Escola de Enginyeria
Departament d'Enginyeria Química

Environmental Science and Technology studies

"Production of proteases from industrial wastes through solid-state fermentation at different scales. Potential applications."

PhD Thesis

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Antoni Sánchez Ferrer y **Teresa Gea Leiva** profesores titulares del Departamento de Ingeniería Química de la Universitat Autònoma de Barcelona,

Certifican:

que, la bioquímica **Juliana Abraham** ha realizado bajo nuestra dirección el trabajo, con el título "Production of proteases from industrial wastes through solid-state fermentation at different scales. Potential applications", que se presenta en esta memoria, la cual constituye su Tesis para optar al Grado de Doctor en Filosofía en Ciencia y Tecnología Ambiental por la Universitat Autònoma de Barcelona.

Y para que se tenga conocimiento y conste a los efectos oportunos, presentamos en la Escola d'Enginyeria de la Universitat Autònoma de Barcelona la citada Tesis, firmando el presente certificado.

Bellaterra, Septiembre de 2014.

Dr. Antoni Sánchez Ferrer

Dra. Teresa Gea Leiva

A mis padres y a mi hermana,
por su presencia infinita incluso en la distancia

A Dani,
por acompañarne en este viaje

It is good to have an end to journey toward, but it is the journey that matters in the end.

U.K Le Guin

Everything must go somewhere. There is no "waste" in nature and there is no "away" to which things can be thrown. The Closing circle: Nature, Man and Technology (1971).

Barry Commoner


¿QUÉ TE PASÓ ENRIQUETA? ¿TE CAÍSTE?
NO... ESTOY VIENDO TODO LO QUE TENGO.
¿CÓMO? ¿QUÉ?
TODO ESTO TENGO. EL CIELO, LA TIERRA, EL AIRE... TODO ESTO ES NUESTRO.
SOMOS RICOS.
SEP.

por Liniers

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Overview of the Thesis

An environmental-friendly biotechnological process is proposed in this work to reduce the negative impact of the increasing industrial residues due to the faster growth of the world population in the last decades. It consists on the valorization of nitrogen-rich local residues, such as soy fiber, hair waste and coffee husk, by solid-state fermentation (SSF) to obtain a biological catalyst such as proteases. SSF experiments were undertaken in 0.5, 4.5, 10 and 55 L near-to-adiabatic reactors along 1, 2 and 3 weeks. Continuous aeration was provided to ensure the prevalence of aerobic conditions and the biological activity was measured by monitoring the oxygen concentration during the assay. Alkaline proteases were produced as a consequence of the degradation of these materials, formerly considered residues, by the microorganisms developed. It was no necessary to sterilize the materials and no inoculation of pure microorganisms was needed for the development of the process. However, a specific inoculation with *Bacillus thuringiensis* was also evaluated to improve the protease production. Moreover, the final organic material obtained in the process presented a stability degree similar to that of compost and could be used as a soil amendment. Consequently, addressing the SSF under these conditions, the scale-up of the process is presented as an easy one with promising results.

The highest activity of the alkaline proteases in crude extracts was determined at 3, 7 or 14 days of the process according to the nature of the resources assayed. Partial biochemical characterization of the crude extracts was also carried out. Potential application of the extracted proteases has been successfully studied on dehairing cowhides, representing a significant advantage over the chemical process. Also, a preliminary study on kinetically controlled synthesis of oligopeptides has been performed in a research stay at an abroad laboratory in The United States.

Furthermore, the gaseous emissions as well as the energy consumed along the process were evaluated to estimate the environmental and economic sustainability of the process.

This thesis represents the beginning of a new research line in the Composting Research Group (GICOM) at the Deparment of Chemical Engineering of the Universitat Autònoma de Barcelona on the use of SSF with organic used materials as a suitable tool to valorize them while generating new products as enzymes of high value.

Resumen de la Tesis

En este Trabajo se propone un proceso biotecnológico respetuoso con el medio ambiente para reducir el impacto negativo del aumento de los residuos industriales, debido a la aceleración del crecimiento de la población mundial en las últimas décadas. Consiste en la valorización de residuos locales ricos en nitrógeno, como la fibra de soja, residuos de pelo y cáscara de café, por fermentación en estado sólido (SSF) para obtener catalizadores biológicos, tales como proteasas. Se llevaron a cabo experimentos de SSF en 0,5, 4,5, 10 y 55 L en reactores en condiciones adiabáticas a lo largo 1, 2 y 3 semanas. Se proporcionó aireación continua para asegurar la prevalencia de condiciones aeróbicas y la actividad biológica se midió mediante el control de la concentración de oxígeno durante el ensayo. Como consecuencia de la degradación de estos materiales, antes considerados residuos, por los microrganismos desarrollados se produjeron proteasas alcalinas. No fue necesario esterilizar los materiales como tampoco la inoculación de microorganismos puros para el desarrollo del proceso. Sin embargo, se evaluó también la inoculación con *Bacillus thuringiensis* para mejorar la producción de proteasas. Por otra parte, el material orgánico final obtenido en el proceso presentó un grado de estabilidad similar a la de compost pudiendo ser utilizado como una enmienda del suelo. En consecuencia, llevando a cabo la SSF en estas condiciones, el escalado del proceso se presenta como una tarea fácil con resultados prometedores.

La mayor actividad de las proteasas alcalinas en extractos crudos se determinó a 3, 7 o 14 días del proceso de acuerdo con la naturaleza de los residuos usados. También se llevó a cabo una caracterización bioquímica parcial de los extractos crudos. La aplicación potencial de las proteasas extraídas se ha estudiado con éxito en el depilado de pieles de vaca, lo que representa una ventaja significativa sobre el proceso químico. Además, un estudio preliminar sobre la síntesis controlada cinéticamente de oligopéptidos se ha realizado en una estancia de investigación en un laboratorio en los Estados Unidos (Rensselaer Polytechnic Institute).

Por otro lado, se evaluaron las emisiones gaseosas así como la energía consumida a lo largo del proceso para estimar la sostenibilidad económica y medioambiental del proceso.

Esta tesis representa el comienzo de una nueva línea de investigación en el Grupo de Investigación de Compostaje en el Deparment de Ingeniería Química de la Universitat Autònoma de Barcelona sobre el uso de SSF con materiales orgánicos como una herramienta adecuada para valorizarlos asi como también se generan nuevos productos como enzimas de alto valor.

Resum de la Tesi

En aquest treball es proposa un procés biotecnològic respectuós del medi ambient per reduir l'impacte negatiu de l'augment dels residus industrials, a causa de l'acceleració del creixement de la població mundial en les últimes dècades. Consisteix en la valorització de residus locals riques en nitrogen, com la fibra de soja, els residus de pel i closca de cafè, per fermentació en estat sòlid (SSF) per obtenir un catalitzador biològic, com ara proteases. Es van dur a terme experiments de SSF en 0,5, 4,5, 10 i 55 L reactors en condicions adiabàtiques al llarg dels 1, 2 i 3 setmanes. Es va proporcionar ventilació contínua per assegurar la prevalença de condicions aeròbiques i l'activitat biològica es va mesurar mitjançant el control de la concentració d'oxigen durant l'assaig. Es van produir proteases alcalines com a conseqüència de la degradació d'aquests materials, abans considerats residus, pels microorganismes desenvolupats. No va ser necessari esterilitzar els materials ni la inoculació de microorganismes purs per al desenvolupament del procés. No obstant això, una inoculació específic amb *Bacillus thuringiensis* es va avaluar també per millorar la producció de proteasa. D'altra banda, el material orgànic final obtingut en el procés presentar un grau d'estabilitat similar a la de compost i podria ser utilitzat com una esmena del sòl. En conseqüència, dirigint-se a la SSF en aquestes condicions, l'escalat del procés es presenta com una tasca fàcil amb resultats prometedors.

La major activitat de les proteases alcalines en extractes crus es va determinar a 3, 7 o 14 dies del procés d'acord amb la naturalesa dels recursos assajats. També es va dur a terme una caracterització bioquímica parcial dels extractes crus. L'aplicació potencial de les proteases extretes s'ha estudiat amb èxit en depilat de pells de vaca, el que representa un avantatge significatiu sobre el procés químic. A més, un estudi preliminar sobre la síntesi cinèticament controlat de oligopèptids s'ha realitzat en una estada de recerca en un laboratori a l'estranger als Estats Units.

A més, les emissions gasoses, així com l'energia consumida al llarg del procés es van avaluar per estimar la sostenibilitat econòmica i mediambiental del procés.

Aquesta tesi representa el començament d'una nova línia de recerca en el Grup de Recerca de Compostatge al Deparment d'Enginyeria Química de la Universitat Autònoma de Barcelona sobre l'ús de SSF amb materials orgànics utilitzats com una eina adequada per valoritzar-los i generar nous productes com enzims d'alt valor afegit.

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List of Abbreviations

Abbreviation	Definition	Units
AFP	Air filled porosity	%
BCA	Bicinchoninic acid assay	
BD	Bulk density	kg L^{-1}
BSA	Bovine serum albumin	
Bt	*Bacillus thuringiensis*	
C	Carbon	% w/w dry basis
CH_4	Methane	mg absolute value kg Mg^{-1} relative value
CRI/AT	Cumulative oxygen consumption	g O_2 kg^{-1} DM
DM	Dry matter	% w/w, wet basis
DRI	Dynamic respirometric index	g O_2 kg^{-1} DM h^{-1}
F_n	Fermentation	
FAS	Free air space	% v/v
KC	Kinetically controlled manner	
^{1}H-NMR	Proton Nuclear Magnetic Resonance	
MALDI-TOF	Matrix-Assisted Laser Desorption-Ionization/Time-Of-Flight	
MW	Molecular weight	kDa
nm	Measure of wavelength (nanometers)	nm
N	Nitrogen	
NH_3	Ammonia	mg absolute value kg Mg^{-1} relative value
NH_4^+	Ammonium	%, dry basis
N_2O	Nitrous oxide	mg absolute value kg Mg^{-1} relative value
O_2	Oxygen	% v/v
OM	Organic matter	% w/w, dry basis
PA	Protease activity	U g^{-1} DM, U mL^{-1}
PAGE	Polyacrylamide gel electrophoresis	

PBR	Packed bed reactor	
QRIE	Quality and respitation index efficiency	
RIE ec	Respiration index efficiency	kJ (g O_2 kg^{-1} OM h^{-1})$^{-1}$
SDS	Sodium dodecyl sulfate	
SEM	Scanning electron microscopy	
SmF	Submerged fermentation	
sOUR	Specific oxygen uptake rate	g O_2 kg^{-1} DM h^{-1}
SP	Soluble protein	mg mL^{-1}
sPA	Specific protease activity	U mg^{-1} SP
SSF	Solid-state fermentation	
T	Temperature	ºC
TC	Thermodynamically controlled manner	
TKN	Total Kjeldahl nitrogen	% w/w, dry basis
TOC	Total organic carbon	% w/w, dry basis
TS	Total solids	% w/w, wet basis
VOCs	Volatile organic compounds	mg absolute value
		kg Mg^{-1} relative value
VS	Volatile solids	% w/w, dry basis
WC	Water content	% w/w, wet basis

Chapter1

Introduction

Part of this chapter has been submitted for publication to Critical Reviews in Environmental Science and Technology. From wastes to high value added products: Novel aspects of SSF in the production of enzymes.

El-Bakry, M., Abraham, J., Cerda, A., Barrena, R., Ponsá, S., Gea, T., Sanchez, A.

1.1. Environmental awareness of worldwide increasing wastes

The faster development of the world population in modern societies brought together an excessive increment in the production and consumption of a wide variety of materials. In consequence an increase in uses of natural resources and generation of wastes from different natures was produced and still continues rising.

Nowadays, an increasing environmental awareness is growing-up all over the world and also strong policies have been developed in many countries for the management and treatment of wastes, aiming to reduce environmental damage and negatives effects on health and life quality in ecosystems. In this regard, the strategies promote the use of organic wastes, avoiding losses of materials (less consumption of new resources) and leading to reduction in energy demands. Over and over, the concepts of reduce, re-use and recycle are being taken into account for the reduction of wastes generation and also the energy-saving concept is being remarkably considered. Authorities form the European Commission, The United States of America, Japan and China among others are including laws for general waste prevention, waste recycling and biological treatments for the reduction of final disposal in landfills. Concretely, the European Commission and its Waste Framework Directive (2008/98/CE) introduces and defines basic concepts such as waste: any substance or object, which the holder discards or intends or is, required to discard**.** It distinguishes between urban and industrial wastes depending on the origin where they are generated, among other classification of wastes and also demands that all the waste generated must be treated in a way that protect the environment and human health by preventing or reducing its adverse impact. Hence, the waste prevention is the preferred option in the waste hierarchy stablished. Then, biological treatment plants are recommended for organic waste valorization, being the main destination.

Biological treatment processes to treat organic wastes such as composting and anaerobic digestion have been widely performed all over the world (Haug, 1993). These treatments are useful to stabilize the biodegradable organic matter, to reduce the volume of the waste and destroy their pathogens. Besides, the production of biogas for energy is possible in the case of anaerobic digestion. The final product obtained in these processes can be used as a fertilizer or soil amendment regarding its quality (Haug, 1993).

Otherwise, the disposal of these organic materials in landfills without the appropriate management produces different gaseous emissions, some of them known as greenhouse gases

like methane (CH_4), carbon dioxide (CO_2) and nitrous oxide (N_2O). These gases emissions build-up in the atmosphere can remain for several years and warm the climate, leading to many other changes around the world. Consequently, the suitable reduction and stabilization of the organic wastes also contributes to decrease these hazardous emissions in the environment.

In Spain, accordingly with Waste Directive (2008/98/CE), the competent authorities settle the law of residues (10/1998) and also established a "Integrated National Plan of residues 2008-2015" which goals are the suitable management and the biological treatment of municipal solid waste, wastes coming from agriculture and industry classified as non-hazardous, among others. In this matter, biological treatment plants based on composting and/or anaerobic digestion processes are being widely constructed.

1.1.1 Actual situation on local industrial residues

In Catalonia, the annual production of urban and industrial residues overcomes the 4 and 5 millions of tons, respectively while the generation residues from agricultural, fishing and livestock activities rises 15 millions of tons in Spain (INE 2010, ARC 2011).

The leather industry is a crucial economical sector in Catalonia (Spain), with a turnover of 170 million euros per year and more than 800 direct jobs. The leather tanner's union in Igualada (Barcelona, Spain) comprises 40 companies and treats 100.000 kg of leather daily (14.000 m^2/d). Around 5.000 ton per year of hair wastes are produced. The union built a plant to treat the wastewater where the production of sludge rises over 13.000 ton per year. The sludge is currently biodried and landfilled.

The leather manufacturing industry has been facing high demands for process optimization and for reducing environmental impacts in the past decades (Kanth et al., 2009). Due to recent regulations, the adoption of hair-save unhairing techniques has initially solved the problem of the production of high amounts of organic pollutants that typically ended-up in wastewater (Valeika et al., 2009). However, this fact implies the production of another solid by-product to be managed and treated. Additionally, conventional dehairing processes use a large amount of fresh water, alkaline substances and sodium hydrosulfide that increases the chemical and biochemical oxygen demand and the dissolved solids in effluents during the process (Thanikaivelan et al., 2004).

Regarding the management of hair wastes, previous research has effectively tested co-composting techniques to biodegrade hair waste and sludge (Pagans et al., 2006, Barrena et al., 2007 a,b).

On the other hand, regarding food processing industries, at least 30% of the incoming raw materials become wastes rather than a valued product. Hence, the food and agricultural industries also produces large amounts of wastes and include peels of fruit and vegetables, husk and bran from grains, brewery grains and sludge from all the process. Since all of the food wastes are almost 100% organic every ton of food waste means 4.5 tons of CO_2 emissions (Kosseva and Webb, 2013), impacting negatively the environment if their final disposal are landfills.

Although soy products originated in the Orient, nowadays they are commonly found on dining tables and in kitchens all over the world. In Catalonia, although is not a region where soybean is particularly cultivated, it is exported from other countries for the production of soy products such as soymilk and derivatives thereof. Concretely, a local factory produces soymilk and tofu and generates 20 tons a week of soy residues that remains after the grain processing. These soy residues are currently treated by composting and/or used for livestock feed. Furthermore, these soy residues are rich in water-insoluble ingredients including, in the majority, fiber but also protein, fat, starch and sugar, which could allow them to be potentially used as high quality media for microorganism's fermentation (Hiesh and Yang, 2004).

Likewise, coffee is one of the most favourite drink in the world and so an important agricultural merchandise. In this industry, almost 90.5% of all raw material used for coffee preparation corresponds to leftovers (Murthy and Madhava Naidu, 2012). Coffee husk is the main by-product generated in the dry coffee grain processing. In a local factory these residues produced are moslty used for compost production.

Coffee husk is mainly constituted by the coffee berry, pulp and parchment of the coffee grain. It is a product rich in carbohydrates (72.3%) including fiber and also contains proteins (7.0%), ash (5.4%) and moisture (15.0%) (Murthy and Madhava Naidu, 2012) that makes this waste a perfect substrate to generate value added products such as enzymes.

Therefore, the use of such wastes is an environment-friendly method for waste management that enhances the economic value of these materials and also avoids their disposal to soil or landfill that causes serious environmental problems. Further investigation and development of

potential value-added processes for reuse the organic wastes are to be investigated and developed.

1.2 Introduction to solid-state fermentation (SSF)

1.2.1 What is SSF? Comparison with other types of fermentations. Uses.

Solid-state fermentation (SSF) has been defined as the fermentation process that involves a solid matrix, namely the substrate, and is performed in the absence or near absence of free water (Pandey et al., 1999, Salihu et al., 2012); however, the substrate must possess enough moisture to support the growth and metabolism of the microorganisms. SSF is a three-phase heterogeneous process. Figure 1.1 shows the different phases involved in this process such as the moist solid particles, the gas phase (in between the particles) and a minimum of visible water.

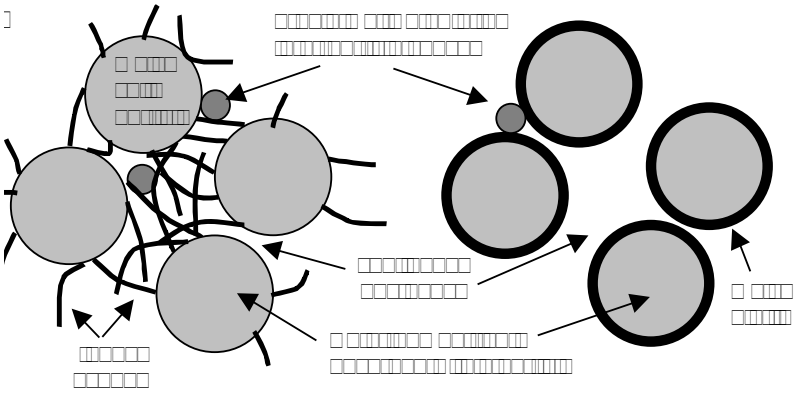

Figure 1.1. SSF system phases involved. A case of a filamentous fungus (left side) and a unicellular microorganism (right side) are shown. Source: Mitchell et al., 2006.

The substrate would act either as a source of nutrients or as an inert support, which has a nutrient solution absorbed within the matrix (Rodriguez-Couto and San Román, 2006). This technique reproduces the natural microbiological processes like composting and ensiling, which are fermentations in aerobic and anaerobic conditions, respectively.

A more general term, ‘solid-substrate fermentation’, was described before by Moo-Young et al. (1983), to indicate any type of fermentation process that implicates solids, suspensions of solid particles in a continuous liquid phase and even trickling-filter systems. Hence, solid-state

fermentation is one type of solid-substrate fermentation. This research is focused on solid-state fermentations and particularly in aerobic conditions.

Traditionally, SSF was developed and extensively used by societies thousands of years ago, to transform and produce food such as cheese or bread, among others. In the majority of the cases, the fermentation was done in order to preserve the food, improve the nutritional value and even to confer health benefits. The fermentation knowledge has been passed on from generation to generation and it was not since the 20th century when fermentation acquired more scientific basis and it was mainly used with medical purposes.

Recent SSF studies report uses in bioprocess such as biopulping, bioremediation of soils and biodegradation of toxics compounds from the solid matrix (Sayara et al., 2010, Abraham et al., 2014a). Besides, other studies report it uses in the obtention of products like enzymes, biosurfactant, biopesticides, biofuel, organic acids, etc (Pandey et al., 1999, Singhania et al., 2009).

The same products cited above can be also produced in a liquid medium containing the required dissolved nutrients (Colla et al., 2010, Belmessikh et al., 2013). This kind of fermentation is known as submerged fermentation (SmF) and the major benefits correspond to the homogeneity of the culture media and an easy control of parameters like temperature and pH during fermentation. However, there are several advantages of SSF over the use of SmF (Mitchell et al., 2006). It is a simple technology due to its mimicry and closeness with natural habitat of microorganisms, so higher production is often obtained. Moreover, from an environmental and economic point of view, SSF requires lower water and energy demands, and lower volume of effluents is produced compared to SmF (Subramaniyam and Vimala, 2012).

On the other hand, there are some disadvantages regarding SSF like difficulties to monitoring the process parameters, such as pH, heat, moisture and nutrients conditions. These difficulties are because of the low effectively in mixing the solid matrix. Besides, problems with the scale-up related to the heat mass transfer have been referred (Mitchel et al., 2006). Finally, the higher impurity of the product increases the cost of recovery and purification (Subramaniyam and Vimala, 2012). Moreover, another limitation is the possibility of carrying out the process without sterilization of the solid matrix. In consequence, the use of this type of fermentation in industrial processes is not widely applied due to challenges and limitations concerning monitorization, control and scaling-up of the process (Salihu et al., 2012, Sukumaran et al., 2010).

Nowadays, even though this is not a brand new technology and its use is widespread in many ambits, the paradigm is changing because of the substrates used. Recent studies have used residues such as peels of fruits and vegetables, animal waste and waste of plant origin and from food industry also called by-products. These wastes can be used as ideal substrates for the microbial fermentation due to their rich contents of organic components, which are considered as essential sources for carbon and nitrogen (Rodriguez-Couto, 2008). For example, Mahanta et al. (2008) utilized deoiled Jatropa seed cake, waste generated from the biodiesel production process, as a suitable substrate for the succesfull production by SSF of enzymes such as proteases and lipases. Mukherjee et al. (2008) also studied the enzyme production by SSF from different agro-industrial and kitchen residues inoxulated with *Bacillus subtilis*, being the potato peels the best ones to produce proteases and xilanases. Fish flour, a fish processing by-product, mixed with polyurethane foam has been tested by García-Gomez et al. (2009), for the production of a proteolytic extract with similar protein hydrolysis to a commertial enzyme. Belmessikh et al. (2013) has been achieved the production of proteases by *Aspergillus oryzae* from tomato pomace, an inexpensive substrate,

So, using low-cost wastes mainly from the industrial sector to generate other products have several advantages. For instance, the prices of production can be reduced, the energy efficiency of the process enhanced and the consumption of new resources avoided. Likewise, organic wastes can be reutilized to minimize the disposal to landfill (Waste Framework Directive, 2008/98/CE) and contribute to achieve the sustainable consumption and production (SCP) challenge (European Environment Agency).

Conversely, most of the SSF studies have performed the experiments by using few grams of substrates, sterilized conditions, constant temperature, and inoculation of pure microorganisms. Moreover, these solid substrates are grains (with C-N supplementation) and not often residues from the agro-industrial sector.

Addressing SSF process scale-up under these conditions is a challenge because of the inherent difficulties of handling large quantities of solid substrate while avoiding biological contamination and problems related to mass and heat transfer that are associated with the solid processes. An alternative approach to overcome these constraints involves working with a microbial consortium under a dynamic temperature profile including a thermophilic range similar to that of a

composting process. Additionally, when fermentation occurs at high temperatures, thermostable enzymes may be produced as a microorganism's adaptation strategy (Santis et al., 2011).

1.2.2 SSF: parameters and evolution of the process.

As stated before, an SSF process performed with organic solid wastes in aerobic conditions is similar to a composting process. The evolution of a aerobic process such as composting as well as the parameters involved along the process for a suitable degradation and stabilization of the solid matrix have been well studied previously by many authors as well as the Composting Research Group (GICOM) at UAB. Moreover, GICOM, the group in with this thesis was developed, has a long experience in composting of different organic wastes at different scales.

This aerobic process normally is developed in two stages: the decomposition and the mature stage. The decomposition stage occurs at the beginning and corresponds to the transformation of complex biodegradable organic matter into simpler organic and inorganic molecules by microorganisms' metabolic activity; therefore, this stage is characterized by the production of heat (Haug, 1993) and by high oxygen uptake rates. The microorganisms' metabolic activity includes the use of their battery of enzymes to degrade the organic matter and obtain the nutrients necessary to their suitable development.

At initial time, the microorganisms dominant are mesophilic (those that grow best at temperatures around 25 and 45ºC), they use available oxygen (O_2) to oxidize carbon from the solid matrix to obtain energy and materials to build new biomass and so they produce CO_2 and water. This growth developed produce heat and the matrix temperature rises over 45ºC. At these temperatures, the mesophilic microrganisms die or become dormant in case they sporulate, waiting for more suitable conditions. Accordingly, at this point thermophilic microorganisms become active, consuming the materials that are readily available and multiplying rapidly replacing mesophilics in most sections of the material. Besides, temperature can get values up to 70°C. This thermophilic phase is considered very important for the material pathogen inactivation and seed destruction (US EPA). Excessive matrix temperatures (over 70ºC) must be controlled because of its ignition risk, the limitation of microbiological activity and the NH_3 emissions that are enhanced by high temperature and turnings (Soliva, 2001, Pagans et al., 2006).

When sources of readily available carbon are depleted, thermophilic activity decreases and temperature drops. Mesophiles begin to dominate the process once again until all readily available energy sources have been consumed.

At the end of the decomposition stage, due to the slow microbial activity, temperatures start to decrease untill 40°C, the material becomes dry and powery; this corresponds to the maturing stage and the material is considered stabilized (Haug, 1993). Finally the material reaches room temperatures and macroorganisms may appear. This stage is important because is when nitrogen obtained from dead biomas is incorporated into high molecular weight compounds that are resistant to the microbial decomposition, forming the nitrogen reserve (Haug, 1993 Tchobanoglous et al., 1994). This stage is less demanding in terms of O_2 and humidity than the decomposition stage and results in a net loss of total organic matter and inorganic constituents. The main products of the composting process are fully mineralized such as CO_2, H_2O, mineral ions, ash and stabilized organic matter such as humic acids (Haug, 1993).

All the process evolution related to the stages and the microoganisms developed are depicted in Figure 1.2.

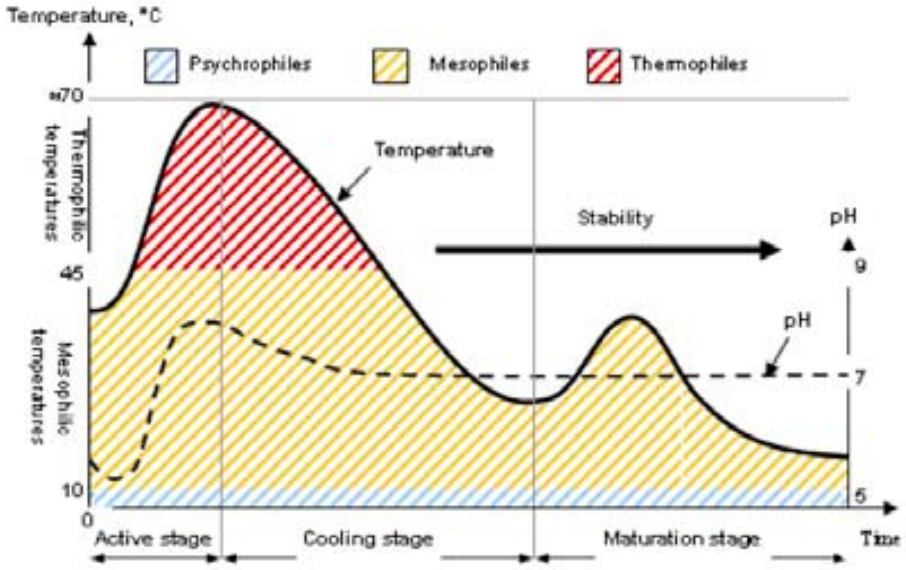

Figure 1.2. Typical temperature and pH evolution in a solid matrix degrading process.

In sum, in this process it is important to maintain the biological, chemical and physical requirements of microorganisms in order to reach the maximum degradation levels through the

stages of the process. For that reason, before the process takes place, sometimes the wastes need to be structured in order to reach the suitable water moisture, adequate porosity and also a proper C/N ratio because mostly of the time, the residues have very different nature, composition and origin.

Previous studies recommended an initial C/N ration of 15 to 30 as an optimum for suitable composting materials (Haug, 1993). Lower values will promote N losses in the form of NH_3 while higher values can slow down the composting process due to lack of nitrogen to support microbiological activity. Some substrates may require additional nutrients to sustain rapid microbial growth rates. Also, water moisture of 40-60 % is recommended for good practices. Even though, these percentages can vary depending on the nature of the material. For instance, high degradation rates have also been reported at low moisture content of 30% when working with fat residues (Gea et al., 2007, Santis et al., 2011).

Besides, the particle size and the porosity of the sample are of importante relevance for the suitable development of the process. For that reason, the free air space (FAS), which is defined as the ratio of air volume to total volume of the sample (Haug, 1993), is also a critical paramenter to measure in initial samples but also during the process to avoid compaction. There is a wide range of FAS recommended optimum values and all seem to be dependent on the type of material to be degraded (Ruggieri, 2008).

Consequently, after the initial characterization of the wastes, they are usually mixed with bulking agent as wood chips (Haug, 1993, Larsen and McCartney 2000, Ruggieri et al. 2008) in order to adjust the previous paramenters described.

Furthermore, regarding the evolution of the process, some other parameters are important to measure or monitoring. In this sense and as stated before, this biological process has an exothermic development due to the degradation of organic matter and so the temperature is one important parameter to measure along the process to evaluate the efficiency of the degradation.

The pH of the solid matrix also changes during the process due to the micrororganisms activity (Figure 1.2) and its measure its of special relevance in order to maintain the optimal values. pH optimum values for the microbial population present in the degrading matrix ranges between 6 and 8 (Haug, 1993).

Air is also required during the aerobic process for several purposes such as supply oxygen for biological decomposition, remove moisture from the solid matrix, remove heat to control process

temperature and remove CO_2 and NH_3 gases (Haug, 1993, Soliva, 2001). The minimum oxygen requirement ranged between 5 to 8%, depending mostly on the chemical composition of the solid matrix. Contrary, less O_2 percentages leads to anaerobic conditions and different evolution of the microrganisms and so different process. In this sense, monitoring the O_2 content within the solid matrix allows operators to control the process by aerations feedbacks.

Additionally, another parameter derived from the oxygen concentracion measured can be calculated. In this sense a dynamic respiration method can be used to estimate the oxygen requirements to achieve biodegradation (Barrena, 2006, Ponsá, 2010b, Pognani, 2011). This method evaluated by these authors gives an on-line process value of the biological activity that is happening during the degradation and is considered one of the most adequate tools for monitoring microorganism's activity. From this method, different indexes can be calculated at different time for a determined sample and a cumulative O_2 consumption. The dynamic respiration index (DRI) and the cumulative oxygen consumption or the total O_2 consumed (CRI/AT_4) are different indexes utilized in the composting field to evaluate the stability of a solid organic material. Both are important and give information about the potential of biodegradavility of an organic material.

In this work, all these paramenters described were used to determine the appropriate initial mixtures and the evolution of the process as well.

1.2.3 SSF Bioreactors. Process Scale-up

There are different types of bioreactors used in SSF processes. They are classified in two categories regarding the quantity of substrate utilized (Durand, 2002):

- At laboratory (lab) and bench-scale: when using quantities of dry solid medium from a few grams up to few kilograms.
- At pilot and industrial-scale: where several kilograms up to several tons are used.

They are also classified into four groups based on the pattern of aeration and/or agitation system used (Mitchell et al., 2006):

- The first group corresponds to the called "tray bioreactors" in which the bed is static or mixed very infrequently; and the air circulates around the bed. The substrate forms a thin layer of a few centimeters deep.

- The second group refers to the "packed-bed bioreactors" in which the bed is static or mixed very infrequently; and the aeration is forced throughout the bed.
- The third group is known as "stirred or rotated drums bioreactors". In this case, the bed is continuously or very frequently mixed while the air is not forced through the bed, but circulates around the bed.
- The last or four group corresponds to the bioreactors in which the bed is agitated and the air passes forcefully through the bed. They are called "gas-solid fluidized beds" or stirred-aerated bioreactors".

All these designs of reactors have their own advantages and disadvantages and the proper selection for the SSF process scale-up should be done according to the quantity and quality of the substrate, the microorganisms used and the product expected.

Others important aspects can influence the process and thus should be taken into account when selecting the reactors and for scaling-up. These aspects correspond to the temperature, the moisture, the pH and the size of the particle. SSF is difficult to scale-up due to the three-phase heterogeneous nature of the substrate and so the existing gradients inside the reactor (Salihu et al., 2012). Likewise, the absence of free water during the SSF process leads to poor heat removal and accessibility of nutrients resulting in slow microbial growth and in consequence, low or no production of the desired product. As discussed above, it is not so easy to monitor the paramenters along the process but in most of the cases are of particular interest to adequately mix the solid matrix and perform the process satisfactorily. For instance, the O_2 consumption and the CO_2 evolution are important measurements since they represent the best way of monitoring the growth of the microorganisms inside the reactor. Moreover, in aerobic systems, the temperature is also important to monitor because the heat generation rate is proportional to the O_2 consumption rate.

In sum, the selection of a proper reactor for scaling-up the process is not so easy to determine from the lab scale studies and consequently a combined experimental and modelling studies should be involved (Mitchell et al., 2006). In this sense, a mathematical modelling of the process has highlighted the effect of denaturalization of the produced enzyme at pilot scale caused by an increase in temperature (Muller dos Santos et al., 2004). These combined studies would solve the problems related to the scale-up of the process for the production of any particular products.

Besides, other authors have posed that modelling studies are difficult to obtain due to the heterogeneous nature of the substrate (Singhania et al., 2009, Ali et al., 2011).

Currently, limited studies have been studied an SSF process at pilot scale. Edwinoliver et al. (2010) scaled-up the lipase production from 10 g to 100 g and 1 kg in tray bioreactors monitoring the temperature and the moisture during the process. The yield obtained was 96 and 83% for 100 g and 1 kg respectively, from the optimum activity obtained with 10 g of substrate. Rodriguez-Fernandez et al. (2012) performed the production of phytases in a stirred drum bioreactor with 2 and 20 kg monitoring the airflow intensity and the porosity of the sample along the process. The similar results in production indicated that both variables should be considered in the scale-up of SSF.

Finally, scaling-up a particular compound produced by SSF might require the study of the technical, environmental and economical feasibilities of the process developed.

1.2.4. Gaseous emissions and environmental impact of the SSF

Although the objective of the SSF process is the valorization and transformation of wastes into less polluting compounds as well as to obtain useful products, the organic wastes treatment inherently comprises some possibly negative environmental impacts, which have been widely studied at the composting field. The release of atmospheric pollutant such as volatile organic compounds (VOCs), CH_4, NH_3, N_2O and odor emissions are the most common emissions produced (Komilis et al. 2004, Cadena et al. 2009a, Colón et al. 2012, Maulini et al. 2013).

Many studies focused on the atmospheric emissions itself while many others studied the life cycle assessment (LCA) which is a methodological tool to determine the environmental impact and energy or resource consumption of products and services over their whole life cycles, which includes the extraction of the raw materials, the production of the product itself, it use use and treatment after disposal as waste (Cadena et al., 2009b)

Regarding the SSF process, a recently study by Gassara et al. (2011) demostrated that for all the possible treatments performed for apple pomace by-products from the fruit industry in Quebec, the production of a enzyme by fermentation is the least polluting option in terms of gaseous emissions, being 906.81 tons CO_2 equivalent per year, followed by animal feed (963.38 tons of CO_2 equivalent per year), incineration (1122.10 tons of CO_2 equivalent per year), composting 1273.00 tons of CO_2 equivalent per year) and landfilling (1841.00 tons of

CO_2 equivalent per year). Ebner et al. (2014) verified that the life cycle greenhouse gases impact associated with the conversion of food scrap waste from a supermarket together with fruit syrup food processing waste into ethanol (EtOH) and the co-products of compost and animal feed, produce 295 L EtOH/dry ton feedstock and 1458 g CO2equivalent per L EtOH.

Althought there is still a lack of studies on gaseous emissions environmental impacts and LCA in SSF processes to this point, the production of enzymes or any other compound by SSF using wastes from another process supposes a sustainable option to be considered.

1.3 Proteases as valued products.

1.3.1 Introduction to proteases

Enzymes are proteins with abilities in catalysis, being responsible of thousands of metabolic processes. The proteins are formed by a number of amino acids that range from 100 to several hundreds of them and are covalently bounded through the peptide bond. The peptide bond is formed between the carbon atom of the carboxyl group of one amino acid and the nitrogen atom of the α-amino group of the following amino acid. Besides, according to the nature of lateral group of the amino acids (R), they can be non-polar (hydrophobic) or polar (charged or uncharged) and their distribution along the proteins determines its behavior (Lehninger, 1993). Figure 1.3 shows the protein structures. The gene that codes for the protein genetically determines the primary structure, and then regarding the sequence of amino acids assembled and its interaction the second structure is formed. The three-dimensional structure of the proteins (tertiary and quaternary structure, native structure) is finally formed by the succesives interactions between the amino acids chain and the sorrounding amino acids and medium. In sum, the structure is genetically determined and also environmentally conditioned. All this structures are necessary to provide the protein its biological functionality.

In the case of the enzymes, they can join substrates and turn into products with high specificity. The enzymes allow a determined reaction by lowering its activation energy. There are several different enzymes according to the catalyzed reaction they are involved in. Among this classification, the proteases (EC 3.4.21-24) are hydrolases that catalyze the cleavage of peptides bonds in proteins (Figure 1.4). Three different synonyms are known to name the peptide bond hydrolases: proteases, peptidases and proteinases. In this particular study, the term protease was

chosen. This highly complex group of enzymes differs in their substrate specificity and catalytic mechanism being Serine, Aspartate, Cysteine, Threonine, Glutamic Acid and Metallo Proteases (Lehninger, 1993). Additionally, these enzymes are also classified into three main categories; alkaline, neutral and acid proteases on the basis of pH range in which their activities are optimal.

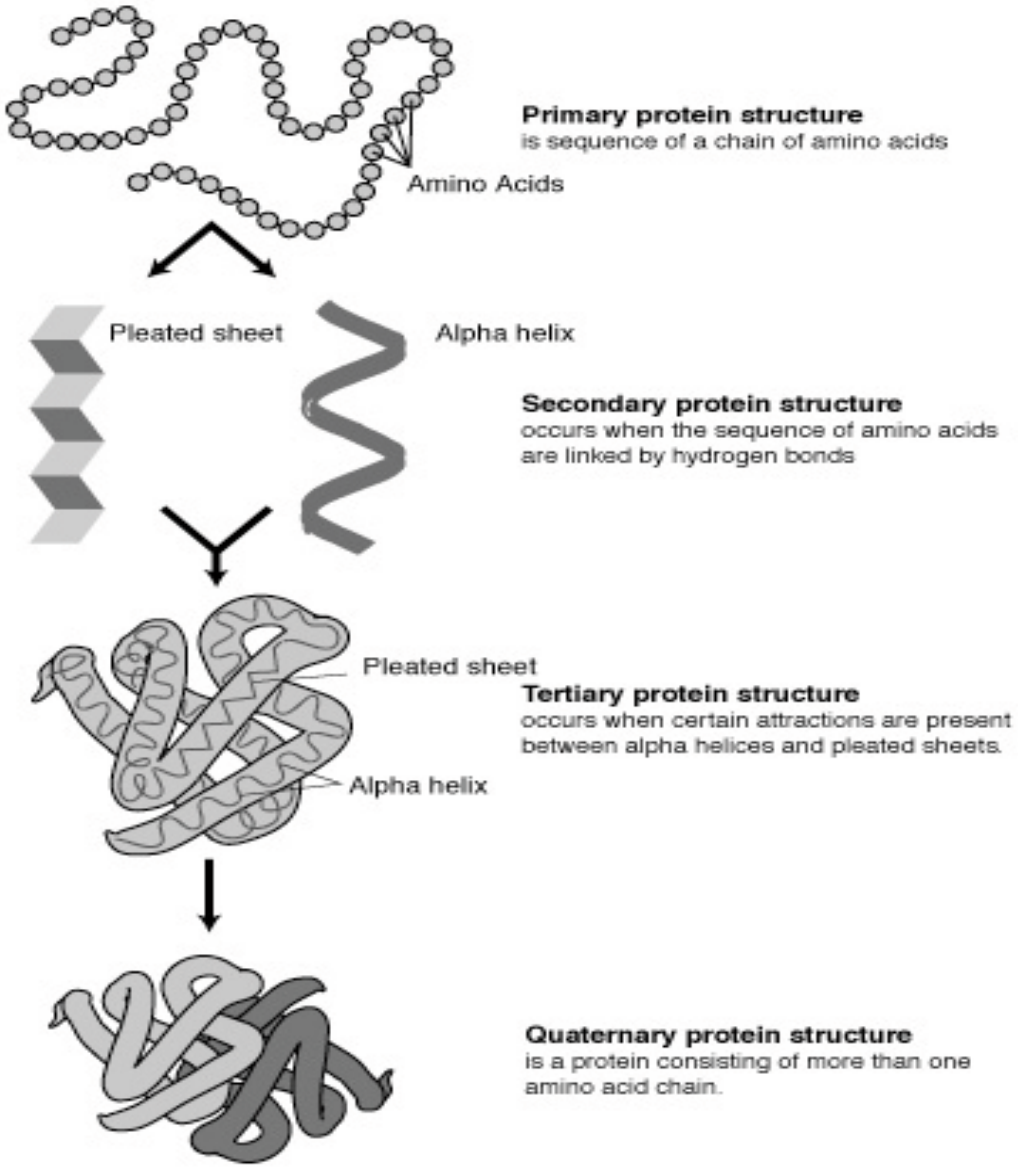

Figure 1.3. Protein structures. Source: National Center for Biotehcnology Information (NCBI).

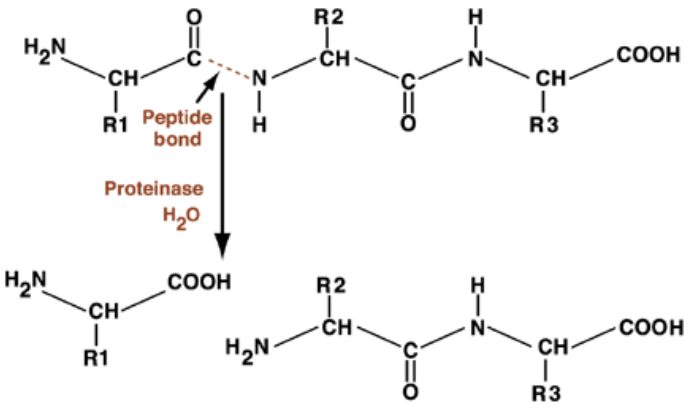

Figure 1.4. Proteases are able to produce the cleavage of peptide bonds by different catalytic mechanisms. Proteinase, peptidade and protease are all synonyms. Source: NCBI. In determined conditions the reaction could be reversible.

1.3.2 Uses of proteases

Enzymes have been used in a wide variety of applications from industrial sectors to household products. Among microbial proteases, alkaline proteases are used in an extensive range of applications such as uses in the detergent formulations due to its stability and compatibility with most of the tested commertial laundry detergents (Mukherjee et al., 2008, Paul et al., 2013) food deproteinization such as shrimp wastes processing from fish industry (Valdez-Peña et al., 2010, Jellouli et al., 2011) pharmaceutical and paper industries uses and goat and buffalo leather dehairing instead of using hazardous chemical (Dayanandan et al., 2003, Riffel et al., 2003, Kandansamy et al., 2012).

Moreover, another interestingly uses studied are their application into soil together with crops wastes to enhance the availability of nutrients and increase soil fertility (Han and He, 2010) and the use of the proteases in the synthesis of oligopeptides with specific functions developing the proteases' reversible reaction of hydrolysis (Qin et al., 2011, Viswanathan et al., 2012). About this particular topic more information will be explained in chapter 8 of this work.

Most of the applications have been proved with proteases obtained by SmF. However, some of them have been recently proved with proteases obtained by SSF. Rai et al. (2009) worked with an alkaline β-keratinase produced by *B. subtilis* that showed significant stability and compatibility with commercial laundry detergents. Boyce and Walsh (2012) used a protease produced by *S. commune* to remove an industrial-like milk fouling deposit from stainless steel. Using this

cleaning in place procedure, suitable cleaning was achieved at 40ºC without the use of environmentally harmful and corrosive chemicals. Further applications of proteases produced by SSF need to be explored.

1.3.3 Production of protease by SSF.

Enzyme production is an increasing field of Biotechnology (Thomas et al., 2013). Furthermore, proteases are the most important enzymes that account for about 60% of the world market of industrial enzymes. They have been produced extensively by SmF and have been commercially available since many years ago (Anwar and Saleemuddin., 1998, Queiroga et al., 2012).

On the other hand, production by SSF has been recently implemented in order to reduce cost and enhance field production (Sandhya et al., 2005, Kumar et al., 2009, Belmessikh et al., 2013). However, there are a few developed studies on solid-state fermentation and the majority was performed with only grams of substrate (lab-scale). Table 1.1 summarizes the state-of-the-art of protease' production by SSF. The substrate utilized (type and amount), the process conditions used (T, pH, water and microrganisms inoculated) and also the protease activity obtained are shown. Noteworthy, the protease activity assay recovered in bibliography is different between each study. The differences were the substrates used (casein, azocasein), the time (10, 15, 20, 60, min) and the temperature (30, 37, 45ºC) of incubation. Besides, the measurement of the absorbance varied regarding the products measured (280, 440, 650, 700 nm) and finally the expressions of the activity are also different between authors (units in μg, μmol, increase in absorbance per mL or g per minute or not). However, they have all different expressions, they are in the table in order to know the magnitude of enzyme activity obtained in each fermentation from different substrates and conditions.

Table 1.1 Summary of different substrates and process conditions to produce proteases by SSF.

Substrate		**Process conditions**					
Type	**Amount (g)**	**T (ºC)**	**pH**	**Water content (%)**	**Microorg.**	**Protease Activity (U g^{-1} DM)**	**References**
Defatted soy bean cake, supplemented	few grams	28	5	55	Penicillium sp.	43 U mL^{-1}	Germano et al. 2003
Whet bran Lentil husk	few grams	37	10	30 40	Bacillus sp.	429.041 168.640	Uyar et al. 2004
Green gram husk	10	33	9	50	Bacillus sp.	9550	Prakasham et al.2006
Jatropha seed cake	5	30	6	50	*Pseudomona aeruginosa*	1818	Mahanta et al. 2008
Potato peel, grass	100	50	8	50	*Bacillus subtilis*	2383	Mukherjee et al.2008
Fish flour, polyurethane foam	30	30	nc	50	*Aspergillus niger*	121 (μmol Tyr/L)	García-Gomez et al. 2009
Tannery solid waste	5	37	6	50	*Synergistes sp.*	755	Kumar et al. 2009
Chicken feather	5	50	8	50	*Bacillus sp.*	95.3	Rai et al. 2009
Wheat bran	few grams	27	acid	60	*Aspergillus oryzae*	$83x10^5$ (U= increase in 0.001 Abs)	Vishwanatha et al.2009
Wheat bran	5	45	nc	60	*Thermomucor indicaeseudaticae*	168 (U mL^{-1}) (U=increase in 0.1 Abs)	Merheb-Dini et al. 2010.
Wheat bran	5	nc	nc	nc	*Myceloiphthora sp.*	19.8 (total units)	Zanphorlin et al. 2011

Wheat bran	10	24-40	nc	50	14 fungal strains	5.05	Boyce and Walsh 2012
Cow dung	5	37	8	50	*Halomonas sp.*	1351	Vijayaraghavan et al. 2012
Tomato pomace	10	30	6.8	60	*Aspergillus oryzae*	21309	Belmessikh et al. 2013

Chapter 2

Research Objectives

Based on the consolidated background of GICOM on the composting of different organic wastes at several scales, this study corresponds to the beginning of a brand new line of research called “From waste to products”. For this reason this research should be understood as a first screening of the process, products and their potential applications.

The main objective of this research is to study the valorization of local solid wastes by using solid-state fermentation process as a tool to stabilize them while obtaining value-added products such as proteases.

In order to go deeper and reach this main objective, several specific ones were also set and are presented below:

- To search for nitrogen-rich local residues to be capable of producing proteases.

- To study and screen the SSF process of each residue and obtain the production profiles of the enzymes generated.

- To study the stability of the solid matrices (to be used as organic amendment for soils), after SSF by the utilization of the respirometric indices.

- To determine, by means of a bibliographic research, the best method to measure protease activity. In addition, to set up the methodology for measuring the proteases activity on solid samples and extracts of these fermented residues.

- To characterize the enzymes obtained in terms of pH and temperature stability, molecular weight, and also the conservation of them until usage.

- To assess how the heterogeneity of solid wastes influences the process at every batch and how consistent SSF is.

- To explore the potential of the proteases obtained by SSF in different applications. Among them, to probe the feasibility of the kinetically controlled synthesis of oligopeptides.

- To evaluate the effect of scale-up in the SSF from grams to kilograms.

- To analyze the environmental impact of the SSF process of the residues studied in terms of gaseous emissions and energy consumed.

- To evaluate, as a first approach, the effect of adding microorganisms for increasing the production of proteases and the quality of the stabilized final material.

Chapter 3

Materials and Methods

Part of this chapter has been described for standard operating procedures and different protocols at the laboratory.

Part of this chapter has been published at Biochemical Engineering Journal 74, 15–19. 2013. Potential of solid-state fermentation of soy fiber residues by native microbial populations for alkaline protease production at bench scale.
Abraham, J., Gea, T., Sanchez, A.

Summary

This chapter describes all the experimental set ups and the analytical methods used to study the solid-state fermentation process during this research. The **Plant I** was modified and enlarged several times along the research time in order to obtain more reproducible results. All these changes are also described in detail. However, **Plant II** was constructed and carried out before this research by previous PhD Thesis. Although all the general analytical methods are described in this chapter, the methodology regarding some specific experiments and materials are reported in detail at the corresponding chapter.

3.1 Materials: Residues, Reactors and pilot plants used for SSF research

3.1.1 Residues

The materials used were soy fiber and coffee husk, which are final residues from the food industry, and hair waste from the tanning industry. All of them were obtained from local industries in Barcelona, Spain. The soy fiber residue is the result of the pressing of soy grains when producing soymilk and derivatives (Natursoy®, Castellterçol, Barcelona, Spain). The coffee husk is the main residue caused by the drying of coffee grains when producing coffee (Marcilla®, Mollet del Vallés, Barcelona, Spain) and the hair waste is generated as a result of a chemical dehairing process in strong alkaline conditions, (Pere de Carme tannery, Igualada, Barcelona, Spain). They were chosen in order to screen for a suitable protein-rich waste for protease production.

Other materials such as compost and wood chips were obtained from a composting treatment plant (Manresa and Torrelles, Barcelona, Spain) and dehydrated fresh sludge from a wastewater treatment plant (Barcelona and Navàs, Barcelona, Spain). Compost and sludge were mixed to the targeted residues described to add microorganisms and water while wood chips were also used as bulking agent to add porosity to the mixtures. Specific details of characterization and quantities are described at each particular chapter.

3.1.2 Plant I

The Plant I was originally composed of 5 Dewar® near-to-adiabatic reactors with 4.5 L working volume. The majority of the experiments along this work were performed in this Plant. During the time of this research, this plant was extended and finally was composed of 12 Dewar® near-to-adiabatic reactors with 4.5 L and 3 reactors with 10 L working volume (Figure 3.1). These static packed-bed reactors (PBR) were modified and conditioned to operate in batch-mode form to carry out the solid-state fermentation process. The vessels were also used and validated in previous composting studies (Gea, 2001). They are thermally isolated, so the influence of ambient temperature can be minimized or even ignored, and equipped with on-line temperature monitoring by temperature sensors (Pt-100, Desin, Spain). The temperature probes were connected to a data acquisition system (MAC 3500, Desin, Spain) and registered by a personal computer. The software used (Proasis®Das-Win 2.1, Desin, Spain) allows monitoring both temperature and oxygen content into the reactors (Figure 3.1 and 3.2).

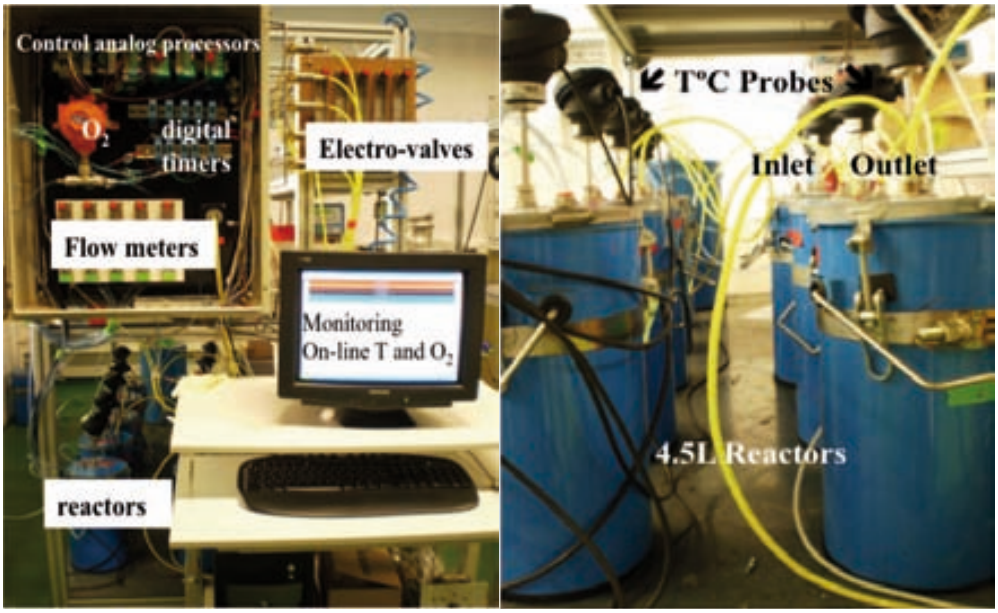

Figure 3.1. Images of Plant I.

Aeration was provided to the reactors according to the process performance to ensure a sufficient oxygen level (> 5 %) and avoid anaerobic conditions. In this context, air was provided to the mixture through a pipeline connected to the bottom of the reactor, where a plastic mesh is placed to ensure a correct distribution of the air through the sample. Exhausted air exited the

reactor through an outlet in the reactor cover (Figure 3.3) and was sent to the oxygen analyzer. Oxygen concentration was measured by an oxygen sensor (Sensortran, Spain), where the inlet of the sensor is connected to the reactor outlet and consequently the oxygen percentage was determined (0-20.9% v/v).

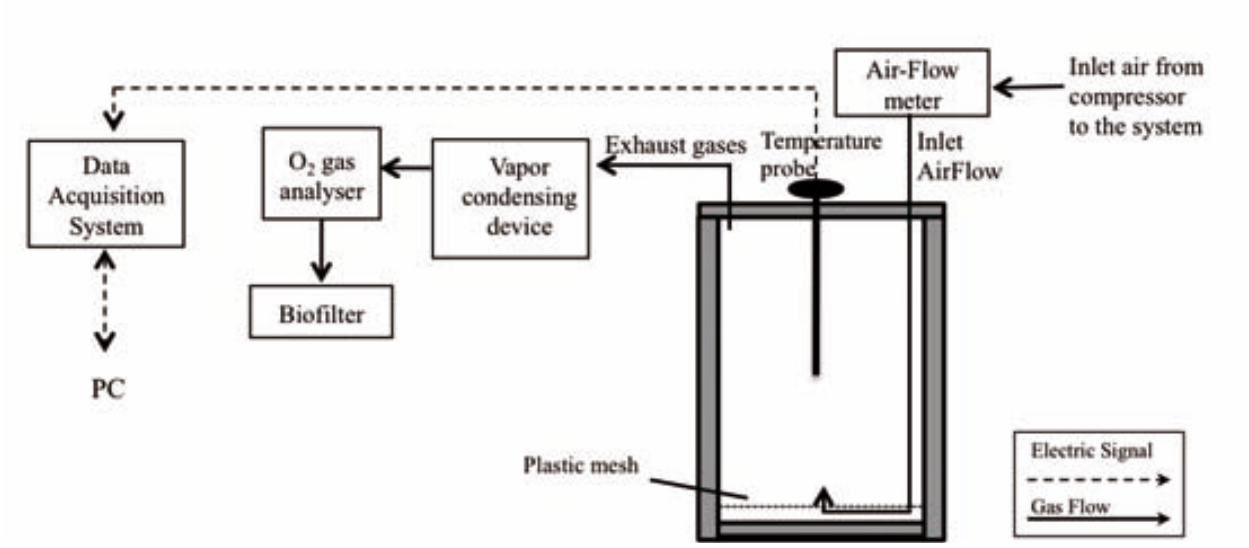

Figure 3.2. Schematic representation of the experimental set up of Plant I.

Between the reactor and the oxygen sensor there is a vapor-condensing device to avoid humidity to reach the sensor and consequently damage it. All the outlet pipelines were connected to a biofilter (10 L Dewar® with mature compost).

Figure 3.3. 4.5 L near-to-adiabatic reactor.

Aeration modes

Initially, intermittent aeration was provided so electrical valves (SMC, Spain) were connected to digital timers (MFZ12DDX-UC, Eltako) in order to adjust the aeration frequencies, and flow meters (0.5-5 NL min^{-1}) (Tecfluid, Spain) were used to measure the airflow rate into the reactor. Afterwards, air flow meters with lower flow range (0.1-0.5 NL min^{-1}) were installed and it was possible to supply a continuous aeration to the reactors. Henceforth, the electrical valves were connected to the digital timers in order to use one oxygen sensor and commutate between each three reactors, so that the oxygen percentage at the reactor's outlet could be determined automatically for each reactor.

Previous studies determined that the gas flow in these 4.5 L reactors behaves similarly to the plug flow with an axial dispersion model, according to the residence time distribution (RTD) method (Puyuelo, 2012). Consequently, an oxygen concentration can be estimated to determine whether the process is performing under aerobic conditions throughout the entire reactor.

Finally, flow meters were changed by mass flow meters to increase the precision of the flow. Also a controller of oxygen uptake rate described and validated by Puyuelo et al. (2010) was used in the last experiment performed. Briefly, this controller is an algorithm developed to achieve an automatic airflow regulation and consequently to optimize the biological activity by means of providing the maximum oxygen uptake rate along the process. This objective was achieved through a control system working in cycles of one hour each, according to the residence time distribution study. After completing a cycle, the oxygen level is revised to avoid percentages below 5% (v/v). If the level of oxygen is less than the limit, the airflow will be increased 50%. If an adequate oxygen level is detected, the system will start to control the oxygen uptake rate (OUR) measurements. Hence, values of flow and OUR, which are related, are re-evaluated by the controller between two consecutive cycles. For both parameters, three situations are possible, for instance, the system determines if the current value is lower than, higher than or equal to the previous value. Different absolute thresholds were established to defined the superior and inferior limits in which the variation of OUR and airflow can be considered negligible. The limit to detect OUR variation was defined as 0.5 % of the maximum OUR by previous studies. The range considered for the airflow measurements was 0.05 L min^{-1}. Figure 3.6 shows the algorithm used.

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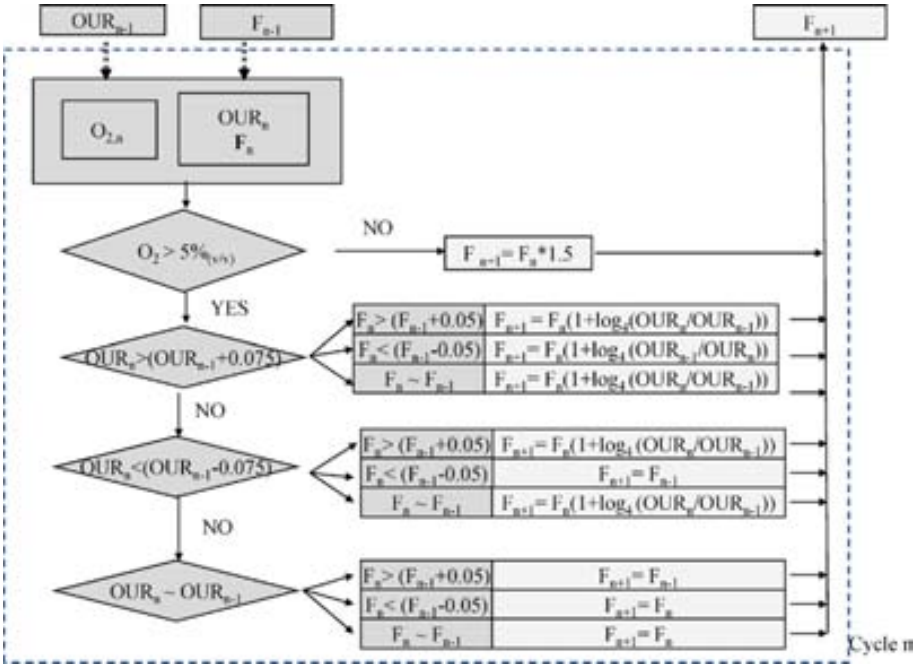

Figure 3.6. Scheme of the control laws for the OUR controller. F, airflow; OUR, oxygen uptake rate (Source: Puyuelo 2010).

3.1.3 Plant II

Figure 3.4 shows a scheme of the Plant II. This experimental assembly is situated in the external part of the Chemical Engineering Department at UAB and allows experiments to be made at bigger scale than Plant I. It consists of 2 Reactors of 50 L working volume, 55 L total volume (R1 and R2, Figure 3.5) and was used for the experiment included in chapter 7 of this thesis.

The entire system was supplied with filtered compressed air, the pressure compressor used was a Decibar 30 Worthington®. Two connectors were placed at the cover of both reactors. One connector was designed to insert the temperature probe at the center and the other one to exit the outgoing gases. A third connector was placed at the bottom of the reactor to inject the inlet air. Exhausted gases exiting from the top of the reactor firstly passed through a water trap and then went to the O_2 sensor (Xgard 501/265/S, Crowcon, England). The electric signal of the sensor was firstly registered by the PAD acquisition system (cDAQ-9172, National Instruments, USA), connected to the PC though a RS-232 serial port and then recorded and shown on a graph using LabView 8.6 Software (National Instruments, USA). A continuous airflow was supplied from the

bottom of the reactor using a plastic mesh. Inlet airflow was automatically controlled and adjusted with a mass flow meter and a controller of oxygen uptake rate described and validated by Puyuelo et al. (2010). Finally, leachate was collected from the bottom of the reactor. A weight scale was at the bottom of the reactor to follow the mass content evolution.

On the other hand, R1 was also designed to allow the measurement in situ of samples' porosity during the process. More information of this analysis is given in the corresponding section (3.2.4).

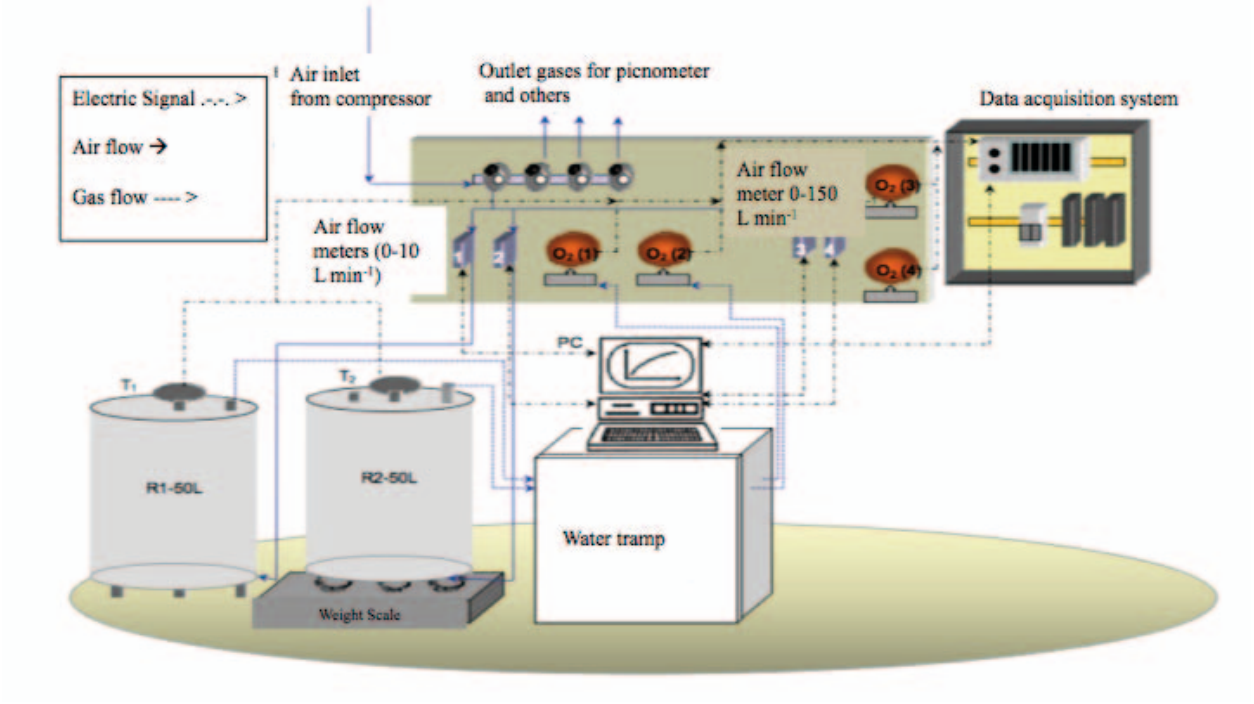

Figure 3.4. Scheme of Plant II (adapted from Puyuelo, 2010).

Figure 3.5. Reactors R1 and R2.

3.2 Specific methods for organic mixtures

3.2.1 Dynamic respiration index (DRI)

The dynamic respirometer was designed, built and well described during previous studies by Ponsà et al. (2010a) and Pognani et al. (2011); based on the methodology proposed by Adani et al. (2006). In few words, this respirometer can analyze 12 samples in a 4 independent lines composed by 3 reactors of 0.5 L each. Every line consists of 3 independent air flow meters, one for each reactor and 3 electro-valves that commutate the exhaust gases to CO_2 and O_2 detectors. The timing used to manage the electro-valves enables to analyze the exhaust flow of each reactor separately. Specific software (Indusoft Web Studio, version 2008, USA) was installed on a personal computer to build a continuous database of the obtained results (Figure 3.7).

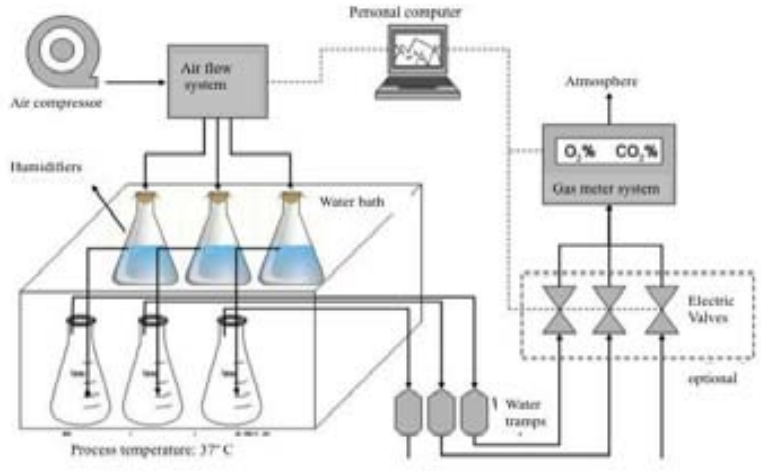

Figure 3.7. Sketch of dynamic respirometer (Source: Pognani et al. 2009).

A 100 g mixture of sample, the residue with addition of an inert (non-biodegradable during the assay time) bulking agent in wet weight ratio of 1:10 (Ponsá, 2010), is introduced in the reactor (Erlenmeyer flask) and incubated at 37ºC. Then, the dynamic respiration index (DRI) and the cumulative respiration index (CRI, also known as AT) can be calculated. The DRI (equation 1) represents the average oxygen uptake rate during the 24 hours of maximum biological activity observed during the respirometric assay and it reports the stability degree of the sample. The CRI_t

(equation 2) represents the cumulative oxygen consumption observed during the t days of maximum respiration activity. Both, DRI and CRI are well known indexes at the composting field (Figure 3.8). In fact, both indexes are useful to evaluate the biodegradability of the samples; however, the CRI also quantifies the biodegradable organic matter content of a given sample. The use of both indexes is presented as the best tool for the characterixaiton of the content of biodegradable organic matter and the estimation of process requirements (Ponsá, 2010b).

$$\text{DRI (mg } O_2 \text{ g}^{-1} \text{ DM h}^{-1}) = \frac{(O2,i - O2,o)x\ Fx\ 31.98\ x\ 60}{22.4\ x\ DM} \quad \text{(equation 1)}$$

$$CRI_t \text{ (mg } O_2 \text{ g}^{-1} \text{ DM)} = \int_{tl}^{tl+t} DRIt.\, dt \quad \text{(equation 2)}$$

Where: ($O_{2,i}$- $O_{2,o}$), is the difference in the oxygen content (volumetric fraction) between airflow in (0.209) and out of the reactor at that given time; F, volumetric airflow measured under normal conditions (1 atm and 273 K) (L min^{-1}); 31.98, is the molecular mass of oxygen (g mol^{-1}); 60, is the conversion factor for minutes to hours; 22.4, is the volume occupied by 1 mol of gas under normal conditions; DM, dry matter of sample loaded in the reactor (g); tl, time when lag phase finishes. Lag phase ends when oxygen uptake rate reaches 25% of the maximum uptake rate calculated as the average of three hours (Federal Government of Germany, 2001).

A dynamic respiration index curve can be built from on-line collected data as shown in figure 3.8.

This dynamic respirometer was used to characterize the initial mixtures assayed, to evaluate the stability of the final material after SSFs and also to assess the SSF at lab scale, as a first approach.

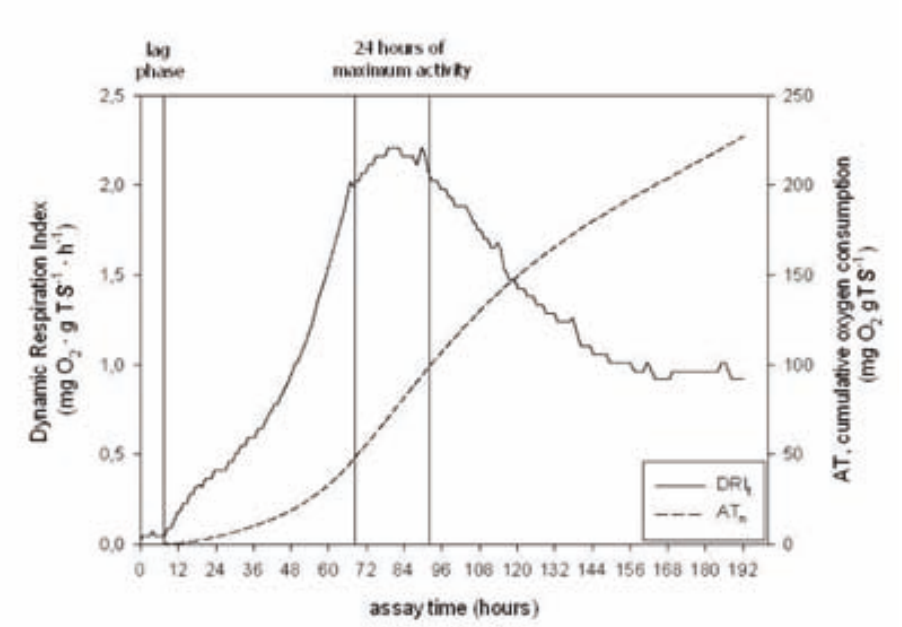

Figure 3.8. Typical curve for dynamic respiration indices' evolution and calculation (Source: Ponsá et al., 2010a).

3.2.2. Oxygen uptake rate (OUR)

The oxygen uptake rate is an indirect measure of biological activity that can be calculated during the SSF process. While DRI is an analytical procedure to assess the stability of an organic sample, OUR is a process parameter that can be calculated with on-line measurements at any given time (Gea et al. 2004). During this research, equation 3 was used to calculate $sOUR_1$ and $sOUR_{24}$, which correspond to the average of specific oxygen uptake rate for 1 hour and 24 hours, respectively.

$$sOUR\ (g\ O_2\ kg^{-1}\ DM\ h^{-1}) = \frac{(\square\square\square\square\square\square\square)\square\square\square\square\square\square\square\square\square\square\square\square\square\square\square}{\square\square\square\square\square\square\square\square\square} \quad (\text{equation 3})$$

Where: sOUR, is specific oxygen uptake rate, $(O_{2,i}- O_{2,o})$, is the difference in the oxygen content (volumetric fraction) between airflow in (0.209) and out of the reactor at that given time; F, volumetric airflow measured under normal conditions (1 atm and 273 K) (L min^{-1}); 31.98, is the molecular mass of oxygen (g mol^{-1}); 60, is the conversion factor for minutes to hours; 22.4, is

the volume occupied by 1 mol of gas under normal conditions; DM, dry matter of sample loaded in the reactor (kg).

Studies at large scale demonstrated that during the process and, due to compaction and degradation of the sample, the reactor behaves as a succession of a plug-dispersion reactor and a mixed reactor. The headspace, between the sample and the ceiling of the reactor, behaves as a proper mixed reactor Tremier et al. (2008). This study indicated that the situation inside the reactor is a mixture of situations, not so easy to determine. Hence, to calculate the sOUR along this research, only the oxygen at the inlet and the oxygen at reactor's outlet were considered, like the input and output in a black box system.

3.2.3 Bulk density (BD)

BD is defined as the weight per unit of volume of sample. BD was calculated on wet basis dividing the sample weight by the sample volume as shown in the following equation:

$$BD = \frac{Ws}{Vs} \quad \text{(equation 4)}$$

where: BD, bulk density (kg L^{-1}); Ws, sample wet weight (kg); Vs, sample volume (L).

3.2.4 Free Air Space (FAS)

Also known as air filled porosity (AFP), it refers to the ratio of gas filled pores volume of the sample (Vg) to total volume of sample (Vs) (Haug, 1993) as presented in next equation:

$$AFP = \frac{Vg}{Vs} \quad \text{(equation 5)}$$

This parameter was determined by using an air pycnometer (Figure 3.9) to maintain the proper air circulation through the organic matrix as proposed in previous studies (Ruggieri et al., 2009). Briefly, the air pycnometer was constructed by Ruggieri (2008). It consists of a gas reservoir chamber containing air at a known pressure and a sample chamber filled with a known volume of material. An air valve connects both chambers. After sealing the system, compressed air from the reservoir chamber is released to the sample chamber by opening the connecting valve and the

pressure is allowed to equilibrate. A pressure-measuring device at the reservoir chamber records the pressure before and after the connecting valve is opened. By using Boyle's law and some assumptions the AFP can be determined as a relation of the two pressures.

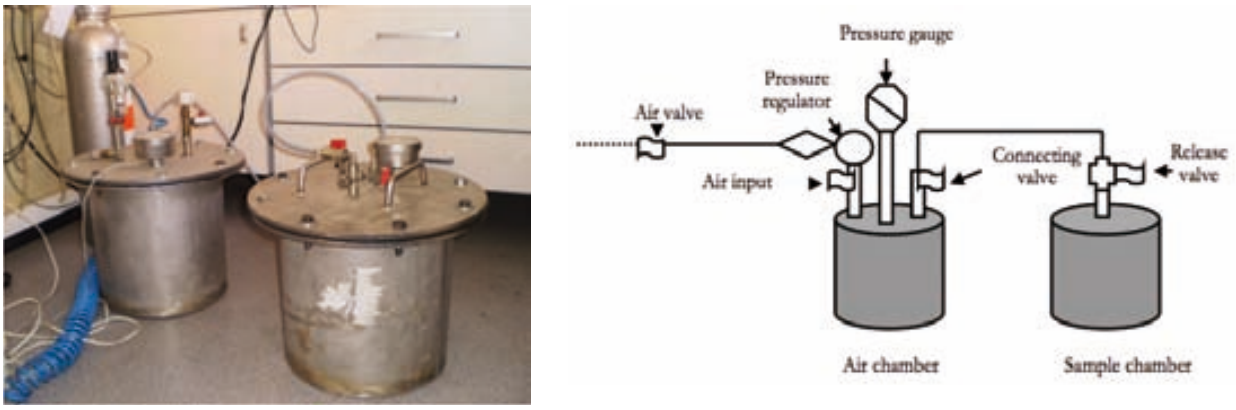

Figure 3.9. Picture of air pycnometer used to determine the AFP in organic solid mixtures (left) and air pycnometer Scheme (Source: Ruggieri 2008) (right).

The gas reservoir chamber was also designed to connect and measure the AFP in situ on the 50 L working volume reactor (R1) at Plant II. Because of this, continuos monitoring of AFP could be obtained during the experiment.

3.2.5 Fist test

This test corresponds to an easy and quick method to approximate the moisture content of the mixture when sampling. It must be confirmed later with the water content assay described below. In sum, the sample is held in the hand and is pressed by closing the fist. If the sample oozes water, is too wet. If opening the hand the sample comes apart, it is too dry. If none of these situations are given, the material has adequate moisture.

3.3 Standard Analytical Methods

Regular parameters were determined according to the standard procedures included in the "Test Methods for the Examination of Composting and Compost" (US Department of Agriculture

and US Composting Council, 2001) when not; it is menctioned the other procedure utilized. All the results were calculated as a mean of three replicates.

3.3.1 Water content (WC) and dry matter (DM, equivalent to total solids, TS)

To calculate the WC and DM samples were oven dried in a capsule at 105ºC for 24 hours, then weighted and calculated by using these following equations:

$$\text{WC (\%)} = \frac{Pi - Pf}{Pi - Po} \times 100 \quad \text{(equation 6)}$$

$$\text{DM (\%)} = 100 - \%\ \text{WC} \quad \text{(equation 7)}$$

where: Pi, initial wet weight of the sample; Pf, final dry weight of the sample; Po, capsule weight.

3.3.2 Organic matter (OM, equivalent to volatile solids content, VS)

OM was analyzed by ignition at 550°C of the dry sample in the presence of excess air for 4 hours, calculated as equation shows below:

$$\text{OM (\%)} = \frac{Pi - Pa}{Pi - Po} \times 100 \text{ (equation 8)}$$

where: Pi, initial weight of the dry sample; Pa, weight of the ashes ; Po, capsule weight

3.3.3 pH and electric conductivity (EC)

pH was determined by mixing a ratio of 1:5 w/v basis of sample and deionized water. The sample was shaken for 30 minutes at room temperature to allow the salts to solubilize in the deionized water. Then centrifuge at 3500 rpm for 10 min and the pH was measured with an electrometric pH meter (Crison®, micropH2001). Also the EC was determined using an electrical conductimeter (Crison®, microCM2100)).

3.3.4 Total Organic Carbon (TOC)

The TOC was determined using an O.I. Analytical Solid TOC Analyser/Win TOC Solids v3.0. The samples were previously dried up and sieved at 0.5 cm.

3.3.5 Total Kjeldahl Nitrogen (TKN)

The TKN was determined using 0.5 g of dried and sieved sample. The sample was digested for 1.5 h at 420°C using 20 mL of concentrated sulphuric acid in 100 mL Kjeldahl tubes using a Bloc Digester 6 (with six tubes capacity) (J.P. Selecta S.A., Barcelona, Spain). Before digestion, a catalyst (Kjeltab®) was added. A Büchi Distillation Unit K-355 (Flawil, CH) was used for sample distillation with an excess of NaOH (35%). The condensate was placed in a conical flask with 100 mL of boric acid (4%) with mixed phenolphthalein indicator. After distillation, titration with HCl was used to measure the amount of nitrogen formed. TKN was calculated using Equation 9:

$$\text{TKN (\%)} = \frac{(V1-V0)x\ N\ x\ 1.4}{DM} \quad \text{(equation 9)}$$

Where: V1, HCl volume consumed (mL) in sample titration; V0, volume of HCl consumed (mL) in control titration; N, normality of the HCl used in determination; and DM, sample weight in dry basis (g).

To calculate the crude protein of the solid sample an approximation was done by multiplying 6.25 to the value of TKN (in fraction).

3.3.6 Soluble N-NH_4^+

This determination is based in a Kjeldahl-like distillation without the digestion part of the sample.

On this regard, the soluble N-NH_4^+ that is originally in the sample could be quantified. For this determination an extract 1:5 as explained in section 3.3.3 was done. Then, 10 mL of the extract was distilled and titrated as explained for Kjeldahl determination. The following equation shows how to calculate this ion concentration:

$$\text{Soluble N-}NH_4^+ \text{ (mg g}^{-1}\text{)} = \frac{(V1-V0)\ x\ N\ x\ 14\ x\ Ve}{10\ x\ DM} \quad \text{(equation 10)}$$

Where: V1, HCl volume consumed (mL) in sample titration; V0, volume of HCl consumed (mL) in control titration; N, normality of the HCl used in determination; Ve, mL of water used to do the extract; 10, mL of extract used in the analysis; and DM, sample weight in dry basis (g).

3.3.7 Fat Content (HEM- Hexane extractable material)

The fat content was measured using a standard Soxhlet method with n-hexane as organic solvent (The U.S. Environmental Protection Agency, Method 9071B). The samples were previously dried up and sieved as in TKN and TOC assays. The extraction was carried out by, mixing the same quantity of dry sample with sodium sulfate anhydrous in a cellulose cartridge, and using Soxhlet E-816 (Büchi) with the solvent n-hexane (≥ 95% of purity, Sigma Aldrich) during 4 hours. Results were presented in percentages on dry matter basis (gravimetric basis).

3.4 Specific Methods for enzymes determination and characterization

3.4.1 Enzyme extraction

Fermented solid material was mixed thoroughly with 50 mM HCl-Tris ((hydroxymethyl) aminomethane) buffer, pH 8.10, in a ratio 1:5 (w: v) for 45 min and the extract was separated by centrifugation at 10000 rpm for 10 min at 4ºC and further filtration through 0.45 μm. The filtered supernatant was used as crude enzyme extract.

3.4.2 Protease Activity Assay

Alkaline protease activity was determined using a method previously described for soils (Alef and Nannipieri, 1995).

The fundamentals are based on the determination of Trichloroacetic acid (TCA)-soluble tyrosine derivatives by the Folin reagent. The amino acid tyrosine, present in the majority of proteins, is liberated along with other amino acids and peptide fragments from Casein, the substrate, during the incubation because of protease activity present. Folin & Ciocalteu Phenol reagent primarily reacts with double bond of molecules to produce a blue colored chromophore, which is

quantifiable and measured as an absorbance value on the spectrophotometer. Absorbance values generated by the activity of the protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the same reagent to correlate changes in absorbance with the amount of tyrosine. It is also necessary to prepare a blank of the sample to subtract the value of absorbance produced by double bond molecules that do not correspond to Tyrosine or another amino acid in the sample.

The recipe and steps are summarized below:

Chemicals and solutions

- **Tris buffer (50 mM, pH 8.1)**

Dissolve 6.05 g Tris in 700 mL distilled water, adjust the pH to 8.1 with HCl and dilute to 1000 mL with distilled water.

- **Sodium Caseinate (2%)**

Suspend 10 g sodium caseinate in warm distilled water (50ºC) to 500 mL (use a stirrer).

The casein should be prepared daily in order to be fresh.

- **Trichloroacetic acid (TCA)(15%)**

Dissolve 75 g TCA in about 300 mL distilled water and dilute to 500 mL with distilled water. Trichloroacetic acid is a strong acid and should be handled with care.

- **Alkaline reagent**

Dilute 60 mL of NaOH (1M) with distilled water before dissolving 50 g $NaCO_3$ anydro in the solution and bring up to 1000 mL with distilled water.

Dissolve 0.5 g $CuSO_4.5H_2O$ in distilled water and dilute to 100 mL with distilled water.

Dissolve 1 g potassium sodium tartrate ($C_4H_4KNaO_6.4\ H_2O$) in distilled water and dilute to 100 mL with distilled water.

Mix 1000 mL of $NaOH/NaCO_3$ solution with 20 mL of $CuSO_4$ solution and 20 mL of potassium sodium tartrate solution.

- **Folin-Ciocalteau reagent (25%)**

Dilute 1 part of the reagent in 3 parts of distilled water. Folin's Phenol Reagent is an acid and should be handled with care.

The alkaline reagent and the Folin-Ciocalteu reagent should be prepared immediately before use.

- **Tyrosine standard solution (500 ug mL^{-1})**

Dissolve 50 mg of tyrosine in Tris buffer and dilute to 100 mL with Tris buffer.

Procedure

1. Place 1 g of mixed sample in a centrifuge tube and add 5 mL of Tris buffer and 5 mL of fresh sodium casein solution. Or place 1 mL of extract described in 3.4.1 in a centrifuge tube, add 4 mL of Tris buffer and 5 mL of fresh sodium casein solution.
2. Mix the contents and incubate for 2 h at 50ºC on a shaking water bath. At the end of incubation, add 5 mL of cold TCA solution and mix the contents thoroughly to stop the reaction.
3. To perform the controls, add 5 mL of sodium casein solution at the end of incubation and immediately before adding the TCA solution.
4. Centrifuge the resulting sample suspensions at 10000 rpm for 10 min.
5. Filter the samples through paper filter. Supernatants can be stored at 4ºC for 5 h but no longer before being analysed for their tyrosine content.
6. Pipette 5 mL of the clear supernatant into tubes, mix with 7.5 mL of the alkaline reagent, and incubate for 15 min at room temperature.
7. Then, add 5 mL of the Folin reagent and measure the absorbance at 700 nm after exactly 1 h of incubation at room temperature (measure several times until the measured value becomes constant).

Calibration curve

Pipette different mL of tyrosine standard solution into centrifuge tubes, bring up to 5 mL with Tris buffer, and add 5 mL of sodium casein solution. Don't add any tyrosine standard to the blank. Then add 5 mL TCA and perform the same way as described above the samples. The standard curve doesn´t need the incubation part. An example of the standard curve for all the determinations during this research is presented on Annex I.

Calculation

Correct the measured absorbance for the controls (Abs sample - Abs control) and calculate as follows:

$$PA\ (U\ g^{-1}\ DM) = \frac{C \times 15}{DM} \quad \text{when use 1 g sample} \qquad \text{(equation 11)}$$

$$PA\ (U\ g^{-1}\ DM) = \frac{C \times 15 \times 5}{DM} \quad \text{when use 1 mL extract} \qquad \text{(equation 12)}$$

$$PA\ (U\ mL^{-1}\ extract) = C \times 15 \qquad \text{(equation 13)}$$

Where: PA, is protease activity; U: units; C, is the measured tyrosine concentration (ug mL^{-1} supernatant); DM, is the dry weight of mixed sample (g); 15, is the final volume of solutions at the assay; 5, factor conversion regarding to the extract done from the solid sample.

One unit of alkaline protease activity (U) was expressed as 1 microgram (μg) of Tyrosine released under the assay conditions (2 h at 50ºC in orbital shaking). The results are expressed at an average of the 3 replicates and also the error calculated as propagation.

Another amounts informed during this research corresponded to sPA and Yield. sPA is the specific activity which is the ratio of the enzymatic activity, protease in this case, to the total amount of proteins. Although this determination is used when quantifying a purification of the enzyme, here is used to evaluate the extraction from the solid matrix and quantify the specific activity of the extracts.

$$sPA\ (U\ mg^{-1}\ SP) = \frac{\text{total activity}}{\text{total proteins}} = \frac{C \times 15 \times 5}{WM \times SP \times 5} \qquad \text{(equation 14)}$$

where: WM, is wet matter; SP, is soluble protein, determined by Bradford assay (mg mL^{-1}).

On the other hand, the Yield is the production yield obtained at the highest peak of protease activity during fermentation and is calculated as follows:

$$Yield\ (\text{total units}) = PA_n\ (U\ g^{-1}\ DM) \times g_n\ DM \text{ in reactor} \qquad \text{(equation 15)}$$

Where: PA_n, is the protease activity at day n; g_n DM, is the g at day n of dry matter of the mixture treated.

3.4.3 Bradford assay - Soluble Protein (SP)

The Bradford reagent can be used to determine the concentration of proteins in solution. The procedure is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. The amount of absorption is proportional to the protein present. The linear concentration range is 0.1-1.4 mg/mL, using bovine serum albumin (BSA) (Sigma Aldrich®) as standard protein (Bradford, 1976).

Procedure

1. Gently mix the Bradford reagent in the bottle and bring to room temperature. (The reagent bottle has to be conserved at 2-4ºC).
2. Prepare protein standards of appropriate concentrations in the same buffer as the unknown samples, by serially diluting the BSA standard (2 mg mL^{-1}).
3. Add 1.5 mL to each spectrophotometer cuvettes and then, 0.05 mL of the standard protein or unknown sample.
4. Mix gently the mixture and let the sample incubate at room temperature for 5 to 45 minutes. Color development begins immediately.
5. Measure the absorbance at 595 nm. The protein dye complex is stable up to 60 minutes. The absorbance of the samples must be recorded before the 60 minutes time limit and within 10 minutes of each other.
6. Determine the protein concentration by comparison of the unknown samples to the standard curve prepared using the protein standards. An example of the standard curve and the calculations done is presented on Annex I.

3.4.4 Polyacrylamide electrophoresis gel (PAGE)

PAGE is a widely known technique to separate proteins according to the polypeptide chain, molecular weight (MW), among other factors. The gel of polyacrylamide serves as a size-selective sieve during separation. The proteins move through a gel because of the electric field applied and simultaneously, the pore structure of the gel allows small proteins to travel more

rapidly than bigger proteins. There are different types of PAGE and the following instructions describe only the ones used during this research.

Native PAGE

In this system, proteins are prepared in non-reducing, non-denaturing sample buffer and electrophoresis is also performed in absence of denaturing and reducing agents (Figure 3.10, native proteins, without changes in thee-dimensional structure). Here, the native charge-to-mass ratio of protein is conserved and so the proteins can also migrate because of charge and not only by MW being sometimes difficult to interpret. However, native PAGE does allow separation of proteins in their active state and can resolve proteins of the same MW (Ornstein, 1964, Davis, 1964).

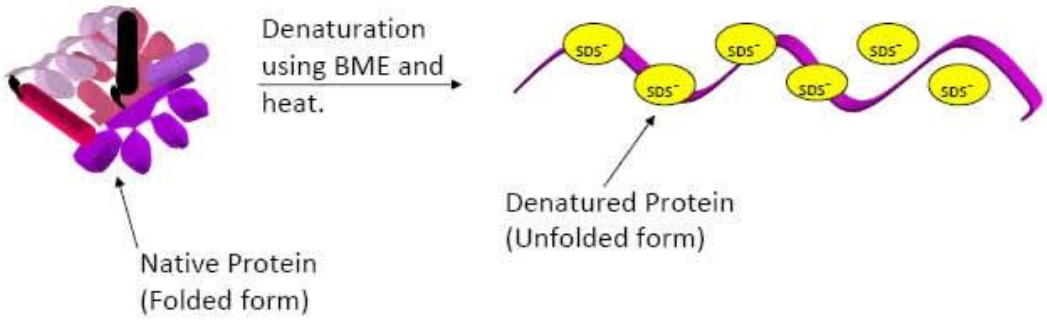

Figure 3.10. Native structural conformation of proteins and denatured protein after treatment. BME: 2-β-mercaptoethanol.

Steps:

1. Prepare the 12% polyacrylamide (w/v) separating gels (Biorad®) inside the vertical electrophoresis chamber and between the two platinum electrodes (Figure 3.11.).
2. Add running buffer Tris/Glycine 1X to complete the circuit (Dissolve 100 mL of buffer 10X in 900 mL of pure distilled water).

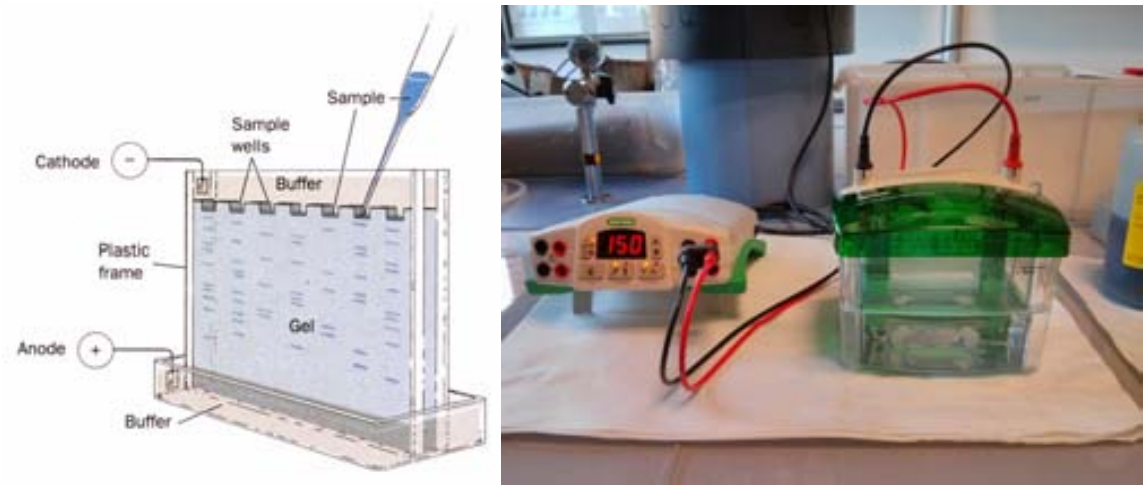

Figure 3.11. Vertical Electrophoresis equipment. Mini Protean Tetra cell (BioRad®)

3. Add 1 part of sample to 2 parts of sample buffer.
4. Load each well with the different samples prepared (15 uL). One well should be a MW marker in order to interpret the results later.
5. Connect power supply and set to 150 V. Stop the electrophoresis when the sample dye front reaches the bottom of the gel (back line).
6. Remove the plastic covers from the gel, wash x3 with pure distilled water and stain accordingly with Bio-Safe™ Coomassie G-250 Stain.

SDS-PAGE

The term SDS refers to sodium dodecyl sulfate. In presence of this detergent and reducing agents, the proteins became fully denatured and dissociate from each other (Figure 3.10). The detergent disrupts hydrophobic interactions between and within proteins. Besides, the SDS binds noncovalently to proteins conferring an overall negative charge in all the proteins, similar charge-to-mass ratio and a long, rod-shape on the proteins instead of a complex tertiary conformation. The reducing agents such as 2-β-mercaptoethanol disrupt intramolecular and intermolecular disulfide bonds. All these characteristics enable the protein to be separated by its size and so that the estimation of MW is possible (Laemmli, 1970).

Same procedure as described above for Native with some differences in steps:

2- Add running buffer Tris/Glycine/SDS 1X.

3- Add 15 uL of sample to 5 uL of sample buffer 4X (Tris-HCl 125mM pH6.8, SDS 4% w/v, bromophenol blue 10% w/v and 2-β-mercaptoethanol 10% v/v)

Heat at 95ºC per 5 min. Vortex, centrifuge and load the sample on the corresponding wells continuing with number 4- of the above description. Use Precision Plus Protein™Dual Color Standards (BioRad®) as a marker.

Zymogram

It is a kind of native PAGE so the sample preserves the native protein conformation, subunit interactions and biological activity. Because of that it is possible to incubate with a determined substrate, in this case with casein, and the protein is separated by MW and then the substrate is broken down, allowing the identification of proteases. After staining, all the gel is stained but leaving clear areas around active proteases.

Although the procedure is the same as in Native PAGE, after running the gel and before staining it is necessary to incubate the gel with casein solution 2% (same as used in proteases activity assay) for 1 h at 50ºC.

3.4.5 Effect of pH and temperature (T) on protease stability

The effects of pH and T on alkaline protease stability were analyzed by a 12 full factorial experimental design with 3 replicates at the center (using the software package Sigmaplot version 10, Systat software Inc.). The temperatures were fixed at 30, 50 and 70ºC, and the pH were 5.00 (Tris-HCl, 1 M), 8.00 (Tris-NaOH, 1 M) and 11.00 (acetic acid- sodium acetate, 1 M) that are the typical ranges of enzymatic activity (Gea et al. 2007, Santis et al. 2011). The objective function selected was the residual alkaline protease activity percentage (RA, referred to the initial activity of the extracts) after one hour of incubation of the crude extracts at the selected T and pH. Table II.1 and II.2 in Annex II shows these experimental conditions selected, togheter with the results obtained for the particular crude extract assayed (results explained in next chapters, 4 and 5).

3.4.6 Scanning electron microscopy (SEM)

The SEM is one of the most versatile instruments available for the examination and analysis of the microstructure morphology and chemical composition characterizations. Image formation in

the SEM is dependent on the acquisition of signals produced from an electron beam and the sample interactions. During the research study several different samples (liquid, lyophilized and solids samples of cow hides) were observed by microscopy at UAB Servei de Microscopia, using Evo ® MA10, and Merlin Compact microscopies, Carl Zeiss®. The majority of the samples can be observed directly through loading them on carbon tape. Figure 3.12 shows the preparation of the liquid samples. Before the analysis by SEM, the samples were air-dried porperly. In the case of the cowhides, the samples were coated with a metal (gold) before analysis.

The samples were visualized with different resolutions and detectors regarding the sample and the object searched. These specifications are described when presenting the corresponding images during this study (Chapter 4, 5 and 9).

Figure 3.12 Preparation of liquid samples for the observation by SEM.

Chapter 4

Potential of solid-state fermentation to obtain proteases from different nitrogen-rich residues. Partial characterization of protease extracts.

Part of this chapter has been published at Biochemical Engineering Journal 74, 15–19. 2013. Potential of solid-state fermentation of soy fiber residues by native microbial populations for alkaline protease production at bench scale.
Abraham, J., Gea, T., Sanchez, A.

Part of this chapter has been presented as co-author on a conference presentation at Red Española de Compostaje Conference, 2011, Spain. El compostaje desde un nuevo paradigma: obtención de productos de valor añadido.
Abraham, J., Artola, A., Barrena, R., Font, X., Gea, T., Sánchez, A., Santís, A.

Summary

In this chapter three different residues obtained from local industries were analyzed at lab scale in order to screen for a suitable biodegradable material to produce proteases. Meanwhile using the residue that produced the highest protease activity, the soy fiber residue, the methodology for determination of these enzymes was settled. Thereafter, experiments at bench scale (1 or 2 kg) were carried out in order to valorize the residues to compost-like products and also to produce valued enzymes such as proteases. Bench scale experiments with soy fiber and coffee husk residues are described in this chapter while the third one, the hair waste, is described at Chapter 5. Residues were assayed with and without addition of compost to compare protease production by native microorganisms together with low-cost microbial consortium added. The soy fiber extracts obtained, which had the highest proteases activity, were biochemically characterized in terms of pH and temperature stability and conservation of extracts, among others.

4.1 Materials

The materials used were soy fiber, coffee husk and hair waste as described in Chapter 3, section 3.1.1. Table 4.1 shows the main characteristics of these residues. Wood chips (10% DM) were added to the materials in a 1:1 (w:w) ratio and acted as a bulking agent to provide the proper porosity (Ruggieri et al. 2009) at 4.5-10 L experiments. Raw sludge (31.9% DM, 82.6% OM and DRI of 4.2 g O_2 kg^{-1} DM h^{-1}) from a local wastewater treatment plant was used to inoculate the hair waste to begin the biodegradation. Otherwise, this residue alone is very difficult to be degraded because of the characteristics of the hydrolyzed hair waste, as described in previous studies (hair waste: raw sludge weight wet ratio of 1:2, Barrena et al. 2007a,b). Compost from a composting plant in Barcelona (83.1% DM, 76% OM and DRI of 1.6 g O_2 kg^{-1} DM h^{-1}) was used as an inoculum for the soy fiber in the 4.5 L reactors experiments and also the coffee husk in 10 L reactors.

4.2 Experiments

0.5 L scale reactors

To screen for a suitable protein-rich waste for protease production, preliminary SSF experiments were assayed in triplicate for 5 days at 37ºC in 500 ml Erlenmeyer flasks with 100 g of sample (Figure 4.1) in the dynamic respirometer described in Chapter 3, section 3.2.1. The three residues were assayed withouth previous sterilization and after 5 days of incubation, the DRI and protease activities were analyzed.

Table 4.1. Characterization of different local residues studied for SSF

Waste	**Hair waste**	**Soy fiber**	**Coffee husk**
Water content (%, db)	59.93 ± 0.01	82.52 ± 0.01	79.43 ± 0.01
Organic matter (%, db)	85.96 ± 0.01	97.72 ± 0.01	92.18 ± 0.01
pH	10.76 ± 0.01	7.37 ± 0.01	7.49 ± 0.01
EC (mS cm^{-1})	5.03 ± 0.01	0.70 ± 0.01	1.26 ± 0.01
Respiration index (g O_2 kg^{-1} DM h^{-1})	3.0 ± 0.4*	5.6 ± 0.8	3.0 ± 0.3
Cumulative oxygen consumption (4 days, g O_2 kg^{-1} DM)	160 ± 12*	374 ± 31	217 ± 41
Total Organic Carbon (% db)	57.2 ± 0.9	67.5 ± 0.9	67.4 ± 0.1
Total Kjeldahl Nitrogen (% db)	12.0 ± 0.1	4.4 ± 0.2	2.7 ± 0.2
C/N ratio	4.8	15.4	24.8
fat content-HEM (% db)	0.86 ± 0.34	5.30 ± 0.04	2.18 ± 0.12

wb: wet basis; db, dry basis; EC, electrical conductivity; * Ratio 1:2 hair waste: raw sludge.

Setting up the Protease Assay

The protease assay utilized was the one described in Chapter 3, section 3. 4. 2. This method established for soil samples was evaluated in order to determine if it was adequate for solid organic wastes (fresh and partially fermented). To set up the methodology, soy fiber residues were used. At final incubation (5 days), the protease assay was determined at different incubation time as 0, 30, 60, 90, 120 and 150 minutes. Also protease activity was determined from solid samples and extracted samples.

Bench scale reactors

Experiments were performed for 14 days at 4.5 L and 10 L reactors at Plant I for soy fiber residues and coffee husk residues, respectively. Continuous aeration at a rate of 0.1 L min^{-1} was provided to the reactors. Although different aeration was explored in soy fiber SSFs; here, only

the SSF experiment with successful results is presented. The information regarding the optimization in aeration and the experiments performed with intermittent aeration are described in detail in Chapter 6. The mixtures were prepared by mixing soy fiber and bulking agent in a wet weight ratio of 1:1 (S) and soy fiber, bulking agent (1:1, w:w) and 10 % of compost (SC) as seen on Figure 4.2. The mixtures were not sterilized prior to SSF. The final weight of the mixture was 1.250 kg for each reactor. Each experiment was carried out in duplicate.

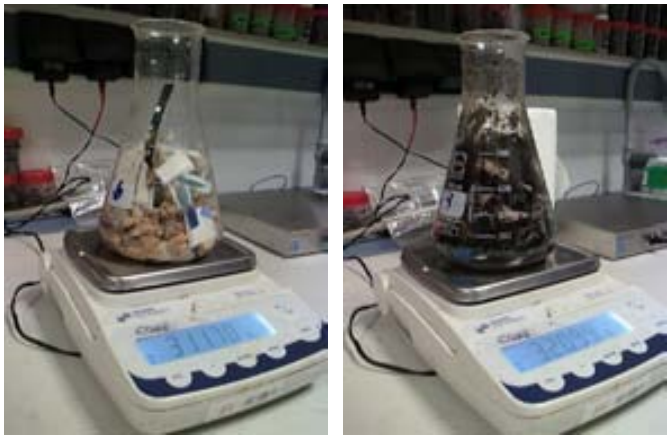

Figure 4.1. SSF starts at laboratory scale. Preparing samples of soy fiber residues (left) and coffee husk (right) for respirometric assay.

Figure 4.2. SSF mixture prepared with soy fiber and bulking agent (left) and soy fiber, bulking agent and 10% of compost (right).

The same ratio and mixtures were used to coffee husk being coffee husk with bulking agent (CH) and coffee husk with bulking agent and 10% of compost as inoculum (CHC). The final weight of the mixture was 2.5 kg for each reactor. Table 4.2 shows the characterization of the

initial SSF mixtures. As noticed by mixing with bulking agent, the residues decrease its humidity and so the mixture had around 50% of WC. The WC of the mixture during the experiment was adjusted within the recommended values by adding tap water if needed. Samples were collected at 0, 3 (after 24 h in thermophilic phase), 7, 10 and 14 days after manual homogenization of the entire mass.

Table 4.2. Characterization of the initial mixtures for SSF process.

Mixture	S	SC	CH	CHC
Water content (%, db)	50.48 ± 0.04	48.76 ± 0.03	56.32 ± 0.01	54.67 ± 0.01
Organic matter (%, db)	93.00 ± 0.03	99.50 ± 0.01	95.90 ± 0.03	91.50 ± 0.04
Bulk Density (kg L^{-1})	0.23	0.23	0.24	0.24
FAS (%)	74	74	78	78

4.3 Results and discussion

Screening materials for protease production

The accurate selection of the substrates is necessary to reach higher quantities of enzymes desired (Rodriguez-Couto, 2008). In this sense, nitrogen-rich residues were selected from local industries to search for proteases. A respirometric study was performed with these three residues to evaluate the biodegradability of each mixture and the best waste capable of protease production. The maximum enzyme production was observed with soy fiber. Figure 4.3 shows the difference between the three residues studied and also two other residues being studied at that moment. The protease production was 37244±1101 U/g DM for soy fiber, 17011±2122 U/g DM for hair waste and 10681±71 U/g DM for the coffee husk. The different levels of protease production among residues could be caused by the chemical composition and biodegradability of the materials related to the duration of the experiment, the pH and C/N ratio, and the different microbial populations that developed. The hair and coffee husk resulted in a lower DRI than soy fiber (Table 4.1). Hair resulted in a high pH and an extremely high N content; however, this fraction might not correspond to an easily biodegradable fraction but, rather, to recalcitrant C and N chemical forms (Ponsà et al., 2011) due to its chemical composition. In addition to a high DRI and CRI_4, that indicate the higher degradability of this material, soy fiber has a considerable

amino acid content and N availability (Fetuga et al., 1974). Therefore, it appears to be the best of the three assayed materials for proteases production. As Pandey et al. (2000) reported, these results indicate that the protease production pattern varied with the type of residue.

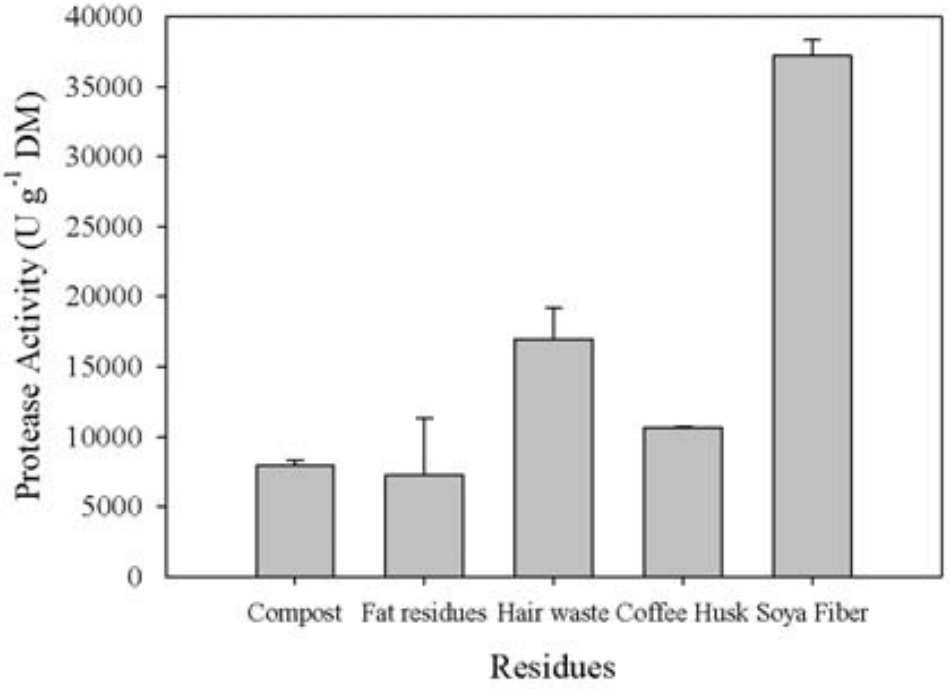

Figure 4.3. Screening of protease activity obtained from different residues by SSF.

Set-up the methodology for proteases by SSF

There are methods that determine proteases activity by using gold nanoparticles, magnetics sensors and casein assays (Guarise et al., 2006, Zhao et al., 2003, Alef and Nannipieri, 1995). As a first approach, the casein assays are useful to screen proteolysis activity in most samples and also are the simplest assays in terms of methodology. Likewise, the spectrophotometry assays normally succeed in reproducibility, flexibility and availability in any laboratory.

The method described by Alef and Nannipieri was developed to determine the proteases activity in soils. Because of that, a battery of experiments was done in order to determine the accurate time to incubate the reaction and check if the quantity of substrate was adequate. The test was performed with 1 g of solid sample obtained in SSF experiments with soy fiber residues. The typical curve's evolution for enzymes is depicted with the values obtained in Figure 4.4, indicating that the quantity of substrate was enough because of the linear behavior. Besides,

within the 2 hours of incubation time the speed of the reaction haven't arrived to the equilibrium suggesting that there is still more substrate to turn into product.

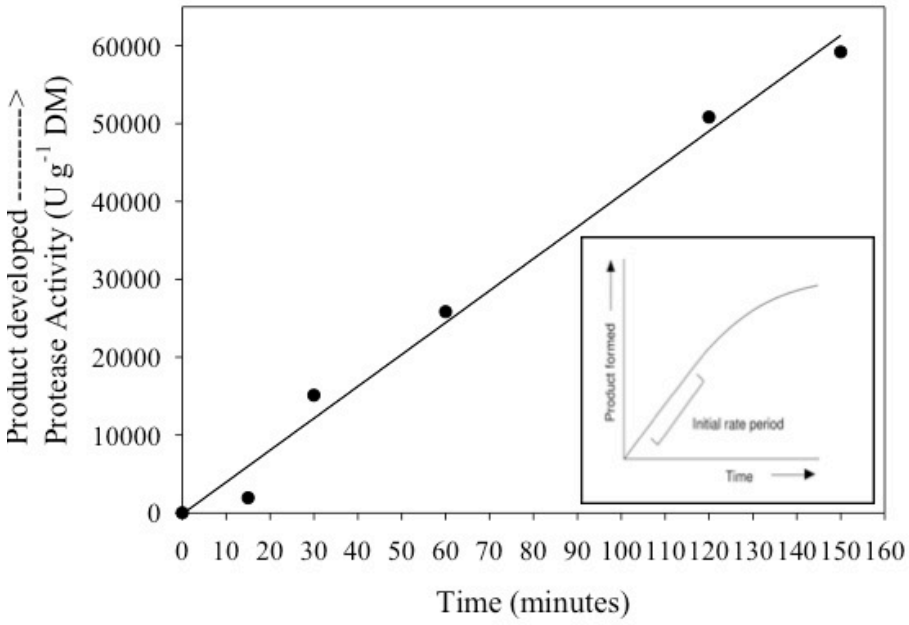

Figure 4.4. Enzyme activity in solid samples related to the incubation time on hydrolytic casein assay. The inside figure corresponds to a progress curve for a typical enzyme reaction.

Moreover, considering the protease activity determined on solid samples and extracts, the activity on solid samples was less than the one determined on extracts samples (7282 U g^{-1} DM and 14680 U g^{-1} DM respectively). These results pointed out that the buffer extraction was a suitable way to recover the desired enzyme from the solid matrix. On one hand, the nature of this kind of enzymes is to be soluble in water and on the other hand, by extracting them with a low ionic strength buffer (50 mM) the solubility is increased because of salting-in effect.

The fact that much more enzyme activity is obtained in the extract than in the solid matrix contributes to the separation of the enzymes, one of the products obtained, and its availability for future uses. Henceforth, the determinations of enzyme activity were realized on crude protease extracts.

Contrarily, the production of another enzyme, a lipase, by SSF from industrial fat residues performed by a colleague indicated that the extraction was not successful due to the nature of the enzyme (Santis, 2013).

SSF trials in 4.5 L reactors with soy fiber

Figure 4.5 shows the SSF process evolution during 14 days at 4.5L reactors for S (up) and SC (down) experiments. The on-line profile of temperature and % oxygen content describes the biological activity while more information such as pH and the alkaline protease activity were punctual determinations when sampling. One replicate is shown as the difference in the temperature profile between replicates was below 5% in both cases (area below temperature curve was 457 and 477ºC day^{-1} for S replicates, 501 and 520ºC day^{-1} for SC replicates). Further discussion on repetition of SSF is presented in chapter 6, 'Comparison of SSF experiments performed at 4.5 L reactors'.

The increase in temperature was caused by the biodegradation of organic matter. A secondary temperature increase was observed after homogenizing the reactor contents when sampling at days 3, 7 and 10. The results of the oxygen concentration gradient indicated that the reactors were fully aerobic during the entire process. The $sOUR_1$ was 1.9 and 1.02 g O_2 kg^{-1} DM h^{-1} for S and SC, respectively.

The highest protease activity coincided with the thermophilic phase, which was higher in experiment S (47331 ±1391 U/g DM) than in SC (18750 ±1596 U/g DM). In many cases, the time required for the optimum protease production by bacteria or fungus may be as long as 48 h to 9 days (Germano et al., 2003, Mahanta et al., 2008, Mukherjee et al., 2008). These results suggested that the inoculation of different microorganisms from the compost did not increase the production of alkaline proteases and that the autochthonous microorganisms developed on soy fiber are more efficient for protease production. These values are similar or bigger than the ones referred by other studies described at Table 1.1 in chapter 1.

The results also showed that enzyme synthesis coincided with the increase in the temperature and pH that are inherent to an adiabatic biodegradation process of biodegradable organic matter (Haug, 1993).

Additionally, the DRI of the final material at 14 days were 0.92 and 0.58 g O_2 kg^{-1} DM h^{-1}, indicating enough stability of the organic matter and the possibility to be used as a soil amendment, as referred by Ponsá et al. (2010b) and Barrena et al. (2014).

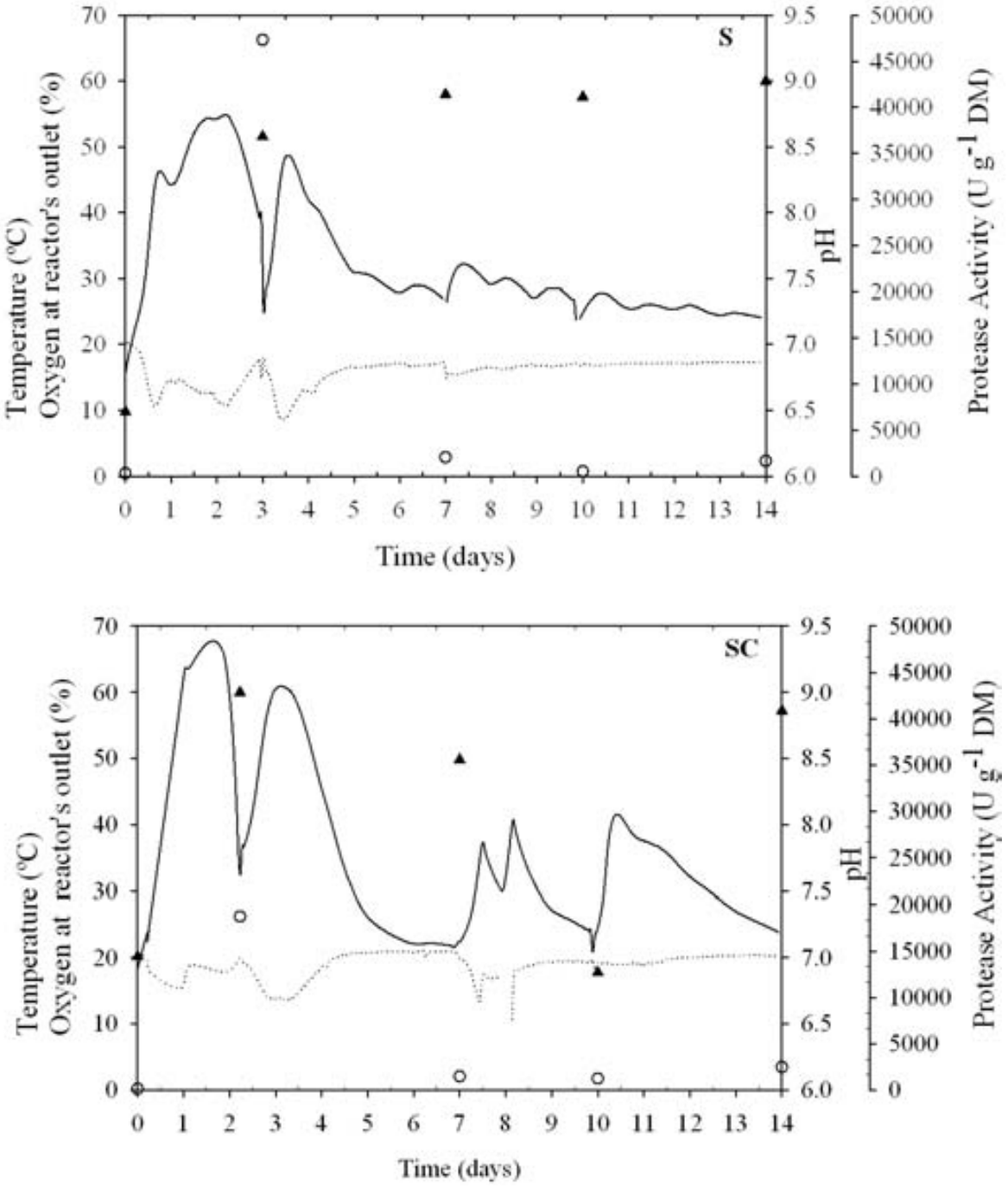

Figure 4.5. Solid-state fermentation profiles of soy fiber (S) and soy fiber with 10% compost (SC). Temperature (—, solid line), oxygen content in exhaust gases (···, dotted line), pH (▲) and protease activity (O).

Biochemical characterization of crude extracts from soy fiber

Effect of pH and T on crude enzyme extracts stability

Temperature and pH are the most relevant environmental factors affecting enzyme performance. These two variables affect enzyme activity and stability as well. The enzyme stability is considered as the capacity of the enzyme to preserve its activity. The denaturation of the enzymes involves structural changes such as covalent and non-covalent bond disruption and changes in their active site so it comprises the folding of the protein and also their three-dimentional structure, altering the enzymatic activity (O'Fágáin 2003).

Figure 4.6 shows the response surface for residual activity as a function of T and pH. Table II.1 in Anex II also shows the experimental conditions tested for each crude extracts and the residual activity. Alkaline protease activity decreased after incubation for all the tested combinations. However, due to the high initial activity level, the enzyme extract still presents a high proteolytic potential.

For the two samples analyzed, the best fitting obtained from the experimental design was a full second-order polynomial function. Equations (4.1) and (4.2) were obtained to describe enzyme stability (best fitting obtained from the normalized experimental values, statistically validated through F test) for S and SC, respectively:

$$\text{Residual activity (\%)} = 24.71+8.33pH-12.17T-0.63pH^2-11.13T^2-5.00pHT \quad \text{(Eq. 4.1, } R^2=0.92\text{)}$$

$$\text{Residual activity (\%)} = 27.63+12.67pH-11.50T-1.63pH^2-6.88T^2-8.23pH\cdot T \quad \text{(Eq. 4.2, } R^2=0.95\text{)}$$

The interaction term pH-T in equations 4.1 and 4.2 highlights the cross effect of pH and temperature on stability. Negative coefficients of T indicate a negative effect of high temperatures on activity.

When the development of the microoganisms in the solid matrix is taking part in alkaline and high T conditions, the enzymes produced could be thermostable and alkaline in nature as a microorganism' strategy for growth (Rai et al., 2009, Santi et al., 2011, Subba Rao et al., 2009, Zanphorlin et al., 2011).

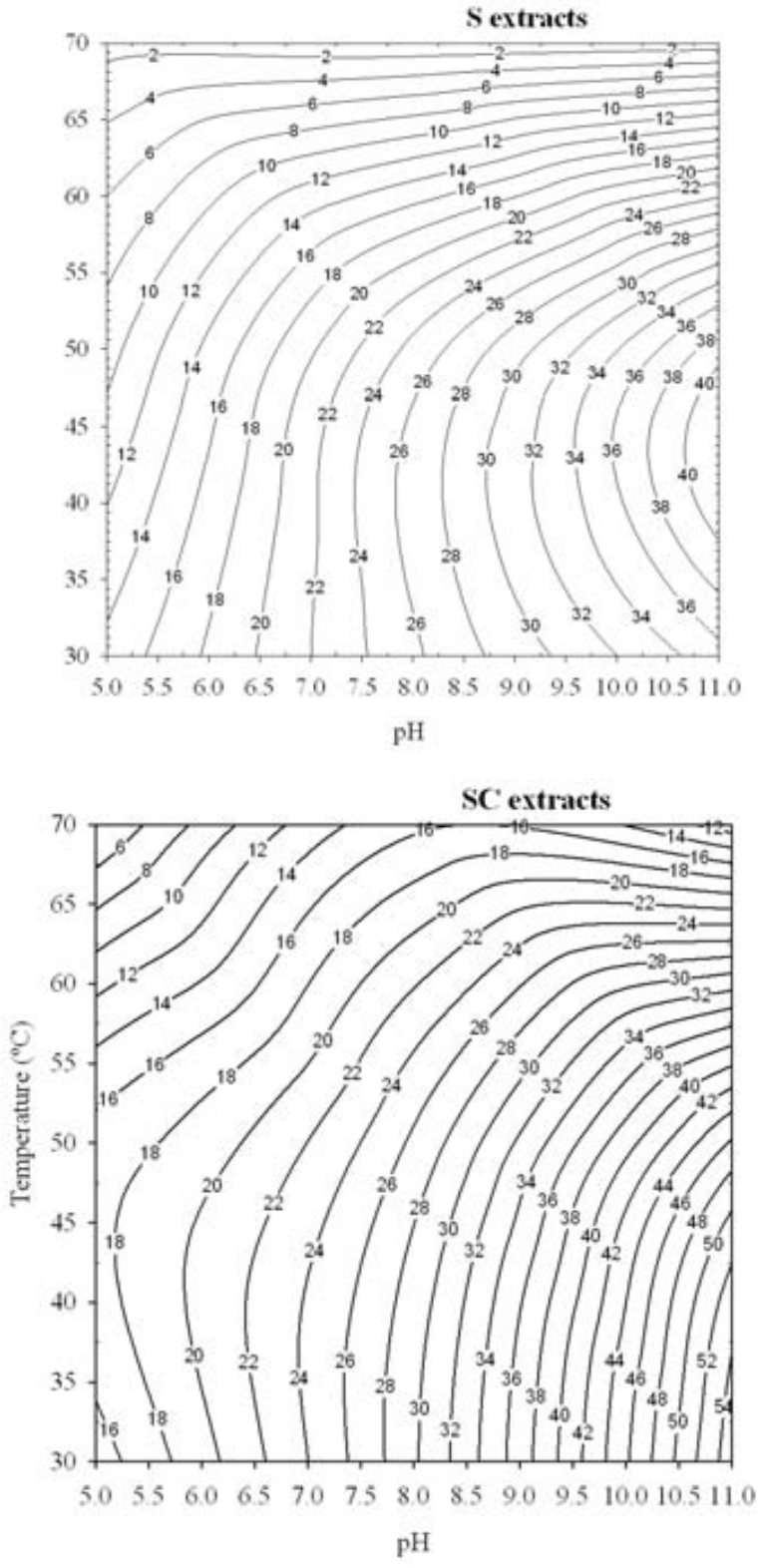

Figure 4.6. Response surface for residual activity (%) in stability assays for S and SC extracts.

As seen in Figure 4.6, the enzyme was more stable at alkaline pH and the optimum was pH 11, confirming that the obtained enzymes were alkaline proteases. Although a thermophilic stable enzyme was expected, the best results for thermal stability were obtained at 43.8ºC for S and 30ºC for SC samples (37% and 51% residual activity at optimal conditions, respectively). The decline in enzyme activity post 1 h incubation might be due to slow denaturation and/or decomposition of protease (Uyar et al. 2004).

Alkaline protease conservation

Many times, after the production of the enzymes and the following utilization it happens a determined time so that they should be stored until use. For that reason, a protocol of conservation of the enzymes until its use was defined by determining the protease activity after days, weeks and months at different temperatures (4, -20, -80 ºC) and also different procedures (store in cold conditions and dehydration under vacuum such as lyophilization).

The results obtained from the conservation methods tested showed that the activity of the alkaline protease at 4ºC and -20ºC was constant for only one week of storage. At -80ºC, the activities were stable (90%) for at least three months (Figure 4.8). For the lyophilized samples, the activity was constant for one month (Figure 4.8). Besides, the protease was not damaged during lyophilization process because of continuing with the same activity. After one month, the stability seemed to be committed because the protease activity increases, indicating the possibility of degradation of the enzymes. An explanation could be that these lyophilized samples were not well conserved because the samples were found with humidity. A suitable storage in a cold and dry place was the next step to increase conservation of lyophilized samples. Other studies have shown that proteases are unstable in several conditions, and that immobilization is the only good alternative for preserving their stability in the long term (Ó'Fágáin 2003, Silva et al. 2006).

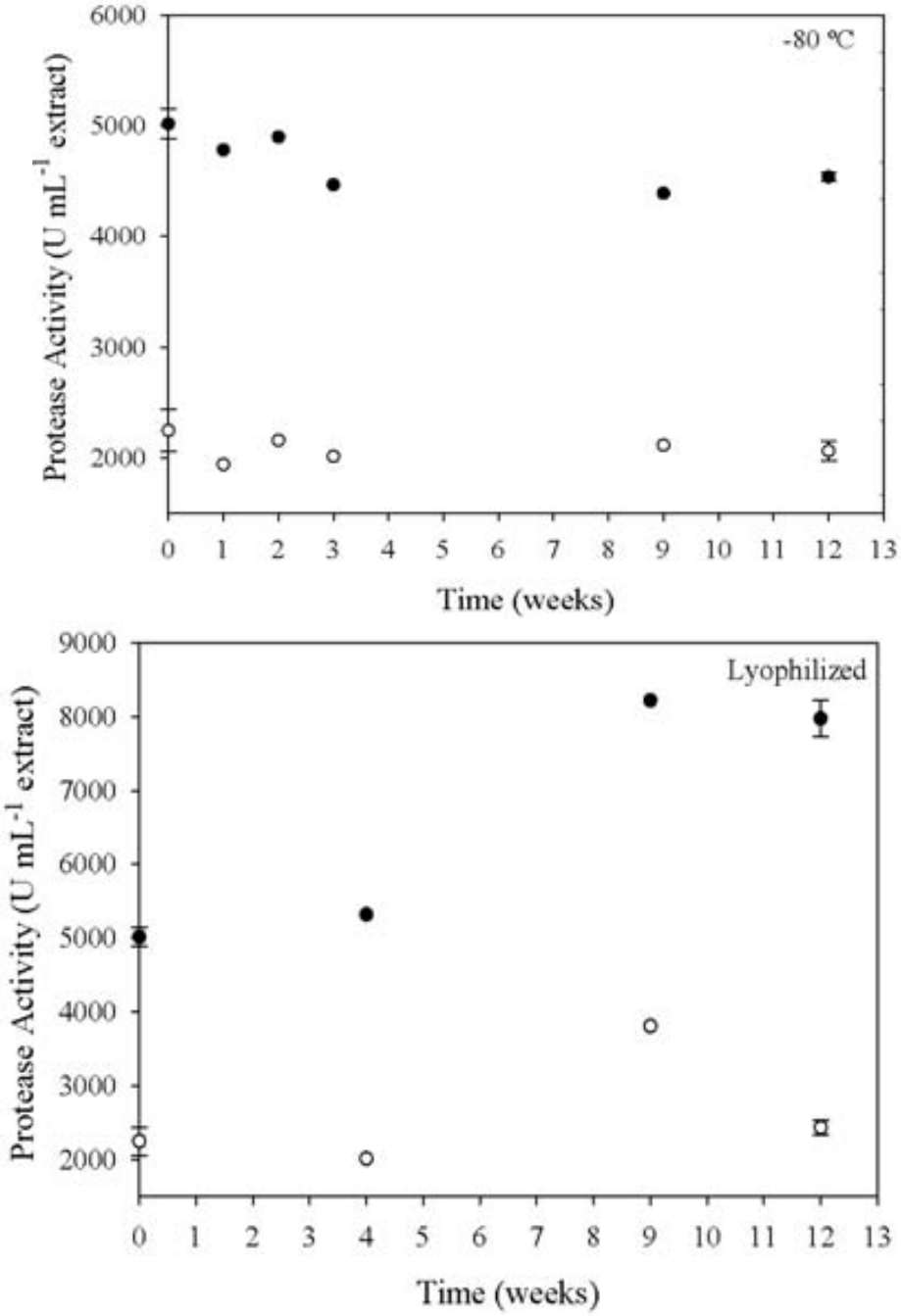

Figure 4.8. Conservation studies for S (full circle) and SC (empty circle) extracts.

Besides, a SEM image was performed with the lyophilized samples. The Figure 4.9 shows the matrix of enzymes present in the lyophilized and also white spots, which represent the presence of inorganic salts. These salts could come from buffer extraction and also another salts extracted

from the process together with enzymes and other soluble particles smaller than 0.45 μm. These salts could play a role in stabilization of the enzymes.

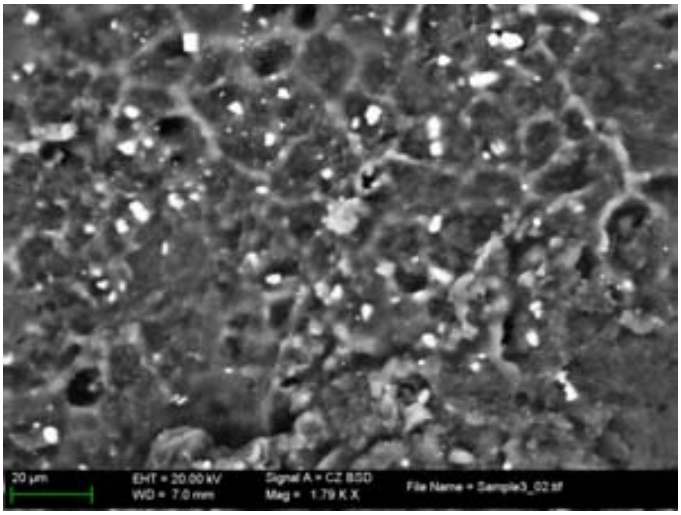

Figure 4.9. SEM image of the lyophilized sample. Magnification: 2000X.

Separation of proteases from extracts using PAGE

Numerous studies have reported the production and the following purification of the enzyme obtained by either SSF or SmF (Rai et al., 2009, Zanhorlin et al., 2011, Jain et al., 2012). In this case, only PAGE was utilized to separate the enzyme/s and identify the possible MW of the enzyme/s generated but no purification was done, only a few experiments in fast protein liquid cromatoghaphy (FLPC) but with no good results due to problems with the ancient machine. Even though the purification of the enzyme is interesting, most of the time it leads to expensive techniques. As commented before, this research is a first approach in production and further application of the proteases so that the aim was to use the extracts without purification to evaluate a low cost production and possible uses. Once good results were obtained, the optimization and purification, if needed, will be developed. Information regarding this point will be explained in conclusions and future research.

The PAGE technique was performed several times in order to determine the type of gel, a proper quantity of sample, sample buffer and the time of running. Also was probed with two different dyes (Coomassie Blue and Silver stain). Several PAGEs were made without good results or without enough clarity to confirm proteases evidence. Generally, this technique is used

with purer and concentrate solutions of the protein desired; however in this case, with a crude extract, it was more difficult to obtain good results and also analyze the information obtained. As an example, figure 4.7 shows results obtained by a SDS-PAGE with lyophilized samples from S and SC, stained with Coomasie Blue. The gels done with extracts presented very weak bands (not shown) while the gel done with the lyophilized samples, showed several clear bands. This fact suggested that the enzymes in the extracts are far diluted and the need to be concentrated by lyophilizing or by another known method but not purification to continue with the idea of reducing costs. On the other hand, the gel with the lyophilized samples showed bands. The lyophilized initial samples (L0) showed high quantity of proteins in the sample and some clear bands, more marked at final samples (L14). The lyophilized thermophilic samples (L3) showed no visible bands and the L14 showed some bands around 100 KDa and 20 KDa. Taking into account the results in protease activity, these bands correspond to proteins and enzymes from extracts that were not the desired enzymes. More assays should be made to obtain a suitable characterization of PM of the proteases obtained by SSF.

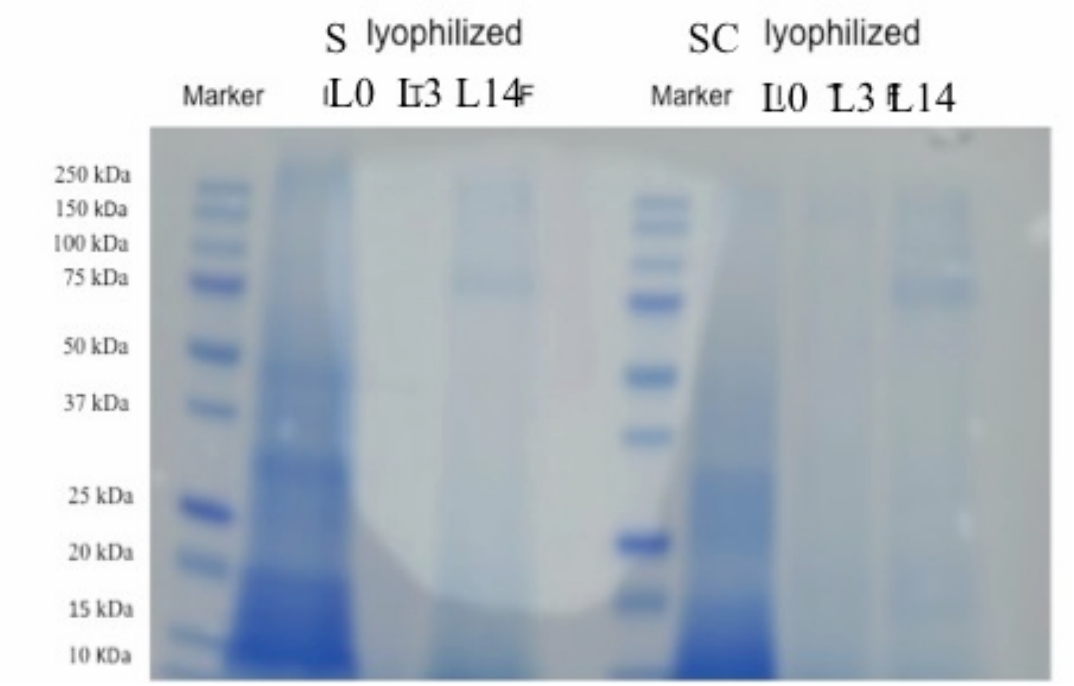

Figure 4.7 SDS-PAGE of S and SC lyophilized samples. M: marker, L0: initial; L3: thermophilic and L14: final lyophilized samples.

SSF trials in 10 L reactors with coffee husk

Although this residue had no high quantities of activity at lab scale, comparing with soy fiber or hair waste, this experiment was performed at bench scale too. The objective was to screen the protease production together with the cellulases production. In this sense, this experiment was performed together with a colleague group who was doing her Msc. research, Cerda-Llanos (2013). Other objective was to assess the effect of the scale-up and no control of temperature with the new 10 L reactors and the OUR controller recently installed. Although this experiment is presented here for better discussion of the results, it was performed at lab after the others with soy fiber and hair waste at 4.5L reactors.

Figure 4.10 shows the evolution of SSF process of CH and CHC respectively, in terms of temperature and % of oxygen content. Also punctual chemical determinations as the pH and the activity of the alkaline protease are shown. The temperature and pH increased as happened in S and SC experiments explained before because of the biodegradation of organic matter by the microorganisms developed as in any typical degradation process.

A secondary temperature increase was observed after homogenizing the reactor contents when sampling at days 3, 7 and 10. The results of the oxygen concentration gradient marked that the reactors were under aerobic conditions during almost all the process. It was observed a decreased until 0% of the oxygen during thermophilic phase in both reactors. However, when this was resolved by adjusting the oxygen controller, the oxygen content began to rise and the thermophilic conditions were restored, which guaranteed metabolic activity. The $sOUR_1$ was 2.71 and 2.09 g O_2 kg^{-1} DM h^{-1} to CH and CHC, respectively.

Regarding the protease activity profile, the highest production was observed at day 7 of SSF being biggest in CH rather than in CHC experiments. The IRD at 7 days of SSF were 0.44 and 0.38 g O_2 kg^{-1} DM h^{-1} for CH and CHC respectively, indicating enough stability of the organic matter.

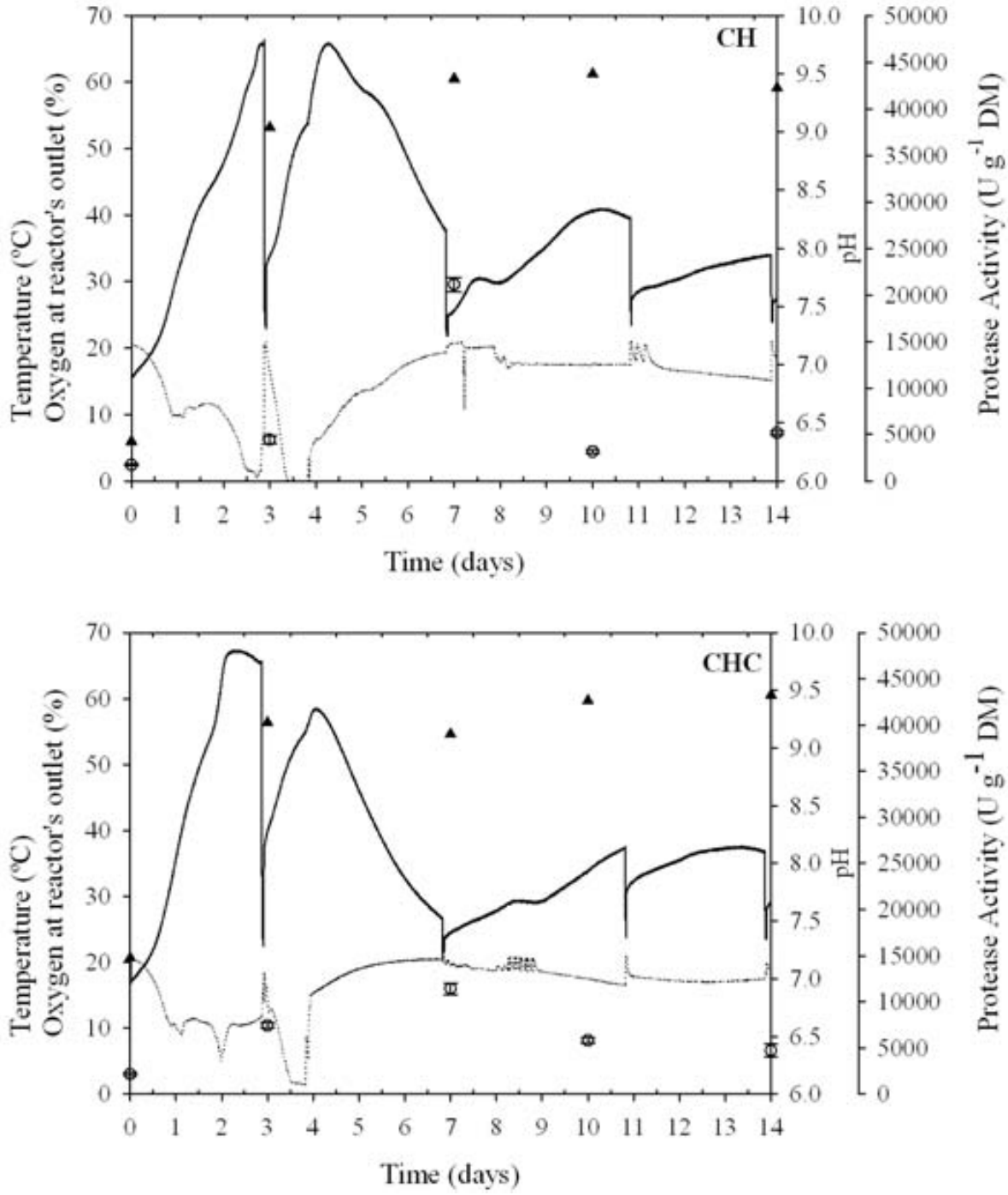

Figure 4.10. Solid-state fermentation profiles of coffee husk (CH) and coffee husk with 10% compost (CHC). Temperature (—, solid line), oxygen content in exhaust gases (···, dotted line), pH (▲) and protease activity (O).

As with soy fiber experiments, the option of adding compost as an inoculum showed no increments in the protease production. Maybe the use of this residue to produce proteases is not adequate, even inoculating microorganisms from compost. Likewise, this residue corresponds to the one with less quantity of nitrogen. However, other metabolites were obtained; the cellulases activities obtained were pretty high (Cerda-Llanos 2013).

A study by Shemekite et al. (2014) revealed that co-composting of coffee husk with cow dung produced higher degradation of organic matter than coffee husk alone or even mixed with vegetables or fruits, and also produced several different enzymes from proteases.

4.4 Conclusions

All the three nitrogen content residues obtained from the local food and tanning industries showed production of proteases while degrading the organic content by SSF in preliminary studies at laboratory scale. However, the soy fiber residues seemed to be the best suitable source for alkaline protease production. In consequence, was used to settle the hydrolytic casein assay conditions. This assay used to determine the protease activity on these residues seemed to be adequate by incubation of 2 hours at 50 ºC with 2% of fresh casein solution.

Furthermore, the protease activity generated during the SSF process was evaluated in solid samples and extracts samples with 50 mM HCl-Tris buffer. More activity (the double) was detected in extracts than in solid samples. Accordingly, the protease activity was determined in extracts samples in the next chapters. This contributes to the separation of one of the product from the solid matrices for future uses in any application.

Later, the same three residues were performed at 4.5 L reactors. However, in this chapter, soy fiber and coffee husk were evaluated while in the next chapter the third one, the hair waste, is described together with an application of the crude enzyme extracts. The soy fiber and coffee husk fermentation results revealed that the SSF was successful without sterilization of the residues, no addition of microorganisms and no temperature control. This suggested that the SSF process could be easily scalable. Also the addition of compost (specific microorganisms) did not increment the protease production with none of these two residues.

On the other hand, the characterization of the soy fiber fermented extracts suggested that this enzyme is more stable at alkaline pH and mesophilic temperatures and can be preserved for at least three months by freezing at -80ºC or by lyophilization. Besides, lyophilization seemed to be

good in order to concentrate the enzymes obtained. Further studies are necessary to confirm the possible uses of this alkaline protease and its full characterization.

Chapter 5

Production of proteases from hair waste and sludge under SSF at 4.5L reactors. Application in dehairing as an alternative to chemical process.

Part of this chapter has been published at Journal of cleaner production 74, 191-198. 2014. Substitution of chemical dehairing by proteases from solid-state fermentation of hair wastes.
Abraham, J., Gea, T., Sanchez, A.

Part of this chapter has been presented as co-author on an international conference presentation; Wastes: Solutions, Treatments and Opportunities. 2013, Portugal. From waste to product: Enzyme production from industrial organic wastes by solid-state fermentation and its use in environmental applications.
Abraham, J., Cerda, A., Santís, A., Artola, A., Barrena, R., Font, X., Gea, T., Sánchez, A.

Summary

This chapter consists on the valorization of hair waste together with sludge from wastewater treatment by solid-state fermentation process to obtain proteases as seen in chapter 4. SSF was undertaken in 4.5 L scale reactors. Neither sterilization of the materials nor inoculations of pure microorganisms were necessary for the development of the process, whereas aeration was provided during the assay to ensure the prevalence of aerobic conditions. Alkaline proteases were produced as a consequence of the degradation of hair by microorganisms present in sludge. Potential application of the extracted proteases from hair wastes and soy fiber residues in dehairing cowhides was proved as an alternative to the chemical dehairing process. The results in the efficiency of the dehairing process were similar to those obtained with the chemical process, when assayed with hair wastes extracts. Thus, avoiding the need of chemical reagents and strong chemical conditions. The entire process permits the substitution of the chemical process of dehairing by an environmentally friendly enzymatic process, closing the organic matter cycle.

5.1. Materials

The materials used in SSF were hair wastes from a local tanning industry and raw sludge (33.02% of DM, 84.30% of OM and DRI of 3.42 g O_2 kg^{-1} DM h^{-1}) from a municipal wastewater treatment plant (both located in Barcelona, Spain). Table 5.1 shows the main characteristics of hair waste. Wood chips were used as bulking agent in SSF experiments. Cowhides from the same tanning industry were used in the dehairing experiments with the proteases obtained from the two different extracts.

5.2 Experiments

Mixtures

Raw sludge was mixed with hair waste in ratio 2:1 (wet weight ratio) to start up the biodegradation as determined in previous studies (Barrena et al., 2007a,b) and also previously explained in Chapter 4. Bulking agent was mixed in a ratio 1:1 (v:v) to provide the proper porosity and to maintain aerobic conditions during the SSF process (Ruggieri et al., 2009). All mixtures were manually prepared with materials at room temperature and were fermented

without prior sterilization. Table 5.1 also shows the main characteristics of the initial mixture. Figure 5.1 shows the appearances of the residues and the mixture before performing the process.

SSF

Approximately 1.5 kg of mixture was fermented for 21 days in 4.5 L near-to-adiabatic reactors, working under near-adiabatic conditions and continuous aeration (0.1 L min^{-1}). Water content of the mixture was adjusted to be within the recommended values by adding tap water during the experiments, when necessary. Samples were collected at 0, after 24 hours of thermophilic temperature (about 3 days), 7, 14 and 21 days of process time after manual homogenization of the entire mass in the reactors to obtain the profile of the process and the proteases production. The experiment was undertaken in triplicate. A duplicate of the process was undertaken after three months of the first SSF experiment to evaluate the reproducibility of the process.

Figure 5.1. Appearance of the residues and the mixture prepared for SSF

Table 5.1. Characterization of the hair waste and the initial mixture assayed.

Parameters	Hair waste	Initial Mixture
Water content (%, wb)	59.9 ± 0.4	44.7 ± 0.6
Organic matter (%, db)	86.0 ± 0.1	88.6 ± 0.9
Dynamic respiration index (g O_2 kg^{-1} DM h^{-1})	3.0 ± 0.4*	1.3 ± 0.2
Cumulative oxygen consumption (4 days, g O_2 kg^{-1} DM)	160 ± 12*	66 ± 0.5
Total Nitrogen Kjeldhal (%, db)	12.0 ± 0.1	8.3 ± 0.5
Total Organic Carbon (%, db)	57.2 ± 0.9	64 ± 3
C/N ratio	4.8	7.7
pH	10.76 ± 0.01	6.85 ± 0.01
EC (mS cm^{-1})	5.03 ± 0.01	2.38 ± 0.01
Free air space (FAS, v/v)	n.a	0.73

EC: electrical conductivity, wb: wet basis, db: dry basis, DM: dry matter. n.a: not analyzed.
* Ratio 1:2 hair waste: raw sludge (w/w).

Dehairing assay of cowhides

Originally the potential on dehairing cowhides experiments were designed according to bibliography (Riffel et al., 2003, Tiwary et al., 2010 and Sundararajan et al., 2011). These experiments were evaluated by testing different conditions and the successful one is following described. Wet-salted cowhides were washed and cut to the same area (10.68 cm^2). Then they were incubated, with 15 mL of enzymatic crude extracts or 15 mL of 50 mM HCl-Tris buffer (pH 8.10) (control) at 37ºC for 24 h on a rotary shaker at 120 rpm. 2% of sodium azide was added to inhibit the microorganisms' growth and to determine only the effect of the enzyme on dehairing. The protease activity and the soluble proteins were determined at 0 and 24 hours of incubation. Hides with the same area were treated sequentially with the following chemicals: sodium carbonate (15 mL, 0.3%, w/v) with a non-ionic surfactant (0.3%, w/v) (soaking, 20 h), calcium hydroxide (50 mL, 1%, w/v, 1 h) and sodium hydrosulfide (50 mL, 1%, w/v, 30 min in orbital agitation) to simulate the chemical dehairing process used in tannery industries. Samples treated with enzymes, chemicals and controls were assayed in triplicate. Dehairing efficiency was evaluated by scraping gently with tweezers all the hides. After this, the determination of the hair

mass for each treatment was evaluated according standard methods for total suspended solids (APHA, 1995). The treatment efficiency for enzyme and control experiments were referred to a percentage of dehairing obtained in the chemical treatment.

The same full factorial experimental design previously described at chapter 3, for protease stability was used to determine the optimal pH and temperature for the dehairing enzymatic process. In this case, pH was 8.5, 10 and 11.5 using the same buffers than those of protease stability and temperature was set to 20, 35 and 50ºC.

Enzymatic activities

Alkaline protease activity was determined as described in chapter 3, section 3.4.2. Other activities such as keratinase and collagenase were also determined in this study. Keratinase activity was determined by using the method described by Rai et al. (2009). 1.0% (w/v) of native sterilized chicken-feather was used as a substrate to incubate the crude extracts at 45ºC for 15 min. Finally, the addition of 0.5 ml of 10% (w/v) ice-cold trichloroacetic acid was added to stop the reaction. Digested protein in the supernatant was determined colorimetrically by using the Folin-Ciocalteu's reagent (Lowry et al., 1951). One unit of keratinase activity (U) is defined as the µg of tyrosine liberated under the assay conditions.

Collagenase activity was determined according to the methodology proposed in previous works (Nilegaonkar et al., 2007). Briefly, it consists of incubating 1 mL of the extract with 4 mg of azocoll reagent (Sigma Aldrich) in buffer HCl-Tris pH=8.10 at 70ºC for 1 hour and 200 rpm. The reaction was stopped with 15% trichloroacetic acid. Then samples were centrifuged at 8500 rpm for 10 min and the absorbance of the supernatant was measured at 520 nm. One collagenase unit is defined, as the amount of enzyme required increasing 0.1 units of absorbance per minute at 70ºC

5.3. Results and Discussion

SSF process performance

Figure 5.2 shows the temperature, the percentage of oxygen at reactor's outlet and pH during the entire SSF experiment at bench scale (21 days) for one of the three replicates. One replicate is shown as the difference in the temperature profile was below 4 % in between replicates. All the

graphics of replicates are presented and discussed in next chapter, Comparison of SSF experiments performed at 4.5 L reactors.

As observed, the temperature and pH profiles were similar to that of composting; in which both parameters rise due to the biodegradation of the organic material, as observed in previous SSF and composting experiments with hair wastes (Barrena et al., 2007a,b). The oxygen levels in the reactor showed the prevalence of the aerobic conditions during the entire process.

The profile of alkaline protease activity determined at the sampling days is also shown in Figure 5.2. The activity was measured in the obtained crude extracts (initial extract: E0, extract day 3: E3, extract day 7: E7, extract day 14: E14 and extract day 21: E21). The highest activity of the enzyme was observed at 14 days of the SSF process in E14. Similar previous experiments using soy fiber residues showed the highest activity at 3 days of SSF, after 24 h of thermophilic temperatures. These results confirm the difference in protease production due to the different biochemical composition of materials when treated by SSF.

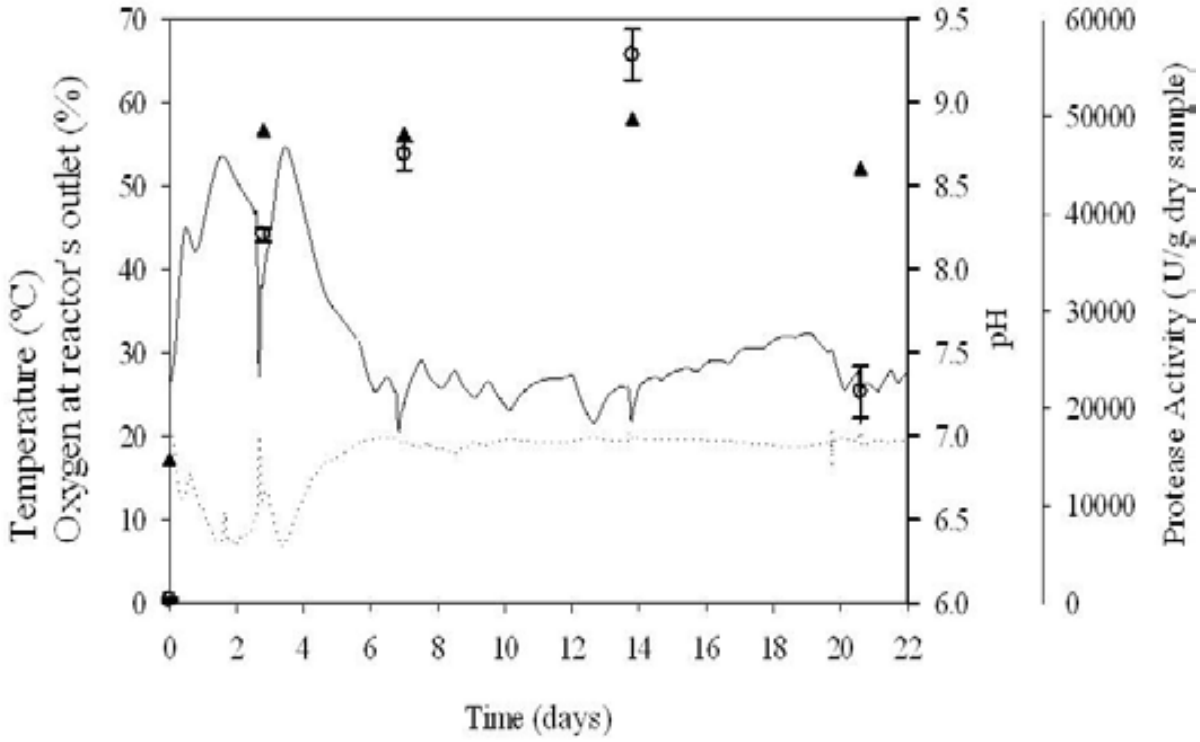

Figure 5.2. Solid-state fermentation profile. Temperature (—, solid line), percentage oxygen content in exhaust gases (···, dotted line), pH (▲) and protease profile (○).

Keratinase activity was determined in the extracts and the results showed practically no keratinase activity increase in the process (E0: 1520 UA/g DM, E3: 1780 UA/g DM; E7: 0 and E14: 400 UA/g DM). These results are in agreement with previous studies on the properties of dehairing proteases (Tiwary and Gupta, 2010) although some other studies have shown the relationship among keratinase and dehairing activities (Pillai et al., 2011, Srinivas and Naik, 2011). Additionally, collagenase activity was not detected during the experiments in agreement with other studies (Nilegaonkar et al., 2007). This activity is undesirable in the dehairing process, as it causes damage on the skin matrix (Jian et al., 2011).

Additionally, the DRI of the mixture was determined at 14 days of SSF process and its value (0.63 $gO_2\,kg^{-1}DM\,h^{-1}$) indicates the complete stability of the residue, as with soy fiber and coffee husk mixtures.

Figure 5.3 shows the aspect of the mixture after 14 days of SSF. Several organisms have been developed covering the mixture with a good looking white layer. Although these microorganisms weren't carefully analyzed, visual aspects suggested that they were the typical ones as developed in a degrading process such as composting (Soliva 2001).

Figure 5.3. Appearance of the mixture when sampling at day 14th of SSF.

Biochemical characterization of crude extracts

Extraction

The solid and liquid samples of day 14 (when the highest alkaline protease activity was detected) were analyzed to determine the enzyme activity in both phases (Table 5.2). As expected, considering the affinity of the enzyme to water and previous results with soy fiber

residues, higher alkaline protease activity was found in the crude extract indicating that buffer extraction is an efficient way to recover and concentrate the desired enzyme from the solid mixture as well.

Table 5.2. Summary of determined protease activity in solid and extract samples.

Sample	**PA (U mL^{-1} extract)**	**PA (U g^{-1} wet sample)**	**Protein (mg g^{-1} wet sample)**	**sPA (U mg^{-1} SP)**
Solid	-	15379±784	170±0.02	90
Extract	6313	31565±1961	0.85±0.01	37135

U: units, PA: protease activity, sPA: specific protease activity.

As seen before with the soy fiber protease samples, the activity measured expressed in U g^{-1} dry or wet samples were the double in extracts samples than in solids samples. In this case, the sPA was also measured in order to obtain a more specific value, and this sPA in extracts samples resulted bigger than solid samples too.

Effect of pH and T on protease stability

Figure 5.4 shows the response surface of the residual activity for the three crude extracts assayed to check their stability under different T and pH conditions, as performed with soy fiber crude protease extracts. Table II.2 in Annex II also shows the experimental conditions tested for each crude extract and the residual activity.

Equations 5.1, 5.2 y 5.3 were obtained to describe enzyme stability (best fitting obtained from the normalized experimental values, statistically validated through F test) for E3, E7 and E14, respectively:

$$\text{Residual Activity (\%)} = 28.86 + 19.3pH - 10.83T + 8.87pH^2 - 14.62T^2 - 8.25pHT \quad \text{(Eq. 5.1, } R^2=0.92\text{)}$$

$$\text{Residual Activity (\%)} = 25.46 + 14.16pH - 11.83T + 4.63pH^2 - 11.37T^2 - 11.5pHT \quad \text{(Eq. 5.2, } R^2=0.96\text{)}$$

$$\text{Residual Activity (\%)} = 29.5 + 16.83pH - 17.16T + 5pH^2 - 14T^2 - 13.25pHT \quad \text{(Eq. 5.3, } R^2=0.96\text{)}$$

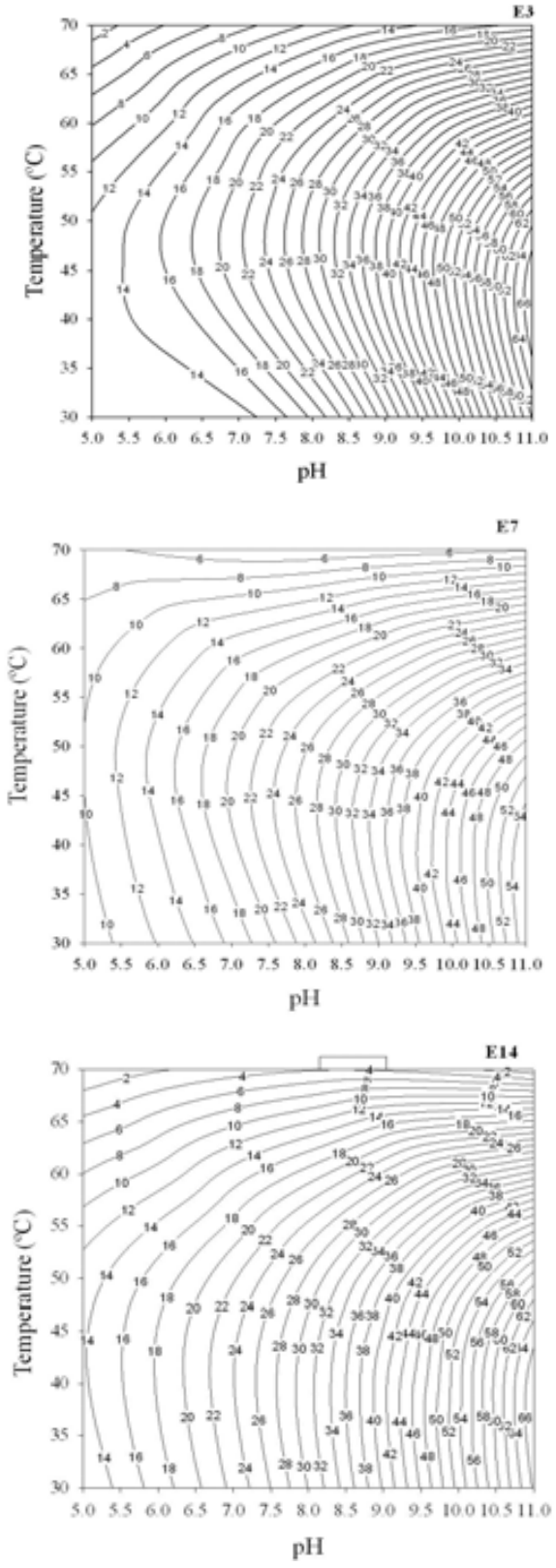

Figure 5.4. Response surface for residual activity (%) in stability assays for E3, E7 and E14.

As with soy fiber extracts the interaction term pH-T in these equations highlights the cross effect of pH and T on stability. Negative coefficients of T indicate a negative effect of high temperatures on activity.

According to the results, enzymes were more stable at pH 11 confirming that the obtained enzymes were alkaline proteases. The best results for thermal stability were obtained at 50ºC, 30ºC and 30ºC for E3, E7 and E14, respectively. Objective function (residual activity) was 63.3, 56.2 and 67.8% at the optimal value, respectively. A thermostable enzyme could be expected if it is produced by microorganisms that grow at thermophilic temperatures as the alkaline protease produced at E3, whereas mesophilic enzymes are to be produced at mesophilic temperatures such as those of E7 and E14 (Abraham et al., 2013, Zanphorlin et al., 2011). In this case, protease in crude extract E3 showed good stability both at mesophilic and thermophilic temperatures.

PAGE

Figure 5.5a shows the Native-PAGE and Figure 5.5b the zymogram for the different crude extracts analyzed (E0, E3, E7, E14 and E21). The same results were obtained in four gels. Even though the bands remained weak as with soy fiber extracts; here, they were marked enough to be distinguished. As observed, several bands appeared with different molecular mass and particularly at E14 the highest quantity of bands was detected. Besides that, the absence of blue color at the zymogram indicates that the bands correspond to that of a catalytic protease activity. These results coincided with the protease activity detected at each extract and the evolution of protease activity (Figure 5.2) and dehairing results. Previous studies (Dayanandan et al., 2003, Nilegaonkar et al., 2007, Kandasamy et al., 2012) have inoculated specific microorganisms to produce selected enzymes, whereas in this work there are different microbial populations growing (probably from the initial microbial consortia from raw sludge) and producing enzymes. For this reason, it should not be expected to find only a defined band in a Native-PAGE.

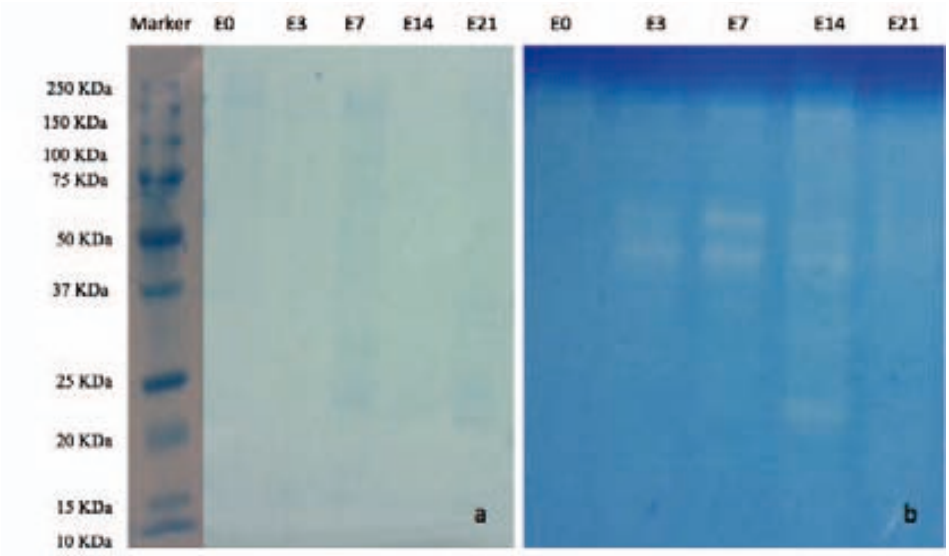

Figura 5.5. Polyacrylamide gel (12%) electrophoresis of crude extracts (15 µL) (n=4). (a) Native-PAGE, (b) Zymogram.

Potential of the crude protease extracts in cowhides dehairing

Figures 5.6 and 5.7 show the procedure carried out to cut and prepare the cowhides and all the steps of the cowhides dehairing process, respectively. Wet-salted cowhides were cut into equal circles and desalted by soaking with tap water during 24 hours.

Figure 5.6. Preparing same areas of cowhides to assay dehairing.

The areas used to evaluate the dehairing were the same between treatments so that the quantity of hair could be assumed the same between cowhides. Consequently, the quantity of hair recovery could be calculated and then, all the hair removed from each treatment related to the chemical treatment.

Table 5.4 presents the results obtained for dehairing treatments using the proteases produced by SSF. After 24 hours of incubation, the hides treated with the E7, E14 and E21 showed an easier removal of hair when mechanically scraped with tweezers compared to control experiment with no protease.

Figure 5.7. Incubating, scraping, filtrating and obtaining the hair mass when dehairing at lab.

Contrary, E3 showed no hair removal as in control experiments. These results suggest that the type of protease produced in the different stages have different dehairing activities. In addition, E14 showed more dehairing activity when compared to the rest of extracts and the same dehairing yield than the chemical process. This confirms that the enzymatic treatment can be a good alternative to chemical dehairing as pointed in others studies carried out on goatskins or buffalo hides (Tiwary and Gupta, 2010, Kandasamy et al., 2012, Saran et al., 2013).

Additionally, there was a good correlation between protease activity and dehairing capacity, as observed when E14 was diluted, which is in accordance with literature (Kandasamy et al., 2012). These results are consistent with other dehairing assays performed with extracts obtained from replications of the SSF process. In this sense, a replicate was done with a lyophilized extract obtained at day 14 of another SSF process performed 3 months later (E14' L, Table 5.4) and the percentage of removal obtained was 98%.

On the other hand, extracts of soy fiber samples at 3 days of SSF were also assayed for dehairing (E3-S, Table 5.4) and no good results were obtained. This extracts had relatively lower activity than the rest of extracts and also obtained low percentage of hair removal. This result suggested that higher activities are required to use the extracts in dehairing but also the proteases obtained from hair waste are more specific for dehairing than those obtained by soy fiber

residues. In this sense, as commented at chapter 1, enzymes have high specificity for the substrate and particularly, in the case of proteases from hair waste, they were generated from the degradation of the hair by the microorganisms with their specific proteases.

Although no mechanisms of dehairing with these enzymes was studied, an explanation would be that the enzyme could cause the weaken of hair's bulb more efficiently than the chemicals.

Table 5.4. Results of dehairing cowhides experiments for control, enzymatic and chemical treatments.

Sample	**Protease activity (U/cm^2)**	**Hair removal (%) with respect to chemical treatment (100%)**
Control	-	43
E3	4775 ± 1084	33
E7	4173 ± 286	81
E14	5491 ± 418	100
E14' (L)	5955 ± 690	98
E21	2181 ±153	58
E3-S	2369 ± 158	45
Chemical	-	100

E14' (L): lyophilized extract obtained after 14 days of SSF in another fermentation batch experiment, E3-S: extract of 3 days of SSF from soy fiber residues.

Figure 5.8a shows the SP detected in control experiments and the different crude extracts assayed in dehairing, whereas Figure 5.8b shows the remaining protease activity after the dehairing process. As observed in Figure 5.8b, the extract maintained the protease activity after 24 hours of incubation in all the cases. SP was determined at 0 and 24 hours of incubation (Figure 5.8a) and the results revealed the capability of the extracts for degrading the SP released during dehairing because of their protease activity content. Contrary, the SP released in the control extracts increased significantly because of the lack of proteases. These results suggested the capacity of the enzymatic treatment for dehairing hides while reducing the organic matter and total solids in effluents from tannery industries as referred by Thanikaivelan et al. (2004).

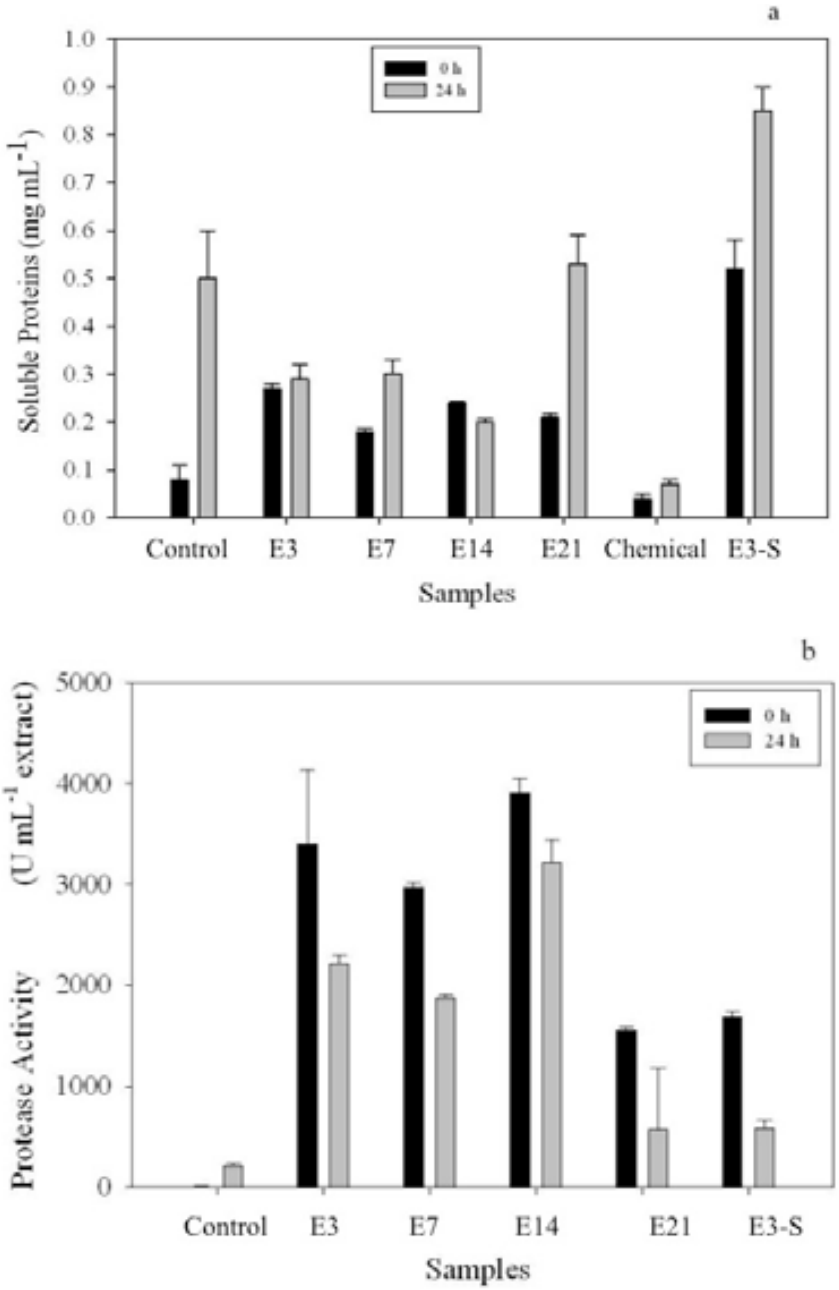

Figure 5.8. a) Soluble proteins in control experiment and different crude extracts assayed at initial and final (24 hours) of the cow hides enzymatic dehairing process. Initial and final soluble proteins levels in the last step of chemical dehairing process are also shown. b) Protease activity before and after dehairing process.

The scanning electron microscope images (SEM) of the unhaired samples after enzymatic and chemical treatments are shown in Figure 5.9 with a magnification of 200X and 500X.

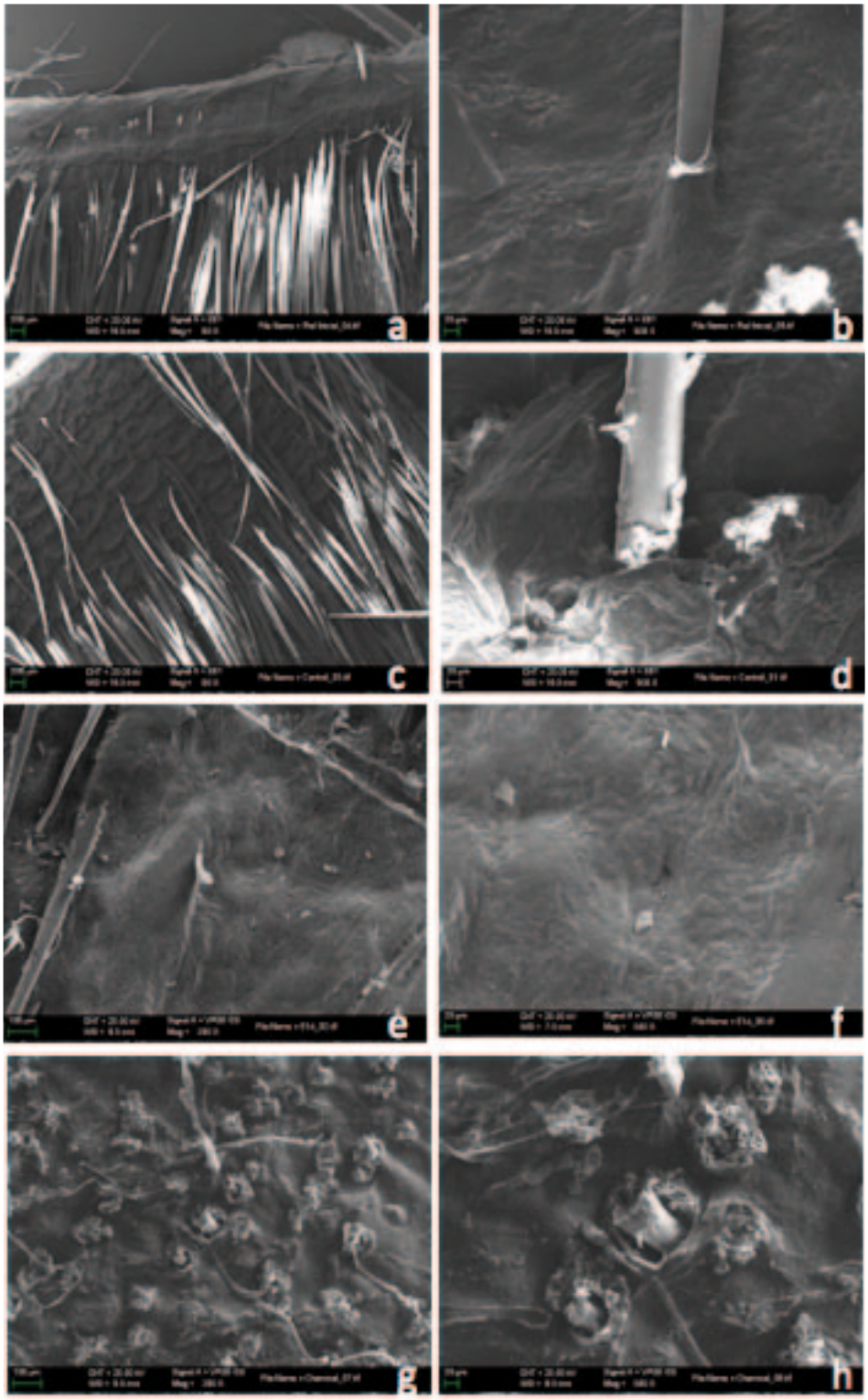

Figure 5.9. Scanning electron microscopy of initial hide (a,b), control hide (c,d), treated E14 hide (e,f) and chemically treated hide (h,g). 200X and 500X for left and right images, respectively.

The enzymatic treated hides appear cleaner and with no superficial damage when compared to the chemical treated hides. The latter also presented clear haircuts by the alkaline chemical compounds instead of being removed from the hair's root. A similar dehairing study by Sundararajan et al. (2011) showed comparable results on SEM images.

Finally, Table 5.5 shows pH and temperature effect on the enzymatic dehairing process with E14. Dehairing appears to be more efficient when increasing pH and temperature in the range of studied parameters; especially when temperatures are around 50ºC and pH around 10. Optimization of the best polynomial fitting to the experimental results showed best dehairing yield at 50ºC and pH 11.5 (objective function value 104%).

Table 5.5. Full factorial experimental design and results for optimal pH and temperature values for the dehairing process with E14 (5491±418 UA/cm^2).

pH	**T (ºC)**	**Hair removal (%) with respect to chemical treatment (100%)**
8.5	20	48.3
10	20	62.1
11.5	20	77.8
8.5	35	52.6
10	35	62.9
11.5	35	70.2
10	35	62.1
10	35	79.3
10	35	73.7
8.5	50	84.7
10	50	99.8
11.5	50	97.6

Feasibility of the new process in real tanning industry

Implementing this alternative cleaner process in real tanning industry appears feasible from an environmental, technical and economical point of view. Saran et al. (2013) have recently demonstrated the enzymatic dehairing and degreasing of skins at real scale using 100 kg of hides.

A mass balance done with the data presented in this study confirms that enough hair and sludge are produced in the region under study (Igualada, Spain) to obtain enough proteases to dehair 14000 m^2/d of leather, without significant protease surplus.

The equipment used for chemical dehairing (stirred tanks or rotating drums and pumps) could be used for the enzymatic process without further modifications. The tanning industry treats the wastewaters in its specific treatment plant, producing sludge that could be used in the SSF process. Proteases could be produced by SSF and further extracted using simple equipment as static bed reactors (used in composting) and stirred tanks, or using sequencing batch reactors (SBR) with a sequence of operations. Manufacturers should certainly invest in the proper equipment for proteases production. However, the savings in chemicals and in waste management are expected to compensate this. A preliminary economical balance results in near 2 million € savings per year by eliminating the chemicals cost and internalizing the solid waste management (total costs, 0.391 €/m^2 leather, including: chemicals cost, 0.168; hair waste management, 0.043; sludge management, 0.180).

5.4. Conclusions

A production of an alkaline protease has been developed by SSF from hair wastes and wastewater sludge at kg scale. The highest protease activity was detected at 14 days of SSF, being 56270 ± 2632 U/g dry sample. The characterization of the crude extract indicates that several proteases had been produced by the microbiota developed and they are relatively stable at mesophilic temperatures. A crude extract with a specific activity of 5491±418 U/cm^2 was successfully assayed in dehairing cowhides as a sustainable alternative while valorizing a by-product of the tanning industry and reducing wastewater pollution.

A cleaner production process for the leather industry is demonstrated on this study. The results of this study open the real possibility of substituting a chemical process such as chemical dehairing, which implies the use of reagents and pollution in wastewater and solid wastes, by a biotechnological process where the substrate is the same waste produced in the dehairing

industry. The economical savings on chemicals and waste management that have been estimated for the Catalonia region also supports the feasibility of implementing this process at real scale.

Nowadays, the Catalonian tannery industry is interested in this feasible technology and more experiments in dehairing cowhides, with proteases obtained by SSF, are going to be developed on their establishment for dehairing. Therefore, this study allowed the continuity of this research by other colleague, who is starting her PhD focusing on the SSF of this particular mixture and enzyme.

Further experiments are to be undertaken at higher scale reactors to obtain reliable information on process yield and process emissions. This will allow for a proper economic and environmental assessment.

Further research should be focused on: i) the process characterization at full scale to conduct a reliable technical, economic and environmental analysis, and ii) the complete characterization of the protease obtained and the possibilities of improving its performance using immobilization techniques together with the overall dehairing process optimization (temperature, dosage, reuse, etc).

Chapter 6

Comparison of SSF experiments performed at 4.5 L reactors.

Part of this chapter is in preparation for submittion to an international SCI journal with the tentative title: Consistency of enzyme activity obtained by SSF at 4.5 L scale reators.

Summary

This chapter summarizes and discusses all the SSFs performed at 4.5 L reactors throughout this research, with soy fiber and hair residues. From the three residues studied in the previous chapters, these two presented the higher production of proteases at lab scale and then at 4.5 L reactors scale. Consequently, these two residues were assayed several times at the 4.5 L reactors in diverse experiments with different objectives. Because the residues were from different batches, their characterization alone and within the mixtures were analyzed. Solid residues mixtures are heterogeneous comparing to liquid mediums used in fermentations and so the objective of this compilation is to know how this heterogeneity influences the process at every fermentation and how consistent the SSF is. Parameters that indicate organic solids degradation such as dry matter and also parameters indicating microbial activity such as temperature, oxygen consumption and the generation of proteases were altogether evaluated in order to obtain a profile of the fermentations. Because of the lack of this topic in bibliography, all this information will be useful to withdraw conclusions and give the necessary information for success in future fermentations.

6.1 Materials

Soy fiber residues and hair waste were used on this study and each one was obtained from same producer but different lots and batches in a manufacturing process. All residues used were collected initially at January 2011. Then, the soy fiber residues were obtained several more times for one year and a half (February 2011 to June 2012) and the hair waste for around one year (June 2012 to July 2013). Table 6.1 shows residues characterization as a range of values.

6.2 Experiments

Soy fiber residues

Experiments were performed for 7 and 14 days at 4.5 L reactors at Plant I. Initially intermittent and then continuous aeration of 0.1 L min^{-1} was provided to the reactors. The information regarding to the Plant I configuration at the beginning and its evolution along this research according to the aeration was completely described at Chapter 3. The mixtures were prepared by mixing soy fiber and bulking agent in a wet weight ratio of 3:1 and 1:1 depending on

the experiment. Accordingly, the final weight of the mixture in the reactor ranged from 1.8 kg to 1.25 kg. The experiments were undertaken alone, in duplicate or triplicate regarding the availability of the reactors at the plant and the research objectives for a given experiment.

Hair residues

Experiments were performed for 21 days at 4.5 L reactors, in triplicate and then in duplicate. Continuous aeration at a rate of 0.1 L min^{-1} was provided to the reactors.

Table 6.1. Characterization of the soy fiber residues (n=7) and hair waste (n=3) when arrived from factory.

Parameters	Soy fiber	Hair waste
Water content (%, db)	80.44 - 83.03	59.93 - 71.37
Organic matter (%, db)	93.45 - 97.72	85.96 - 92.18
pH	5.35 - 7.37	9.8 - 10.76
CE (mS cm^{-1})	0.70 - 0.90	5.03*
Respiration index (g O_2 kg^{-1} DM h^{-1})	4.5 - 5.6	3.0 *
Total Organic Carbon (% db)	67 - 71	45 - 57
Total Kjeldahl Nitrogen (% db)	4.4 - 5.3	6.92 - 12
C/N ratio	13.5 - 15.4	4.8 - 6.5
fat content-HEM (% db)	5.30 - 7.60	0.86*

*Only one sample assayed (n=1)

6.3 Results and discussion

Summary of the SSF process with soy fiber residues

As shown in Table 6.1 soy fiber had very similar characteristics between different batches, but only a notable difference was evidenced in pH. Besides, Table 6.2 shows all fermentations along the research study. Only the first 7 days of SSFs were considered to compare all the fermentations because this period coincided with the main degradation and production of proteases.

Bulk Density (kg L^{-1})	initial DRI (g O_2 kg^{-1} DM mix h^{-1})	initial DRI (g O_2 kg^{-1} DM soy fiber h^{-1})	sOUR max (gO_2 $kg^{-1}DMh^{-1}$)	Area T (°C day^{-1})	Area sOUR (gO_2 $kg^{-1}DM$)	pH (0-7 day)	Dry mass reduction (%)	PA ($Ug^{1}DM$) 3 days	sPA (Umg^{-1} SP)
with ratio 3:1, discontinue aeration, manual measures.									
0.36	-	-	n	250	n	7.2-8.8	21	16420	n.a
				274		7.2-8.5	16	41770	
0.40	3.7±0.2	27.3	n	318	n	5.7-8.8	13	3161	9092
				291		5.7-8.9	16	15434	34400
				276		5.7-9.0	16	3025	4079
0.39	3.8±0.1	27.4	n	311	n	6.4-8.5	23	39783	20940
				298		6.4-8.6	27	18712	10284
with ratio 1:1, lower range flow meters, continuous aeration, on-line measures.									
0.23	2.02±0.09	22.4	1.69	268	5.93	6.5-8.9	13	47331	7839
			2.02	295	7.40	6.5-8.8	13	39312	8798
0.24	2.5±0.1	27.6	-	-	-	n.a	20	26477	7767
							20	19165	5623
0.23	2.34±0.07	26.0	1.78	-	-	n.a	10	42008	7043
0.31	2.4±0.2	26.7	1.45	214	2.82	6.1-7.8	14	24777*	4760
0.29	1.8±0.1	20.0	0.90	246	2.84	6.0-7.9	12	12112	7913
with mass flow meters (higher accuracy on an airflow given)									
0.24	1.6±0.2	17.8	2.23	289	6.20	6.0-9.0	11	22692*	7347

ntation experiments; Fn' , duplicate; Fn'', triplicate; n, no data available ; -, technical problems to obtain data from PC; n.a, not analyzed. * 7 days of SSF.

Firstly, experiments (F_1, F_2 and F_3) were done by mixing 3 times this material with 1 of bulking agent in wet weight to adjust the water content and promote the proper porosity in the mixture as referred by Ruggieri (2008). These SSFs showed high activity of proteases but each replicate differed too much from each other in terms of maximum protease activity. The profiles of temperatures are shown in Figure 6.1 and 6.2. The maximum T achieved varied considerably among replicates for F_2 and F_3. These profiles show very low percentage of oxygen during almost all the thermophilic phases. Also these mixtures presented signs of compaction during the process.

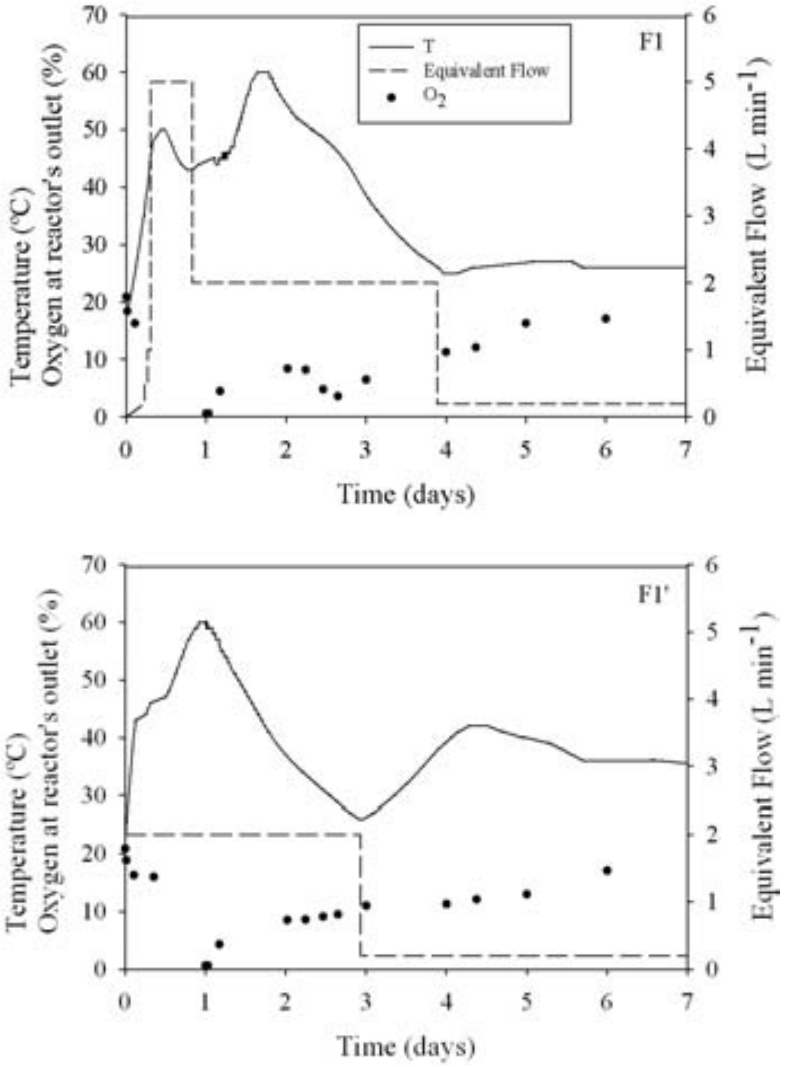

Figure 6.1. Process profile obtained with the initial set-up used for SSF experiments F_1. O_2 amount (%) manually measured.

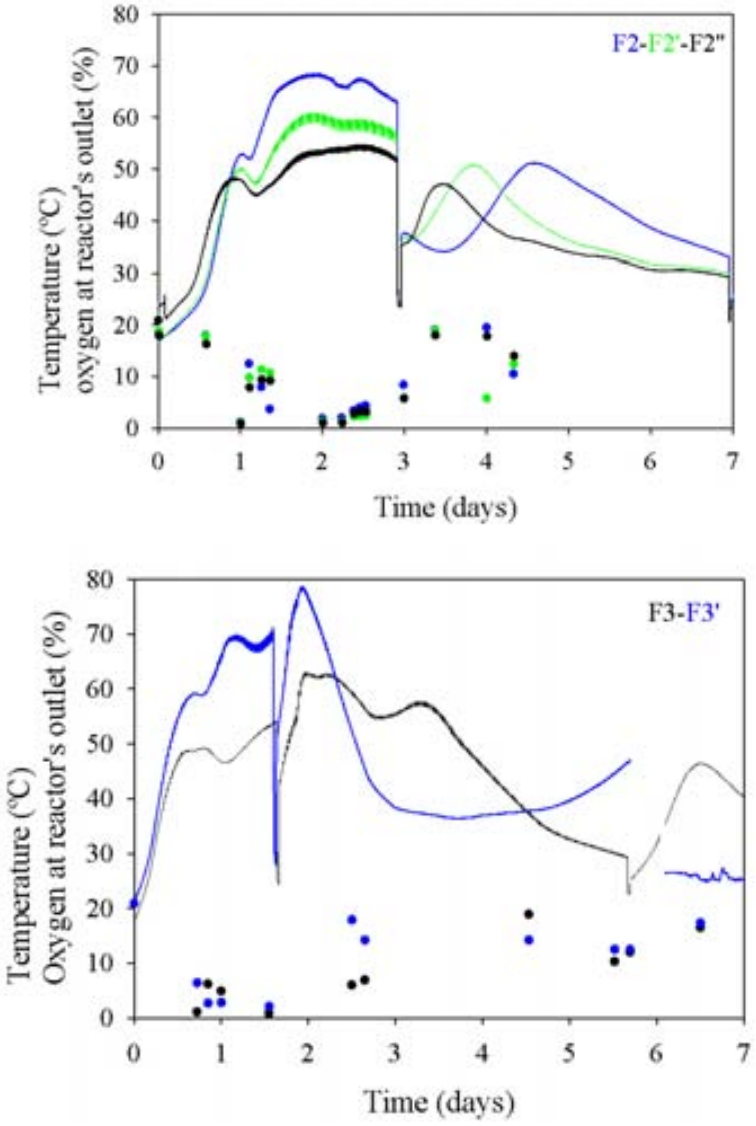

Figure 6.2. Process profile obtained with the initial set-up used for SSF experiments F_2 and F_3. O_2 amount (%) manually measured. Note that equivalent flow in F_2 and F_3 figures is not showed to make clear all the profiles.

This compaction could produce lower or non-homogeneous diffusion of oxygen all over the sample. Hence, preferable paths could be produced between the anoxic compacted zones (Haug 1993). Even though mixed with bulking agent, the mixture still continued to have elevated water content (Table 6.2). Besides, these values were above the higher values recommended (Haug, 1993).

Moreover, the area below T curve, which is related to heat released in the biodegradation, and the area below $sOUR_1$, which is the total oxygen consumed, were calculated for 7 days of SSF by trapezoidal rule (Table 6.2).

After these first results obtained, a lower ratio of 1:1 wet weight was then evaluated to correct the compaction and avoid anoxic zones due to excess of humidity in the samples. Besides, flow meters with lower range were installed and so it was possible to use continuous aeration (0.1 L min^{-1}) and obtain more information of the process. From this moment on, the possibility of measuring both parameters on-line during the fermentations (oxygen and temperature) was useful to follow these indicators of microbiological activity. Therefore, it was possible to know the content of oxygen at reactor's outlet more frequently, and change the airflow if necessary, to maintain the process in aerobic conditions.

The F_4, F_5, F_6, F_7 and F_8 experiments were carried out under these conditions and are also shown at Table 6.2. Moreover, F_9 was performed with mass air flow meters, instead of air flow meters as used with the rest, and so more accuracy in the aeration given to the reactor and in consequence in the sOUR calculation.

Reducing the ratio of soy fiber, logically the mixture reached a lower bulk density (contrary the FAS raised from around 64% to around 74 %). Comparing to previous experiments, no compaction was observed within these mixtures. Moreover, the area below T curve and the area below $sOUR_1$ were also calculated by trapezoidal rule, for these 7 days of SSF experiments. Figure 6.3 shows the temperature and percentage of oxygen profiles at each fermentation. Unfortunately, F5 was not included because of technical problems to obtain data from the computer and F6 because was sacrificed at 3 days of SSF to obtain sample for different analysis.

Replicates F_4 and $F_{4'}$ have similar profiles. However, some differences were found in the following experiments (F_7, F_8 and F_9), especially at the beginning of the fermentation. Replicates of F_4 did not have lag phase meanwhile a lag phase was evidenced in F_8 and F_9 and longer in F_7. At the same time, F_7 and F_8 had lower results in terms of increasing of temperature and consumption of oxygen during the fermentation. Although F_5 profile could not be compared, the maximum temperature registered was around 45ºC and no lag phase was evidenced. In the case of F_6 the maximum temperature registered was around 65ºC and also no lag phase was observed.

Comparing with the initial conditions, a lower continuous aeration seemed to be better than higher intermittent aeration, especially at the thermophilic phase. In this stage, a balance between

the avoidance of anoxic conditions and cool down of the solid matrix is of a notable importance for the correct growth of the microorganisms.

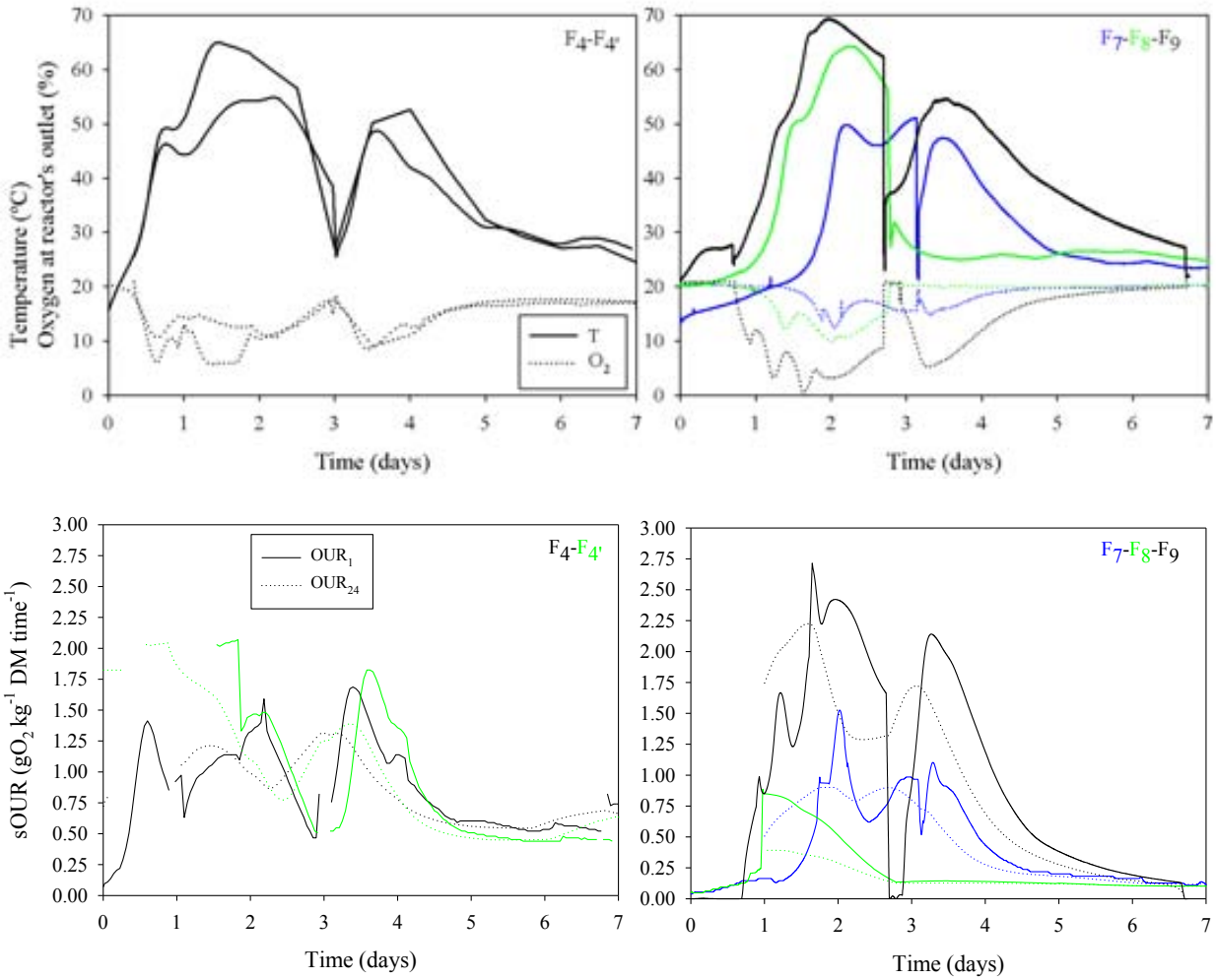

Figure 6.3. Process profile obtained for SSF experiments F_4, $F_{4'}$, F_7, F_8 and F_9. Continuous aeration. On-line measures.

One possible explanation to this could be that the soy fiber of these experiments (F_7, F_8 and F_9) was freeze when arrived from the factory until the reactors were available. As a consequence of freezing and thawing this residue, losses of degradable compounds or on the vitality of microbial communities could happen. Previous analysis with different organic solid residues referred by Pognani et al. (2012) showed lag phase after freezing for one year and also DRI was not altered. Contrary, F_8 and F_9 showed less DRI. Thus, indicating the labile components of soy fiber.

The pH raised during the SSF, reaching alkaline values in all the SSF analyzed, as in any degradation process while mass reduction was evidenced in all the SSF too. In addition, the average of dry mass reduction between the different ratio seemed pretty similar, being 16.57 and 14.13 % for ratio 3:1 and ratio 1:1, respectively.

Regarding the protease activities (Table 6.2), some different values but in the same order of magnitude, were obtained. The higher production of proteases was in most of the cases at around 3 days of SSF, meaning during thermophilic peak. However, the highest activity detected in F_7 and F_9 was obtained after the thermophilic peak (7 days). The highest values were 39312, 42008 and 47331 U g^{-1} DM for $F_{4'}$, F_6 and F_4 respectively. F_5, F_7, F_8 and F_9 had lower protease activities than the rest. Moreover, the specific protease activity (sPA) is shown. The specific activity represents the quantity of protein extracted from the solid matrix that has protease activity. The units of activity per quantity of residue (U g^{-1} DM) and this parameter (U mg^{-1} soluble protein, SP) complement the results in terms of productions and seemed to be useful to compare extracts from different residues. This parameter also shows the selectivity of the extraction and was pretty similar for all the fermentations (ratio 1:1, F_4 to F_9) except for the sPA of F_7.

Finally, F_7 and F_8 showed higher WC and BD than the rest of samples with ratio 1:1. Carefully analysis of all the data obtained was done. However, neither correlation between BD, WC, DRI of initial mixture nor sOUR during the process respect to protease activity was found. Interestingly, no correlation between DRI and sOUR was found. Although no sufficient data was collected to apply appropriate statistics, figure 6.4 shows some results in terms of Box plot of data obtained in all the experiments performed (F_1 to F_9). The box plot graphically depicted groups of data (T area, DM, PA and sPA) based on the five number summary: minimum, first quartile, median, third quartile, and maximum. The vertical lines from the boxes (wiskers) indicated the variability outside the upper and lower quartiles. The individual points corresponded to outliers data indicating variability in the measument or experimental error. There is pretty dispersion in all the values regarding each parameter quantified.

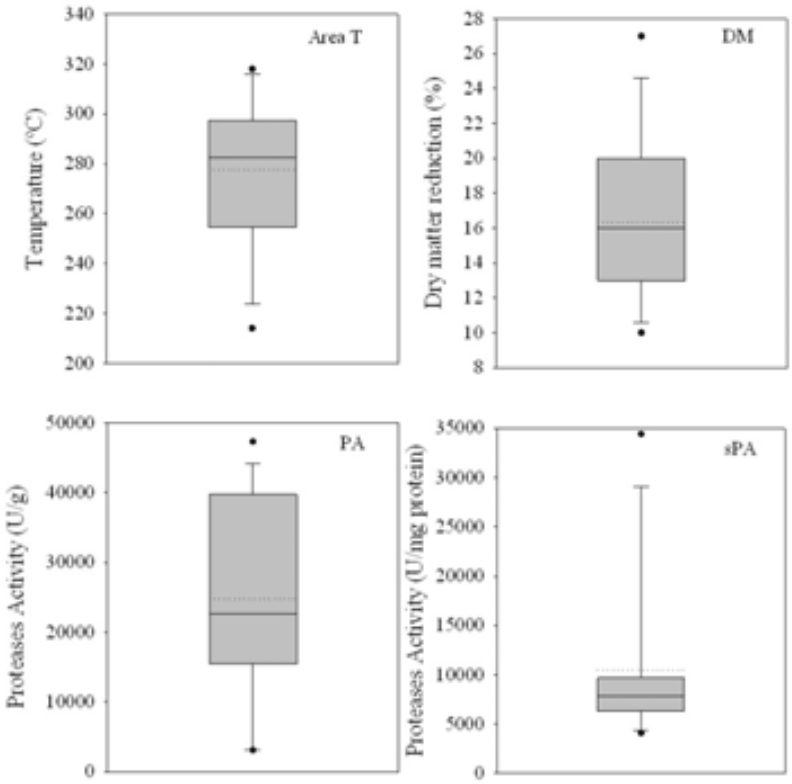

Figure 6.4. Box plot with wiskers of minimum and maximum values obtained in all the fermentations with soy fiber residues (solid line, median; dotted line, mean).

On the other hand, the fact that the higher protease activity was found mostly at the thermophilic peak suggested that the residue is still active in terms of stability after extracting the protease. In chapter 4, soy fiber stability was checked at the last day of SSF (14 days). So that the DRI was evaluated at thermophilic peak of F_5, being 1.9 g O_2 kg^{-1}DM h^{-1}, confirming that after extraction the residue should be stabilized for more days. Besides, the DRI at 7 days was 0.55 g O_2 kg^{-1} DM h^{-1} and so the residue now stabilized would be used as an amendment for agricultural soils.

Summary of the SSF process with hair waste residues

According to the experience obtained with the fermentation of soy fiber, hair waste fermentations were performed. As seen in Table 6.1 the hair waste seemed to have more differences between batches than the soy fiber residues, especially the pH and the TKN. Table

6.3 shows parameters measured during the SSF process. As seen, the mixture prepared had different DRI and BD.

Table 6.3. All the experiments carried out with hair waste. Results obtained during 21 days of SSF.

SSF	Water Content mixture (%)	Bulk Density (kg L^{-1})	initial DRI mix(g O_2 kg^{-1} DM h^{-1})	OUR max (gO_2 kg^{-1}DM h^{-1})	Area T (°C day^{-1})	Area OUR (gO_2 kg^{-1} DM)	pH (0-21 day)	Dry mass reduction (%)	PA U g^{-1}DM (14 days)	sPA (U mg^{-1} SP)
F_1	44.72	0.32	1.3±0.2	1.82	708.74	10.08	6.85 -8.59	32	56270±2632	47260
$F_{1'}$				2.29	794.74	11.57	6.85-8.07	37	60088±1522	35941
$F_{1''}$				1.28	704.92	5.99	n.a	22	n.a	n.a
F_2	52.00	0.27	2.9±0.4	1.74	609.70	6.71	6.75 -8.65	23	58329±1540*	36133
$F_{2'}$				1.43	802.12	7.07	6.75-8.07	21	47351±1222	38210

* 7 days of SSF

However, the fermentations carried out with this residue looked pretty similar in terms of temperature and oxygen content measured at the reactor's outlet. Figure 6.5 (up) shows the temperature and oxygen concentration at reactor's outlet during the 21 days of process for the first fermentation in triplicate and the second fermentation in duplicate (3 months later). Figure 6.5 (down) shows the sOUR consumed during the same experiments. In all the cases, temperatures reached thermophilic ranges within the first day of SSF. After almost 4 days in thermophilic conditions, the temperatures begun to descend. These peaks of temperature matched the highest consumptions of oxygen, as expected. Nevertheless, in none of the cases the mixture reached less than 5 % of oxygen content, confirming that the process was entirely in aerobic conditions in all the experiments. No compaction was observed with these mixtures during the fermentation days.

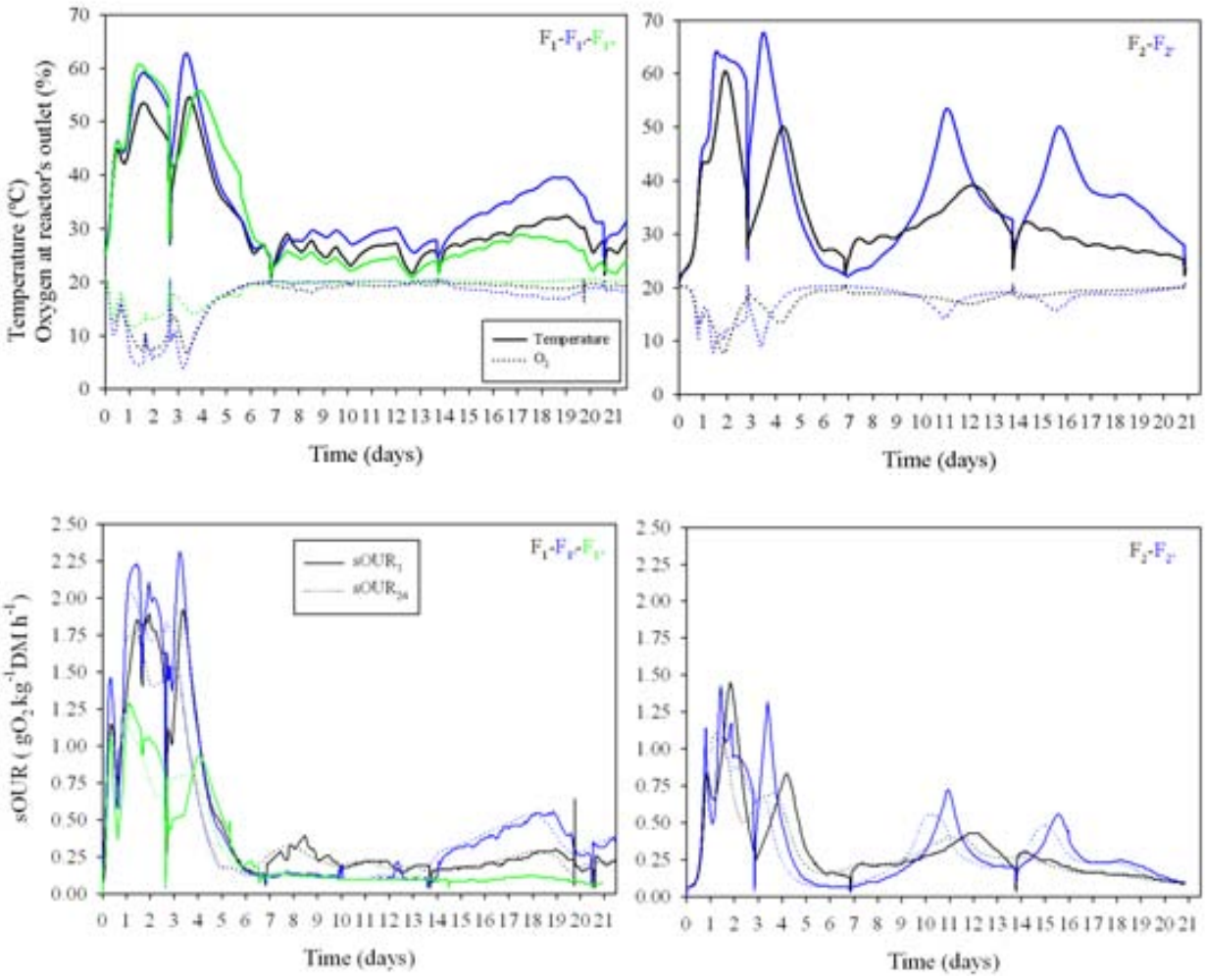

Figure 6.5. Process profile of fermentations corresponding to hair waste SSF experiments.

Differences of process performance in replicates were assessed through the statistical comparison of temperature, area below T curve (ºC day^{-1}), and oxygen consumption, area below oxygen uptake rate (OUR, in g O_2 kg^{-1} DM). T-test and analysis of variance showed no significant differences between experimental trials. Average values plus standard deviation were 724±78 ºC day^{-1} for T curve and 8±2 g O_2 kg^{-1} DM for sOUR curve.

An increase of pH during the process was evidenced as with soy fiber SSFs. The degradation was evidenced also by the percentage of reduction in dry mass of each reactor (Table 6.3). The figure 6.6 shows the mass reduction obtained at each sampling in terms of DM and OM. Besides, another reduction parameter evidenced was the nitrogen from TKN content. These numbers were then multiplied per 6.25 as described in chapter 3, section 3.3.5, to obtain the crude protein

content in the solid matrix. The reduction in crude protein is also shown when measured. A reduction in DM, OM and crude protein is observed particularly at the beginning of the SSF due to the degradation process occurred, followed by a stabilization after 7 or 14 days of SSF. In the case of crude protein, the degradation generates more availability of N for protease production, microorganism's biomass and also volatile nitrogen compounds such as NH_3. In fact, during thermophilic stages characteristics odours derived from nitrogen compounds were smelled. Consequently, in the next experiment this point was taking into account by measuring the gaseous emissions together with the scale-up of the process and is discussed in the next chapter.

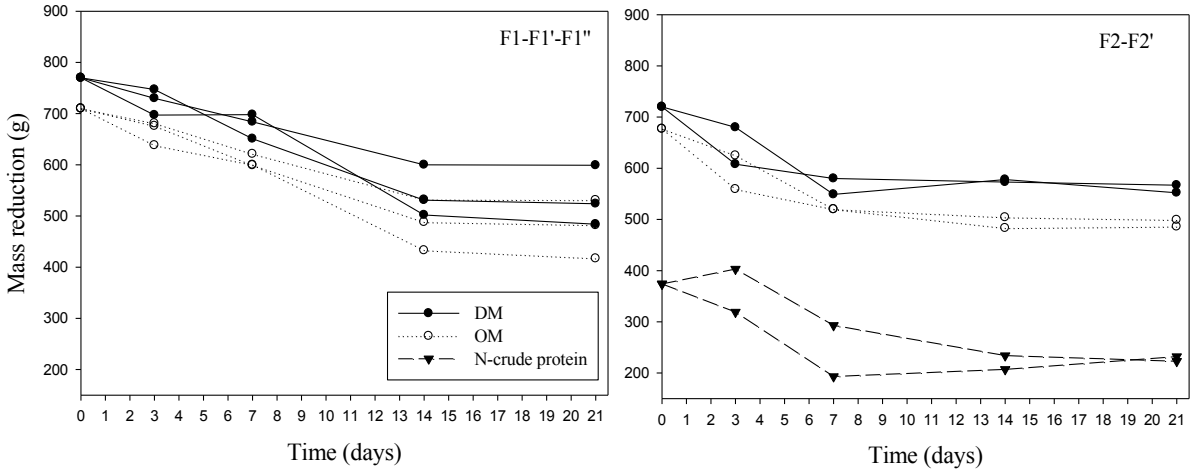

Figure 6.6. Mass reduction evidenced along 21 days of SSF.

During the process the protease activity was also evaluated. The results obtained demonstrated that the major activity was found at 14 days of fermentation in almost all the cases. Only F2 had the maximum at 7 days (Table 6.3). The Student's t-test revealed no significant differences among the protease activities detected in extracts after 14 days of SSF ($p < 0.01$). Although only three points are involved, a possible correlation between sOUR and protease activity was detected. Nevertheless, more feremntations should be performed in order to establish this correlation.

Comparison between SSF from different residues and at different kg scale

Table 6.4 shows the major production obtained during SSF with the three different materials assayed at 4.5 L scale. Although along this research protease activity is expressed in U g^{-1} DM, here different forms of expressions are informed to make possible a comparison with

bibliography. As stated before, (Table 1.1. Chapter 1) there is not a homogeneous expression of protease activity in bibliography. While this comparison is not so easy to do because of the different methodologies and expressions used to determine the activities in each work, these different expressions in the table would help in the comparison. In almost all the cases the production obtained is similar or higher than referred (Table 1.1, Chapter 1).

Table 6.4. Maximum protease activity obtained from the three residues. Different expressions.

	(U mL^{-1})	**(U g^{-1} DM)**	**(U g^{-1}DM min^{-1})**	**(μmol Tyr g^{-1}DMmin^{-1})**
Hair				
$F_{1'}$	5949	60088	501	2.76
F_1	5514	56270	469	2.59
$F_{2'}$	4830	47351	404	2.23
Average	*5431*	*54570*	*457*	*2.53*
Soy fiber				
F_4	5017	47331	394	2.18
F_6	4201	42008	350	1.93
$F_{4'}$	3695	39312	328	1.80
Average	*4303*	*42883*	*357*	*1.97*
Coffee husk				
F_1	1915	21124	176	0.97

U: μg Tyrosine under determined conditions: 50ºC and 2 h incubation.

Furthermore, the protease production yields were obtained for all the residues fermentations calculated as explained in chapter 3, section 3.4.2. The production yield obtained in the best production with soy fiber was $2.95x10^7$ total units while the production yield obtained in the best production with hair waste was $3.79x10^7$ total units. Although the coffee husk had the lower production of these enzymes of the three residues studied and also was performed in another volume reactors (10 L), the production yield obtained was $2.23x10^7$ total units.

On the other hand, Table 6.5 shows the summary of the proteases obtained by SSF with 100 g of substrate and with 1.25 kg (soy fiber), 1.5 kg (hair) and 2.5 kg (coffee husk) of the substrate.

The protease activity from soy fiber and hair are presented as an average of the maximum activity obtained in the different SSF assayed.

Table 6.5 Comparative of proteases obtained by SSF performed at different scales.

Solid substrate	**PA (U g^{-1} DM)**	**PA (U g^{-1} DM)**
SSFs with	*100 grams*	*kilograms*
Coffee husk	10681 ± 71	21124 ± 762 (2.5 kg)
Hair	17011 ± 2122	54570 ± 5937 (1.5 kg)
Soy fiber	37244 ± 1101	42884 ± 4081 (1.25 kg)

As seen, when performed the experiments at lab scale (100g), the best substrate to produce proteases is the soy fiber, followed by the hair and then, the coffee husk. This difference between residues is related with the chemical composition and the biodegradability of the substrates, as discussed in Chapter 4. However, when performed the SSFs with 1-2.5 kg and longer in time, the best substrate for production is the hair, followed by the soy fiber. These results indicated that the biodegradability of the materials is also related with duration of the experiment and so the production of the enzyme. Although the experiments at lab scale were undertaken at constant temperature (37ºC) and the protease activity was determined at 5 days instead of performing a profile of activities along the SSF as with 4.5 L reactors, a comparison between both scales suggested that when using bigger amounts of substrate the production is higher than the experiments performed with grams, in all the cases. Moreover, working with no control of temperature might develop thermophilic microorganisms that could also increase the thermotable protease production as a microrganisms' adaptation strategy.

Then there are a lot of aspects involving protease production and so the quantity of nitrogen in these materials is of important relevance, but also the biodegradability of the materials is critical for the time of production. In this sense, table 6.6 shows the concentration of proteins measured in the extracts obtained from different substrates along the evolution of the SSF. These results showed the quantity of soluble proteins from the beginning and the variations in the process.

Table 6.6. Concentrations of soluble proteins (mg mL^{-1}) measured at initial time and during evolution of process in the different extracts.

Time (days)	**0**	**3**	**7**	**10**	**14**	**21**
Coffee husk	0.11	0.42	0.40	0.31	0.36	-
Hair	0.26	0.14	0.14	-	0.15	0.17
Soy fiber	0.61	0.55	0.43	0.26	0.18	-

The soy fiber is the material with more quantity of soluble proteins at the beginning indicating the availability of the nutrients and the faster degradation, matching with the higher biodegradability of this material and the main production of the proteases, at 3 days of SSF.

The coffee husk started with less quantity of proteins and the increase was detected during the thermophilic phase and at 7 days of SSF, matching the microorganism development and so the main production of proteases obtained. Finally, the quantity of soluble proteins in hair was almost similar during all the process, demonstrating the characteristics of the nutrients in this material. Although the hair has higher N content and it partially hydrolyzed by the chemical treatment in the tannery, this nutrient is taking part of the structural proteins of the hair, namely the keratin. Hence, it seemed to be degraded slower than the other materials and the main production of enzymes was detected on 14^{th} day of SSF.

6.4 Conclusions

All the SSF with soy fiber residues and hair wastes at 4.5 L working volume reactors were compared. In the case of soy fiber, two different ratio of mixture was probed for soy fiber residues. The one suitable to reduce WC in sample and avoid compaction seemed to be 1:1 in wet weight ratio. This mixture was successfully degraded and protease activity produced was principally at thermophilic peak, being the higher value obtained 47331±1391 U/g DM sample and the average between the higher values of production in 3 batches was 42883± 4080. Even though this residue is very homogeneous between different batches, after preparing the sample by mixing with the bulking agent, the fermentations followed very different patterns. Besides, the residue was conserved in the freeze until some of the fermentations could be carried out and this would influence the process.

Thanks to all the experience gained with the soy fiber residues and the evolution of the plant in terms of aireation all the fermentations with hair wastes were performed efficaciously. Contrary, these fermentations were more similar in profiles of T and O_2. This mixture is also successfully degraded and protease activity produced was principally at 14 days of SSF, being the higher value obtained 60088±1522 U/g DM sample and the average between the higher values of production in 3 batches was 54933±5937 U/g DM.

On the other hand, the option of using lower continuous aeration and on-line measurements allowed maintaining aerobic conditions during thermophilic stages and following the T and O_2 profiles during fermentations, respectively. Also allowed to calculate the sOUR, which could be useful for determining if the process is developing well and so the proteases are being produced. In this sense, the hair wastes experiments showed a tendency that the major production of proteases is obtained with major sOUR, but more experiments should be performed to establish a correlation if any.

From the comparison between all fermentations, the hair wastes seems to have lower availability of materials due to its chemical composition and also similar profiles of production were obtained; while the soy fiber has more availability of the nutrients and higher biodegradability and probably less chance to be reproducible between SSFs.

These results open the authentic possibility of use this technology as a tool to valorize the residues and also obtain proteases at lower cost and requirements than the SmF. The fact that the materials are originally residues without sterilization and no addition of a particular microorganism is necessary to produce enzymes suggested that the SSF process would be easily scalable at low costs. On the other hand, when the process is performed under optimized conditions for the mixture (ratio utilized, FAS, among others) and continuos aireation, the variability of the protease activity measured between batches in the same experiment was less than 5% of standard deviation and between different experiment in time was 10% standard devation and 4 % of standard error. This variability between different batches is subject to the characteristics of the residue and its origin. In these sense, a characterization before SSF should be important for adjusting the ratio of bulking agent added. These enzyme activity results suggested that the production by SSF is reproducible for these conditions.

Additionally, a life cycle assessment should be done in order to evaluate the gaseous emissions, the energy needed and the environmental impact of this process.

Due to these results obtained with the three residues, the following step was to assay the mixture of hair wastes at 50 L reactor scale. The challenge is how possible the scale-up is, considering that no control of temperature and no inoculation of pure microorganisms are required on this SSF. Moreover, another objective is to evaluate the gaseous emissions emitted and the energy consumed during the process.

Chapter 7

SSF of hair waste and sludge at 50 L reactor and gaseous emissions involved during the process.

Part of this chapter has been presented as an oral presentation at an international conference: ORBIT: Organic Resources and Biological Treatment: 2014, Hungary. Protease production from organic residues by SSF and their potential uses.
Abraham, J., Gea, T. Sanchez, A.

Part of this chapter together with other results about emissions by SSF has been submitted to a intenational SCI Journal. Gaseous emissions during the solid-state fermentation of different wastes for enzyme production.

Part of this chapter together with other results discussing the scale-up effect is in preparation for submittion at an international SCI journal.

Summary

This chapter presents the SSF at 50 L working volume reactors of hair waste together with sludge from wastewater treatment. As demonstrated in previous chapters, the SSF of this mixture is a good alternative to produce proteases from wastes while stabilizing a significant quantity of residues generated by the tanning industry. The main objective of this work is to study the SSF of this mixture in a bigger scale than the assayed before to assess the scale effect. The second objective was to measure the gaseous emissions in order to evaluate the environmental impact of the process. For that reason, the analysis of emissions was done in collaboration with a colleague of the group, Caterina Maulini, who is doing her PhD in gaseous emissions produced along the composting/SSF processes.

7.1 Materials

The materials used in this SSF were hair wastes from a local tanning industry and raw sludge (15.41 % of DM, 80.60 % of OM and DRI of 2.02 g O_2 kg^{-1} DM h^{-1}) from a municipal wastewater treatment plant (Navàs, Spain). Table 7.1 shows the main characteristics of hair waste and also the main characteristic of the initial mixtures. Wood chips were used as bulking agent.

7.2 Experiments

Mixtures

The same-procedure described at chapter 6 was used. Briefly, raw sludge was mixed with hair waste in ratio 2:1 (wet weight ratio) and bulking agent was mixed in a ratio 1:1 (v:v). All mixtures were manually prepared with the materials at room temperature and without previous sterilization. The table 7.1 also shows the main characteristics of the initial mixture.

SSF

Approximately 25 kg of wet mixture were fermented for 21 days in 50 L reactors, working under adiabatic conditions and continuous aeration. The experiment was carried out by duplicate (R1 and R2) at Plant II. It was not necessary to add tap water to the mixtures along the experiment because of the water content of the mixtures. Samples were collected at 0, after 24 hours of thermophilic temperature (about 2 days), 4, 7, 10, 14 and 21 days of process time after

manual homogenization of the entire mass to obtain the profile of the process and the proteases production.

Table 7.1. Characterization of the hair waste and the initial mixture assayed.

Parameters	Hair waste	Initial Mixture
Water content (%, wb)	64 ± 1	64 ± 4
Organic matter (%, db)	86 ± 1	90 ± 1
Dynamic respiration index (g O_2 kg^{-1} DM h^{-1})	n.a	1.67 ± 0.05
Cumulative oxygen consumption (4 days, g O_2 kg^{-1} DM)	n.a	88.42 ± 4.05
Total Nitrogen Kjeldahl (%, db)	6.9 ± 0.1	7.68 ± 0.02
Total Organic Carbon (%, db)	45 ± 0.5	50 ± 0.6
C/N ratio	6.5	6.5
pH	7.96 ± 0.01	7.40 ± 0.01
EC (mS cm^{-1})	2.83 ± 0.01	2.00 ± 0.01
Free air space (FAS, v/v)	n.a	0.71

EC: electrical conductivity, wb: wet basis, db: dry basis, DM: dry matter. n.a: not analyzed.

* Ratio 1:2 hair waste: raw sludge (w/w).

Gaseous emissions analysis

The daily evolution of volatile organic compounds (VOCs), methane (CH_4), nitrous oxide (N_2O) and ammonia (NH_3) emissions were evaluated for each mixture assayed. The samples were collected from reactors' outlet in 1 L Tedlar® bags and determined by gas chromatography (Agilent Technologies 6890N Network GC system, Madrid, Spain) as referred by Colón et al 2012.

Energy consumption

The total energy consumption (E) was estimated from the total air supplied during the SSF process and using a conversion factor (396 KJ/m^3, manufacturer's data) to transform the air consumed into energy as described by Puyuelo et al. (2012). The calculation of the resources consumption on this SSF process was done by taking into account the yield of the process

calculated as the reduction or stabilization of organic matter proposed as RIE and QRIE by Colon et al. (2012) and calculated as follows:

$$\text{RIE} = \frac{\text{E}}{\text{DRI initial} - \text{DRI final}}$$

$$\text{QRIE} = \frac{E}{DRI\ initial - DRI\ final}\ x\ DRI\ final\ product$$

where: RIE, is the energy consumption associated to the RIE (kJ (mg O_2 g^{-1}OM h^{-1})$^{-1}$); E , is the total energy consumption along the experimental time (kJ) and DRI reduction as DRI initial - DRI final, is the reduction obtained during the experiment (mg O2 g^{-1} OM h^{-1}); QRIE, the same as RIE but taking into account the quality of the final stabilized product.

These indexes were explained and used by Colón et al. (2012) in order to compare different biological treatments in 4 urban solid wastes treatment plants located in Catalonia. Concisely, the value of the final product DRI is only a measure of product quality no matter the waste origin, plant performance, among others, while DRI reduction is an intermediate value that considers all these factors and permits the calculation of advanced environmental impact indices such as RIE and QRIE. Consequently, RIE index is of high relevance because they allow calculating the energy consumed in the process regarding the stabilization of the material and in the case of QRIE is a specific index in which the quality of the final product is also included.

Likewise, these indexes are also used here to calculate the energy and resources involved in SSF process.

Another index calculated to determine the energy consumption regarding the enzyme activity as described previously by Santis (2013), was:

$$\text{EEA}_{\text{n}} = \frac{\text{En}}{\text{total PAn}}$$

where: EEA, is efficiency of enzymatic activity at day n (J U^{-1}); E, is the energy consumed until day n; total PA_n, is the total protease activity at day n (U g^{-1} DM).

7.3 Results and discussion

SSF process performance

The figure 7.1 shows the SSF process in the R1 and R2 reactors. As the previous SSF figures, these figures represent some of the physical parameters which were monitored online, such as the temperature and the oxygen content at reactor's outlet. It is also represented in the same figure punctual chemical parameters that were determined when sampling, such as the pH and the activity of the proteases. The latter represents the proteases produced by the microorganisms initially from sludge and/or developed along the process. The temperature profile seemed to be similar in both reactors (area below temperature curve were 951 ºC day^{-1} for R1 and 878 ºC day^{-1} for R2, which represents less than 8 % of difference). Besides, no lag phase was detected, as temperatures increased since the beginning of the experiment and reached thermophilic temperatures within the first day of SSF. The first thermophilic peak, which is the highest, coincided with the highest consumption of oxygen. The mixture at this experiment followed the same temperature profile that those of experiments at 4.5L reactors (Chapter 5, figure 5.2) but at 50 L scale the temperatures reached were higher (70-73 ºC compared to 55-60 ºC) and kept longer.

The process was entirely in aerobic conditions, especially in R1, regarding to the oxygen data obtained at reactor's outlet. In R2, lower values of oxygen (around 3%) were obtained between days 2 and 3 of SSF, because of a constraint with the oxygen controller. As a consequence, the increase in temperature after the first sampling was lower than that of R1. However, the recovering of the oxygen concentration and the following increment of temperature showed that the aerobic conditions were restored. Figure 7.2 shows the $sOUR_1$ and the airflow.

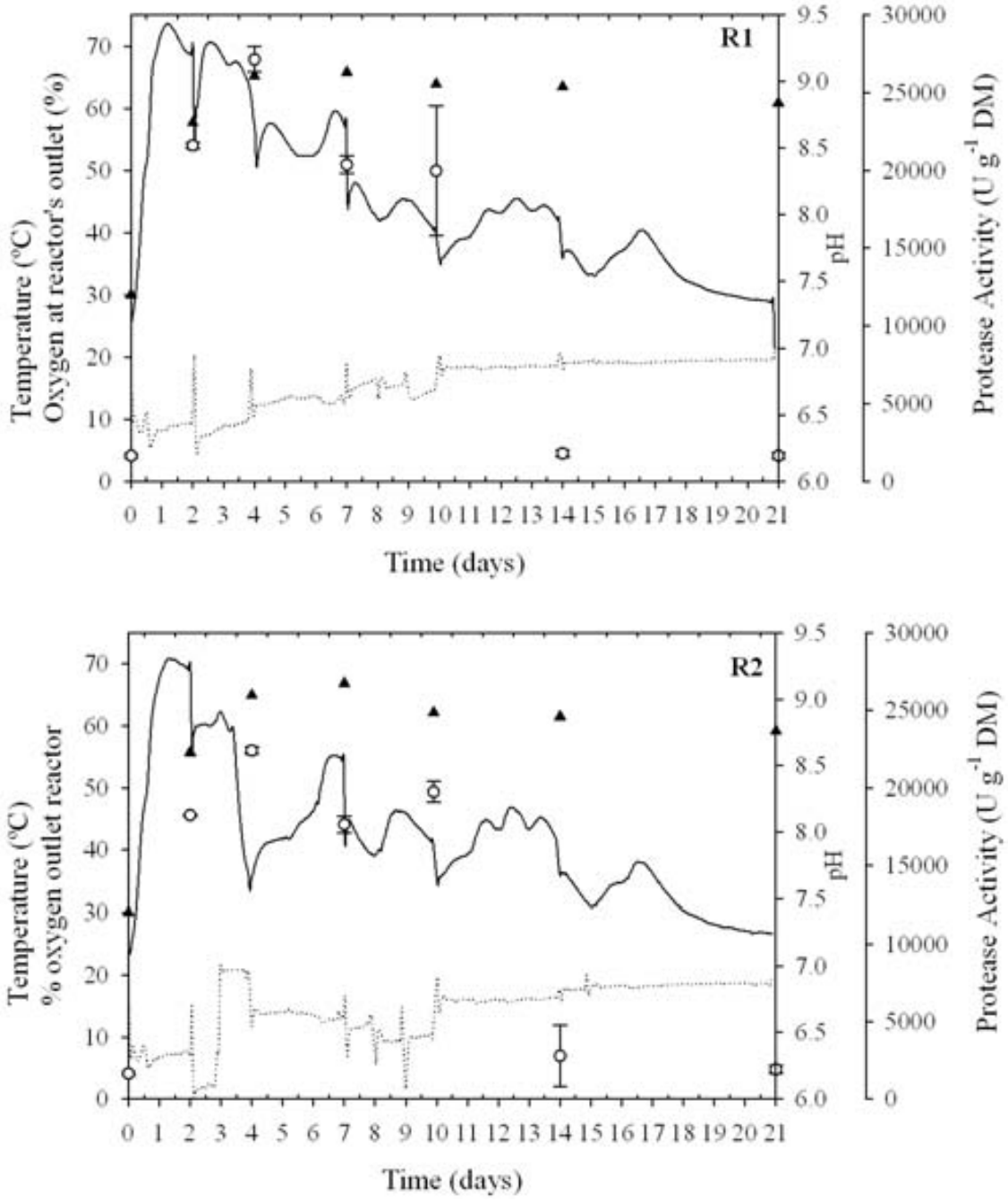

Figure 7.1. Solid-state fermentation profile of R1-50 L and R2-50L working volume reactor. Temperature (—, solid line), percentage oxygen content in exhaust gases (···, dotted line), pH (▲) and protease profile (○).

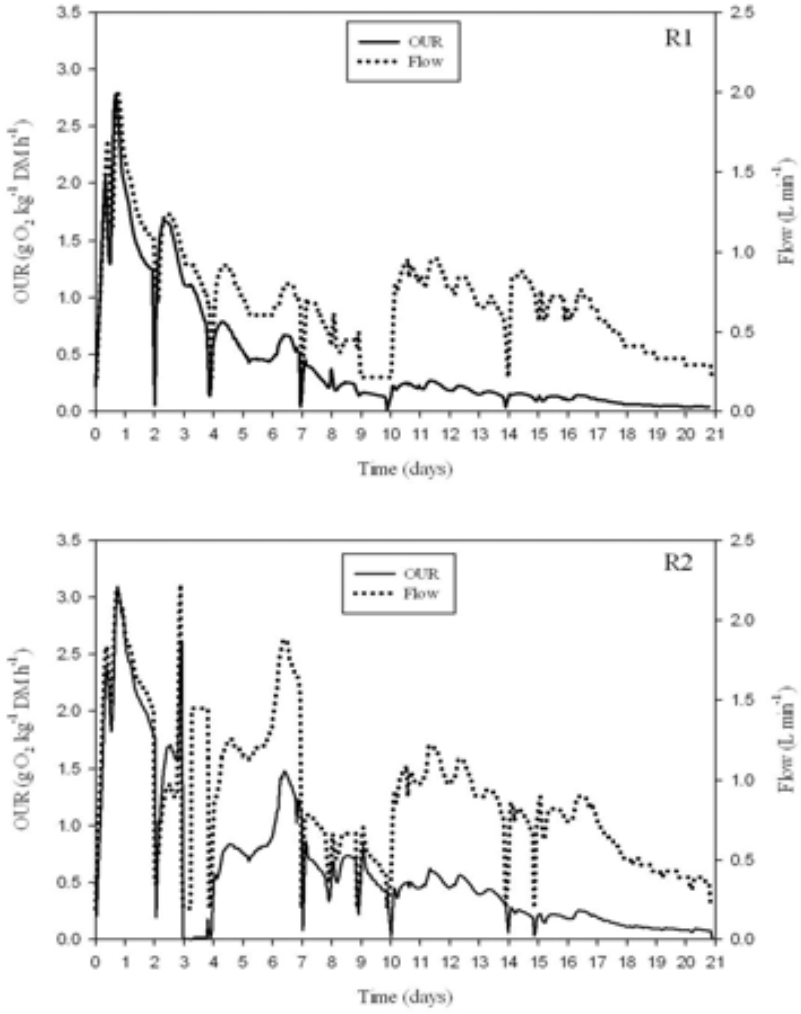

Figure 7.2. Flow received by each reactor at any given time and the corresponding oxygen uptake rate obtained.

The increment in the temperature especially at the beginning of the SSF, showed at figure 7.1, matched an important increment in the oxygen consumption, shown in Figure 7.2. Likewise, these sOUR values show the microorganism's development by consuming oxygen and also the C, N and other sources from the solid material. The maximum OUR_1 detected was 2.8 and 3.3 g O_2 Kg DM^{-1} h^{-1}, for R1 and R2 respectively, which are higher than previous studies at 4.5 L reactors. At 4.5 L reactors the $sOUR_1$ max was between 1.29 and 2.31 g O_2 kg^{-1} DM h^{-1}. At 50 L experiments, the controller changed the flow to achieve the maximum sOUR at each hour while at 4.5 L experiments the flow is constant (0.1 L min^{-1}).

In order to supply the proper quantity of oxygen and following the corresponding algorithm, the controller provided different flows for each reactor along the process. The flow in the two reactors was almost similar, being the maximum flow applied by the controller 2.01 and 2.23 L min^{-1} for R1 and R2, respectively. Besides, the total air supplied was 2446 and 2992 L kg^{-1} for R1 and R2 during 21 days of SSF respectively. At 4.5L reactors the total air supplied was 2623 L kg^{-1} for 21 days.

Regarding the pH (Figure 7.1), an increment was detected during the fermentation as seen in other degradation processes and also similar to previous pH values obtained at 4.5L trials.

The protease production profile (figure 7.1) is pretty similar in both reactors and presents some differences from the ones obtained at 4.5 L. At 4.5 L scale, high level of activity was maintained for 2 weeks, reaching the maximum at day 14 and falling after that. Here, the maximum activity was detected on day 4 and high levels were kept for 10 days. This variation may be due to some differences encountered in the initial hair waste, such as the pH (9.8/10.78 compared to 7.98), and the initial moisture of the mixture due to the higher WC of the sludge (around 67 % WC compared to 85%). Finally, the production yield obtained in this fermentation at 50 L working volume reactors were $1.9x10^8$ at day 4 and $1.3x10^8$ at day 10 of SSF total units for R1 while $1.7x10^8$ for day 4 and $1.3x10^8$ for 10 days of SSF total units for R2.

On the other hand, to follow the changes in porosity of the mixture during SSF, the AFP was measured in situ at R1 (Figure 7.3) and the results are shown in figure 7.4. Although are punctual measures, the graphic in figure 7.4 is done with dotted line so that the sequence of the AFP determination during the time could be understood. Some days have two measurements and correspond to the AFP before and after sampling respectively.

Figure 7.3. Measuring the AFP in situ in R1.

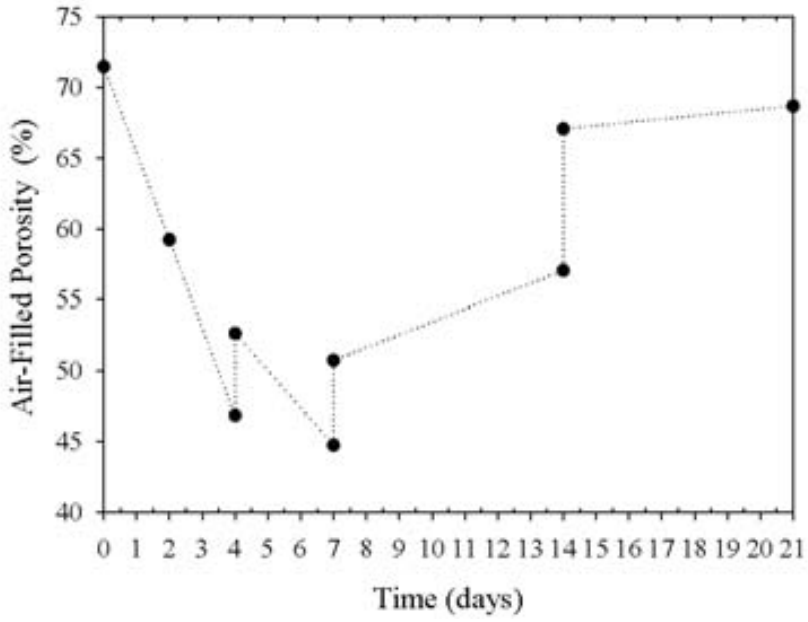

Figure 7.4. AFP (%) during SSF in R1 reactor.

The AFP was initially 71% which is over the recommended values (40-60 %), but bigger initial AFP are acceptable regarding to the biochemical composition and the mechanical resistance of the mixtures treated (Ruggieri, 2008). During the fermentation and even that an appropriate mixture was used (Barrena et al., 2007a,b) a decreasing of AFP is observed because

of an expected volume reduction and compaction of the sample. An increase in AFP was observed after each sampling. The reason why is because when sampling the mixture was mixed and the porosity increased. Although the AFP decreased during the first days, it was kept between recommended values all along the experiment and this is due to the physical structure and the mechanical strength provided by the bulking agent to the mixture (Ruggieri, 2008). After the 7th day, stabilization and also an increment is detected due to the degradation of the organic matter.

On the other hand, the reduction in weight during the SSF process showed the degradation and subsequent stabilization of the material. The total dry weight reduction of the mixtures was 36 % for R1 and 35% for R2. The organic matter reduction was 24 % for R1 and 30% for R2.

Besides, another reduction parameter evidenced was the nitrogen from TKN content. This TKN includes the organic and inorganic forms of nitrogen, so that ammonium quantities were also determined during the process and were properly subtracted. These numbers were then multiplied per 6.25 as described before, to obtain the crude protein content in the solid matrix. Figure 7.5 shows the mass reduction evidenced in terms of organic matter, dry matter and the crude protein content during the process. The TKN and crude protein reductions were 40 % - 32 % for R1 and 46 % - 37 % for R2, respectively. These reductions corresponded to the degradation of the solid matrix, especially proteins from hair, into other solid nitrogenous compounds and also to the production of volatile nitrogen compounds, such as N_2O, NH_3 and subsequent emissions, which are described and analyzed below.

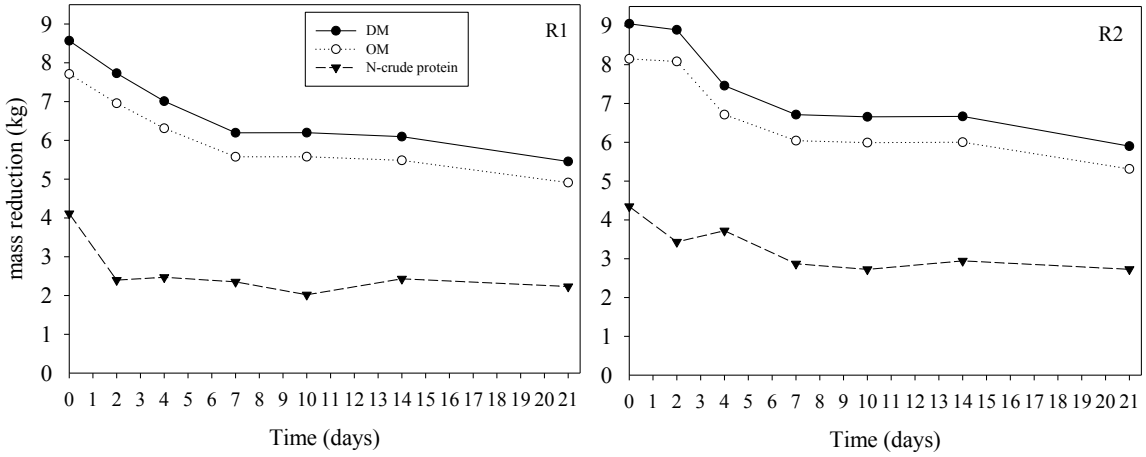

Figure 7.5. Mass reduction evidenced along the 21 days of SSF.

Finally, the DRI obtained at the end of the experiment gave values into the stability degrees, being 0.83 and 0.32 g O_2 kg^{-1} DM h^{-1} for R1 and R2 respectively.

Gaseous emissions

VOC evolution

The VOCs, such as terpenes, alcohols, ketones and amines among others, are associated with particular odors during the process and will be emitted even if the process is undertaken properly. VOCs emissions are related to process heating and so the main generation of VOCs is detected normally during the transition from mesophilic to thermophilic phase, as referred by Komilis et al. (2004) and Maulini et al. (2013).

In figure 7.6, the production of VOCs is shown. The generation of these gases was detected since the beginning of the SSF, matching the changes in temperature. They increased quickly and then, they almost stabilized from 7th day of SSF to the final. Both reactors had very similar emissions. The total kg of VOCs per (megagramo) Mg of residue treated was 0.097 and 0.10 to R1 and R2 respectively. These results coincided in order with previous studies on composting raw sludge by Maulini et al. (2013).

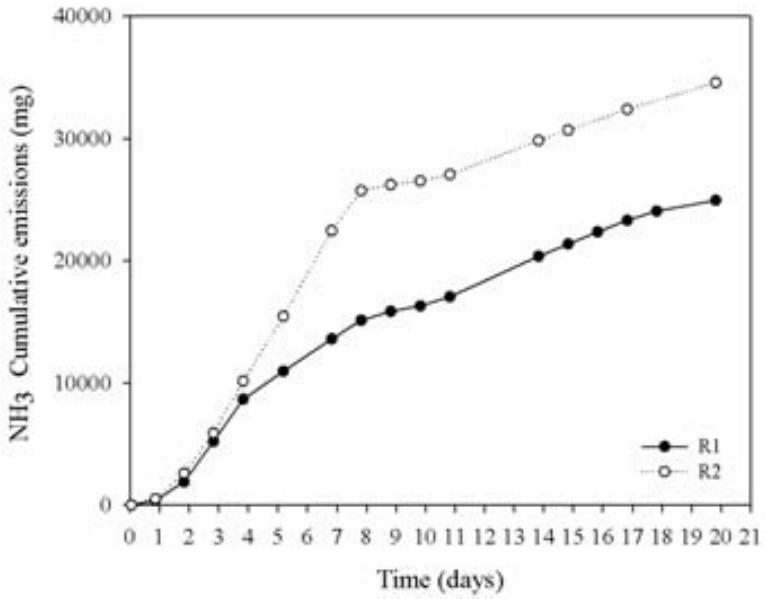

Figure 7.6. Cumulative VOCs emissions during 21 SSF days at 50 L reactors, R1 and R2.

CH_4 evolution

Methane is one of the considered greenhouse gases (GHGs) and is produced by microorganisms in anaerobic conditions. Normally and due to the aerobic features of this process, CH_4 emissions should not be produced. However if they are present, indicates possibility of anaerobic zones. Sometimes, these zones are because of elevated moisture or insufficient porosity of the mixture and others due to lower aeration. In this experiment, the porosity and the aeration seemed to be adequate, as shown before in figures 7.1 and 7.4, but maybe the elevated biodegradability of the organic matter produced fast oxygen depletion especially at the beginning. Figure 7.7 shows both-profiles obtained. The total kg of CH_4 per Mg of residue treated was 0.0018 and 0.0014 to R1 and R2 respectively. These expected low values confirmed that no anaerobic zones were predominant in the mixture and that the conditions are mostly aerobic in the entire reactor. Although, both reactors raised similar emissions, the differences in the profile could be due to the different regime of the flow and oxygen uptake rate pattern evidenced at figure 7.2. Besides, similar pattern of emissions but higher in quantity were referred by Maulini et al. (2013) for the same raw sludge alone.

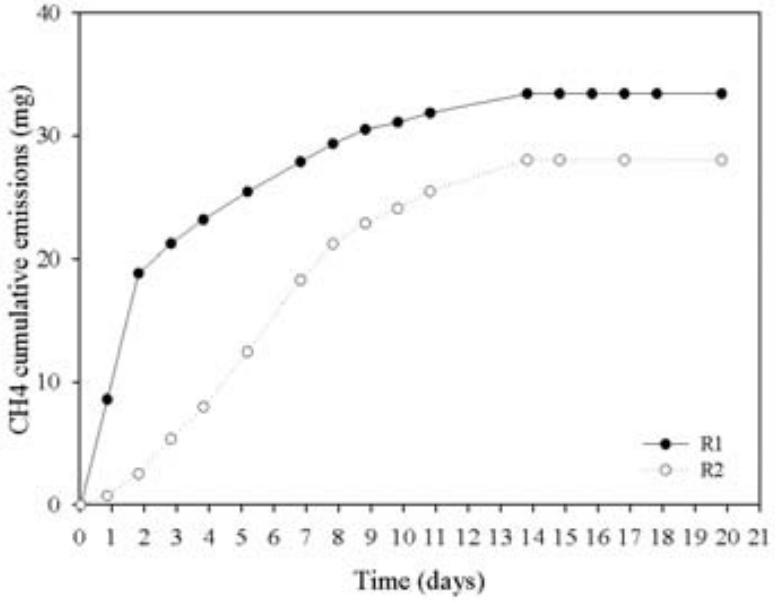

Figure 7.7. Cumulative CH_4 emissions during 21 SSF days at 50 L reactors, R1 and R2.

NH_3 evolution

NH_3 emissions are related to the biochemical composition, such as the ammonium and the organic nitrogen content, in the mixture assayed. Besides, it depends on the temperature, pH and aeration during the assay. Pagans et al. (2006) demonstrated that the higher emissions were produced during the thermophilic phases. Jiang et al. (2011) showed that an high aeration could influence the values of the emissions due to striping effect, which corresponds to dragging the gas through the sample due to forced aeration in the system. Finally, pH can influence because alkaline medium can favor the conversion of NH_4^+, mostly from the degradation of proteins, into NH_3 and the consequent emissions. Figure 7.8 shows both profiles obtained.

The total kg of NH_3 per Mg of residue treated was 1.3 and 1.7 to R1 and R2 respectively. These values are higher than the ones obtained in previous experiments with raw sludge alone (Maulini et al., 2013), being 0.87 and 0.28 for R1 and R2 respectively. Despite this experiment was performed for only 13 days compared to 21 days, these higher values probably corresponded to other nitrogen contribution, meaning to the degradation of hair waste.

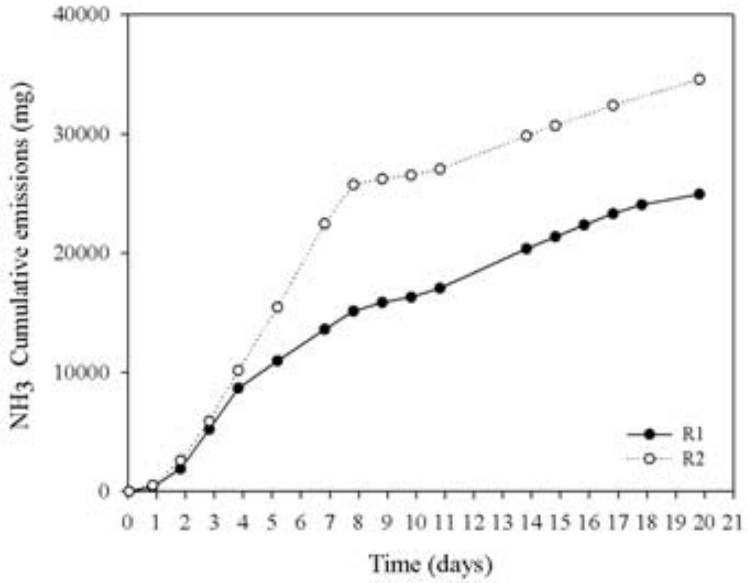

Figure 7.8. Cumulative NH_3 emissions during 21 SSF days at 50 L reactors, R1 and R2.

Hence, this emission was calculated for 13 days and gave 1.1 and 1.5 kg Mg^{-1} for R1 and R2 respectively, still higher than the raw sludge alone. Likewise, this emission has different pattern from the one with raw sludge alone, showing continuous increment along the SSF process. In this

case, the cumulative emissions show two different slopes in the curve. One could correspond to the raw sludge degradation while the second one to the degradation of hair wastes, due to its lower biodegradability nature. The difference between the emissions in both reactors could be explained by the difference in the flow rate during the process. The flow in R2 was higher than R1 and so, the drag of the gas was more important in R2 than in R1.

Moreover, these values are a bit lower than previous studies with the same residue analyzed by Pagans et al. (2006).

N_2O evolution

Known as another GHG is produced and emitted when nitrification and denitrification occurs during the biological transformation of nitrogen. This gas has stronger potential to contribute in the greenhouse effect (more than 300 times the CO_2 does) so it is important to be evaluated when study the environmental impact of any given process. Figure 7.9 shows both values obtained. The total kg of N_2O per Mg of residue treated was 0.00069 and 0.0015 to R1 and R2 respectively. Additionally, these emissions did not coincide with the NH_3 ones, meaning that the higher emissions were before the latter. In this assay maximum emission coincided with maximum temperature as referred by Maulini et al. 2013 for raw sludge. Besides, the values obtained were lower than referred in the same study.

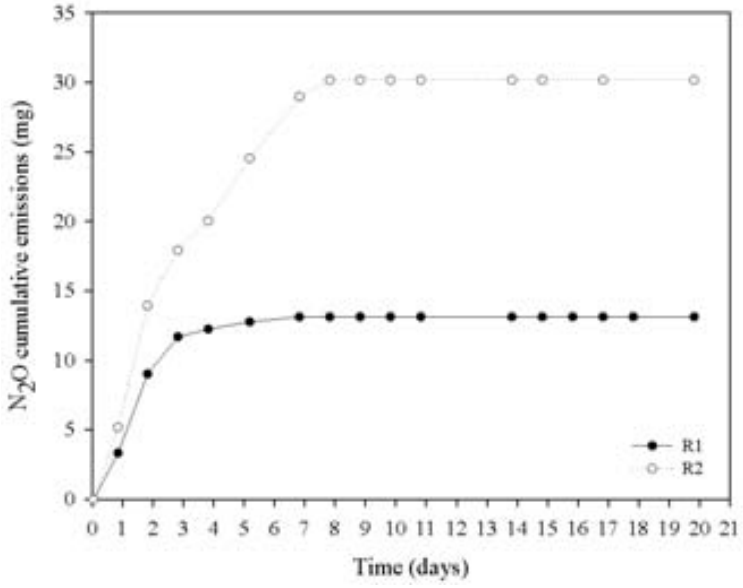

Figure 7.9. Cumulative N_2O emissions during 21 SSF days at 50 L reactors, R1 and R2.

Energy consumption

The reduction index efficiency calculated as RIE was $1.03x10^3$ and $7.97x10^2$ kJ (mg O_2 g^{-1}OM $h^{-1})^{-1}$ and the QRIE was $1.06x10^3$ and $2.92x10^2$ kJ for R1 and R2, respectively for 1 kg of dry mixture treated. The average between both reactors are $9.11x10^2$ kJ (mg O_2 g^{-1}OM $h^{-1})^{-1}$ and $6.76x10^2$ kJ for RIE and QRIE, respectively. These values indicate the energy consumed for the valorization of this material and would be used to compare with other biological treatments. For instance, these values are similar to the ones obtained by Puyuelo (2012) and lower than the obtained by Santís (2013) in previous studies at the same pilot plant (Plant II) with solid urban residues for 2 weeks and fat residues for 4 weeks, respectively. Moreover, in Table 7.2 these indexes are compared with the composting of raw sludge alone in 13 days and organic fraction of municipal solid wastes (OFMSW) in 21 days undertaken by Maulini et al (2013, 2014) in the same plant. Although there is a lot of dispersion between the emissions measured in both reactors (R1 and R2), the average of results indicated that the SSF process requires less energy for treatment than the composting process for raw slude (RIE= $9.11x10^2$ compared to $1.46x10^3$ kJ (mg O_2 g^{-1}OM $h^{-1})^{-1}$).

Even though the RS experiment was performed in 13 days and the mixture with hair wastes were performed for 21 days, the emissions of VOCs, CH_4 and N_2O were lower for the latter. However, the NH_3 calculated for 13 days is still higher than RS alone, as discussed before.

Regarding the experiment with OFMSW, this experiment was also undertaken for 21 days and all the emissions were higher than the SSF process, the NH_3 emissions had an important desviation between replicates; however, they are also higher than SSF process.

On the other hand, the enzymatic activity efficiency calculated were $4.3x10^{-8}$ and $6.6x10^{-8}$ J U^{-1} for 4 and 10 days of SSF in R1, respectively; while $6.3x10^{-8}$ and $8.2x10^{-8}$ J U^{-1} for 4 and 10 days of SSF in R2. These values are lower than the values obtained by Santis (2013) in the SSF for production of lipases.

Table 7.2. Comparative between hair wastes: raw sludge mixture, raw sludge alone and organic fraction of municipal solid wastes experiments. All the results are referred to 1 kg solid treated.

Reactor	VOCs (kg Mg^{-1})	CH_4 (kg Mg^{-1})	NH_3 (kg Mg^{-1})	N_2O (kg Mg^{-1})	Total Air (L)	RIE kJ(gO_2kg^1 $OMh^{-1})^{-1}$	QRIE kJ
R1 HW-RS	9.70E-02	1.80E-03	1.30E+00	6.90E-04	2446	1.03E+3	1.03E+3
R2 HW-RS	1.00E-01	1.40E-03	1.70E+00	1.50E-03	2992	7.97E+2	7.97E+2
R1-RS	2.10E-01	1.40E-02	8.70E-01	6.40E-03	3165	2.36E+3	2.53E+3
R2-RS	1.40E-01	1.20E-02	2.80E-01	2.70E-03	1574	5.67E+2	2.83E+2
R1-OFMSW	6.43E-01	7.40E-01	3.49E-00	1.38E-02	nc	nc	nc
R2-OFMSW	4.25E-01	6.31E-01	1.24E-00	8.85E-03	nc	nc	nc

HW, hair waste; RS, raw sludge; OFMSW: organic fraction municipal solid wastes; VOCs, volatile organic compounds; RIE, environmental impact and energy consumed regarding the organic matter reduction. QRIE: idem RIE but regarding the quality of final product. Nc: not calculated.

Mass balance

The total solid nitrogen suffered transformations during the process as described; taking part in the mineralization of the nitrogen in any other forms, in the constitution of microorganisms structures, in the production of enzymes and also in volatile compounds, which were emitted. However, the N-balance that corresponds to the variations in solid nitrogen content and the nitrogenous emissions during the process was very different (solid nitrogen was much higher than volatile nitrogen). The kg of volatile nitrogenous compounds produced was the 50 % (R1) and 60 % (R2) of the reduction of the total solid nitrogen, indicating the possibility of losses in the quantification of the emissions.

7.4 Conclusions

A bigger quantity of mixture of hair waste and raw sludge (24-25 kg compared to 1.5 kg) were assayed at 50 L working volume reactors located at Plant II. The degradation and later stabilization of the mixture and also the production of proteases analyzed were successful in both reactors. The degradation part was evidenced by the OUR rate, the temperature and pH increment, but also with the values of mixture dry weight, organic matter and crude protein reductions, which were 33 %, 25% and 42 % average from the initial mixture, respectively. Additionally, low DRI were determined at the final of the process, indicating the stability of the

material studied. Regarding the enzyme production, protease activity was quantified and the maximum obtained coincided with the maximum thermophilic peak. Although lower yield than in 4.5 L reactors trials were obtained, an analysis of the emissions produced during the process and the energy consumption indexes were calculated. This study i help in the evaluation of the environmental impact of the process and would be compared lately with other techniques of production. According to the data of emissions obtained and compared with bibliography, only the NH_3 emissions were higher because of the composition of the mixture and the conditions of the process. However, by using biological techniques such as biofilters, these emissions can be abated (Alfonsín et al. 2013). Moreover, the emissions emitted were lower than the ones in a composting process of organic fraction of municipal solid waste, indicating that the SSF is probably more efficient than composting in terms of emissions.

Although this mixture was proven successfully in the dehairing of cowhides, another application was explored considering the possibility of these enzymes to catalyze the revesible reaction, which is the synthesis of oligopeptides.

The proteases are also desiderable catalysts for performing synthesis and have been considered to fulfill the requisites for environmentally innocuous manufacturing, sustainable development and green chemistry (Illanés 2008, Ageitos et al.2013).

In this context, meaning the production of proteases from residues without sterilization and low environmental impact of production, as seen in this chapter, it would be interesting to explore the possibility of using this enzyme in the creation of other products under sustainable conditions as well.

Chapter 8

Preliminary study on the uses of proteases on kinetically controlled synthesis of polypeptides.

Summary

This chapter describes the research done at the Center for Biocatalysis and Bioprocessing of Macromolecules at Rensselaer Polytechnic Institute (RPI), NY-USA. Formerly located at New York University-Polytechnic Engineering School (NYU-POLY). When I arrived there, the lab had just moved to RPI, but Professor Gross' company, Synthezyme, still remained at NYU-POLY so that I began the stay there for one month and then, moved to RPI, when permissions at the labs were ready. Despite of the difficulties at the beginning in terms of permissions and laboratories, it was a very stimulating and interesting experience in both scientific and human aspects. Despite of the difficulties at the beginning in terms of permissions and laboratories, it was a very stimulating and interesting experience in both scientific and human aspects. I also learnt a lot there.

The main objective of this stay was to learn about proteases catalyzed synthesis to peptides with control of sequence. Moreover, **t**o probe the potential of the enzymes obtained by SSF in previous fermentations in the synthesis of oligopeptides, which could be also used for other environmental application such as the phytochelating-like peptides studied by the hosting group (Visnawathan et al., 2012). The oligopepetide consists in an alternating glutamic acid and cysteine residues whith availability for chelating heavy metals and is formed by synthesis under the fundaments of green chemistry.

8.1 Brief Introduction

As explained in chapter 1, Introduction, proteases are able to catalyze the hydrolysis of peptides. There are several classes of proteolytic enzymes (Serine, Aspartate, Cysteine, Threonine, Glutamic Acid and Metallo Proteases) regarding to its catalytic mechanism and substrate specificity. Proteolytic processing can be limited by the specificity of the protease, the accessibility to the susceptible peptide bond of the substrate, the required activation of an enzyme precursor and the activation of protease inhibitors or a combination of these factors. Additionally, the hydrolysis reaction can be reversed. Peptide bonds can be synthesized using proteases in either a thermodynamically controlled (TC) or a kinetically controlled (KC) manner. The kinetically manner is a green and clean chemical reaction mostly possible, faster and the conversion is often higher than the TC. In the kinetically mechanism model, the substrate should

be activated in order to interact with the enzyme forming a Michaelis-Menten complex. Contrary, in the TC the substrate is not activated and so depends on the pH of the medium and the pKa of the amino acid to allow the reaction take place. Then, this complex is competitively deacylated by water and a nucleophile. If the nucleophile is an amino acid or oligopeptide, a new peptide bond is formed. Precipitation of product from the reaction solution during synthesis further shifts the equilibrium toward peptide formation. Simultaneously, pH medium of the reaction changes due to the release of chlorhydric acid (HCl). Because a covalent complex must be formed, only serine and cysteine proteases can be used for this strategy. Figure 8.1, resumes the reactions explained before with a particular amino acid activated as L-Glutamic Acid Diethyl Ester Hydrochloride.

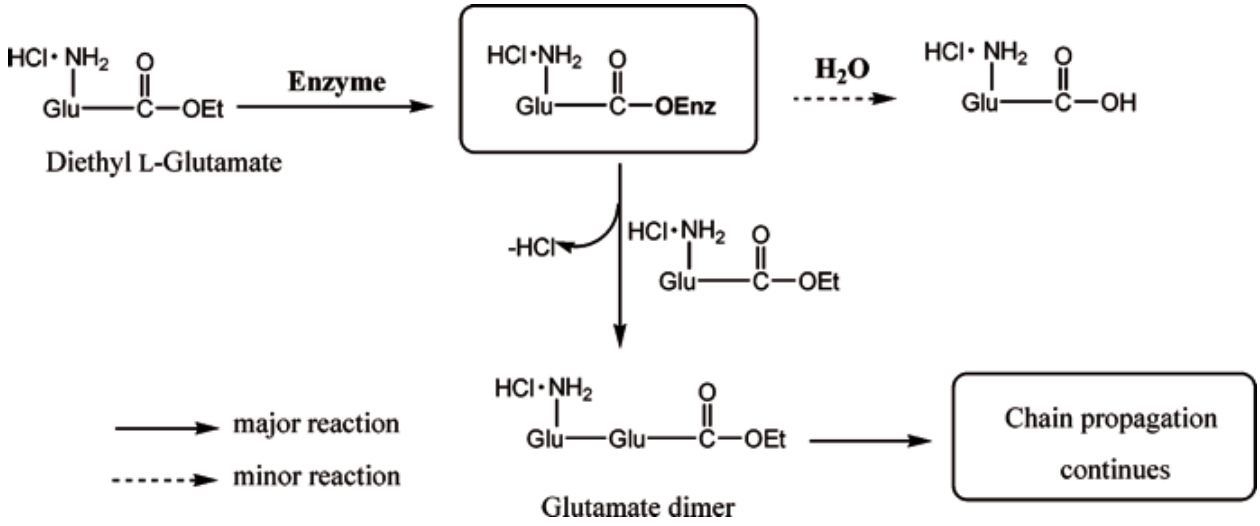

Figure 8.1. Kinetically controlled oligopeptides Synthesis Scheme (Source: Li et al. 2006).

8.2 Materials

The following materials were used:

- Commercial enzymes such as Trypsin, Papain, a-Chymotrypsin, Bromelain, Proteinase K and Protease.
- Extracts containing proteases obtained by SSF from hair waste (Gicomzim H corresponds to thermophilic peak extract and Gicomzim H' to 10 days fermentation process), and from soy residues at the thermophilic peak of fermentation (Gicomzim S). Both produced at GICOM-UAB, described in the previous chapters.

The commercial as well as the extract from SSF were a powder obtained by lyophilization. Reagents for the different assays are described below.

8.3 Methods

8.3.1 Protease Activity Assay Nº2

In this assay, as in protease assay described at Chapter 3 section 3.4.2, casein acts as a substrate and Folin & Ciocalteau's as reagent too. Because of that both assays share the same fundamentals. The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease. The recipe and steps are summarized below:

Chemicals and solutions

- **50 mM Potassium Phosphate Buffer, pH 7.5.**

Prepare by using 11.4 mg mL^{-1} of potassium phosphate dibasic, trihydrate in purified water and adjusting pH with 1M HCl. This solution is placed at 37°C prior to use.

- **0.65% weight/volume casein solution.**

Prepare by mixing 6.5 mg mL^{-1} of the 50 mM potassium phosphate buffer. The solution temperature is gradually increased with gentle stirring to 80-85 °C for about 10 minutes until a homogenous dispersion is achieved. It is very important not to boil the solution. The pH is then adjusted if necessary.

- **110 mM Trichloroacetic acid (TCA) solution.**

Dilute a 6.1N stock 1:55 with purified water.

- **0.5 M Folin & Ciocalteu's, or Folin's Phenol Reagent**.

- 500 mM Sodium Carbonate solution

Prepared by using 53 mg mL^{-1} of anhydrous sodium carbonate in purified water.

- **Enzyme diluent solution**

Consists of 10 mM Sodium Acetate Buffer with 5 mM Calcium, pH 7.5, at 37°C. This solution is what we use to dissolve solid protease samples or dilute enzyme solutions.

- **1.1 mM L-tyrosine Standard stock solution**

Prepare using 0.2 mg mL^{-1} L-tyrosine in purified water. This solution is diluted further to make our standard curve.

Procedure

For each enzyme test, 4 vials are needed. One vial is used as a blank, and three others are used to assay activity of three dilutions of the protease.

1. To each set of four vials add 5mL of 0.65% casein solution, and let them equilibrate in a water bath at 37°C for about 5 minutes. Then, add the enzyme solution to test to the three of the test sample vials, but not the blank. Mix them by swirling and incubate for 37°C for exactly ten minutes.
2. Add the 5 mL of the TCA reagent to each tube to stop the reaction. Then an appropriate volume of enzyme solution is added to each tube, even the blank, so that the final volume of enzyme solution in each tube is 1 ml. Then, incubate the solutions at 37°C for 30 minutes.
3. Filter each of the test solutions and the blank using a 0.45 um polyethersulfone syringe filter.
4. Add 5mL of sodium carbonate, and 1 mL of Folin's reagent is added immediately afterwards. The vials are then mixed by swirling and incubated at 37 ºC for 30 minutes.
5. After this incubation, 2 mL of filtered solution is measured by using a spectrophotometer at 660 nm (Figure 8.2).

Calibration curve

To set up the tyrosine standard dilutions (Figure 8.2), use 6 vials and add the 1.1 mM tyrosine standard stock solutions with the following volumes in mL: 0.05, 0.10, 0.20, 0.40, 0.50. Don't add any tyrosine standard to the blank. Once the tyrosine standard solution has been added, add an appropriate volume of purified water to each of the standards to bring the volume to 2 mL. Perform the same way as described before for samples. An example of the standard curve for the determination is presented on Annex I.

Calculation

Correct the measured absorbance for the controls (Abs sample - Abs control). From the standard curve the activity of protease samples can be determined in terms of Units, which is the amount in micromoles of tyrosine equivalents released from casein per minute. Should be calculated as follows:

$$PA\ (U\ mL^{-1}) = \frac{\text{micromol of Tyrosine released x 11}}{1 \text{ x } 10 \text{ x } 2} \quad \text{(equation 16)}$$

where: PA, is protease activity; U, units; 11, Total volume (in mL) of assay; 10, Time of assay (in minutes); 1, Volume (in mL) of enzyme used; 2, Volume (in mL) used in colorimetric determination.

$$PA\ (U\ mg^{-1}\ solid) = \frac{U\ mL{-}1\ enzyme}{mg\ solid\ mL{-}1\ enzyme} \text{ (equation 17)}$$

Figure 8.2. Calibration curve for protease assay (left) and spectrophotometer used during the assay (right).

8.3.2 Bicinchoninic acid Assay (BCA) - Soluble protein

The principle of the BCA assay relies on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the protein present. It has been shown that cysteine, cystine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to Cu^{1+}. BCA forms a purple-blue complex with

Cu^{1+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins.

Procedure

1. Mix 20 parts of the BCA Working Reagent* with 1 part of a protein sample.
2. * The BCA Working Reagent is prepared by mixing 50 parts of Reagent A with 1 part of Reagent B. Mix the BCA Working Reagent until it is light green in color.
3. Use 0.1 mL of a protein sample (standard or unknown sample) and 2 mL of the prepared BCA Working Reagent. The standard consists of a known concentration of bovine serum albumin (BSA) and for the blanks use buffer solution.
4. Prepare standards of different concentrations. These BSA protein standards can range from 200–1,000 mg mL^{-1} (20–100 mg of total protein).
5. Vortex gently for thorough mixing. The total liquid volume in the test tube is 2.1 mL.
6. Incubate at 37 °C for 30 minutes.
7. If required, allow the tubes to cool to room temperature.
8. Transfer the reaction solutions into a cuvette. Measure the absorbance of the solution at 562 nm.
9. Calculate protein content by using the standard curve with BSA protein standards. An example of the standard curve for the determination is also presented on Annex I.

8.3.3 General Procedure for Protease-Catalyzed Oligopeptides Synthesis

1. Dissolve 0.5 M of amino acid in 5 mL of phosphate buffer solution (PBS) 0.6 M set at a determined pH in a 50 mL tube. In this case, pH= 8, 9 and 10 were used.
2. Add the enzyme to be tested.
3. Stir gently in a water bath at 40ºC for 3 hours.
4. After this period of time, add acid to stop the reaction and cool the mixture to room temperature by adding 20 mL of cool deionized water.
5. Separate the insoluble product by centrifugation 4000 rpm.
6. Lyophilize the resulting insoluble product in order to analyze it by MALDI-TOF and H-NMR. Figure 8.3 (right).

Automatic pH control was performed using a Tiamo titration control system and Metrohm CH9101 dosing unit. The dosing solution (1 M and 6 M) was added at 0.05 - 0.1 uL min^{-1}, and the frequency at which the probe check the pH was set to 1s. All data was registered on the PC as shown in Figure 8.3 (left).

Figure 8.3. Hardware and Software Instruments used for Proteases catalyzed synthesis reactions (left) and lyophilizer machine (right) at RPI.

L-Glutamic Acid Diethyl Ester Hydrochloride, L-Alanine Ethyl Ester Hydrochloride, L-Leucine Ethyl Ester Hydrochloride and L-Phenylalanine Ethyl Ester Hydrochloride were used to screen the synthesis activity of UAB samples. The amino acids used were activated/protected by the form of ethyl ester as they are good leaving groups/protectors. Besides that, the ester group plays an important role in the active site of the enzyme. The enzyme is supposed to join to this kind of groups. The same amino acids catalyzed by papain were assayed in order to compare the products obtained by UAB samples. The quantity of enzyme utilized was selected according to group's works. All the procedure used and results were also compared with the last-mentioned works, such as Li et al., 2006, Li et al., 2008, Viswanathan et al., 2010, Viswanathan et al., 2012, and Qin et al., 2011.

8.4 Instrumental Methods to determine peptides

8.4.1 MALDI-TOF

Is a soft ionization technique used in mass spectrometry. Its name is because of the abbreviation of Matrix-Assisted Laser Desorption/Ionization and its associated detector, Time-Of-Flight.

This technique allows the analysis of biomolecules (polymers such as DNA, proteins, peptides and sugars) and large organic molecules, which tend to be fragile and fragment when ionized by more conventional ionization methods. The following figure 8.4 resumes the fundamentals of the technique:

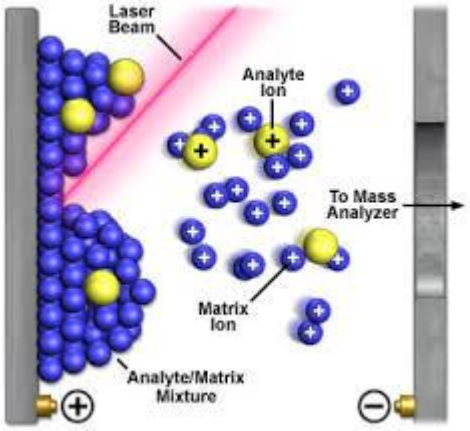

Figure 8.4. Fundamentals of the MALDI-TOF

Protocol of preparing samples: Dried-Droplet Method

Solution A: 10mL DMSO (dimethyl sulfoxide) and 10 uL TFA 0.1% (trifluoroacetic acid).

Solution B: 6.66 mL distilled water, 3.33mL ACN (acetonitrile) and 10 uL TFA.

Solution C: CCA (a-Cyano-4-hydroxycinnamic-acid) matrix saturated solution. Centrifuge after vortexing 15mg in 1 mL solution B.

1. Prepare sample solution 1 with sample and solution A (6 mg lyophilized sample x 0.5 mL^{-1} solution A)
2. Prepare sample solution 2 by diluting 10 uL of sample solution 1 with 240 uL of solution B.
3. Dilute 10 uL of sample solution 2 with 10 uL of solution C.

4. This solution is spotted onto a MALDI plate (usually a metal plate designed for this purpose). The solvents vaporize, leaving only the recrystallized matrix, but now with desired molecules embedded into MALDI crystals. The matrix and desired molecules are said to be co-crystallized.
5. Analyze the samples.

The OmniFlex MALDI-TOF instrument (Bruker Daltonics, Inc.) was operated in a positive ion reflector mode with an accelerating potential of +20 kV.

8.4.2 ^{1}H-NMR

Proton Nuclear Magnetic Resonance or H-NMR is a phenomenon, which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin.

When a sample of magnetically active nuclei is placed into an external magnetic field, the magnetic fields of these nuclei align themselves with the external field into various orientations. Each of these spin-states will be nearly equally populated with a slight excess in lower energy levels. During the experiment, electromagnetic radiation is applied to the sample with energy exactly equivalent to the energy separation of two adjacent spin states. Some of the energy is absorbed and the alignment of one nucleus' magnetic field reorients from a lower energy to a higher energy alignment (spin transition).

The signal in NMR spectroscopy results from the difference between the energy absorbed by the spins which make a transition from the lower energy state to the higher energy state, and the energy emitted by the spins which simultaneously make a transition from the higher energy state to the lower energy state. The signal is thus proportional to the population difference between the states. NMR is a rather sensitive spectroscopy since it is capable of detecting these very small population differences. It is the resonance, or exchange of energy at a specific frequency between the spins and the spectrometer, which gives NMR its sensitivity.

By sweeping the frequency, hence the energy, of the applied electromagnetic radiation, a plot of frequency versus energy absorption can be generated. This is the NMR spectrum.

Protocol of preparing samples

1. Prepare a solution with 10 mg sample in 1 mL DMSO. For UAB extracts, because of its impurities, the double of sample were used in order to obtain a good signal.
2. Place into a NMR tube.
3. Analyze by the ^{1}H-NMR.

8.5 Results and discussion

Proteases activities and Protein Content

As observed in the Table 8.1 the activities of the commercial proteases are logically higher than the SSF obtained proteases. The samples from SSF were separated from the solid fermented matrix and concentrated by lyophilizing although the samples were not pure, as seen on chapter 4. Salts and ions from the buffer and the fermentation process were not removed. Besides, another possible enzymes or proteins, part of the microorganisms or produced by them, could still remain there.

Assays, protease activity and protein content, are different from the ones used to work at UAB during this research, described at Chapter 3, section 3.4.2 and 3.4.3. In the case of the protease activities, the volume of the reaction and the temperature are the two principal differences. Meanwhile in the protein content assay, BCA respect to Bradford assay, the difference is related to the reagent used to determine the content of protein. The BCA assay is less susceptible to detergents and it is applicable over a broad range of protein concentrations. This is an interesting point to be carefully examined (by my colleagues at UAB lab), when working with other enzymes as lipases or other products and using some detergents in the extraction method. An amount of Triton X-100 until 5 % is compatible with BCA; contrary Bradford is compatible with less than 0.1 % of Tritón X-100. In addition to protein determination in solution, the BCA protein assay has other applications, including determination of protein covalently bound to agarose supports and protein adsorbed to multiwell plates. The use of this assay would be remarkable in case of continuing with immobilizations of proteases in the future.

Table 8.1 Casein hydrolytic activities and protein content of commercial and SSF obtained proteases.

Enzymes	Source	Name	Supplier	Cat#	PA (UmL^{-1})	PA (Umg^{-1} DM)	sPA (Umg^{-1} SP)
Papain	S	1P	CalBio Chem	5125	0.227	1.136	4.06
Papain	S	2P	CalBio Chem	5125	0.216	1.082	
Papain	S	3P	CalBio Chem	5125	0.127	0.634	
Papain	S	4P	Sigma	P3375	0.155	0.775	
Papain	RPI	6P	CalBio Chem	5125	0.179	0.897	3.08
Bromelain	S	1B	CalBio Chem	203761	0.064	0.318	
Bromelain	S	2B	Sigma	B4882	0.176	0.878	
Bromelain	S	3B	Sigma	B4882	0.073	0.367	
chymotrypsin	S	1A	Sigma	C4129	0.337	1.684	
chymotrypsin	RPI	2A	Chem-II	1783	1.136	5.680	5.68
Trypsin	S/RPI	1T	Sigma	T9201	0.662	3.310	
Trypsin	S	2T	Sigma	T7409	0.333	1.666	
Proteinase K	S	1K	Sigma	P2308	1.199	5.995	
Proteinase K	S	2K	Sigma	P2308	1.116	5.578	
Protease	S	-	Sigma	P5380	0.969	4.847	
Gicomzim S	UAB	S	SSF	May'13	0.022	0.110	1.09
Gicomzim H	UAB	H	SSF	3July'13	0.020	0.100	1.64
Gicomzim H	UAB	Hbis	SSF	3July'13	0.014	0.070	1.02
Gicomzim H’	UAB	H’	SSF	11July'13	0.017	0.085	1.23

S: Synthezyme, Dr. Gross’ company. RPI: Rensselaer Polytechnic Institute.

Instead, that all the commercial enzymes activities were determined, the subsequent assays were done only with Papain for several reasons. This enzyme is one of the most used and well-known by the hosting group and also cheaper than the others. The reaction with L-Glutamic Acid Diethyl Ester Hydrochloride was an easy start for a beginner on this topic, within only a couple of months to perform the experiments. Moreover, extracts Gicomzim H and S were not completely characterized in terms of type of proteases, specificity of substrate, and more. This was necessary as a first screen.

Analysis of Synthesis reaction by a commercial enzyme and Gicomzim S and H

The first assay was to reproduce the papain catalyzed synthesis Gross' Group studied. The product obtained with papain under established conditions (Li et al. 2006) was the same as assayed. This first reaction was assayed in order to familiarize with the procedure and learn about the reaction of synthesis catalyzed by proteases. Moreover, it was assayed with less quantity of Papain (4 mg mL^{-1} instead of 20 mg mL^{-1}) in order to compare afterwards with extracts Gicomzim, which had less activity. The structural analysis revealed that the product was the one expected. The ^{1}H-NMR spectrum of the precipitated product is displayed at Figure 8.5. Peaks positions and assignments are the same (b) as described for oligo (γ-ethyl-L-glutamate) at different references indeed Li et al. 2006 (a). In Figure 8.6, MALDI-TOF spectrum is shown and coincided with the product characterized by ^{1}H-NMR. In Figure 8.6 (a) and (b), 157 m/z units corresponding to the mass of oligo (γ-ethyl-L-glutamate) repeat units are the separations between the series of peaks. The mass peaks observed relate to polymerization degree (DP) values of 7 to 11. The major peaks were accompanied by a series of lower intensity peaks with m/z less by 28, which corresponds to hydrolysis of one ester group either at a chain end or a pendant group along oligo (γ-ethyl-L-glutamate). Matsumura et al. 1999, Uyama et al. 2002, Li et al 2006 among others referred that oligomers formed using Papain, Bromelain and a-Chymotrypsin are mostly a-linked.

The same reaction was probed with extracts Gicomzim S and H, at pH 8, 9 and 10 so as to determine the optimum working pH for these proteases. Previous studies on this extracts have revealed that the pH for hydrolysis was alkaline, being pH=11 the optimum (Abraham et al., 2013, Abraham et al., 2014b). No precipitate was obtained in any of the pH assayed. Besides,

analysis by ^{1}H-NMR of the lyophilized solution revealed that at pH higher than 8 the esters of amino acids were hydrolyzed (Figure 8.7a and 8.7b). Comparing both figures (Fig. 8.5 and Fig 8.7) is possible to see that peaks, which correspond to the ethyl ester group of the molecule, were not encountered on Figure 8.7 (peaks A and E of Figure 8.5 a).

There were two possible explanations: alkaline solutions hydrolyzed the esters so that the activate substrate was no more available to move towards the synthesis reaction; and the second possibility was that the extracts contained some other enzymes that produced the hydrolysis of the esters as a lipase. This activity was determined by one member of the hosting group and the results obtained indicated that no esterase activity were in the extracts. As a result of these first assays, it was decided to continue working at pH= 8.

Besides, the results at MALDI-TOF with S and H revealed that not too much quantity of product was formed. Moreover, a lot of peaks with different mass than expected were obtained (data not shown). These results also suggested that the enzyme from the extract H and S should be purified in order to avoid interferences when analyzing the results. On the other hand, the MALDI-TOF spectrum of the extract H and S before reaction showed a lot of different peaks and suggested a lot of impurities (data not shown). These impurities could correspond to salts, ions and other molecules due to the extraction process, as seen in previous chapters. According to references, higher concentration of enzyme is necessary for synthesis.

Afterwards, another strategy followed was to try the fast ultrafiltration with Millipore® tubes. According to the molecular mass obtained in previous electrophoresis studies, Amicon Ultra-15 10K unit were chosen to eliminate impurities and also amino acids with a hydrophobic side chain as L-Phenylalanine, L-Alanine and L-Leucine were assayed to favor a precipitate product that could be indeed easily separated. A member of the group taught the procedure of ultracentrifugation-diafiltration. Despite of the fact that this procedure requires a variety of steps and only a few were done, it allowed increasing the protein content up to the double, maintaining the protease activity. This method could be a good route to develop in future fermentations in order to obtain more purified samples after extracting from the fermented solids.

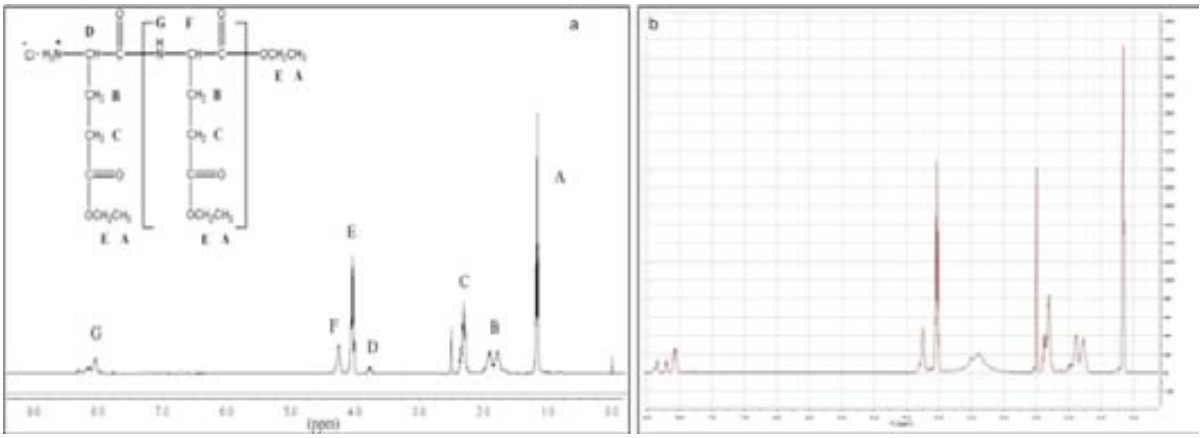

Figure 8.5 ^{1}H-NMR (300MHz,DMSO-d6) spectrum of oligo (γ-ethyl-L-glutamate) synthesized using a) 0.5M L-glutamic acid diethyl ester hydrochloride, 8 mg mL^{-1} Papain, 0.9 M phosphate buffer, at 40ºC (from Li et al. 2006). b) 4 mg mL^{-1} Papain.

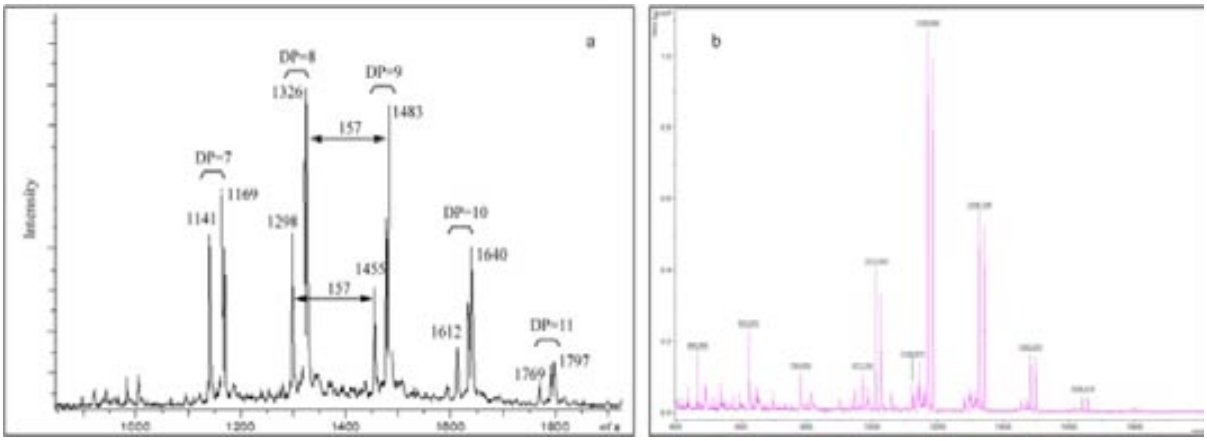

Figure 8.6 MALDI-TOF spectrum of oligo (γ-ethyl-L-glutamate) synthesized using 0.5 M L-glutamic acid diethyl ester hydrochloride at 40 ºC a) with 8 mg mL^{-1} catalyst (from Li et al. 2006) b) with 4 mg mL^{-1}.

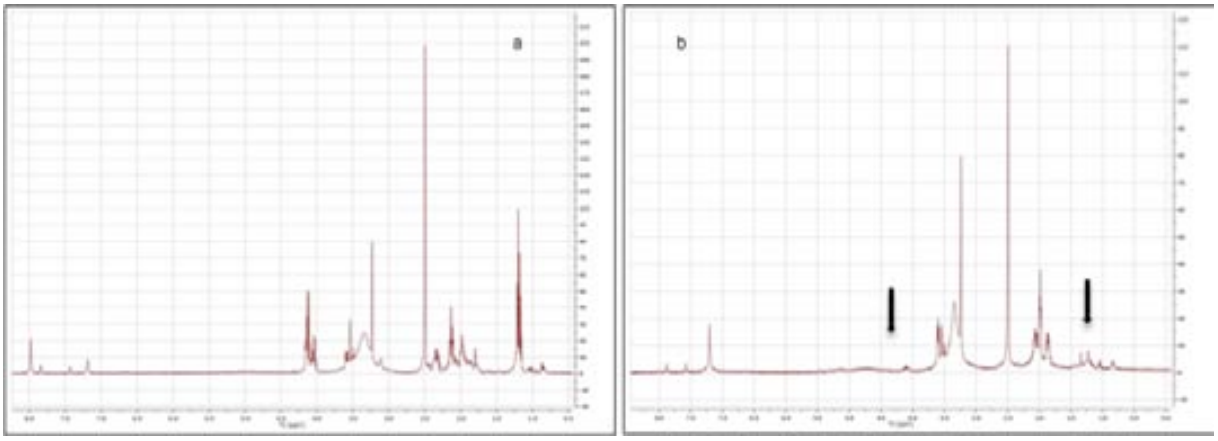

Figure 8.7 ^{1}H-NMR (300MHz,DMSO-d6) spectrum of lyophilized solution products of S at pH=8 (a) and (b) at pH=9. H spectrums are the same as for S.

To compare and analyze the supposed products obtained by extracts Gicomzim S and H with the hydrophobic amino acids, the same reaction was performed with Papain (Figure 8.8).

Samples from UAB were also performed with this amino acid using both extracts: with ultracentrifugation (Su and H'u) and without (S, H and H'). All the results obtained are presented in table 8.2. As shown, at the samples with ultracentrifugation the peaks that appeared, especially in H'u, were similar to those obtained with Papain. However, with less area, meaning less quantity of products. The separations between the series of peaks are repeat units of 147 m/z, which corresponds to the mass of oligo (L-Phenylalanine). The mass peaks correspond to DP values from 2 to 6.

Table 8.2. Peaks related to m/z obtained by MALDI-TOF analysis to different samples by using 0.5 M of L-Phenylalanine ethyl ester hydrochloride at 40ºC with 200 mg of S, H and H' and 150 mg of Su and Hu. Only one form was found, as Phe-OCH_2CH_3Na.

DP	Papain	S	H	H'	Su	H'u
2	363		363	363		363
3	511				510	510
4	657	656			657	657
5	804					805
6	951					951
7	1098					

DP, polymerization degree; S, H and H' SSF extracts samples without ultrafiltration; Su H'u, SSF extract samples with ultrafiltration.

At the same time, another two activated amino acids were tested. L-Alanine Ethyl Ester Hydrochloride and L-Leucine Ethyl Ester Hydrochloride were assayed with S. The second amino acid was evaluated increasing the quantity of enzyme. Another possible variable to test is the quantity of catalyst assayed. The results obtained with L-Alanine showed several peaks but none of them were similar to those of Papain. In contrast, results obtained with L-Leucine and higher quantity of catalyst showed some promising results (Table 8.3). There are two or three peaks that appeared in two different forms when using the extract Gicomzim S and comparing with papain products.

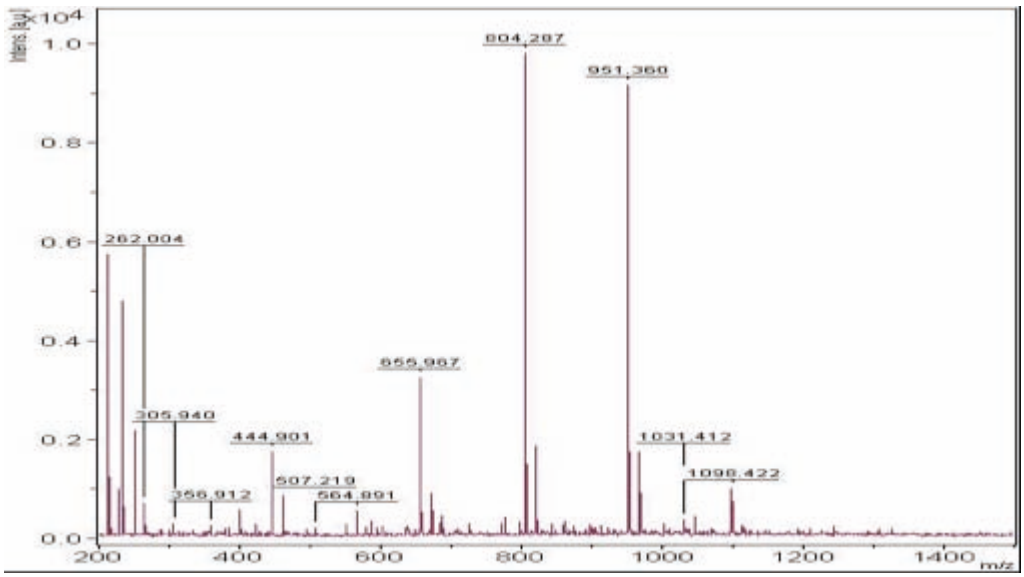

Figure 8.8. MALDI-TOF spectrum of oligo L-Phenylalanine synthesized using 0.5 M of this amino acid ester hydrochloride at 40 ºC with 4 mg/mL catalyst (Papain).

Table 8.3. Peaks related to mass (m/z) obtained by MALDI-TOF analysis to different samples by using 0.5 M of L-Leucine Ethyl Ester Hydrochloride at 40ºC with 500 mg of extract Gicomzim S

DP	Papain Leu-OCH_2CH_3	S Leu-OCH_2CH_3	Papain Leu-OHNa	S Leu-OHNa
4	635		607	
5	748	747	719	719
6	861	861	833	833
7	974		946	946
8	1087		1059	
9	1200			
10	1313			

DP: polymerization degree; S, extract from SSF of soy fiber residues.

8.6 Conclusions

Instead of the reduced time, a preliminary novel study on the synthesis of oligopeptides catalyzed by enzymes obtained by SSF could be proved as a first approach. Also, the fact that the hosting group worked with concentrated and pure enzymes was a useful advantage to learn about them and compare with the opposite situation that is having a crude extract sample.

Because it is well known that only serine and cysteine proteases can do this Michaelis-Menten complex, so these preliminary results suggested that this kind of enzymes might be present in these extracts from soy fiber and hair residues. However, this is not a proper procedure to know the class of the enzymes. As a previous step for synthesis an inhibition assay should be done in order to confirm the class of the proteolytic enzyme available in the extracts and indeed its specificity.

Likewise, due to the initial data obtained is it possible to use the extracts in synthesis but further investigations should be done. These following research would be the concentration of the extracts in order to obtain more activity per mg, the evaluation of the suitable quantity of extract to be used, the reaction time required, the careful investigation of the oligopeptide to be synthesized with uses in environmental purposes.

Moreover, the concentration and better separation of the enzymes obtained in the extracts should be considered due to use them on this particular application. As seen before, the crude extract with a high quantity of enzyme performed well in the dehairing process. However, in this case it is necessary to use the extracts as concentrated as possible to successfully proceed with the synthesis of a particular oligopeptide.

In sum, the synthesis of oligopeptides with this enzyme obtained by SSF from non-sterilized residues and without inoculation of a particular strain is very important to obtain another product that could be produced for a particular microrganisms but in sterilized conditions.

In consequence, further investigations are to be undertaken to explore this first approach. In this sense, the PhD colleague who is continuing with the production of proteases will also examine more in detail this application.

Chapter 9

Inoculation of a particular microorganism to produce proteases

Part of this chapter has been discussed and written for the elaboration of a research project (AGEC 2012), Generalitat de Catalunya.

Summary

This chapter is a further study on the SSF process with soy fiber residues because of its availability of C and N and high biodegradability. As demonstrated in previous experiments, this residue without sterilization reached the stabilization after 7 days of SSF process at bench scale. Besides, the inoculation of a consortium of microorganisms of those of compost was not a good alternative to obtain more proteases from soy fiber residues. In these experiments, a specific microorganism producer of proteases and also used as a bioinsecticide in agriculture was inoculated and evaluated. The objective was to valorize the residue by SSF and also increase the quality of the final material both with proteases as well as with its spores. In this sense, the challenge was to make growth this specific bacterium together with other microorganism (residue without sterilization) and so enhance the production of proteases and also the quality of the final amendment with spores of a bioinsecticide.

The following experiments were done in collaboration with a group colleague, Cindy Ballardo Matos, who is starting her PhD in SSF and continuing with this research.

9.1 Brief Introduction

The majority of the protease production studies on SSF have been performed with *Bacillus sp*. as seen before in Table 1.1 (Chapter 1) and they have also been extensively produced by SmF (Sundararajan et al. 20011, Jellouli et al. 2011). So far, proteases produced by the *Bacillus* especies are referred as one of the best commercial alkaline proteases (Singh et al., 2004).

Furthermore, some especies of this genus are also producers of other metabolites with potential uses. Concretely, *Bacillus thuringiensis* (Bt) is a facultative anaerobic gram-positive bacterium present in soil, water and plant surfaces. It is a spore former meaning that in adverse conditions the microrganisms has the capacity to become dormant until the conditions are favorable for grow again. It is also a producer of a toxin, a paraesporal crystal protein (δ-endotoxin). The toxin along with the spores have a great potential to cause mortality of insects belonging to different orders such as Diptera, Coleoptera and Lepidoptera. These insects are pests that destroy more than 40% of the world's food, forage, and fiber production. These toxins are encoded in Cry gens which confeir their names. They are secreted as protoxins and then cleaved

to be toxins; their mechanisms of action are also well-known and explained elsewhere (Brar et al. 2006). Figure 9.1 shows the different types of prototoxins/toxins and their molecular weights.

These biopesticides used in biological control of plagues, are an environmentally safe alternative to synthetics pesticides and have been used in Europe and worldwide for many years for food crops and forestry pests (FAO, 2007, Chandler et al., 2011). Due to limitations in the specific regulation for the product marketing and its use, they are not extensively used; however, their use is increasing nowadays (Chandler et al., 2011).

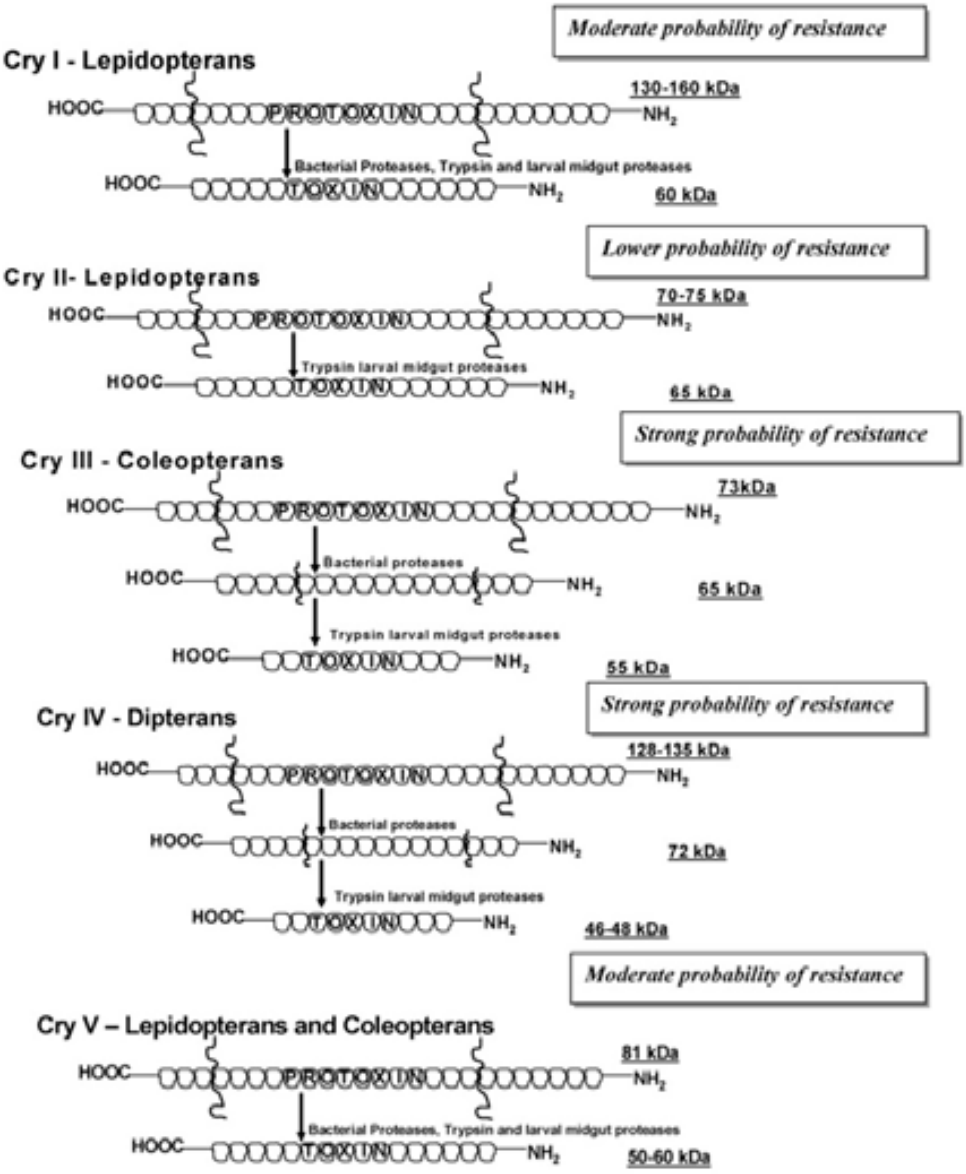

Figure 9.1. Cry I, II, III, IV and V toxins generated by Bt and its corresponding molecular weight. Source: Brar et al. 2006.

The production of Bt by SmF and SSF has been studied successfully under sterile conditions (Vimala Devi et al., 2005, El-Bendary, 2006, Zhang et al., 2013). Interestingly, Brar et al. (2006) and Yezza et al. (2006b) have produced Bt by SmF from wastewater slugde and a correlation between the toxicity of Bt and the protease activity produced was found. They suggested that monitoring the protease activity would be a simple indirect evaluation of toxicity produced during the fermentation.

9.2 Materials

The strain used was *Bacillus thuringiensis* subspecie kurstaki HD-73 ATCC® 35866, (Bt). Powder media to obtain liquid media for the growing of this microorganism was provided by Oxoid®.

9.3 Experiments

Microorganism

The bacterial strain was restored from the ATCC® lyophilized strain as described in ATCC® guide. After that, a seed lot system was prepared with glycerol 10% and conserved at -80ºC. When needed, one thawed Bt strain from seed lot system was cultured in nutrient agar plate for 1 day at 30 ºC and then conserved at 4 ºC until use it.

SmF

To prepare the SmF experiment, a loop from the agar plate was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of sterilized nutrient agar broth. The flask was then incubated with agitation for 20 h 30ºC.

SSF

The inoculum obtained by SmF was then centrifuged and the pellet inoculated in 100 g of soy fiber and bulking agent using the ratio obtained previously with this residue at lab scale. Firstly, sterile conditions were proved in order to understand the growth of this bacterium in this solid media, afterwards non-sterile conditions were probed.

Extraction

To extract the Btk cells from the solid matrix a sterile solution of NaCl 0.85 % (w:v) was used in a ratio 1:10 (w:v). The proteases were extracted as before with HCl-Tris, pH=8.1, ratio 1:5. Also the extraction with NaCl 0.85% and pure water, both in ratio 1:10 (w:v) were evaluated for protease extraction.

Estimation of cells and spores count

To determine total cell count (CC) in sterile conditions, the samples were serially diluted with sterile saline solution, NaCl 0.85% (w:v). 0.1 mL of the appropriate dilution were plated on nutrient agar plate and incubated at 30 ºC for 24 h to allow the development of the colonies. For spore count (SC), the same appropriate dilution was heated at 80 ºC for 10 min and then chilled on ice for 5 min. This shock of temperature allows the vegetative cells to lyse and liberate the spores already formed in the bacterial cells. Then, this solution was also plated in nutrient agar as for cell count. All the counts were done by triplicate and results were expressed as colony forming units per mL (CFU mL^{-1}).

9.4 Results and discussion

SmF experiments at lab scale

The Bt was successfully cultured in liquid media. The growth was assayed at 30ºC with (130 rpm) and without agitation. Figure 9.2 shows the exponential growth under both conditions. The growth of the Bt was measured by measuring the turbidity of the solutions by absorbance at 660 nm. As seen, the best growth was found in experiments with agitation because of the diffusion of the oxygen all over the medium. This experiment was performed because the best situation referred for the Bt was without agitation as its natural habitat, the soil. Thus, the standardization of the inoculum was settled in order to obtain the same quantity of inoculum every time a new experiment was performed.

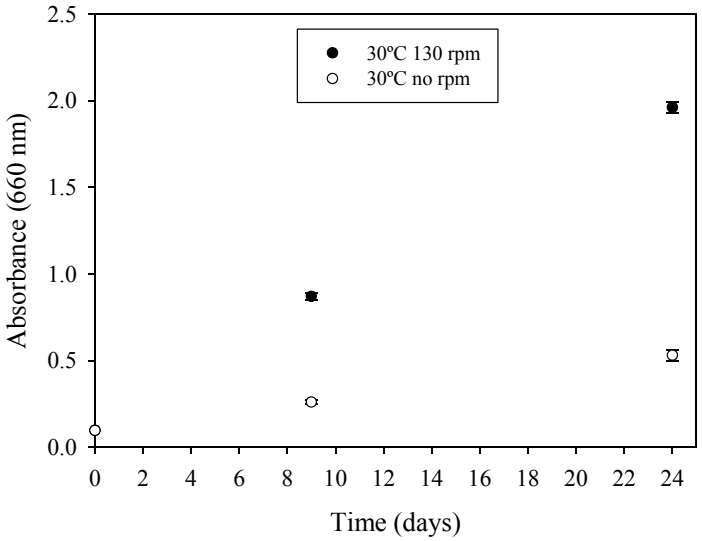

Figure 9.2. Growth of Bt in liquid media with and without agitation. Note that the Absorbance rises up to 2 because of the correction of the dilution assayed to measure.

The figure 9.3 shows the appearances of Bt at 40X optical microscopy (left). Note its rod shape, some bacillus appear in pairs or more. The figure 9.2 (right) shows the SEM image at 500X of the same culture. A characteristic form of the bacteria's grown in the liquid media was observed.

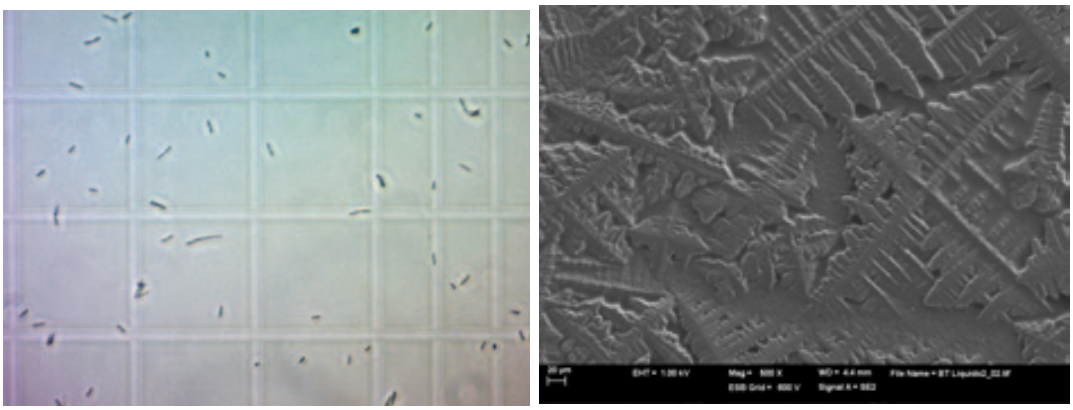

Figure 9.3. Bt at optical microscopy 40X (left). SEM image from the liquid culture 500X(right).

Moreover, the protease activity obtained by SmF was determined, being 1008 U mL^{-1}. The protease activity related to the mass of powder from nutrient agar (the substrate) used was 40315 ± 987 U g^{-1} DM.

SSF experiments at lab scale

Firstly, the SSF experiments were performed with sterile conditions to check if the Bt was able to grow in this particular substrate and the second experiment was performed without sterilization in order to evaluate the possible growth of Bt competing with other microorganisms and so avoiding the sterilization of the materials as in previous experiments. Moreover, in this particular case the capacity of the Bt to generate spores in adverse conditions is a strategy to explore.

Table 9.1 shows the characterization of the soy fiber. Because of the previous results, the soy fiber used was no longer frozen between experiments and so fresh residues were sent from the factory before each single experiment was carried out.

Table 9.1 Soy fiber residues characterization.

Waste	**Soy fiber**
Water content (%, db)	83.78 ± 0.01
Organic matter (%, db)	95.89 ± 0.1
pH	6.12 ± 0.01
EC (mS cm^{-1})	0.97 ± 0.01
DRI (g O_2 kg^{-1} DM h^{-1})	4.7 ± 0.2
Total Organic Carbon (% db)	53.2 ± 0.5
Total Kjeldahl Nitrogen (% db)	4.6 ± 0.07
C/N ratio	11.5

This experiment was performed per triplicate (Bt1, Bt2 and Bt3) for 6 days at 30 ºC and the inoculum used was 5 % (v:w). Figure 9.4 shows the oxygen content consumed (left up side) and the corresponding sOUR for each treatment (the rest of figures). The profile of the oxygen and the sOUR followed very similar patterns, although at the beginning there was a little difference in Bt3 probably due to any difference in the inoculum. Although all the inoculums were 5 % (v:w) probably they did not have the same amount of viable cells or probably some difference regarding the heterogeneity of the mixture and so this Bt developed less than the others. The peak evidenced at day 3 in Bt1 is because of the sampling. The sOUR1 was 2.69, 2.84 and 2.95 g O_2

kg $^{-1}$ DM for Bt1, Bt2 and Bt3, respectively. These sOUR are higher to those obtained with soy fiber performed before and explained in Table 6.2. at chapter 6.

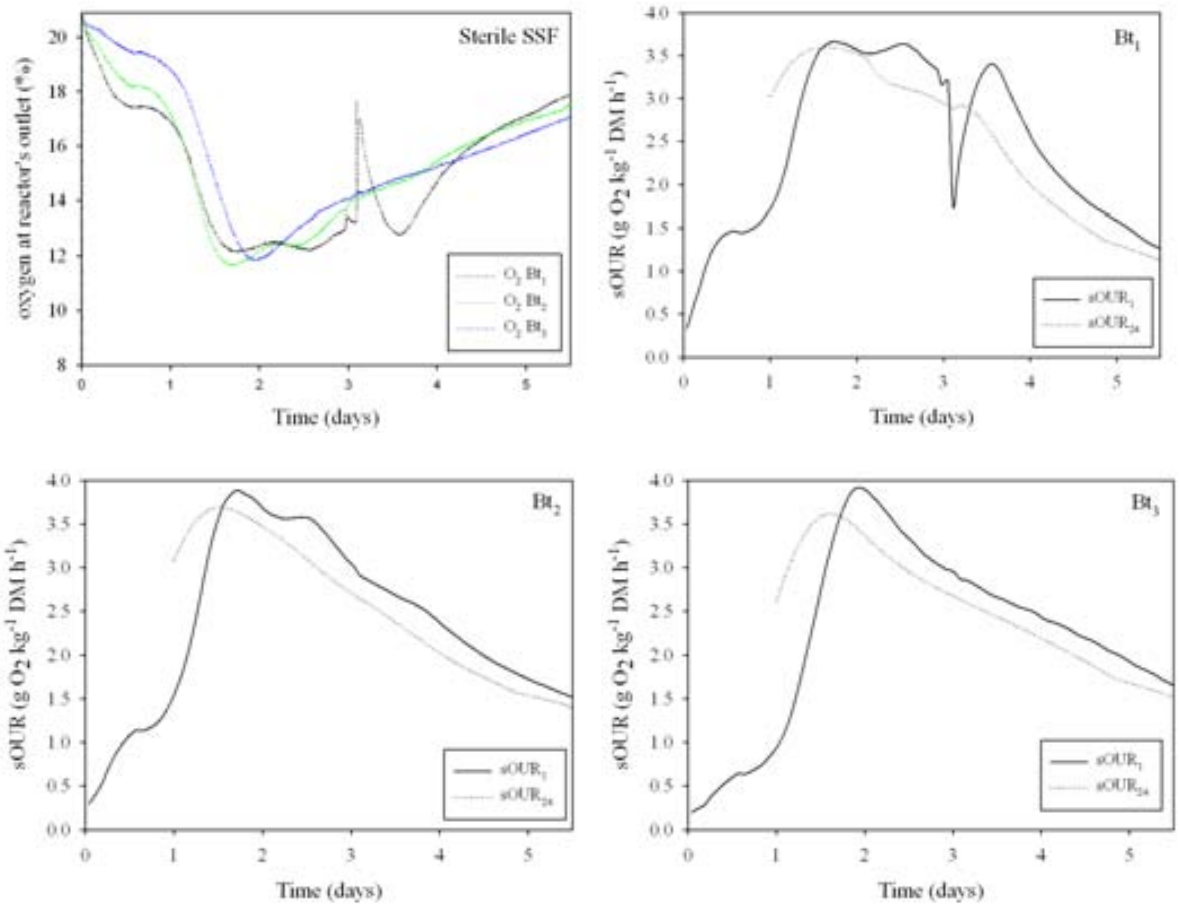

Figure 9.4. Oxygen content (left up side) and sOUR in sterile SSF experiment.

At 3 days of process only one reactor was sampled to determine the protease activity and also to count cell and spores. The same measurements were done at final stage, 6 days of SSF. The protease activity was measured in two different extracts; the first one with HCl-Tris buffer, as performed until now in this research, and the second one with NaCl 0.85% (w:v). The latter was described by Yezza et al. (2006a) and Zhuang et al. (2011) to determine the cells and spores present in the medium. So, from this extracts the protease activity and the cells and spores count were also determined. Table 9.2 shows the protease activity obtained in these extracts. These results showed that the protease activity obtained by SSF is pretty higher than the obtained in the SmF at the same scale in sterile conditions (40315 ± 987 U g^{-1} DM). The best production was

found at 6 days of SSF. Moreover, both solutions (HCl-Tris and NaCl 0.85%) are useful to extract the protease from the solid matrix. In addition, bigger protease activities were measured in NaCl 0.85 % extracts, being the average 142056 U g^{-1} DM compared to 202625 U g^{-1} DM at 6 days of SSF with Tris buffer and NaCl 0.85 % respectively.

Both values, at 3 and 6 days of SSF, are pretty higher than the values obtained in previous experiments at lab scale with the same residue in non-sterile conditions and without inoculation, described at Chapter 4 (37244 ± 1101 U g^{-1} DM).

Table 9.2. Protease activity production on sterile SSF experiments.

	PA (U g^{-1} DM)	
	Extracts	
Reactors	**HCl-Tris buffer**	**NaCl 0.85%**
Bt1, 3 days	109387±3156	153889±3820
Bt1, 6 days	171685±3762	162809±2137
Bt2, 6 days	126381±3148	195962±2159
Bt3, 6 days	128102±4346	249074±2760

Regarding the CC, the CFU were higher than the initial inoculum ($2.4x10^8$ UFC mL^{-1} compared to $1x10^{11}$ CFU mL^{-1}), indicating the adequately development in this solid matrix. Besides, almost the same quantity of spores (SC) was counted at initial and final stage ($1.4x10^{10}$ CFU mL^{-1}).

Figure 9.5 shows the SEM images obtained from the extracts. The form of the bacteria's growth in this media seemed similar to that of SmF.

The objective of performing the SEM images was to identify the toxin produced by the Bt. These toxins have particular forms and have been recognized by SEM in bibliography. However, no cristal were identified in this experiment.

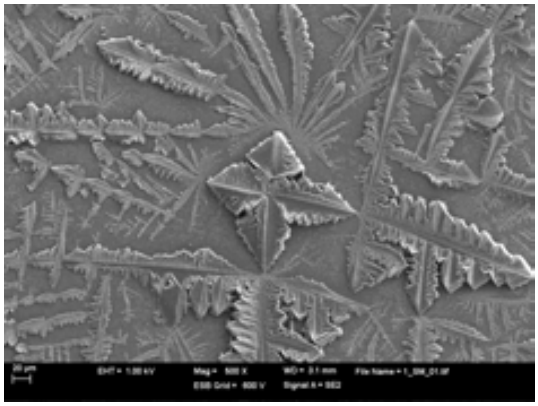

Figure 9.5 SEM image of Bt from solid culture. 500X.

On the other hand, SDS-PAGE assay was performed with the samples and two different stains, Coomasie Blue and Silver stain. Also, two different quantities of sample were assayed with Coomasie Blue (18 and 30 μL) because of the difficulties to see the band in previous assays, discussed at Chapter 4. From both gels stained with Coomasie Blue different bands were identified (not shown). The same bands were identified and also with more intensity in the gel stained with Silver because of the higher sensibility of this staining. The latter is presented at Figure 9.6. Samples were proven per duplicate, Bt corresponds to Bt1 at first sampling (3 days), Bt1f, Bt2f and Bt3f correspond to the different reactors at final stage (6 days). Bands at 100, 70-75, around 50 and more than 25 kDa were found, among others.

Afterwards, the band with MW around 70-75 kDa, MW similar to one of the Bt toxin expected, was cut from the gel stained with Coomaise Blue and posterior identification. MALDI-TOF-MS analyses were carried out in the UAB Proteomics facility, a member of ProteoRed-ISCIII network. It was not possible to analyze the bands stained with silver because of the incompatibility of this staining with the MALDI-TOF technique. Results indicated that the quantity of the band was not enough to be identified. Report from Proteomics facility is presented in Anex III.

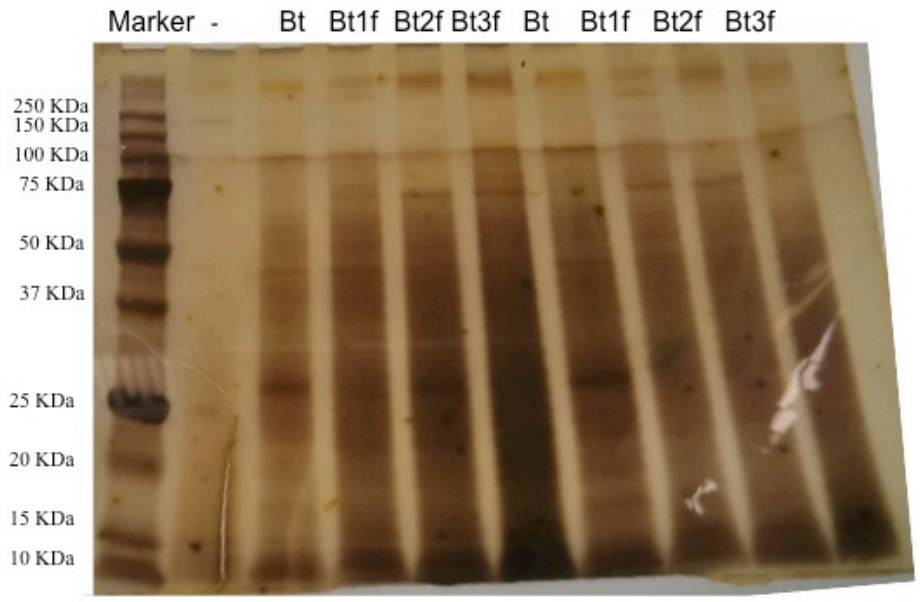

Figure 9.6. Silver stained SDS-PAGE with extracts at 3 days (Bt) and extracts at 6 days, final (f).

The second experiment was performed without sterilization and 12 % (v:w) inoculum of Bt. The amount of inoculum was incremented this time because the results obtained in sterile conditions (the growth was not high as expected) and because of previous experiments with the same soy fiber residues and the inoculation of *Bacillus subtilis* for the production of a biofloculant (Zulkeflee and Sanchez, 2014).

Due to the non-sterile conditions of this experiment, the evaluation of cell and spores to identify the development of the Bt by plating in agar were not realized. Contrary, the difference in growth was evaluated by taking into account only the respiration index, which represents as commented before, a measure of the biological activity.

Figure 9.7 shows the oxygen content consumed (left) and the corresponding sOUR for each treatment (right). As seen in the graphics, at the beginning the Bt could grow successfully, the oxygen and sOUR is marked higher than the control, but after that (day 2 until the final), probably other microorganisms competed for the nutrients and oxygen with the Bt, reaching same values of oxygen. At the beginning, the profile of the oxygen and the sOUR followed had less oxygen consumption and consequently sOUR for the control (Ctrl, without inoculation), as expected. The peak evidenced at 3 days in Bt1 and control is because of the sampling. The $sOUR_1$ was 4.98 and 4.07 and 4.44 g O_2 kg $^{-1}$ DM h^{-1} for Bt1, Bt2 and control, respectively. The

areas below sOUR curve were 10.95, 10.20 and 11.49 g O_2 kg $^{-1}$ DM. No differences between the $sOUR_1$ and OUR area were found. Nevertheless, these values are higher than the sOUR values obtained in sterile conditions.

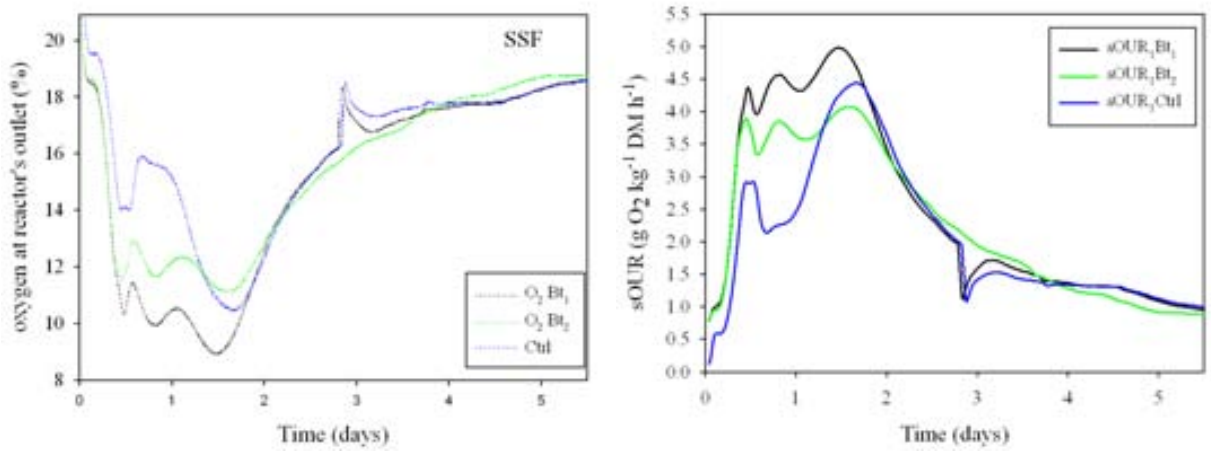

Figure 9.7. Oxygen content (left) and sOUR in non-sterile SSF experiment.

Moreover, the proteases activities obtained after 3 and 6 days of SSF process and extracted with NaCl 0.85 % solution were analyzed and are presented in table 9.3. This time, an extraction with pure water at 6 days of SSF was also evaluated. The protease activity at control reactor was similar or even higher than the reactor with Bt at both days of fermentation. In addition, the protease activity detected in pure water extracts was also lower than the one obtained with NaCl, probably due to lack of salt content that can buffered or stabilize the enzymes in the extract. In the current experiment, the sampling should be done around 1 day of SSF in order to see differences in the production of proteases. Interestingly, due to previous and current results, a profile production should be determined for proteases activity in no sterile conditions. However, Bt produces different types of proteases during exponential growth but also the post-exponential and stationary phases as referred by Yezza et al. (2006b, Brar et al. 2006).

Table 9.3. Protease activity production on non-sterile SSF experiments.

	PA (U g^{-1} DM)	
	Extracts	
Reactors	**NaCl 0.85%**	**pure water**
Bt1, 3 days	73918±2205	-
Control, 3 days	77549±2205	-
Bt1, 6 days	59656±2007	51399±1112
Control, 6 days	67108±2815	64082±4177

Also a SDS-PAGE was performed (Figure 9.8) with extracts of NaCl 0.85 % and pure water. In addition, the extracts were concentrated with AMICON® tubes as learned at RPI (Chapter 8). The concentration was done in order to obtain more bands to be identified by sequenciation. Moreover, only Coomasie Blue stain was used because of the compatibility with the proteomic technique. The same marker for Biorad® and also commercial BSA was used because of its MW= 66 kDa which is near the MW of one of the toxins expected. Moreover, knowing the concentration of this protein a quantification of the band could be then estimated.

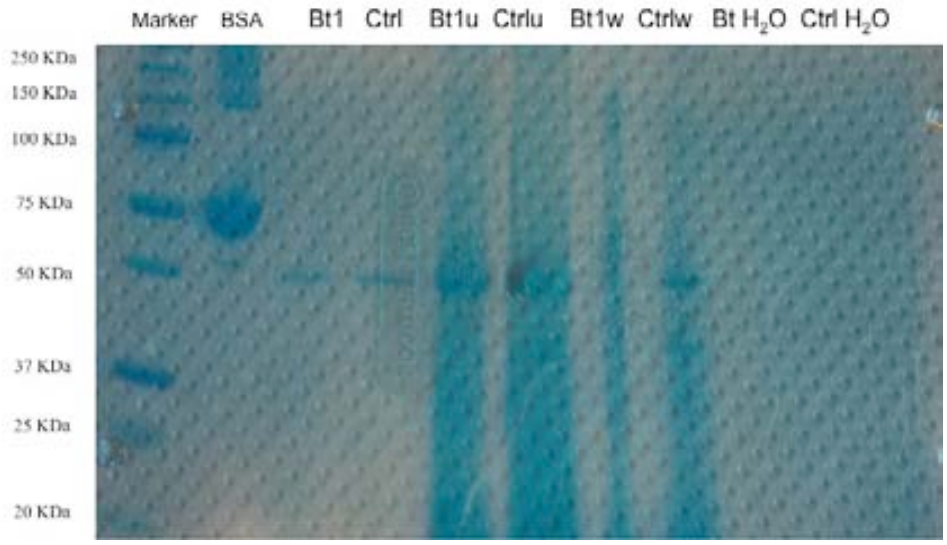

Figure 9.8. Coomasie Blue stained SDS-PAGE with Bt1 and Control (Ctrl) extracts with NaCl 0.85% and pure water (H_2O), u: extracts ultrafiltrated, w: wash obtained from the AMICON tube.

In this gel, a clear visualization of a band at around 50 kDa is observed with the extracts of Bt1 and the control (Ctrl) as well. The same bands but higher in intensity are observed in the ultrafiltrated extracts (Bt1u and Ctrlu). Also the washed extracts (Btw and Ctrlw) after the ultrafiltration-dialfiltration were evaluated in order to the see the correct concentration of the desired proteins. Finally, in the last two wells the samples extracted with pure water did not give clear bands. Although this band found (50 kDa) could be one of the toxins expected, did not correspond to one of them, because it appeared in both treatments (with and without inoculation). However, MALDI-TOF analyses were performed in case it was a protease. Report from Proteomics facility is also presented in Anex III and indicates that the band corresponds to another enzyme differnet from a protease, an alkaline phosphatase.

9.5 Conclusions

This study described the production of proteases in solid and in liquid media using a specific protease producer strain. The value of proteases obtained by SmF was 40315 ± 987 U g^{-1} DM, that is near the protease activity obtained in previous experiments at lab scale (37244 ± 1101 U g^{-1} DM), without sterilization of the substrate and with no inoculation. Additionally, inoculation strategy gave good results in protease production when using this substrate in sterile conditions, being 142056 ± 25674 U g^{-1} DM the average between the 3 reactors. The protease production was 3.5 times higher in SSF than in SmF performed in sterile condition and therefore the highest efficiency of solid-state compared to liquid culture for enzyme production. This study is in agreement with other studies reported (Belmessikh et al 2013). Also, validates that the use of this substrate produces higher proteases than SmF and reduces the cost of their production while contributing to the minimization of waste disposal.

On the other hand, several bands obtained by SDS-PAGE appeared similar in molecular weight to those of the Bt toxin (Ozcan et al. 2010) and proteases referred in bibliography (Pillai et al., 2011, Zanphorlin et al., 2012). Unfortunately, the bands weren't concentrated enough to be identified by sequenciation of the oligopeptides (MALDI-TOF tecnique).

Contrary, in the same experiments with non-sterile conditions, the production of proteases was lower than expected (comparing to a control with no inoculation), indicating that the Bt was probably competing with other microorganisms for nutrients and oxygen. Besides, SDS-PAGE revealed a band that could not be identified neither as a protease nor as a toxin.

Further studies on the evaluation of the inoculum, its quantity and the identification of the Bt among the development of other microorganisms in non-sterile conditions are been studied. Moreover, the identification of the growth of Bt by other methods instead of the OUR and the uses of the same residues as a inoculum istead of using SmF to obtain it.

Chapter 10

Conclusions and future research

The **conclusions** reached from the results obtained in this research are detailed below:

- The three local "residues" selected due to its N content that were soy fiber and coffee husk from food industries and hydrolyzed hair from tannery industry, were successfully stabilized through SSF at lab scale as well as presented production of protease, the biocatalyst desired.

- The methodology to extract and determine the enzyme activity was successfully settled for these materials. Whereas protease activity determination from solids and crude extracts samples was possible, the best was performed with the extracts (2 times more activity, expressed in U g-1 DM). Moreover, the extraction was also effective in terms of separation of the enzyme from the solid matrix for later application.

- The production of proteases by SSF process was proven feasible in near-to-adiabatic packed bed 4.5 L reactors. The protease production obtained was higher than experiments at lab scale and even than reported in bibliography. Neither need of sterilization of the materials nor inoculation of a particular strain were necessary. The on-line monitoring of the temperature and oxygen allowed to follow-up the entire aerobic process in terms of growth developed. Moreover, it was possible to calculate the oxygen uptake rate (OUR). All these statements demonstrated that the SSF under these conditions is potentially easily scalable for protease production.

- The protease profiles were studied for the three materials along 2 or 3 weeks of SSF at 4.5 L reactors and they were very different between each residue. For instance, the highest production of protease was at 3, 7 and 14 days for soy fiber, coffe husk and hair waste respectively; demonstrating that the protease production pattern varied with the biochemical composition and biodegradability of the materials. However, they all produced the enzyme desired; the best productions were obtained with soy fiber and hair, being 42884 and 54933 U g^{-1} DM, respectively.

- The inoculation with consortiums of microorganisms such as those of compost was proved with soy fiber and coffee husk with no increment in protease production. In these both materials the authoctonous microoganisms developed during the fermentation were enough for the appropriate degradation. However, the inoculation with micoorganisms present in raw sludge was successful for the suitable degradation of the hair.

- It was possible to partial characterize the crude extracts by means of pH and T stability, to establish a protocol of conservation until use of the enzymes produced. Besides, the identification of different enzymes presented due to the different molecular weights found was possible by PAGE assays. The latter was possible especially with extracts from hair.

- The protease crude extracts were consequently evaluated in dehairing of cowhides with no good results for soy fiber crude extracts and with successful results when using the hair extracts, probably due to the specificity of the enzyme obtained. Using these extracts for dehairing suggests the avoidance of large quantities of water and hazardous reagents. Moreover, this study opens the real possibility of assessing the dehairing with bigger areas of cowhides in the tannery.

- A novel preliminary study on kinetically controlled synthesis of oligopeptides was also performed by using the crude protease extracts with promising results. In these experiments the capacity of the enzyme to perform the reverse reaction under determined conditions was explored. This application would be of relevant importance to produce specific oligopeptides from protease, avoiding particular inoculations and high cost procedures in production of oligopeptides with determined functions.

- A study at pilot scale was possible to perform with hair waste and studied the gaseous emissions and energy consumption involved in the process for a following life cycle assessment (LCA) evaluation. In this experiment, the emissions were below the recommended values for volatile organic compounds (VOCs), methane (CH_4) and nitrous oxide (N_2O); however, the emissions of NH_3 were high especially at the thermophilic phase. Furthermore, these values were lower than the emissions obtained in composting

process of raw sludge and organic fraction of municipal solid wastes at the same pilot scale, indicating that the SSF process would be the biological treatment more efficient, in terms of emissions.

- The inoculation of soy fiber with *Bacillus thuringiensis*, a specific protease producer strain and its potential as a bioinsectide, was performed successfully, in sterile conditions, to produce more proteases and enhance the quality of the final amendment. Also the production of protease by this microorganism was 3.5 times higher by SSF than SmF.

Specifically, this study open 3 different routes described below that are already being studied:

1) The optimization of the extraction of the enzyme produced by SSF of hair for future application in dehairing at real scale in tannery and also to go further in the kinetically synthesis of oligopeptides.
2) The increase in the production of proteases and the quality of the final admentment with *Bacillus thuringiensis* by SSF in non-sterile conditions.
3) The enhancing in the production of protease by inoculating thermophilic strains as another strategy to obtain more enzymes without sterilizing the residues.

Regarding the possible immobilization of the protease for conservation as discussing in Chapter 4 and also for future uses, one possibility would be the use nanoparticles of iron (Fe_3O_4) as studied successfully by Xin et al. (2010). In this case, this topic would mix two lines of research in which the group has knowledge.

Furthermore, the classification of the enzyme according to its catalytic mechanism before continuing with the oligopeptide synthesis, an inhibition experiment would be interesting. This experiment would be done by incubating the extracts with different inhibitors such as EDTA, iodoacetic acid, phenylmethylsulfonyl fluoride (PMSF) among others (all inhibitors of proteases regarding their specificity) and measure the protease activity before and after incubation. For example, the decrease in the activity after incubation with PMSF will detemine that the enzyme is a serine protease; while decreases in the activity after incubation with iodoacetic acid will detemine that the enzyme is a cysteine protease and so. Moreover, this would be double checked

by doing a zymogram. In this case, a zymogram will be performed with the extracts before and after incubation with the inhibitors. Consequently, clear bands will appear in all the extracts before incubation because of the protease activity and no clear bands will appear in the extract after the incubation with the correct inhibitor.

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Annexes

I. Calibration curves

Calibration curve and calculation done for the protease activity assay modified for SSF of residues described on Chapter 3, section 3.4.2. Only one example is showed. Every set up of a new experiment, the calibration curve was re-done.

Table I.1. Standard curve prepared from 500 ug mL^{-1} Tyrosine Standard solutions.

Nº Tube	Standard (mL)	Buffer (mL)	Concentration (ug/mL)
0	0.00	5.00	0.00
1	0.25	4.75	8.30
2	0.50	4.5	16.70
3	0.75	4.25	25.00
4	1.00	4.00	33.30
5	1.25	3.75	41.70
6	1.50	3.50	50.00
7	1.75	3.25	58.30
8	2.00	3.00	66.70
9	2.25	2.75	75.00
10	2.50	2.5	83.30

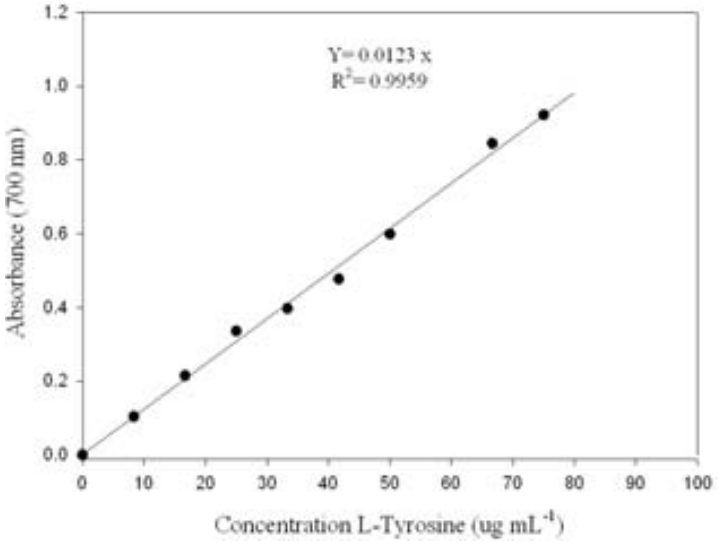


Figure I.1. Standard curve for Protease Activity. Absorbance = f (concentration)

Absorbance (700 nm) = 0.0125 * Concentration (ug mL^{-1})

Calibration curve and calculation done for the soluble protein content - Bradford assay described on Chapter 3, section 3.4.3. Only one example is showed. Every set up of a new experiment, the calibration curve was re-done.

Table I.2. Standard Curve prepared from 2 mg mL^{-1} bovine serum albumin (Sigma Aldrich ®)

Tube	C (mg/mL)	V (uL) Std	V(uL) water	Bradford (mL)
Blank	0.0	0	-	1.5
0	0.0	0	50	1.5
1	0.2	5	45	1.5
2	0.4	10	40	1.5
3	0.6	15	35	1.5
4	0.8	20	30	1.5
5	1.0	25	25	1.5
6	1.2	30	20	1.5
7	1.4	35	15	1.5

where: C, is concentration in mg mL^{-1}; V, is volume in microliter (uL); Std, is standard protein; Abs, is Absorbance at 595 nanometers (nm).

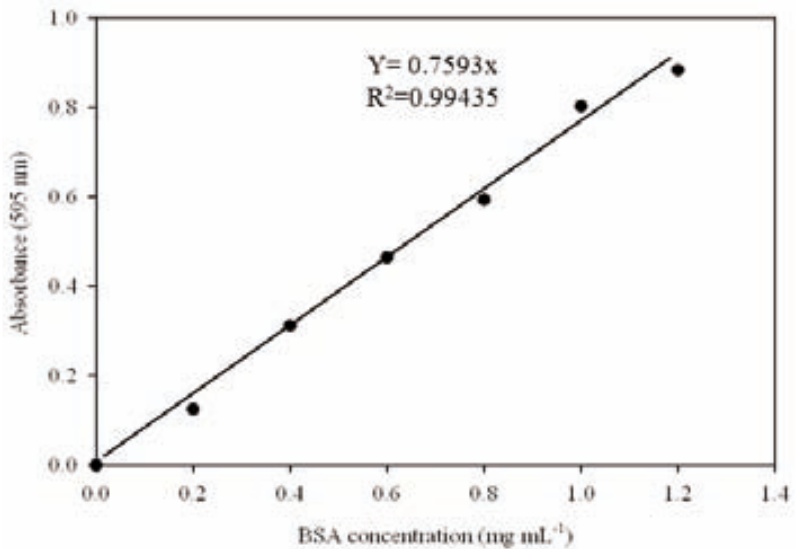


Figure I.2. Standard curve for soluble protein content. Absorbance = f (concentration)

Absorbance (595 nm) = 0.7593* concentration (mg mL^{-1})

Calibration curve and calculation done for the protease activity assay used during research stay described on Chapter 8, section 8.3.1.

Table I.3. Standard curve prepared from 1.1 mM L- Tyrosine Standard solution.

Nº Tube	**Standard (mL)**	**Water (mL)**	**micromol L-Tyrosine**
0	0.00	2.00	0.000
1	0.05	1.95	0.055
2	0.10	1.90	0.111
3	0.20	1.80	0.221
4	0.40	1.60	0.442
5	0.50	1.00	0.553

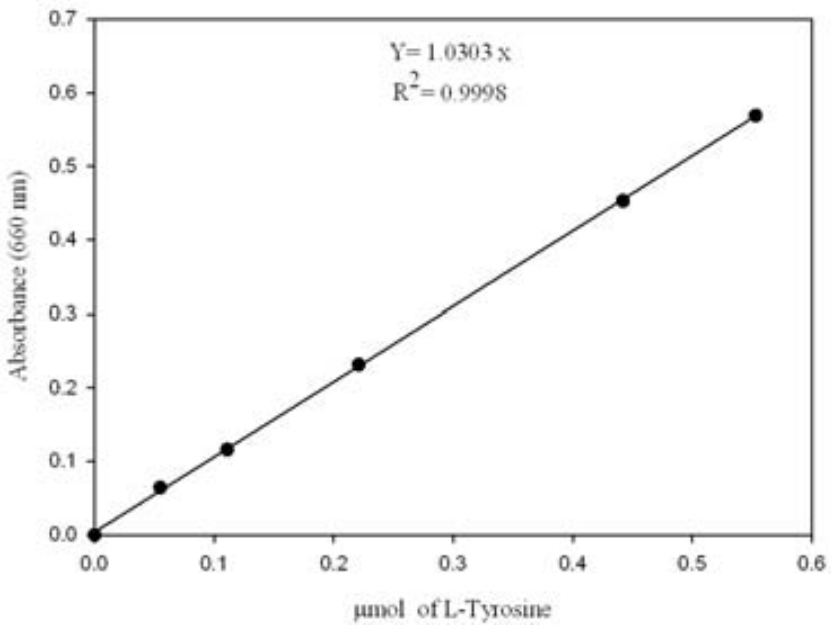


Figure I.3. Standard curve for Protease Activity. Absorbance = f (umoles L-Tyrosine)

Absorbance (660 nm) = 1.0303 * micromol of L-Tyrosine

Calibration curve and calculation done for the soluble protein content - BCA assay described on Chapter 8, section 8.3.2.

Table I.4. Standard Curve prepared from 1 mg mL^{-1} bovine serum albumin (Sigma Aldrich ®)

Tube	C (mg/mL)	V (uL) Std	V(uL) water	BCA (mL)
0	0.0	0	100	2
1	0.2	20	80	2
2	0.4	40	60	2
3	0.6	60	40	2
4	0.8	80	20	2
5	1.0	100	-	2

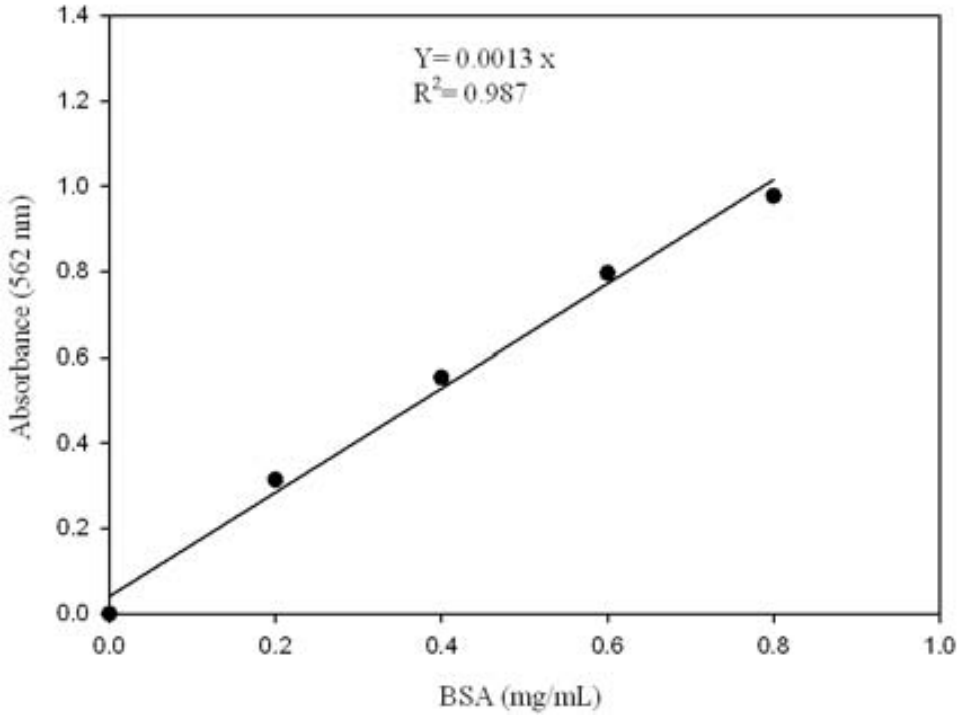


Figure I.4. Standard curve for soluble protein content. Absorbance = f (concentration)

Absorbance (562 nm) = 0.0013* concentration (mg mL^{-1})

II. pH and T factorial design experiments

Table II.1. Full factorial experimental design and results for stability analysis of the soy fiber and soy fiber with compost crude extracts.

pH	T (ºC)	RA (%) F	RA (%) FC
5	30	15	15
5	50	9	17
5	70	1	4
8	30	26	30
8	50	21	25
8	70	1	16
11	30	35	55
11	50	39	46
11	70	1	11
8	50	26	27
8	50	27	26
8	50	25	28

T, temperature; RA, residual activity; F, soy fiber extracts; FC soy fiber +compost extracts.

Table II.2. Full factorial experimental design and results for stability analysis of hair waste crude extracts.

pH	T (ºC)	RA (%) E3	RA (%) E7	RA (%) E14
5	30	13	9	12
5	50	12	10	13
5	70	1	6	0
8	30	18	23	30
8	50	29	26	32
8	70	10	4	4
11	30	62	55	66
11	50	63	49	59
11	70	17	6	1
8	50	28	25	27
8	50	28	28	28
8	50	31	24	28

T, temperature; RA, residual activity; E3, E7, E14, extracts day 3,7 and 14 respectively.

III. Proteomics analyses

SP-006B	Informe Resultados Espectrometría de masas: PMF	Servei de Proteòmica
UAB Universitat Autònoma de Barcelona / sePBioEs Servei de Proteomica i Biologia Estructural	CODIGO INFORME:	JAbraham_070214
	REALIZADO POR:	Sebastian Trejo

MUESTRAS

CÓDIGO SERVICIO	CÓDIGO CLIENTE	CÓDIGO SERVICIO	CÓDIGO CLIENTE
Bt	SP584		

CONDICIONES EXPERIMENTALES

1- Digestión tríptica de las bandas de gel:

a) *Destinción: Bicarbonato amónico 50mM / ACN 50%*

b) *Reducción: DTT 20 mM /20 min /60ºC*

c) *Alquilación: Iodoacetamida 25 mM / 15 min / 37ºC*

d) *Digestión con tripsina: 25 ng/muestra (sequencing grade-Promega) 3h /37ºC*

e) *Elución: H_2O:ACN (1:1)+0.2%TFA*

2- Preparación muestra para su análisis por espectrometría de masas MALDI-TOF:

Placa: *Ground steel*

Matriz: *Ácido α-4-hidroxicinámico (hcca)*

Equipo: *MALDI-TOF UltrafleXtreme (Bruker Daltonics)*

Se mezcla 1:1 muestra:matriz (0.5μL:0.5μL) y se deposita 1 μL sobre una placa ground steel. Se analiza la muestra usando un método en modo reflectrón y un voltaje de aceleración de 25kv. Los análisis se calibran usando calibradores externos

3- Observaciones: no se detectan péptidos, probablemente por una limitación en la cantidad inicial de muestra.

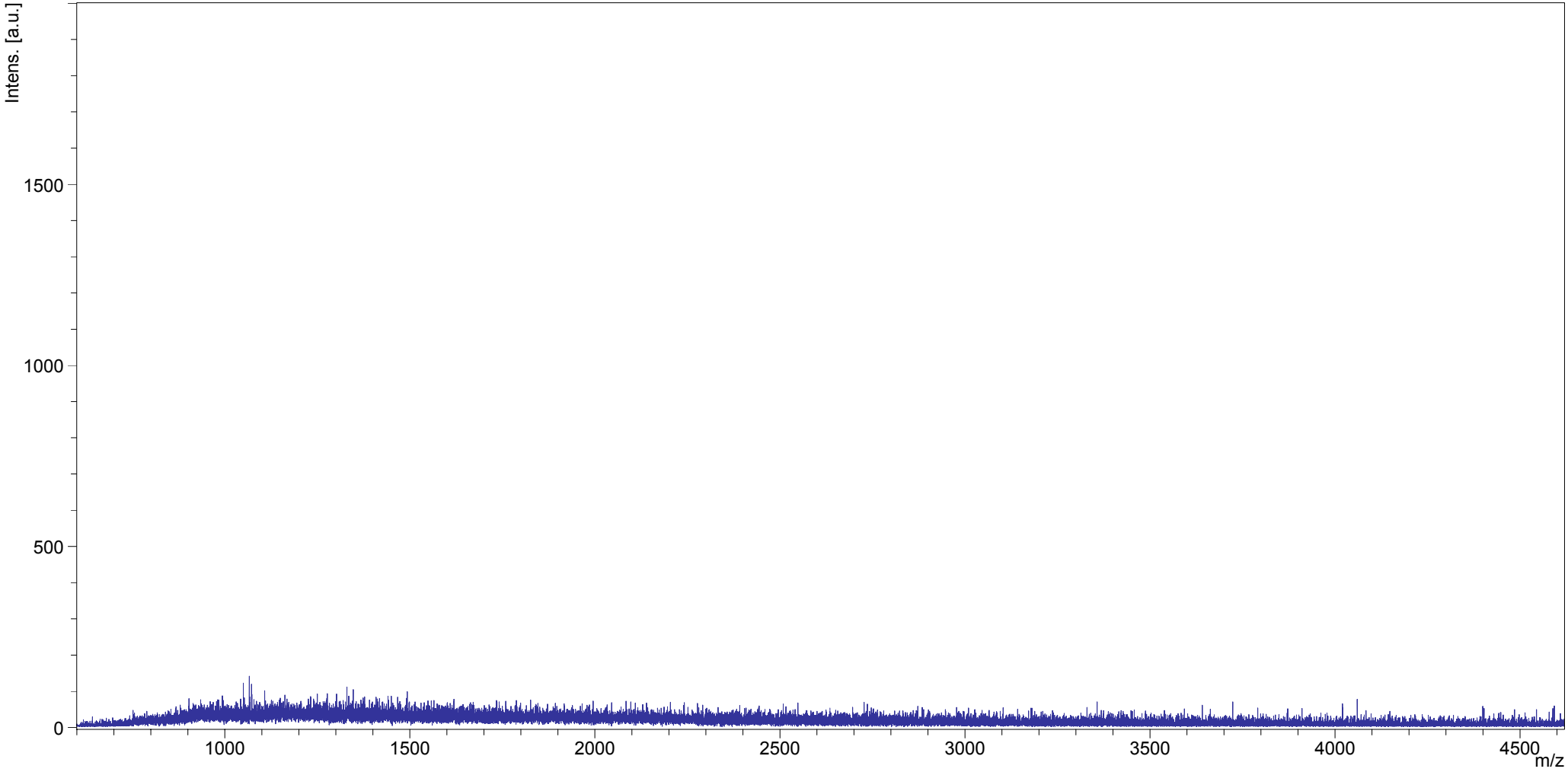
AGRADECIMIENTOS

Se agradecería a los usuarios que en las publicaciones o documentos dónde se haga referencia a los resultados aquí descritos, se haga constar que los análisis se han realizado en el Servei de Proteòmica de la UAB, el cual pertenece a Proteored, el Insituto Nacional de Proteómica-Instituto de Salud Carlos III, mediante una frase similar a la siguiente:

"The MALDI-TOF MS analyses were carried out in the Proteomics facility from UAB, a member of ProteoRed-ISCIII network"

Comment 1 SP584

Comment 2 ZipTip C18



SP-006B	Informe Resultados Espectrometría de masas: PMF	Servei de Proteòmica
UAB Universitat Autònoma de Barcelona / sePBioEs Servei de Proteomica i Biologia Estructural	CODIGO INFORME:	JAbraham_270314
	REALIZADO POR:	Sebastian Trejo

MUESTRAS

CÓDIGO SERVICIO	CÓDIGO CLIENTE	CÓDIGO SERVICIO	CÓDIGO CLIENTE
Muestra 1	SP622		

CONDICIONES EXPERIMENTALES

1- Digestión tríptica de las bandas de gel:

a) *Destinción: Bicarbonato amónico 50mM / ACN 50%*

b) *Reducción: DTT 20 mM /20 min /60ºC*

c) *Alquilación: Iodoacetamida 25 mM / 15 min / 37ºC*

d) *Digestión con tripsina: 25 ng/muestra (sequencing grade-Promega) 3h /37ºC*

e) *Elución: H_2O:ACN (1:1)+0.2%TFA*

2- Preparación muestra para su análisis por espectrometría de masas MALDI-TOF:

Placa: *Ground steel*

Matriz: *Ácido α-4-hidroxicinámico (hcca)*

Equipo: *MALDI-TOF UltrafleXtreme (Bruker Daltonics)*

Se mezcla 1:1 muestra:matriz (0.5μL:0.5μL) y se deposita 1 μL sobre una placa ground steel. Se analiza la muestra usando un método en modo reflectrón y un voltaje de aceleración de 25kv. Los análisis se calibran usando calibradores externos

3- Observaciones: no se detectan péptidos, probablemente por una limitación en la cantidad inicial de muestra.

AGRADECIMIENTOS

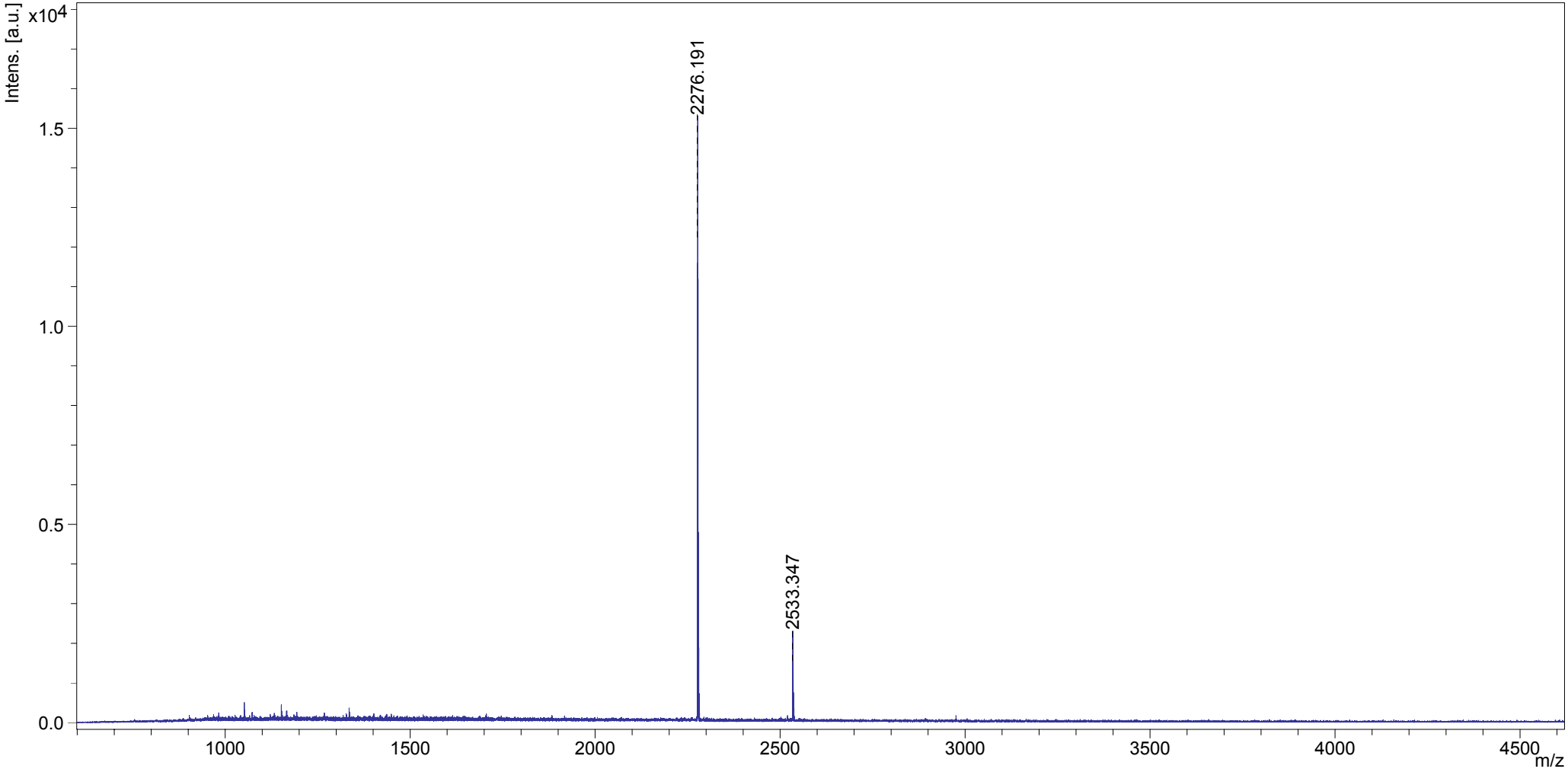
Se agradecería a los usuarios que en las publicaciones o documentos dónde se haga referencia a los resultados aquí descritos, se haga constar que los análisis se han realizado en el Servei de Proteòmica de la UAB, el cual pertenece a Proteored, el Insituto Nacional de Proteómica-Instituto de Salud Carlos III, mediante una frase similar a la siguiente:

"The MALDI-TOF MS analyses were carried out in the Proteomics facility from UAB, a member of ProteoRed-ISCIII network"

\\ULTRAFLEXTREME\Data\MALDI_USERS\1_SePBioEs\JAbrahamson\140326\0_M5\1\1SRef

Comment 1 SP621

Comment 2 HCCA



SP-006B	Informe Resultados Espectrometría de masas: PMF	Servei de Proteòmica
UAB Universitat Autònoma de Barcelona / sePBioEs Servei de Proteomica i Biologia Estructural	CODIGO INFORME:	JAbraham_270314
	REALIZADO POR:	Sebastian Trejo

MUESTRAS

CÓDIGO SERVICIO	CÓDIGO CLIENTE	CÓDIGO SERVICIO	CÓDIGO CLIENTE
Muestra 1	SP622		

CONDICIONES EXPERIMENTALES

1- Digestión tríptica de las bandas de gel:

a) *Destinción: Bicarbonato amónico 50mM / ACN 50%*

b) *Reducción: DTT 20 mM /20 min /60ºC*

c) *Alquilación: Iodoacetamida 25 mM / 15 min / 37ºC*

d) *Digestión con tripsina: 25 ng/muestra (sequencing grade-Promega) 3h /37ºC*

e) *Elución: H_2O:ACN (1:1)+0.2%TFA*

2- Preparación muestra para su análisis por espectrometría de masas MALDI-TOF:

Placa: *Ground steel*

Matriz: *Ácido α-4-hidroxicinámico (hcca)*

Equipo: *MALDI-TOF UltrafleXtreme (Bruker Daltonics)*

Se mezcla 1:1 muestra:matriz (0.5μL:0.5μL) y se deposita 1 μL sobre una placa ground steel. Se analiza la muestra usando un método en modo reflectrón y un voltaje de aceleración de 25kv. Los análisis se calibran usando calibradores externos

3- Observaciones: no se detectan péptidos, probablemente por una limitación en la cantidad inicial de muestra.

AGRADECIMIENTOS

Se agradecería a los usuarios que en las publicaciones o documentos dónde se haga referencia a los resultados aquí descritos, se haga constar que los análisis se han realizado en el Servei de Proteòmica de la UAB, el cual pertenece a Proteored, el Insituto Nacional de Proteómica-Instituto de Salud Carlos III, mediante una frase similar a la siguiente:

"The MALDI-TOF MS analyses were carried out in the Proteomics facility from UAB, a member of ProteoRed-ISCIII network"

G:\SePBioEs\MALDI\2014\TGea\JAbrahamson\140327\0_M5\1\2276.1600.LIFT\1SRef

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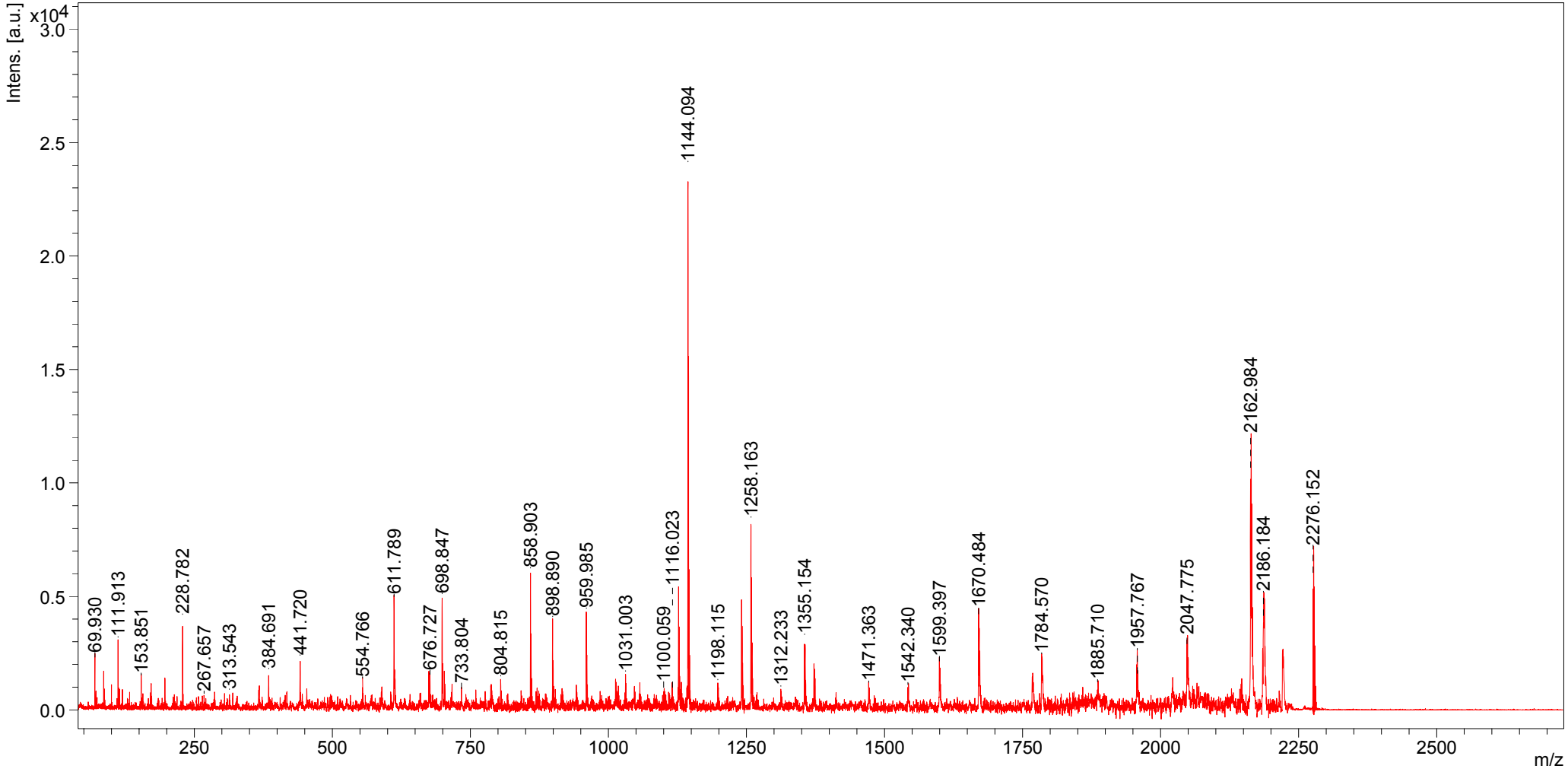
Comment 2 SP622 HCCA



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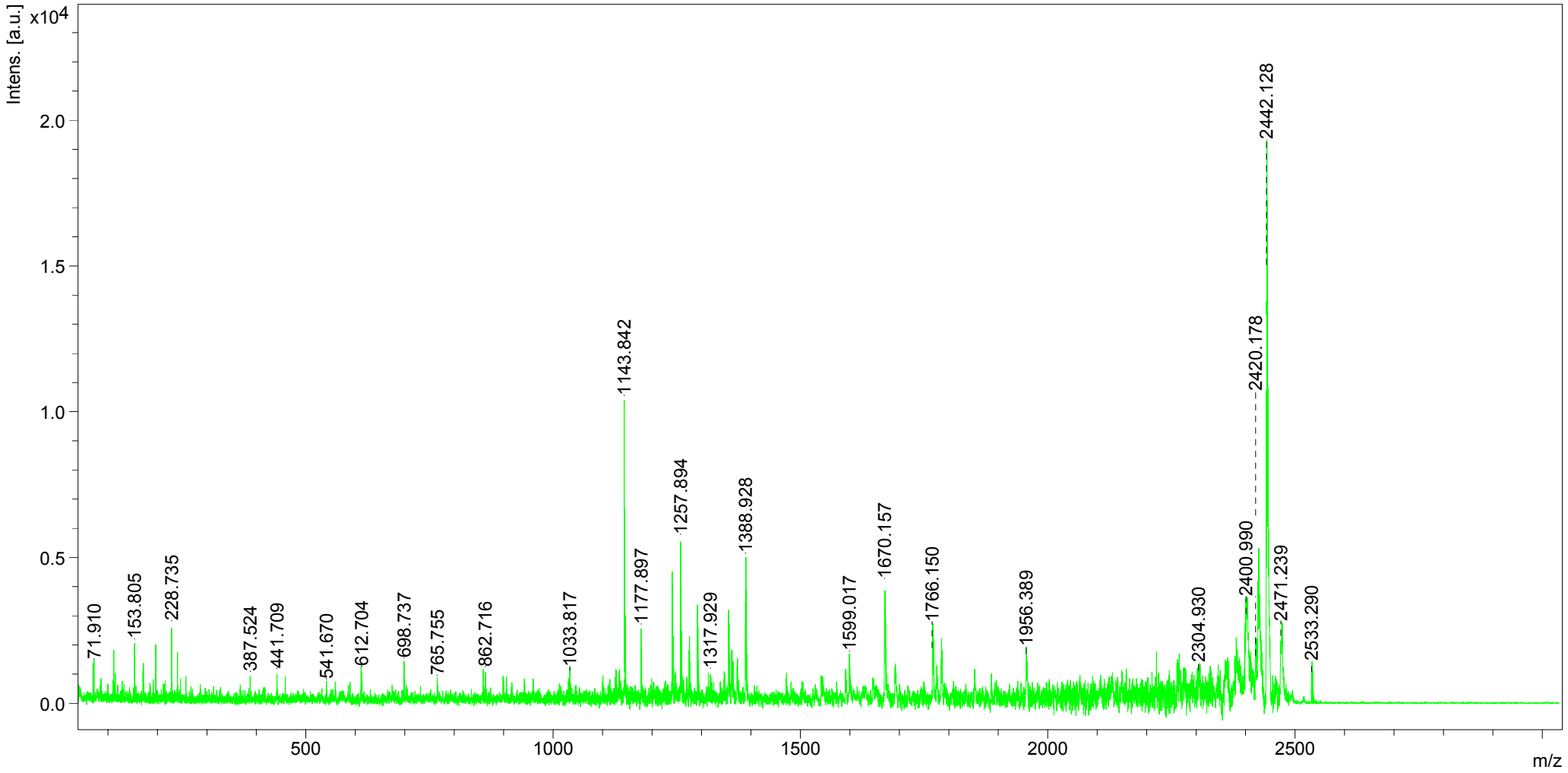
Comment 2 SP622 HCCA

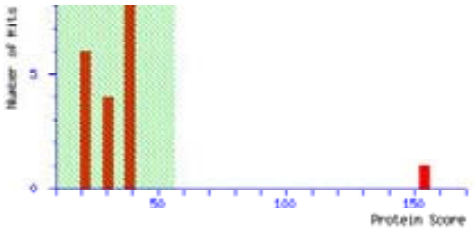


Mascot Search Results

```
User            : SAT
Email           : sebatrejo@gmail.com
Search title    : SP622 MSMS
MS data file    : DATA.TXT
Database        : NCBInr 20140323 (38032689 sequences; 13525028931 residues)
Timestamp       : 28 Mar 2014 at 14:40:10 GMT
Protein hits    : gi|194365985 alkaline phosphatase [Stenotrophomonas maltophilia R551-3]
                  gi|493100150 integral membrane protein, partial [Streptomyces gancidicus]
                  gi|185132459 fatty acid-binding protein, heart [Oncorhynchus mykiss]
                  gi|571922808 glycosyl transferase family 1 [Afipia sp. P52-10]
                  gi|548021632 s-adenosylmethionine synthase [Sutterella sp. CAG:397]
                  gi|532038943 PREDICTED: proenkephalin-A [Microtus ochrogaster]
                  gi|17987072  alanyl-tRNA synthetase [Brucella melitensis bv. 1 str. 16M]
                  gi|516221145 hypothetical protein [Nocardiopsis chromatogenes]
                  gi|158425467 ParB-like partition protein [Azorhizobium caulinodans ORS 571]
                  gi|34786818  p24 protein [Human immunodeficiency virus 1]
```

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 56 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As [Peptide Summary] Help

Significance threshold p< [0.05] Max. number of hits [10]

Standard scoring ◉ MudPIT scoring ○ Ions score or expect cut-off [0] Show sub-sets [0]

Show pop-ups ◉ Suppress pop-ups ○ Sort unassigned [Decreasing Score] Require bold red ☐

Preferred taxonomy [All entries]

Overview Table

Click on column header to jump to entry in results list.
Move mouse over any indicator to highlight identical peptides.
Click on an indicator to see details of individual match.
Use check boxes to select sub-set of queries for new search.

Mouse over: [-Query-] [-Accession-] [-Sequence-]

Hit:	1	2	3	4	5	6	7	8	9	10
☑ 1051.7692 (1+)										
☑ 1151.7904 (1+)										
☑ 1165.8037 (1+)										
☑ 1334.9565 (1+)										
☑ 2276.1912 (1+)	●	●	●	●	●	●	●	●	●	●
☑ 2533.3471 (1+)										

Select All | Select None | Search Selected | ☐ **Error tolerant**

1. gi|194365985 **Mass:** 40590 **Score:** 154 **Matches:** 1(1) **Sequences:** 1(1)
alkaline phosphatase [Stenotrophomonas maltophilia R551-3]
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2275.1015	0.0824	0	154	1.2e-11	1	U	R.FATATNAGAVNNPSTCSGIGRPL.- + Carbamidomethyl (C)

Proteins matching the same set of peptides:
gi|498171181 **Mass:** 40540 **Score:** 154 **Matches:** 1(1) **Sequences:** 1(1)
alkaline phosphatase [Xanthomonadaceae]
gi|518167560 **Mass:** 40514 **Score:** 154 **Matches:** 1(1) **Sequences:** 1(1)
alkaline phosphatase [Stenotrophomonas maltophilia]

---

2. gi|493100150 **Mass:** 27487 **Score:** 42 **Matches:** 1(0) **Sequences:** 1(0)
integral membrane protein, partial [Streptomyces gancidicus]
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2275.1444	0.0395	0	42	1.6	2	U	R.AAVAGVYDAVAALLDAAGSDGATAR.R

3. gi|185132459 **Mass:** 14520 **Score:** 42 **Matches:** 1(0) **Sequences:** 1(0)
fatty acid-binding protein, heart [Oncorhynchus mykiss]
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2274.2141	0.9698	0	42	1.9	3	U	R.QVGGMTKPTTIIEVAGDTVTLK.T + Oxidation (M)

Proteins matching the same set of peptides:
gi|185134100 **Mass:** 14621 **Score:** 42 **Matches:** 1(0) **Sequences:** 1(0)
muscle fatty acid binding protein [Salmo salar]

4. gi|571922808 **Score:** 39 **Matches:** 1(0) **Sequences:** 1(0)
glycosyl transferase family 1 [Afipia sp. P52-10]
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2275.1056	0.0783	0	39	3.5	4	U	R.TVLAGIAADFSPDVIHCNGYR.E + Carbamidomethyl (C)

5. gi|548021632 **Score:** 38 **Matches:** 1(0) **Sequences:** 1(0)
s-adenosylmethionine synthase [Sutterella sp. CAG:397]
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2274.0620	1.1219	0	38	4.2	5	U	K.VADQISDAVLDACLAQDPMSR.V + Carbamidomethyl (C)

6. gi|532038943 **Score:** 36 **Matches:** 1(0) **Sequences:** 1(0)
PREDICTED: proenkephalin-A [Microtus ochrogaster]
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2274.0912	1.0927	0	36	7.6	7	U	K.DLLQMSKPELPWDSPDMLK.D + 2 Oxidation (M)

7. gi|17987072 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella melitensis bv. 1 str. 16M]
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2275.1445	0.0394	0	35	8.5	8	U	K.LALGGGSSDGGSAVEAVNGVNFLGK.I

Proteins matching the same set of peptides:
gi|225852697 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella melitensis ATCC 23457]
gi|490823605 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella melitensis]
gi|493130345 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella melitensis]
gi|493157564 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella sp. 63/311]
gi|493217024 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella suis]
gi|493298386 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella melitensis]
gi|493307961 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella melitensis]
gi|493692127 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella ceti]
gi|495781030 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella inopinata]
gi|496220115 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella sp. NVSL 07-0026]
gi|496221874 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella]
gi|496823323 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella sp. BO2]
gi|516360230 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella melitensis]
gi|560146994 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella ceti TE10759-12]
gi|594563398 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
alanyl-tRNA synthetase [Brucella melitensis 548]

8. gi|516221145 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
hypothetical protein [Nocardiopsis chromatogenes]
☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2275.1301	0.0538	0	35	8.8	9	U	R.LVTVNCMALGPQGADGSVTTLR.D + Carbamidomethyl (C); O:

9. gi|158425467 **Score:** 35 **Matches:** 1(0) **Sequences:** 1(0)
ParB-like partition protein [Azorhizobium caulinodans ORS 571]

☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2274.1215	1.0624	0	35	9	10	U	R.AVDLDMAAAGWRPTVDNFLGR.V

---

10. gi|34786818 **Score:** 31 **Matches:** 1(0) **Sequences:** 1(0)
p24 protein [Human immunodeficiency virus 1]

☐ Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
5	2276.1912	2275.1839	2275.0647	0.1192	0	36	7.5	6	U	R.GLGPGATLEEMMTACQGVGGPGVK.A + Oxidation (M)

Peptide matches not assigned to protein hits: (no details means no match)

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
☑ 6	2533.3471	2532.3398	2531.2247	1.1151	0	29	24	1		TIENTPALTCVPNTSQDVCDIIK + Carbamidomethyl (C)
☑ 1	*1051.7692*	*1050.7619*								
☑ 2	*1151.7904*	*1150.7831*								
☑ 3	*1165.8037*	*1164.7964*								
☑ 4	*1334.9565*	*1333.9492*								

Search Parameters

Type of search : MS/MS Ion Search
Enzyme : Trypsin
Variable modifications : Carbamidomethyl (C),Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 0.2 Da (# ^{13}C = 1)
Fragment Mass Tolerance: ± 0.8 Da
Max Missed Cleavages : 0
Instrument type : MALDI-TOF-TOF
Number of queries : 6

Mascot: http://www.matrixscience.com/