask and reactor, the improvement in DH and PR can be explained by i) improvement in the mass and/or heat transfer allowing homogeneous enzyme distribution, reducing potentially high local product concentrations and avoiding concentration gradients, and ii) particle size reduction as a consequence of the high speed, thus increasing the surface area available for the enzymes attack, and consequently, higher efficiency of the enzymatic hydrolysis (Elmalimadi et al., 2017; Kadić et al., 2014; Mussatto et al., 2008). The lower DH at lower agitation speed in the orbital shaker could be explained by insufficient mass/heat transfer and inhomogeneous enzyme distribution. In partial agreement with these findings, Zaghloul et al., (2011) reported an enhanced level of soluble proteins with similar levels of secreted alkaline protease enzyme but a higher agitation speed (700 rpm performed better than 500 rpm), attributing this to a satisfactory contact between the enzyme and the substrate in all the reactor. However, a lower amount of NH_2-free amino groups were present in the hydrolysate when the process was conducted at 700 rpm (Zaghloul et al., 2011). In some cases, high impeller rotation speeds (> 500 rpm) were reported to favor the formation of foam, decreasing the DH (Elmalimadi et al., 2017; Nouri et al., 1997). Foam formation was not observed in the hydrolysis of PDF with either Alcalase 2.4L or Neutrase 0.8L.

Besides agitation, pH control could have influenced the enzymatic hydrolysis, specifically the process with Alcalase 2.4L. In the shake flask experiments pH was manually adjusted around 8 every 2 h (during the first 12 h of hydrolysis). The hydrolysis carried out in stirred batch-reactor was equipped with an automatic pH controller, stabilizing it around a set point equal to 8.0 ± 0.1. Fluctuations of the pH in both systems are shown in Figure S3.1 (Supplementary data). The ups and downs in pH value observed in the enzymatic hydrolysis performed in the shake flasks could have led to progressive inactivation of the enzyme. Additionally, unlike the process carried out in the reactor where pH was kept at optimal pH for Alcalase 2.4L, shake flask experiments also suffer temperature changes while pH was adjusted out of the incubation system. Given these facts, we can speculate that the combination of agitation speed and pH plays a paramount role in the enzymatic hydrolysis of PDF with Alcalase 2.4L.

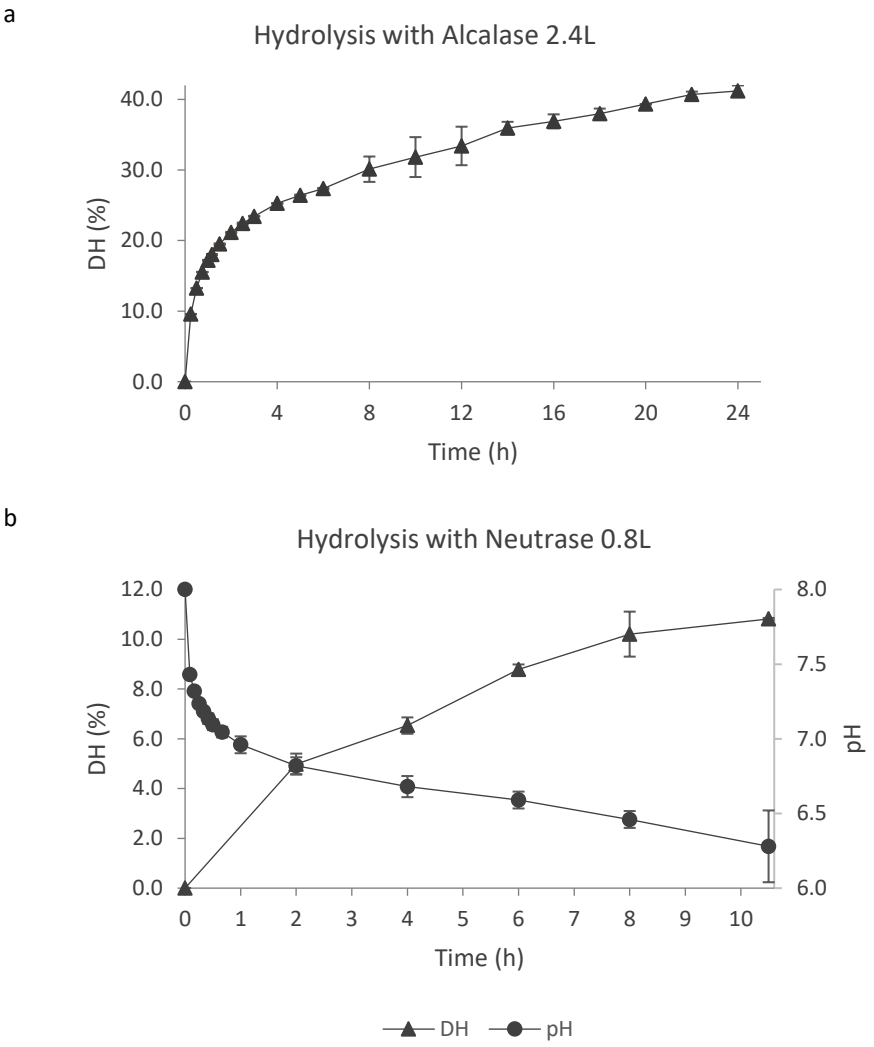

Figure 3.13. Evolution of hydrolysis of PDF in the reactor using a) Alcalase 2.4L (measured by pH-stat method) and b) Neutrase 0.8L (measured by OPA method). The pH evolution was recorded for Neutrase 0.8L.

The ultrafiltrated hydrolysates, RHA and RHN, containing approximately 76.2 ± 2.6% of protein, were subjected to SEC, in order to estimate the size distribution of peptides and for comparison with HA and HN. As displayed in Figure 3.14, the hydrolysates obtained in the reactor with Alcalase 2.4L (RHA) showed to contain a slightly higher relative percentage of peptides < 1 kDa compared to HA (83.2% *vs.* 78.2%). A similar trend was observed for RHN, although the differences between RHN and HN were not so evident. As previously pointed out in the present study, the focus of the produced protein hydrolysates is to integrate them into microbiological growth media. Given the specificity of the enzymes used, similar amino acids profiles were expected. Nevertheless, not only the amino acid composition but the form of the amino acids (peptides or free amino acids) determines the biological value of a particular protein hydrolysate (Ummadi & Curic-Bawden, 2010). With this in mind, due to the high abundance of small peptides, the obtained hydrolysates represent a nitrogen source of good nutritional quality for a wide

range of microorganisms without proteolytic system. Although there is no consensus about the optimal protein source for each microorganism, a balanced combination of dipeptides, tripeptides, and oligopeptides would be an optimal solution (Ummadi & Curic-Bawden, 2010).

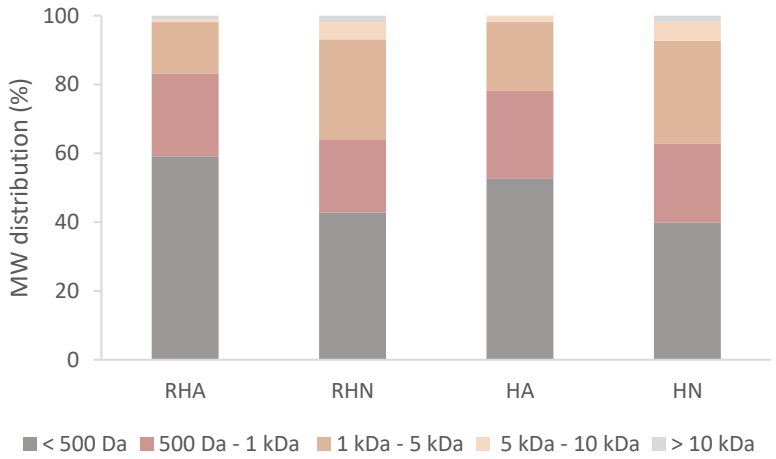

Figure 3.14. MW distribution of final hydrolysates obtained in Alcalase 2.4L and Neutrase 0.8L in the shake flasks (HA and HN) and in the reactor (RHA and RHN).

Conclusively, data indicates that the processes have been scaled-up successfully, achieving higher DH, PR, and an increase in the relative abundance of small and medium peptides thanks to a properly designated reactor setup and efficient mixing.

4. Conclusions

The present study showed that through enzymatic hydrolysis, the protein fraction present in *fines* (PDF), can be recovered as freeze-dried protein hydrolysates. Enzymatic hydrolysis of PDF was optimized using two commercial proteases, Alcalase 2.4L and Neutrase 0.8L, the former proving higher efficiency in digesting PDF. Three factors, E/S ratio, pH, and time were studied for PDF hydrolysis with Alcalase 2.4L. The conditions yielding the higher DH, 21.5 ± 1.4%, were E/S ratio 5%, pH-controlled around 8, and incubation time 24 h. Whereas RSM was selected for optimization with Neutrase 0.8L, integrating E/S ratio, time, initial pH, and temperature as input variables that might influence DH. The results from RSM showed that PDF hydrolysis process was mainly influenced by E/S ratio and time. DH was maximized at 7.2% when the four variables were set as E/S ratio 15%, initial pH 8, time 10.5 h, and temperature 40 °C.

The hydrolysates prepared from PDF with Alcalase 2.4L and Neutrase 0.8L present significantly higher protein content and lower ash and fat content than PDF, which adds stability during storage. SDS-PAGE and SEC profiles revealed that both hydrolysates contain a large proportion of low molecular weight peptides (< 5 kDa). Albeit a similarity was revealed between PDF and the hydrolysates for most of the amino acids, the hydrolysates present a balanced amino acid composition. FTIR spectrum analysis has shown that the structure of the PDF changed after the enzymatic processing. The volatiles profiles were also influenced by the proteolysis due to the formation of free amino acids and other low molecular weight compounds.

Finally, the process was scaled-up under the selected conditions, and higher efficiencies were obtained with both proteases, attributing this effect mainly to pH control and agitation. Moreover, the process proposed was easy to scale-up.

These results imply that insoluble wastes from animal processing plants and the rendering industry can be turned into soluble protein hydrolysates by proper enzymatic treatments for non-feed applications.

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6. Supplementary data

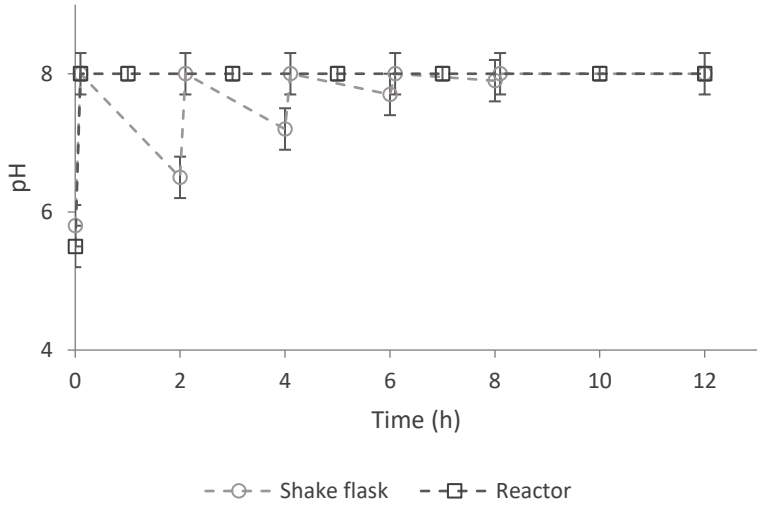


Figure S3.1. pH evolution over the enzymatic hydrolysis with Alcalase 2.4L in the shake flask and the reactor.

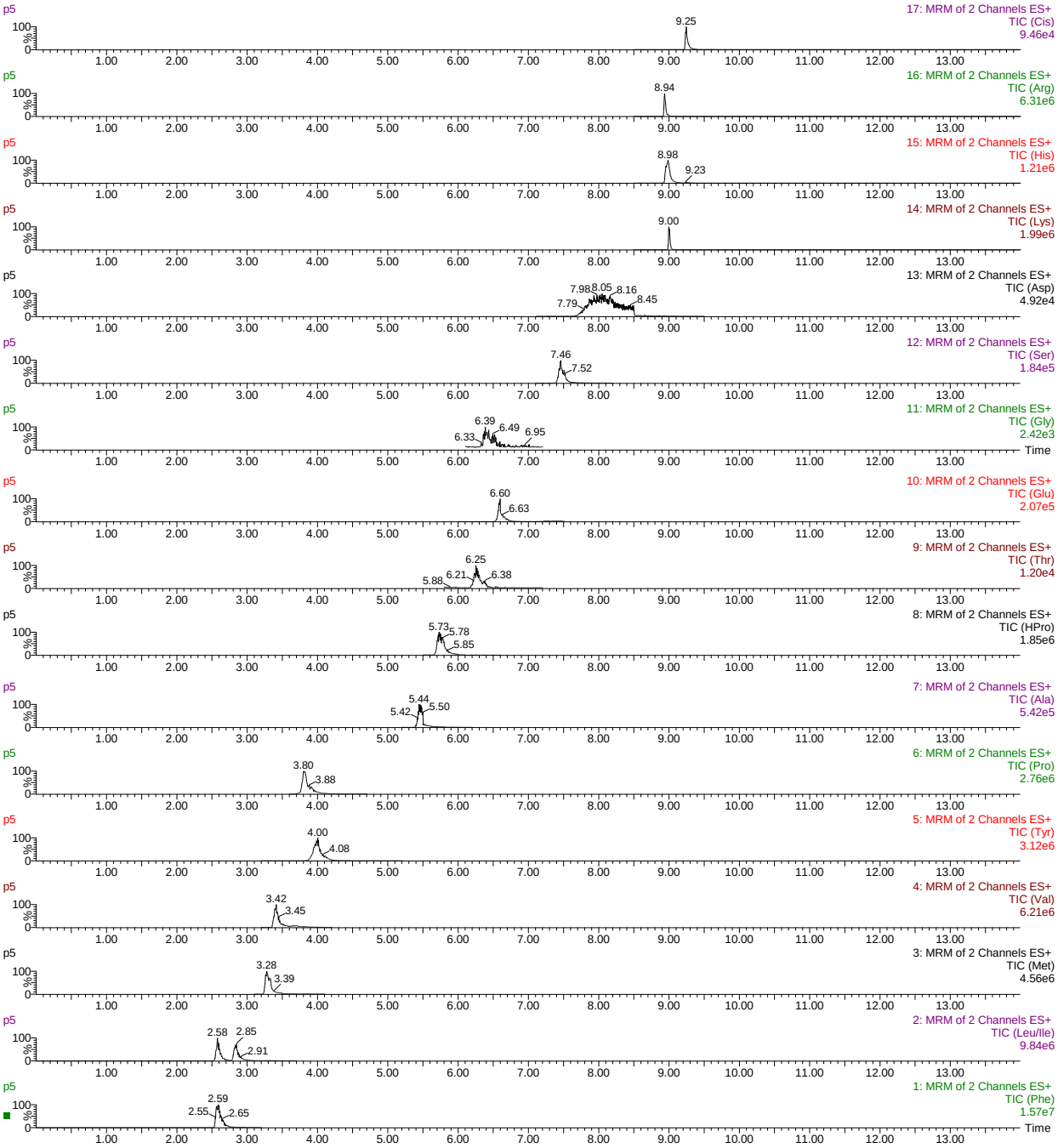


Figure S3.2. Chromatograms of the standards used for the quantification of the 18 amino acids.

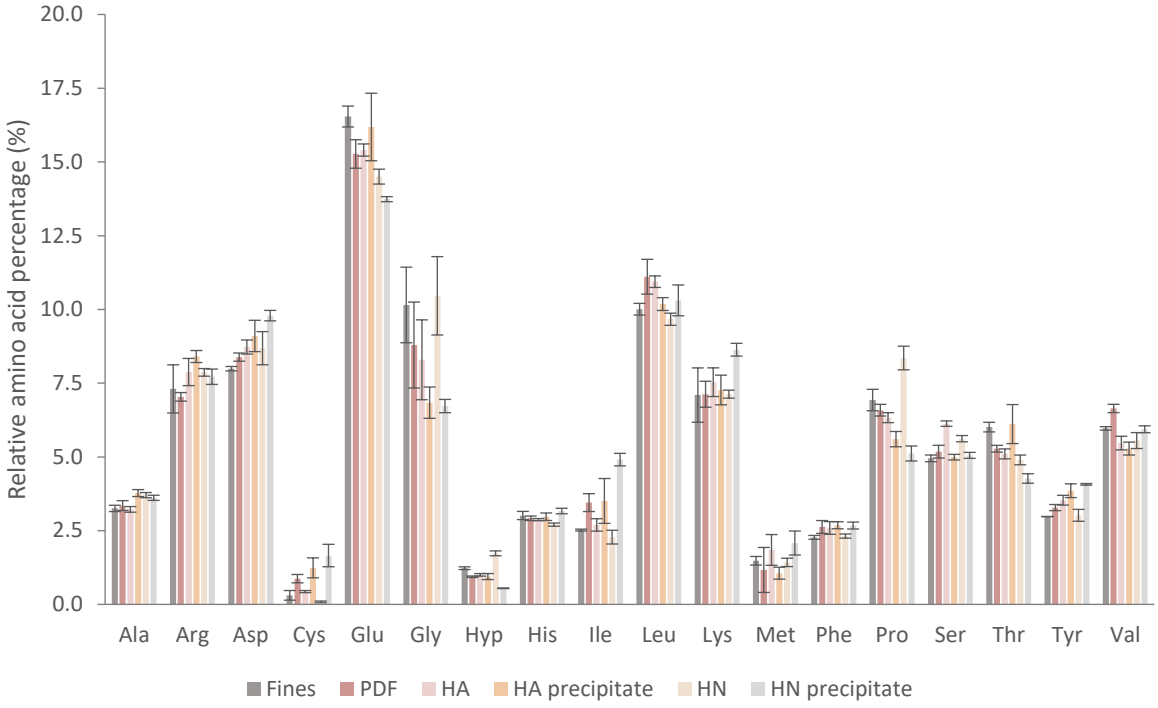
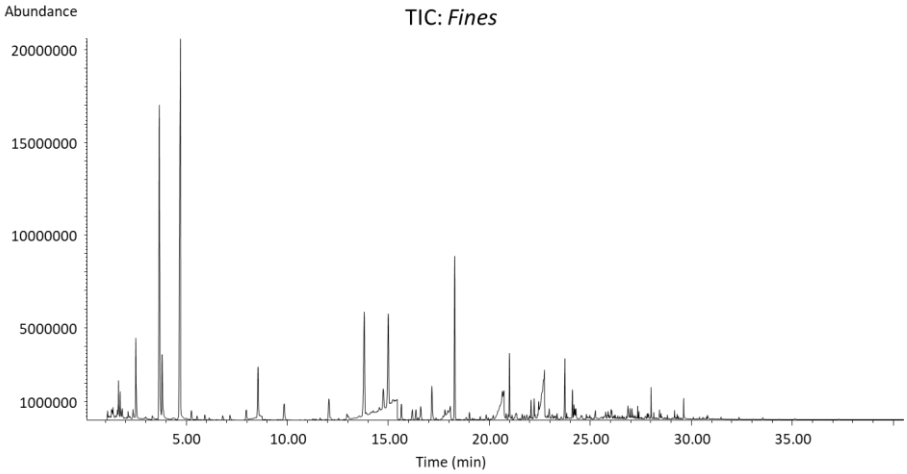


Figure S3.3. Relative percentage of each amino acid in *fines*, partially defatted *fines* (PDF), Alcalase 2.4L hydrolysate (HA), HA precipitate, Neutrase 0.8L hydrolysate (HN), and HN precipitate.

Table S3.1.Proximate composition and amino acid concentration in MBM.

Reference		(Johnson et al., 1998)	(Karakas et al., 2001)			(Ravindran et al., 2002)	(Ravindran et al., 2005)	(Adedokun & Adeola, 2005)	(Hendriks et al., 2006)	(Sriperm et al., 2011)	(Li et al., 2011)	(Kim et al., 2012)	(Muir et al., 2013)	(Moutinho et al., 2017)	This work
Animal			Swine					Swine						70% Swine	Swine
Country		US	Denmark			New Zealand	New Zealand	US			China			Spain	Spain
									% (DM basis)						
CP (N x 6.25)		55.0	66.9	58.6	44.4	55.6	54.0	53.8	58.4	54.8	52.0	54.3	50.3	53.1	45.8
Fat		10.0	11.2	11.2	10.7	10.6		11.6	10.8				6.0	15.3	37.2
DM		97.0	97.8	98.4	98.3	95.4	95.5	99.1	95.8	95.1	96.1		93.4	97	97.2
Ash		24.1	20.9	29.3	43	27.8		26.1	29.4				36.1	26.9	12.2
Amino acids									**mg/g (DM basis)**						
Essential	Arg	37.9	41.1	38.1	30.8	41.1	38.6	37.2	40.0	36.5	38.2	36.7	37.8	37.2	36.3
	His	12.3	11.4	9.9	6.4	10.8	8.9	10.6	10.5	9.6	12.4	10.3	10.5	10.8	15.0
	Ile	19.3	17.8	15.2	10.3	16.6	15.0	16.2	17.4	16.6	20.9	15.2	14.4	10.1	12.56
	Leu	42.3	37.7	31.9	22.3	36.1	34.1	33.3	32.3	34.4	37.0	32.7	30.5	26.2	49.8
	Lys	33.4	31.2	28	19.9	30.6	27.5	28.5	29.7	26.8	32.9	29.3	27.3	25.6	35.5
	Met	10.4	7.1	7.4	5	8.8	10.0	8.3	9.5	7. 8	11.4	7.5	8.0	7.5	7.3
	Phe	23.6	19.3	17.8	12.8	19.5	17.5	17.7	16.2	20.6	19.2	17.5	16.1	15.8	11.3
	Thr	23.3	19.9	16.8	11.6	20.2	19.2	17.7	18.0	18.6	25.2	16.1	18.1	16.8	29.9
	Val	27.5	25.5	21.2	15.3	25.3	22.2	23.7	23.0	24.3	23.2	22.9	20.6	17.6	29.7
Semi	Cys	7.5	4.6	3.4	2.2	3.9	4.0	5.8	4.0	7.2	5.1	4.7		7.3	1.5
	Tyr	15.2	14.7	13.0	8.6	13.8	12.2	12.8	13.5	8.9	15.1	12.8	10.5	11.2	14.8
Non-essential	Ala	41.1	41.8	36.6	30.2	43.1	40.1	35.9	42.3	37.6	49.7	38.3	41.6	36.8	16.2
	Asx	53.1	45.1	39.0	28.6	45.0	41.8	39.9	43.6	40.6	54.9	40.3	36.7	50.8	39.8
	Glx	75.0	74.5	61.4	47.0	69.3	68.0	70.2	64.1	63.5	71.4	64.7	64.0	69.8	82.4
	Gly	62.2	64.3	53.6	47.2	72.5	72.8	68.2	72.9	66.7	90.2	71.1	77.9	75.4	50.8
	Hyp							26.4			30.0				6.1
	Pro	42.0	47.9	49.6	37.7	46.7	45.2	42.0	43.8	45.0	61.0	39.0		42.7	34.5
	Ser	24.0	28.2	21.0	15.9	22.6	21.7	20.1	19.3	25.5	21.6	17.8	22.4	22.8	24.7
	Trp		2.8	2.7	1.6			3.8		3.7	4.1	3.6			
AA N						80.8	73.19		77.6	77.9					71.4
Sum AA		550.1	534.9	466.6	353.4	525.9	498.8	518.3	500.1	493.7	622.7	480.5	436.4	484.5	498.3


TIC: *Fines*
Abundance
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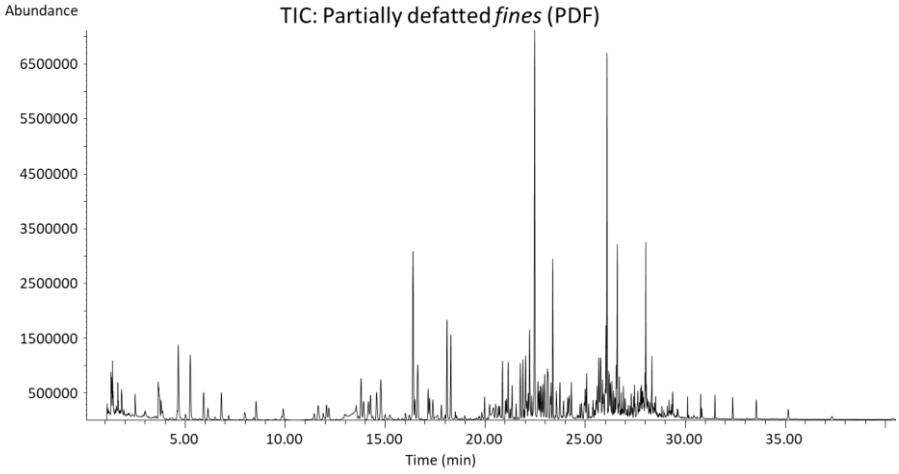
10000000
5000000
1000000
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Time (min)


TIC: Partially defatted *fines* (PDF)
Abundance
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2500000
1500000
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5.00
10.00
15.00
20.00
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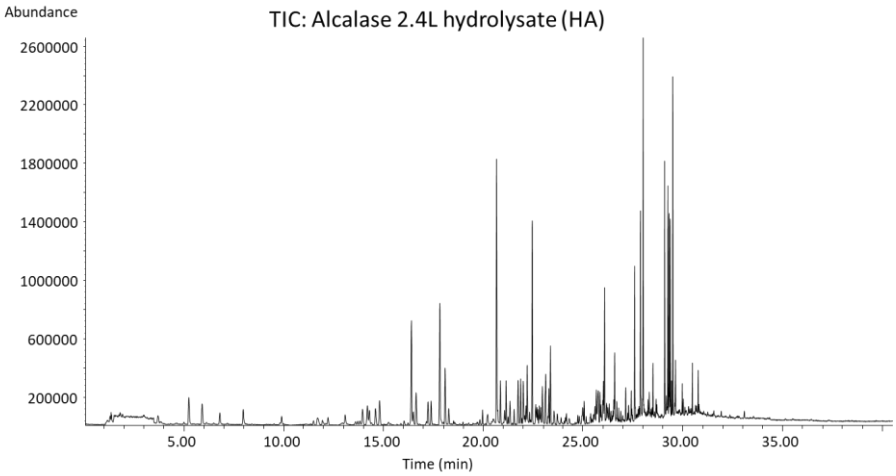
30.00
35.00
Time (min)


TIC: Alcalase 2.4L hydrolysate (HA)
Abundance
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20.00
25.00
30.00
35.00
Time (min)

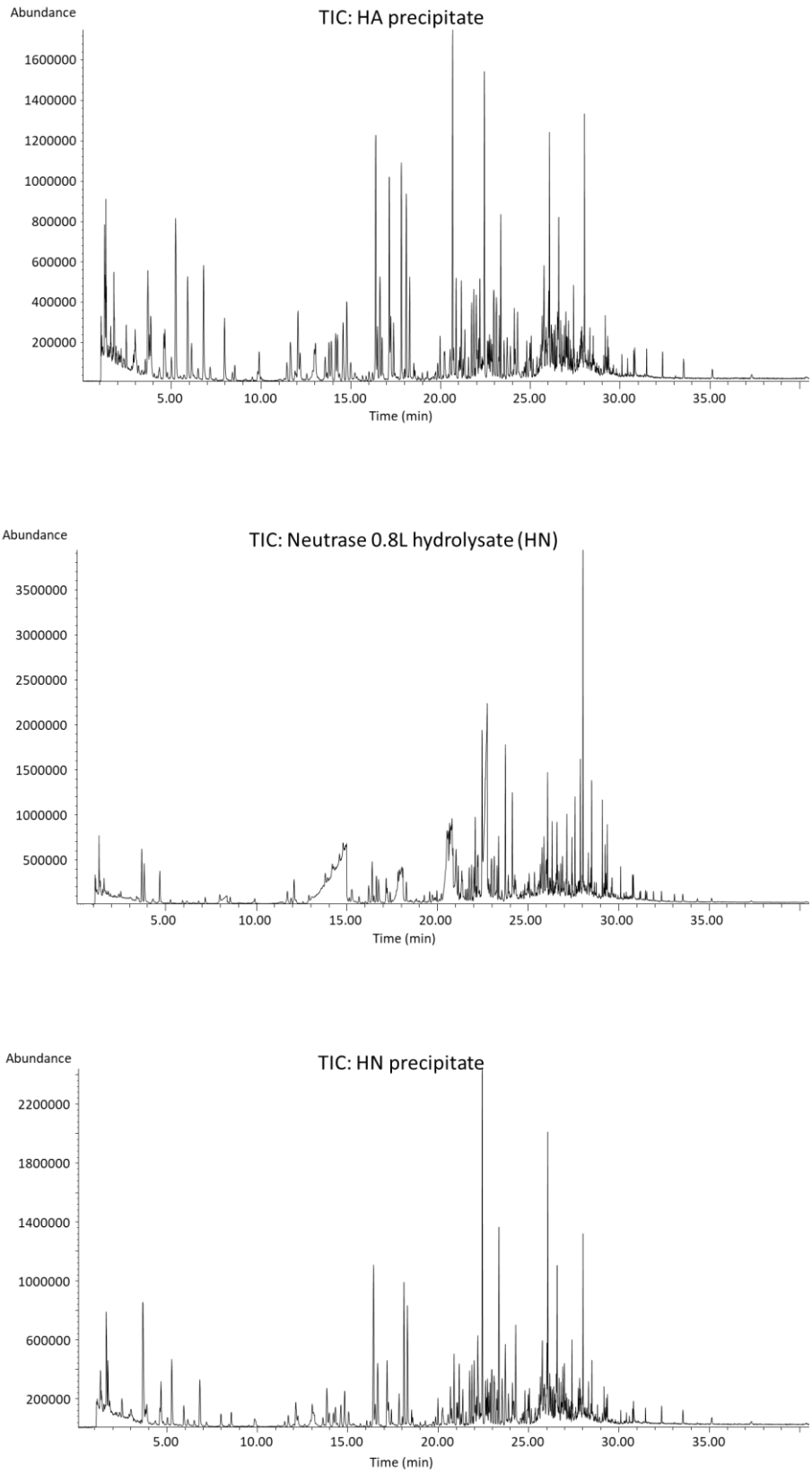

Figure S3.4. Chromatograms of the volatiles profiles.

Chapter 4. Utilization of protein hydrolysates in bacteria culture

1. Background

Bacterial species are of high interest in the industry due to their vast application potential. A wide range of products can be obtained, such as enzymes (Mabrouk et al., 1999; Kirk et al., 2002), bulk chemicals (Zeng & Biebl, 2002), polyesters (Lenz & Marchessault, 2005), starter cultures (Ummadi & Curic-Bawden, 2010), nanoparticles (Carrasco et al., 2021), etc. However, despite extensive studies and demonstration of the feasibility of production on a small scale, only a reduced number of processes have been scaled-up to industrial production.

It is known that the growth and productivity of a microorganism are strongly influenced by medium components (Hammami et al., 2018). In this sense, variations in the nutritional nitrogen requirements are observed among species, and even strains, to achieve maximum growth and/or productivity (Ummadi & Curic-Bawden, 2010). Besides this, there is no formula to determine the exact requirements, and it requires a great deal of trial and error. Although no medium has been designed for all species and applications, Luria-Bertani (LB) broth, a nutritional rich media, is one of the most referenced bacterial media for growth and maintenance of *Escherichia coli* and other non-fastidious species because it permits fast and good growth yields (Sezonov et al., 2007).

Since *Escherichia coli* (*E. coli*) and *Pseudomonas putida* (*P. putida*) were used as model microorganisms in our laboratories, they were selected to study the ability of the obtained protein hydrolysates to support their bacterial growth. Besides this, to have more extensive knowledge about the applicability of the peptones produced, *Bacillus cereus* (*B. cereus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were also included in this study. *E. coli,* widely used in biotechnology (Dufossé et al., 2001), is characterized by its rapid proliferation and ability to utilize amino acids and peptides as a sole source of carbon and nitrogen without the need for glucose addition (Stiborova et al., 2016). Similarly, there is a growing interest in *P. putida* for its potential application in biotechnology and low nutritional requirements. It has been proven to be an excellent bacterial host to produce polymers (polyhydroxyalkanoates and alginates), bulk chemicals (*cis*,*cis*-muconic acid), drugs (polyketides and non-ribosomal peptides), and also can be used as a biocontrol agent and plant growth-promoting and in bioremediation (Weimer et al., 2020). On the other hand, *B. cereus* and *S. aureus* are frequently involved in foodborne diseases and isolated in human samples (Lanciotti et al., 2001), and therefore their growth is of interest for clinical purposes. According to the wide range of applications, the culture of these microorganisms shows an increasing demand for low-cost microbial media. Given that usually the most expensive component is the nitrogen source, approaching the obtention of peptones from economical/low-cost sources is of great interest (Djellouli et al., 2017; Safari et al., 2012).

In the present study, two low-cost nitrogen sources obtained from *fines*, a valueless meat by-product, were screened in view of their effect on bacteria growth, and the results were compared with the growth using commercial peptones. The selected bacteria were: two Gram-negative (*E. coli* and *P. putida*) and two Gram-positive (*S. aureus* and *B. cereus*).

2. Materials and methods

2.1. Materials

Tryptone, sodium chloride (NaCl), yeast extract, and agar were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alcalase 2.4L hydrolysate (HA) and Neutrase 0.8 hydrolysate (HN) were produced following the methodology described in Chapter 3 Section 2.5.

2.2. Microorganisms, media, and inoculum preparation

Four bacterial species were employed. *E. coli* BL21 (DE3), *S. aureus* ATCC 25923, and *B. cereus* NCIMB 7464 were obtained from the culture collection of the University of Lleida. *P. putida* EM42 was kindly provided by the Technical University of Denmark. LB medium was used as reference medium. The strains were conserved in glycerol at -80 °C and replicated in LB agar plates and LB broth medium for the assays. LB agar was composed of the following (g/L): tryptone, 10; yeast extract, 5; NaCl, 5; and agar, 12. LB broth contained (g/L): tryptone, 10; yeast extract, 5; and NaCl, 10. The pH of all the media was adjusted at 7 before autoclaving at 121 °C for 20 min.

First, a pre-culture was prepared by adding one single colony from an LB agar plate to 250 mL shake flasks containing 100 mL of LB broth. The culture of *E. coli* was grown at 37 °C and 160 rpm overnight, while *S. aureus*, *B. cereus*, and *P. putida* were incubated at 30 °C. After 16 - 20 h the culture was centrifuged, the supernatant discarded, and the pellet was resuspended in 0.9 % NaCl solution, ready to be used as inoculum. The optical density at wavelength 600 nm (OD_{600}) was used to measure inoculum growth.

2.3. Growth curves

To assess the effect of commercial and newly produced peptones on different bacteria growth, the resuspended pre-culture was diluted to OD_{600} ~ 0.05 in 25 or 50 mL of modified LB media according to Table 4.1. Peptic digest of meat (commercial meat peptone) was used as reference peptone instead of tryptone in LB medium (LB-C). As the N content was similar for all the peptones, the commercial meat

peptone in the LB media was replaced by the same amount (weight) of the enzymatically produced protein hydrolysates (HA and HN) while all other components of the media were not modified (LB-HA and LB-HN). A media without peptone was prepared to distinguish the peptone effect (LB-WP). The pH of all the media was adjusted at 7 before autoclaving at 121 °C for 20 min.

The microorganisms were incubated in an orbital shaker at 160 rpm and 37 °C for *E. coli* and 30 °C for *S. aureus*, *B. cereus*, and *P. putida*.

Growth was monitored by measuring the OD_{600} in a PerkinElmer Lambda XLS or Shimadzu UV-1800 UV Spectrophotometer at specific intervals by sampling 0.5 or 1 mL aliquots. Prior to measurements, the aliquots were centrifuged, the pellets were washed twice with sterile Milli-Q® water and resuspended to the adequate dilution. Each growth curve was obtained from three individual independent experiments.

Table 4.1. Composition of medium (g/L) formulated for the evaluation of HA and HN.

Compound	**Medium (g/L)**			
	LB-C	**LB-WP**	**LB-HA**	**LB-HN**
Commercial meat peptone	10	-	-	-
HA	-	-	10	-
HN	-	-	-	10
Yeast extract	5	5	5	5
NaCl	10	10	10	10

LB-C: Luria-Bertani medium control; LB-WP: Luria-Bertani medium without peptone; LB-HA: Luria-Bertani medium containing Alcalase 2.4L hydrolysate (HA); and LB-HN: Luria-Bertani medium containing Neutrase 0.8L hydrolysate (HN)

2.4. Statistical analysis

All the experiments were performed in triplicate. The presented results are the mean of triplicates and the standard deviations are shown as error bars in the figures. Data handling and statistics were performed using Excel software package (Microsoft Excel, 2013).

3. Results and discussion

3.1. Bacterial growth

The capability of the protein hydrolysates from *fines* to replace commercial peptone in LB medium was assessed by measuring the growth of the cells. As it is widely reported that excessive concentration of nitrogen source in media might cause significant inhibition of cell growth (Taskin et al., 2016; Ummadi & Curic-Bawden, 2010), the commercial peptone was replaced by the same amount of the prepared protein hydrolysates.

As widely known, the bacterial growth curve shows four phases (Figure 4.1). In the first, during the lag phase, cells are adapting to the new environment. The lag phase duration depends on the strain and medium and varied from 2 to 6 h. However, the length of the lag phase can be controlled by the inoculum size and the type of medium. Following the lag phase, growth is exponential, and the cells grow until reaching the stationary phase. During the growth phase, the specific growth rate is approximately the maximum specific growth rate. The time required to attain the stationary phase is variable for each strain and cultivation medium. The stationary phase is caused by nutrients depletion or inhibitory compound accumulation. Although at this point cells still grow and divide, thanks to dying cells that lyse and serve as a source of nutrients, the stationary phase can be defined as a state of no net growth. Finally, cells enter in death or decline phase, which is characterized by a loss of viable cells (Doran, 2013; Maier, 2009).

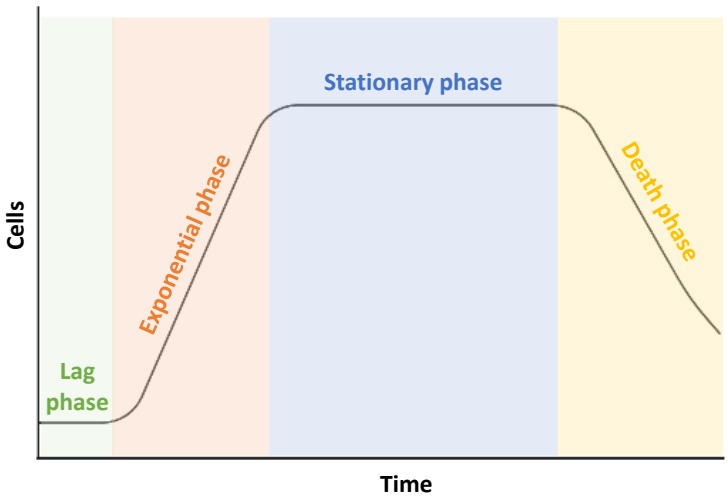

Figure 4.1. A typical growth curve for a bacterial population.

3.2. Growth comparison

Growth curves obtained for *E. coli* are shown in Figure 4.2. Almost identical curves were observed for the different media containing peptones, demonstrating the potential of the produced protein hydrolysates to be used as nitrogen source for this strain. Although HA and HN have different amino acid and peptide profiles, *E. coli* did not show a preference for a specific nitrogen source. The OD_{600} are in concordance with the ones reported by Martone et al., (2005), who grew *E. coli* in LB medium and a medium containing an FPH (fish protein hydrolysate), achieving an OD_{600} of 1 after 7.5 h of cultivation. However, Aspmo et al., (2005) reported different growth curves for *E. coli* depending on the nitrogen source, observing a lower OD_{600} for tryptone, which is usually used in LB medium. These authors attributed this phenomenon to pH/buffering effects induced by the buffer capacity of the peptones. Our findings might indicate that the *E. coli* strain used was not sensitive to the differences among the nitrogen sources supplied, and neither correlation was observed between the size of the peptides and the growth. The medium without peptones, containing only yeast extract as N source, showed more unsatisfactory performance. This was reasonably expected and can be explained by the lower amount of nutrients added to the medium.

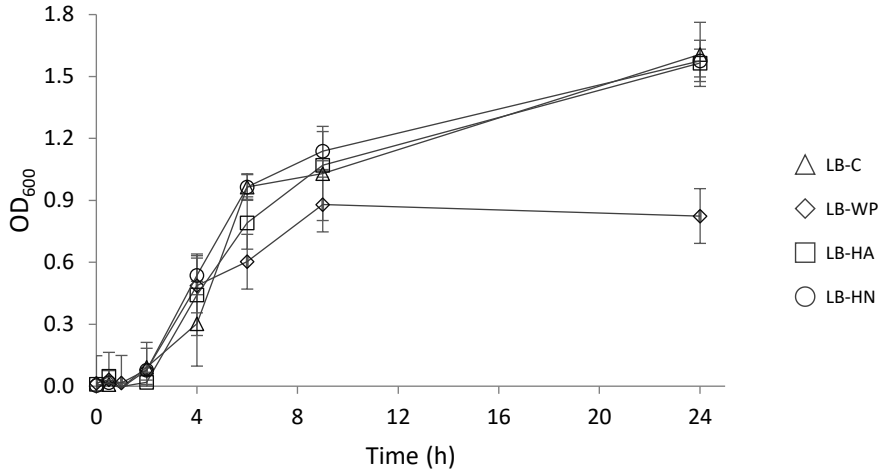

Figure 4.2. Effect of different nitrogen sources in the growth of *E. coli*. LB-C: Luria-Bertani medium control; LB-WP: Luria-Bertani medium without peptone; LB-HA: Luria-Bertani medium containing Alcalase 2.4L hydrolysate; and LB-HN: Luria-Bertani medium containing Neutrase 0.8L hydrolysate. Refer to Table 4.1 for media composition.

Growth curves obtained for *S. aureus* are shown in Figure 4.3. In that case, the effect of the type of peptone is evident, showing that HA and HN outperformed the commercial meat peptone. These observations might be related to the presence of specific peptides in the produced protein hydrolysates or factors other than amino acid supply (Lapeña et al., 2018). Similar growth curves were obtained by Klompong et al., (2012), who observed the higher cell density in media containing FPH obtained with alcalase and flavourzyme with a DH of 25%, comparable to HA. Besides this, the amount of ash in HA and

HN could also positively impact the growth, as this reflects the amount of minerals and these serve as the main source of mineral elements needed for microbial activities (Taskin & Kurbanoglu, 2011).

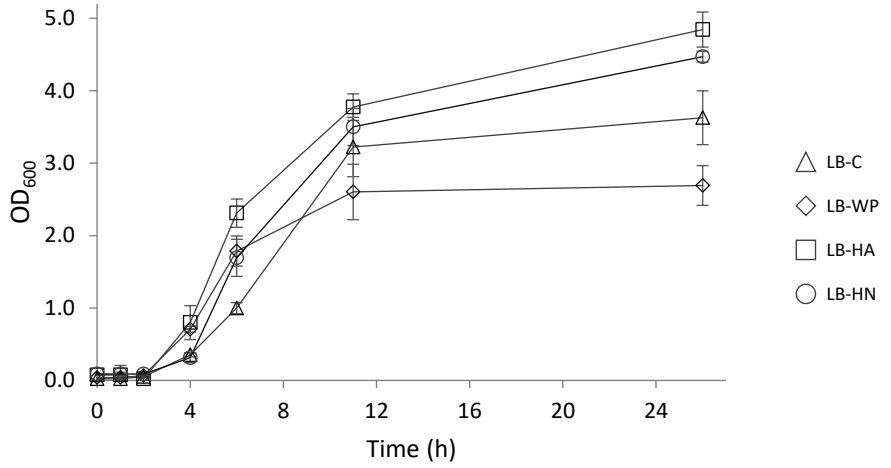

Figure 4.3. Effect of different nitrogen sources in the growth of *S. aureus*. LB-C: Luria-Bertani medium control; LB-WP: Luria-Bertani medium without peptone; LB-HA: Luria-Bertani medium containing Alcalase 2.4L hydrolysate; and LB-HN: Luria-Bertani medium containing Neutrase 0.8L hydrolysate. Refer to Table 4.1 for media composition.

Growth curves obtained for *B. cereus* are shown in Figure 4.4. Slight variability was observed among the growth media tested, and even the medium without peptones performed well. There is no clear evidence of the peptone effect in the growth of this microorganism. A possible explanation is that the growth of this strain was not inhibited by nutrients depletion, and there were enough nutrients even when peptone was removed (LB-WP) to support growth. It should be noted the longer adaptation phase of this strain to LB-HN, although once growth started the growth rate did not show large differences.

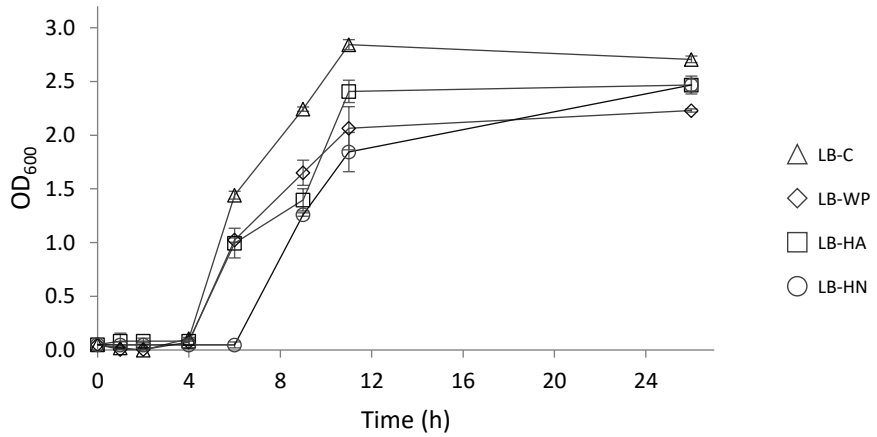

Figure 4.4. Effect of different nitrogen sources in the growth of *B. cereus*. LB-C: Luria-Bertani medium control; LB-WP: Luria-Bertani medium without peptone; LB-HA: Luria-Bertani medium containing Alcalase 2.4L hydrolysate; and LB-HN: Luria-Bertani medium containing Neutrase 0.8L hydrolysate. Refer to Table 4.1 for media composition.

Growth curves for *P. putida* are shown in Figure 4.5. The media containing peptones supported well the growth of this bacteria. Although no differences were observed among the media after 6 h of cultivation, after 8 h a higher OD_{600} was given in the medium containing HA. These experiments clearly manifest that despite using the same raw material, the properties of the protein hydrolysate, which were affected by the enzyme, clearly influenced growth performance. This behavior was previously observed by several authors (Klompong et al., 2012; Safari et al., 2012).

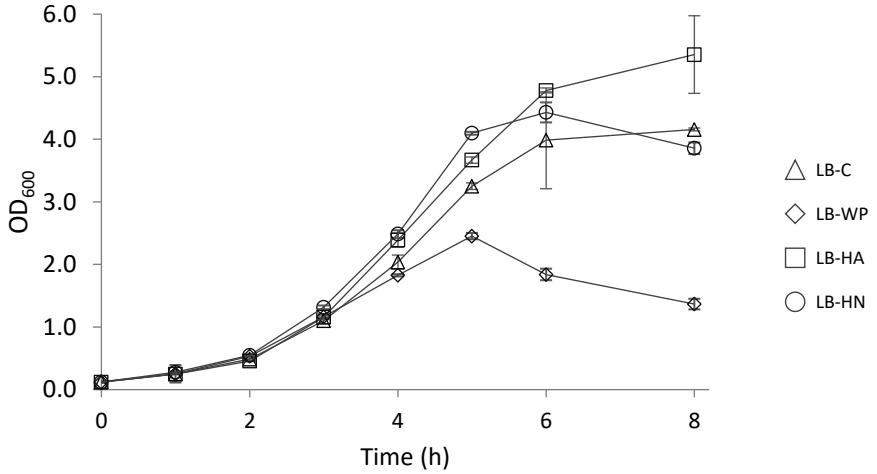

Figure 4.5. Effect of different nitrogen sources in the growth of *P. putida*. LB-C: Luria-Bertani medium control; LB-WP: Luria-Bertani medium without peptone; LB-HA: Luria-Bertani medium containing Alcalase 2.4L hydrolysate; and LB-HN: Luria-Bertani medium containing Neutrase 0.8L hydrolysate. Refer to Table 4.1 for media composition.

Although a specie-specific effect of protein hydrolysates HA and HN on growth performance was observed, the results demonstrated their capacity to substitute commercial peptones by supporting bacterial growth while producing analogous profiles to commercial ones or even outperforming them. The media containing only yeast extract and NaCl (LB-WP) permitted only limited bacterial growth, observing a lower maximum OD_{600} for *E. coli*, *S. aureus*, and *P. putida*. However, a lower impact was observed for *B. cereus*, which gave similar results compared to media supplemented with peptones. This reveals that the growth performance is affected by peptone choice and for some bacterial species peptones are essential to achieve high biomass concentrations, a requirement in biotechnological processes, as it greatly influences production.

The present findings are exciting and promising, as there is a growing interest in the use of microbial biomass for biotechnological and industrial purposes. For instance, the large knowledge about *P. putida*, together with the intrinsic biochemical capabilities has made a reputation for being a promising microbial chassis for the bioindustry, offering potential for a wide range of industrial applications (Weimer et al., 2020). The exploitation of renewable feedstocks, such as lignocellulose, and waste streams to produce

value-added chemicals using this bacterium, or others of interest, will lead to a high demand for economical nitrogen sources to sustain the growth and to achieve high biomass concentrations. Besides this, it is worth highlighting that the contribution of protein hydrolysates is more than merely supplying nitrogen sources, as they can enhance productivities by some unknown mechanisms (Pasupuleti et al., 2010). The produced peptones supported well the growth of all the bacteria tested. As peptone is the most expensive ingredient in the growth media, the utilization of protein hydrolysates from a valueless raw material will suppose a significant decrease in the process cost.

4. Conclusions

In the present study, two low-cost protein hydrolysates prepared in our laboratory were evaluated as nitrogen source on bacterial growth. Four bacterial species were selected given their interest in biotechnology. In general, a typical growth curve was observed for all the species. Moreover, the tested bacteria strains displayed good or even better performance in LB medium prepared with HA and HN as nitrogen sources compared to the control. Thus, these results demonstrate that these low-cost peptones represent an attractive alternative to supplement growth media and to obtain high biomass. Given that peptone is usually the most expensive ingredient in the growth media, the replacement of commercially available protein hydrolysates by peptones from a valueless raw material will suppose a significant decrease in the process cost.

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Chapter 5. Assessment of various nitrogen sources in yeast fermentation

The results described in the chapter have been obtained at PROSYS Research Centre - DTU Chemical Engineering (Technical University of Denmark).

1. Background

Nutritional requirements of microorganisms used in fermentation processes are as complex and varied as the microorganism types. Media formulation is essential to satisfy the requirements of the fermenting organism while achieving proper chemical balance at the lowest cost and still allow easy processing. In addition to the carbon source, the nitrogen source is generally the next most plentiful substance for the fermentation media (Kampen, 2014).

Saccharomyces cerevisiae (*S. cerevisiae*) is of great importance for several biotechnological processes due to its fermentation capacity and its resilience to adverse conditions of pH and osmolarity (Parapouli et al., 2020). Hence, it is commonly used in industrial fermentation processes (Brandberg et al., 2007; Karagöz & Özkan, 2014). In addition, the topology of the metabolic network of *S. cerevisiae* is one of the best known (Crépin et al., 2017). Currently, the main fields of application involving *S. cerevisiae* are food, beverage, and biofuel production industries (Parapouli et al., 2020).

Bio-based ethanol is a sustainable energy source with a high potential to replace fossil fuels such as diesel and gasoline (Li et al., 2017; Raposo et al., 2017). It aims to be cost-competitive, environmentally friendly, and self-sustaining compared to its petrochemical equivalents (Otero et al., 2007). In the last decade, world ethanol production for transport fuel has increased substantially, and it is the most popular alcoholic biofuel available in the market (Li et al., 2017; Vohra et al., 2014). The main feedstocks used for 1st generation ethanol production are sugarcane and corn, which compete for food production. Lignocellulosic biomass from agriculture (like bagasse and straw) and forestry (like wood waste) residues is the most abundant form of biomass (Zhang et al., 2017). Currently, fuel ethanol production from lignocellulosic feedstocks, known as 2nd generation ethanol (2G ethanol), is a promising candidate to become the main type of renewable liquid fuel, and it is one of the largest areas of biotechnological research (Almeida et al., 2009; Barahona et al., 2019; Knudsen & Rønnow, 2020; Pereira et al., 2015; Robak & Balcerek, 2018; Singh & Bishnoi, 2013; Vohra et al., 2014; Westman et al., 2014). 2G ethanol production process does not compete against food and feed supplies and has potential environmental benefits. In addition to ethanol production, lignocellulosic biomass is a source of weak acids (such as acetic, formic, or levulinic acids), phenolic compounds (such as vanillin or 4-hydroxybenzoic acid), and furaldehydes (such as furfural and 5-hydroxymethylfurfural) (Almeida et al., 2009; Palmqvist et al., 1999).

Ethanol production from lignocellulosic biomass requires 1) pre-treatment to expose the cellulosic fibers, 2) saccharification of biomass to fermentable sugars, and 3) fermentation of sugars into ethanol (Cabaneros Lopez et al., 2019). However, successful and economically viable 2G ethanol production is still

challenging due to several hurdles: pre-treatment methods cost and effectivity, hydrolysis enzymes costs and performance, efficient conversion of hexoses and pentoses to ethanol, and media formulation.

S. cerevisiae is regarded as one of the most promising industrial microorganisms for the exploitation of 2G ethanol due to its ethanol-producing capacity and high tolerance to ethanol and the inhibitory compounds present in the lignocellulosic hydrolysates (Palmqvist & Hahn-Hägerdal, 2000a; Tomás-Pejó et al., 2008; Tran et al., 2020). Hydrolysates from lignocellulosic materials are generally low in nutrients and nitrogen (Jørgensen, 2009; Tomás-Pejó et al., 2012), and therefore nitrogen supplementation is necessary to facilitate yeast growth (short lag phase, high growth rate, and high biomass), and fermentation performance (fast and complete fermentation). To address commercial bio-based ethanol production feasibility, the growth media cost should be kept at a minimum. Although *S. cerevisiae* is able to use a wide range of nitrogen sources (Beltran et al., 2004; Li et al., 2017), yeast extract - peptone (YP) is a conventional nutrient employed in yeast growth. Media supplementation components can imply more than 30% of the total production cost; thus, it has a very strong effect on the economy of the industrial ethanol production process (Tomás-Pejó et al., 2012). Alternatively, engineering strains to require less nutritional supplementation is important for the expansion of bio-based ethanol production (Jarboe et al., 2007). Hence, an approach to reduce cellulosic ethanol production costs significantly would be the replacement of expensive nitrogen sources by low-cost nitrogen sources capable of supporting yeast growth, achieving high biomass, and enhanced ethanol production.

In this context, this study aimed to investigate the effect of previously obtained protein hydrolysates on growth media of two *S. cerevisiae* strains, *S. cerevisiae* Ethanol Red® and *S. cerevisiae* CEN.PK XXX, with high interest in the ethanol industry. First, yeast strains growth (optical density at 600 nm (OD_{600}), lag phase (λ), and growth rate (μ)) and fermentation performance (yield, productivity, and ethanol formation) were evaluated in synthetic media. Then, as *S. cerevisiae* CEN.PK XXX has the capacity to degrade both dextrose and xylose, it appeared as an attractive strain in the fermentation of lignocellulosic biomass. A wheat straw hydrolysate containing both dextrose and xylose was used in a fermentation setup to screen the effect of different nitrogen sources, in addition to the protein hydrolysates. Nitrogen source ability to improve fermentation was evaluated based on ethanol yield and productivity, as these parameters are of interest to minimize fermentation time and maximize ethanol production.

2. Materials and methods

2.1. Materials

Dextrose, xylose, ammonium sulfate, and agar were purchased from Sigma-Aldrich (St. Louis, MO, USA). Yeast extract and peptone from casein were purchased from Microbiology Fermentech (Merk, New Jersey, USA). The hydrolysates obtained with Alcalase 2.4L in the reactor (RHA) and with Neutrase 0.8 L (RHN) were produced following the methodology described in Chapter 3 Section 2.6.

The pre-treated wheat straw hydrolysate (WSH) was supplied by Lund University. The composition of wheat straw hydrolysate was (g/L): dextrose, 44: xylose, 22; and acetic acid, 3.05.

2.2. Microorganisms, media, and inoculum preparation

S. cerevisiae Ethanol Red® purchased from Leaf (Lesaffre Advanced Fermentations, Marcq-en-Baroeul, France) and a xylose-consuming *S. cerevisiae* strain, CEN.PK XXX (Westman et al., 2014) were employed. The strains were conserved in 20% glycerol at -80 °C. *S. cerevisiae* Ethanol Red® was replicated in Yeast Peptone Dextrose (YPD)-agar plate and YPD broth, while *S. cerevisiae* CEN.PK XXX was replicated in Yeast Peptone Dextrose Xylose (YPDX)-agar plates and YPDX broth media for the assays. YPD was composed of the following (g/L): yeast extract, 10; casein peptone, 20; and dextrose, 20. YPDX contained (g/L): yeast extract, 10; casein peptone, 20; dextrose, 20; and xylose, 20. The pH of all the media was adjusted at 6 before autoclaving at 121 °C for 20 min. Dextrose, xylose, and YP solutions were autoclaved separately.

One milliliter from a glycerol stock of *S. cerevisiae* Ethanol Red® was grown in 250 mL shake flasks containing 100 mL of YPD medium and *S. cerevisiae* CEN.PK XXX was grown in YPDX medium at 30 °C and 180 rpm for 16 and 36 h, respectively. After 1000-fold dilution, the yeast strains were plated in a solid YPD or YPDX-agar plate and incubated at 30 °C for 16 or 36 h. A pre-culture was prepared by adding one single colony from the agar plate to 250 mL shake flasks containing 100 mL of YPD or YPDX broth. The culture was grown at 30 °C and 180 rpm. After 12 or 36 h the cells were harvested by centrifugation, washed, and resuspended in a sterile 0.9% NaCl solution, ready to be used as inoculum.

2.3. Growth curves and measurement of extracellular metabolites

For assessing the growth of the different yeast strains in the media containing commercial and the newly produced peptones, the resuspended pre-culture was diluted to OD_{600} ~ 0.20 - 0.25 in 100 mL of modified YPD or YPDX media according to Table 5.1. This media was considered as control (YPD/YPDX). As the N

content was similar for all the peptones, the commercial meat peptone in the YPD/YPDX media was replaced by the same amount (weight) by the enzymatically produced protein hydrolysates while all other compounds were the same (YD-RHA/YDX-RHA and YD-RHN/YDX-RHN). A media without peptone was also prepared (YD/YDX). The pH of all the media was adjusted at 6 before autoclaving at 121 °C for 20 min.

Table 5.1. Composition of the culture medium (g/L) tested based on YPD and YPDX for evaluation of RHA and RHN.

Compound	**Culture medium (g/L)**							
	***S. cerevisiae* Ethanol Red®**				***S. cerevisiae* CEN.PK XXX**			
	YPD	YD	YD-RHA	YD-RHN	YPDX	YDX	YDX-RHA	YPDX-RHN
Dextrose	20	20	20	20	20	20	20	20
Xylose	-	-	-	-	20	20	20	20
Meat peptone	20	-	-	-	20	-	-	-
RHA	-	-	20	-	-	-	20	-
RHN	-	-	-	20	-	-	-	20
Yeast extract	10	10	10	10	10	10	10	10

YPD: Yeast Peptone Dextrose medium; YD: Yeast Dextrose medium; YD-RHA: Yeast Dextrose medium containing Alcalase 2.4L hydrolysate produced in the reactor; YD-RHN: Yeast Dextrose medium containing Neutrase 0.8L hydrolysate produced in the reactor; YPDX: Yeast Peptone Dextrose Xylose medium; YDX: Yeast Dextrose Xylose medium; YDX-RHA: Yeast Dextrose Xylose medium containing Alcalase 2.4L hydrolysate produced in the reactor; and YDX-RHN: Yeast Dextrose Xylose medium containing Neutrase 0.8L hydrolysate produced in the reactor

At pre-established times, two aliquots were manually withdrawn. The first sample was used to monitor the OD_{600} and the second one was used for analysis with high-performance liquid chromatography (HPLC) to determine the consumption and production of extracellular metabolites.

2.4. Lignocellulosic biomass fermentation and N source test

For nitrogen source screening tests, the pre-treated WSH medium was supplemented with various organic and inorganic nitrogen sources, according to Table 5.2. All fermentations were carried out with the same batch of pre-treated WSH containing 44 g/L dextrose and 22 g/L xylose. The process was carried out in 250 mL shake flasks containing 100 mL of wheat straw media covered with sterile cotton wool to prevent contamination. The medium was mixed with an amount of organic or inorganic nitrogen source that would yield the same nitrogen content (Table 5.2) and pH was adjusted at 5. The WSH supplemented was inoculated with *S. cerevisiae* CEN.PK XXX (inoculum was prepared as explained in Section 2.2) to yield an OD_{600} of 1. Fermentations were carried out at 30 °C and 180 rpm. Samples were collected at specific times and prepared for HPLC analysis.

Table 5.2. Nitrogen sources tested, their respective N content, and the added amount.

N source	N (%)	Amount added (g/L)
Urea	46.64	2.6
Casein	12 – 14	10
Ammonium sulfate	21.2	5.7
RHA	12.3	10
RHN	12.1	10

RHA: Alcalase 2.4L hydrolysate produced in the reactor and RHN: Neutrase 0.8L hydrolysate produced in the reactor

Experimental design. Production of 2G ethanol.

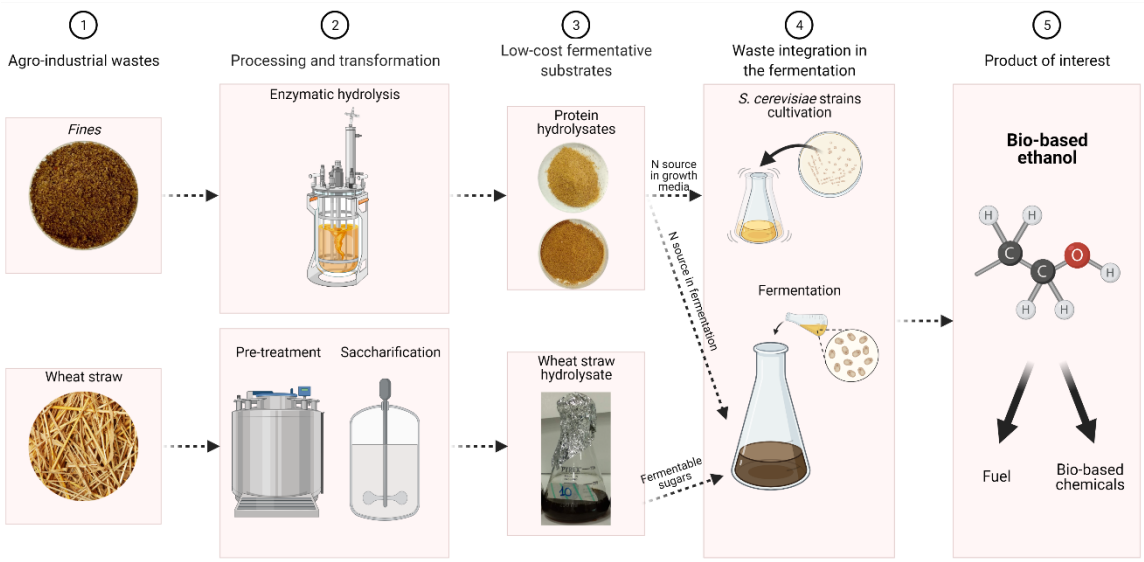

Figure 5.1. General overview of the bioethanol production process integrating animal and agricultural wastes. Created with BioRender.com.

2.5. Analytical methods

2.5.1. Cell density measurements

Cell growth was directly monitored by measuring OD_{600} of the culture broth at 600 nm using a Shimadzu UV-1800 UV Spectrophotometer (Germany). Growth curves gave access to determine λ, μ, and maximum OD_{600} (OD_{max}).

2.5.2. Fermentation profile – High-Performance Liquid Chromatography coupled with Refractive Index detector (HPLC-RI)

Dextrose, xylose, ethanol, and glycerol were determined by HPLC using an Ultimate 3000 HPLC instrument equipped (Thermo Scientific, Massachusetts, USA) with 4 UV/VIS and a refractive index (RI) detector (ERC RefractoMax 520, Prague, Czech Republic). The samples were filtered through a 0.20 µm cellulose acetate filter (Labsolute, Renningen, Germany), acidified by adding 25 µL of 5M H_2SO_4 to 475 µL of sample, and analyzed. The separation was achieved on an Aminex HPX-87H column (300 x 7.8 mm, 9 µm) (BIORAD, California, USA) at 50 °C. The mobile phase consisted of 5 mM H_2SO_4 aqueous solution and the flow rate was 0.6 mL/min. Each run was completed in 25 min. All the compounds studied were identified by their retention times and quantified based on calibration curves derived from the respective standards. Data acquisition and quantification were performed with the software Chromeleon 6.8.

2.6. Calculations

- The specific growth rate (µ) was determined by linear regression, plotting Ln (OD_{600}) *vs.* incubation time, corresponding to the slope of the exponential growth phase.
- The ethanol yield on consumed sugars ($Y_{EtOH/CS}$) was calculated using Equation 5.1:

$$Y = \frac{ethanol\ produced\ (g/L)}{glucose + xylose\ consumed\ (g/L)}$$

Equation 5.1

- The volumetric ethanol productivity was calculated using Equation 5.2:

$$Productivity\ (g/L/h) = \frac{ethanol\ produced\ (g/L)}{time\ (h)^a}$$

Equation 5.2

[a] when dextrose was totally consumed and xylose dropped below 5 g/L.

2.7. Statistical analysis

Data handling were performed using Excel software package (Microsoft Excel, 2013). Statistical analysis was carried out using JMP 14 Pro software (Statistical Discovery™ from SAS, Cary, NC, USA). Tukey's

honestly significantly different (HSD) test with a significance level of 0.05 was used for means comparison. The results were expressed as mean ± standard deviation.

3. Results and discussion

3.1. Growth characteristics

The capability of the protein hydrolysates from *fines* to replace commercial peptones added in YPD and YPDX media was assessed by measuring the growth and the consumption/production of extracellular molecules. The microorganisms used were *S. cerevisiae* Ethanol Red®, which is the industry standard in first and second generation ethanol plants (Gronchi et al., 2019) with the ability to ferment dextrose and tolerate high ethanol concentrations and *S. cerevisiae* CEN.PK XXX, a xylose-fermenting strain engineered by Westman et al., (2014).

The growth curves of *S. cerevisiae* Ethanol Red® and *S. cerevisiae* CEN.PK XXX are displayed in Figure 5.2 and

Figure 5.3, respectively. The λ, μ, and OD_{max} of both strains were compared during growth in culture media containing different complex nitrogen sources. Growth profiles were significantly different between both strains.

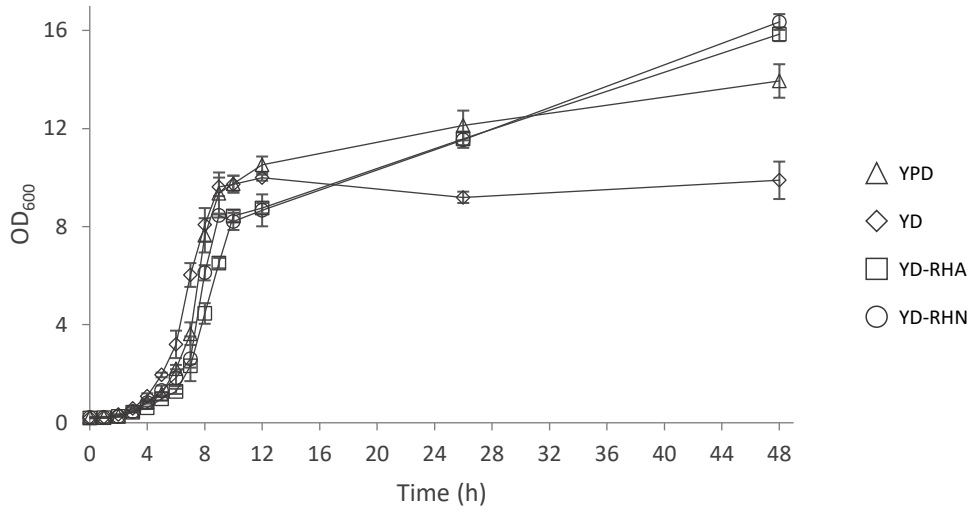

Figure 5.2. Growth curves of *S. cerevisiae* Ethanol Red®. YPD: Yeast Peptone Dextrose medium; YD: Yeast Dextrose medium; YD-RHA: Yeast Dextrose medium containing Alcalase 2.4L hydrolysate produced in the reactor; and YD-RHN: Yeast Dextrose medium containing Neutrase 0.8L hydrolysate produced in the reactor. Refer to Table 5.1 for media composition.

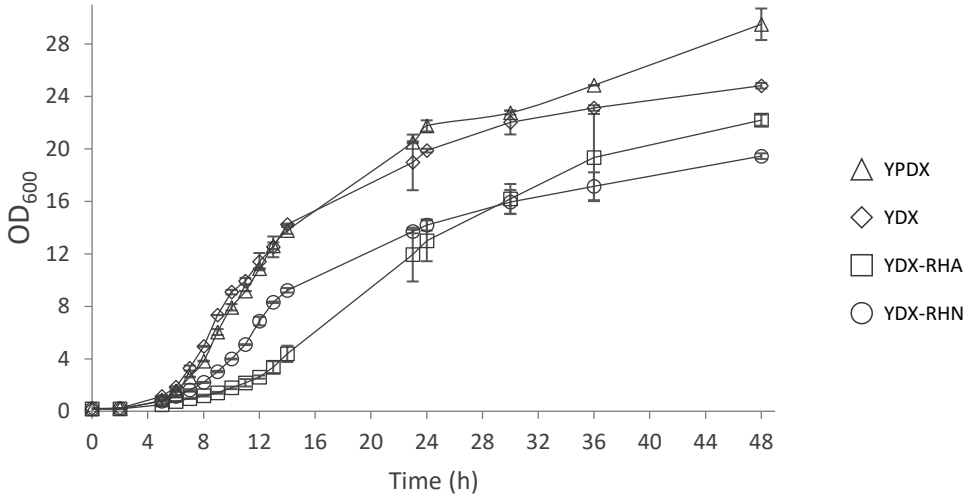

Figure 5.3. Growth curves of *S. cerevisiae* CEN.PK XXX. YPDX: Yeast Peptone Dextrose Xylose medium; YDX: Yeast Dextrose Xylose medium; YDX-RHA: Yeast Dextrose Xylose medium containing Alcalase 2.4L hydrolysate produced in the reactor; and YDX-RHN: Yeast Dextrose Xylose medium containing Neutrase 0.8L hydrolysate produced in the reactor. Refer to Table 5.1 for media composition.

3.1.1. Lag phase

The lag phase corresponds to physiological adaptation to the media. Although the same inoculum size was used, *S. cerevisiae* Ethanol Red® showed a lag phase of nearly 2 – 3 h, while *S. cerevisiae* CEN.PK XXX showed a more extensive lag phase of 5 to 8 h (Table 5.3), depending on the media formulation. When comparing *S. cerevisiae* Ethanol Red® lag phase in the control experiment (YPD) with the experiments where peptone was replaced (YD-RHA and YD-RHN) no significant differences were observed. However, the removal of peptone decreased the lag phase by -1. Similarly, *S. cerevisiae* CEN.PK XXX decreased the lag phase by -1 when the peptone was removed; nevertheless, the length of the lag phase increased by +3 in the presence of RHA. This clearly indicated that the presence of this protein hydrolysate involved more preparation to meet the new culture requirements, suggesting that the presence of different peptides induced a reorganization and physiological shift to synthesize the appropriate enzymes. On the other hand, the shorter lag phase in the culture medium without peptones might be explained by the faster acclimatization of both yeast strains due to the absence of new compounds and sufficient nutrients for growth. Arguably, the yeast extract provided enough nitrogen for growth. Although lag phase is highly dependent on size and cell cycle of the inoculum, medium formulation, and strain (Ginovart et al., 2011; Vermeersch et al., 2019), similar lag phase values were reported in the literature for yeast in the absence of inhibitors, ranging from one up to several hours (Aspmo et al., 2005; Cabañeros López, 2020; Klompong et al., 2012; Wallace-Salinas & Gorwa-Grauslund, 2013).

In addition to the differences in the lag phase, the strain CEN.PK XXX showed a diauxic growth pattern, characterized by growth in two phases (Figure 5.3). This behavior is usually observed in the presence of two sugars on the growth media, resulting in a faster growth as a consequence of the fast metabolization of the preferred one, followed by a lag phase while activating the machinery to metabolize the second one, and finally, the metabolization of the second sugar (Gopinarayanan & Nair, 2019; Lane et al., 2018). The second growth phase after the lag phase was slowed down for all the nitrogen sources tested, which might be due to the difficulties of this strain in converting xylose when compared with dextrose.

Table 5.3. Lag phase (λ) and specific growth rate (μ) in the different media tested.

Medium	***S. cerevisiae* Ethanol Red®**		**Medium**	***S. cerevisiae* CEN.PK XXX**	
	λ (h)	**μ (h^{-1})**		**λ (h)**	**μ (h^{-1})**
YPD	3	0.536 ± 0.009^{b}	**YPDX**	5	0.480 ± 0.001^{a}
YD	2	0.575 ± 0.023^{a}	**YDX**	4	0.474 ± 0.012^{a}
YD-RHA	3	0.460 ± 0.012^{c}	**YDX-RHA**	8	0.213 ± 0.019^{c}
YD-RHN	3	0.488 ± 0.008^{c}	**YDX-RHN**	5	0.295 ± 0.010^{b}

Means in the same column not connected by the same letter are significantly different (Tukey HSD test, $\alpha = 0.05$)
YPD: Yeast Peptone Dextrose medium; YD: Yeast Dextrose medium; YD-RHA: Yeast Dextrose medium containing Alcalase 2.4L hydrolysate produced in the reactor; YD-RHN: Yeast Dextrose medium containing Neutrase 0.8L hydrolysate produced in the reactor; YPDX: Yeast Peptone Dextrose Xylose medium; YDX: Yeast Dextrose Xylose medium; YDX-RHA: Yeast Dextrose Xylose medium containing Alcalase 2.4L hydrolysate produced in the reactor; and YDX-RHN: Yeast Dextrose Xylose medium containing Neutrase 0.8L hydrolysate produced in the reactor

3.1.2. Specific growth rate and OD_{max}

As displayed in Table 5.3, the specific growth rate of *S. cerevisiae* Ethanol Red® was higher than that of *S. cerevisiae* CEN.PK XXX. However, replacing the commercial peptone with RHA and RHN resulted in a reduction in the specific growth rate, regardless of the strain. These variations in the specific growth rate were consistent with data from the literature (Albers et al., 1996), who observed significant differences depending on the N source.

Despite a lower μ in the growth media containing RHA and RHN, significantly higher OD_{max} values were observed for *S. cerevisiae* Ethanol Red® in the media containing these hydrolysates. Therefore, media containing RHA and RHN could be used as growth media to produce a higher amount of biomass. Besides this, these hydrolysates can be considered as a good nitrogen source, given the short lag phase, the intermediate specific growth rate, and the higher OD_{max} (Su et al., 2020). The promising results might be thanks to the high content of arginine and glutamine/glutamate in the hydrolysates, the preferred nitrogen source of *S. cerevisiae* for *de novo* synthesis of amino acids (Crépin et al., 2017; Hofman-Bang,

1999; Magasanik & Kaiser, 2002; Ter Schure et al., 2000). Moreover, it is well known that *S. cerevisiae* cells are prompt to grow and ferment when multiple amino acids are available instead of single amino acids (Kemsawasd et al., 2015). Interestingly, a higher μ was observed for the YD medium compared to the control. This result might lie in the fact that dextrose and yeast extract provided sufficient nutrients to support lag and exponential phases. However, the lowest OD_{max} was observed in this medium, which might be due to the depletion of nutrients to support further biomass production. Based on this data, although this yeast strain prefers yeast extract as the nitrogen source, once this is consumed, the yeast takes advantage of the available resources, in this specific case peptone, to continue growing.

Conversely, growth curves of *S. cerevisiae* CEN.PK XXX showed that μ was reduced near to half in YDX-RHA and YDX-RHN compared to the control. Moreover, OD_{max} values were significantly lower, which indicates that this strain cannot assimilate the nitrogen present in RHA and RHN, observing even an inhibitory effect. The growth observed can be attributed to the yeast extract; therefore RHA and RHN can be considered a poor nitrogen source for this *Saccharomyces* yeast strain. Aspmo et al., (2005) found similar results when testing the growth of *S. cerevisiae* ATCC 18824 on a media supplemented with different nitrogen sources. They reported the yeast extract as the preferred commercial substrate for the yeast compared to other 9 nitrogen sources, including a protein hydrolysate prepared with Alcalase 2.4L.

3.2. Fermentation profile of *S. cerevisiae* Ethanol Red® under different N sources

The nitrogen source is one of the most important factors that affect the aerobic growth of *S. cerevisiae*, hence ethanol production (Li et al., 2017). The time course of dextrose consumption and ethanol production of *S. cerevisiae* Ethanol Red® in the different media are shown in Figure 5.4, while ethanol yields and volumetric productivities are presented in Table 5.4. The trends of dextrose consumption and ethanol production among all media were similar, with slight differences. Faster dextrose consumption and ethanol production was observed in the absence of peptone in the media, reaching a productivity of 1.01 g/(L h) (Table 5.4). The amount of yeast extract added might have been enough to sustain fermentation. YPD and YD-RHN exhibited a similar behavior, as dextrose consumption and ethanol production curves were overlapped, attaining a productivity of 0.86 g/(L h). The slowest dextrose consumption was observed in YD-RHA medium; nevertheless, the productivity was not significantly different from the control. As all the fermentations were finished in a maximum time of 10 h, when comparing conversion of dextrose and growth rate in this time frame we can observe that fermentation was associated with the growth rate. Albers et al., (1996) observed similar patterns when growing *S. cerevisiae* under different N sources. Since nitrogen utilization affects the fermentative activity of yeast

(Crépin et al., 2017), we can speculate that the differences in the productivities are mainly due to the presence of different nitrogen sources.

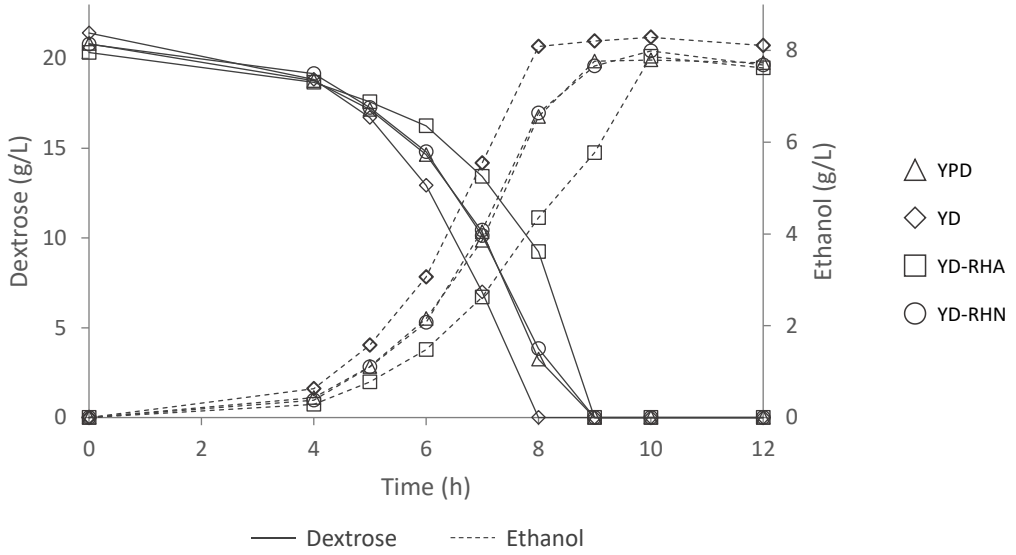

Figure 5.4. Dextrose consumption and ethanol production along the fermentation with *S. cerevisiae* Ethanol Red® using different N sources. YPD: Yeast Peptone Dextrose medium; YD: Yeast Dextrose medium; YD-RHA: Yeast Dextrose medium containing Alcalase 2.4L hydrolysate produced in the reactor; and YD-RHN: Yeast Dextrose medium containing Neutrase 0.8L hydrolysate produced in the reactor. Refer to Table 5.1 for media composition.

Despite the significant influence of the newly obtained protein hydrolysates on the specific growth rate (Table 5.3), the ethanol yields on dextrose (Table 5.4) were not affected, even higher amounts were produced compared to the control (YPD). In the study by Barahona et al., (2019), higher yields were reported, ranging from 0.46 to 0.49, values closer to the theoretical maximum yield, 0.511 g ethanol/g consumed dextrose.

Table 5.4. Ethanol yield ($Y_{ethanol/consumed\ sugars}$) and productivity in the different media tested.

Medium	*S. cerevisiae* Ethanol Red®	
	$Y_{EtOH/CS}$	Productivity (g/(L h))
YPD	0.374 ± 0.001[b]	0.863 ± 0.002[b]
YD	0.387 ± 0.001[a]	1.011 ± 0.066[a]
YD-RHA	0.387 ± 0.002[a]	0.851 ± 0.053[b]
YD-RHN	0.384 ± 0.003[a]	0.787 ± 0.004[b]

Means not connected by the same letter are significantly different (Tukey HSD test, $p < 0.05$)
YPD: Yeast Peptone Dextrose medium; YD: Yeast Dextrose medium; YD-RHA: Yeast Dextrose medium containing Alcalase 2.4L hydrolysate produced in the reactor; and YD-RHN: Yeast Dextrose medium containing Neutrase 0.8L hydrolysate produced in the reactor

Glycerol plays a vital role in the osmotic regulation of the cell. One of its functions is the maintenance of intracellular redox balance. Previous results showed the positive effect of high concentrations of amino acids in reducing glycerol while enhancing ethanol yield (Albers et al., 1996). A similar trend in glycerol production was observed in the tested culture media (Figure 5.5). Throughout the fermentation process, a higher amount of glycerol was produced during dextrose consumption, a phase that is related to cell growth, and no further production was observed in the stationary phase. The highest concentration of glycerol was observed in YPD, with 0.53 g/L, followed by YD-RHA (0.43 g/L), YD (0.25 g/L), and YD-RHN (0.16 g/L). These low concentrations reflect that dextrose was mainly used for ethanol production. Interestingly, YPD media was characterized by the lowest yield and the highest glycerol concentration. However, based on all the data, no correlation between glycerol and ethanol yield was observed, which agrees with the previous results reported by Jørgensen, (2009).

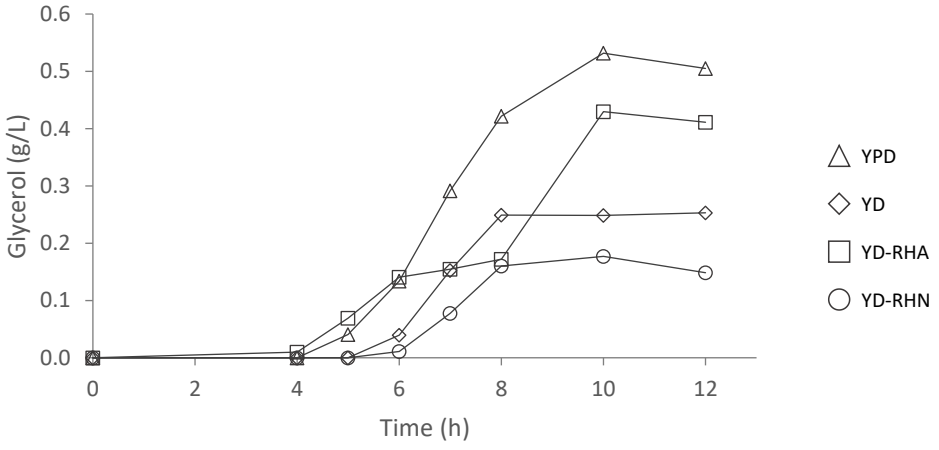

Figure 5.5. Glycerol production along the fermentation with *S. cerevisiae* Ethanol Red® using different N sources. YPDX: Yeast Peptone Dextrose Xylose medium; YDX: Yeast Dextrose Xylose medium; YDX-RHA: Yeast Dextrose Xylose medium containing Alcalase 2.4L hydrolysate produced in the reactor; and YDX-RHN: Yeast Dextrose Xylose medium containing Neutrase 0.8L hydrolysate produced in the reactor. Refer to Table 5.1 for media composition.

3.3. Fermentation profile of *S. cerevisiae* CEN.PK XXX under different N sources

The time profiles of sugar consumption and ethanol production obtained with *S. cerevisiae* CEN.PK XXX are displayed in Figure 5.7, while ethanol yields and volumetric productivities are presented in Table 5.5. Sugar consumption curves were characterized by a pronounced fast metabolization of C_6 - sugar (dextrose), within the first 15 h of fermentation, followed by a slower C_5 – sugar (xylose) conversion immediately after dextrose depletion. This strain showed a poor capacity for the simultaneous co-fermentation of dextrose and xylose in the tested media. YPDX and YDX media showed similar trends in dextrose consumption. However, slightly faster xylose metabolization was observed in the YDX medium, yielding significantly higher productivity, 0.61 g/(L h).

The preference of *S. cerevisiae* for yeast extract compared to other organic and inorganic nitrogen sources has been previously reported (Barahona et al., 2019; Li et al., 2017). This might be associated with the fact that yeast extract not only supplies nitrogen, but also vitamins, growth factors, and nucleic acids (Barahona et al., 2019). However, it is more expensive and not viable at industrial scale for bio-based ethanol production. Conversely, the fermentation performance of the yeast in the media containing RHA and RHN was lower, despite the presence of the yeast extract. These observations indicated that the amino acids uptake was somehow altered due to the presence of two different nitrogen sources simultaneously, suggesting that RHA and RHN are intermediate nitrogen sources for *S. cerevisiae* CEN.PK XXX strain. Both dextrose and xylose were consumed slowly; hence more time was necessary to achieve a similar ethanol concentration compared to the control. Consequently, the ethanol volumetric productivity was significantly reduced (0.58 g/(L h) in YPDX *vs.* 0.35 g/(L h) in YD-RHA/RHN), which correlates with the significantly lower specific growth rate.

Table 5.5. Ethanol yield ($Y_{ethanol/consumed\ sugars}$) and productivities in the different media tested.

Medium	***S. cerevisiae* CEN.PK XXX**	
	$Y_{EtOH/CS}$	**Productivity (g/(L h))**
YPDX	0.342 ± 0.001[b]	0.576 ± 0.001[b]
YDX	0.370 ± 0.003[a]	0.607 ± 0.004[a]
YDX-RHA	0.318 ± 0.004[c]	0.356 ± 0.005[c]
YDX-RHN	0.342 ± 0.004[b]	0.353 ± 0.004[c]

Means not connected by the same letter are significantly different (Tukey HSD test, $p < 0.05$)
YPDX: Yeast Peptone Dextrose Xylose medium; YDX: Yeast Dextrose Xylose medium; YDX-RHA: Yeast Dextrose Xylose medium containing Alcalase 2.4L hydrolysate produced in the reactor; and YDX-RHN: Yeast Dextrose Xylose medium containing Neutrase 0.8L hydrolysate produced in the reactor

The experiments with this strain showed a significant decrease in the ethanol yield in the YDX-RHA medium compared to control, while no significant differences were observed between YPDX and YDX-RHN media. Ethanol yields on consumable sugars were less influenced by the culture medium than specific growth rate and productivity. Provided that a similar effect was observed for both strains, this indicates that growth was more susceptible to the effect of the nitrogen source than the ethanol yield. Similar findings were reported by Palmqvist & Hahn-Hägerdal, (2000b), who pointed out that the observed decrease in specific growth rate caused by inhibitors (furfural, 5-hydroxymethylfurfural, etc.) did not imply a lower ethanol production. Although no inhibitors were added to the media tested in the present study, an analogy between the nitrogen source and the inhibitors behavior can be made.

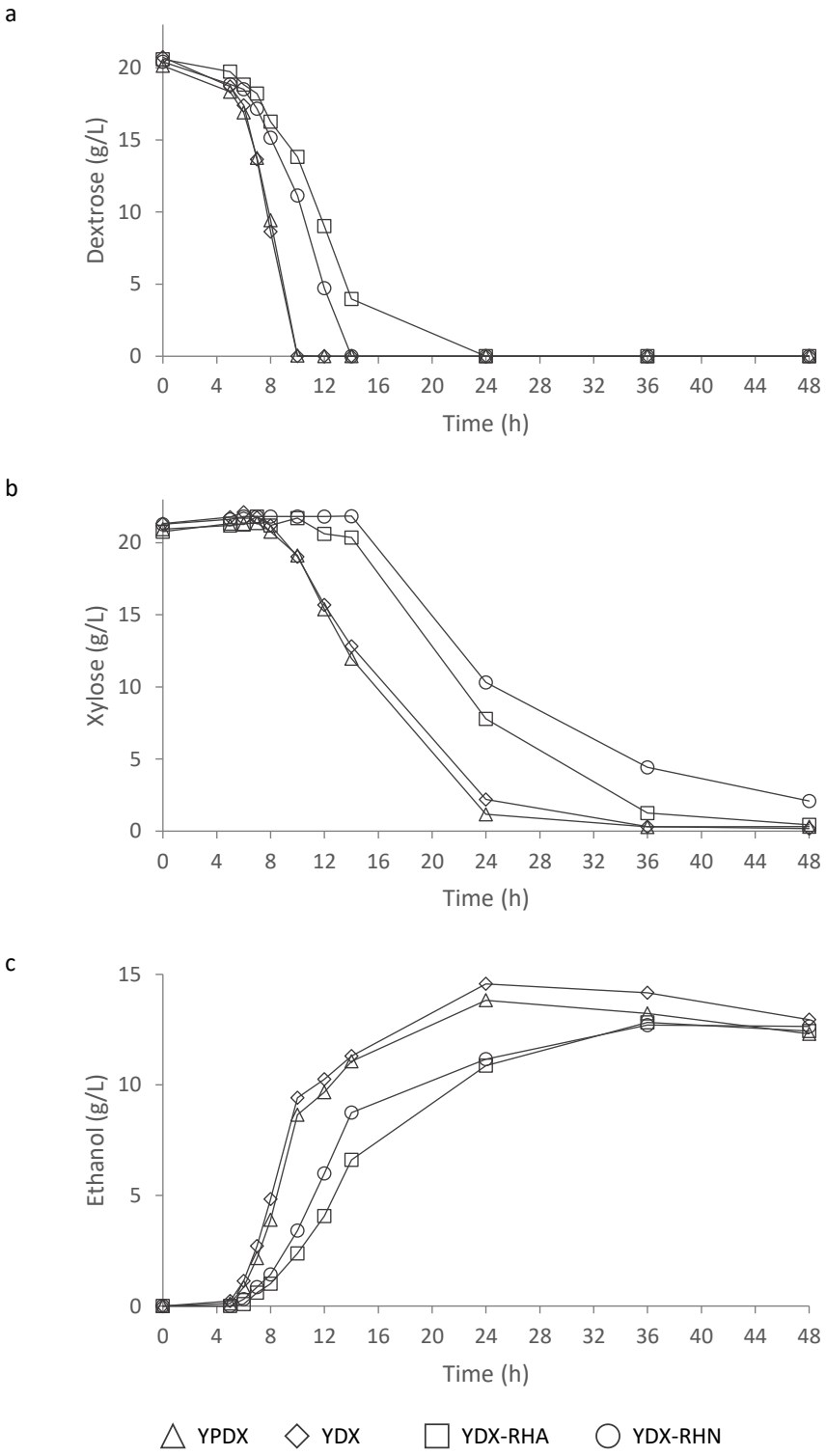

Figure 5.6. a) Dextrose and b) xylose consumption, and c) ethanol production curves with *S. cerevisiae* CEN.PK XXX. YPDX: Yeast Peptone Dextrose Xylose medium; YDX: Yeast Dextrose Xylose medium; YDX-RHA: Yeast Dextrose Xylose medium containing Alcalase 2.4L hydrolysate produced in the reactor; and YDX-RHN: Yeast Dextrose Xylose medium containing Neutrase 0.8L hydrolysate produced in the reactor. Refer to Table 5.1 for media composition.

An increased glycerol synthesis was observed for the *S. cerevisiae* CEN.PK XXX compared to *S. cerevisiae* Ethanol Red®, which might be a response to general stress caused by the higher sugar concentration (Nissen et al., 2000). However, no correlation was found between ethanol yield and glycerol concentration. Surprisingly, in that case, a higher concentration of glycerol was observed in the medium with YDX-RHA, followed by YPDX. Both strains showed significantly lower glycerol concentration in YDX and YDX-RHN, which clearly indicates the effect of the N source in product formation. A decrease in glycerol concentration was observed once dextrose was depleted, which might be due to glycerol consumption as C source.

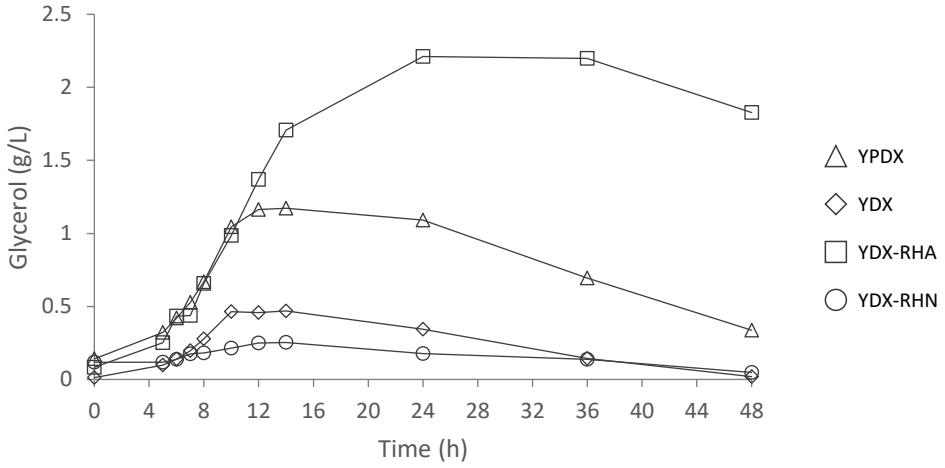

Figure 5.7. Glycerol production along the fermentation with *S. cerevisiae* CEN.PK XXX using different N sources. YPDX: Yeast Peptone Dextrose Xylose medium; YDX: Yeast Dextrose Xylose medium; YDX-RHA: Yeast Dextrose Xylose medium containing Alcalase 2.4L hydrolysate produced in the reactor; YDX-RHN: Yeast Dextrose Xylose medium containing Neutrase 0.8L hydrolysate produced in the reactor. Refer to Table 5.1 for media composition.

The present results demonstrate that growth and fermentation performance of different *Saccharomyces* strains indeed is affected by different N sources differently. Similar findings were reported by Barahona et al., (2019), observing significant differences in dextrose consumption among different *Saccharomyces* strains and N sources.

3.4. Effect of N source on the fermentation of hydrolyzed lignocellulosic biomass

Hydrolysates from lignocellulosic material are generally low in nutrients and nitrogen. Therefore, there is great potential in improving the fermentation performance of lignocellulosic material by supplementation with optimal nutrients. The effect of different N sources on the fermentation performance (dextrose consumption, ethanol yield, and volumetric ethanol productivity) of a lignocellulosic hydrolysate was investigated. The five nitrogen sources tested were selected from those commonly used for yeast media

formulation (Table S5.1 in Supplementary data), while capability in fermenting both dextrose and xylose of *S. cerevisiae* CEN.PK XXX strain made this the most promising candidate to be assayed in the fermentation of WSH.

To assess the nitrogen effect, comparable levels of total elemental nitrogen were added within the different N sources instead of comparable levels as whole molecules. This approach was selected to ensure that the amount of nitrogen did not constitute a limiting factor in the experiments, rendering 1200 mg/L regardless of the nitrogen source, which is above the recommended minimum level for *S. cerevisiae* to complete fermentation at a normal rate (120 – 140 mg/L) (Kemsawasd et al., 2015).

3.4.1. Dextrose and xylose consumption

As a result of different nutrient addition in the WSH, a great variation was observed in the yeast performance. Although dextrose exhaustion was observed in all supplemented media, dextrose and xylose showed different conversion rates for the different N sources tested (Figure 5.8). The fastest sugar consumption was observed in the media supplemented with casein peptone, corresponding to the higher volumetric ethanol productivity, 0.827 g/(L h). Besides this, some co-consumption of both sugars, dextrose and xylose, was displayed by the cells, observing a decrease of 3.25 g/L in the xylose concentration concomitant with the consumption of all the dextrose (Figure 5.8a and b). This finding is in agreement with the reported by Westman et al., (2014), who carried out fermentations with the same strain under similar dextrose concentrations and 2-fold xylose concentrations, observing approximately a reduction of 6.5 g/L in xylose concentration. Given these facts, this phenomenon might not be observed in the synthetic medium due to the lower dextrose concentration assayed.

Unexpectedly, the media supplemented with urea showed faster sugar consumption (Figure 5.8a and b) and ethanol production (Figure 5.8c) than those supplemented with ammonium sulfate, RHA, and RHN. Given the nature of RHA and RHN, similar performance compared to the control (WSH supplemented with casein) was expected. However, dextrose consumption was slower. As comparable N levels were added and there was no nitrogen limitation, these observations indicate some difficulties for yeast in assimilating the N present in these hydrolysates.

Not surprisingly, the absence of an external N source in the lignocellulosic substrate imposed the harshest conditions for yeast growth, leading to no fermentation of the present sugars. The small amount of ethanol observed (0.65 g/L) after 45 h of fermentation could be related to autophagy, an adaptive response triggered by nitrogen starvation conditions that allow survival of the cells due to recycling of building blocks resulting from its own digestion and metabolization of deadwood proteins into amino

acids that can be utilized in subsequent protein synthesis pathways in the absence of a nutrient supply (Barahona et al., 2019; Cebollero & Gonzalez, 2006; Kohda et al., 2007). This observation confirms the poor nutritional quality of the wheat straw hydrolysate, previously reported by Jørgensen, (2009) and Tomás-Pejó et al., (2012).

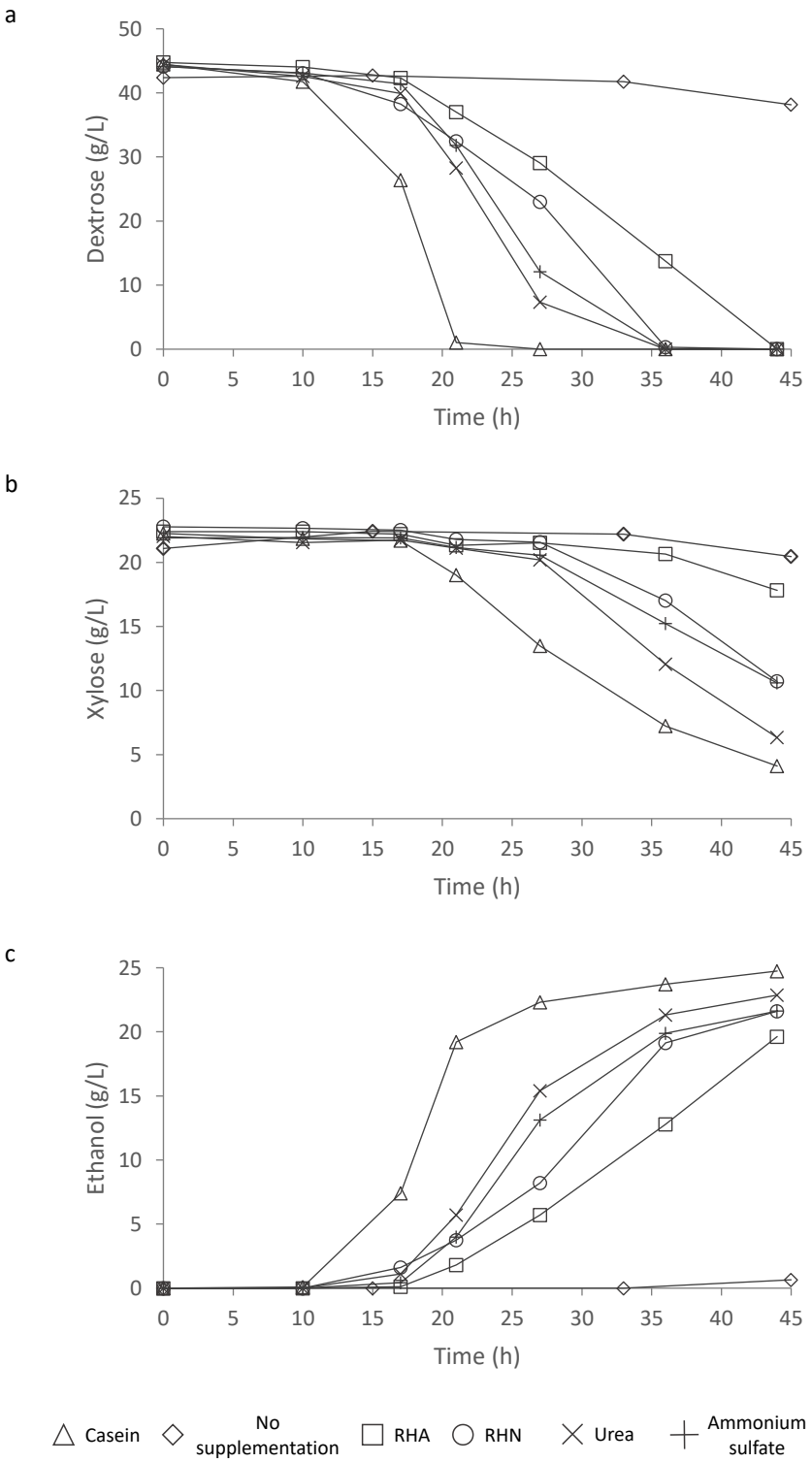

Figure 5.8. Dextrose and xylose consumption (a and b, respectively) and ethanol production (c) curves obtained with *S. cerevisiae* CEN.PK XXX on wheat straw hydrolysates supplemented with different N sources.

3.4.2. Ethanol yields and productivities

As shown in Table 5.6, supplementation with casein resulted in the highest ethanol production (24.75 ± 0.15 g/L), followed by urea with maximum ethanol production of 22.87 ± 0.50 g/L within 44 h of fermentation and a productivity of 0.59 g/(L h). The maximum ethanol concentration achieved when supplementing with ammonium sulfate, RHN, and RHA were 21.62 ± 0.31 g/L, 21.61 ± 0.14 g/L, and 19.63 ± 0.22 g/L, respectively, with productivities between 0.45 and 0.53 g/(L h). Although no significant differences in maximum ethanol concentration and ethanol productivity were observed between the media containing ammonium sulfate or RHN, the values obtained were significantly lower compared to casein and urea. In a previous study (Jørgensen, 2009), when comparing fermentation with urea, peptone, yeast extract, ammonium sulfate, and corn steep liquor in wheat straw hydrolysate fermentation, urea and ammonium sulfate addition did not significantly improve fermentation rate. Furthermore, when comparing fermentation with urea or ammonium sulfate with that without nitrogen addition, similar ethanol concentrations were reached, indicating the availability of some nitrogenous compounds in WSH and poor performance of these organic and inorganic N sources, which might be related to a general deficiency of essential nutrients and vitamins in the WSH (Jørgensen, 2009). Conversely, Li et al., (2017), Raposo et al., (2017), and Knudsen & Rønnow, (2020) have reported that urea could be a promising nitrogen source in the production of 2G ethanol.

Table 5.6. Yields and productivities obtained under supplementation of WSH with different N sources.

N source	***S. cerevisiae* CEN.PK XXX**		
	Max. ethanol (g/L)	**$Y_{EtOH/CS}$**	**Productivity (g/(L h))***
Urea	22.87 ± 0.50^{b}	0.380 ± 0.011^{a}	0.592 ± 0.004^{b}
Casein	24.75 ± 0.15^{a}	0.395 ± 0.003^{a}	0.827 ± 0.010^{a}
Ammonium sulfate	21.62 ± 0.31^{c}	0.390 ± 0.010^{a}	0.553 ± 0.002^{c}
RHA	19.63 ± 0.22^{d}	0.395 ± 0.003^{a}	0.446 ± 0.005^{d}
RHN	21.61 ± 0.14^{c}	0.385 ± 0.003^{a}	0.532 ± 0.002^{c}
No supplementation	0.65^{e}	0.134^{b}	0.014^{e}

Levels not connected by the same letter are significantly different (Tukey HSD test, $p < 0.05$)
RHA: Alcalase 2.4L hydrolysate produced in the reactor and RHN: Neutrase 0.8L hydrolysate produced in the reactor
* productivity was calculated when no dextrose was detected in the fermentation medium

Despite the significant differences in dextrose uptake and ethanol productivity among the different N sources tested, no significant differences were observed when comparing ethanol yields. Hence, regardless the nitrogen source, the *S. cerevisiae* strain used dextrose and xylose for similar purposes. An ethanol yield of 0.39 g of ethanol produced/g of sugar consumed was obtained, which corresponds to an

efficiency of 76% (based on the theoretical maximum yield), evidencing that about 25% of all the consumed sugar was used for biomass or other unknown purposes.

The obtained results evidence the potential of increasing ethanol production by optimizing the nitrogen source. Although the protein hydrolysates did not render equally to the commercial peptones in terms of dextrose uptake and productivity, there exists a huge potential for optimization. As pointed out by Li et al., (2017), nitrogen concentration has a significant effect on ethanol yield and efficiency. For instance, they reported an improved yield and efficiency when supplementing liquefied corn starch with 2.5% of peptone, while in the present study the WSH was supplemented with only 1%.

4. Conclusions

In this work, two nitrogen sources derived from animal by-products were proposed and assayed in yeast culture and sustainable production of 2G ethanol. The protein hydrolysates showed different activities depending on the strain. Differences were revealed in the lag phase, specific growth rate, and OD_{max} depending on both the nature of the nitrogen source and the yeast strain. In the synthetic media, *S. cerevisiae* Ethanol Red® displayed good growth performance for the nitrogen sources tested and a narrower variance in their characteristics according to the nitrogen source. However, *S. cerevisiae* CEN.PK XXX exhibited lower growth capacity and productivity in the media containing RHA and RHN.

The performance of the nitrogen sources tested in a wheat straw hydrolysate showed high variability. Although similar ethanol yields were observed when compared to casein, and even urea, a cheap nitrogen source, the supplementation with RHA and RHN resulted in significatively lower productivities compared. Bearing in mind that the present study includes one concentration of nitrogen, there is potential in improving productivities by optimizing the dose and screening synergic effect among different nitrogen sources (by mixing expensive and low-cost N sources) to reduce growth media cost.

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6. Supplementary data

Table S5.1. Comparative study of lignocellulosic biomass fermentation with *S. cerevisiae* strains.

Strain	Supplementation (g/L)	Inoculum size	Yield (EtOH/CS)	Efficiency (%)	Productivity (g/(L h))	Fermentation time (h)	Reference
S. cerevisiae strain F12	$(NH_4)_2SO_4$, 5	1 g/L		92	0.13	50	(Tomás-Pejó et al., 2008)
Saccharomyces cerevisiae	Screening of different N sources in combination or separately $MgSO_4$; $(NH_4)_2SO_4$; Urea; Corn steep liquor; Yeast extract; and Peptone	0.33 g/kg (1.2 g DM/kg)	Depends on N source	Depends on N source 99[a]	Depends on N source 1.16[a]	Depends on N source	(Jørgensen, 2009)
S. cerevisiae MTCC 174	Nutrient solution	3.3%	0.48		0.45	36	(Singh & Bishnoi, 2013)
S. cerevisiae Red Star Ethanol Red	$(NH_4)_2SO_4$, 25 – 400 mM Urea, 50 – 400 mM Peptone, 0.5 – 3% Yeast extract, 0.3 – 3.5%	1 mL/g	Depends on N source	Depends on N source 84.2[a]	Depends on N source	Depends on N source	(Li et al., 2017)
S. cerevisiae strains	Screening of different N sources $(NH_4)_3PO_4$; NH_3; and Yeast hydrolysate	0.1%	Depends on N source 0.49[a]	Depends on N source 96.72[a]	Depends on N source	Depends on N source	(Barahona et al., 2019)
S. cerevisiae NCIM 3570	Yeast extract, 3 Malt extract, 3 Peptone, 5	1.15 g/L	0.30		0.95	34.43	(Patel et al., 2019)
S. cerevisiae cV-110	Urea, 3	1 g DW/L		90	1.1		(Knudsen & Rønnow, 2020)

[a] maximum value reported regardless of the N source

Chapter 6. Effect of nitrogen dose and source on 5-hydroxymethylfurfural biotransformation using fungi as biocatalysts

1. Background

Biocatalysis has emerged as a promising alternative to chemical approaches and it has many benefits to offer (Xu et al., 2018). Traditional chemical methods are not very specific processes, and a high number of steps, high-cost chemicals, and extreme conditions are required to enhance selectivity and purity, which difficult economic feasibility (Lalanne et al., 2021; Millán et al., 2021). In contrast, biocatalytic processes are performed under milder conditions and more environmentally friendly reaction conditions (Domínguez de María & Guajardo, 2017). Biocatalytic processes can be performed as whole cells biotransformation or using isolated enzymes (Sheldon, 2012). One of the major bottlenecks for microbial enzyme production is the high cost, mainly due to the cost of the growth substrate, which represents around 30 - 40% of the production costs (Parrado et al., 2014). Similarly, employing whole cells as a biocatalyst requires specific nutritional requirements to obtain energy for the biosynthesis of cellular matter and products in cell operation, maintenance, and reproduction (Kampen, 2014). Among the growth media ingredients, organic nitrogen sources, such as protein hydrolysates, yeast extract, or beef extract (Chen et al., 2021; Feldman et al., 2015; Godan et al., 2019; Hossain et al., 2017; Koopman et al., 2010; Pan et al., 2020; Zhang et al., 2017) tend to be the most expensive components. In addition, in some cases, elevated cells dosages (200 mg/mL) are necessary to alleviate the substrate's inhibition and increase productivities (Chen et al., 2021), thus significantly increasing the process cost. Therefore, addressing the lower-priced nitrogen sources will have a great impact on future industrial feasibility.

The compound 5-hydroxymethylfurfural (HMF) is a platform chemical that can be converted into a wide range of high-value derivatives, such as 2-methylfuran (MF), 2,5-di(hydroxymethyl)furan (DHMF), 2,5-diformylfuran (DFF), 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), 5-formyl-2-furancarboxylic acid (FFCA), and 2,5-furandicarboxylic acid (FDCA) (Hu et al., 2017; Lalanne et al., 2021). Among them, DHMF has great potential as an intermediate for the manufacturing of various interesting compounds, such as fuels, resins, fibers, foams, drugs, and crown esters (Hu et al., 2017), while DFF has applications in therapeutics, fungicides, polymers, and material science (Domínguez de María & Guajardo, 2017; Lalanne et al., 2021).

Currently, industrially HMF-based derivatives are synthesized via catalytic approaches, such as hydrogenation, oxidation, or other reactions (Hu et al., 2017). Due to the well-known toxicity of HMF towards cells, it is still challenging the use of whole cells as biocatalysts in HMF biotransformation (Almeida et al., 2009; Cang et al., 2019). However, in the last years, significant advances have been performed using isolated enzymes and whole-cell biocatalysts in HMF biotransformation (Chang et al., 2021; Chen et al., 2021; He et al., 2018; Pan et al., 2020; Paukner et al., 2015; Xu et al., 2018; Yuan et al.,

2018). The biocatalytic reduction of HMF has been previously described using yeast (Li et al., 2017; Liu et al., 2004; Ra et al., 2013; Xu et al., 2018), bacteria (Chang et al., 2021; He et al., 2018; Zhang et al., 2012), and fungi (Chen et al., 2021; Feldman et al., 2015; Millán et al., 2021; Ran et al., 2014). However, HMF oxidation to DFF has not been reported using whole cells.

Since biocatalytic biotransformation of 5-hydroxymethylfurfural (HMF) using *Fusarium* (*F.*) strains was approached in our laboratory, the effects of the obtained protein hydrolysates from *fines* on this process were studied.

In this context, we hypothesized that the use of low-cost protein hydrolysates from rendered proteins might represent a good alternative to currently used nitrogen sources in the biocatalytic conversion of HMF, such as yeast extract or peptones, helping to lower fermentation cost and making the process economically and industrially viable. In the present work, we investigated the effect of the two low-cost animal-derived protein hydrolysates (HA and HN) obtained from *fines* in the biotransformation capability of several *Fusarium* strains.

2. Materials and methods

2.1. Materials

Malt extract and meat peptone were purchased from Biokar Diagnostics (Solabia Group, Pantin, France). Peptone from soybean was purchased from Acros Organics (Geel, Belgium). The HMF (98%) was purchased from Fluorochem Ltd. (Hadfield, UK). The DHMF (97%) was purchased from Apollo Scientific (Stockport, UK). Glucose, agar, 5-acetoxymethyl-2-furaldehyde, MF, and DFF were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethyl acetate was purchased from Honeywell (Morristown, USA). Protein hydrolysates with Alcalase 2.4L (HA) and Neutrase 0.8L (HN) were produced following the methodology described in Chapter 3 Section 2.5.

2.2. Microorganisms, media, and inoculum preparation

Seven *Fusarium* strains were employed for the assays. *F. sporotrichioides* (B3), *F. tricinctum* (T263), *F. poae* (G1), *F. chlamydosporium* (T773), and *F. sambucinum* (B6) were obtained from the culture collection of the Food Technology Department (University of Lleida). *F. culmorum* EAN 51 (CECT2148) was obtained from the Spanish Type Culture Collection. *F. striatum* was isolated in our laboratory as reported by Millán et al., (2021).

The *Fusarium* strains were maintained at 4 °C by replications on Malt Extract Agar (MEA) containing the following (g/L): malt extract, 20; glucose, 20; peptone, 1; and agar, 15. Prior to the experiments, the strains were grown in MEA at 28 °C for seven days for activation.

2.3. HMF biotransformation: Screening

Biotransformation experiments were carried out by inoculating 3 discs of 8 mm from the Petri dishes containing the activated strains into 50 mL flasks containing 15 mL of the culture media according to Table 6.1. The pH of all the media was adjusted at 7 with 1M NaOH before autoclaving at 121 °C for 20 min. The flasks were incubated in an orbital shaker (Nahita®) at 28 °C and 160 rpm. After 72 h, HMF was added to the broth containing the fungus, obtaining a final concentration of substrate of 50 mM.

Table 6.1. Composition of the culture medium (g/L) tested based on ME for the evaluation of HA and HN.

Compound	**Culture medium (g/L)**		
	ME	**ME-50HA**	**ME-50HN**
Malt extract	20	10	10
Glucose	20	20	20
Soybean peptone	1	-	-
HA	-	11	-
HN	-	-	11

ME: Malt Extract medium; ME-50HA: Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate; and ME-50HN: Malt Extract medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate

2.4. Nitrogen dose and source effect on *F. sambucinum* HMF biotransformation

In a standard experiment, 3 discs of 8 mm of the activated *F. sambucinum* in MEA were inoculated into flasks containing 15 mL of culture media according to Table 6.2. The pH of all the media was adjusted at 7 with 1M NaOH before autoclaving at 121 °C for 20 min. After 3 days of growth at 28 °C and 160 rpm, HMF was added to obtain a concentration of 50 mM.

Table 6.2. Composition of the culture medium (g/L) tested based on ME for the evaluation of HA.

Compound	Culture medium (g/L)					
	ME	**ME-WP**	**ME-50WP**	**ME-50Meat**	**ME-HA**	**ME-50HA**
Malt extract	20	20	10	10	20	10
Glucose	20	20	20	20	20	20
Soybean peptone	1	-	-	-	-	-
Meat peptone	-	-	-	11	-	-
HA	-	-	-	-	1	11

ME: Malt Extract medium; ME-WP: Malt Extract medium without peptone; ME-50WP: Malt Extract medium without peptone and 50% of malt extract; ME-50Meat: Malt Extract medium containing meat peptone; ME-HA: Malt Extract medium containing Alcalase 2.4L hydrolysate; and ME-50HA: Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate

2.5. Nitrogen dose and source effect on *F. striatum* sporulation and HMF biotransformation

2.5.1. Sporulation

The induction of *F. striatum* sporulation was tested in seven different media, according to Table 6.3. The activated fungus was inoculated in all the culture media and incubated at 28 °C in the presence or absence of visible light. Growth was monitored visually after 3 and 6 days. After 10 days of growth, spores were counted in a Neubauer chamber.

Table 6.3. Composition of the culture medium (g/L) tested based on MEA for the evaluation of HA and HN.

Compound	Culture medium (g/L)						
	MEA	**MEA-WP**	**MEA-50WP**	**MEA-HA**	**MEA-50HA**	**MEA-HN**	**MEA-50HN**
Malt extract	20	20	10	20	10	20	10
Glucose	20	20	20	20	20	20	20
Soybean peptone	1	-	-	-	-	-	-
HA	-	-	-	1	11	-	-
HN	-	-	-	-	-	1	11
Agar	15	15	15	15	15	15	15

MEA: Malt Extract Agar medium; MEA-WP: Malt Extract Agar medium without peptone; MEA-50WP: Malt Extract Agar medium without peptone and 50% of malt extract; MEA-HA: Malt Extract Agar medium containing Alcalase 2.4L hydrolysate; MEA-50HA: Malt Extract Agar medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate; MEA-HN: Malt Extract Agar medium containing Neutrase 0.8L hydrolysate; and MEA-50HN: Malt Extract Agar medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate

2.5.2. HMF biotransformation

2.5.2.1. Substrate feeding with 75 mM HMF

In a standard experiment, 3 discs of 8 mm or a suspension of spores (2.2 x 10^6 spores/mL) of *F. striatum* were inoculated into flasks containing 15 mL of culture media according to Table 6.4. The culture medium ME was inoculated with spores from MEA, ME-50HA with spores from MEA-50HA, and ME-50HN with spores from MEA-50HN, all spores obtained in the presence of light. The pH of all the media was adjusted at 7 with 1M NaOH before autoclaving at 121 °C for 20 min. After 3 days of growth at 28 °C and 160 rpm, HMF was added to obtain a concentration of 75 mM. After 48 h from the first addition, a second addition was performed.

Table 6.4. Composition of the culture medium (g/L) tested based on ME for the evaluation of HA and HN.

Compound	**Culture medium (g/L)**		
	ME	**ME-50HA**	**ME-50HN**
Malt extract	20	10	10
Glucose	20	20	20
Soybean peptone	1	-	-
HA	-	11	-
HN	-	-	11

ME: Malt Extract medium; ME-50HA: Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate; ME-50HN: Malt Extract medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate; HA: Alcalase 2.4L hydrolysate; and HN: Neutrase 0.8L hydrolysate

2.5.2.2. Substrate feeding with 45 mM HMF

In a standard experiment, a suspension of spores (2.2 x 10^6 spores/mL) of *F. striatum* was inoculated into flasks containing 15 mL of culture media according to Table 6.5. All the culture media tested were inoculated with spores from fungus grown in MEA. The pH of all the media was adjusted at 7 with 1 M NaOH before autoclaving at 121 °C for 20 min. After 3 days of growth at 28 °C and 160 rpm, HMF was added to obtain a concentration of 45 mM. Subsequent additions after 24, 48, and 72 h from the first addition were performed.

Table 6.5. Composition of the culture medium (g/L) tested based on ME for the evaluation of HA and HN.

Compound	Culture medium (g/L)				
	ME	ME-50WP	ME-50Meat	ME-50HA	ME-50HN
Malt extract	20	10	10	10	10
Glucose	20	20	20	20	20
Soybean peptone	1	-	-	-	-
Meat peptone	-	-	11	-	-
HA	-	-	-	11	-
HN	-	-	-	-	11

ME: Malt Extract medium; ME-50WP: Malt Extract medium without peptone and 50% of malt extract; ME-50Meat: Malt Extract medium containing meat peptone; ME-50HA: Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate; ME-50HN: Malt Extract medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate; HA: Alcalase 2.4L hydrolysate; and HN: Neutrase 0.8L hydrolysate

2.6. GC-FID analysis

HMF and its derivatives (MF, DHMF, and DFF) were identified and quantified by GC-FID following the methodology previously described by Millán et al., (2021). Briefly, aqueous aliquots were extracted with ethyl acetate. Analyses were carried out with an Agilent 7890GC (Agilent Technologies, Palo Alto, CA, USA) coupled to an FID detector (Agilent Technologies, Palo Alto, CA, USA). An FFAP column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) from Agilent was used at a constant flow using hydrogen as carrier gas. Injector temperature was 230 °C and the oven program was set as follows: initial temperature of 100 °C held for 1 min, increased to 240 °C by a ramp of 20 °C/min, and held for 5 min. Calibration curves were performed periodically for the quantification of the compounds and 5-acetoxymethyl-2-furaldehyde was used as internal standard.

2.7. Statistical analysis

All the experiments were performed at least in duplicate. The presented results are the mean of replicates and the standard deviations are shown as error bars in the figures. Data handling were performed using Excel software package (Microsoft Excel, 2013). Statistical analysis was carried out using JMP Pro 14 statistical software (Statistical Discovery™ from SAS, Cary, NC, USA). Tukey's honestly significant difference (HSD) test with a significance level of 0.05 was used for means comparison.

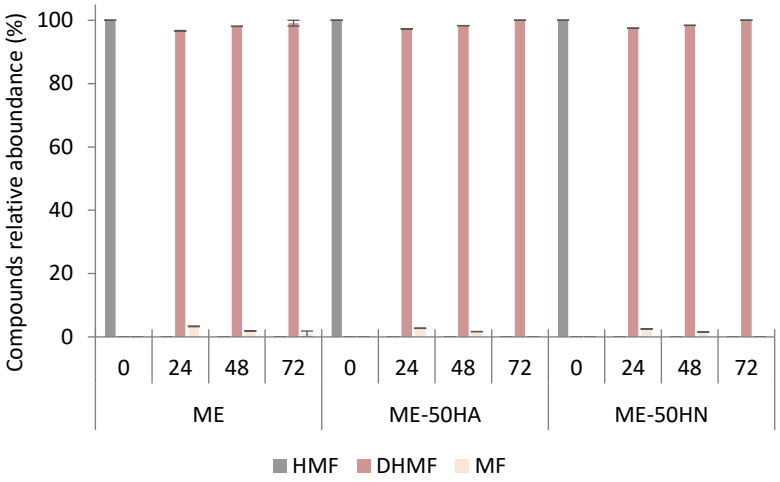
3. Results and discussion

3.1. *Fusarium* strains screening

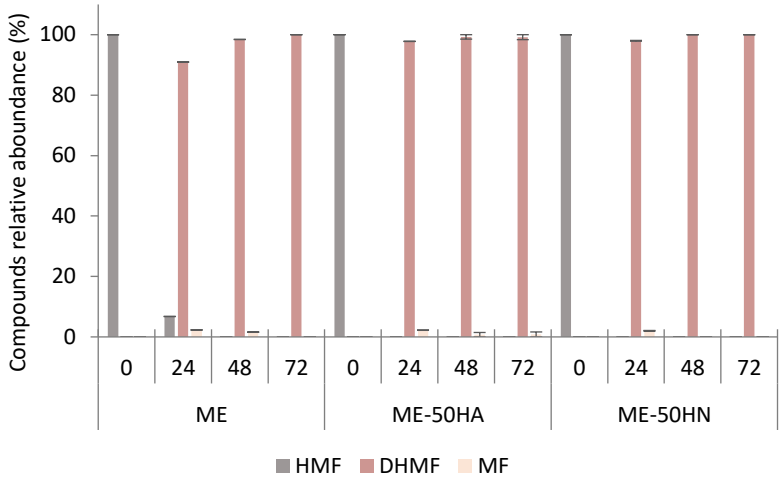
HMF is an inhibitory toxic compound towards microorganisms that causes damage to cells walls (He et al., 2018; Li et al., 2017). The biotransformation performance of HMF into added value derivates by seven *Fusarium* strains cultivated in ME culture medium and media amended with two animal-derived protein hydrolysates produced in our laboratory was evaluated. It should be noted that the composition of the culture media prepared in HA and HN was considerably different compared to ME. Not only the commercial peptones added in ME but also 50% of the malt extract were replaced by the newly produced peptones, therefore substituting the two most expensive ingredients of the media by the hydrolysates. In the present study, their metabolic ability was examined by adding HMF at 50 mM. The results presented for ME culture medium have been previously published by our group (Millán et al., 2021), and the experiments carried out with ME-HA and ME-HN were performed simultaneously.

As shown in Figure 6.1, all the strains were able to metabolize HMF measurably. *F. sporotrichioides*, *F. tricinctum*, *F. poae*, and *F. striatum* could biotransform almost 50 mM of HMF into DHMF and MF within 24 h. On the whole, the source and the amount of nitrogen did not affect the performance of these fungi strains, observing similar concentrations of products in all the tested culture media. Conversely, *F. chlamydosporium*, *F. sambucinum*, and *F. culmorum* showed more difficulties in transforming or degrading HMF. Furthermore, the amount and source of nitrogen had a significant effect on the obtained products.

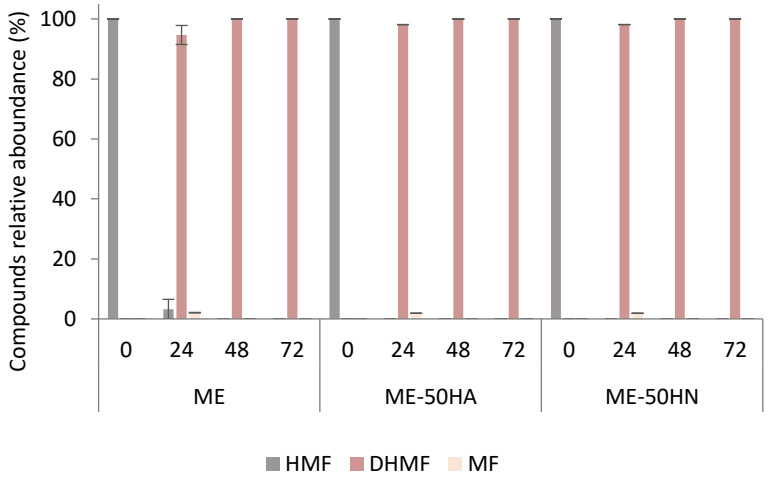
a


Compounds relative abundance (%)
100
80
60
40
20
0
0 24 48 72 0 24 48 72 0 24 48 72
ME
ME-50HA
ME-50HN
HMF DHMF MF

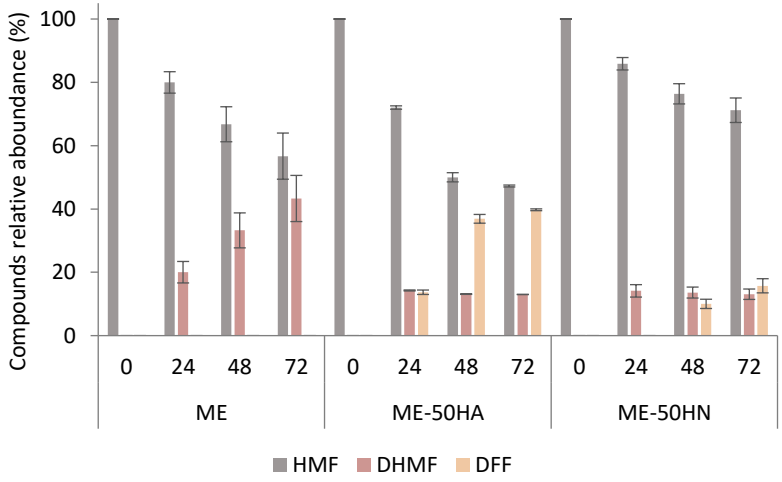
b


Compounds relative abundance (%)
100
80
60
40
20
0
0 24 48 72 0 24 48 72 0 24 48 72
ME
ME-50HA
ME-50HN
HMF DHMF MF

c


Compounds relative abundance (%)
100
80
60
40
20
0
0 24 48 72 0 24 48 72 0 24 48 72
ME
ME-50HA
ME-50HN
HMF DHMF MF

d


Compounds relative abundance (%)
100
80
60
40
20
0
0 24 48 72 0 24 48 72 0 24 48 72
ME
ME-50HA
ME-50HN
HMF DHMF DFF

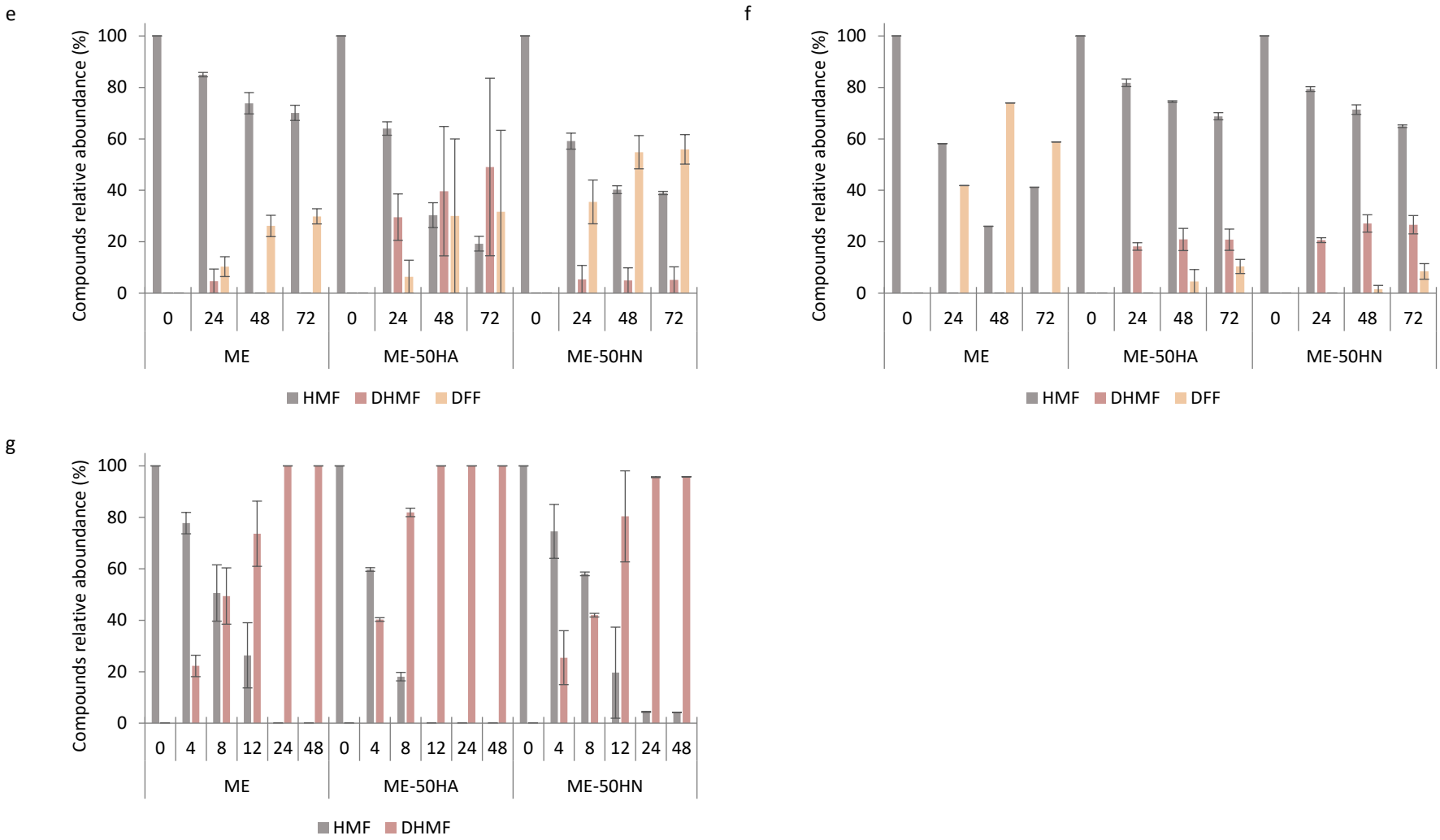


Figure 6.1. Biotransformation of 50 mM of HMF using: a) *F. sporotrichioides*; b) *F. tricinctum*; c) *F. poae*; d) *F. chlamydosporium*; e) *F. sambucinum*; f) *F. culmorum*; and g) *F. striatum*. Culture media employed were: ME: Malt Extract medium; ME-50HA: Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate; and ME-50HN: Malt Extract medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate. Refer to Table 6.1 for media composition.

In the medium ME, *F. chlamydosporium* metabolized only 20% of the HMF within the first 24 h, and even after 72 h of reaction 60% of the HMF was not transformed, rendering DHMF as the main product (Figure 6.1d). Whereas, in the medium containing HA the amount of HMF metabolized was significantly higher compared to ME (Figure 6.1d). Moreover, two products were identified, DFF and DHMF, indicating that in the presence of animal-derived protein hydrolysate, this fungi strain can both oxidize and reduce HMF. Although conversion of HMF was significantly lower in the medium containing HN compared to the control (ME) and ME-HA, both DFF and DHMF were detected, confirming the effect of the N source and amount on the metabolization of HMF.

F. sambucinum exhibited different behavior depending on the culture media, observing significantly higher biotransformation of HMF when the fungus was grown in ME-HA and ME-HN (Figure 6.1e). Only 30% of the HMF was metabolized within 72 h in ME, while in ME-HA and ME-HN around 80% and 62% were converted, respectively (Figure 6.1e). Besides this, as in the case of *F. chlamydosporium*, the presence of HA and HN in the culture medium led to the formation of oxidation and reduction derivatives of HMF. However, in this case, DHMF and DFF were also detected in ME medium within 24 h. After 48 and 72 h only DFF was detected in ME, indicating that the DHMF produced within 24 h was further oxidized into DFF. Meanwhile, in the culture media containing HA and HN, DHMF and DFF were detected throughout all the reaction time. It should be highlighted that the large deviations observed for ME-HA medium can be explained as follows: in two of the replicates both DFF and DHMF were detected after 72 h, while in the other replicates, only DHMF was detected. Therefore, these observations suggest that this fungus has the capability to reduce and oxidize the different compounds present in the broth. The large deviations observed, specifically in the culture medium containing HA, might be explained by the constant changing of the substrate and products profile, which could not be strictly followed due to the ample time intervals when sampling. However, as stated by Papagianni, (2004), it is complicated to deduce unequivocal general relationship among process variables, product formation, and fungal morphology since too many parameters influence these interrelationships. Besides this, the role of many of them is still not fully understood.

F*. culmorum* showed higher biotransformation ability in ME than ME-HA and ME-HN. After 48 h, 26% of the HMF remained in the ME medium, while in ME-HA and ME-HN even after 72 h about 69% was remaining. Moreover, significant differences were observed among the yielded products. In ME this fungus strain transformed all the metabolized HMF into DFF while the addition of animal-derived protein hydrolysates induced the biotransformation of HMF into DHMF and DFF (Figure 6.1f).

According to the results mentioned above, all the *Fusarium* strains tested biotransformed HMF to some extent, regardless of the culture medium. The catalytic performances of the whole cells tested indicate

that: i) all the strains possess the genes to express the necessary enzymes to metabolize toxic HMF into less toxic compounds such as DHMF and DFF (Martins et al., 2020), ii) the obtained protein hydrolysates in our laboratory represent a promising alternative to commercial peptones for most of the strains, and iii) the selection of an adequate nitrogen source, as well as the amount, can significantly influence enzyme and metabolite production, as most of the strains showed different behavior depending on the culture medium. The enzymes catalyzing the reduction of HMF into DHMF are described in various microorganisms. Martins et al., (2020) and Ran et al., (2014) reported an aldehyde oxidoreductase responsible for the reduction of HMF into DHMF in the filamentous fungi *Pleurotus ostreatus* and *Aspergillus nidulans*. However, the whole-cell oxidation of HMF into DFF is not described. Some *Fusarium* species have shown the capability to produce galactose oxidase (Gasparotto et al., 2006; Paukner et al., 2015), an enzyme that catalyzes the oxidation of HMF into DFF with high yields (Qin et al., 2015). Arguably, the modification of the nitrogen source and together with the amount influence the enzymatic expression of the fungi that can produce galactose oxidase, which have the capability of both oxidizing and reducing HMF (*F. chlamydosporum, F. sambucinum*, *and F. culmorum*).

To the best of our knowledge, the HMF concentration assayed in this study (50 mM) is considerably higher compared to those values reported in the literature for filamentous fungi (Ran et al., 2014). In addition, significant differences were observed in the rendered products depending on the medium formulation. However, further assays were necessary to determine if the biotransformation of HMF is dependent on the amount of nitrogen, the source of nitrogen, or both. Given these facts, *F. sambucinum* and *F. striatum* were selected to provide an insight on the effect of the nitrogen source in HMF metabolization.

3.2. *Fusarium sambucinum*

3.2.1. Effect of nitrogen source

F. sambucinum was selected to test the effect of nitrogen source due to the high effect on HMF metabolization rate and the products obtained as a consequence of the biotransformation of HMF (Figure 6.1). The protein hydrolysate HA was selected for the assays given the good performance exhibited in the screening. The obtained results suggested that nutrients might influence the mechanisms to withstand HMF. The effect of nitrogen source on HMF biotransformation performance of *F. sambucinum* was evaluated in culture media containing a commercial or the own produced protein hydrolysate at the same level. *F. sambucinum* was grown in ME culture medium, ME without peptone (ME-WP), and in a culture medium where soybean peptone was replaced by HA (ME-HA). The results of the effect of nitrogen source on HMF consumption and products detection are shown in Figure 6.2. As shown in Figure 6.2a, no

remarkable differences were observed among the control and culture medium ME-HA or the one without peptone (ME-WP) in the HMF consumption. This suggested that *F. sambucinum* HMF consumption rate was not affected by the nitrogen source, demonstrating its capability to metabolize HMF without peptone in the culture medium. It should be noted that the malt extract contains 5% of protein, which represents 1 g/L of protein; and therefore, the amount of N added with the malt extract might be enough. Therefore, when the nitrogen source was present at low concentrations, it did not affect considerably the HMF consumption rate.

Although similar patterns were observed for HMF metabolization, several differences were observed in the product formation. *F. sambucinum* reduced HMF to MF and DHMF, and different concentrations were observed depending on the culture medium (Figure 6.2b and c). DHMF concentration ranged from 13.10 to 22.30 mM, while MF from 1.39 to 1.80 mM within the different media. Once formed, the products were persistent in the culture broth, suggesting that the products were stable and not further degraded. When the fungus strain was grown in ME-HA, both MF and DHMF concentrations were significantly lower compared to ME and ME-WP. Given that a similar amount of HMF was metabolized, it can be speculated that this fact might be due to the formation of other by-products that could not be detected with the employed methodology, such as HMFCA, FFCA, FDCA, the acids resulting from the oxidation of HMF.

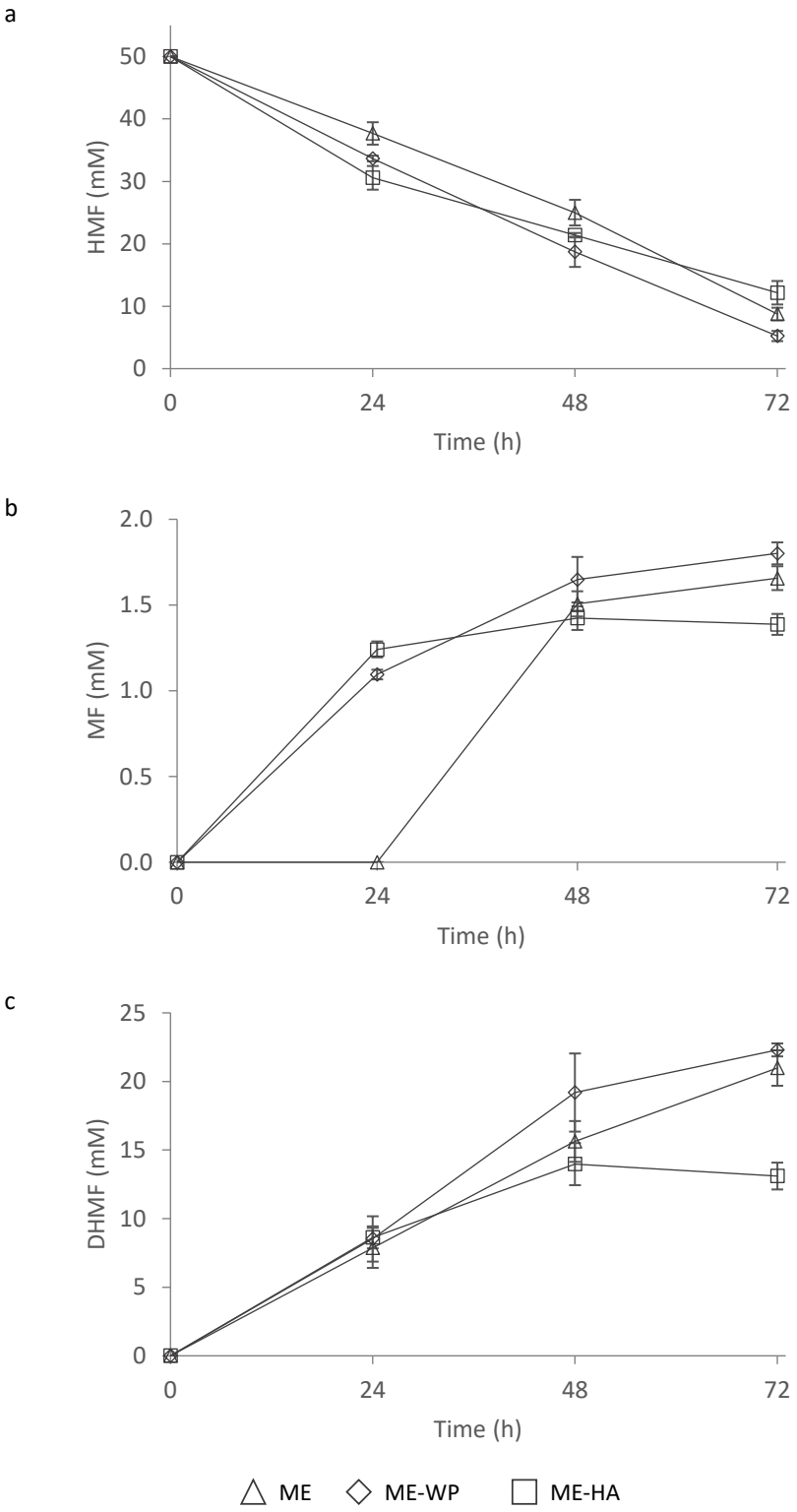


Figure 6.2. HMF metabolization and products formation by *F. sambucinum*. ME: Malt Extract medium; ME-WP: Malt Extract medium without peptone; and ME-50HA: Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate. Refer to Table 6.2 for media composition.

3.2.2. Effect of nitrogen dose and source

To check if HMF degradation and product formation were nitrogen dose-dependent, protein hydrolysate content in the culture media was increased, replacing 50% of the malt extract. The main reason to try to replace malt extract is the high price of this component. Besides this, to assess the performance of the obtained protein hydrolysate in our laboratory, a commercial meat hydrolysate was used instead of soybean peptone in the control. For comparison purposes, the results obtained previously with ME (Figure 6.2) were included.

As shown in Figure 6.3, significant differences in HMF metabolization and products synthesized were observed among the treatments. Figure 6.3a shows the effect of the protein hydrolysates and malt extract removal in HMF metabolization. It was found that the removal of peptone and half of the malt extract exerted a significant effect on the catalytic performance of *F. sambucinum* cells, particularly compared to the culture medium containing a high amount of peptone. Within the first 24 h, the reaction in ME-50WP worked similarly to ME; however, afterward HMF metabolization rate was slowed down. Given the carbon source excess in the broth and the slow metabolization of this nutrient by other *Fusarium* strains (Millán et al., 2021), arguably, the nitrogen depletion might be limiting HMF metabolization by *F. sambucinum*. When peptone was added to the culture medium, initial reaction rates were improved (1.17 g/(L h) *vs.* 0.48 g/(L h); calculated at 24 h). Therefore, the assumption that nitrogen could be the limiting nutrient was proven. In addition to the effect on the consumption rate, nitrogen supplementation increased the amount of HMF metabolized. For instance, within 72 h of reaction, *F. sambucinum* grown in ME-50WP reduced the initial HMF concentration to half, whereas the cells in ME-50HA completely metabolized HMF within 48 h. Despite that the culture media ME-50HA and ME-50Meat were supplemented with a similar amount of protein hydrolysate, significant differences were observed in HMF consumption after 24 h of reaction. The reaction rate of cells in ME-50Meat was slowed down, and the complete HMF metabolization was not observed even within 72 h of reaction (there was 8 mM of HMF remaining). This suggested that *F. sambucinum* might have a preference for certain peptides or a combination of amino acids and highlighted the effect of the nitrogen source on the reaction.

In addition to the differences in HMF metabolization, the effect of the amount and the source of nitrogen on the metabolic pathways involved in products synthesis was evaluated. As shown in the Figure 6.3b, c, and d, the substitution of the malt extract by a nitrogen source or his removal had a significant effect on the capability of *F. sambucinum* to reduce or oxidize HMF. The addition of peptone at a low concentration and nitrogen resources scarcity induced the reduction of HMF to MF and DHMF. However, animal-derived protein hydrolysate addition at high concentration altered the enzymes profile,

observing HMF oxidation and reduction derivatives simultaneously in the initial stage. Specifically, supplementation of culture medium with a commercial meat peptone yielded 5 mM of MF and 9 mM of DFF. DHMF was also detected at a very low concentration, but it was not stable in time. Surprisingly, the addition of a high amount of HA as nitrogen source and the removal of 50% of the malt extract enhanced the HMF-oxidative activity, achieving a DFF yield of 80% after 48 h of reaction with a good selectivity (> 90%).

Although there exists uncertainty in the metabolic pathways for HMF oxidation and reduction, Li et al., (2017) and Xu et al., (2018) reported the importance of glucose in the biocatalytic HMF reduction with *Meyerozyma guilliermondii* SC1103 cells. The efficient regeneration of the reduced form of nicotinamide adenine dinucleotide phosphate cofactor (NAD(P)H), required for HMF reduction by various aldehyde reductases, depends on glucose concentration. In the same line, Zhang et al., (2017) reported that DHMF was synthesized more readily by *Comamonas testosteroni* SC1588 in the presence of nutrients (especially carbon sources), while lower yields were observed with resting cells, suggesting the same reasons as Li et al., (2017) and Xu et al., (2018). Given the above observations, reduction of malt extract content jointly with an adequate nitrogen source might have triggered alterations in metabolic pathways, facilitating the synthesis of the oxidized product. These observations reveal that the amount of nitrogen and C/N ratio are sensitive parameters affecting HMF metabolization by *F. sambucinum.* These differences might be due to the dependence of enzymatic systems involved in HMF degradation on nutrients. To the best of our knowledge, HMF oxidation to DFF has not been reported using microorganisms as biocatalysts.

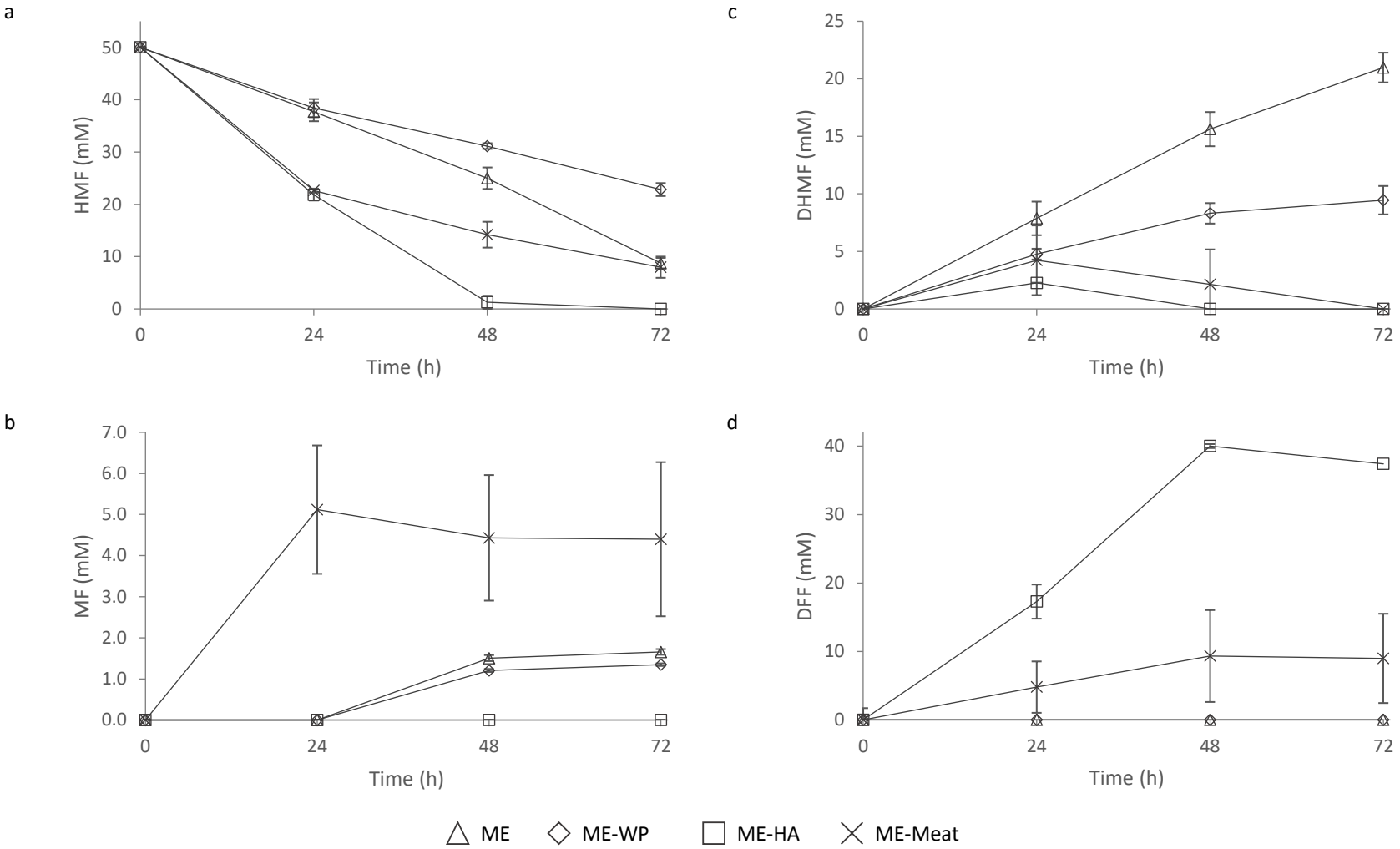


Figure 6.3. HMF metabolization (a) and derivatives: MF (b), DHMF (c), and DFF (d) in different culture media. ME: Malt Extract medium; ME-50WP: Malt Extract medium without peptone, ME-50HA: Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate; and ME-50Meat: Malt Extract medium containing meat peptone. Refer to Table 6.2 for media composition.

3.3. *Fusarium striatum*

3.3.1. Effect of nitrogen source on *F. striatum* sporulation

In the present study, the animal-derived protein hydrolysates were evaluated as nitrogen source in *F. striatum* growth and sporulation. Colony diameter and sporulation were evaluated under different nutrients and light regimes, as starvation or nutrient depletion and exposure to light have been reported to successfully induce sporulation in some fungi species (Su et al., 2012).

As shown in Figure S6.1 (Supplementary data) all the media supported the growth sufficiently, reflecting that all the media provided favorable growth conditions. Similar colony diameters were observed in all the culture media after 6 days. As shown in Figure 6.4, the composition of the culture medium and the interaction of both variables tested had a significant effect on sporulation. MEA, the standard medium used in our laboratory for *F. striatum* growth, resulted in the production of 2.2 x 10^6 spores/mL in the presence of light with no significant differences when it was grown in the dark. Surprisingly, the removal of nutrients from the culture media (MEA-WP and MEA-50WP) did not stimulate fungal sporulation, regardless of the light regime. Nevertheless, it should be noted that particularly in the case of filamentous fungi, findings cannot be readily extrapolated (Sharma & Pandey, 2010). The addition of protein hydrolysate HN did not affect significantly sporulation compared to MEA. Conversely, culture media amended with HA at a high concentration, replacing commercial peptones and half of the malt extract, was effective in inducing sporulation of *F. striatum*, observing a three-fold increase compared to MEA. In addition, light presence induced sporulation in MEA-50HA significatively, showing an increase from 5.5 x 10^6 spores/mL in the dark to 6.9 x 10^6 spores/mL in the presence of visible light. It should be noted that the culture media MEA-HA incubated in the darkness induced sporulation. Similar results were reported by Lazarotto et al., (2014) in the sporulation of *F. chlamydosporium* species complex, showing that the light regime only affected sporulation in some culture media and continuous light induced greater sporulation than other photoperiod regimes. Based on these results the light effect remains unclear. Furthermore, the knowledge in the sporulation process of *Fusarium* species is very limited (Li et al., 2006).

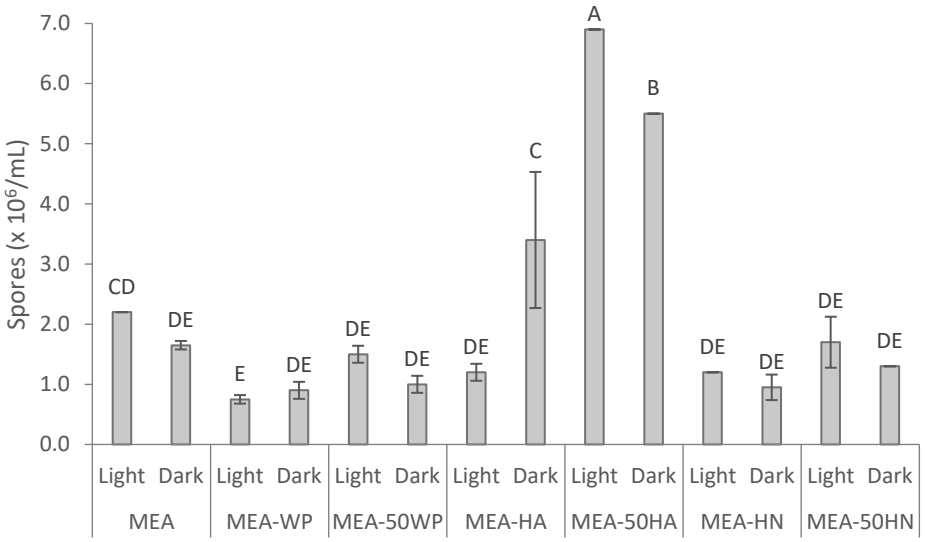


Figure 6.4. Effect of medium composition and presence or absence of visible light on sporulation of *F. striatum*. MEA: Malt Extract Agar medium; MEA-WP: Malt Extract Agar medium without peptone; MEA-50WP: Malt Extract Agar medium without peptone and 50% of malt extract; MEA-HA: Malt Extract Agar medium containing Alcalase 2.4L hydrolysate; MEA-50HA: Malt Extract Agar medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate; MEA-HN: Malt Extract Agar medium containing Neutrase 0.8L hydrolysate; and MEA-50HN: Malt Extract Agar medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate. Refer to Table 6.3 for culture media components. Different letters indicate significant differences among culture media ($p < 0.05$).

Microscopic examination revealed that spores morphology was influenced by nutrient and light presence (Figure 6.5). In the absence of visible light only microconidia were produced, regardless of the nutritional conditions. However, depending on the culture media, light presence induced the production of two types of conidia, micro- and macroconidia (Figure 6.5). Three out of the seven tested media showed both types of conidia in the presence of light, MEA-50HN, MEA-HA, and MEA-50HA. MEA-50HN and MEA-HA showed small, oval-shaped, and single or two-celled microconidia and no septate and slightly curved macroconidia were observed. Meanwhile, in MEA-50HA oval microconidia and 3-5-septated and gently dorsiventrally curved macroconidia were observed (Chehri et al., 2015; Sandoval-Denis et al., 2019). Li et al., (2006) suggested that a reduction of the cell wall integrity increased the size of conidia by altering turgor pressure. Although both types of conidia were observed in some culture media, microconidia were more abundant than macroconidia. The production of both micro and macroconidia is a usual phenomenon in *Fusarium* (Li et al., 2006).

Our experiments showed that nutrient limitation was not effective in inducing sporulation of *F. striatum*. Neither visible light can be regarded as an important stimulus for sporulation in most of the culture media. However, protein hydrolysate HA added at high concentrations has demonstrated effectiveness in inducing sporulation.

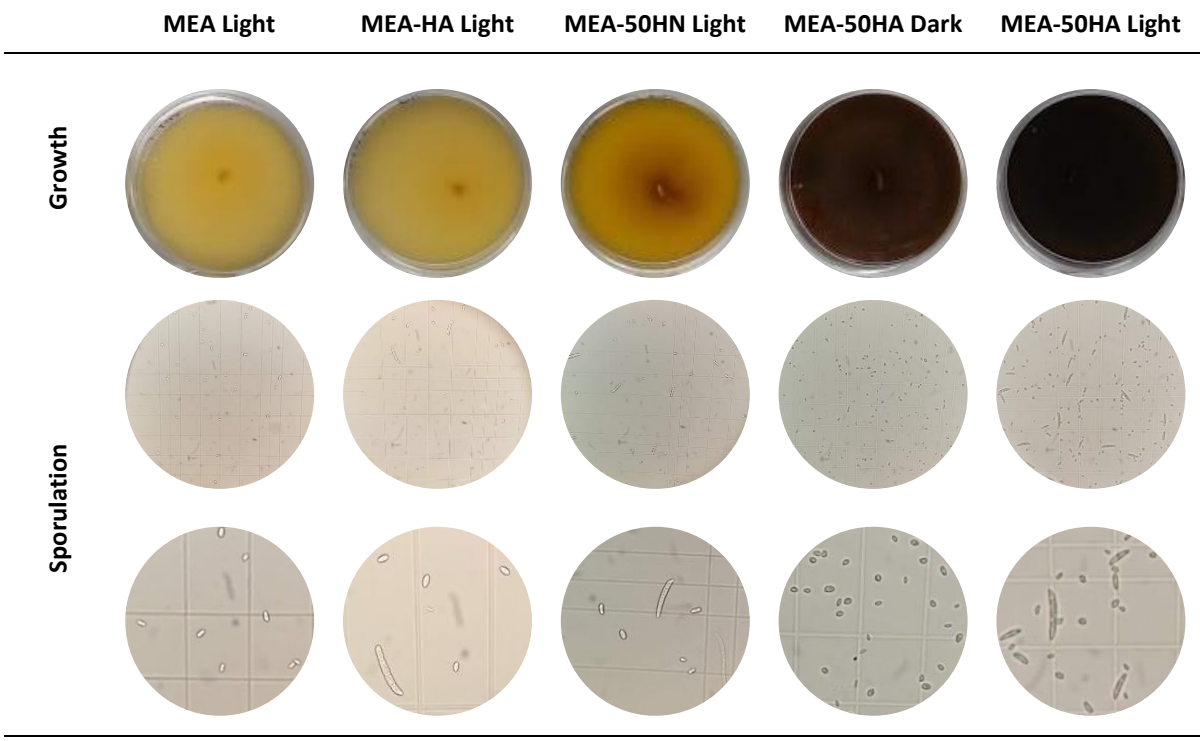


Figure 6.5. Cultural and morphological characters of *F. striatum* in MEA, MEA-HA, MEA-50HN, and MEA-50HA. MEA: Malt Extract Agar medium; MEA-HA: Malt Extract Agar medium containing Alcalase 2.4L hydrolysate; MEA-50HN: Malt Extract Agar medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate; and MEA-50HA: Malt Extract Agar medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate. Refer to Table 6.3 for culture media components.

3.3.2. Effect of inoculum type in HMF biotransformation

Inoculation with spores and discs are standard methods used when working with filamentous fungi (Feldman et al., 2015; Ran et al., 2014). Spores inoculation presents several advantages, as higher reproducibility and control over the amount of catalyst added compared to fungal agar discs inoculation (Millán et al., 2021). In addition, inoculum nature, as well as medium constituents and culture conditions might affect fungal morphological form when grown in submerged culture. Process performance might be highly influenced by a particular morphology (Papagianni, 2004).

The effects of the inoculum type and the culture composition were evaluated in HMF biotransformation with *F. striatum*. Although satisfactory results were obtained in the screening with 50 mM of HMF with discs, Millán et al., (2021) reported higher toxicity toward this filamentous fungus when an HMF concentration over 75 mM was added to the ME culture medium. Therefore, assessment of inoculum type and culture composition was carried out using a substrate-feeding approach, which allowed to i) overcome the toxicity effect and ii) increase final HMF derivatives concentration. The HMF and DHMF

concentration were monitored and HMF was added again after 48 h from the first addition. The results of the biotransformation of 2.6 mmol of HMF under the different conditions are shown in Figure 6.6.

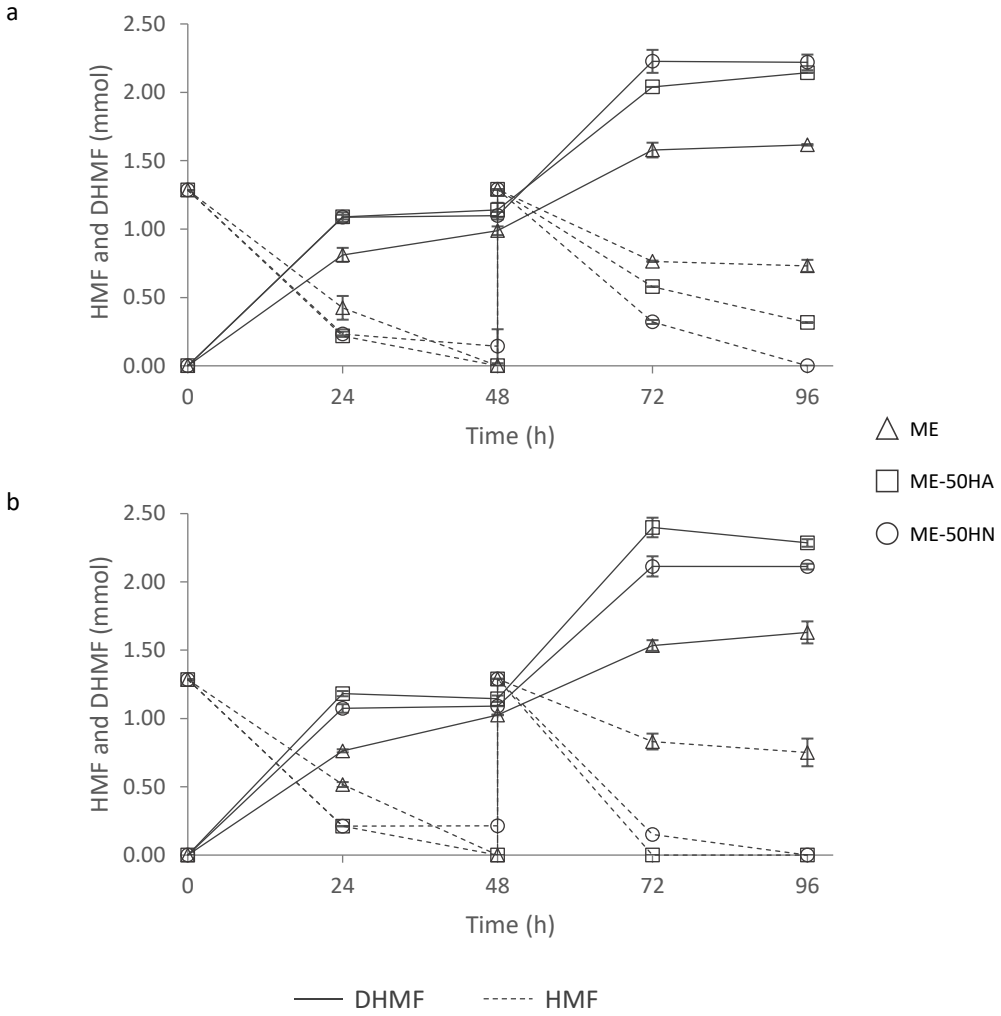


Figure 6.6. HMF reduction to DHMF with a) discs and b) spores. ME: Malt Extract medium, ME-50HA: Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate, and ME-50HN: Malt Extract medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate. Refer to Table 6.4 for culture media components.

The effect of the two variables, inoculum type and culture medium, was determined with two-way ANOVA at 24 and 72 h of reaction time. The inoculum type did not show a significant effect in HMF reduction to DHMF. Nevertheless, the culture medium and the interaction of both variables had a significant effect. Although the same number of spores (2.2×10^6 spores) and discs (3 discs) were inoculated in the culture media tested, *F. striatum* cells grown in ME-50HA and ME-50HN media performed significantly better than in ME. It should be highlighted that the volume of the spore suspension required to inoculate the same number of spores was remarkably lower in the ME-50HA medium (0.32 mL *vs.* 1 mL). This was due to the higher sporulation observed for *F. striatum* grown in MEA-50HA (Figure 6.4). In addition, the inoculation with spores led to significantly higher DHMF yields (88 ± 1.4 %) compared to discs (75 ± 2.5 %)

in ME-50HA medium after both additions (yields at 72 h). The HMF metabolization rate by *F. striatum* grown in ME-50HA medium is comparable to the one reported by Millán et al., (2021) in ME with an addition of 4.0 x 10^6 spores to biotransform 2.6 mmol of HMF. These observations indicate that culture medium supplementation with a low-cost protein hydrolysate, such as HA, sustains better fungal growth, requiring a smaller inoculum size. This will suppose an opportunity to reduce costs. These results indicate that the culture media highly influenced HMF metabolization, which might be due to cells growth, and the inoculum type effect was dependent on the culture medium. Given the promising results obtained with the inoculation of spores in the ME-50HA medium, this inoculum type was selected for further experiments.

3.3.3. Effect of nitrogen source in HMF biotransformation

In the previous substrate-feeding approach, *F. striatum* showed the capacity to metabolize almost 1 mmol within 24 h in ME-50HA and 0.86 mmol in ME. Based on these observations, four consecutive HMF additions of 45 mM (0.86 mmol) in time intervals of 24 h were carried out. For comparison purposes, all the culture media tested were inoculated with spores from fungi grown in MEA. The results are shown in Figure 6.7. *F. striatum* showed the capability to metabolize almost all the HMF in every addition performed. After 96 h of reaction, the remaining HMF ranged from 0.32 to 0.44 mmol, depending on the culture medium. It should be noted that HMF was never completely metabolized, leading to successive accumulation, around 0.1 mmol every 24 h.

Within 72 h of reaction, *F. striatum* reduced HMF to DHMF at a constant rate, achieving around 2.5 mmol of DHMF in the reaction media (Figure 6.7). After the fourth HMF addition, although HMF degradation rate was not reduced (90% of the HMF added was consumed), the DHMF production was slowed down, observing that only 30% was biotransformed to DHMF. HMF could have been used as carbon source or biotransformed to other non-detectable products with the methodology employed. These observations might be related to the *F. striatum* cells tolerance towards DHMF.

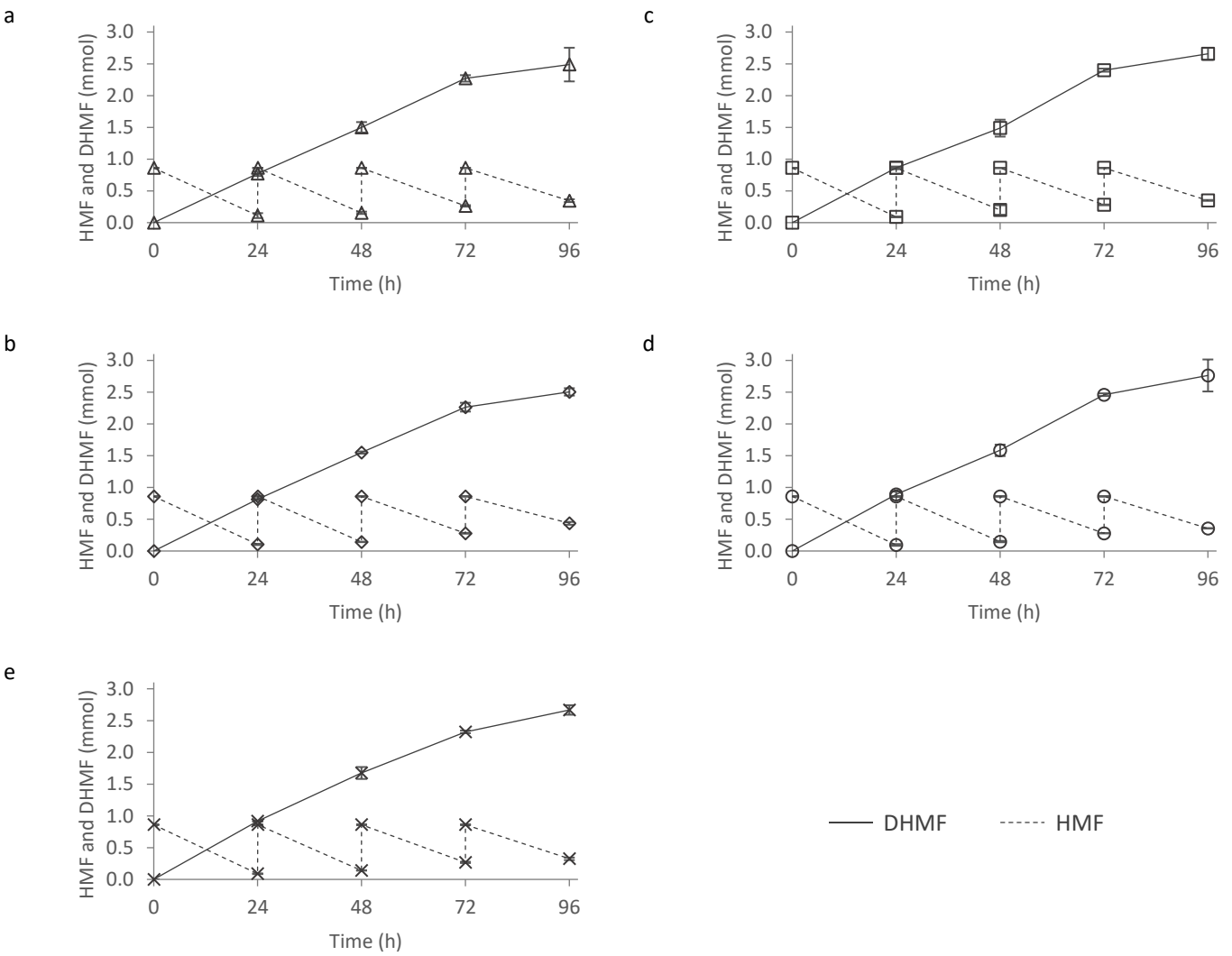


Figure 6.7. Substrate approach with 0.86 mmol additions. The culture media assayed were the following: a) ME, b) ME-50WP, c) ME-50HA, d) ME-50HN, and e) ME-50Meat. ME: Malt Extract medium; ME-50WP: Malt Extract medium without peptone and 50% of malt extract; ME-50HA: Malt Extract medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate, ME-50HN: Malt Extract medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate, and ME-50Meat: Malt Extract medium containing meat peptone. Refer to Table 6.5 for culture media components.

Although DHMF is reported to be non-toxic and highly tolerated by a wide range of microorganisms, the damage caused to proteins, nucleic acids, and cells organelles by the successive additions of HMF jointly with the high concentration of DHMF might have led to irreversible damage (Domínguez de María & Guajardo, 2017). It can also be due to the long reaction times or the accumulation of DHMF inside the cells after long incubation times (Martins et al., 2020). Statistical analysis showed that significant differences were observed after 72 h of reaction time among the culture media. *F. striatum* grown in ME-HN and ME-HA culture media yielded more DHMF compared to ME-WP culture medium. However, not significant differences were observed among ME, ME-50Meat, and ME-WP. This indicated that *F. striatum* was able to reduce HMF into DHMF without the addition of peptones, arguably due to the low toxicity of the HMF towards the cells at the concentration added. Note that although significantly better results were obtained in ME-HA and ME-HN, these differences are lower than in the previous assay in which HMF was added at a concentration of 75 mM. Therefore, the addition of the hydrolysates HA and HN to the media increases the tolerance of *F. striatum* towards high concentrations of the substrate, but their positive effect on the DHMF production decreases for less toxic levels of HMF.

4. Conclusions

In the present study, two low-cost protein hydrolysates prepared in our laboratory were evaluated as nitrogen source and compared with commercial ones in the biocatalytic transformation of HMF by several *Fusarium* strains. HA and HN showed to be suitable nitrogen sources for most of the *Fusarium* strains tested, observing similar performance compared to commercial peptones. In the case of *F. sambucinum*, when the peptone from soybean was substituted by HA or HN there were no differences in the metabolization of HMF by the fungus. However, when the nitrogen amount was increased, differences started to appear among the different nitrogen sources. The addition of protein hydrolysate HA highly influenced HMF metabolization, describing for the first time the whole-cell oxidation of HMF into DFF. In the case of *F. striatum*, the addition of HA stimulated sporulation significantly. Moreover, it seems to support better fungal growth, tolerating better higher HMF concentrations.

Therefore, we have demonstrated that the animal-derived protein hydrolysates may be a promising alternative to commercial ones in HMF biotransformation using *Fusarium* whole-cells as biocatalyst. In addition, the nitrogen source highly influences the metabolization of HMF if it represents a substantial part of the fungi growth medium.

5. References

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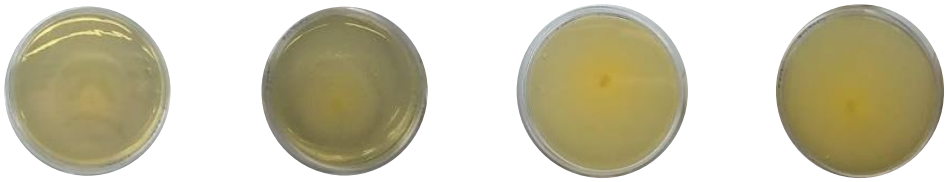
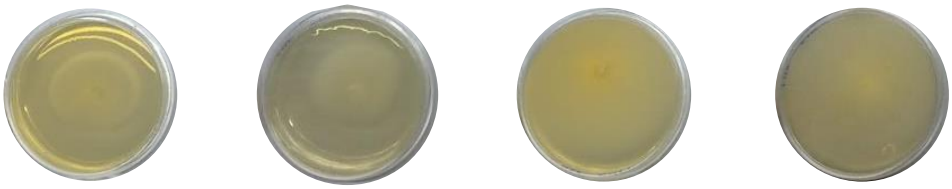
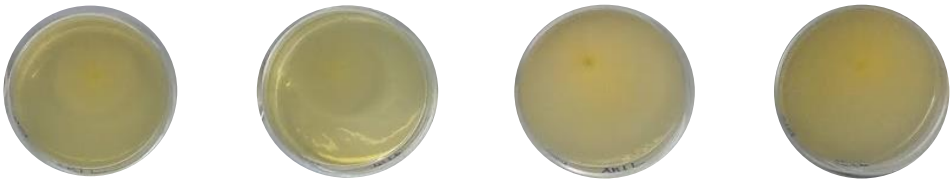
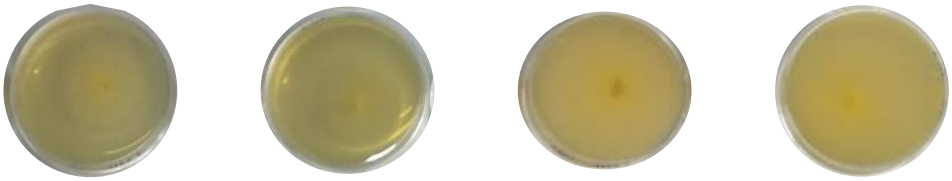
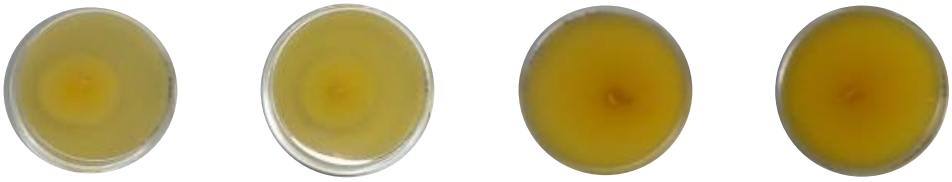
Culture medium	3 days		6 days	
	Light	Darkness	Light	Darkness
MEA				
MEA-WP				
MEA-50WP				
MEA-HA				
MEA-50HA				
MEA-HN				
MEA-50HN				

Figure S6.1. *Fusarium striatum* growth in Petri dishes containing different culture media after 3 and 6 days. MEA: Malt Extract Agar medium; MEA-WP: Malt Extract Agar medium without peptone; MEA-50WP: Malt Extract Agar medium without peptone and 50% of malt extract; MEA-HA: Malt Extract Agar medium containing Alcalase 2.4L hydrolysate; MEA-50HA: Malt Extract Agar medium with 50% of malt extract replaced by Alcalase 2.4L hydrolysate; MEA-HN: Malt Extract Agar medium containing Neutrase 0.8L hydrolysate; MEA-50HN: Malt Extract Agar medium with 50% of malt extract replaced by Neutrase 0.8L hydrolysate.

Chapter 7. Protein hydrolysates as nitrogen suppliers *in vitro* plant culture

1. Background

Plant species can absorb and assimilate nitrogen (N) from several compounds, including nitrate (NO_3^-), ammonium (NH_4^+), urea, and amino acids. However, regardless of the N source available, the NO_3^- form of N is the main form absorbed by plants. Most soils do not have sufficient N available, representing one of the most important limiting factors to support desired productivities. Hence, maximizing crop yields depends on the addition of fertilizers, leading to severe environmental problems worldwide due to their high mobility and facility to leach into groundwater (Liu et al., 2008; Santi et al., 2017; Sestili et al., 2018). Nowadays, the world's horticulture systems face the need to balance two requirements: i) increase the supply of food produced on the available farmlands; intensive-farm-management activities, and ii) reduce agriculture's impact on human health and the environment (Colla et al., 2015, 2017). In view of this situation, biostimulants, an emerging class of crop management products, represent a promising alternative to fertilizers. They have significant potential for improving physiological processes in plants and enhancing germination, growth, and stress tolerance (Calvo et al., 2014; da Silva, 2018; Santi et al., 2017; Ugena et al., 2018). du Jardin, (2015) have described seven main categories of plant biostimulants: humic and fluvic acids, protein hydrolysates and other N-containing compounds, seaweed extracts and botanicals, chitosan and other biopolymers, inorganic compounds, beneficial fungi, and beneficial bacteria.

Protein hydrolysates are defined as "*mixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources using partial hydrolysis*" (Colla et al., 2015). Protein hydrolysates-based biostimulants applied to plants through foliar application or soil/substrate drenching have demonstrated several positive effects (Calvo et al., 2014), such as enhanced biomass production and N assimilation in leafy radish (Liu et al., 2008), promotion of root growth and micronutrient accumulation on maize seedlings (Santi et al., 2017), increased root growth and plant biomass on tomato plants (Sestili et al., 2018), increase in the grain yield of winter wheat (Popko et al., 2018), enhancing yield and nutritive value of perennial wall-rocket (Caruso et al., 2020), increased root and leaf growth and morphological changes in root architecture in maize seedlings (Ertani et al., 2009), more extensive root apparatus and increased N assimilation on corn, pea, and tomato (Colla et al., 2014), etc. The improvement in growth performance has been attributed principally to i) iron (Fe), carbon (C), and N metabolism (genes involved in NO_3^-, NH_4^+, and amino acids transporters were differentially regulated in an N-dependent manner under protein hydrolysates supply), ii) nutrient uptake, water, and nutrient use efficiencies for both macro and microelements, and iii) induction of plant defense responses and increased plant tolerance towards abiotic stresses, such as salinity or oxidative conditions (Bulgari et al., 2019; Calvo et al., 2014; Colla et al.,

2015; Sestili et al., 2018). In addition, specific peptides have exhibited hormone-like activity, as auxin and gibberellin activities, thus promoting crop yields (Colla et al., 2015). Furthermore, enhancing N utilization can reduce N losses through leaching and runoff (Liu et al., 2008).

In addition to the ability of several protein hydrolysates to stimulate plant growth, yield, resilience, and counteracting abiotic stresses, recycling of animal derived-wastes into useful agricultural products can lead to enhance matter and energy conservation simultaneously leading to environmental benefit (Calvo et al., 2014; Corte et al., 2014).

Considering this background, this study aimed to determine the effect of animal-derived protein hydrolysate in germination and at the early stage of growth of tomato and radish plants. The protein hydrolysates were applied at two levels, and their effect was assessed based on dry plant biomass, dry root weight, and chlorophyll and proline contents.

2. Materials and methods

2.1. Plant material and treatments

Seeds of *Raphanus sativus* var. Redondo Punta Blanca-Roja-Iceberg (radish) and *Lycopersicon esculentum* var. Tres Cantos (tomato) were used for the experiments. Seeds were surface-sterilized by soaking with 2% sodium hypochlorite solution containing a few drops of Tween-20 for 1 min 30 sec at room temperature. Then, seeds were rinsed twice with sterile distilled water and used for *in vitro* culture tests under sterile conditions.

A control and three treatments (Table 7.1) for each species were carried out in order to evaluate the effect of protein hydrolysates on the germination of seeds and plant growth. The treatments were derived from the medium Murashige and Skoog (MS) (control), a medium containing extra potassium (added in the form of KNO_3) (treatment 1), and media where 25% or 50% of the inorganic nitrogen source, NH_4NO_3, was substituted by the protein hydrolysate prepared with Neutrase 0.8L (HN) (treatments 2 and 3, respectively) prepared as described in Chapter 3 Section 2.5 with a small modification; KOH was used to adjust the pH instead of NaOH. The pH of all media was adjusted to 5.7 prior to autoclave.

Table 7.1. Chemical composition of the media used in the experiments for tomato and radish plants growth.

		Control and treatments			
		Control	**Treatment 1**	**Treatment 2**	**Treatment 3**
Macronutrients (g/L)	NH_4NO_3	1.65	1.65	1.24	0.83
	Protein hydrolysate	-	-	1.3	2.6
	KNO_3	1.9	2.017	1.9	1.9
	$CaCl_2 \cdot 2H_2O$	0.44	0.44	0.44	0.44
	$MgSO_4 \cdot 7H_2O$	0.37	0.37	0.37	0.37
	KH_2PO_4	0.17	0.17	0.17	0.17
Micronutrients (mg/L)	$MnSO_4 \cdot H_2O$	16.9	16.9	16.9	16.9
	$ZnSO_4 \cdot 7H_2O$	8.6	8.6	8.6	8.6
	H_3BO_3	6.2	6.2	6.2	6.2
	$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25
	KI	0.83	0.83	0.83	0.83
	$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025
	$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.025
	FeNa-EDTA	36.7	36.7	36.7	36.7
Vitamins and organic constituents (mg/L)	*myo*-Inositol	100	100	100	100
	Nicotinic acid	0.5	0.5	0.5	0.5
	Indoleacetic acid	2	2	2	2
	Thiamine · HCl	0.1	0.1	0.1	0.1
	Pyridoxine ·HCl	0.5	0.5	0.5	0.5
	Glycine	2	2	2	2
	Sucrose	30000	30000	30000	30000

2.2. *In vitro* culture test

2.2.1. Germination and seedling growth

Twenty-five sterilized seeds were layered over soaked filter paper in 100 x 15 mm polystyrene Petri dishes containing 4 mL of the different media (Table 7.1). A total of 125 seeds were subjected to each treatment (25 seeds x 5 replicates). Germination was carried out in a climatic chamber at 23 ± 2 °C, under 90 – 110 µmol s^{-1} m^{-2} light intensity (Sylvania Gro-Lux F58W/GRO-T8) and 16 h light photoperiod.

The seed germination and sprout development were visually monitored regularly. At the termination of each experiment, 5 days for radish and 10 days for tomato, germination and dry weight of plants, including aerial plant and roots, were measured. Germination was calculated by the following Equation 7.1:

$$Germination\ (\%) = \frac{Germinated\ seeds}{Placed\ seeds} \times 100$$

Equation 7.1

2.2.2. Plant growth

The sterilized seeds were sown in culture tubes containing 25 mL of the different media supplemented with a Gelrite™ gelling agent. A total of 80 seeds per treatment were sown. Plants were grown in a climatic chamber at 23 ± 2 °C, under 90 – 110 µmol s^{-1} m^{-2} light intensity (Sylvania Gro-Lux F58W/GRO-T8) and 16 h light photoperiod. The plants were visually monitored. After 21 days, plant material was harvested. The final biomass of each plant was determined by measuring the fresh weight and dry weight of remaining aerial plant parts and roots in a ventilated oven at 60 °C until constant weight. Chlorophyll and proline content of leaves were recorded.

2.2.3. Chlorophyll content

Among five and twelve leaves from different plants were randomly selected and chlorophyll content was measured with a SPAD 502 chlorophyll meter (Minolta, Japan) for each treatment.

2.2.4. Proline content

Proline content in leaves was determined following the methodology described by Bates et al., (1973). Briefly, 100 mg of fresh leaves were mixed with 2 mL of 3% sulfosalicylic acid and grounded. The leaf homogenate was incubated with acetic acid and acidic ninhydrin reagent (1:1:1) at 90 °C for 30 min. Then, 2 mL of toluene were added to the cooled down mixture and were vigorously vortexed. The absorbance of the resulting upper phase was measured at 524 nm in a spectrophotometer Helios Gamma (Thermo Electron Corporation, England). A standard curve (0 – 30 µg/mL) obtained with L-proline was used for proline concentration determinations. Results were expressed as µg of proline per g of fresh weight (FW).

2.3. Statistical analysis

Treatment comparisons for germination and growth data were subjected to one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons among treatments and control with a significance level of $p < 0.05$. Statistical analysis was carried out using JMP Pro statistical software (Version 14; Statistical Discovery™ from SAS, Cary, NC, USA).

3. Results and discussion

3.1. Germination and seedling growth

Germination tests have been widely applied to terrestrial plants (Hillis et al., 2011) and are routinely performed previous to more laborious assays. The visual aspect of radish and tomato seeds are shown in Figure 7.1 and Figure 7.2, respectively, while the average percentage germination and plant dry matter in the conducted treatments are displayed in Figure 7.3.

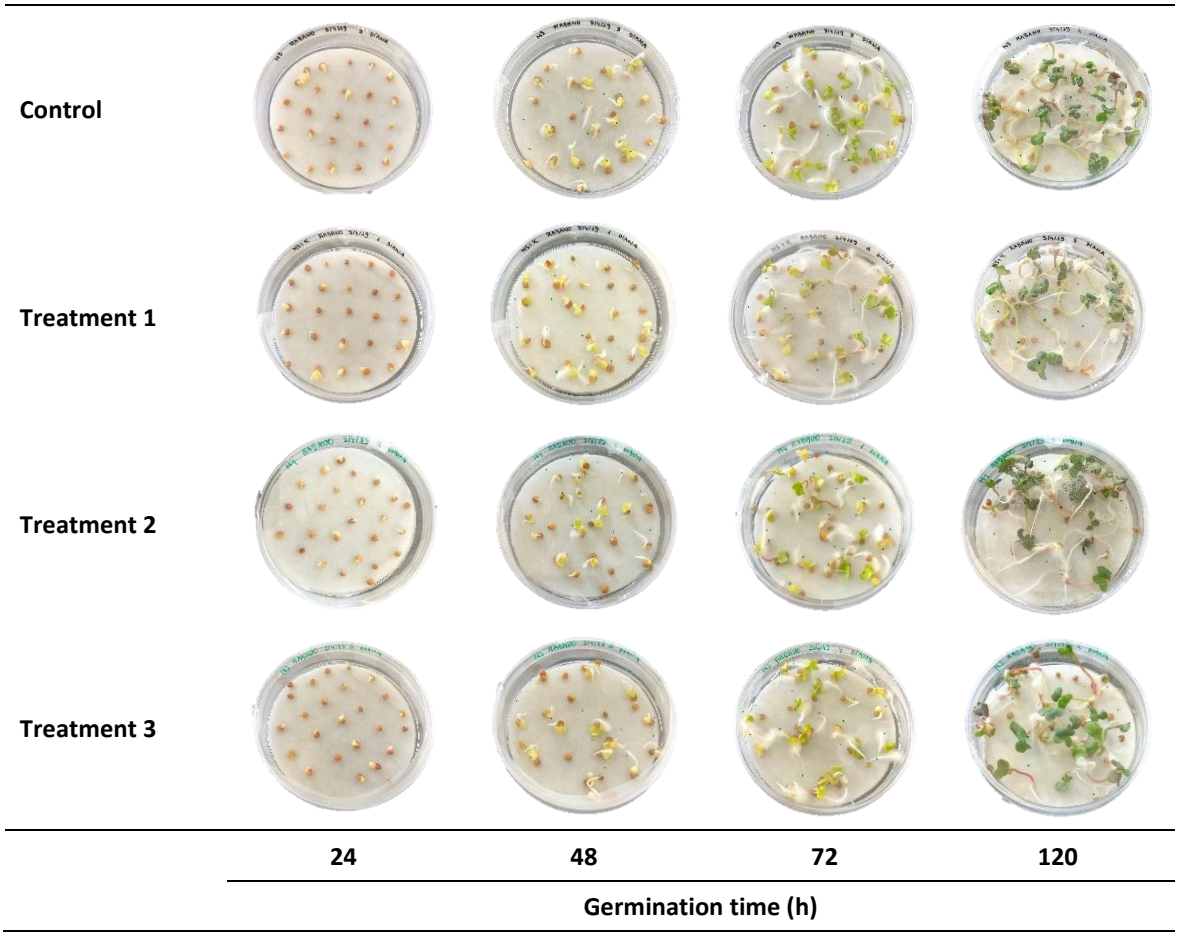


Figure 7.1. Radish seeds germination under the control and the three treatments.

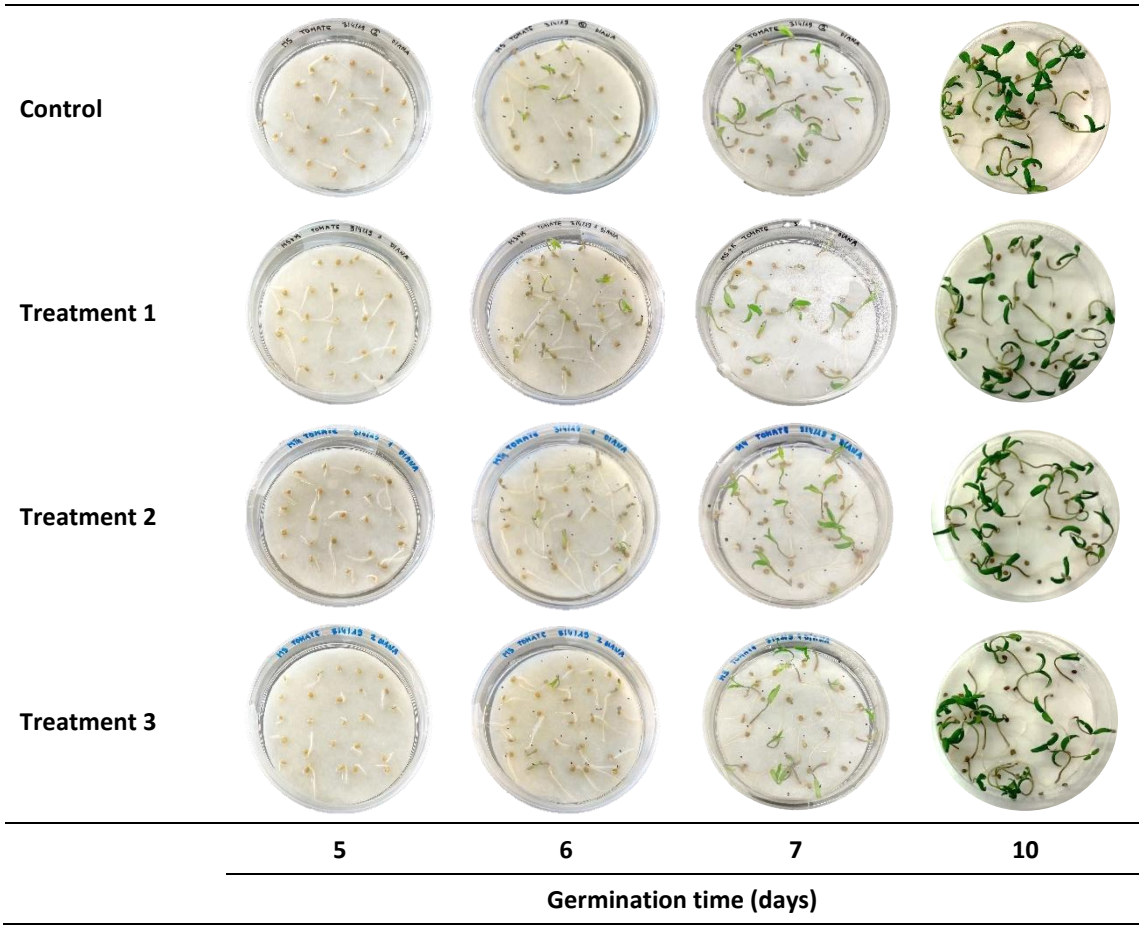


Figure 7.2. Tomato seeds germination under the control and the three treatments.

In the present work, the protein hydrolysate was studied at two levels. Given that substitution of ammonium nitrate lead to the addition of a considerable amount of K, a culture medium containing Murashige and Skoog (MS) with an extra amount of K was included in the study for the evaluation of the effect of the protein hydrolysate.

Germination of seeds in the control culture medium was over 90% for radish and around 85% for tomato (Figure 7.3a). A longer exposure time was needed for the tomato seeds to obtain consistent germination rates (> 80%). Radish had a germination rate > 80% after 3 days, while tomato achieved a similar rate after 6 days. No significant differences were observed in the germination rates among the treatments regardless of the specie, indicating that the seeds did not show sensitivity to the culture medium containing extra K, and neither to the protein hydrolysate. Besides this, it was observed that the different treatments applied did not cause any effect on plant dry weight (Figure 7.3b), nor apparent visual effects on sprouted seeds (Figure 7.1 and Figure 7.2). As previously highlighted by Hillis et al., (2011), germination is a highly conserved process, with many of the required nutrients stored and available for seedling emergence.

Therefore, tests based on germination are not enough sensitive and satisfactory to differentiate relevant effects on plant development (Serrano-Ruíz et al., 2018).

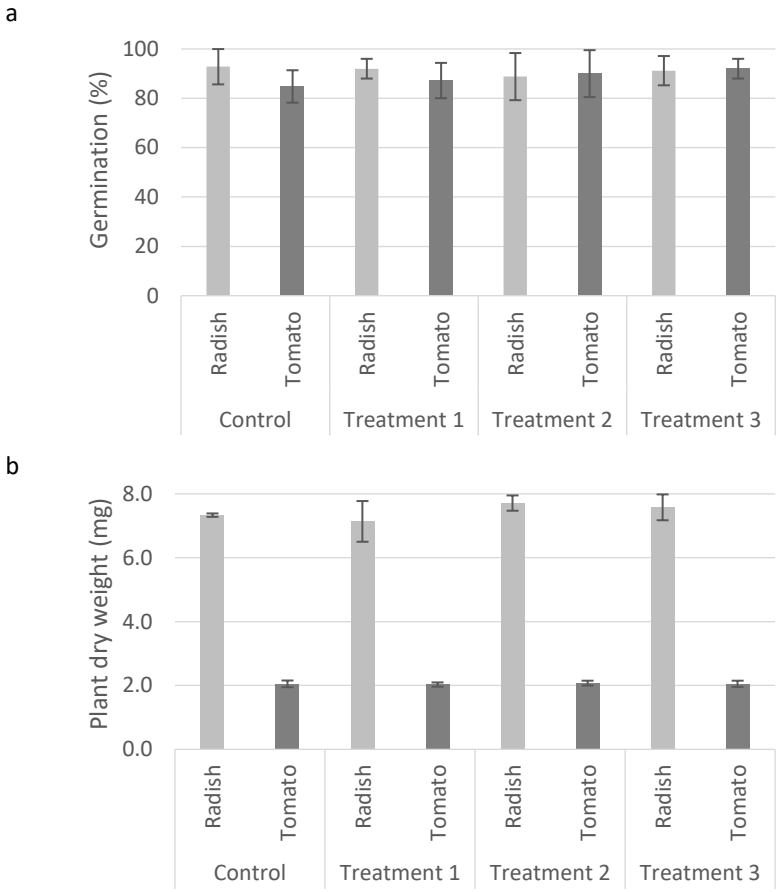


Figure 7.3 a) Germination rate and b) Plant dry weight of radish and tomato seeds after 5 and days 10.

No inhibition and neither improvement in the germination rate was observed in the presence of MS with extra K (treatment 1), or with the mixture of amino acids compared to the control. Therefore, further assays to determine the effect on root development, leaf quality, and stress response were needed.

3.2. Radish plant growth

Radish plant development and plant visual aspect were monitored during and at the end of the culture time, 21 days (Figure 7.4). The addition of 25 or 50% of the nitrogen source as a protein hydrolysate affected leaves, stems, and roots differently.

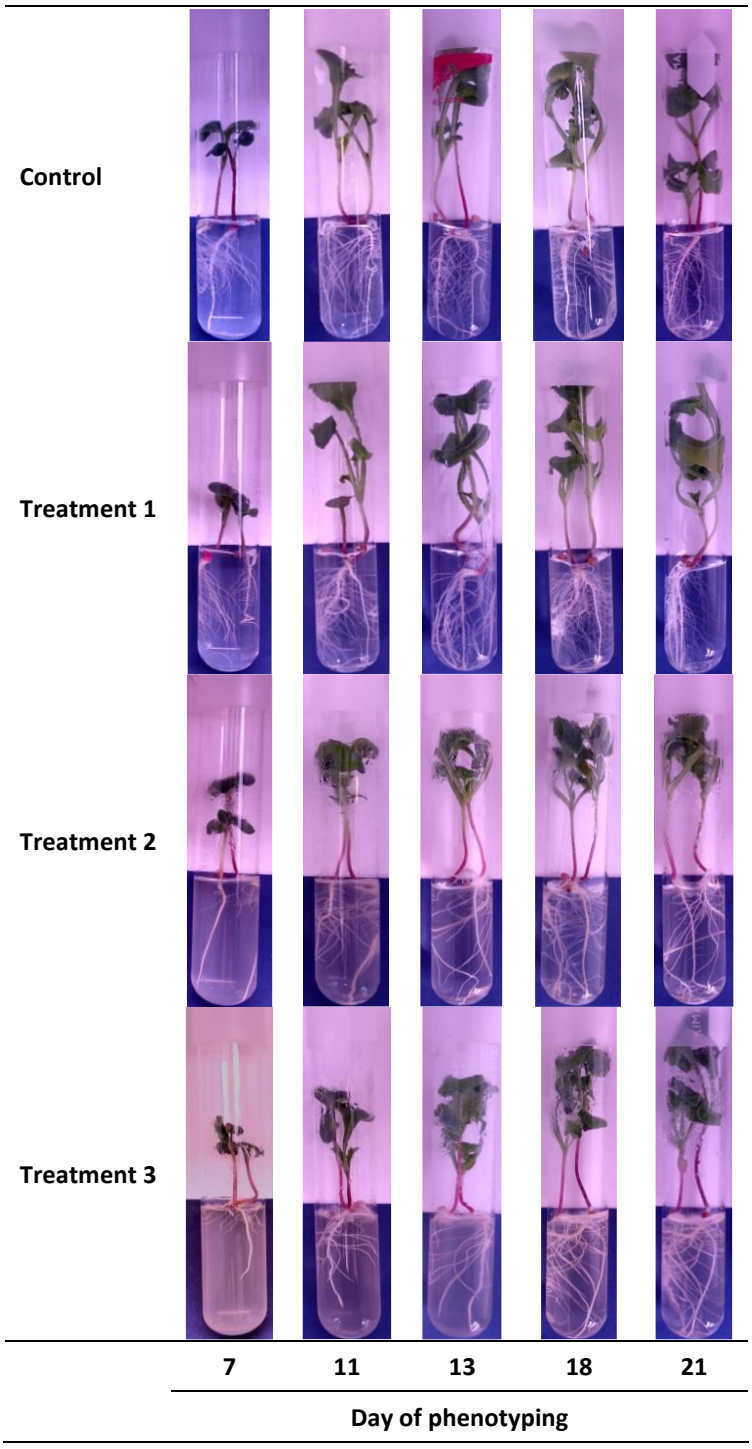


Figure 7.4. Radish plants at regular times of in vitro growth under the different treatments assayed.

The effect of the different treatments on shoot and root dry weight is shown in Figure 7.5. Although no significant differences were observed on shoot dry weight (Figure 7.5a), indicating that leaves and stem development were not altered, protein hydrolysates markedly modify the structure and color of the root system (Figure 7.4).

The root system developed quite well under all the treatments, but the differences in the root system were still visible at the end of the culture time. The roots of plants grown in control and treatment 1 were thin, white, and widely branched, while plants growing with organic nitrogen source had shorter, darker, and yellowish and thicker primary and secondary roots compared to the control and the treatment 1. Hence, roots were not affected by the extra K added in the culture medium, but by the peptides and amino acids present in the hydrolysate. Furthermore, the plants under treatment 3, the one containing the highest amount of amino acid and peptides, promoted thicker main and lateral roots compared to treatment 2. These differences in the roots are reflected in the root dry weight (Figure 7.5b). Root dry weight of treatment 3 exhibited an increase of 13% compared to the control. As the root system architecture is principally regulated by nutrient and water uptake efficiency (Arif et al., 2019; Colla et al., 2014), these results suggested that the protein hydrolysate stimulated root growth due to an increase in nitrogen assimilation. Colla et al., (2014) reported that plant-derived protein hydrolysate increased significantly shoot, root dry weight, root length, and root area in tomato plants, suggesting that a more robust and extensive root system can lead to an increase in plant productivity. In analogy with the previous observations, peptides and amino acids found in plant-derived protein hydrolysates have been reported to play an important role as signaling molecules in regulating plant development (hormone-like activity) (De Pascale et al., 2017). However, in the present study, the shoot dry weight of plants under treatment 2 and 3 was not significantly higher compared to the control. Therefore, the increase of the diameter of the root might be an adaptive mechanism of stress tolerance (Arif et al., 2019).

Chlorophyll content, representative of the efficiency of chlorophyll biosynthesis, was not significantly affected by the treatment compared to the control (Figure 7.5c). Proline is widely used as a marker for several stress conditions. Proline accumulation in leaves has demonstrated to improve tolerance to abiotic stress (Colla et al., 2014). The content of proline in the fresh leaves ranged from 732 to 1004 μg/g of fresh weight. However, no significant differences were observed among the control and the three treatments.

These results suggest that the partial replacement of NH_4NO_3 by amino acids and peptides did not exhibit significant effects on the shoot system. Whereas the adaptation of the roots system architecture to the available nutrients allowed the normal growth of the plants, not compromising the aerial plant development.

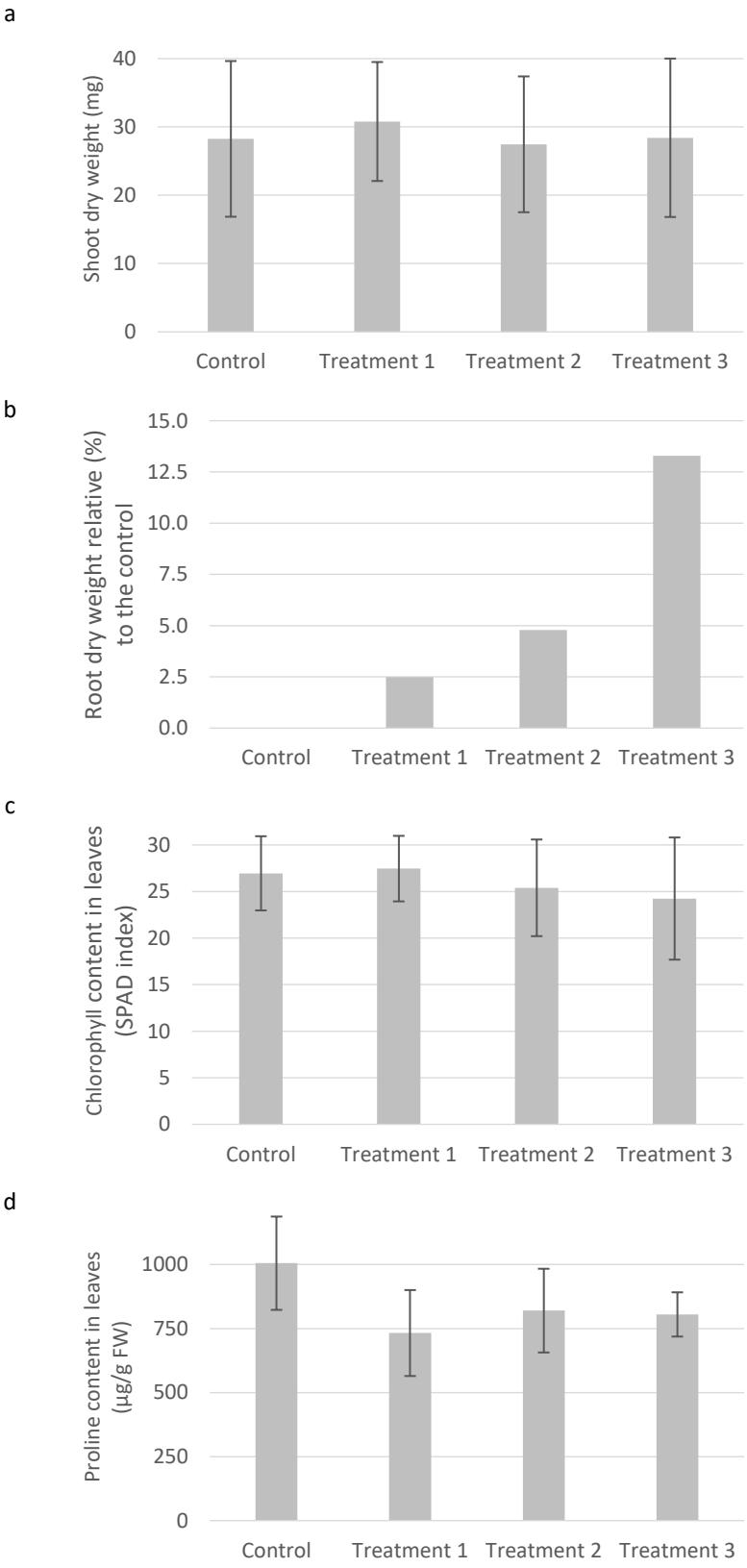


Figure 7.5. Radish plants growth after 21 days in the different culture media. a) Shoot dry weight, b) Root dry weight relative to control, c) Chlorophyll content in the fresh leaves, and d) Proline content in the fresh leaves. The asterisk (*) indicates a significant difference for treatments as compared to control according to Dunnett's test ($p < 0.05$).

3.3. Tomato plant growth

Tomato plant development and plant visual aspect were monitored during and at the end of the culture time, 21 days (Figure 7.6).

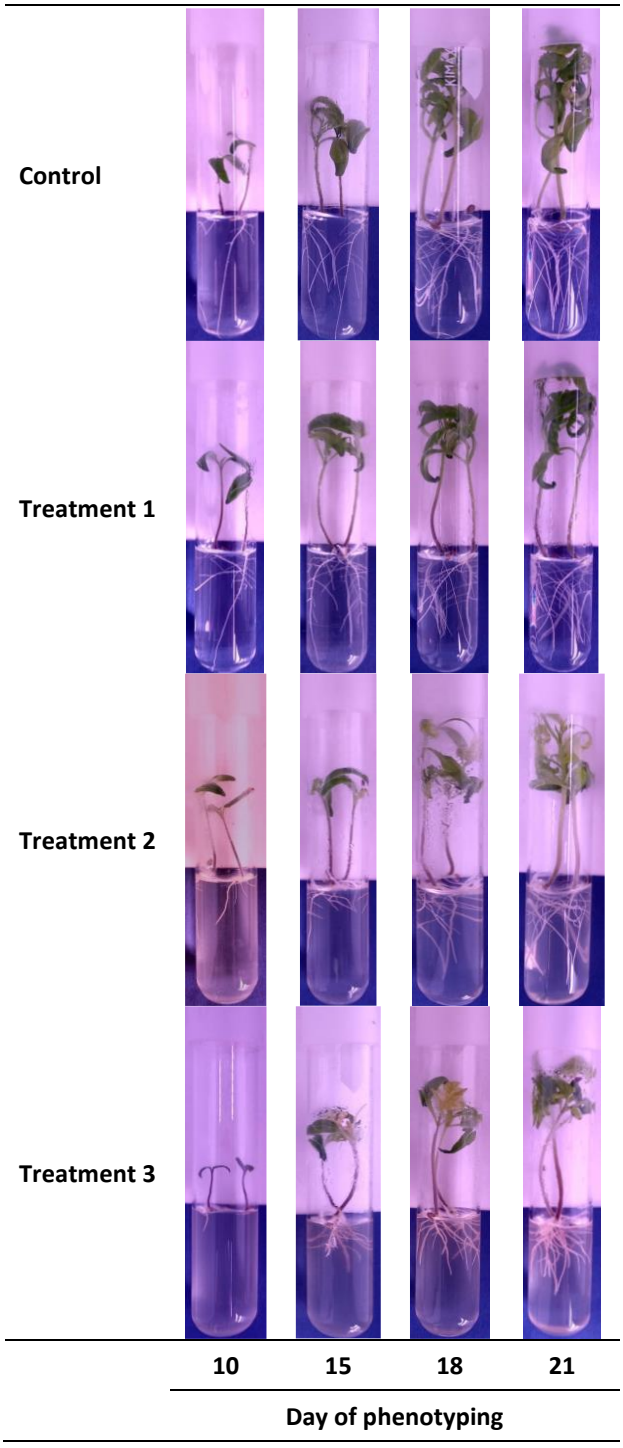


Figure 7.6. *In vitro* growth of tomato plants with MS media (control) and under three different treatments.

The effect of the protein hydrolysates on the aerial plant and root system development was evaluated by recording the dry weight of both aerial plant and root. The effect of the different treatments is shown in Figure 7.7. The obtained results showed that the type of treatment significantly affected the shoot dry weight (Figure 7.7a). Replacement of 50% of NH_4NO_3 by animal-derived protein hydrolysate (treatment 3) inhibited the growth of the shoot compared to the control. Nonetheless, the plants grown under treatment 2 (only 25% of NH_4NO_3 was substituted) did not show significant differences in shoot dry weight compared to the control. Although no significant differences between treatments 2 and 3 regarding shoot dry weight were observed, the visual look of plants differed between these treatments (Figure 7.6). The stem of plants grown under treatment 3 exhibited a more intense red color compared to the other treatments, associated with the synthesis and accumulation of anthocyanins. The synthesis of these pigments by tomato plants is usually observed when undergoing environmental stress, similarly to the proline, both associated with oxidative stress [c]

Root growth and morphology were also affected by the treatments. Although root dry weight of plants under treatments 2 and 3 increased compared to the control (Figure 7.7b), root elongation was strongly inhibited (Figure 7.6), developing numerous small lateral roots. In addition to length inhibition, the mixture of amino acids and peptides affected root diameter and color, leading to thicker and yellowish roots compared to the control. All these observations reflect that the effects are dose-dependent; a higher amount of protein hydrolysate exerted more negative effects on shoot and root systems. Growth inhibition caused by the presence of animal-derived protein hydrolysates might be due to their content in free amino acids and salts (Colla et al., 2014). Na concentration can also negatively affect soil physical, chemical, and biological properties, and thereby crop production and quality (Parrado et al., 2008). Despite the protein hydrolysates used in this study were prepared with KOH instead of NaOH to avoid Na addition, approximately 12.5 mg of Na were detected per g of protein hydrolysate (data not shown), representing 1.4 mM Na in the treatment 3 culture media. Cuartero & Fernández-Muñoz, (1999) reported that nitrogen uptake by tomato plants is not affected at relatively low salt concentrations (70 mM NaCl). Thus, the small amount of Na supplied in the culture media could not affect the shoot and roots development of tomato plants, reflecting that the effects observed were mainly due to the protein composition.

The undergoing stress situation suffered by tomato plants in the presence of protein hydrolysates was not reflected by the chlorophyll content, observing no significant differences between the control and the treatments (Figure 7.7c).

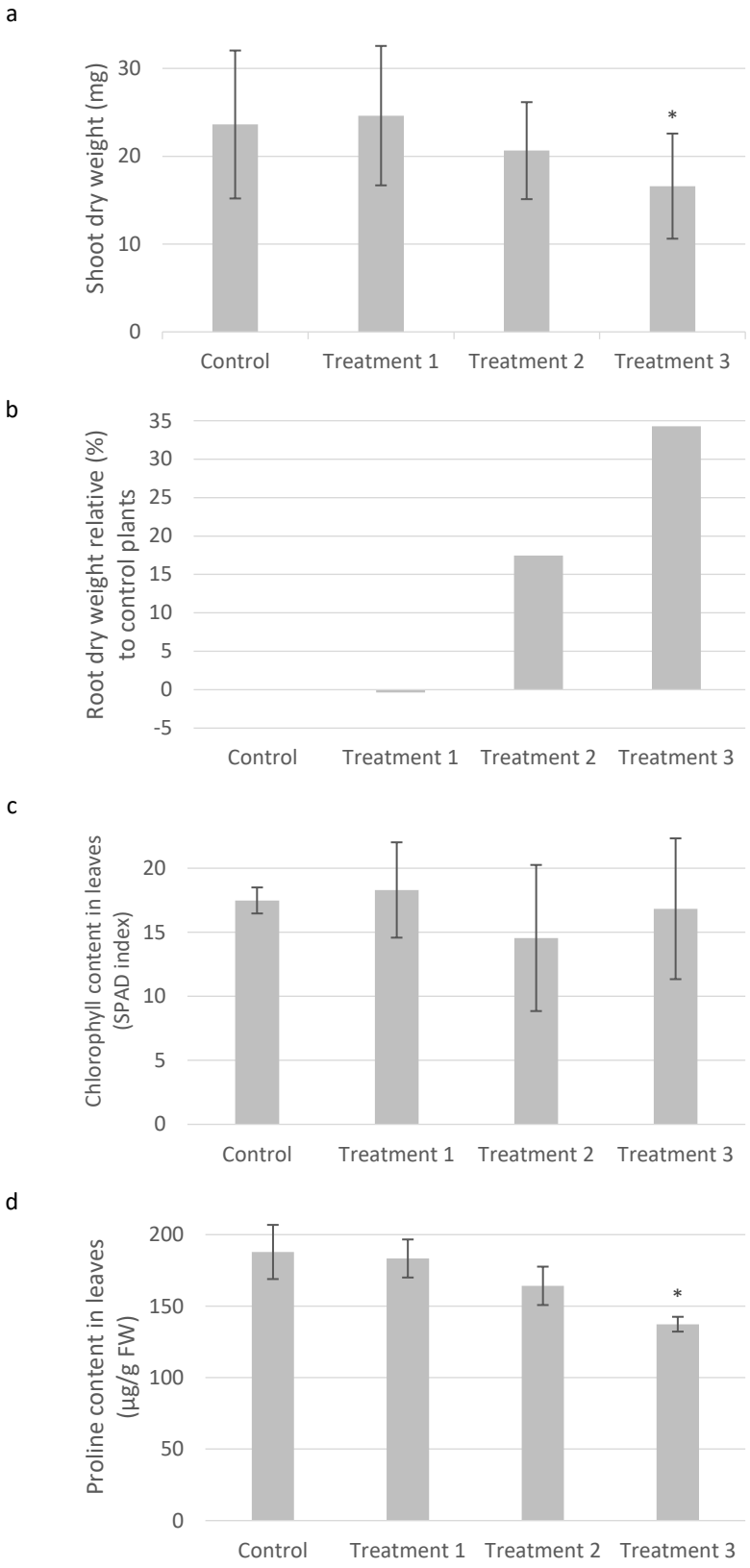


Figure 7.7. Tomato plants growth after 21 days in the different culture media. a) Shoot dry weight, b) Root dry weight relative to control, c) Chlorophyll content in the fresh leaves, and d) Proline content in the fresh leaves. The asterisk (*) indicates a significant difference for treatments as compared to control according to Dunnett's test ($p < 0.05$).

Tomato plants showed higher sensitivity to the animal-derived protein hydrolysate than radish plants. The *in vitro* germination assays did not show remarkable differences in germination rate and plant dry biomass, proving that these tests are not sensitive enough to discern protein hydrolysate effects. Whereas, *in vitro* plant growth assays showed significant inhibitory effects on shoot development and root dry weight in tomato plants when 50% of the inorganic N source was substituted by protein hydrolysate. However, radish plants were not significantly affected. Although a wide variety of studies have demonstrated specific positive effects of protein hydrolysate on plant performance, Calvo et al., (2014) pointed out that mixed results were observed in the most recent studies regarding yield enhancement and nutrient uptake. Besides this, Miller et al., (2007) have previously reported that NO_3^- and NH_4^+ uptake and transporters expression of plant roots can be down-regulated by an external supply of amino acid or tissue concentration of amino acids; also when mixed N sources are supplied and the relation NO_3^-/ NH_4^+ vary. Therefore, the adverse effects observed for plants grown in treatments 2 and 3 were N source related. Analysis of transcriptional changes in genes encoding nitrate, ammonium, and amino acids transporters are necessary to provide insight on the tested animal-derived protein hydrolysate effects.

4. Conclusions

In the present study, the biostimulant effect of the enzymatically produced protein hydrolysate on tomato and radish plants has not been demonstrated. The application of the animal-derived protein hydrolysates did not exhibit significant effects on seed germination. Furthermore, *in vitro* growth experiments showed significant effects only in tomato plants, observing inhibitory effects in root elongation. Further research is necessary to understand the action mechanism of this protein hydrolysate in plants. The reasons for the low effectiveness of the protein hydrolysate assayed might lie in their composition, the concentrations used, or the application method.

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General discussion

Slaughterhouses, as well as the processing of meat, generate large amounts ABPs, which pose a cost for the meat processing sector as well as an important environmental issue. Therefore, ABPs management and disposal following strict regulations becomes imperative. Although there exist several industrial systems to treat ABPs resulting in low-value applications, there is a growing awareness that most times these by-products are under-utilized. In this regard, research has been attempted to produce new products with a significant added-value and/or strong economic potential, capable of covering processing and disposal costs while reducing environmental damage (Mora et al., 2014; Toldrá et al., 2012). In general, ABPs as well as rendered meals and other wastes, are excellent sources of protein and lipids, macromolecules that can be transformed into high-value products if optimally exploited.

As stated in Hypothesis and objectives section, this Doctoral Thesis aimed at *fines* valorization, an unexploited material generated during rendering which has not received research attention. In this regard, we focused the scope of this thesis on fat and protein recovery from *fines*, as these are the main constituents of *fines* and they are projected as valuable components. Considering the opportunities of adding value to this waste together with the need for proper utilization, it appeared to be more valuable to extract the principal components separately. The recovery of these macromolecules can be addressed with sustainable methodologies, which are of special interest in the current global framework. Based on these premises, the first and second sections were devoted to the recovery of lipids and protein, respectively. To close the loop, the last section of this thesis was focused on demonstrating the potential of application of the protein fraction as a nitrogen source. An overview of the main results obtained in *fines* valorizations will be presented and discussed in this section.

Fat recovery

The starting point of this work consisted of the characterization of *fines* (Chapter 2). According to our results, the fat content of *fines* can suppose more than 37% of their dry weight. Given the interest in lipidic fraction together with the emphasis on a green extraction methodology, different methodologies were employed to isolate the lipids and then evaluated. The Eco-Scale approach was applied to select the most sustainable methodology, while also considering the extraction yields. As expected, the Soxhlet method, a reference method widely used, together with the Folch method, resulted in the highest yields (100%). In contrast, aqueous-based methods had lower recovery (42 – 60%) compared to the reference method. Due to growing environmental concerns over the use of hazardous organic solvents, the Eco-Scale approach was used to allocate penalty points based on well-defined criteria, such as reagents hazard, energy, waste generated, etc. This revealed that aqueous extraction at high temperature ranked as an excellent green process (Eco-Scale score = 91), resulting in simultaneous production of crude fat and a

protein concentrate (PDF). Furthermore, the extraction with water makes both the lipid and protein fractions suitable for other processes. In line with these observations, water, and aqueous-based solvent systems, have represented an increased choice to replace conventional solvents (Gai et al., 2013; Jiao et al., 2014; Rosenthal et al., 1996; Zhang & Wang, 2016). Besides this, utilization of water for industrial applications will be more cost-effective since the investments relating to solvent recovery, process safety, and solvent loss control systems will be lower.

Finally, the consistency of the extracted animal fat has been improved in order to meet the renderer requirements. A semi-solid fat was prepared by adding CaO and water following molar ratio 5/1/1, respectively. The change introduced will expand the applicability options.

Protein recovery

Along with Chapter 3, the focus was on the recovery of the protein. The high protein content (57%) of PDF together with the reduction in fat content supposed an advantage for the enzymatic hydrolysis, the technique selected for recovery of protein fraction. Enzymatic hydrolysis has been widely applied in animal co-products and by-products valorization (Fu et al., 2019; Lapeña et al., 2018; Pagán et al., 2013; Zhang et al., 2013). However, the solubilization and hydrolysis of the thermal-treated materials can be challenging.

Two commercially available enzymes were selected based on the literature review, Alcalase 2.4L and Neutrase 0.8L (Dey & Dora, 2014; Kurozawa et al., 2008; Solaiman et al., 2011). Several parameters were considered to find the optimal conditions to maximize the DH. The effect of pH, E/S ratio, and time were optimized to achieve the higher DH with Alcalase 2.4L. We observed that controlling the pH value at 8 was crucial to obtain a high DH. Regarding the E/S ratio, 5% provided the higher DH, observing that doubling enzyme concentration did not further improve DH. An incubation time of 24 h was enough to achieve a DH of 21.5%. Attempting to decrease the number of experiments required for optimization, in the case of Neutrase 0.8L the effect of selected variables was first assessed using one-factor-at-a-time (OFAT) followed by RSM. The relevance of E/S ratio, incubation time, initial pH value, and temperature, as well as their interactions on PDF hydrolysis, were determined using CCD. The model showed a good fit in experimental data, displaying that DH was highly influenced by the E/S ratio and incubation time. The maximum DH predicted with the generated model was 8.1%. Under optimal conditions, E/S ratio 15%, time 10.5 h, initial pH 8, and temperature 40 °C, the maximum DH achieved was 7.2%, demonstrating the potential of this approach. The usefulness of this statistical technique has been previously highlighted (Singh et al., 2017; Yolmeh & Jafari, 2017; Zhang et al., 2016). Regardless of the strategy used to maximize DH, Alcalase 2.4L proved to be more efficient in degrading the protein fraction. Indeed, some authors

such as Dey & Dora (2014); Fu et al. (2018); and Shahidi et al. (1995) also described the higher efficiency of alcalase over neutrase in hydrolyzing proteins. The broad catalytic specifications of alcalase together with the lower cost compared to neutrase provide an incentive for its use in industrial applications.

The produced protein hydrolysates employing optimal conditions were characterized. The main features to be highlighted as a consequence of the enzymatic hydrolysis are:

- Increase in protein content (from 57.7 to 70%).
- Decrease in fat (from 18.9 to 2.6 – 12.9%) and ash (from 16.9 to 6.5 – 9.8%) contents.
- Large proportion peptides smaller than 5 kDa (90 – 97%).
- A similar amino acid profile, predominated by Glx, Leu, Gly, and Asx.
- Significant changes in functional groups and volatiles profile, due to the formation of free amino acids and other low molecular weight compounds.

Furthermore, the process was successfully scaled-up in a bench-scale bioreactor, showing encouraging results for further optimization.

In view of the characteristics above mentioned, the produced protein hydrolysates from *fines* were considered suitable candidates to be used as nitrogen source in microbiological growth media and *in vitro* plant culture. Besides this, these hydrolysates can be regarded as cheap renewable nitrogen source, which is of interest for industrial scale production of microorganisms.

Protein hydrolysates applicability potential

In the last part of this Doctoral Thesis (Chapter 4, Chapter 5, Chapter 6, and Chapter 7), we focused on harnessing the potential of the produced protein hydrolysates from *fines*. Considering that the growth and productivity of microorganisms are strongly influenced by medium components, and usually the most expensive component is the nitrogen source, approaching the utilization of low-cost protein hydrolysates in growth media was of great interest (Pasupuleti & Demain, 2010). Besides this, protein hydrolysates have demonstrated several positive effects on plant growth performance (Colla et al., 2017).

Chapter 4 involved the study of the ability of the produced protein hydrolysates in supporting the growth of four bacterial species and their comparison with commercial ones. *E. coli*, *P. putida*, *B. cereus*, and *P. aeruginosa* were selected for this purpose. The protein hydrolysates from *fines* displayed good performance, resulting in similar OD_{600}, even outperformed commercial ones in some cases, representing

a low-cost encouraging alternative. Meanwhile, peptone removal from the growth media showed poorer performance, with the exception of *B. cereus.*

Chapter 5 involved the study of the effect of the protein hydrolysates on *S. cerevisiae* Ethanol Red® and *S. cerevisiae* CEN.PK XXX growth and fermenting capability. Besides this, the integration of two different treated agro-industrial wastes, wheat straw hydrolysates and the above-mentioned protein hydrolysates, for bio-based ethanol production was approached. First, the protein hydrolysates from *fines* supported well growth and fermentation using *S. cerevisiae* Ethanol Red®. However, *S. cerevisiae* CEN.PK XXX showed more difficulties of adaptation to these nitrogen sources compared to the commercial ones. Given that hydrolysates from lignocellulosic material are generally low in nutrients and nitrogen, the integration of wheat straw hydrolysate and protein hydrolysates for ethanol production was assessed. Although this strategy allowed the production of ethanol and not significant differences were observed for ethanol yield (0.395 g ethanol/g consumed sugar), faster sugar consumption and higher productivities were showed when supplementing with expensive commercial nitrogen sources, such as casein. However, it is worth mentioning that these preliminary results, together with other studies (Barahona et al., 2019; Li et al., 2017), suggest that there is room for improvement.

Chapter 6 involved the study of the effect of the protein hydrolysates in the biocatalytic conversion of HMF using different *Fusarium* strains. All the *Fusarium* strains tested biotransformed HMF to some extent, regardless of the nitrogen source added in the culture medium. Nevertheless, the nitrogen sources, as well as the amount, significantly influenced strains behavior, observing different patterns in HMF biotransformation and derivatives formation, suggesting that the addition of the protein hydrolysates influenced the metabolic pathways of the fungi. *F. sambucinum* displayed a higher HMF metabolization rate when HA was added to the growth media replacing 50% el malt extract compared to conventional media (1.17 g/(L h) *vs.* 0.48 g/(L h)). In addition, HA enhanced HMF-oxidative activity, achieving a DFF yield of 80%, whereas DHMF was the main product in the presence of other N sources. The cultivation of *F. striatum* under diverse nutrients and light regimes showed significant differences in sporulation induction, being HA (replacing 50% of malt extract) the most effective. Moreover, *F. striatum* cells grown in ME-50HA and ME-50HN performed significantly better than in ME, yielding more DHMF.

Chapter 7 involved the study of the biostimulant effect of the protein hydrolysates on tomato and radish plants development. Although there exists a wide variety of studies demonstrating specific positive effects of protein hydrolysate on plant performance (Colla et al., 2017, 2014; du Jardin, 2015; Sestili et al., 2018), in the present study, the application of the animal-derived protein hydrolysates was not effective in stimulating plant growth.

As there is a growing interest in the use of microbial biomass for biotechnological and industrial purposes and one of the major bottlenecks is the growth medium costs, these observations were considered of interest and promising. Hence, enzymatic hydrolysis has been demonstrated to be an adequate method for the valorization of *fines* protein fraction.

Further research is necessary to understand the action mechanism of these protein hydrolysates.

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General conclusions

From the results obtained in this Doctoral Thesis, the following conclusions have been established in relation to the objectives initially set.

Objective I. To determine the amount of valuable compounds to be extracted from the *fines*.

1. The characterization of *fines*, an unexploited waste from the rendering industry, showed that the main components were protein (45.8%) and fat (37.2%), followed by ash (12.2%). Protein and fats can be transformed into high-value products if optimally exploited.

Objective II. To ascertain the optimal fat extraction methodology to promote a cleaner alternative based on the Eco-Scale approach and improve fat consistency.

2. The evaluation of five extraction methods for lipids recovery from *fines* revealed significant differences in methods performance. Whereas Soxhlet (reference method) and Folch methods revealed the highest extraction yields, aqueous extraction at high temperature (80 °C) was proposed as an excellent green process based on the Eco-Scale approach achieving the highest score (Eco-Scale score = 91).
3. The consistency of the extracted fat was modified using CaO and water at moderate temperature, obtaining a partially solidified product. This change provided wider utility for management, increasing product acceptability and simultaneously, an expected benefit for animals when integrated as an ingredient in their diet.

Objective III. To optimize a methodology for the enzymatic hydrolysis of the protein fraction of *fines* and perform the characterization of protein hydrolysates.

4. The partially defatted protein fraction was hydrolyzed using two commercial enzymes and recovered as freeze-dried protein hydrolysates. Although both enzymes Alcalase 2.4L and Neutrase 0.8L, showed good efficiency in hydrolyzing PDF, Alcalase 2.4L proved higher efficiency.
5. The higher DH with Alcalase 2.4L (21.5%) was achieved through optimization of E/S ratio, pH, and time.
6. Hydrolysis of Neutrase 0.8L was optimized through RSM using a CCD integrating E/S ratio, time, initial pH, and temperature as input variables, resulting in a maximum DH of 7.2%.
7. The protein hydrolysates prepared with both enzymes in the optimized conditions showed significantly higher protein content, and lower ash and fat content as compared to *fines*.

8. The enzymatic hydrolysis did not modify the amino acid profile significantly, observing a similarity among the substrate and the hydrolysates for most of the amino acids.
9. SDS-PAGE and SEC profiles revealed that both hydrolysates contain a large proportion of low and medium molecular weight peptides (< 5 kDa).
10. FTIR spectrum analysis has shown that the functional groups changed as a consequence of the enzymatic processing.
11. The volatiles profiles were strongly influenced by the enzymatic process as well as the enzyme used.
12. The feasibility of scaling-up the process was demonstrated, observing higher efficiencies attributable to pH control and better agitation.

Objective IV. To prove the feasibility of using the produced protein hydrolysates as substitutes of commercial protein hydrolysates in microbiological growth media or other nitrogen sources.

13. The protein hydrolysates obtained from *fines* showed excellent capacity in supporting bacterial growth.
14. The performance of the protein hydrolysates when supplemented in fermentation medium showed variability depending on the yeast strain. In addition, integration of protein hydrolysates together with wheat straw hydrolysates was achieved for bio-based ethanol production process.
15. The protein hydrolysates showed to be suitable nitrogen sources for most of the *Fusarium* strains tested, observing strain-dependent effects in HMF metabolization.
16. The application of the protein hydrolysates did not stimulate tomato and radish plants growth.
17. Given that peptone is usually the most expensive ingredient in the growth media, formulating culture and fermentation media with low-cost protein hydrolysates could be a boost in industrial processes involving microorganisms.

Future perspectives

The results obtained in this Doctoral Thesis have shown that by-products and wastes from animal processing plants and rendering industry can be turned into soluble protein hydrolysates by proper enzymatic treatments for non-feed applications. Nevertheless, there is a lack of information about the applicability of this specific process at the industrial level. Therefore, further investigation is required when considering innovative value addition for *fines*, including:

- To evaluate the scalability of the proposed process and the feasibility of successful direct scale-up to production.
- To perform a techno-economic analysis (TEA) of animal-derived protein hydrolysates production through enzymatic hydrolysis of *fines* for biotechnological applications of microorganisms. The commercial viability could be assessed while identifying the economic "hotspots" to guide future development.
- To evaluate the potential of the protein hydrolysates in the production of microbial enzymes, since cost reduction is a major drive in enzyme manufacturing and it is estimated that approximately 30 - 40% of the production cost of many industrial enzymes comes from the cost of the fermentation broth.
- To evaluate the performance of the protein hydrolysates in fermentation processes with lactic acid bacteria (LAB) and culture of other fastidious strains.
- To establish and optimize a strategy for protein recovery from *fines* using this as carbon and nitrogen sources by a microorganism-mediated biological process while producing enzymes of commercial interest and protein hydrolysate (Figure 1).

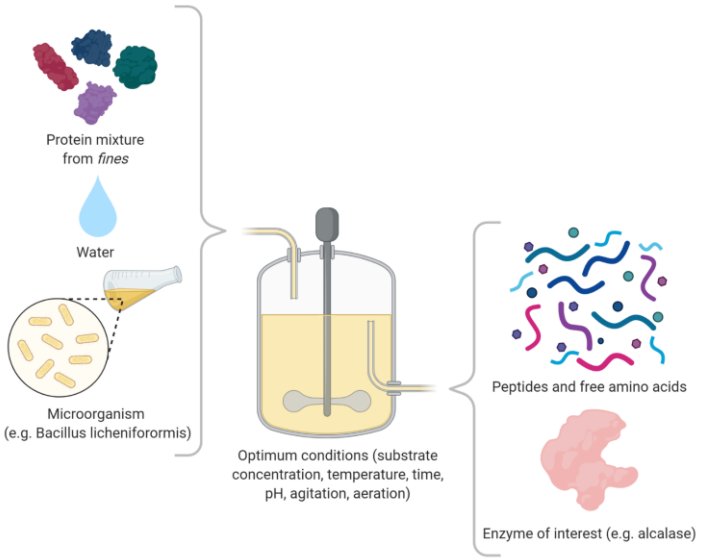

Figure 1. An approach to generate value-added products from waste: Production of protein hydrolysates and protease enzyme through an integrated strategy. Created with BioRender.com.