Universitat Autònoma de Barcelona

SUSTAINABLE CARBOHYDRASE PRODUCTION USING ORGANIC WASTES THROUGH SOLID-STATE FERMENTATION: OPERATIONAL STRATEGIES AND MICROBIAL COMMUNITIES ASSESSMENT

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PhD Thesis

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Antoni Sánchez Ferrer i Teresa Gea Leiva, professors titulars del Departament d' Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

Certifican:

Que la enginyera bioquímica i màster en estudis ambientals Alejandra Patricia Cerda Llanos ha realitzat sota la nostra direcció el treball amb títol "Sustainable carbohydrase production using organic wastes through solid-state fermentation: Operational strategies and microbial communities assessment", que es presenta en aquesta memòria i que constitueix la seva tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I perquè en prengueu coneixement i consti els efectes oportuns, es presenta a l'Escola de Enginyeria de la Universitat Autònoma de Barcelona l'esmentada tesi, signant el present certificat.

Dr. Antoni Sánchez Ferrer

Dra Teresa Gea Leiva

Bellaterra, Febrer 2017.

To my mother.

*"Real **education** must ultimately be limited to men/women who insist on knowing, the rest is mere sheep-herding"*- Ezra Pound.

*"Sólo con el **corazón** se puede ver bien, lo esencial es invisible para los ojos*"- Antoine de Saint-Exupèry.

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Summary

According to the solid-state fermentation (SSF) research line of the composting research group GICOM, the main goal of this thesis is to develop a sustainable process for the production of carbohydrases using organic wastes in a representative scale. To achieve this goal, several organic wastes were screened on its potential to produce cellulase at laboratory scale. Also, compost was added as a complex mixture of biomass in order to provide cellulose-degrading microorganisms. After the selection of the two best substrates: orange peels and coffee husk using 0.5 L reactors, the scale up was assessed in 10 and 50 L reactors. All SSF were undertaken in near-adiabatic conditions using thermally isolated reactors with a continuous monitoring of the biological activity reflected as specific oxygen uptake rate (sOUR) and temperature.

The main challenges to overcome in SSF using organic wastes is on one hand the possibility to work in a continuous configuration and, on the other hand, the reproducibility of the proposed process. These two aspects were assessed in this thesis.

A first approach towards a continuous process was carried out for the production of amylase using bread waste and soy fiber as substrate and adding *Thermomyces sp* as inoculum. Three different strategies were developed in a sequential batch operation by removing part of the substrate in three diffeerent stages of the process: during maximum sOUR, maximum amylase activity and maximum amylase activity with a posterior enzymatic extraction. An increase of above 200% in amylase activity production was obtained in all the assessed strategies.

Due to the success of the amylase production in a continuous configuration, the next step was to use the operational conditions obtained at laboratory scale for cellulase production and develop a sequential batch operation in 4.5L reactors. An initial compost addition as inoculum was considered only at the beginning of the fermentation. After reaching the maximum cellulase production the system started working in cycles, removing one part of the substrate and replacing it for fresh substrate. Two substrate exchange ratios were evaluated: 90% and 50%. Using this configuration, a continuous process was carried out, with a continuous production of cellulase and valorization of organic wastes and eliminating the requirement for fresh inoculum in each cycle. The sequential batch operation resulted in the successful production of cellulase with sustained values around 8-9 filter paper units per grams of dry matter in both configurations. In addition. the microbial communities present at the end of this operation was characterized, identifying several cellulose, lignin and hemicellulose degraders. This fermented solid was described as a specialized inoculum able to colonize the solid matrix of the reactor and provides the opportunity to develop a reproducible process.

Finally, the reproducibility and consistency of the process was assessed in triplicates in a 50 L reactor using the specialized inoculum and coffee husk as the substrate. In addition the gaseous emissions were measured in order to fully understand the process and to determine the requirement of a gas treatment unit. Results showed that the process in consistent and robust with minor emissions of pollutants.

Resumen

De acuerdo con la línea de investigación de fermentación en estado sólido (FES) que actualmente se desarrolla en el grupo de investigación de compostaje (GICOM) el objetivo principal de esta tesis es desarrollar un proceso sustentable y a una escala representativa para la producción de celulasas utilizando residuos orgánicos. Con el fin de lograr este objetivo se analizaron diferentes residuos orgánicos y su potencial para la producción de celulasas a escala de laboratorio. Además, se utilizó compost como inoculo mixto, con el objetivo de incorporar el matriz sólida microorganismos capaces de degradar material lignocelulósicos. Luego de la selección de los mejores substratos en reactores de 0.5 L, se llevó a cabo el escalamiento del proceso en reactores de 10 L and 50 L. Todos los experimentos fueron desarrollados en condiciones cercanas a adiabáticas utilizando reactores aislados térmicamente. Durante el desarrollo del proceso, se realizó la monitorización de la actividad biológica reflejada como la velocidad especifica de consumo de oxígeno (sVCO) y temperatura.

El principal desafío a superar cuando se trabaja con FES y residuos orgánicos son, por un lado, el desarrollo de un proceso en continuo y, por otro lado, que el proceso desarrollado sea reproducible y consistente. Ambas temáticas fueron abordadas durante el desarrollo de la tesis.

Un primer acercamiento a un proceso en continuo se desarrolló para la producción de amilasas utilizando fibra de soja y residuos de pan como substrato y *Thermomyces sp* como inoculo. Se evaluaron tres estrategias de operación en reactores secuenciales, retirando del reactor una parte del sólido fermentado en el momento de: máxima sVCO y máxima actividad de amilasas. En esta última se evaluó la reinoculación con y sin extracción enzimática. En todas las estrategias se obtuvo un incremento por sobre el 200%.

Debido al éxito de la operación en continuo para la producción de amilasas, se desarrolló un proceso similar para la producción de celulasas utilizando las condiciones operacionales desarrolladas a escala laboratorio. Inicialmente se consideró la incorporación de compost como inóculo (10%) y cascarilla de café como substrato (90%). Después de conseguir el máximo de actividad de celulasas, el sistema se continuó operando en ciclos, substrayendo una cantidad determinada de material y reemplazándolo por substrato fresco. Se trabajó con tasas de intercambio de 50% y 90%. Finalmente se pudo desarrollar exitosamente un proceso en continuo para la producción de celulasas, logrando producciones sostenidas en el tiempo entre 8-9 unidades de actividad por gramos de materia seca. Adicionalmente, se caracterizó el sólido fermentado obtenido al final de la fermentación con la finalidad de identificar las poblaciones microbianas presentes. En este sólido se identificaron una amplia gama de bacterias y hongos capaces de degradar material lignocelulósico, por lo que se catalogó como un inóculo especializado que podría ser utilizado con la finalidad de desarrollar un proceso reproducible; sin necesidad de la adición continua de nuevo inóculo.

Finalmente, se evaluaron a escala piloto (50 L) la reproducibilidad y consistencia del proceso utilizando el inóculo especializado y cascarilla de café como substrato en triplicados. Adicionalmente, se analizaron las emisiones gaseosas generadas durante el proceso con la finalidad de determinar los posibles requerimientos de una unidad de tratamiento de gases. Los resultados obtenidos en estos experimentos mostraron que tanto la dinámica del proceso como la producción enzimática fueron consistentes y robustas. Además, el proceso puede ser catalogado como amigable con el medioambiente, debido a la valorización de residuos orgánicos, así como también debido a los bajos factores de emisión generados por el sistema.

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CHAPTER 1. INTRODUCTION

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1.1 Introduction: Towards a circular economy

In the last years, the worldwide solid waste production has been estimated to be near 10 billion tonnes, half of which is produced by industrialized countries. However, the wastes production in non developed countries has also increased during the last decades, with people moving from rural to urban areas, thus increasing the rate of hazardous and industrial wastes (UNEP, 2015).

These solid wastes are generally disposed in controlled or, in the worst cases, uncontrolled landfills, incinerated or destined for animal feed, representing great environmental problems with its inherent financial losses. These associated costs are mainly related to the high cost of transport in the case of landfill disposal, the high amounts of water and energy inputs for incineration and in the case of animal feed, the obvious suitability of every waste for animal consumption (Liguori et al., 2013). Therefore, there is an urgent need for the development of more sustainable programs of waste management, with the main objective to reduce associated environmental and monetary costs. In this context, a beneficial waste management is based on circular economy that strives to reduce the waste generation and to exploit the renewable resources (Allesch & Brunner, 2014). One of the most interesting alternatives relies on the biorefinery concept, which is defined as the sustainable conversion of biomass into a range of marketable bio-based products (platform chemicals, additive molecules for the chemical industry, biomaterials, etc.) and/or bioenergy (biofuels, electricity, heat, etc.) (Jacquet et al., 2015).

In this context, lignocellulosic biomass is considered as the major promising renewable resource, due to its great availability worldwide and its great potential to generate bio-based products and bioenergy. Therefore, lignocellulosic wastes should be considered as "renewable resources" that can be reused to generate valuable and marketable products, replacing the exhaustible fossil-based resources (Jacques et al., 2015). In parallel, there has been an increasing interest for the reduction of fossil fuel consumption and the development of new sustainable biofuels mainly obtained from this lignocellulosic biomass (Liguori et al., 2013). There is such a great interest in renewable energy that it has been reported that this type of energy supplied a 19.1% of the global energy request in 2013, and furthermore, this contribution is continued to expand in the next years.

In summary, there are two main concerns nowadays, on one hand it is the necessity of replacing fossil fuels for biofuels and, on the other hand, there is the new biorefinery concept for the sustainable management of organic solid wastes. Considering all these aspects and in response to these two main concerns, bioethanol appears as an interesting alternative. Bioethanol can be obtained using lignocellulosic wastes as cheap and widely available substrates. Assessments on bioethanol production have proven that the main cost associated to this process is cellulase production,

accounting up to 40% of total costs (Arora et al., 2015). It is for this reason that most of the current efforts on these issues are focused on the optimization of cellulase production and the development of novel and cost-effective technologies to allow it.

1.2 Lignocellulosic materials

Lignocellulose is the largest biomass feedstock on Earth and one of the most studied raw materials. This material is the main components of the cell wall of plants, accounting up to 90% of its dry weight. These types of materials include agricultural residues (e.g stoves, cobs, husks, straws), industrial wastes (e.g sawdust, paper mills discards), urban and domestic solid wastes (sewage, organic fraction of the solid wastes), among others (Martins et al., 2011).

The lignocellulosic feedstock generally consists of three main structures: cellulose, hemicellulose and lignin. A scheme of the structure of the cell wall of plants is presented in Figure 1.1.

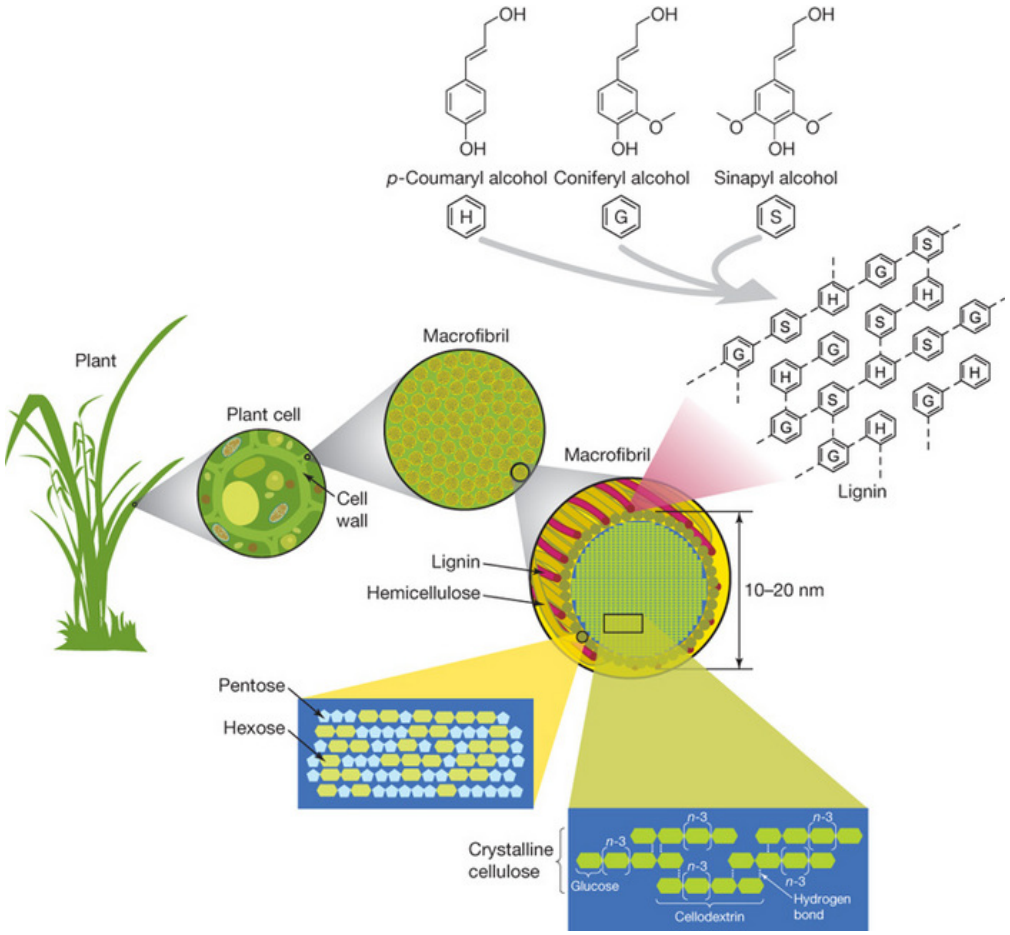

Figure 1.1. Model of molecular structure of the main constituents of lignocellulosic material. Source: Rubin (2008).

In plant derivates, these components constitute the supportive skeleton of the plant body. Cellulose forms fine fibers to constitute the cell wall network skeleton. Hemicellulose and lignin are the filler and binder among the fibers. This entire structure contributes to tensile strength and it is responsible for the chemical resistance, providing hydrophobic conditions.

1.2.1 Cellulose

Cellulose is the major component in lignocellulose, accounting between 35-50% of the total cell wall. Chemically it is a linear polymeric chain formed by several D-glucose molecules bound together by β-1,4-glycosidic bonds, which can be generally represented by the formula $(C_6H_{10}O_5)_n$. The number of glucose unit ranges within hundreds and thousands.

All alternate D-glucose residues in the same cellulose chain are rotated 180º. One glucose unit is the monomeric unit of cellulose and the dimer, cellobiose, is the repetitive structural unit of the cellulose. This structure is polarized: there is a non reducing group at one of its ends and a reducing group at the opposite end (Varrot et al., 2003).

Cellulose has several glucose units organized in long and unbranched chains caused by $–CH_2OH$ groups alternating above and below the plane of rings. The lack of side chains lets cellulose molecules form organized structures. Cellulose has both crystallized (higher packing density) and amorphous (lower packing density) regions. Hydrogen bonds and van der Waals forces connect cellulose chains in the crystallized region into structures named elementary fibril. Aggregates of these structures are called microfibrils, which stacked together make up the fibrils, which gives the cellulose fibers (Rojas et al., 2015) (Figure 1.2).

In these cellulose fibers, crystalline and amorphous regions alternate. The crystalline regions are very cohesive, with rigid structure, formed by the parallel configuration of linear chains, which results in the formation of intermolecular hydrogen bonds. This set-up contributes to the insolubility and low reactivity of cellulose, making it more resistant to acid hydrolysis and modifying fiber elasticity. The amorphous regions are formed by cellulose chains with weaker organization, being more accessible to enzymes and susceptible to hydrolysis.

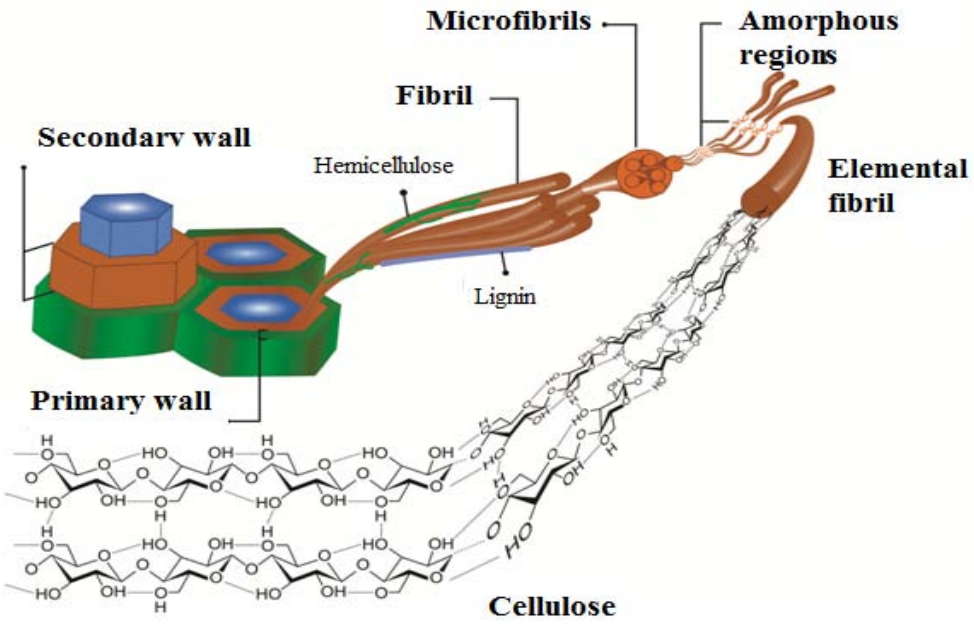

Figure 1.2. Plant cell wall structure and microfibril cross-section (strands of cellulose molecules embedded in a matrix of hemicellulose and lignin). Source: Rojas et al. (2015).

1.2.2 Hemicellulose

Hemicellulose is the second most abundant renewable organic material on Earth, next to cellulose. In the conversion of lignocellulosic biomass into biofuels, the utilization of hemicellulose as a by-product is essential to make posible the biotransformation into value added products.

Hemicellulose is a plant-derived polysaccharide consisting of many sugars monomers of pentoses (D-xyloses and D-arabinose), hexoses (D-glucose, D-galactose and L-rhamnose) and another uronic acids as a branched chain connected to the base chain such as glucuronic acid and D-galacturonic acids. This polysaccharide contains from 200 to 700 monosaccharides residues (Varrot et al., 2003).

Then, hemicellulose is defined as an heteropolymer, which classification depends on the type of the constituent monomer. Hemicellulose can be classified into xyloglucans, xylans, mannans and β-(1→3,1→4)-glucans. Also galactans, arabinans and arabinogalactans can be included in the hemicellulose group, even though they do not share the equatorial β-(1→4)-linked backbone structure. In this way, hemicellulose form and structure will depend on the source, either from fruity or woody origin (Bidlack et al., 1992).The branched and amorphous structure of hemicellulose, in addition to their low molecular weight, make these polysaccharides readily metabolizable (Li et al., 2010). This is important, considering further cellulose hydrolysis, for which it is mandatory to remove

big amount of hemicellulose, thus improving accessibility of cellulases for its substrate (Agbor et al., 2011).

1.2.3 Lignin

Lignin is probably the most complex and least characterized molecular group among the wood components. It is a recalcitrant aromatic polymer, without defined repetitive units. Its precursors are three p-hydroxycinnamyl alcohols or monolignols (*p*-coumaryl, coniferyl and sinapyl) and their acylated forms (Martínez et al., 2008).

Although these precursors are phenolic compounds, the polymer is basically non-phenolic due to the high frequency of ether linkages between the phenolic position (C4) and a side-chain (or aromatic ring) carbon of the *p*-hydroxyphenyl propenoid precursors, strongly predominant in the growing polymer. Unlike cellulose and hemicelluloses, the lignin polymerization mechanism (based on resonant radical coupling) results in a complex three-dimensional network.

The main purpose of the lignin is to provide strength and water permeability to plants, but also to protect them from pathogens. This protection prevents the action of microorganisms and hydrolytic enzymes hindering further cellulose and hemicellulose hydrolysis (Martins et al., 2011).

1.3 Cellulases

Cellulases are a group of enzymes of great industrial importance, especially regarding bioethanol production. These enzymes are considered as components of the enzymatic system for the hydrolysis of plant cell wall. Cellulase is a complex group of enzymes comprised of several enzymatic activities, each containing more similar enzymes of the same class. Furthermore, cellulolytic enzymes often possess the ability to react on a variety of substrates.

1.3.1 Structure and function

Structurally, cellulases are modular enzymes composed of functionally discrete units, referred to as modules or domains. In general, cellulases structure normally consists of one catalytic domain and one carbohydrate binding module, which is usually joined to the catalytic domain by an often glycosylated peptide (Davies & Henrissat, 1995).

The carbohydrate binding domain main role is to favor the enzyme to cellulose contact by increasing at the same time the effective enzyme concentration and the time the enzyme will spend in the near proximity of the substrate. The absence of carbohydrate binding domain, i.e enzyme with only the main catalytic domain, still have the ability to join to cellulose; however with less affinity for the substrate (Rabinovich et al., 2002).

All cellulolytic enzymes belong to the *o*-glycosyl hydrolases (EC 3.2.1), hydrolyzing glycosidic bonds between two or more carbohydrates, or between carbohydrate and non-carbohydrate. Classically, cellulases have been classified according to the substrate specificity in two groups: endo- and exo-glucanases. A third class of enzymes working together in synergy with cellulase are β- glucosidase.

a) Endoglucanases (EC 3.2.1.4): This group of enzymes, also named endo-1,4-β-D-glucanases or endocellulase, acts on a random sector on the amorphous cellulose chain generating different sized oligosaccharides. The main objective of this group of enzymes is the reduction of the polymerization degree of the cellulose chain, making it more available for further hydrolysis.

b) Exoglucanase (EC 3.2.1.91): This group of enzymes is also named exo-1,4-β-D glucanases, cellobiohydrolases or exocellulases. These enzymes act from the end of the cellulose polysaccharide chain, processing along the polymer chain while releasing cellobiose as a main product (Davies & Henrissat, 1995). Small amounts of glucose and celotriose can be also released to the media in early stages of hydrolysis. One of the main features of these enzymes is their ability to act on microcrystalline cellulose (Teeri, 1997). This is of special relevance considering the structural resemblance of microcristalline cellulose to a pretreated real lignocellulosic substrate (Percival-Zhang et al., 2006).

c) β-glucosidase: This enzyme catalyzes the hydrolysis of short soluble oligosaccharides and cellobiose into glucose. This reaction takes place on the liquid phase of the media, which is one important difference when comparing with the action of endo and exoglucanases. Both enzymes act on the surface of cellulose. It is for this reason that β-glucosidase is often not included in the main cellulase group. A schematic figure on the full cellulase action is presented in Figure 1.3.

Final products of the full cellulose degradation are glucose and cellobiose. It is of great importance the removal of these compounds, due to their significant non-competitive inhibitory effect on endo- and exo-glucanases. It has been reported that β-glucosidase action is the main bottleneck for optimal cellulose degradation (Gusakov & Sinitsyn, 1992).

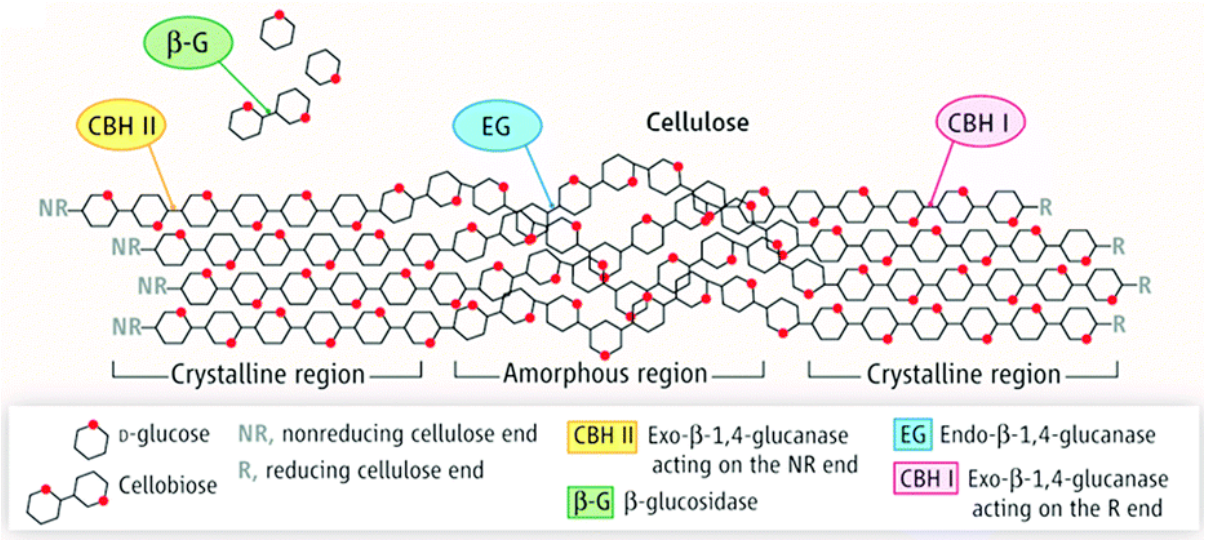

Figure 1.3. Schematic diagram of the action of the three enzymatic groups of cellulases. Source: Ratanakhanokchai & Sakka (2013)**.**

Many microorganisms are able to produce the set of enzymes able to degrade cellulose, among them, bacteria and fungi (Kuhad et al., 2016). These microorganisms can be either aerobic or anaerobic and be able to produce cellulases under mesophillic and thermophillic conditions. Among them, the most studied genera are *Trichoderma*, *Aspergillus*, *Clostridium* and *Cellullomonas* (El-Bakry et al., 2015).

1.3.2 Factors affecting cellulose hydrolysis

When working with lignocellulosic materials, there are several challenges to overcome in order to fully hydrolyze this complex solid matrix. These can include: the origin of the enzymes, thermal inactivation, adsorption or end product inhibition.

The effective enzymatic hydrolysis of cellulose largely depends on the origin of the enzymatic complex, i.e from bacteria or fungi, and the mode of action of the cellulases. On one hand, considering the origin of the enzymes, the enzymatic systems of fungi and bacteria present great differences on their structure and on the characteristics of the produced enzymes (Kuhad et al., 2011). On the other hand, considering the action of enzymes, endoglucanases action mainly takes place in less ordered and inner regions of cellulose, thus contributing to the reduction of polymerization degree. Also, exoglucanases hydrolyzes from the end chain, releasing cellobiose, which have almost negligible effect on polymerization degree reduction. These joint actions can affect the enzymatic hydrolysis on different substrates (Yang et al., 2011). In addition, the action of these enzymes and

potential synergisms between cellulases (or even hemicellulases, such as xylanase) can significantly improve hydrolysis rates on complex substrates (Kostylev & Wilson, 2012). However, it has been stated that authors tend to oversimplify the synergistic effect among cellulases and hemicellulases, which is not a trivial matter or even a fully understood process. This is due to the fact that there is no current evidence that synergism requires interaction between the synergizing cellulases, since cellulases from unrelated organisms often show cross synergism (Berghem & Pettersson, 1973; Kostylev & Wilson, 2012).

The complexity in degradation of lignocellulose results from the three dimensional structure of the plant cell walls and the complex interactions between different components. Access to cellulose is hampered by hemicellulose as well as lignin. Some authors have established that lignin poses the greatest obstacle to cellulose degradation, with hemicellulose having a lesser impact (Van Dyk & Pletschke, 2012). In order to access cellulose structure for hydrolysis, it is mandatory the removal of lignin. Hydrolizates of this material has been proven to present detrimental effect in enzymatic hydrolysis, enzyme recycling and will affect directly the enzymatic extraction in productive processes (Brethauer & Wyman, 2010; Li et al., 2016c). Lignin adsorption is an irreversible and non-productive process formed by hydrophobic interactions, electrostatic and hydrogen bonding interactions, which leads to a continuous loss of enzymatic activity during the hydrolytic process (Pareek et al., 2013). Many studies have been performed In order to reduce the adsorption of cellulases into lignin. Chemical pretreatments such as steam explosion are commonly used for lignin solubilization; however these treatments are expensive, energy consuming and environmentally hazardous (Li et al., 2016c).

In contrast with the non-productive adsorption of cellulase into lignin hydrolyzates, there is productive adsorption of cellulase into cellulose. The insoluble characteristics of cellulose make enzyme/substrate adsorption mandatory to fulfil hydrolysis purposes. Furthermore, adsorption has been found to be one of the important parameters that govern the enzymatic hydrolysis rate of cellulose (Gusakov & Sinitsyn, 1992). It has been shown that cellulase adsorption is facilitated by the carbohydrate binding modules, although, in some cases, catalytic domain can also specifically adsorb to cellulose independently of carbohydrate binding modules. The efficiency of the adsorption process varies with the source of cellulose and the pretreatment methods and it is correlated with the crystallinity and the specific surface area of cellulose substrates. For practical use of cellulosic materials, the cellulose structural properties and their effects on cellulase adsorption and the rate of hydrolysis must be taken into consideration (Lee et al., 1982).

The enzymes of the cellulolytic complex may be subjected to catabolic repression by the final product of hydrolysis. In this sense, β-glucosidase is responsible for preventing accumulation of cellobiose,

controlling the overall rate of the cellulolytic hydrolysis reaction, exhibiting a crucial effect on the enzymatic degradation of cellulose (Leite et al., 2008). Some authors reported that high-substrate consistency hydrolysis with supplementation of hemicellulose could be a practical solution for minimizing end product-inhibition effects while producing a hydrolyzate with high glucose concentration (Xiao et al., 2004).

1.3.3 Industrial significance

Cellulases and xylanases have major and numerous industrial applications, such as in pulp and paper manufacturing, as well as in the textile industry for polishing of fabrics and laundry detergents for improving fabric softness (Kuhad et al., 2011; Mansour et al., 2016). For example, Das et al. (2013) used cellulases produced through an optimized solid-state fermentation for the deinking of waste pulp of laser printed paper, that is, mainly the removal of chromophores and hydrophobic compounds. In addition, cellulases are used in the extraction process of fruit and vegetable juices, starch processing and formulations used for animal feeds (Dhillon et al., 2012a; Singhania et al., 2009). Cellulases also have found promising applications for non-specific hydrolysis of chitosan to produce chitooligosaccharides with low molecular weight, which showed high antibacterial activity (Xia et al., 2008).

From biotechnological perspectives, the most important and recent application of cellulases is bioenergy production. Cellulases are used to hydrolyze cellulosic wastes to sugars that can be fermented to bioethanol or biogas, depending on the process conditions and microorganisms (Kuhad et al., 2016). It is shown that there is a wide potential to develop a simple biological process to produce bioethanol from a variety of lignocellulosic substrates, that is, by hydrolyzing and fermenting carbohydrates, which are considered as waste materials produced in huge amounts especially in the agro-industrial sector. The major constraint in the bioethanol process is precisely the costs of cellulase production according to several studies (Arora et al., 2015; Lever, 2015). In order to reduce downstream processing of the enzymatic compounds, some authors (Lever et al., 2010; Liu et al., 2011; Rocha et al., 2013) directly applied the unprocessed cellulases obtained by SSF into hydrolytic processes of organic wastes for further bioethanol production.

For biogas production and in the context of solid wastes, hydrolysis step is considered as rate limiting and, therefore, it is considered as crucial for the efficiency: the more the substrate is hydrolyzed, the greater is the amount of methane produced (Zverlov et al., 2015). The rate of decomposition during the hydrolysis stage greatly depends on the amount and efficiency of hydrolytic enzymes produced by the bacterial consortium. Moreover, the hydrolysis of cellulose (and hemicellulose) takes place slower

than other complex compounds, such as proteins (Kuhad et al., 2016), thus establishing the importance of these enzymes in the process.

In both bioethanol and biogas production, process integration is perhaps one of the most important approaches for the design of more intensive and cost-effective process configurations. Since most of the current technologies only use a part of the organic wastes for obtaining bioproducts, using the whole feedstock or optimally utilizing all the fractions present in the biomass will enhance the economic and environmental feasibility of biofuels and co-products by a better understanding of relationships between plant (bio)chemistry and potential bioproducts (Joyce & Stewart, 2012).

1.4 Solid-State Fermentation

In the context of circular economy, the most promising technology to produce low cost enzymatic products using lignocellulosic biomass, is solid-state fermentation. Solid-state fermentation (SSF) has been defined by many researchers. These definitions agree in the fact that this process involves solids in the absence or near absence of free water (Chen, 2013; Thomas et al., 2013). In the SSF concept, the solid substrate should possess enough moisture in absorbed or complex form to support microbial growth and metabolic activities (Rabinovich et al., 2002; Singhania et al., 2010).

SSF is a three-phase system consisting of the continuous gas phase, the liquid film, and the solid phase. It should be noted that there is no obvious relationship between the water content of the substrate and the content of free water. Because of the strong hydraulic holding ability of some solid substrates, such as that of the sugar beet plant material, even if the water content of the substrate is more than 80%, there is seldom free water in the solid substrate. Consequently, the content of water cannot be defined as the only standard of solid fermentation (Chen, 2013).

SSF has been classified in two categories depending on the type of the used microorganism: i) natural SSF and ii) pure culture SSF; according to Krishna (2005). The conventional industrial processes mostly involve pure cultures for the production of targeted products. On the other hand, mixed cultures are preferred in the bioconversion processes of agro-industrial residues, such as composting and ensiling.

In recent years, SSF has shown great potential in the development of several bioprocesses and products. This technology has its own advantages over conventional submerged fermentation (SmF) as presented in Table 1.1.

Table 1.1. Comparison between SSF and SmF. Source: El-Bakry et al., (2015).

Factor	SSF	SmF
Substrate	Cheap and available e.g. organic wastes	Expensive media ingredients
Inoculum	Not necessary	Essential
Aseptic conditions	Not needed	Essential
Moisture	No free water	Liquid media required
Agitation	Very difficult	Easy
Process control (T, pH)	Difficult	Easy
Enzyme yield	High, due to high solid content	Low, due to low solid content
Downstream processing	Can be easy, cheap	Difficult, expensive
Liquid waste	Low quantities	High quantities
Scale up	Difficult, new designs required	Easy, commercial equipments available
Volume and costs of equipment	Small reactors can be used, low cost	Large scale reactors are required, high cost

As stated, the potential of SSF is to provide the cultured microorganism an environment as close as possible to natural environment where usually they exist and from where they are isolated. This is apparently the main factor why microbes perform well and give higher products yields in SSF when compared with SmF carried out at optimal conditions for microorganisms growth (Thomas et al., 2013). As mentioned above, the use of agro-industrial residues and by-products as substrates in SSF processes adds economic value to these materials and solves the problem of their disposal or treatment. In this context, SSF has been useful for the production of several value added products, using organic wastes as the substrate, as presented in Table 1.2.

As observed in Table 1.2, most of the research carried out in SSF is performed using few grams of substrate. In addition, most of these studies have been performed under initial sterile conditions, using specific microorganisms and systems with controlled temperature. Taking into account these conditions, the scaling up of the process appears as an extremely difficult issue, considering the widely known constraints of working with large amounts of solid substrates and the costs associated to the conventional operational conditions. Most of these issues have been partially addressed by our research group, working in a representative scale and taking into account the heterogeneous nature of the solid organic wastes.

The composting research group, GICOM, has en extensive experience on the study of composting processes. Using this "know-how" and in order to achieve a cost-effective and sustainable SSF process, our research group has proposed to work in a self-heating process, under near adiabatic conditions using either native or externally added microbiota to non sterile substrates.

Table 1.2.Products obtained using organic wastes as substrates by means of SSF.

Substrate	Microorganism	Product	Amount (g)	Reference
Sugarcane bagasse	*Kluyveromyces marxianus*	Inulinase	2,000	(Astolfi et al., 2011)
Winterization oil cake	Native microbiota	Lipases	2,500	(Santis-Navarro et al., 2011)
Cotton waste	*Aspergillus* spp.	Hydrolytic and oxidative enzymes	5-20	(Bansal et al., 2012; Liu et al., 2011)
Wheat waste	*Aspergillus* spp.	Cellulases, xylanases	5-40	(Bansal et al., 2012; Dhillon et al., 2012b)
Hair waste	Native microbiota	Proteases	2,000	(Abraham et al., 2013)
Soy waste	Native microbiota	Proteases	2,000	(Abraham et al., 2013)
Orange peels	Native microbiota	Cellulases	15,000	(Maulini-Duran et al., 2015)
Empty palm fruit bunches and palm kernel cake	Isolated oleaginous fungi	Cellulase and lipids	< 1	(Cheirsilp & Kitcha, 2015)
Apple pomace	Yeast mixture	Aromas	6,800	(Rodríguez Madrera et al., 2015)
Plum pomace/ Distillery wastes	*Aspergillus níger / Rhizopus oligosporus*	Antioxidants	15	(Dulf et al., 2016)
Aguamiel	*Aspergillus oryzae*	Fructooligosacharides/ Fructosyltransferase	n.r	(Muñiz-Márquez et al., 2016)
Winterization oil cake	*Starmerella bombicola*	Sophorolipids	100	(Jiménez-Peñalver et al., 2016)

1.4.1 Process description

The proposal for a more sustainable SSF consisted of a process that follows the same pattern than the composting process. This process is mainly comprised by two stages: high rate degradation stage and curing, as presented in Figure 1.4.

a) High rate degradation stage: In general, the hydrolysis of complex organic matter into simple organic and inorganic molecules takes place during this stage. This hydrolysis in carried out by microorganisms at its highest metabolic activity, which is reflected in a high oxygen uptake rate. Also, in this stage heat is produced as a result of the metabolic activity, since these are exothermic biological processes (Haug, 1993). In the early stages of the process, mesophillic conditions are present, therefore, mesophillic microorganisms thrive. These microorganisms use available oxygen to oxidize carbon from the solid matrix into biomass, energy and other products, with the consequent CO_2 and water production. As the heat is generated due to metabolic activity, the temperature starts to increase. When the system achieves 45ºC, the mesophilic microorganisms either die or remain with basal metabolism until more suitable conditions are presented. At the same time, thermophilic microorganisms are favoured, and quickly start to consume the readily available materials and to colonize the system. Once this material is fully consumed, the biological activity of thermophilic microorganisms decreases, which generates a decrease in the temperature, also named cooling stage (Haug, 1993). This reduction provides the proper conditions for mesophilic microorganisms, which start to re-colonize the reactor and continue the process.

The thermophilic stage is important for the composting process due to the sanitation that provides to the solid matrix that will be fully composted. When considering a SSF productive process, thermophilic conditions could led to a production of thermostable enzymatic compounds, as reported by Santis-Navarro et al. (2011). However, temperature rising above 70ºC could derive in a severe reduction of microorganisms populations, which may affect the productivity of the process. In addition, at high temperatures an increase in ammonia emissions can occur, mainly generated from stripping during ammonium degradation (Pagans et al., 2006). This is important from the environmental point of view and further assessment of the gaseous stream treatment.

b) Curing: After the high rate degradation stage, the temperature reaches mesophilic levels, where curing stage begins. During this stage, the oxygen requirements are notably lower than in the high rate stage, which results in a net loss of organic and inorganic matter. The importance of this stage is the formation of high molecular compounds resistant to biodegradation from dead biomass (Haug, 1993). At the end of this stage, the solid material will be fully stabilized, with generation of CO_2, water, mineral ions and stabilized organic matter, mostly in the form of humic acids.

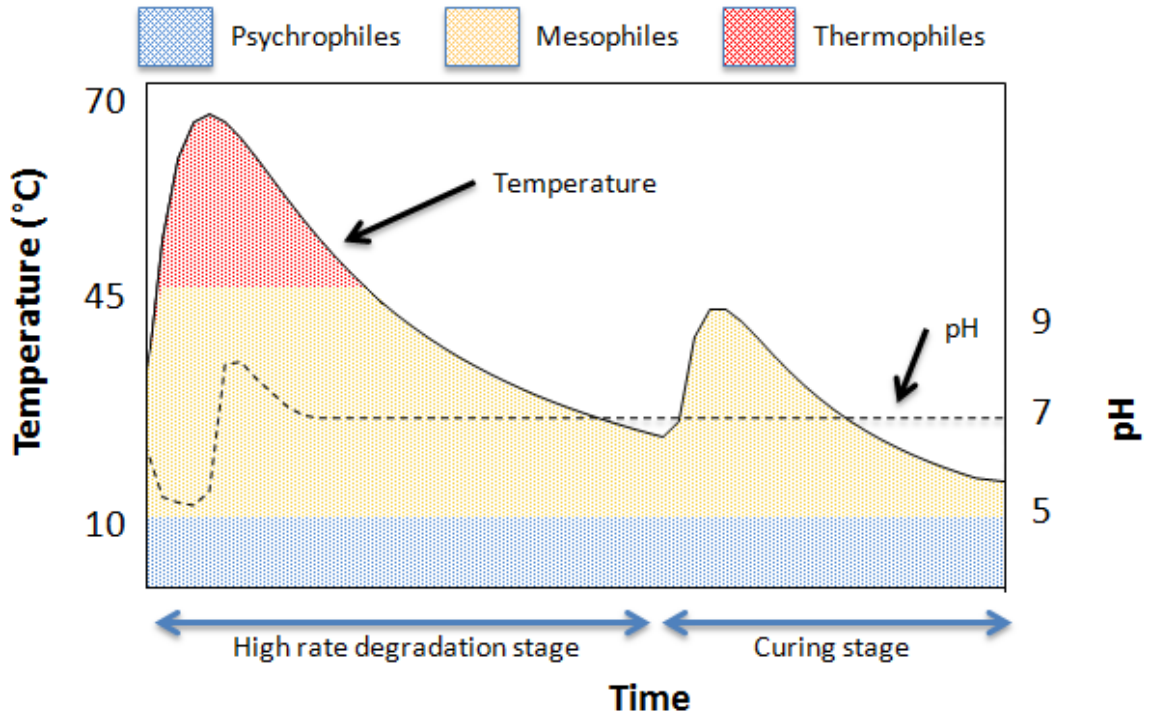

Figure 1.4. Schematic temperature profile and main characteristics of a classic composting process.

Two processes for the production of hydrolytic enzymes have been studied by means of a self-heating process working under near adiabatic conditions. In this sense, lipases and proteases production took place during the high rate degradation stage (Abraham et al., 2013; Santis-Navarro et al., 2011). This may imply that for hydrolytic enzymes production, the curing stage is probably not relevant. The SSF should be then stopped in the high rate stage to extract the maximum enzymatic activity. Fermented solids could be further stabilized to a compost-like product.

1.4.2 Environmental aspects

As any other productive process, SSF development should take into consideration environmental aspects. The most representative aspects are disposal or treatment of the produced solid wastes and released the gaseous emissions.

When a targeted compound is obtained by means of SSF, it is often extracted from the solid matrix. This extraction generates a waste stream of fermented spent solid, that will require stabilization for a potential utilization as new substrate (Lever, 2015; Thomas et al., 2013). This new substrate can be

treated in order to obtain an organic soil amendment or even bioenergy, such as biogas or bioethanol. These processes would close the cycle of organic matter, in accordance to the circular economy paradigm (Lever, 2015). In this context, Abraham et al. (2013) worked with a SSF process for proteases production. Proteases were obtained after extraction from the solid matrix, generating a fermented spent solid as a waste stream. This waste was post-treated, achieving full stabilization after 14 days of composting. Also, Maulini-Duran et al. (2015) assessed the stabilization of fermented spent solids from SSF for proteases, cellulases and lipases, achieving similar results.

A step forward on the complete reuse of wastes with environmental and economic assessment was taken by Lever (2015). In this study, a full ethanol plant was designed using the "zero waste" approach as shown in Figure 1.5. This author compared the use of commercial cellulases and cellulases produced by SSF using wheat straw and fermented solid from ethanol fermentation. The produced fermented solids from SSF were stabilized by anaerobic digestion with the consequent biogas production and further composting process, with full solid recycling. This study showed that the proposed process resulted in high energy yield ratios, a net surplus of on-site heat and electricity, and substantial reductions in the energy required to produce and transport the cellulase compared to commercial preparations. Recycling fermented solids from ethanol fermentation as substrate for cellulase production resulted in a reduction in energy consumption and a decrease in energy yield ratio compared to using fresh wheat straw.

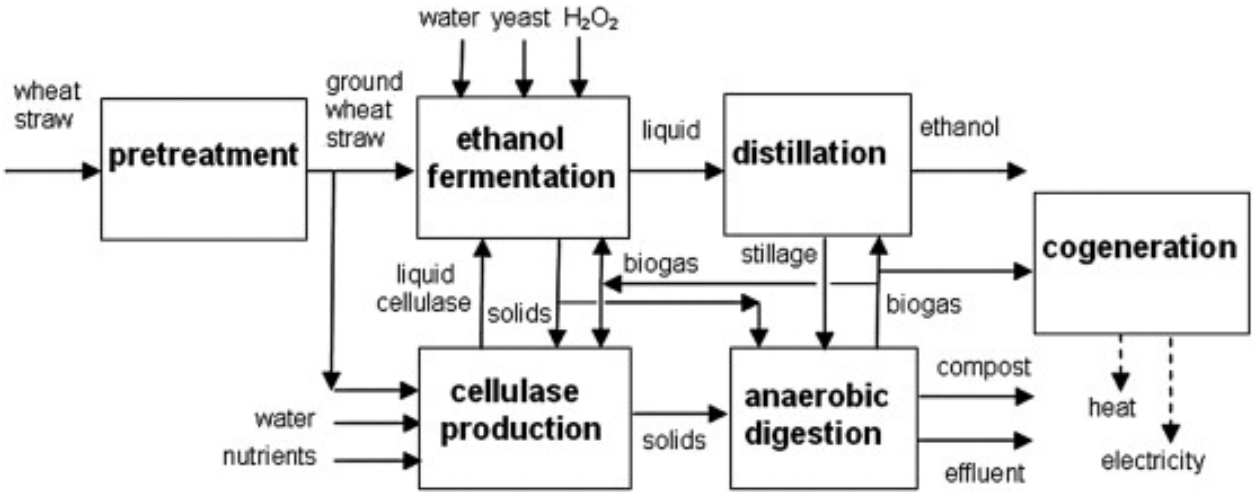

Figure 1.5. Unit operations for proposed cellulose to ethanol conversion process. Source: (Lever, 2015).

One other aspect to consider is the gaseous emissions. Gaseous emissions play an important role in the environmental impact of processes used to manage solid organic substrates. Greenhouse gases

(GHG), ammonia (NH_3) and volatile organic compounds (VOC) are the main gases emitted during SSF processes (Maulini-Duran et al., 2015). Also, CH_4 and N_2O are often released, which present a global warming potential (GWP) of 34 and 298 kg CO_2 equivalents, respectively (IPCC, 2013).

Ammonia is not considered to be a direct GHG, but contributes to global warming because once deposited in the soil, it can be converted into N_2O after nitrification and denitrification reactions (Wunderlin et al., 2013). Due to its high GWP, N_2O can contribute strongly to the carbon footprint, thus it is important to understand its formation during the SSF.

In spite of the great importance of this subject, there are only two reported works on the gaseous emission for SSF process (Gassara et al., 2011; Maulini-Duran et al., 2015). While Maulini-Duran et al., (2015) assessed gaseous emissions of three different SSF processes, Gassara et al. (2011) explored the effect of different waste management systems of apple pomace and the gaseous emissions produced. The different scenarios evaluated were incineration, landfill; animal feed; enzyme production by SSF and composting. After a life cycle assessment study, these authors showed that enzyme production (906.81 tons of CO_2 equivalent per year) and animal feed (963 tons of CO_2 equivalent per year) were the least polluting options in terms of GHG emissions followed by incineration (1122 tons of CO_2 equivalent per year), composting (1273tons of CO_2 equivalent per year) and landfill (1841 tons of CO_2equivalent per year). These results confirm another advantage of SSF, not only as an effective productive process, but as an environmentally friendly process. For this reason, more studies on gaseous emissions are required.

1.5 Cellulase production by SSF

As mentioned in the previous sections, cellulases are a group of enzymes with high biotechnological interest. The possibility to obtain this enzyme by means of SSF using organic solid wastes is very interesting due to the economical and environmental advantages.

Cellulase production is generally represented as three enzymatic activities of filter paper (FPase) for cellobiohydrolase or exoglucanase, carboxymethylcellulase (CMCase) for endoglucanase and β-glucosidase (BGase) for cellobiase or β -glucosidase. These enzymatic activities are normally expressed as units of activity per grams of dry matter, i.e. FPU g^{-1}DM for FPase and U g^{-1}DM for CMCase and BGase respectively, when produced by SSF.

1.5.1 Process conditions

The most utilized substrates for cellulase production are agricultural wastes (soybean, wheat, rice, corn, cotton, sugarcane bagasse), fruits (apple, orange and banana peels), as well as residues from wood industries (softwood or hardwood) as shown in Table 3.

In most of the work developed on SSF using organic solid wastes, the addition of nutrients is often required in order to stimulate the growth of a single or mixed microorganisms. However, in some cases, depending on the nature of the substrate, there is no need of exogenous nutrients addition, as reported by Dhillon et al. (2011) when using wheat bran as substrate. In this sense, many researchers have worked on evaluating mixtures of substrates aiming to either increase cellulase production or, at least, achieve a reduction of additional nutrient supplementation (Brijwani et al., 2010; El-Bakry et al., 2015; Soni et al., 2010).

In addition to the substrate, it is crucial, as in any biological process, to select the proper microorganism(s). In this context, bacteria and fungi are able to use cellulose as primary carbon source; however, bacteria are incapable to fully degrade crystalline cellulose since their cellulase system is incomplete. For fungi the situation is different, these microorganisms are able to completely degrade this structure. It is for this reason that many fungi are studied in recent research works, especially from the genera *Trichoderma* and *Aspergillus*. Some species of these two fungal genera have been reported as the most important cellulase producer microorganisms applied to SSF processes (Farinas, 2015; Hansen et al., 2015).

It is important to mention that some fungi can produce preferently one enzyme over the others. For instance, *Thrichoderma reesei* is well known for its potential for the hyper-production of cellulase, but usually has low activity or is deficient in the production of β-glucosidase (Brijwani et al., 2010; Brijwani et al., 2011). The β-glucosidase hydrolyzes cellobiose, which otherwise will inhibit cellulase joint action and will result in overall low substrate hydrolysis rate. It is for this reason that some authors have made great efforts to improve inoculum and substrates mixtures to increase β-glucosidase production (Dhillon et al., 2012a; Dhillon et al., 2011; Hu et al., 2011; Kaur et al., 2012; Liu et al., 2011).

In terms of operational parameters for cellulase production, there are critical aspects to take into account: availability of oxygen, temperature, pH, water activity and moisture, bed properties and nature of the substrate have been reported as the most important parameters (Mekala et al., 2008; Olofsson et al., 2008; Van Dyk & Pletschke, 2012). In this sense, many authors have targeted their research on the optimization of these parameters by means of response surface methodology and

mathematical modelling techniques (Brijwani et al., 2011; Das et al., 2013; Dhillon et al., 2012a; Khanahmadi et al., 2006).

It has been reported that optimal pH range for cellulase production is between 4-8, depending on which cellulose activity is measured and the microorganism used (Bahrin et al., 2012; Lee et al., 2011; Mekala et al., 2008; Soni et al., 2010; Yang et al., 2012). In general, pH has a soft effect on exoglucanase and β-glucosidase activity within most of experimental research. Robustness against changes of pH from its initial value during enzyme production would be benefitial in shielding any effect on enzyme activity due to pH fluctuations during the productive process. For instance, Brijwani et al. (2010) operated SSF for cellulase production using wheat bran as substrate and using *Trichoderma reseei* as inoculum. This fermentation started at optimum conditions for the inoculum, however the process was carried out without pH control. The microorganism was able not only to survive, but to sustain growth and enzymatic production even working at high alkaline conditions. Also, many authors have stated that cellulase stability at different pH is wide and it has been reported in a ratio between 4-8, maintaining a residual activity in the range of 60-100% (Bansal et al., 2012; Hu et al., 2011; Liu et al., 2011). Above the mentioned pH, the range of cellulase activity decreases drastically.

The process temperature used during the process is an important factor affecting microbial growth and activity and thus, enzyme production in SSF. Commonly, temperature ranges between 25-35°C (Das et al., 2013); however, cellulolytic activity has been observed in a wide range of values, as presented in Table 3. Most of the maximum cellulase production has been found at 30°C, but appreciable production has been obtained in thermophillic conditions. Temperatures in the range of 40-50°C have reported to produce thermostable cellulases with enzymatic activities ranging between 224-526 U g^{-1}DM for CMCase, 7.2-144.6 FPU g^{-1}DM for FPase and 22.4 -30 U g^{-1}DM for β-glucosidase (Bansal et al., 2012; Liu et al., 2011).

Another important aspect to consider is the moisture content during SSF. It has been well established that both high and low moisture content can directly affect the productivity of the SSF. High moisture content decreases porosity, promoting compaction and lowering oxygen transfer. On the other hand, lower moisture content causes a reduction on the solubility of nutrients of the solid substrate, a lower degree of sweeling and higher water tension (Anto et al., 2006). Optimal moisture content ranges between 40-60% (Bansal et al., 2012; Chandra et al., 2007; Das et al., 2013; Dhillon et al., 2012b; Liu, 2007; Soni et al., 2010; Sukumaran et al., 2009) using different lignocellulosic substrates. Regardless, some authors have obtained higher levels at 70%-80% moisture content reaching FPase activity values between 2.5-5.0 FPU g^{-1}DM (Lee et al., 2011; Liu et al., 2011).

As discussed in section 1.3.2, there are many factors affecting cellulose hydrolysis that will require assessment in order to achieve proper cellulase production. Some authors have worked in different strategies to achieve that goal, such as alkaline or acid pretreatment or inductors addition (Bansal et al., 2012; Dhillon et al., 2012a; Sanghvi et al., 2010; Yang et al., 2012). Regarding the pretreatments, Bansal et al. (2012) determined that cellulase production using different raw materials was lower than when using the pretreated substrates. This fact was attributed to the higher lignin content and the firm binding, making cellulose less accessible to microorganisms. Also, the highest cellulase production has been found when using alkaline pretreatment. Acid pretreatment solubilized hemicellulose structure, releasing monomers, furfural and other volatile compounds which, along with lignin, produce a negative environment for an organism to grow, thereby lowering productivities and yields of the process. In contrast, Sanghvi et al. (2010), using the same pretreatment, showed no improvement on cellulase production, which was attributed to a decrease in mycelia growth on the media, which led to a rapid decrease on enzyme production. In this sense, the hemicellulase production is often study along with cellulase production, in order to obtain a mixed enzymatic pool that can potentially improve hydrolysis in a bioethanol production process. Table 3 shows several cellulase producing processes and most of them included hemicellulase production represented as xylanase.

Higher cellulase activity values were obtained by adding inductors (Dhillon et al., 2012a), reaching values of 224-526.3 U g^{-1}DM for CMCase, 30.4-144.6 FPU g^{-1}DM for FPase, 99 U g^{-1}DM for β-glucosidase and 3106 U g^{-1}DM of xylanase. However, the feasibility to implement the addition of inductors, depends on economical aspects such as availability and cost of the selected inductor.

All the above mentioned researches have been made on laboratory scale in a single batch configuration, using amounts of substrate between 5 to 400 g (Table 1.3). Using low amounts of heterogeneous material provides a non easily scalable system as it can avoid compaction problems and its consequent poor oxygen transfer, which are very important factors to take into account when scaling up and reproducibility issues are assessed.

Table 1.3. Summary of latest cellulase production by solid state fermentation using wastes as substrate. RS: rice straw, RH: rice husk, WB: wheat bran, SH: soybean hull, SCB: sugar cane bagasse, AP: apple pomace, BSG: brewers spent grain., T: temperature, M: moisture, FPase: filter paper, CMCase: endocellulase, BGase: β-glucosidase and Xyl: xylanase.

Inoculum	**Substrate**	**Process conditions**				**Cellulase activity**	**Reference**
Type	**Type**	**Scale (SSF time)**	**pH**	**T (°C)**	**M (%)**	**Max. Activity (U g^{-1}DM)**	
T. reesei *A.oryzae*	SH	Deep bed reactor	5.0	30	70	FPase 5.4 CMCase 58.6 BGase 18.4 Xyl 242	(Brijwani et al., 2011)
T. reesei *A.oryzae*	SH:WB	Static tray reactor (96h)	5.0	30	70	FPase 10.8 CMCase 100.7 BGase 10.7	(Brijwani et al., 2010)
T. reseei *A. niger*	RS:WB	Tray (120h)	-	30	-	FPase 35.8 CMCase 132.3 BGase 33.7 Xyl 3106	(Dhillon et al., 2011)
A. niger	AP:RH (48h)	Lab	-	30	75	FPase 133 CMCase 172	(Dhillon et al., 2012b)
T. reesei	RS: SCB (72h)	Lab	-	30	40-57	FPase 4.6 CMCase 135.4 BGase 21.5	(Sukumaran et al., 2009)
Trichoderma sp. *GIM 3.0010*	AP	Lab (120h)	-	30	70	FPase 1-7.6	(Sun et al., 2010)
Trichoderma veride *sp.3.2942*	RH:WB	Lab (120h)	-	30	-	FPase 6.3	(Hu et al., 2011)

Table 1.3. (Continued). RS: rice straw, RH: rice husk, WB: wheat bran, SH: soybean hull, SCB: sugar cane bagasse, AP: apple pomace, BSG: brewers spent grain., T: temperature, M: moisture, FPase: filter paper, CMCase: endocellulase, BGase: β-glucosidase and Xyl: xylanase.

Inoculum	**Substrate**	**Process conditions**				**Cellulase activity**	**Reference**
Type	**Type**	**Scale (SSF time)**	**pH**	**T (°C)**	**M (%)**	**Max Activity (U g^{-1}DM)**	
A.niger NRRL 567	AP:BSG	Lab (96h)	-	30	-	FPase 71.1 CMCase 103.9 BGase 55.9 Xyl 1618.1	(Dhillon et al., 2012a)
A.niger NRRL 567	AP:BSG Lactoserum	Lab (96h)	-	30	-	FPase 96.7 CMCase 134.4 BGase 90.1 Xyl 2619.5	(Dhillon et al., 2012a)
A. niger NRRL 2001	AP:BSG	Lab (96h)	-	30	-	FPase 85.6 CMCase 107.7 BGase 32.8 Xyl 1110.9	(Dhillon et al., 2012a)
A. niger NRRL 2001	AP:BSG Lactoserum	Lab (96h)	-	30	-	FPase 130.4 CMCase 148.9 BGase 90.1 Xyl 2281.7	(Dhillon et al., 2012a)
A. niger NS-2	Corn cob	Lab (96h)	6.5	30	60	FPase 3.1 CMCase 10 BGase 1.8	(Bansal et al., 2012)
A. niger NS-2	Carrot peelings	Lab (96h)	6.5	30	60	FPase 0.3 CMCase 4.9 BGase 5.0	(Bansal et al., 2012)

Table 1.3. (Continued). RS: rice straw, RH: rice husk, WB: wheat bran, SH: soybean hull, SCB: sugar cane bagasse, AP: apple pomace, BSG: brewers spent grain., T: temperature, M: moisture, FPase: filter paper, CMCase: endocellulase, BGase: β-glucosidase and Xyl: xylanase.

Inoculum	**Substrate**	**Process conditions**				**Cellulase activity**	**Reference**
Type	**Type**	**Scale (SSF time)**	**pH**	**T (°C)**	**M (%)**	**Max Activity (U g^{-1}DM)**	
A. niger NS-2	Kitchen waste	Lab (96h)	6.5	30	60	FPase 10.3 CMCase 48.6 BGase 19.5	(Bansal et al., 2012)
A. niger NS-2	RH	Lab (96h)	6.5	30	60	FPase 3.1 CMCase 14.1 BGase 10.6	(Bansal et al., 2012)
A. fumigatus	Vinegar industry waste	Lab	4.0	50	80	FPase 144.6 CMCase 526.3	(Liu et al., 2011)
A. fumigatus	RS	Lab(5d)	7.0	45	75	FPase 3.4 CMCase 98.5 BGase 250.9 Xyl 2782	(Soni et al., 2010)
A. fumigatus	WB	Lab	7.0	45	75	FPase 0.67 CMCase 20 BGase 99.4 Xyl 1722	(Soni et al., 2010)
T.reesei CBS 439.92	WS	Lab	-	30	-	FPase 0.6	(Lever et al., 2010)
A. fumigatus	RS:WB (5d)	Lab	7.0	45	75	FPase 2.1 CMCase 40.9 BGase 243.7 Xyl 444	(Soni et al., 2010)

In summary, it is remarkable to mention that most of the research performed on cellulase production by SSF has been carried out using pure strains and working under mesophillic conditions. In addition, as observed in Table 3, the most common configuration is batch and using small amounts of substrate. In this sense, there is a great challenge in optimize all of these aspects in order to develop a more sustainable SSF process.

1.5.2 Novel microbiological approaches: Strain development and Metagenomics

Cellulases are expressed by a wide spectrum of microorganisms in nature. Screening and isolation of cellulase-producing microorganisms from different environments is one of the most important ways to produce novel cellulases. These newly screened microbes are sources of new cellulase genes with diverse properties (Juturu & Wu, 2014).

In this context, Jing et al. (2015) searched for efficient fungal producers of cellulases in a natural decay system, using a screening process containing hydrolyzed sugarcane bagasse as carbon source. They successfully isolated 12 fungal species, which showed considerable amounts of exoglucanase activity. These species were identified as *Penicillium oxalicum* strains and were assessed as inoculum for pretreated sugarcane bagasse for further bioethanol production with promising results. Another successful report was the presented by Ang et al., (2013). These authors were able to isolate a fungal strain form cow dung. This strain was identified as *Aspergillus fumigatus* SK1 and was evaluated as inoculum in a SSF process using oil palm trunk as substrate. Cellulase activities obtained were 54.3, 3.4, 4.5 and 418.7 U g^{-1}DM substrates for endoglucanase (CMCase), exoglucanase (FPase), β-glucosidase and xylanase respectively. These results are in the middle range of the reported in the literature, as seen in Table 3. Many similar approaches are reported by Juturu & Wu (2014); interestingly, most of them aimed to optimize cellulose degradation for bioethanol production.

Although the above mentioned approaches presented good results, the extreme complexity of natural environments makes culture-based approaches extremely time and labor-consuming. Furthermore, the results are based on phenotypic characteristics, which can be imprecise and/or inconclusive (Wang et al., 2009). In this sense, many authors are currently studying novel techniques, such as strain development, heterologous cellulase expression or metagenomics (Ray & Behera, 2017). The most promising tool for non-sterile SSF application is metagenomics.

Metagenomics, the study of genetic material recovered directly from environmental samples, has the potential to exploit the inexhaustible source of novel biocatalysts trapped in genomes of unculturable

microorganisms. This technology is a useful technique for the monitoring of microbial communities in a SSF process or to discover enzymes from highly diverse environments (Li et al., 2016a).

To the best of our knowledge, there are only two research papers published using the metagenomics approach to follow a multispecie SSF (Li et al., 2016b; Wang et al., 2016). Both of these reports were focused on the study of patterns/dynamics of the populations and their functionality in the productive process. Li et al. (2016) reported the effect of temperature of SSF for cereal starters production at industrial scale. Using this technique, the authors obtained a deep understanding of the process that allowed the effective control of the fermentative process by adjusting relevant environmental parameters. On the other hand, Wang et al. (2016) assessed the influence of the different microorganisms on the aroma and flavour production, during the SSF of a mixed substrate (wheat bran, mash alcohol and chaff) for chinese vinegar production. The authors reported that an improvement of the quality of the product can be obtained by working with the dataset generated by the metagenomics of the process.

GICOM proposal of SSF includes a microbial succession of microorganism characteristic of the composting process. There are a few reports of the follow up of microbial communities during the composting of lignocellulosic materials (Langarica-Fuentes et al., 2014; López-González et al., 2015a; López-González et al., 2015b).

López-González et al. (2015a) reported the microbial population succession and the enzymatic activities expressed throughout the composting process of lignocellulosic material. In this work, more than 4000 strains were isolated, enzymatically characterized, and identified by partial sequencing of 16S rRNA. Mesophilic bacteria were found during the entire process, while thermophilic conditions gathered the highest total counts and spore-forming bacteria prevailed at the high rate degradation stage. The authors also found that cellulolytic and ligninolytic bacteria appeared during thermophilic stage but also appeared in considerable amounts at the end of the process. Furthermore, López-González et al. (2015b) found that cellulolytic activity (obtained from cultured isolates) expressed by the fungal community was also observed during thermophilic stage. In this sense, stable compost could be a potential source of cellulase degraders for further isolation or direct application.

Although these reports are very promising, they are carried out using cultured microorganism, which leave the uncultured microorganism without analysis. Due to the potential of utilization as source of cellulolytic microorganism, it is interesting to assess the changes in uncultured microorganisms population using a metagenomics approach.

1.5.3 Challenges

Once the proper inoculum, substrate and process conditions are selected and optimized, one of the major challenges of developing a reproducible SSF processes is to work at a larger scale.

Most of the research performed has been carried out at laboratory scale (Table 3), usually working in flasks for microorganism growth, which can be convenient for substrate/inoculum screening but provides no useful information for posterior scaling up. In Table 1.4, a comparative of experiments performed in lab and large scale is presented. In this sense, besides oxygen transfer, factors that need to be considered include temperature, water content, morphology of microorganisms (especially in the case of fungi) and sterilization requirements (Durand, 2003). Several reactor designs have been proposed for cellulolytic enzyme production, including tray-type, packed-bed, and horizontal rotating drum bioreactors, amongst others (Mitchell et al., 2006; Thomas et al., 2013). Each one of these designs has its own advantages and disadvantages, which suggested the necessity to develop novel bioreactors with better design in order to solve the major problems related to the scale-up processes for the production of enzymes through SSF.

There are few recent studies on the overcoming of some of the disadvantages and limitations regarding the SSF scaling-up processes. An extensive analysis on the design and operation of bioreactors in SSF has been published by Thomas et al. (2013). Cellulase production using these designs has been successfully carried out by several researchers (Brijwani et al., 2011; Dhillon et al., 2011; Hansen et al., 2015). Even these approaches are a good alternative, there is still a requirement for temperature control and heat removal. One different approach has been reported by GICOM research group for protease and lipase (Abraham et al., 2013; Santis-Navarro et al., 2011). In these approaches, the temperature was not controlled and enzymes were produced in a batch fermentation process similar to that of composting. The temperature rose to thermophilic values due to heat released and decreased to ambient values during the fermentation of the readily biodegradable matter.

Another challenge to overcome is the possibility to develop a process in a continuous configuration. Although Abraham et al. (2013) and Santis-Navarro et al. (2011) approaches were successful and assessed in a pilot scale (Maulini-Duran et al., 2015), they were developed in a batch configuration which could prevent the process of being economically feasible. Very few attempts have been reported on continuous SSF. Two reports have been published on enzyme production in batch and fed-batch configuration for inulinase (Astolfi et al., 2011) and cellulase (Cheirsilp & Kitcha, 2015). Both researches proved that enzymatic production was sustained in time and, in some cases, enhanced. These results were very promising for enzyme production and lignocellulosic waste management, however only the inulinase experiment was performed at a representative scale (2 Kg).

Cellulase experiments were carried out using less than 1 g of material, which is not representative for further scale up. In this context, it is necessary to undertake more studies to develop a continuous system for enzymatic production.

Table 1.4. Conditions needed for lab scale vs. large or commercial scale SSF. Source: El-Bakry et al. (2015).

Condition	Lab scale	Large scale
pH control	Possible by pH adjustment	Not possible
T control, Heat removal	Easy, e.g. water bath	Possible by aeration, costly, presence of T gradients.
Solid handling	Very easy	Very difficult
Inoculation	Easy, not expensive	High costs, difficult homogenization
Agitation	Very easy	Possible but costly, e.g rotatory drums
Aeration	Sufficient	Moderate-high, expensive

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CHAPTER 2. RESEARCH OBJECTIVES

Based on the extensive background on cellulase production by SSF provided in Chapter 1, it can be summarized that the main challenges to overcome in any SSF process are the scale-up and the operation in a continuous configuration. For this reason, further studies must be performed on these subjects in order to develop a more sustainable and consistent process carried out in a representative scale.

In this sense, the main objective of this phD thesis is to develop a sustainable and reproducible cellulase production process by means of solid-state fermentation using a low cost substrate, such as the organic solid wastes.

In order to achieve the main objective, several specific objectives were developed and are presented below.

- To perform a screening of several organic wastes at laboratory scale, in order to assess its potential as cellulase source.

- To assess the scale-up of selected cellulase production processes, following cellulase production profile and the gaseous emissions generated.

- To demonstrate the technical feasibility of a continuous solid-state fermentation focusing on the development of operational strategies. Sequential batch operation was assessed with the main objective to obtain a long term operation and at the same time to specialize the biomass to degrade lignocellulosic materials. To achieve this goal operational strategies for the production of carbohydrases: amylase and cellulase were assessed.

- To produce and to characterize an specialized a cellulose-degrading mixed inoculum. To characterize this inoculum next generation DNA sequencing must be applied.

- To perform the cellulase production in a pilot scale solid-state fermentation using the specialized mixed inoculum. Cellulase production profile and gaseous emissions were followed in order to assess reproducibility.

Due to the promising results obtained during this thesis, another phD student, María Marín, is currently working on the optimization of the enzymatic extraction step and the stabilization of the fermented solids generated, by means of composting and anaerobic digestion processes.

CHAPTER 3. MATERIALS AND METHODS

3.1. Materials: Substrates and reactors set-up

3.1.1 Substrates

The materials used during the development of the thesis were: coffee husk (CH), soy fiber (SF), orange (OP) and lemon peels (L), almond hulls (AH), pre-cooked bread waste (BW) and olive oil cake (OOC). All of these materials are wastes from the food industry. The substrates were obtained from local industries in Barcelona, Spain. Coffee husk is the main waste generated by the coffee production (Marcilla®, Mollet del Vallès, Barcelona, Spain). Specifically, coffee husk is obtained during the drying of coffee grains. Soy fiber a by product obtained during soy milk and derivatives production (Natursoy®, Castellterçol, Barcelona, Spain). This waste is generated as a result of the pressing of soy grain during the process. The rest of the materials were kindly provided by local markets. All of these materials were evaluated as substrate for cellulase production.

3.1.2 Inoculum

Compost was assessed as potential inoculum for cellulase production. This material was obtained from the municipal solid waste treatment plant Ecoparc II (Montcada i Reixac, Barcelona, Spain).

3.1.3 Other materials

Wood chips were added into the substrate/inoculum mixture as bulking agent, in order to provide porosity to the mixture. This material was obtained from a composting treatment plant (Manresa, Barcelona, Spain). Specific details of all materials are provided in each chapter.

3.2 SSF reactors

3.2.1 Laboratory scale set-up

The respirometer set-up was designed and built according to Ponsá et al., (2010) and Pognani et al., (2011). Briefly, the respirometric system consists of 12 lines, each able to carry out 3 independent fermentation using 0.5L reactor. These reactors were Erlenmeyer flasks with an inner system that allowed the proper air circulation on the solid matrix. The inlet of the gas was connected to a silicone tube that go across de reactor from top to bottom. At the bottom of the reactor and connected to the silicone the an air diffuser is set. In addition to this system, a metallic net was set at the bottom of the reactor with two main objectives: the first is to hold the solid material and the second is to provide that the air freely is distributed throughout the solid. Also, each reactor is provided of one mass airflow meters, one electro-valve 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 from each reactor separately. A specific software was developed by GICOM research group to allow the on-line

monitoring and the continuous storage of the experimental data (Figure 3.1). A schematic diagram of the respirometric system is presented in Figure 3.2.

Figure 3.1. Screenshot of the software for online monitoring of different operational parameters.

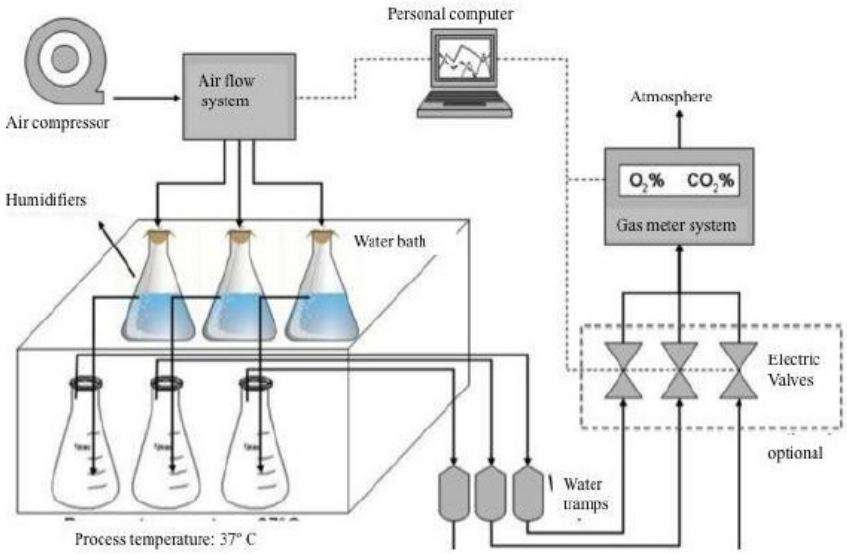

Figure 3.2. Schematic diagram of respirometer used for lab-scale solid-state fermentation. Source: Pognani, (2011).

Using this system, each reactor is able to contain a maximum of 100 g of total mixture, including substrate, inoculum and bulking agent. Also, as the reactors are submerged in a thermostatic bath, the temperature during the fermentations are fixed on one value. The entire screening of substrates for cellulase production by means of solid-state fermentation was carried out using this set-up (Chapter 4).

3.2.2 Bench scale set-up

Solid-state fermentation in bench scale were performed in either 4.5 L or 10 L air-tight packed-bed reactors, thermally isolated to work under near adiabatic conditions. Experimental set-up is presented in Figure 3.3. Air was continuously supplied to the reactors by means of a mass airflow controller (Bronkhorst, Spain). Airflow was automatically adjusted by a feedback controller in order to maintain oxygen content in the reactor in levels above 5% and avoid anaerobic conditions. In this context, air flow was supplied through a pipeline from the bottom of the reactor where, by means of a plastic diffusor, the air circulated through the solid bed of the reactor. The exit of the exhausted gas is located on the top of the reactor, and led to a oxygen analyzer. The oxygen content was measured using a oxygen sensor (Sensortran, Spain) in a range of (0-20.9%).

Between the reactor and the oxygen sensor there is a vapor-condensing device to avoid humidity to reach the sensor and consequently damage. All outlet pipelines were connected to a biofilter.

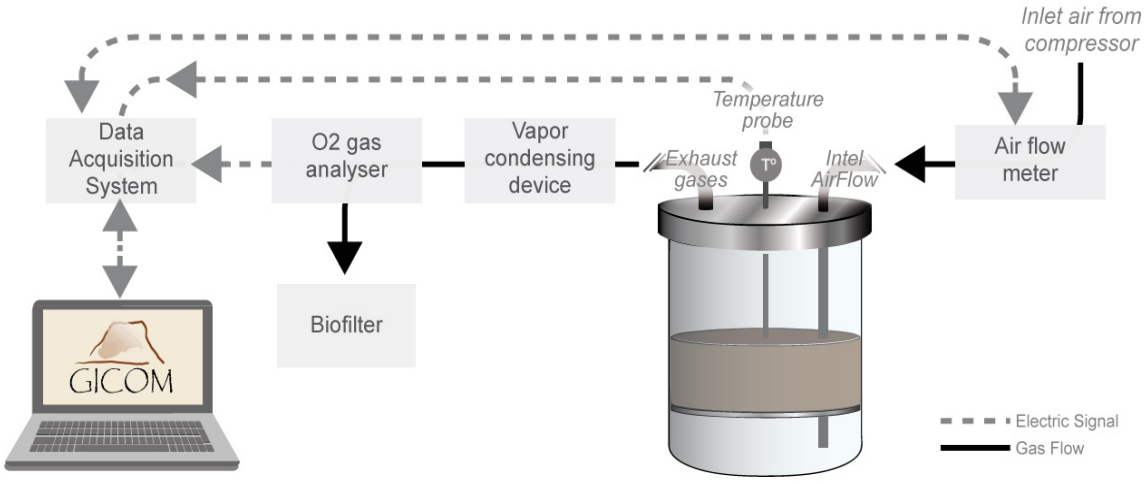

Figure 3.3. Experimental set-up of the bench scale solid-state fermentation system.

During all fermentations airflow, temperature and oxygen content were continuously monitored. Monitorization was performed by a self-made acquisition and control system based on Arduino® and self-made software. The control system is fully described in section 3.4 of this chapter.

3.2.3 Pilot scale set-up

This system is located outside of the Chemical, Biological and Environmental Engineering Department at UAB. It consists of 2 reactors of 55 L total volume of which 50 L is the working volume (shown in Figure 3.3). These reactors were used in all experiments at pilot scale.

The system was supplied with filtered compressed air provided by a pressure compressor Decibar 30 Worthington®. Two connectors were place at the cover of both reactors. One of the connectors was designed to hold and insert the temperature probe and the other one to insert the outlet pipeline of exhausted gases. Another pipeline is set at the bottom of the reactor, which allowed the injection of 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).

During all fermentations airflow, temperature and oxygen content were continuously monitored. Monitorization was performed by a self-made acquisition and control system based on Arduino® and self-made software. The control system is fully described in section 3.4 of this chapter.

Figure 3.5. Picture of pilot reactors of 50-L of working volume.

3.3 Specific methods for the monitoring of cellulase production process.

3.3.1 Specific Oxygen Uptake Rate and Cumulative Oxygen Consumption.

Specific oxygen uptake rate (sOUR) was calculated on-line for continuous monitoring in order to provide more accurate information on biological activity, according to:

$$sOUR = F \cdot (0.209 - y_{O_2}) \cdot \frac{P \cdot 32 \cdot 60 \cdot 10^3}{R \cdot T \cdot DW \cdot 10^3} \quad \text{Equation (1)}$$

where: sOUR is the specific Oxygen Uptake Rate (mg O_2 g^{-1} DM h^{-1}); F, airflow (mL min^{-1}); yO_2, is the oxygen molar fraction in the exhaust gases (mol O_2 mol^{-1}); P, pressure of the system assumed constant at 101325 Pa; 32, oxygen molecular weight (g O_2 mol O_2^{-1}); 60, conversion factor from minute to hour; 10^3, conversion factor from mL to L; R, ideal gas constant (8310 Pa L K^{-1} mol^{-1}); T, temperature at which F is measured (K); DW, initial dry weight of solids in the reactor (g); 10^3, conversion factor from g to mg. Moreover, the area below the sOUR curve was also determined, which represents the cumulative oxygen consumption (COC) during the process (Ponsá et al., 2010). These parameters will provide information on the overall biological activity and one of the main energy requirements for cellulases production.

3.3.2 Enzyme extraction

Cellulases were extracted by adding 150 mL of citrate buffer (0.05 M, pH 4.8) to 10 g of fermented solids in a 250 mL Erlenmeyer flask and mixing thoroughly on a magnetic stirrer for 30 min at room temperature. The mixture was separated by centrifugation at 10000 rpm for 10 min, followed by filtration with a 0.45 μm filter. The remaining supernatant was used for cellulase activity determination (Dhillon et al., 2012).

3.3.3 Cellulase activity

The components of the cellulase system were measured in terms of carboxymethyl cellulase (CMCase), filter paper activity (FPase) and β -glucosidase (BGase) according to the methods recommended by IUPAC (Ghose, 1987).

FPase is the most accepted method to reflect total cellulase action. The main advantage of this procedure is that requires simple reagents and equipments; however, it has been reported as a very laborious and time-consuming method, requiring many manual manipulations which lead to non-reproducible results (Coward-Kelly et al., 2003). Also, it is important to consider possible mass transfer problems when filter paper is used and possible non adequate contact surface of the paper strips in the reaction medium. In spite of this considerations, until this day remains as the most utilized method to describe cellulase activity. For these reasons microcrystalline cellulose (SC) was

evaluated as a possible substrate to replace the filter paper; however, for the difficulties to make proper comparison with reported studies, filter paper was used during the entire thesis.. These results are shown in Appendix III.

FPase and CMCase assay are based on the reducing sugars measurements. The final products were measured using dinitrosalicylic acid (DNS) reagent (Miller, 1959). In the case of BGase, the final product obtained is glucose, this was measured using a YSI 2700 Select Biochemistry Analyser (John Morris Scientific, Perth). One unit of the CMCase (U), FPase (FPU) and BGase (U) were expressed as being equivalent to the amount of enzyme that releases 1μmol of glucose from CMC, Whatman filter paper and cellobiose respectively in 0.05 M citrate buffer, pH 4.8 at the determined time of assay.

In all cases, enzymatic activity production has been expressed with respect to the dry matter content, i.e, FPU g^{-1} DM for FPase and U g^{-1} DM for CMCase and BGase.

Reagents

Citrate Buffer: All cellulase assays were carried out in 0.05 M citrate buffer pH 4.8. This buffer was made by adding 210 g of citric acid monohydrate (C_6 H_8 0_7 * H_20) to 750 mL distilled water and mix. Then, NaOH was added until pH reached 4.3 and diluted to 1000 mL and check pH. If necessary, NaOH until pH equals 4.5. This is 1 M citrate buffer at pH 4.5. When diluted to 0.05 M, pH should be 4.8-5.

DNS Reagent: Prepare a solution adding 10.6 g of 3,5-dinitrosalicylic acid (DNS) and 19.8 g of NaOH to 1416 mL of distilled water and mix. Once the solution is fully dissolved add 306 g of sodium potassium tartrate, 7.6 mL of phenol (if phenol is solid, melt at 50°C) and 8.3 g sodium metabisulfite, then mix.

Procedure

All of the following procedures are described as reported by Ghose, (1987).

a) Filter Paper Activity Assay (FPase).

Substrate: Filter paper strips (6 x 1 x 1 cms).

Procedure:

- Add 1.0 ml 0.05 M Na-citrate buffer, pH 4.8 and 0.5 mL of properly diluted enzyme, to a test tube of volume at least 25 ml. Add one filter paper strip and mix.

- Along with the samples, prepare controls for substrate and enzyme. Substrate control is prepared by replacing the enzyme volume for distilled water. Enzyme control is prepared by replacing substrate volume for distilled water. All controls and samples must be prepared at least in triplicates.

- Incubate all controls and samples at 50°C for 60 min.

- Add 3.0 mL DNS and mix. Boil for exactly 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, controls and the spectro zero should be boiled together.

- After boiling, transfer to a cold water bath. Add 20 mL deionized or distilled water. Mix by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion.

- Measure against the spectro zero at 540 nm.

Calibration curve:

Substrate volume is replaced by deionized or distilled water. Enzyme volume is replaced with glucose standards at several different concentrations. Then, follow the same procedure that for activity measurement. An example of the obtained calibration curves is presented in Appendix I.

Calculations

The calculations were carried out using the following equation:

$$FPase\ (FPU\ g^{-1}DM)\ = \frac{(C_{sample})*D*E*100}{0.18*60*DM} \qquad \text{(Equation 2)}$$

where, C_{sample} is the concentration of reducing sugars in g L^{-1} , D is the dilution factor of the enzymatic extract, E is the extraction factor in (g of fermented solid/ mL of buffer) and DM is the dry matter content of the fermented solid.

b) Carboxymethylcellulase activity Assay (CMCase).

Substrate: Carboxymethylcellulose 2% prepared using 0.05 M Na-citrate buffer at pH 4.8.

Procedure:

- Add 0.5 ml of carboxymethylcellulose solution and 0.5 mL of properly diluted enzyme extract, to a test tube of volume at least 25 ml and mix.

- Along with the samples, prepare controls for substrate and enzyme. Substrate control is prepared by replacing the enzyme volume for distilled water. Enzyme control is prepared by replacing substrate volume for distilled water. All controls and samples must be prepared at least in triplicates.

- Incubate all controls and samples at 50°C for 60 min.

- Add 3.0 mL DNS and mix. Boil for exactly 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, controls and the spectro zero should be boiled together. After boiling, transfer to a cold water bath. Add 20 ml deionized or distilled water. Mix by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion.

- Measure against the spectro zero at 540 nm.

Calibration curve:

Substrate volume is replaced by deionized or distilled water. Enzyme volume is replaced with glucose standards at several different concentrations. Then, follow the same procedure that for activity measurement. An example of the obtained calibration curves is presented in Appendix II.

Calculations

The calculations were carried out using the following equation:

$$CMCase\ (U\ g^{-1}DM)\ = \frac{(C_{sample})*D*E*100}{0.18*30*DM} \qquad \text{(Equation 3)}$$

where, C_{sample} is the concentracion of reducing sugars in g L^{-1} , D is the dilution factor of the enzymatic extract, E is the extraction factor in (g of fermented solid/ mL of buffer) and DM is the dry matter content of the fermented solid.

c) β-glucosidase activity Assay (BGase)

Substrate: Cellobiose 15mM prepared using 0.05 M Na-citrate buffer at pH 4.8.

Procedure:

- Add 1.0 ml of cellobiose solution and 1.0 mL of properly diluted enzyme extract, to a test tube of volume at least 25 ml and mix.

- Along with the samples, prepare controls for substrate and enzyme. Substrate control is prepared by replacing the enzyme volume for distilled water. Enzyme control is prepared by replacing substrate volume for distilled water. All controls and samples must be prepared at least in triplicates.

- Incubate all controls and samples at 50°C for 30 min.

- Transfer the tube to a cold water bath and determine glucose produced using YSI 2700 Select Biochemistry Analyser.

Calculations

The calculations were carried out using the following equation:

$$BGase\ (U\ g^{-1}DM) = \frac{(C_{sample})*D*E*100}{0.18*30*DM} \quad \text{Equation (4)}$$

where, C_{sample} is the concentracion of reducing sugars in g L^{-1} , D is the dilution factor of the enzymatic extract, E is the extraction factor in (g of fermented solid/ mL of buffer) and DM is the dry matter content of the fermented solid.

3.3.4 Microbial characterization

Identification of the microbial population was performed in several solid samples, obtained from fermentation processes, using next generation sequencing. The aim of this analysis was to determine the potential variation on biodiversity and to characterize biomass obtained during enzyme production.

Total DNA was extracted and purified using PowerSoil™ DNA Isolation Kit (MoBio Laboratories, USA) according to provider's specifications. DNA samples were checked for concentration and quality using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware; USA) (López-González et al., 2014).

Bacterial 16S rRNA hypervariable regions V3-V4 and Fungal ITS1-ITS3 were targeted. Later sequencing of the extracted DNA and bioinformatics were performed on MiSeq platform by Life Sequencing S.A (Valencia, Spain).

3.3.5. Gaseous emissions determination

All gaseous emissions measurements were developed according to the reported by Colón et al., (2012) and volatile organic compounds determination was carried out as described by Maulini-Durán et al., (2014).

a) Ammonia determination

Ammonia measurements were performed using an ammonia sensor iTX T82 (Oakdale, PA, USA), which have a range of measurement of 0-200 ppmv. The sensor has to be set inside an hermetically sealed recipient. This recipient has two valves to serve as gas inlet and outlet, this system allows the proper circulation of the airflow. This system has to be located at the gas outlet of the reactor, before the water trap. Ammonia measurement is obtained once the sensor stops fluctuating.

b) Methane, nitrous oxide and volatile organic compounds determination

Conditions

As for methane (CH_4) and nitrous oxide (N_2O) determination, these measurements were carried out by gas chromatography. Results are expressed in parts per million in volume (ppmv). Details of the equipment and operational conditions for the analysis is provided in Table 3.1 and Table 3.2 for methane and nitrous oxide, respectively.

Table 3.1. Specifications of the equipment and conditions of analysis for methane determination.

Equipment	
Cromatograph	Agilent Tech. 6890N GC System
Column	HP-PLOT 30m·0.53mm·40µm (Agilent Tech.)
Detector	Flame ionization detector (FID)
Conditions	
Injector temperature (ºC)	240
Detector temperature (ºC)	250
Oven Temperature (ºC)	Isothermal process at 60ºC
Carrier gas	Nitrogen at 4 psi and split 1:2
Time of analysis (min)	4
Injection volume (µL)	500

VOC analysis was performed by gas chromatography at specific conditions, which are presented in Table 3.3. Quantification of these gases was carried out including all organic compounds present on the gaseous stream, including methane content, but without any further identification. Then, the total VOC value is obtained by subtracting the methane content to the total of organic compounds detected by the chromatograph. VOC concentration is obtained in units of concentration expressed as milligrams of total carbonaceous compounds per cubic meters of gas (mg C_{total}/m^3).

Calibration curves

The calibration is carried out by using a standard gas with known concentration, in order to determine the response of the method and therefore, to be able to construct a calibration curve. The used standard gases were n-hexane (Spigno et al., 2003), methane and nitrous oxide (purity of 99.99%) for VOC. methane and nitrous oxide, respectively.

Calculations

The chromatograph provide de measurement on the gases concentration integrating the area obtained in each injection by means of Chromeleon^{TM} Software.

Table 3.2. Specifications of the equipment and conditions of analysis for nitrous oxide determination.

Equipment	
Cromatograph	Agilent Tech. 6890N GC System
Column	HP-PLOT 30m·0.53mm·40µm (Agilent Tech.)
Detector	Electron capture detector (ECD)
Conditions	
Injector temperature (ºC)	120
Detector temperature (ºC)	345
Oven Temperature (ºC)	Isothermal process at 60ºC
Carrier gas	Nitrogen at 4 psi and split 1:2
Time of analysis (min)	4
Injection volume (µL)	500

c) VOC identification and quantification

A sample from each process was taken in a 250 mL glass gas collector. VOC characterization was performed using air samples analyzed by SPME (Solid Phase Micro Extraction)/GC–MS, as previously reported by other authors (Orzi et al., 2010). A manual SPME device with divinylbenzene (DVB)/Carboxen/ polydimethylsiloxane (PDMS) 50–30 µ m fiber from Supelco (Bellefonte, PA, USA) was used. The compounds were adsorbed from the air samples by exposing the fiber (preconditioned for 1 h at 270 C, as suggested by the supplier) to the sample in the glass gas collector for 30 min at room temperature. A solution of deuterated p-xylene in methanol was used as internal standard (IS). VOC characterization was performed using a Gas Chromatograph (Agilent 5975C)

coupled with a 7890 Series GC/MSD. Volatile compounds were separated using a capillary column for VOC (Agilent Technologies DB-624) measuring 60 m x 0.25 mm with a film thickness of 1.40 µm. Carrier gas was helium at a flow rate of 0.8 mL min^{-1}. VOC were desorbed by exposing the fiber in the GC injection port for 3 min at 250ºC. A 0.75 mm internal diameter glass liner was used, and the injection port was in splitless mode

Table 3.3. Specifications of the equipment and conditions of analysis for volatile organic compounds determination.

Equipment	
Cromatograph	Agilent Tech. 6890N GC System
Column	Tracsil TRB-1, 2m·0.53mm·3µm (Teknokroma)
Detector	Flame ionization detector (FID)
Conditions	
Injector temperature (ºC)	250
Detector temperature (ºC)	250
Oven Temperature (ºC)	Isothermal process at 200ºC
Carrier gas	Helium at 1.5 psi and splitless
Time of analysis (min)	1
Injection volume (µL)	250s

The temperature program was isothermal for 2 min at 50ºC, raised to 170ºC at a rate of 3ºC min^{-1} and, finally, to 230ºC at a rate of 8ºC min^{-1} . The transfer line to the mass spectrometer was maintained at 235ºC. The mass spectra were obtained by electron ionization at 70 eV, a multiplier voltage of 1379 V and collecting data over the mass range of 33–300. Deuterated p-xylene was used to determine the fiber and GC– MS response factors for 15 typical compounds emitted in composting processes according to the literature (Scaglia et al., 2011)These 15 compounds were diluted in methanol at the same concentration as deuterated p-xylene. This solution (10 µL) was injected into the glass gas collector with 10 µL of deuterated p-xylene in methanol solution. The fiber was exposed for 30 min to the resulting solution and injected into the GC–MS using the same method as described above. The area obtained for each compound was compared to deuterated p-xylene to determine each response factor. The aim of determining these response factors is to increase the reliability of the quantitative analysis. Compounds were identified by comparing their mass spectra with the mass spectra contained in the NIST (USA) 98 library. A semi-quantitative analysis for all the identified compounds was performed by direct comparison with the internal standard. Quantitative analysis was

performed for m-xylene, n-decane, alphapinene, beta-pinene, limonene, toluene, dimethyl disulfide, hexanal, styrene, cyclohexanone, nonanal, decanal, eucalyptol, pyridine and 2-pentanone. These compounds have been the most common VOC found in previous experiments (Maulini-Duran et al., 2014), representing different VOC families.

3.4 Aeration control system

Two different control systems were used during the development of this thesis: oxygen feedback control and sOUR control.

3.4.1 Oxygen feedback control

The controller was based on the airflow manipulation by means of the oxygen content measured in the exhaust gas. It was necessary to establish an O_2 set point to maintain the system in favorable conditions. The oxygen set point was fixed between 11.5 and 12.5% (v/v) and considered as the optimum oxygen content . Emulating the controllers used at industrial level, the controller applied a high airflow for oxygen levels below 11.5% and a low airflow for measures over 12.5%, whereas the controller did not take action when the measure was within this range. The highest and lowest airflows depended on the dynamic of every assessed raw material and the type of reactor (0.5, 4.5, 10 or 50 L). Because of the system's slow dynamics the closed loop applied was set up to work in cycles of 15 min.

3.4.2 Oxygen Uptake Rate (sOUR) control

In this case, the measured variable is the sOUR (determined using the online measured oxygen content) and the manipulated variable is the airflow. The sOUR is a parameter that provides direct information on the biological activity of the reactors, it is for this reason that the main goal of this control is to maximize this parameter. More details on the development of this control strategy is published by Puyuelo et al., (2010). A full schematic diagram of the control system is presented in Figure 3.4.

Briefly, the maximization of the sOUR 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 specific oxygen uptake rate (sOUR) measurements. Hence, values of flow and sOUR, 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 sOUR and airflow can be considered negligible. The limit to detect sOUR variation was defined as 0.5 % of the maximum sOUR by previous studies. The range considered for the airflow measurements was 0.05 L min^{-1}.

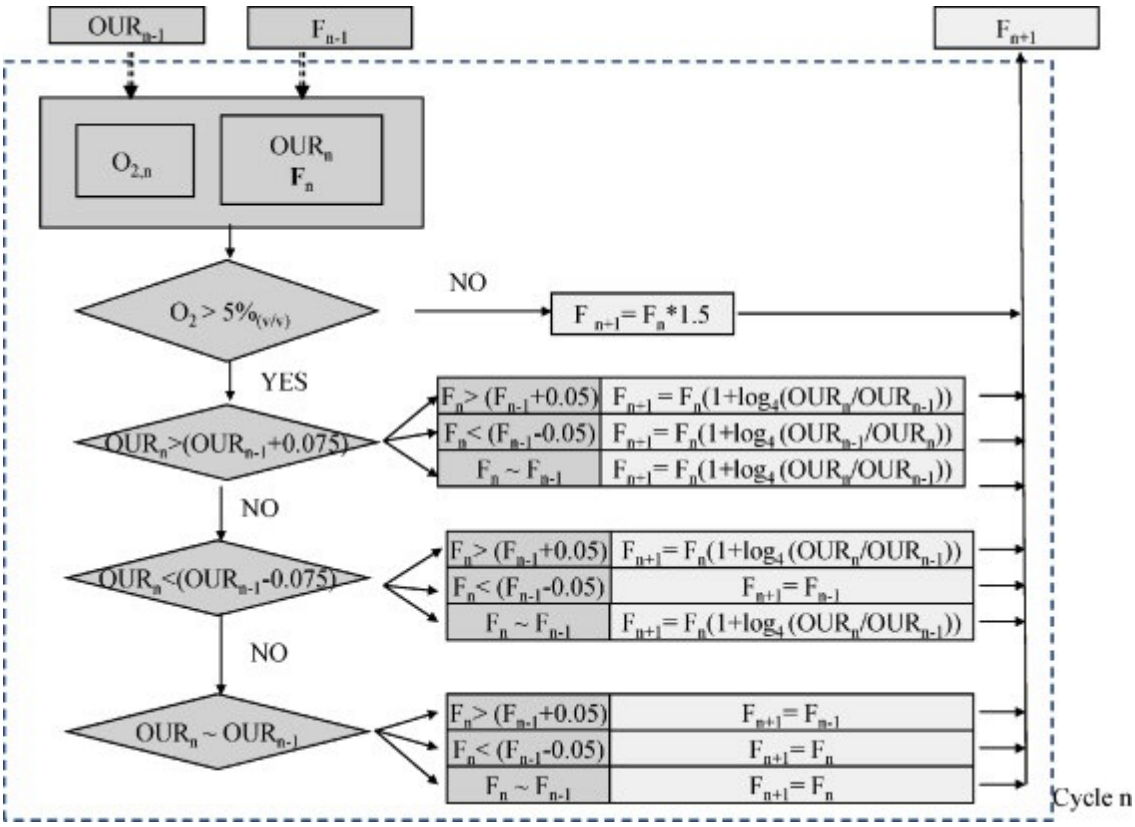

Figure 3.4. Scheme of control laws for the OUR controller. *F*: airflow; OUR: oxygen uptake rate. Source: Puyuelo et al., (2010).

3.5 Standard methods

Routine methods were determined according to standard procedures included in the "Test Methods for the Examination of Composting and Compost" (US Department of Agriculture and US Composting Council, 2001). If it is not included in this reference, the original reference will be provided. All results are calculated using triplicates in all cases.

3.5.1 Moisture and Dry matter content.

To determine moisture (M) and dry matter content (DM, equivalent to total solids), a determined amount of solid sample is set on a previously weighted dry capsule and dried in an oven at 105ºC during 24h, then weighted and calculated according to the following equations:

$$M(\%) = \frac{(Wi - Wf)}{(Wi - Wo)} * 100$$

$$DM(\%) = 100 - \%M$$

(Equation 5)

where Wo is the weight of the dry capsule, Wi is the initial wet weight of the sample and Wf is the final dry weight of the sample.

3.5.2 Organic matter content.

To determine the organic matter content (OM, equivalent to volatile solids), the dried solid sample is submitted to ignition at 550ºC during 4h and it is calculated as follows:

$$M(\%) = \frac{(Wi - Wa)}{(Wi - Wo)} * 100$$

(Equation 6)

where, Wo is the weight of the dry capsule, Wi is the initial dry weight of the sample and Wa is the final weight of the ashes, obtained after ignition.

3.5.3 pH

pH was determined by mixing a ratio of 1:5 w/v of sample into distilled water. The sample was shaken at room temperature during 30 min to allow the salts to solubilize into the liquid phase. Then centrifuge at 3500 rpm during 10 min and measured. pH was measured using an electronic pH-meter (Crinsom®, micro CM2100).

3.5.4 Fiber content.

The fibers content were determined using the method reported by Van Soest et al. (1991). According to these authors, cellulose content is the result of the difference between neutro detergent fibers (NDF) and acid detergent fibers (ADF). Also, hemicellulose content is corresponds to the difference between ADF and lignin content (ADL).

a) Neutro detergent fibers (NDF)

Its determination is based on the solubility of the fiber components to dodecyl sodium sulphate at neutral pH. The soluble components of the cell wall such as starch and simple sugars are solubilized by the detergent, while recalcitrant components such as cellulose, hemicellulose and lignin remain in the solid matrix.

Reagent: NDF reagent was made using 1.86 g EDTA, 0.68 g $Na_2B_4O_7 \cdot 10H_2O$, 3g sodium lauryl sulphate, 1 g of 2-etoxyethanol, 0.456 g of Na_2HPO_4 and 95 mL of H_2O. Then adjusted pH to 7.0 using H_3PO_4.

Procedure:

- Weight 1 g of wet sample and add 100 mL of NDF reagent, 2mL of decahydronaphthalene and 0.5 mL (1.316 g) of Na_2SO_3

-Autoclave for 15 minutes at 121ºC.

- Filtrate using 0.45 μm glass fiber filter (previously dried and weighted).

- Wash with 500 mL of hot water, then distilled water and finally with ketone, in order to remove pigments and water residues.

- Dry during 4 h at 100ºC. When the sample is temperate, weight.

Calculations

The calculations were carried out using the following equation:

$$NDF(\%) = \frac{(Wi - Wf)}{(Wi)} * 100$$

$$Cellulose(\%) = NDF - ADF \qquad \text{(Equation 7)}$$

where, Wi is the initial weight of the wet sample and Wf is the final dry sample.

b) Acid detergent fibers (ADF)

Its determination is based on the cellular components solubility in a cethylmethyl ammonium bromide solution. The residue obtained after the treatment contains mainly cellulose and lignin.

Reagent: ADF reagent was made my mixing 2 g of cethylmethyl ammonium bromide and 100 mL H_2SO_4 1 N.

Procedure:

- Weight 1 g of wet sample in an Erlenmeyer flask and add 100 mL of ADF reagent and 2 mL of decahydronaphthalene.

- Autoclave for 15 minutes at 121ºC.

- Filtrate using 0.45 µm glass fiber filter (previously dried and weighted).

- Wash with 500 mL of hot water, then distilled water and finally with ketone, in order to remove pigments and water residues.

- Dry during 4 h at 100ºC. When the sample is temperate, weight.

Calculations

The calculations were carried out using the following equation:

$$ADF(\%) = \frac{(Wi - Wf)}{(Wi)} * 100$$

$$Hemicellulose(\%) = ADF - ADL \qquad \text{(Equation 8)}$$

where, Wi is the initial weight of the wet sample and Wf is the final dry sample.

c) Lignin (ADL)

- The dry solid obtained as result for ADF determination is used for the ADL determination.

- Cover the solid with sulfuric acid 72% and wait for 1 h. Remove the acid and repite 3 times.

- Filtrate using 0.45 µm glass fiber filter (previously dried and weighted) and wash with 500 mL of hot water.

- Dry until constants weight at 105ºC (W_1).

- Submit the dried sample to ignition at 550ºC and weight (W_2).

Calculations

The calculations were carried out using the following equation:

$$ADL(\%) = \frac{(W_1 - W_2)}{(Wi)} * 100$$

$$Lignin(\%) = ADL \qquad \text{(Equation 9)}$$

where, Wi is the initial weight of the wet sample and Wf is the final dry sample.

References

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CHAPTER 4. CELLULASE PRODUCTION BY SOLID-STATE FERMENTATION USING ORGANIC WASTES AY LAB SCALE AND SCALE-UP.

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Introduction

In this chapter four different residues provided by several local industries were screened in order to determine its suitability for cellulase production at lab scale. The raw materials that provided highest cellulase production, coffee husk and orange peels, were assessed as substrates at a larger scale using 10-L reactors (3-4 Kg). The main objective of working at this scale is to submit the solid mixture to a self-heating process and to assessed the scalability effect when compared with the previous screening. A final scale-up of these processes were carried out in a pilot scale, working in a 50-L reactor (15-20 Kg). In these experiments, in addition to the cellulase production profile, the released gaseous emissions were measured in order to partially assess the potential environmental impact of the SSF. The experiments showed in this chapter were carried out as a joint venture with Pedro Jimenez and in collaboration with Caterina Maulini.

4.1 Methodology

4.1.1 Screening of raw materials for cellulase production

A screening of several organic wastes was performed in order to determine their potential for cellulase production. Orange (OP) and lemon (L) peels, olive oil cake (OOC) and coffee husk (CH) were analyzed. Also, wood chips (WC) and almond hull (AH) were assessed as alternative bulking agents. A full characterization of all the materials is presented in Table 4.1. Compost (C) was added in all cases as inoculum in order to provide microbial diversity to the solid mixture (López-Gonzalez et al., 2015b).

The fermentations were carried out using a mixture comprising each waste as substrate and compost as inoculum in a 90:10 (w/w) ratio. In addition bulking agent was added to provide porosity and improve the oxygen transfer in the solid bed. Each fermentation was performed in triplicates during 4 days using the experimental set-up described in section 3.1.4. Operational conditions of the fermentations were constant, with a temperature of 37ºC and airflow of 20 mL min^{-1}. Two samplings were performed, one at the moment of maximum sOUR and another at the end of the fermentation. Cellulase activities of FPase, CMCase and BGase were measured in all samples taken from the reactors. In addition to the cellulase activities and sOUR monitoring, several physical-chemical parameters were measured: pH, dry matter, organic matter, reducing sugars and glucose.

Table 4.5. Characterization of all evaluated wastes. (*) Mean value ± standard deviation (n=3), (**)s.d< 6% n.m: not measured.

Parameter	**OP**	**L**	**OOC**	**AH**	**CH**	**C**
pH (1:5)	4.64±0.02	5.4±0.01	4.50±0.01	5.96±0.01	6.5±0.01	7.96±0.02
CE (mS cm^{-1})	0.67±0.01	0.85±0.01	2.38±0.10	0.49±0.01	n.m	6.25±0.20
Wet basis(%)						
Moisture	82.3±0.2	80.75±0.04	75.7±0.3	12.9±0.3	59.9±0.4	34.29±0.01
Dry matter	17.7±0.2	19.25±0.04	24.3±0.3	87.1±0.3	40.1±0.4	64.71±0.01
Dry basis (%)						
Organic matter	98±2	95.85±0.01	95.2±0.3	99.01±0.37	90.22±0.08	n.m
Reducing sugars	37±1	36.3±0.2	2.51±0.01	0.5±0.01	0.65±0.01	n.m
Glucose	6±0.01	6±0.01	0.29±0.01	0.08±0.01	0.02±0.01	n.m
Total C**	44.5		55	48	80.1	n.m
Total H**	6.2		7.6	6	n.m	n.m
Total N**	1.01		2	0.5	3.5	n.m
C/N ratio	44		27.5	96	22.9	n.m

4.1.2. SSF scale-up assessment

From the screening described in the previous section, two raw materials were selected for further assessment of the process scale up.

In all the fermentations performed in near-adiabatic reactors, the selected wastes were evaluated as substrates using compost as inoculum in a 90:10 (w/w) ratio. Also the selected bulking agent was added in a 1:1 (v:v) ratio to each fermentation. In a first stage, the fermentations were carried out using the system described in section 3.1.5 using 10-L reactors. The system was operated using the sOUR control as described in section 3.4.2 starting with an initial airflow of 100 mL min^{-1}. During the fermentations, solid samples were taken starting at the moment of maximum sOUR to a final of 6 sampling, in order to determine the cellulase production profile and the specific substrate profile, reflected as reducing sugars and glucose.

In addition to the 10-L SSF, the selected wastes were assessed as raw materials in a pilot scale, using the system described in section 3.1.6 and performed in duplicates. The system was operated also using the sOUR control as described in section 3.4.2 starting with an initial airflow of 200 mL min^{-1}. In this case, also the substrate : inoculum ratio was 90:10 (w/w) and the bulking agent ratio was 1:1 (v/v). Cellulase activity, reducing sugars and glucose profiles were measured during the sampling. Also, total gaseous emissions of CH_4, N_2O, VOCs and NH_3 were measured. 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). The composition of the VOCs emissions was also determined and quantified. Both of these procedures were carried out according to the method described by Colon et al., (2012) (Chapter 3, section3.3.5).

4.2 Results

4.2.1 Screening of raw materials for cellulase production

Maximum biological activity detected in the fermentations is presented in Table 4.2 expressed as sOUR. It is possible to observe that a moderate (to high) biological activity was obtained using all the different substrates. The main differences can be found when is considered the characterization of the raw materials or even the initial mixture. It this sense, the residues coming from a direct agricultural source such as the orange and lemon peel presented a high content of readily metabolizable compounds such as reducing sugars and glucose as seen in Table 4.1. In contrast, coffee husk presented the lowest sOUR and also showed the lowest content of soluble materials. Then, it is possible that the high sOUR values obtained for orange and lemon peels and also olive oil cake are related to the initial content of soluble components.

In Table 4.2 is also presented the time when the maximum sOUR was obtained. It is clearly observed that when using orange peels as substrate and wood chips as bulking agent the fermentation time is longer than in the rest of experiments. This can be due to the initial acidic pH of the mixture (pH= 4.9), which could delay the starting up of the process as reported by Sundberg et al., (2013).

In contrast, when almond hulls are used as bulking agent, the time of fermentation decreases, reaching the maximum sOUR in only 1 day. This fact can be attributed also to the pH. In this case, the initial mixture had a initial pH of 5.5, which is significantly higher that when using wood chips as bulking agent. Almond hulls used as a bulking agent presented positive results, in terms of biological (Table 4.2) and enzymatic activity, which can be due to the high antioxidant content and the cell-protective characteristics of this residue (Moosavi Dolatabadi et al., 2015).

Table 4.6. sOUR values and time of fermentation obtained at the moment of maximum cellulase activity, working at lab scale at 37ºC.

Waste	sOUR (mgO_2 $g^{-1}DM$ h^{-1})	Time (d)
Orange peel + Wood chips	3.5 ± 0.3	2
Orange peel + Almond hull	2.3 ± 0.1	1
Lemon peel + Almond hull	2.5 ± 0.1	1
Coffee husk + Wood chips	1.6± 0.1	1
Olive oil cake +Wood chips	3.1 ± 0.1	1

During the process it was observed a rapid reduction until negligible values of the reducing sugars and glucose (data not presented). The only exception relies on the experiment carried out with orange peels and almond hulls, where an accumulation of nearly 30% of glucose occurred. It has to be considered that reducing sugars and glucose content in the fermentation represents a net value of the process, which includes hydrolysis of polysaccharides and consumption of the released sugars. It was expected that most of the glucose would be immediately consumed by microorganisms, however in OP and AH experiments an accumulation this monosaccharide took place, which can be of interest considering further studies regarding bioethanol production.

Figure 4.2 presents the three cellulase activity values obtained in all experiments. It is clearly observed that highest FPase activity was achieved by the fermentation using as the substrate coffee husk followed by orange peels, with 6.4±0.7 and 1.6±0.5 FPU $g^{-1}DM$, respectively. The rest of the assessed substrates obtained values below the unit of activity. Orange peels is commonly used as s substrate for pectinase or pectine derivates production for its availability and composition (Grohmann & Baldwin, 1992; Li et al., 2016), however, a few reports are published on the cellulase production using OP (Díaz et al., 2011) obtaining low enzymatic level of production. As for coffee husk, this residue is mainly used as fertilizer or animal feed but the usage is limited due to the high amount of organic material and phenolic compounds (antioxidants) (Belitz et al., 2009). For this reason, most of the coffee husk is further stabilized by means of composting (Shemekite et al., 2014a).

CMCase ranged in all cases between 0.5-1.5 U $g^{-1}DM$, which is in the lower range of the reported (Table 1.3, section 1.5.1). BGase, in all cases was also produced in small amounts in comparison to literature, with activity values below 0.2 U $g^{-1}DM$ (Table 1.3, section 1.5.1).

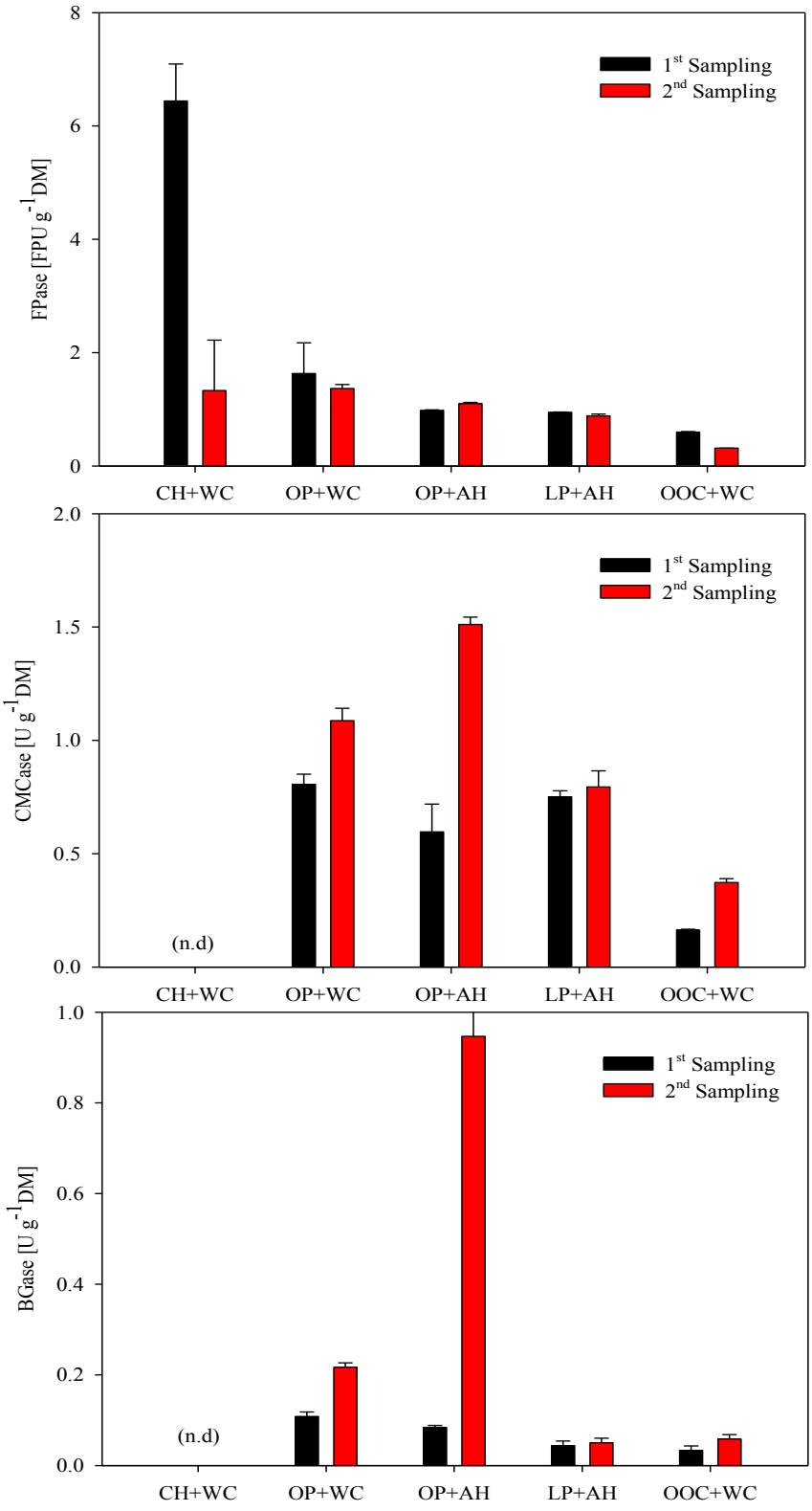

Figure 4.6. FPase, CMCase and BGase activity values obtained by SSF at 37ºC using different substrates and compost as inoculum. The 1st sampling corresponds to the moment of maximum sOUR and the 2nd sampling was at the end of the fermentation. n.d. not determined.

The most interesting result was obtained by the experiments using OP and AH, where a considerable BGase production of 0.111±0.003 U g^{-1}DM was obtained in comparison with the results obtained using the different substrates.

From these results, CH and OP were selected as the best substrates for further scale up assessment

4.2.2 SSF trials using orange peels as substrate

a) Operation in 10-L reactor

Process

This set of experiments were performed using the orange peels as a substrate and stabilized compost as the inoculum. Also, due to the good results obtained in the previous section, almond hulls were used as the bulking agent. Initial characterization of the raw materials and starting mixture for the 10 L reactors are presented in Table 4.3.

Table 4.3. Characterization of raw materials and initial mixture of 10-L OP reactor

Parameter	OP	Compost	OP 10 L
Moisture (%)	82.3	35.3	62.0
Dry Matter (%)	17.7	64.7	38.0
Organic matter (%)	97.8	-	87.0
Total organic carbon (%)	-	-	27.0
C/N ratio	-		15.0
pH	4.6	7.9	6.4

As seen in Table 4.3 , the starting pH of the process was 6.4. Shortly after the fermentation started, there was a substantial drop in pH values due mainly to the organic acids released to the media, which is expected in these processes. After that, a sustained increment of pH was obtained, reaching alkaline values of 8.4 after 9 days of fermentation.

C/N ratio was 15 and 22 at the beginning and at the end of the fermentation, respectively. According to literature, the optimum C/N ratio is around 20 or above (Puyuelo et al., 2011), depending on the substrate. In this experiment, the initial C/N ratio is lower than the reported as optimum. The values of carbon and nitrogen content observed in this experiment for this mixture are very different when compared with the values obtained during the screening of the raw materials (Table 4.1, section 4.1).

This can be due to the composition of the used compost or even to the heterogeneity of the mixture itself., i.e some bulking agent traces may be present in the analyzed mixture.

In Figure 4.2a the full profiles of temperature and sOUR of process are presented. The SSF presented an initial lag phase of nearly one day in the mesophilic range of temperature. After that, temperature started to increase until 55-60°C between days 5 and 9 of the process. During the thermophilic stage, highest sOUR was detected, reaching a maximum value of 5.6 mg g^{-1}DM h^{-1} at day 7 of fermentation.

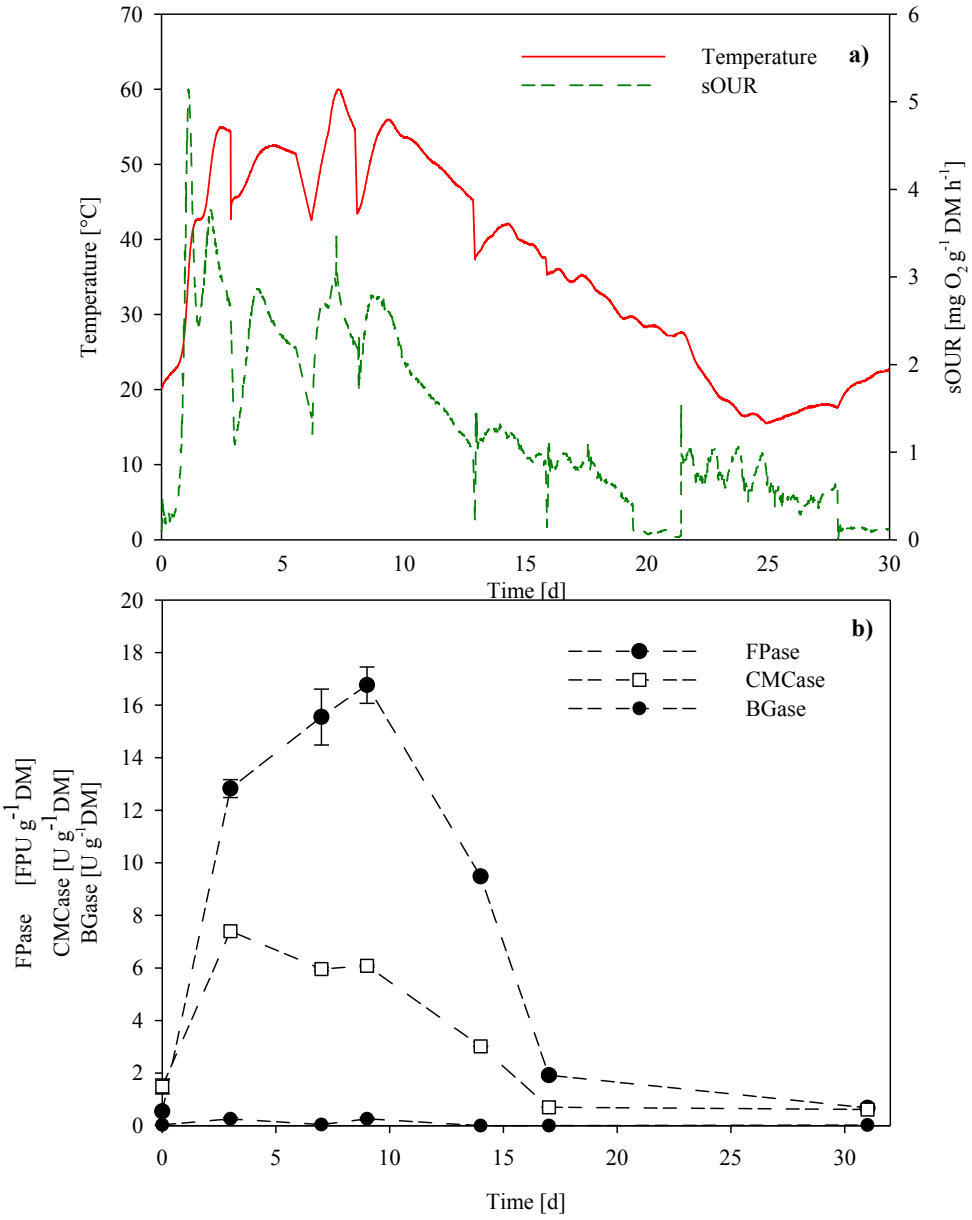

Figure 4.2. Profiles of a) Temperature and sOUR and b) enzymatic activity production for 10-L reactors using orange peels as substrate and compost as the inoculum.

At the same time when maximum sOUR was found, maximum temperature was achieved, reaching a value of 60ºC. In this moment, the reactor was supplied with a maximum airflow of 1000 mL min^{-1} (which is the maximum airflow possible using this configuration). In spite of that, oxygen content drop below the recommended 10% to ensure full aerobic conditions (Ruggieri et al., 2009), reaching values lower than 5%. This fact increases the possibility of the generation of anaerobic zones in the solid matrix of the reactor. This, in a way, can affect the biodegradation process and the enzymatic production.

After day 10 of operation, sOUR and temperature profiles presented a decreasing trend until reaching mesophilic conditions and a low sOUR value, indicating a stabilization on biological activity. This process achieved an overall COC of 285 g g^{-1}DM.

Enzymatic activity production

In terms of enzymatic activity production, the complete profile is presented in Figure 4.2b. Regarding cellulase activity production, it is possible to observe that FPase presented a high production, with a peak of production during thermophilic stage that reached nearly 16 FPU g^{-1}DM. This value is in accordance to the reported range of cellulase activity production in SSF, which is 1-144 FPU g^{-1}DM (Dhillon et al., 2012b; El-Bakry et al., 2015; Mansour et al., 2016). However , it is important to remark that for the measurement of cellulase activity there are several methods available with great differences in the procedure for enzymatic determination and its consequents results (Percival Zhang et al., 2006).

Regarding CMCase activity, the peak of production was obtained at day 3 of operation where the system was already in fully thermophilic conditions. After that moment, the enzymatic production decreased until almost negligible values in the 17th day of operation. The production of this enzyme resulted in values several times lower than the obtained in other SSF using organic wastes as seen in Table 1.3 section 1.5.1. CMCase reflects the endoglucanase activity which randomly bounds to any section of the cellulose structure, reducing the polymerization degree of the cellulose chain. Also, when working under the conditions presented in this SSF, it has to be taken into account that there are several microorganisms working together and in competition for the same substrates. In addition, considering that microorganisms excrete enzymatic compounds only when it is required, i.e when soluble and easily metabolizable substrates are depleted; it is likely to assume that CMCase can have a different profile (and peak) than the other cellulolytic components. In Figure 4.2b is clearly observed than CMCase peak and complete profile is different that the observed for FPase and BGase.

Another reflection of these facts are the end products of cellulolytic action. Glucose and reducing sugars content rapidly decreased during the process. At the beginning of the SSF reducing sugars and

glucose content were near 0.3 g g^{-1}DM and 0.065 g g^{-1}DM respectively. After the first sampling at day 3 of fermentation, nearly 50 and 80% of the reducing sugars and glucose were consumed, disappearing completely by day 9 in the case of glucose and day 17 in the case of reducing sugars.

Finally, for BGase activity, this also seems to be produced according to the requirements of the microorganisms. It is observed that, in comparison with the other enzymes, there is no particular trend in the production of BGase, obtaining two peaks of activity in days 5 and 9, with values of enzymatic activity of roughly 0.4 U g^{-1}DM.

In addition to the different trends among the cellulolytic components, it is clearly observed that maximum production of FPase activity matches with the maximum sOUR and temperature. According to Ghose, (1987) and Percival-Zhang et al., (2006), the FPase activity using filter paper as substrate provides better information than other synthetic substrates of the potential hydrolytic effect of cellulase on different real substrates. It is for this reason that most authors use this measurement as a way to reflect a "total cellulase" measurement. In this sense, for the experiments performed in 10-L reactors using coffee husk as the substrate only FPase was measured.

b) Operation in 50-L reactor.

Process

This experiment has two main differences with the 10L reactors: the first is the use of non-stabilized digested compost as inoculum and the second is that in this case wood chips was used as bulking agent. Duplicates of the reactors of the experiment were made (R1 and R2). Initial characterization is presented in Table 4.4.

Table 4.4. Characterization of raw materials and initial mixture of 50-L OP reactor

Parameter	OP	Compost 50L	OP 50L
Moisture (%)	82.3	29.9	58.1
Dry Matter (%)	17.7	70.1	41.9
Organic matter (%)			89.9
Bulk Density (g/L)			420.0
Air filled porosity (%)			74.0
C/N ratio			26.4
pH	4.6	6.6	5.9

Figure 4.3 are presented the sOUR and temperature profiles of the pilot SSF (R1 and R2) using orange peels as the substrate. In this figure it is possible to observe that, in general, all trends resulted in very similar behavior. However slight differences can be detected in the temperature and sOUR profiles that can be attributed to the intrinsic variability of the orange peel mixture, or even to the differences in the design of the reactors itself. In this sense, thermophilic conditions were achieved in the third day of process, although maximum temperature was not achieved until the twelfth day (R1: 67.4ºC; R2: 61.2ºC). The delay in temperature peaking, in comparison with the 10-L reactor, may be due to different factors: the initial acidic pH of the mixture (5.9, Table 4.4) rose during the process reaching a final value of 8.5; the lack of homogeneity of the initial mixture was overcome through successive mixing when sampling, contributing to mass transfer between the different materials inside the reactor; finally, a potential inhibition by the presence of some toxic component, such as limonene that was stripped off the reactor by the continuous aeration. As expected, the highest sOUR values (average of R1 and R2: 1.55±0.21 mgO_2 g^{-1}DM h^{-1}, representing a coefficient of variation of 13%) matched with the maximum temperature achieved (highest microbial activity). Also, the obtained average COC of this fermentation was 243.9±19.7 mgO_2 g^{-1}DM, representing a variation coefficient of 8%.

Enzymatic activity production

In Figure 4.4a is presented the full profile of cellulase activity and Figure 4.4b presents the reducing sugars and glucose profiles during the fermentations. All values are expressed as the average value of the two pilot reactors (R1 and R2).

FPase activity presented a continuous increase during the fermentation, peaking at 5.3±0.3 FPU g^{-1}DM during maximum sOUR period. Afterwards, the enzymatic production decreased until values below 2 units of activity at the end of the fermentation. The slight disassociation of maximum enzymatic activity with sOUR maximum can be attributed to a small differences among the reactors performance.

CMCase activity profile is slightly different than the obtained for FPase, the maximum activity was found at early stages of the process (at day 5 of fermentation) with a value of 3.531±0.003 U g^{-1}DM. When thermophilic stage started, CMCase action increased. It seems reasonable to hypothesized that endocellulase production started earlier than FPase production, due to its role on depolymerization of the cellulose structure and the lack of readily metabolizable sugars.

According to the results obtained at lab scale with OP as substrate, BGase activity was expected to have a significant production and an accumulation in glucose content. Despite these findings, as seen in Figure 4.4, BGase presented a small peak of production during the moment of maximum biological

activity; however the magnitude of the obtained BGase activity surpassed the lab and bench scale experiments, with a value of 0.48±0.07 U g^{-1}DM. In terms of reducing sugars and glucose profile, both reactors presented similar trends. A rapid reduction of both substrates was observed, which by day 7 of operation were almost zero in both reactors. This behavior was practically identical as obtained in 10L reactor.

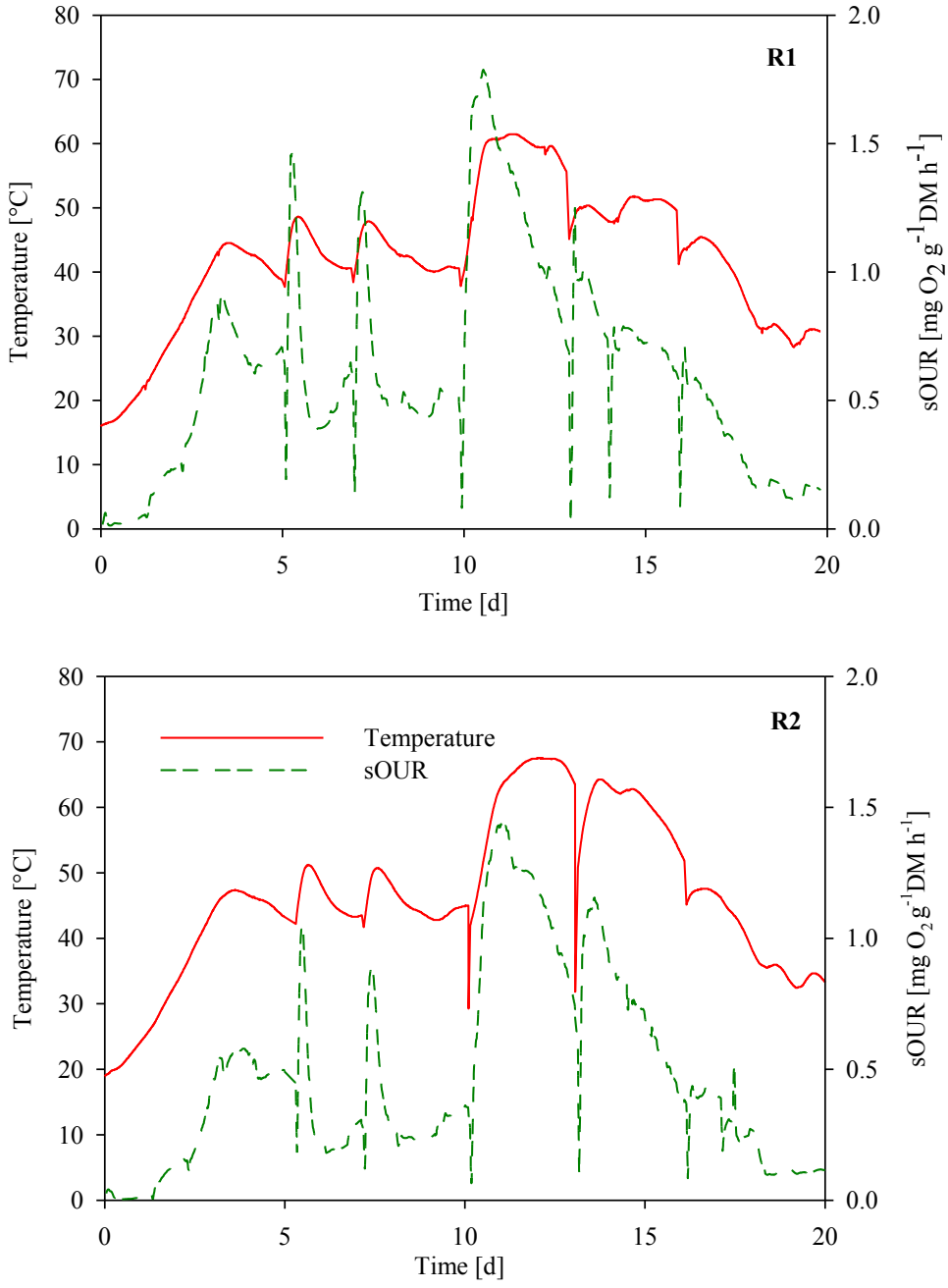

Figure 4.3. Temperature and sOUR profiles for 50-L reactors R1 and R2, using orange peels as substrate and compost as the inoculum.

It has to be remarked the fact that even when working with large amounts of solid wastes in a pilot scale and in duplicates, the obtained results are satisfactory in terms of the variation coefficient, due to the high intrinsic variability of the process itself.

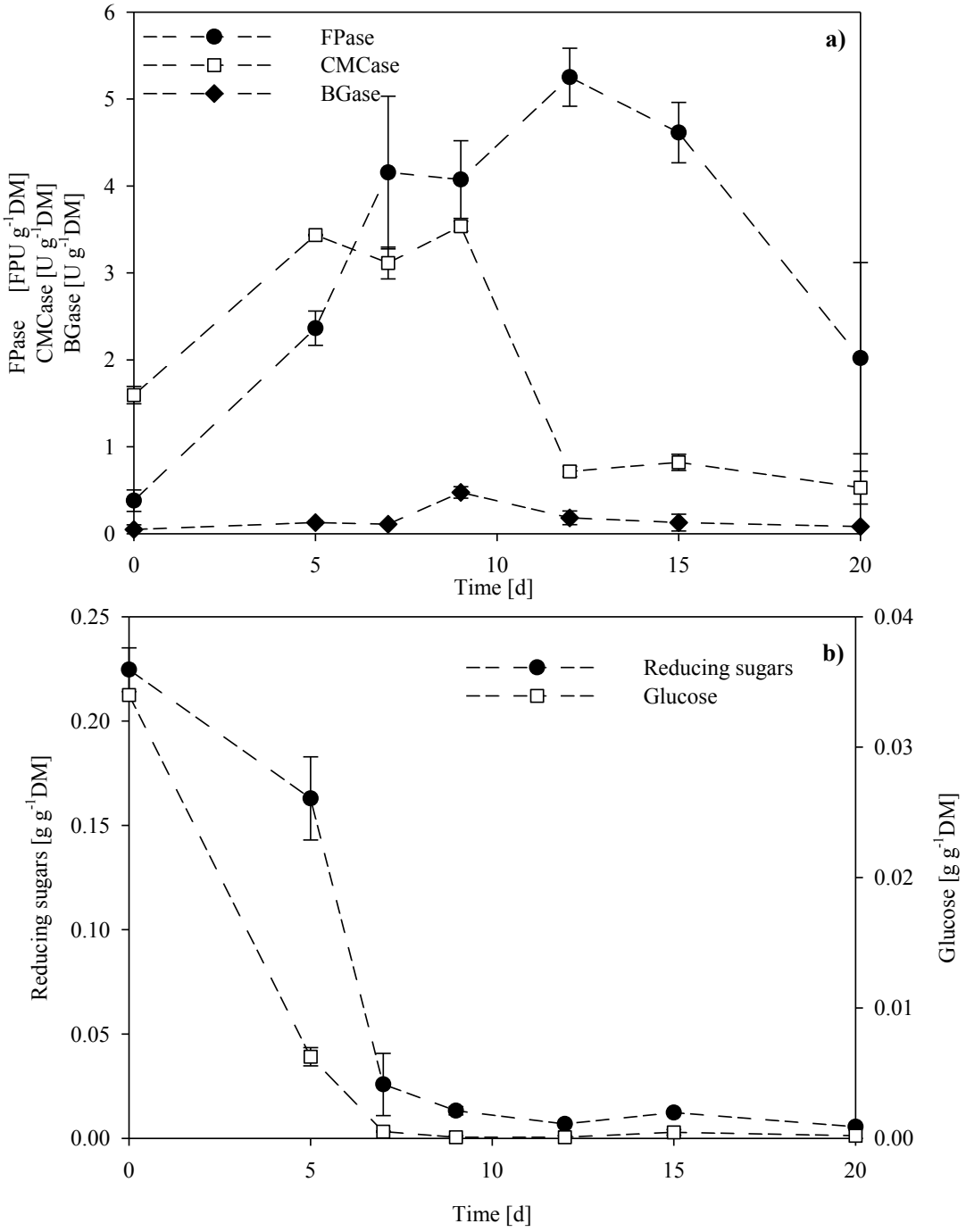

Figure 4.4. a) FPase, CMCase and BGase profiles and b) Reducing sugars and glucose profiles. Values obtained as an average between the two 50-L reactors R1 and R2, using orange peels as substrate and compost as the inoculum.

Table 4.5 shows a summary of the enzymatic production obtained in the experiments at the three different scales. After a one-way ANOVA analysis it can be observed that there are significant differences in the results obtained. It appears that when the SSF is performed in reactors of 10-L the enzymatic production is higher than in other scales. It was expected that in the SSF performed in 0.5 L reactors the cellulase production was lower than at higher scales due to it was performed at fixed temperature and using small amounts of substrate.

Table 4.5. Summary of the maximum filter paper activity (FPase) obtained in the three set of experiments carried out using range peels as substrate. Values of each enzymatic activity that do not share a letter are significantly different according to a one-way ANOVA analysis.

Experiments OP	FPase (FPU g^{-1} DM)
Reactor 0.5 L	1.6±0.5 (A)
Reactor 10 L	16.8±0.7 (B)
Reactor 50 L	5.3±0.3 (C)

It is possible that when working at the highest scale, some compacting issues appeared due to fungal growth (Godoy et al., 2014) which could have led to a decrease in enzymatic production and the generation of anaerobic/anoxic zones in the solid matrix. These contacting issues could have triggered proliferation of anaerobic microorganisms and thus promoting the gaseous emissions of pollutants such as methane of nitrous oxide. This point will be assessed in the following section.

Gaseous emissions

During orange peels (OP) solid state fermentation processes, measurements were made to determine gaseous emissions. Volatile organic compounds (VOC), methane (CH_4,), nitrous oxide (N_2O) and ammonia (NH_3) were monitored. Table 4.6 presents the emission factors obtained from solid state fermentation of orange peels. Also, the composition at family level of VOCs was analyzed and the results are presented in Table 4.7. The emission factors and VOCs percentages presented the same order of magnitude in both reactors

Table 4.6. Emission factors obtained in 50-L pilot SSF using orange peels (OP) as substrate.

	Kg VOC Mg^{-1}OP	Kg CH_4 Mg^{-1}OP	Kg N_2O Mg^{-1}OP
R1	21.6	0.0120	0.0024
R2	13.4	0.0164	0.0066
Average±s.d	18±6	0.014±0.003	0.002±0.001

Table 4.7. Percentages of different VOCs families emitted during orange peels (OP) solid state fermentation in 50L reactors.

VOC family	**R1 (%)**	**R2(%)**	**Average ±s.d**
Furans	0.1	0.2	0.2±0.1
Ester	0.9	2.9	1.9±1.4
Volatile Fatty Acids	0.3	1.2	0.8±0.6
Alcohols	4.0	6.1	5.1±1.5
Alyphatic hydrocarbons	0.2	1.2	0.7±0.7
Aldehydes	0.1	0.6	0.4±0.4
Ketones	1.2	2.0	1.6±0.6
Nitrogen containing compounds	0	0.1	$0.1^{0.1}$
Aromatic hydrocarbons	0.4	2.4	$1.4^{1.4}$
Terpenes	92.8	83.3	88.1±6.7
Sulphur containing compounds	0	0	0

Maximum methane production was obtained during thermophilic stage, during day 10 and 13 of operation coinciding with sOUR maximum (Figure 4.5). Also, at the beginning of the fermentation (at day 5), a small methane peak was observed. This can be due to the low initial pH in the reactors or to a low air flow supply, which could have led to the generation of anaerobic zones. No ammonia was emitted during this experiment, this was probably due to the low nitrogen content in the initial mixture (1.4%).

Nitrous oxide and VOCs also peaked during highest biological activity, in the thermophilic stage. The main N_2O emissions released during the process was found during the first week, as has been previously reported for the aerobic degradation processes of other wastes (El Kader et al., 2007). However, other authors related the inhibition of N_2O production with thermophilic temperatures (Fukumoto et al., 2003). In Figure 4.3 it can be observed that thermophilic temperatures were achieved after the 10^{th} of process pointing that N_2O was emitted even at thermophilic temperatures. N_2O emission factors present the lowest values among the analyzed contaminants, as found in similar biological processes (Maulini- Duran et al., 2013). In spite of that, from an environmental impact point of view the contribution of N_2O to global warming is significantly higher than CO_2 or CH_4. Additionally, it should be highlighted that N_2O presents the highest deviation obtained between emission factors calculated for the two replicates. Along with methane emissions, VOCs also peaked during the first days of operation. Probably, initial airflow supply generated stripping of the volatile compounds across the solid matrix.

Regarding VOCs composition, all families emitted during the process are presented in Table 4.7. Terpenes were the most emitted family of VOCs during the entire process, near 90% in both reactors. The main compound emitted resulted to be limonene, which has been reported as antimicrobial (Martin, 2010). The latter could have influenced in the biological activity during early stages of the process and created a "lag phase" until day 5 of operation of the reactors. After that, an increase of air flow supply took place, which could stimulate limonene releasing by stripping. Total emissions for these compounds are presented in Table 4.8

Table 4.8. Total emission of selected individual volatile organic compounds

Reactors	α- pinene Kg Mg^{-1}OP	β- pinene Kg Mg^{-1}OP	Limonene Kg Mg^{-1}OP
R1	0.017	0.029	0.43
R2	0.015	0.029	0.34
Average±s.d	0.016±0.001	0.029	0.39±0.06

Another important VOC released were α- and β-pinene, which are reported to be associated with emissions generated of woody materials (Maulini-Duran et al., 2014). These compounds are also reported to be related with the bulking agent selection (Büyüksönmez & Evans, 2007).

4.2.3 SSF trials using coffee husk as substrate

a) Operation in 10-L reactor

Process

Figure 4.5 shows the profile of the process evolution of the solid state-fermentation of the coffee husk using compost as the inoculum. It can be seen that the temperature profile followed a typical composting pattern (Haug, 1993). After a brief lag phase, temperature rapidly began to increase up to 65°C, which can be attributed to the abundant and active indigenous microorganisms in the raw materials. Thermophilic conditions were maintained for about one day and a half; after that, temperature decreased to mesophilic values until the end of the experiment. Also, secondary temperature increases were observed after homogenizing the reactor contents when sampling at days 3, 7, 10, 13 and 16.

During the thermophilic phase, it was observed a decrease of oxygen in the reactor until near 0% (data not shown), which could have produced a loss of microbial activity, however after an adjustment in the airflow, the oxygen content began to rise and the thermophilic conditions were restored, which ensured metabolic activity (García et al., 2012). Also, it is important to mention that from the thermophilic phase until the end of the operation of the reactor, pH was found around 9, which has been found to be normal for lignocellulosic materials (García et al., 2012). Finally, maximum sOUR obtained was 3.0 $mgO_2\ g^{-1}DM\ h^{-1}$ with a total COC of 1305 $mgO_2\ g^{-1}DM$ in 25 days of operation.

Enzymatic activity production

Figure 4.5 also shows the cellulase production profile along the SSF process. Filter paper activity reached a maximum of 8.6 ±0.6 FPU $g^{-1}DM$ at the third day of operation. Cellulase production using this system is in the reported range of cellulase production as seen in Table 1.3 section 1.5.1, although most of these research has been performed in sterile conditions, mesophilic temperatures (30-35°C) and variable moisture content (60-90%).

The highest cellulase activity was obtained at day 3 of fermentation, during the thermophilic phase as reported in previous researches (Huang et al., 2010). This would be logical considering that is during this stage that higher metabolic activity takes place (Barrena Gómez et al., 2005; Huang et al., 2010). There are few researches on thermostable cellulases and most of them are related to modified microorganisms at controlled and optimized operational conditions (Liu et al., 2011). Cellulase activities obtained for thermostable enzymes ranged between 40-100 FPU $g^{-1}DM$ (Liu et al., 2011).

A decrease of cellulase production was observed after the third day peak, which is probably due to the irreversible adsorption of the enzyme onto cellulose or lignin or even to the depletion of nutrients

(Gusakov & Sinitsyn, 1992; Olofsson et al., 2008; Xiao et al., 2004). With the aim to achieve higher amount of cellulase different alternatives have been evaluated to enhance cellulase production such as alkali or acid pretreatment or inductors addition (Bansal et al., 2012; Dhillon et al., 2012a).

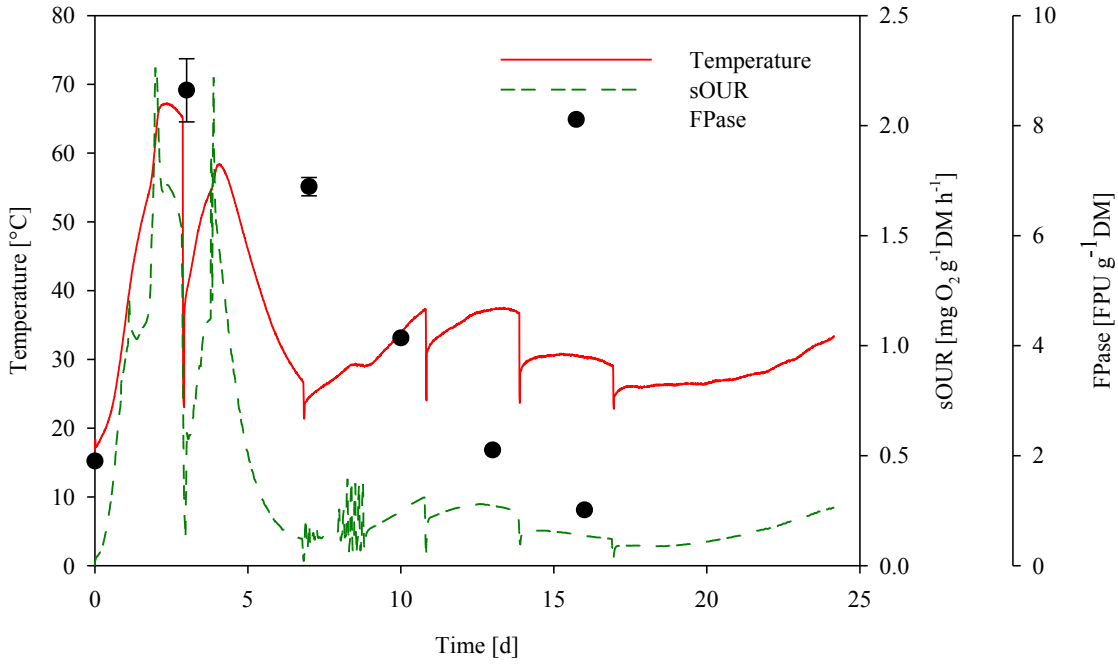

Figure 4.4. Filter paper activity (FPase), temperature and sOUR profiles for 10-L reactors using coffee husk as substrate and compost as the inoculum.

b) Operation in 50-L reactor

Process

Initial characterization of the raw materials and initial mixture for the experiments in 50L reactors of coffee husk are presented in Table 4.9.

In Figure 4.5 temperature and sOUR profiles are presented for the two pilot SSF using coffee husk as substrate (R1 and R2). During the development of these experiments, an aeration problem arose in R2, which lead to an irregular air flow and malfunction of the airflow controller. Is it for this reason that sOUR profile in R2 (Figure 4.5) presents a long period of noise since day 2 of operation. In spite of these operational difficulties, both fermentations showed a clearly similar trend in both monitored parameters.

Table 4.9. Characterization of raw materials and initial mixture of 50L CH reactor.

Parameter	CH	Compost 50L	CH 50L
Moisture (%)	59.8	36.9	59.5
Dry Matter (%)	40.1	63.1	40.5
Organic matter (%)	90.2		90.0
Bulk Density (g/L)	238.1		358.0
Air filled porosity (%)	78.9		77.2
C/N ratio	22.8		13.5
pH	6.3	7.5	7.2

Maximum temperature was achieved in day 2 of operation, reaching values of 66.5 and 75.3 in R1 and R2 respectively. Fully thermophilic stage lasted nearly 3 days in both reactors, after that, mesophilic conditions were reached until the end of the fermentation. It is possible that temperatures as high as the obtained in R2 could negatively affect the process, in terms of reducing the microbial diversity and activity which will directly affect the enzymatic activity production (Arora et al., 2015; Ekinci et al., 2004).

Regarding sOUR, maximum values were obtained also during thermophilic stage, in day 2 of operation reaching values of 2.5 and 3.3 mg O_2 g^{-1}DM $h^{-1}$for R1 and R2 respectively. Similarly to the obtained in the pilot experiments with orange peels, the average results did not present a big dispersion. In this sense, the average sOUR of the process is 2.9 ±0.6 mg O_2 g^{-1}DM h^{-1} with a variation coefficient of 20%. Additionally, the average cumulative oxygen consumption between R1 and R2 was 171± 26 mg O_2 g^{-1}DM, which a variation coefficient of 15%.

During this fermentation C/N ratio was also monitored. The initial C/N value was 13.5, which is a low value in comparison with another substrates and far from the recommended 25 (Jiang et al., 2011). The entire process achieved a total C/N ratio reduction of 13.5% in 15 days of operation. This reduction may seem small, however, authors working in co composting with coffee husk obtained nearly 14.2% reduction in 32 days of operation and with an initial C/N ratio of 17.5 (Shemekite et al., 2014b). Low C/N ratio in the present fermentations, are mainly due to unusually low carbon content in the initial mixture. C content was roughly 40% . This is not comparable with obtained 64% in previous fermentations (C/N ratio:24.8) (Abraham et al., 2013). The intrinsic variability of coffee

husk and compost used in these fermentations and of course a lack of homogeneity in the sampling could have affected these measurements.

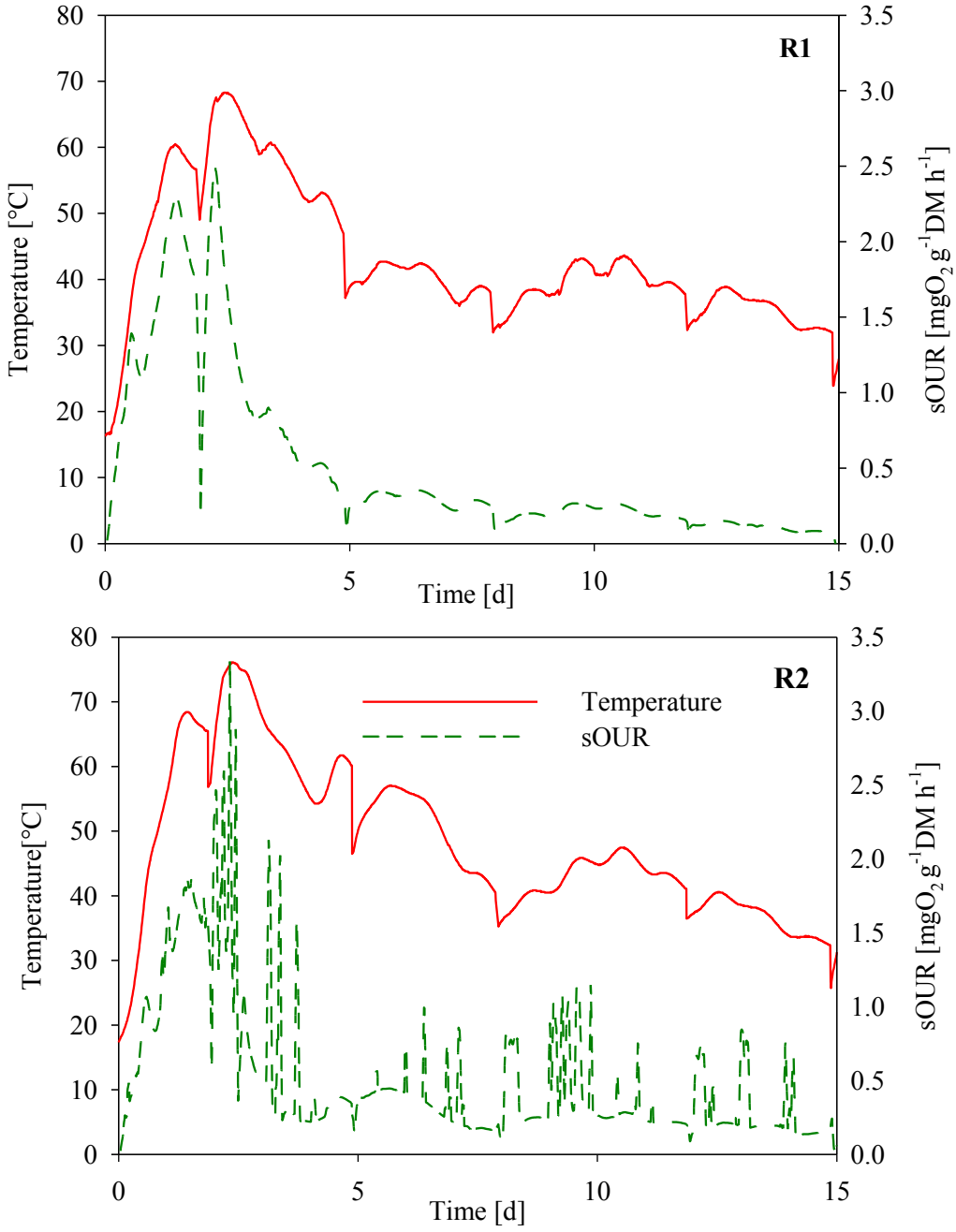

Figure 4.5. Temperature and sOUR profiles for 50-L reactors R1 and R2, using coffee husk as substrate and compost as the inoculum

Enzymatic activity production

In Figure 4.6a shows the average cellulases production profile obtained in the 50 L SSF. Also Figure 4.6b presents the reducing sugars and glucose content during the fermentation in the reactor.

Enzymatic profile of the group of cellulases show clearly a maximum during the time of maximum sOUR and temperature, which is in accordance to the previous experiments carried out in 10 L and 0.5 L reactors. Regarding FPase activity, it reached average values of nearly 9.5±0.7 FPU g^{-1}DM. These values are similar to the obtained in the 10 L SSF which can, in a way, imply that the scale factor is not of great importance when working under the specified conditions ans usinf this specific substrate. In Table 4.10 is summarized the cellulase production at the three different scales. The ANOVA analysis confirms that there is no significant differences between the results obtained at 10 or 50 L.

Table 4.10. Summary of the maximum filter paper activity (FPase) obtained in the three set of experiments carried out using coffee husk as substrate. Values of each enzymatic activity that do not share a letter are significantly different according to a one-way ANOVA analysis.

Experiments CH	FPase (FPU g^{-1} DM)
Reactor 0.5 L	6.4±0.7 (A)
Reactor 10 L	8.6 ±0.6 (B)
Reactor 50 L	9.5±0.7 (B)

The lack of an scale impact on enzymatic production is in accordance to the obtained by Astolfi et al., (2011). They assessed the scale impact on enzymatic production using on one hand 5g and on the other hand, 2 Kg of substrate, resulting in statistically similar activity values.

As mentioned before, several differences in enzymatic production among the replicates could have been generated due to aeration difficulties, specifically due to air supply fluctuations and the generation of anaerobic/anoxic conditions (Rodriguez-Fernandez et al., 2012). In the same context, Rodriguez- Fernandez et al., (2012) has also stated that proper airflow supply is a key factor when a scale-up is considered. In spite of that, the enzymatic production in the replicates are very similar. As for FPase, the variation coefficient obtained as between nearly 4 and 40%, where the maximum dispersion was found at the moment of maximum fluctuation in airflow which could have lead to the mentioned differences. However, it is important to mention that obtained FPase values at pilot-scale

are found in the upper bound of the reported activities (Table 1.3) obtained in sterile conditions, mesophilic temperatures (30-35°C) and variable moisture content (60-90%).

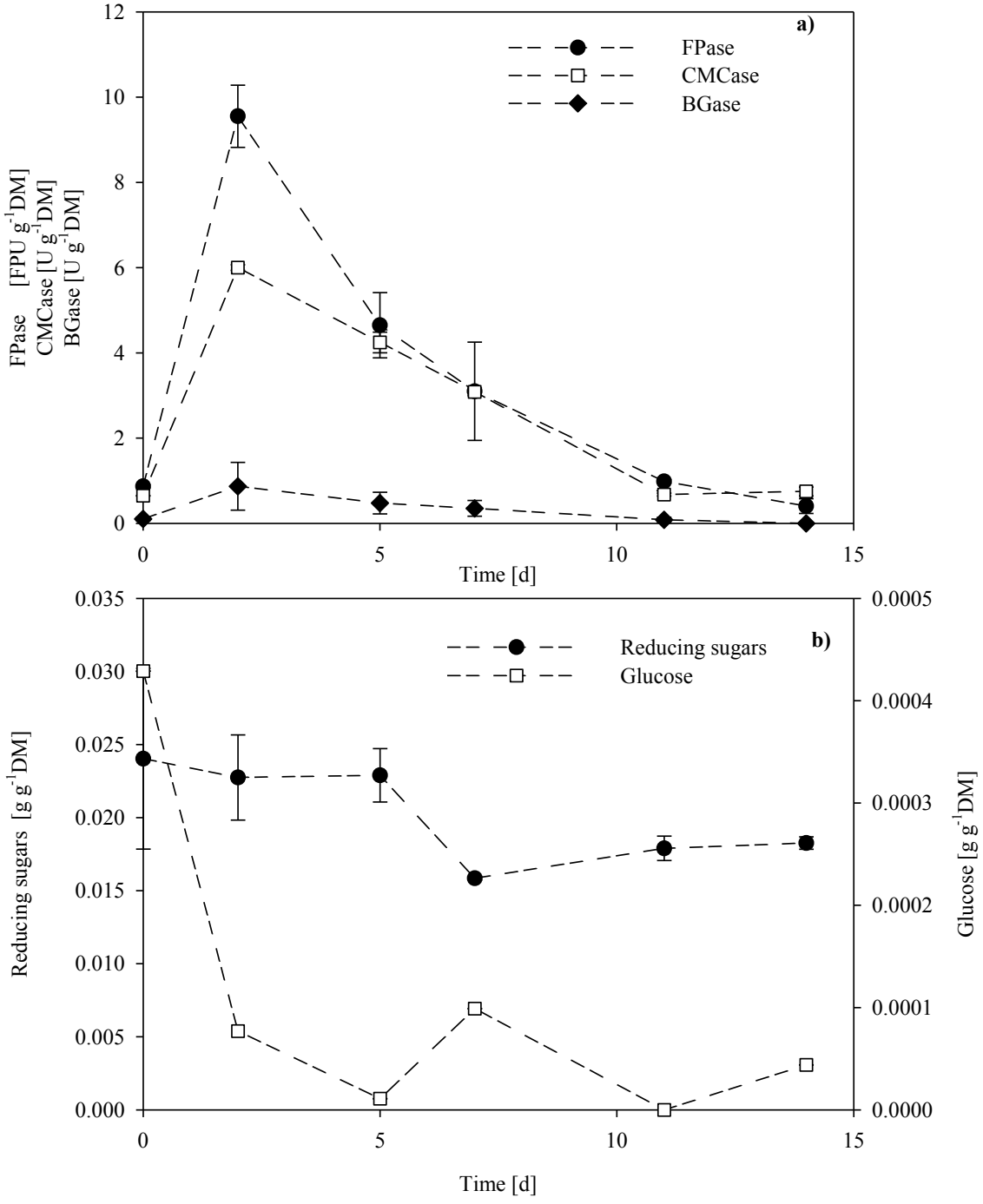

Figure 4.6. a) FPase, CMCase and BGase profiles and b) Reducing sugars and glucose profiles. Values obtained as an average between the two 50-L reactors R1 and R2, using coffee husk as substrate and compost as the inoculum.

BGase production profile is the process where major differences were founds in the replicates. The coefficients of variation in all samplings were above 40%, which suppose a high dispersion of the production between the processes. In addition to the above mentioned airflow difficulties, it is possible that the differences could have been due to performance of the reactors or even the sampling for enzymatic measurements were lacking of homogeneity. Another main factor to consider in cellulase activity production is temperature. Cellulases optimum temperature ranges between 50-55°C, for cellulase of fungal origin. High temperatures obtained in R2 could influence BGase activity, in comparison with R1.

Also it is important to consider the microorganisms who are able to produce cellulases and that are present in both reactors. Some authors have reported that even knowing that bacteria and fungi are able to use cellulose as primary carbon source, bacteria are incapable of degrading crystalline cellulose since their cellulase systems are incomplete. This is a different situation for fungi, that are able to degrade completely this structure (Brijwani et al., 2011).

In this context, it also has been reported by Shatemike et al., (2014) that fungi are not able to thrive under rough environmental conditions such as the early stage of this SSF or even in a composting process, it is for this reason that fungi appears at latter stage of this process and cellulases activity appears at day 32 of composting. However other authors (Lever et al., 2010), has proven to increase cellulase activity and fungi growth in a SSF process by using fermented solids as inoculum. The latter, supports the fact that using compost as an initial mixed inoculum provides different microorganisms (among them fungi and yeasts) that are able to quickly colonize the reactor. In addition, coffee husk provides an ideal environment for fungi to thrive

Figure 4.8b shows the average reducing sugars and glucose profile of the pilot SSF. The glucose profile, shows a rapid consumption at day 2 of operation which left almost negligible amounts of glucose detected in the reactor at day 5. During this stage the reducing sugar content remained almost unchanged until day 7 of fermentation where reducing sugars started to be consumed and an slight glucose accumulation was observed. In contrast of the findings on the OP experiments, the reducing sugars content did not disappeared of the solid matrix, but only decreased in a 33% . This can be a reflection of CMCase or FPase action which could have release hydrolyzates of cellulose (or hemicellulose) with reducing ends exposed to the media.

Gaseous emissions

As it is possible to observe, all emissions presented maximum production during thermophilic stage. In the case of VOCs trends resulted similar in both reactors and obtained similar emission factors, as shown in Table 4.11, with values of 0.14 and 0.12 Kg Mg^{-1}CH.

Nitrous acid production, presented high production at the first days of fermentation, during thermophilic stage; after that, nitrous acid was not significantly produced. It has been reported that a temperature, nitrogen content and aeration rates influences nitrous oxide emissions and found to be produced mainly during early stages of composting processes (He et al., 2001). Also alkaline pH values enhances nitrous acid production, and considering that since day 2 of operation pH ranges between 8.5-9.2, it was expected an increase of nitrous acid. In this case in particular, emission factor obtained was 0,04 and 0,017 Kg Mg^{-1}CH for R1 and R2. It is possible that fluctuations in aeration rates in R1, could have influenced higher emissions in all evaluated compounds.

On the other hand, methane production presented different trends in both reactors. R1, presented a fast methane peak during thermophilic stage, however after that, no significant methane production was observed, even when aeration rate was low (400 mL min^{-1}) during the last stage of the fermentation. In R2, a higher methane production was achieved. At the beginning of the fermentation high rate of methane production was observed until day 5 of operation. However, instead of stop producing methane as in R1, it kept increasing, although at lower rate. Emission factors in R1 and R2 were 0.0007 and 0.0019 Kg Mg^{-1}CH respectively.

It is possible that, considering methane emissions, anaerobic or anoxic zones could have been generated in R2. This could be confirmed by low oxygen level in the reactor during thermophilic stage, lower oxygen consumption and maybe for insufficient aeration during this period.

Regarding VOCs composition, all families emitted during the process are presented in Table 4.12.

Table 4.11. Emission factors obtained in coffee husk (CH) solid state fermentation in 50L reactors.

Reactors	VOC Kg Mg^{-1} CH	CH_4 Kg Mg^{-1} CH	N_2O Kg Mg^{-1} CH
R1	0.14	0.0007	0.04
R2	0.12	0.0019	0.02
Average	0.13±0.014	0.0013±0.0008	0.02±0.02

As in orange peel experiments, terpenes are the main family emitted during coffee husk solid state fermentation, with 61.6 and 66.6% in R1 and R2. This can be expected due to coffee husk composition includes terpenes as a main responsible for flavour and aromatic in this residue (Ray & Ward, 2008). In the first stages of the fermentation, a variety of nearly 10 different terpenes were

emitted, however after thermophilic stage three compounds were mainly produced: limonene, camphene (an isomeric form of limonene) and α-β- pinene. In Table 4.13 total emission of selected compounds are presented. All these components are related to flavour properties in coffee, however, pinene forms can be also related to bulking agent and its woody origin.

Second most important VOC emitted were ketones, with an average of 15.8% in both reactors. This was expected, considering coffee husk volatile fraction composition, which includes nearly 20% of ketones (van Dam & Harmsen, 2010).

Table 4.12. Percentages of different VOCs families emitted in coffee husk (CH) solid state fermentation in 50L reactors.

VOC family	R1 (%)	R2(%)	Average
Furans	0.52	0.16	0.34±0.25
Ester	0.00	0.00	0.0±0.0
Volatile Fatty Acids	4.21	0.00	2.10±2.98
Alcohols	0.74	2.55	1.65±1.28
Alyphatic hydrocarbons	5.69	7.28	6.49±1.12
Aldehydes	4.85	0.00	2.43±3.43
Ketones	15.70	15.95	15.28±0.18
Nitrogen containing compounds	1.44	2.00	1.72±0.39
Aromatic hydrocarbons	5.26	4.95	5.10±0.22
Terpenes	61.6	66.6	64.1±3.54
Sulphur containing compounds	0.01	0.00	0.005±0.007
Halogenated compounds	0.00	0.51	0.26±0.36

Table 4.13. Total emission of selected individual volatile organic compounds.

Reactors	α- pinene Kg Mg^{-1}CH	β- pinene Kg Mg^{-1}CH	Limonene Kg Mg^{-1}CH
R1	0.0016	0.0002	0.0011
R2	0.0010	0.0001	0.0011
Average	0.0013±0.0004	0.0002±0.0001	0.0011

4.3 Conclusion

It was possible to determine at lab scale that orange peels and coffee husk were the most promising raw materials to use as substrate for solid state fermentation for cellulase production. When the scale up was assessed in a 10 L reactor, a decrease of nearly 50% was observed in cellulase production. Finally when the pilot scale experiments were performed, the enzymatic production in comparison with the 10 L SSF was sustained, achieving a maximum value of 5.3±0.3 and 9.5±0.7 FPU g^{-1}DM for orange peels and coffee husk, respectively. The emission factors of both orange peels and coffee husk are significantly lower than the observed in similar processes. In both pilot fermentation the main emitted gases were the VOCs which is probably due to the high carbonaceous material and phenolic compounds present in both raw materials. Among all VOCs, the terpenes were the family with the highest emission factor, followed by α and β- pinene, which are compounds related to lignocellulosic material degradation. The success of both fermentations, especially using coffee husk as the substrate in terms of enzymatic production and environmental impact makes this substrate selected for further research and optimization.

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CHAPTER 5. TOWARDS A COMPETITIVE SOLID-STATE FERMENTATION FOR ENZYME PRODUCTION

Part of this chapter has been submitted for publication in Science of the Total Environment. "Towards a competitive solid state fermentation: cellulases production from coffee husk by sequential batch operation and role of microbial diversity".

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Summary

In this chapter are presented operational strategies for the development of a continuous SSF for cellulase production. As mentioned in Chapter 1, one of the main constrains of SSF is the difficulty to work in a continuous configuration. In this sense, in section 5.1 is presented the development of a sequential batch operation for cellulase using different substrate exchange ratios.

5.1 Towards a competitive solid state fermentation: cellulases production from coffee husk by sequential batch operation and role of microbial diversity.

5.1.1 Materials

Coffee husk (CH) was kindly provided by Marcilla S.A (Mollet del Vallés, Barcelona, Spain) and compost (C, from source-selected organic fraction of municipal solid waste) was obtained from the municipal solid waste treatment plant Ecoparc de Montcada (Montcada, Barcelona, Spain) as explained in chapter 3 section 3.1.1. All materials were stored frozen (-18ºC) until use. The full characterization of both raw materials and the initial and final composition of reactors is presented in Table 5.1.

5.1.2 Experimental procedure

a) Selection of inoculum size

Preliminary SSF experiments were performed in order to assess the inoculum size and the time for maximum cellulase production. Non sterile coffee husk was mixed with compost as inoculum in 0, 10, 20, 50 and 100% (w/w) ratio. Wood chips were added in a 1:1 (v/v) ratio as bulking agent. Fermentation was carried out using the system described in chapter 3 section 3.2.1 SSF were performed in triplicates for 4 days at 37ºC using 90 g of the mixture CH and C using continuous aeration of 20 mL min^{-1} and oxygen monitoring.

b) Sequential batch operation for cellulase production

Fermentations were performed in 4.5L air-tight packed-bed reactors (adapted Dewar® vessels), thermally isolated to work under near-to-adiabatic conditions (Dewar® vessels provide excellent thermal isolation, minor heat exchange with the surroundings take place through the airflow (in and out) and partially through the lid). Experimental set-up is presented in Chapter 3, section 3.2.2 (Figure 3.3).

A self-made acquisition and control system was used based on Arduino® and self-made software. Air was continuously supplied to the reactors by means of a mass flow controller (Bronkhorst, Spain). Airflow was automatically adjusted by a feedback controller (aeration range 15.8 – 31.6 L kg^{-1} DM h^{-1}, set point 11.5% oxygen in exhaust gas) as described in chapter 3 section 3.4.1. Airflow, temperature and oxygen content were continuously monitored.

The initial mixture for the first batch in both strategies contained 90% (wb) of fresh coffee husk as substrate and 10%(wb) of compost as mixed inoculum. Compost was added in order to provide active biomass and mainly for the potential incorporation of diverse communities able to degrade lignocellulosic materials, as reported in recent studies (Lopez-Gonzalez et al., 2014). Wood chips were added as bulking agent in a ratio of 1:1 (v/v) in order to provide enough porosity to promote proper oxygen transfer (Ruggieri et al., 2009). Fermentations were performed with a total weight of 1.2 kg per batch.

Table 5.1. Characterization of coffee husk (CH), compost (C), initial mixtures and final fermented solids for SB50 and SB90. wb: wet basis; db:drybasis

Parameter	Coffee Husk	Compost	SB50 initial	SB90 initial	SB50 Final	SB90 final
Moisture (% wb)	60.8 ± 0.4	34.9 ± 0.3	61.2 ±1.1	62.0 ± 1.3	55.0 ± 1.1	55.0 ± 3.1
pH	6.51 ± 0.01	7.22 ± 0.01	6.45 ± 0.01	6.43 ± 0.01	9.11 ± 0.01	9.07 ± 0.01
Cellulose (%, db)	25.7 ± 0.2	9.8 ± 1.2	23.4 ± 0.8	27.9 ± 0.4	28.6 ± 0.5	23.5 ± 0.4
Hemicellulose (%,db)	14.6 ± 0.1	10.2 ± 0.1	13.2 ± 0.5	13.3 ± 0.3	13.2 ± 0.5	13.3 ± 0.5
Lignin (%, db)	17.6 ± 0.5	13.6 ± 1.2	20.4 ± 0.6	21.8 ± 0.6	16.5 ± 0.7	18.3 ± 0.6

Maximum cellulases activity was achieved in 48h and thus the solid retention time (SRT) was established in 48h. Sequential batch operation was performed in two different configurations:

SB90: 90% of the wet fermented solids were removed from the reactor at the end of the fermentation (every 48h) and used as product for final analysis. The remaining 10% of the fermented solids acted as inoculum to start a new batch, using 90% fresh coffee husk. Six sequential batches (12 days) were performed to allow the microbial community to develop and to assess the reproducibility of the cellulases production.

SB50: 50% of the wet fermented solids were removed from the reactor every 24h and used as product for further analysis. In this case, the remaining 50% of the fermented solid was used as inoculum to start a new batch, with the addition of 50% of fresh coffee husk. SB50 operated for 18 days.

Both strategies performed until steady operation resulting in 12 days for SB90 and 18 days for SB50. During these processes, a continuous on-line monitoring of temperature and sOUR was carried out. Sampling was always performed after a complete manual homogenization of the fermented solids to obtain a full representative sample and prior to feeding the reactor with fresh substrate. From these solid samples, measurements of elemental composition (C,H,O and N), fiber content (cellulose, lignin and hemicellulose), microbial diversity, enzymatic activity and other routine methods (Chapter 3, section 3.3) were determined.

c) Cellulase production profile using specialized inoculum

The final fermented solids obtained at the end of both sequential batch operations SB90 and SB50 were used to inoculate fresh coffee husk in a 10% (w/w) ratio for a new single batch to analyze whether the selected biomass modified the process dynamics and the cellulase production profile. Experiments were performed in the 4.5L bioreactors with a total mass of 1.2 kg and samples were collected after complete homogenization of the reactors at 0, 24, 36, 40 and 60h.

5.1.3 Specific measurements for biodiversity

Identification of the microbial population was performed in the raw materials and in the final samples from both SB50 and SB90 operational strategies using next generation sequencing. The aim of this analysis was to determine the potential variation on biodiversity and to characterize the specialized biomass obtained during sequential batch operations.

Total DNA was extracted and purified using PowerSoil™ DNA Isolation Kit (MoBio Laboratories, USA) according to provider's specifications. DNA samples were checked for concentration and quality using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware; USA) (Lopez-Gonzalez et al., 2014).

Bacterial 16S rRNA hypervariable regions V3-V4 and Fungal ITS1-ITS3 were targeted. Later sequencing of the extracted DNA and bioinformatics were performed on MiSeq platform by Life Sequencing S.A (Valencia, Spain).

Shannon-Wiener (H´) and qualitative (IS) and quantitative (ISquant) Sorensen´s-Dice biodiversity indices were estimated according to the following equations:

$$H' = -\sum p_i log_2 p_i$$

where pi = ni/N, ni= relative abundance of the ith species and N =Σn, and

$$I_S = \frac{2c}{a+b} \quad ; \quad I_{Squant} = \frac{2pN}{aN+bN}$$

where a = number of species in sample 1, b = number of species in sample 2, c = number of species shared by the two samples, pN = sum of the lower of the two abundances recorded for species found in the two samples, aN = number of individuals in sample 1, bN = number of individuals in sample.

5.1.4 Results

a) Selection of inoculum size

Figure 5.1a shows the profiles of the cellulase activity obtained when using different inoculum sizes. Maximum cellulase activity was found in the first day of operation in all cases. It can be noticed that the same trend is observed in all experiments; a rapid increase in cellulase activity in day one followed by a gradual drop until the end of the fermentation. The maximum cellulase activity was statistically similar for samples with 10 and 20% added compost, reaching 7.0±0.3 and 7.5±0.5 FPU g^{-1} DM respectively. After day one, cellulase production decreased achieving values around 0.18 FPU g^{-1}DM by the end of the fermentation. Adding 50% compost dramatically reduced cellulases production until almost negligible values below values obtained with only CH or only C.

In previous studies, the maximum enzymatic activity of amylases or proteases was not related to the maximum biological activity measured as sOUR in similar systems (El-Bakry et al., 2016, Cerda et al., 2016). However, for cellulases the opposite pattern was observed. The maximum cellulase production was found on the peak of biological activity, i.e. during maximum sOUR for all the assessed inoculum ratios (data not shown). As an example, Figure 5.1b shows a full sOUR profile for the sample using 10% of compost as inoculum. In this Figure, it can be observed that the maximum cellulase activity was detected during the most active biooxidative stage of the process, as other authors have also stated (Jurado et al., 2014). Similar results were observed in batch experiments performed in 4.5 L bioreactors where a process time of 48h was fixed for maximum sOUR and cellulase production.

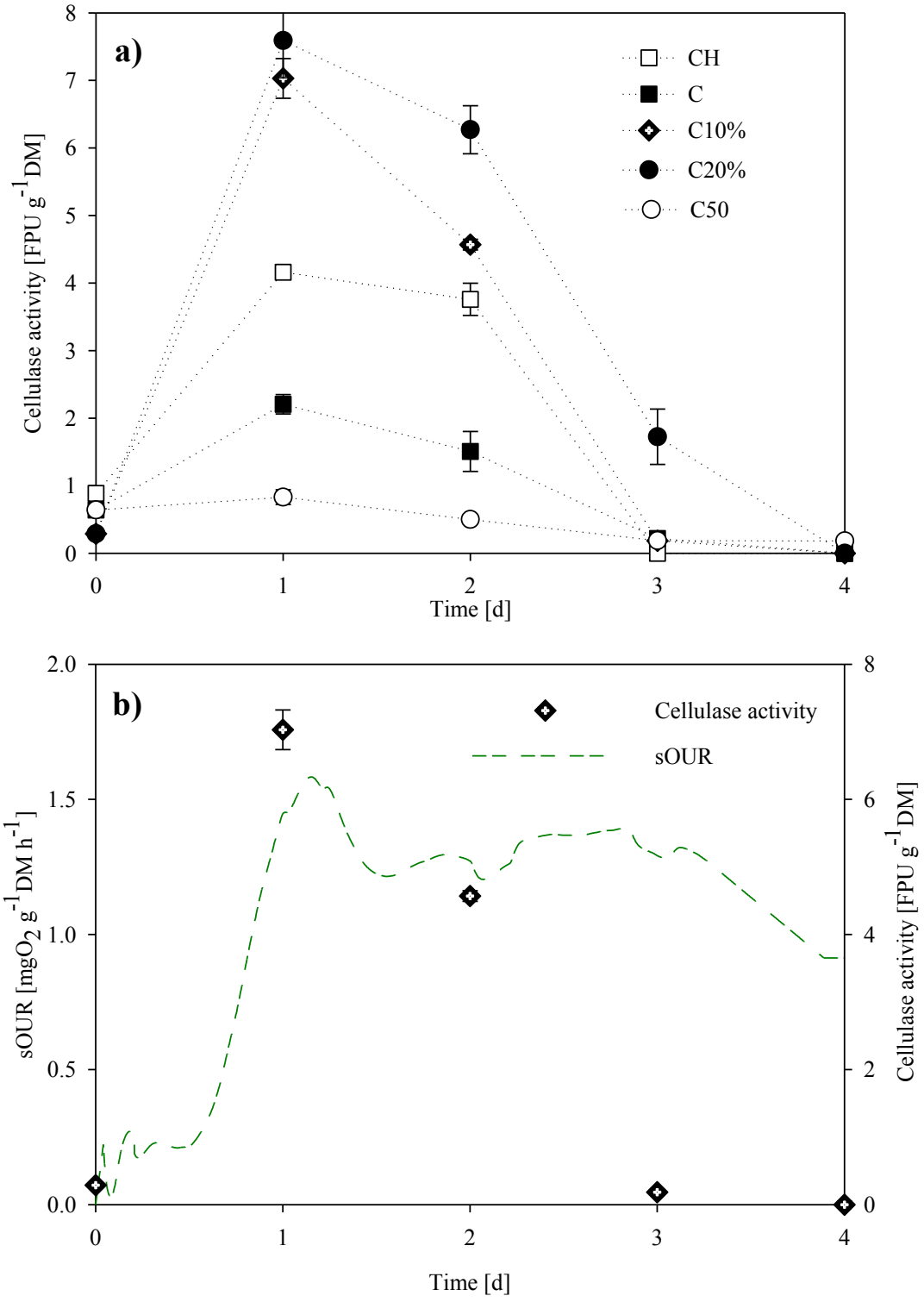

Figure 5.1. SSF of coffee husk using compost as inoculum at lab scale and 37ºC during 4 days. a) Profile of cellulase production using different inoculum sizes and b) profiles of sOUR and cellulase production using 10% of inoculum (CH: coffee husk; C:compost).

b) Sequential batch operation for cellulase production

The operation of bioreactors working under SB90 and SB50 conditions is presented in Figures 5.2 and 5.3 respectively. In both cases, an initial 2-day fermentation process was performed until the maximum sOUR was achieved adding compost in 10% ratio as a mixed inoculum. Final average sOUR and cellulases activity in these initial fermentations for both SB50 and SB90 strategies were 2.84±0.66 mgO_2 g^{-1}DM $h^{-1}$and 7.6± 3.0 FPU g^{-1}DM respectively. A similar temperature profile was obtained, with a maximum of 62ºC in both cases. The characterization of the initial and final fermented solids for both tested strategies is presented in Table 5.1.

Six sequential batches were performed (12 days of operation) for the SB90 strategy as presented in Figure 5.2. Operation was consistent and maximum sOUR and final cellulase activity remained stable among the different batches, with an average sOUR of 2.6 ±0.1 mgO_2 g^{-1}DM $h^{-1}$and 9.0±0.8 FPU g^{-1}DM. No statistical differences were observed for cellulase activity among the six batches. The average COC was 87 ±6 mgO_2 g^{-1}DM per batch (48h).

For SB50 strategy the fermentation was performed for a total process time of 18 days (Figure 5.3). After the first substrate change the sOUR achieved at the end of the batch (24h) was 68% lower although cellulase activity was statistically similar to that of the initial batch. After two batches, maximum sOUR performed consistently to an average value of 3.4 ± 0.2 mgO_2 g^{-1}DM h^{-1} (alterations in days 11 and 15 correspond to failures in the aeration system and the process quickly recovered from this). The average COC was 58 ± 6 mgO_2 g^{-1}DM per batch (24h). Contrary to the sOUR dynamics, cellulase production was not consistent. Cellulase activity of final extracts dropped gradually to 2.1 ± 1.0 FPU g^{-1}DM on day 10, to increase after day 13 to final values of 10 FPU g^{-1}DM on day 18, statistically similar to the yields obtained with the SB90 strategy.

Partial cellulose degradation of 3.1% and 3.2% was found for SB50 and SB90 in 24 and 48h respectively, which is in line with the values found in literature. Hemicellulose degradation of nearly 2%was observed in both SB50 and SB90. Lignin degradation was 2.5% and 0.8% for SB50 and SB90. These results are comparable to those reported by Umasaravanan et al. (2011) that observed only a 6% cellulose reduction and 3.1% lignin reduction in 21 days using sugarcane bagasse and rice straw. Other authors reported high cellulose degradation in SSF of lignocellulosic materials, for instance, 18% in 3 days using olive wastes (Salgado et al., 2015) or 48% degradation in 23 days using high-cellulose paper waste (Das et al., 1998).

Lack of standardized cellulase activity determination makes difficult to provide a proper comparison with reported results. Even more, the most common substrate used for cellulase activity, filter paper, generates great concerns on reproducibility and accuracy, especially in mediums with low β -

glucosidase production (Coward et al., 2003). However, cellulase activity obtained in this work is located in the range of reported researches, between 1-25 FPU g^{-1}DM for small bioreactors under sterile and mesophilic conditions (Behera et al., 2016).

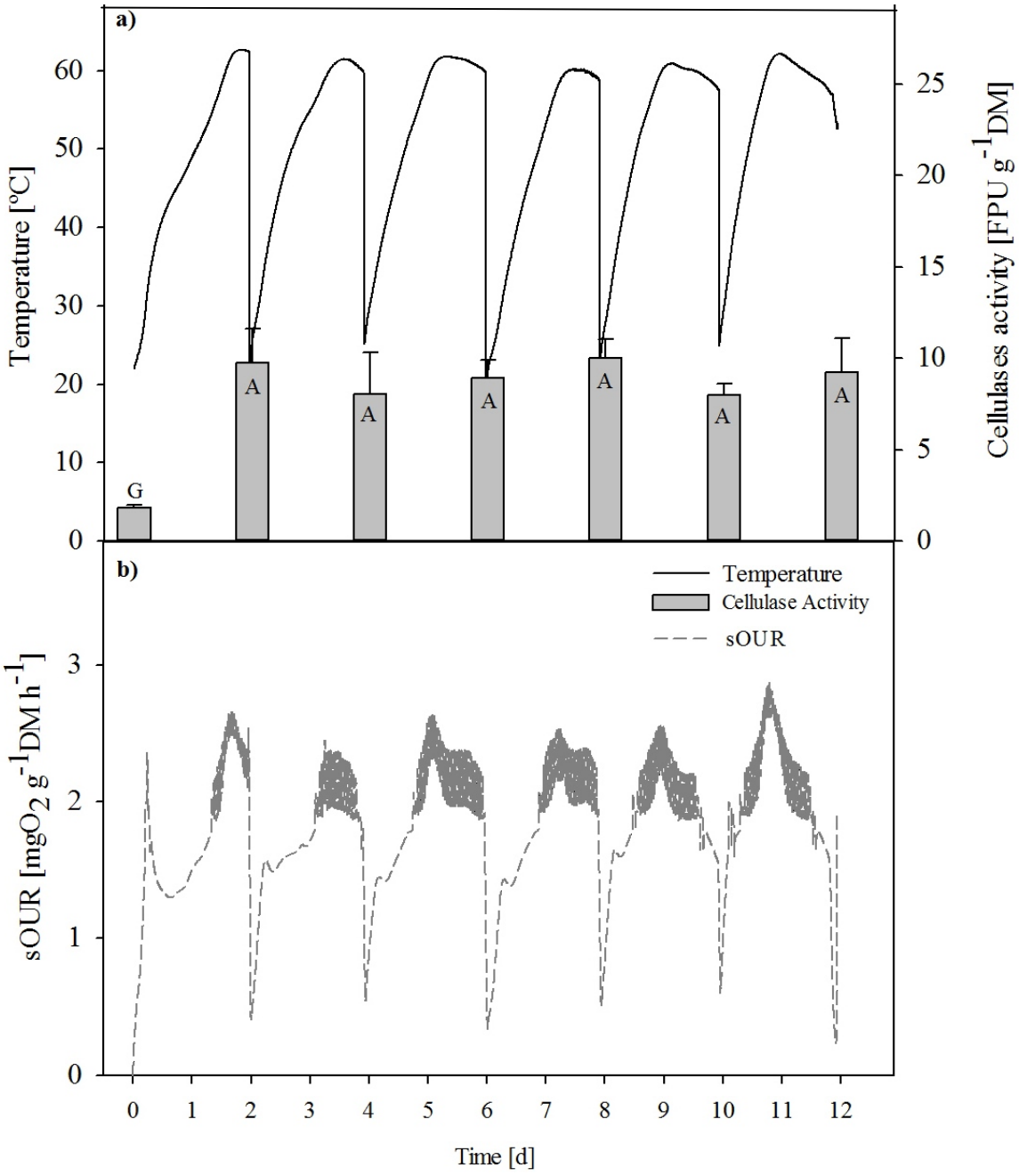

Figure 5.2.Process follow up of sequential batch operation SB90 of coffee husk for cellulase production. In a) temperature and cellulase activity and b) sOUR profiles are presented. Bars that do not share a letter are significantly different.

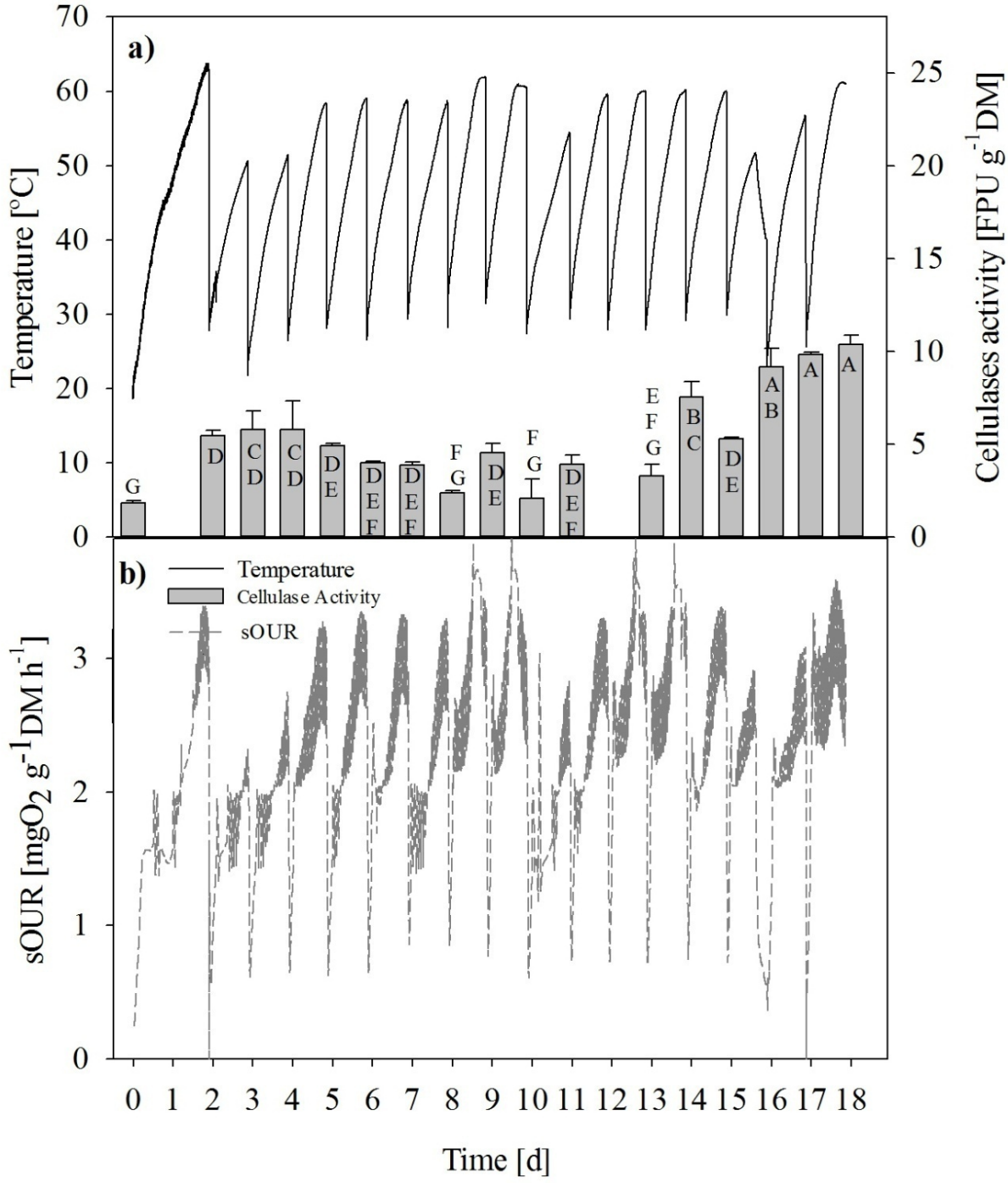

Figure 5.3. Process follow up of sequential batch operation SB50 of coffee husk for cellulase production. In a) temperature and cellulase activity and b) sOUR profiles are presented. Bars that do not share a letter are significantly different.

As it can be observed in Fig. 5.2 and 5.3, similar final cellulase yields were obtained for both strategies. Also in both cases sOUR profile was displaced and the maximum was reached sooner in comparison with the two first batches with compost. As previously discussed, the maximum cellulase activity coincided with the maximum sOUR when using compost as inoculum.

According to sOUR profiles, a reduction in lag phase was accomplished and global biological activity was higher with the specialized biomass obtained at the end of the sequential operation fermentation (final batches) compared to the first batch using compost as inoculum. In general terms, a reduction of the lag phase and an increase in biological activity were expected as a result of inoculation and specialization of biomass (Jurado et al., 2015). This suggests growth stimulation of the microbial communities due to the inoculation of well adapted microbiota. Moreover, it has been stated that inoculation of mixed inoculums not only improved the process on its physical chemical parameters, but also stimulated microbiota growth and microbial diversity during the process (Ishii et al., 2000, Liu et al., 2011).

An enhancement of cellulases production was expected due to biomass specialization. Cellulase production is regulated by an inhibition system, in which cellobiose and glucose inhibits β -glucosidase (Kuhad et al., 2016). These readily metabolizable sugars released to the medium are easily consumed by the microorganisms present in the fermentation, which is one of the advantages of working with SSF. In this sense, it seems reasonable to assume that cellulase production was affected by other circumstances, such as the release of inhibitory compounds generated as the results of lignin degradation (Brijwani et al., 2011), non-productive adsorption to lignin hydrolysates (Akimkulova et al., 2016) or even to depletion of different mineral sources (Salgado et al., 2015).

A single research has been found on sequential batch in SSF for lipids and cellulases production by oleaginous fungi (Cheirsilp et al., 2015), but this study was performed in a different scale (<1g) and under sterile conditions. However, the authors found that at different substrate exchange ratio (>50%), cellulases production could be sustained in values ranging 1.8-2.3 FPU g^{-1}DM, although no increment on its activity was found, in accordance with the findings described in this work.

c) Cellulase production profile using specialized inoculum.

The final fermented solids with selected microbiota obtained for each strategy were stored frozen and later used to inoculate one batch each (per triplicate). The complete cellulase, temperature and sOUR profiles are presented in Figure 5.4. Maximum FPase of 9.8 ± 2.4 FPU g^{-1}DM from SB90 and 4.8±1.1 FPU g^{-1}DM from SB50 were reached at day 2 using inoculum. Although both systems reached maximum sOUR values around 4 mgO_2 g^{-1}DM h^{-1},sOUR dynamics were different with both

inoculums. sOUR peaked sooner in SB50 (24h) while total oxygen consumed was similar in both cases (137 ± 22 and 148 ± 38 mgO_2 g^{-1}DM for SB90 and SB50 respectively).

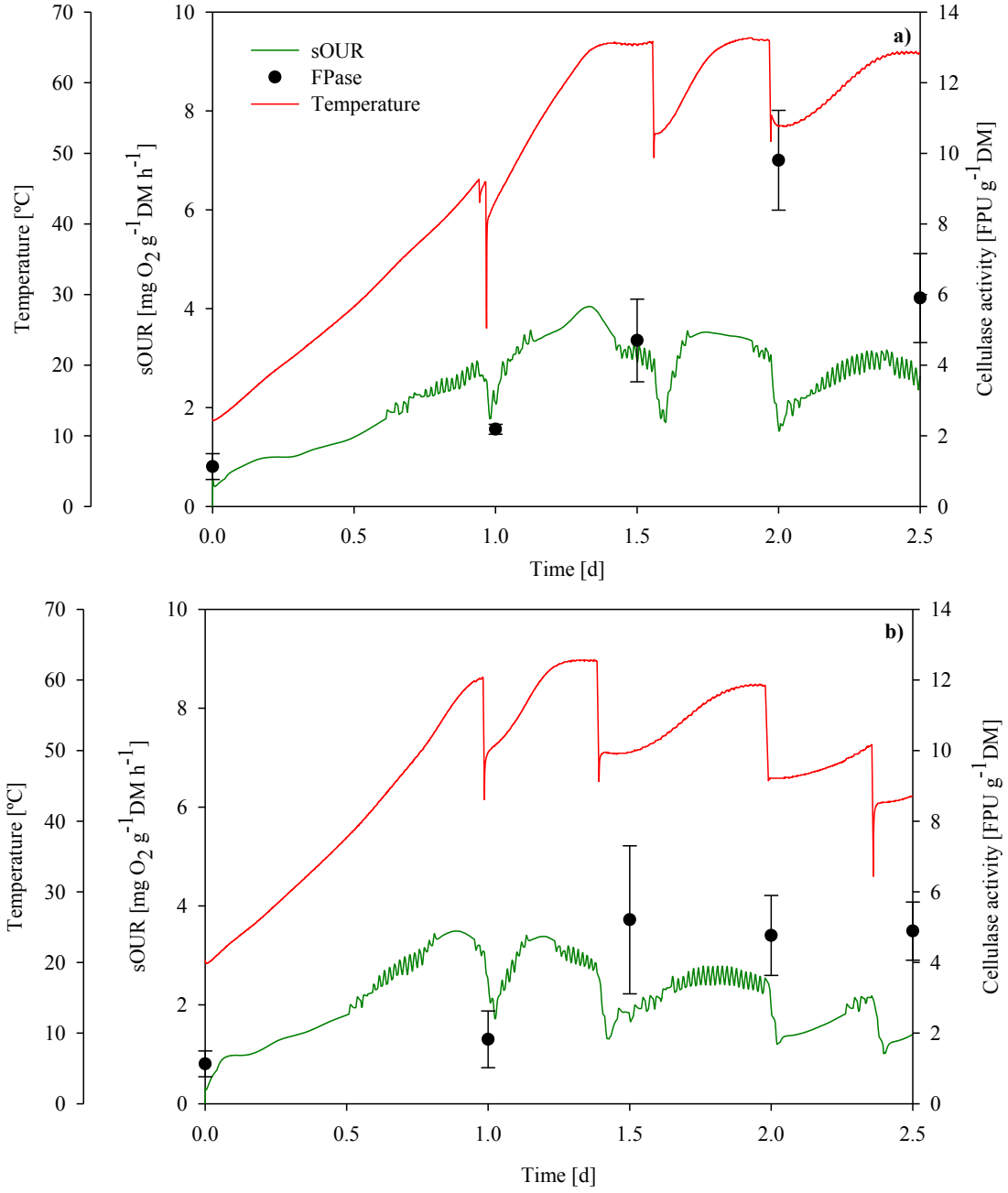

Figure 5.4. sOUR, temperature and filter paper activity profiles using as inoculum the fermented solid obtained at the end of a) SB90 and b) SB50 operation.

In spite of that fact, maximum sOUR values were sustained during the fermentation, only starting to decay after day two of operation. Also in both cases maximum cellulase activity was achieved during the sOUR peak, reaching values of nearly 10 and 5 FPU g^{-1}DM for SB90 and SB50 respectively. The

fact that sOUR and enzymatic production profile presented slight differences comparing when using compost as inoculum is a demonstration of the different dynamics of the new microbial population.

In this case, a reduction in lag phase was accomplished with the specialized biomass obtained at the end of the fermentation compared to the first batch using compost as inoculum.

Both cellulases profiles obtained with the specialized inoculums presented values according to the obtained during the fermentations, even when a smaller size inoculum was added; which was the case of SB50.

As mentioned in section 5.1.3b, it is expected a reduction in the lag phase and an increase on the duration of the biological activity when an inoculation is performed. This can be easily observed in Figures 5.4a and b, where in both configuration the lag phase was reduced to less than 10h and thermophilic conditions and maximum sOUR was sustained until the end of the batch.

d) Microbial communities characterization

Bacteria

A total of 22 bacterial families were detected in SB50 and SB90 with an abundance above 1% as presented in Figure 5.5. Only those families present in C and CH that were also found in SB50 and SB90 final products are depicted for C and CH in order to better illustrate the origin of the bacterial community present in the final products. Other families that did not thrive along the fermentations have been ignored for clarity purposes.

SB50 and SB90 presented a uniform distribution, with roughly 15 and 20% of less than 1% of relative abundance, and a relative high biodiversity as indicated by Shannon-Wiener index, which reached levels greater than 3 for this taxonomic level (3.43 and 3.07, respectively). Taking into account the values found for both raw materials (3.75 for C and 1.73 for CH), bacterial communities in two final products seem to be more influenced by compost, despite its lower percentage at the initial mixture. Sorensen´s-Dice indices, qualitative and quantitative (Figure 5.6), strengthen this assessment, since higher values and, consequently, greater similarities were obtained when comparisons were established between either both final products and C.

According to these results and those obtained from preliminary assay at lab scale (Section 5.1.3a), CH would be a proper and more efficient substrate to promote cellulase activity on account of its high cellulose content, but the bacterial cellulolytic community in compost dominates the structure of the final products. Compost is the result of a complex process characterized by the presence of recalcitrant and non-readily degradable substrates.

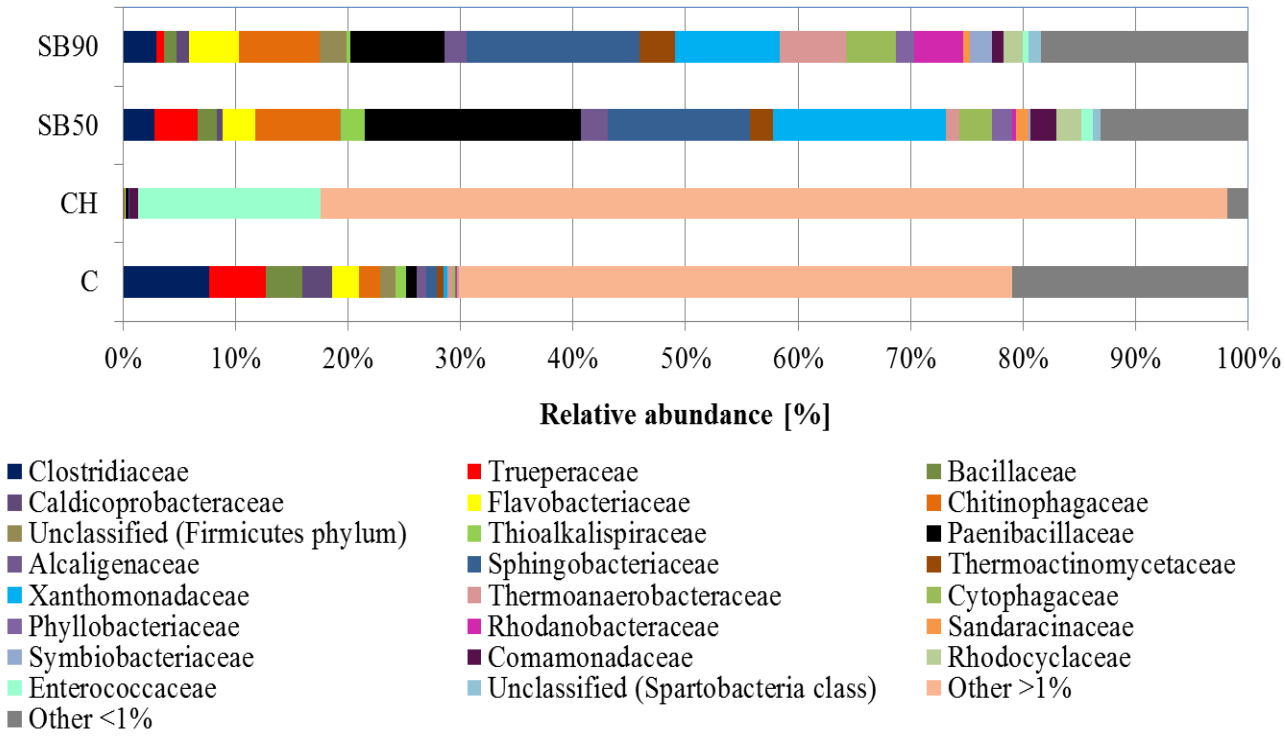

Figure 5.5. Bacterial distributions at the family level according to the16s sequencing for Compost (C), Coffee Husk (CH) and the final product of reactors SB50 and SB90. Only families with a relative abundance >1% in either SB50 or SB90 are depicted. Families detected with a relative abundance < 1% in these two samples are grouped as "others".

From a biological point of view, this results in a very strong competitive selection of the microorganisms able to carry out all the necessary transformations. Thus, a concentration effect takes place throughout the process (Lopez-Gonzalez et al., 2014) that promotes the presence of a more specialized microbiota in the final product, adapted to those specific nutritional conditions that are typical in composting, among them, cellulolytic microorganisms.

The lower similarity between the bacterial population associated to CH and both final products is clearly evidenced by the fact that the most abundant families in CH, *Pseudomonadaceae* (33.7%), *Leuconostocaceae* (22.6%), *Enterobacteriaceae* (17.7%) and *Enterococcaceae* (16.3%) (Table S1), were sparingly represented in the latter. Thus, only the latest was found with a relative presence over 1% in SB50. On the other hand, in C nearly 80% of all families were a part of *Firmicutes*, *Proteobacteria* and *Actinobacteria* phyla, which have been found to be predominant in compost from lignocellulosic materials (Zhang et al., 2016). *Clostridiaceae* was the most predominant family in C present in 7.7%, which was present in both SB50 and SB90. It is important to point out that *Pseudomonaceae* and *Rhodospirillaceae* families were more abundant in the CH and C respectively,

however their species were not able to thrive at the fermentations conditions of SB50 and SB90. Amounts of these families on final fermentation products were below 1%.

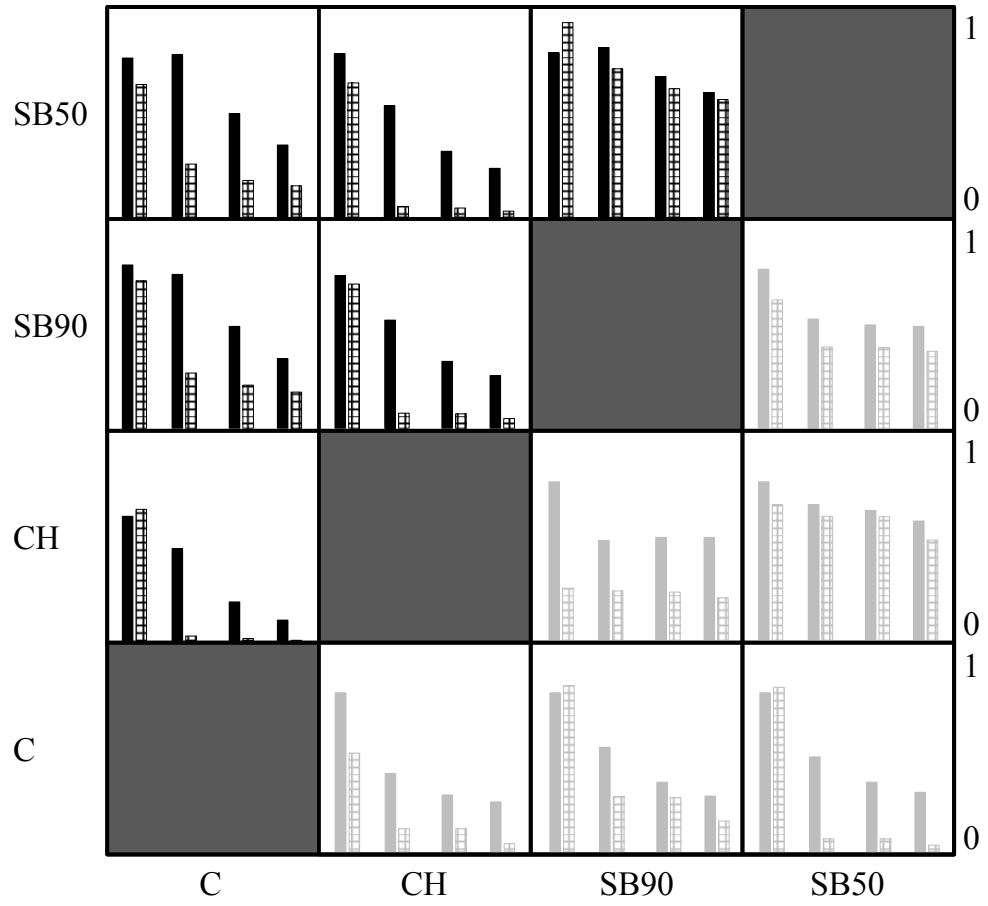

Figure 5.6. Sorensen´s-Dice indices for prokaryotic (black) and eukaryotic (grey) microbiota associated to raw materials and final products (Compost (C), Coffee Husk (CH) and the final product of reactors SB50 and SB90). Every quadrant shows both qualitative (■, ■) and quantitative (⊞, ⊞) indices for all taxonomic levels (from left to right: phylum, family, genus and specie).

SB50 and SB90 resulted in similar bacterial distribution, in which compost influence as inoculum is clearly reflected. *Sphingobacteriaceae, Paenibacillaceae* and *Xanthomonadaceae* families together were present in a discrete 2.2% in C, however, they represented a 47.2 and 33.1% in SB50 and SB90 respectively. From these families, several species have been identified during the early stage of lignocellulosic material composting process (Lopez-Gonzalez et al., 2015a).

From the dominant families, *Pseudoxanthonomas taiwanensis* and *Sphingobacterium composti* appeared as most abundant identified species in SB50 and SB90. Relative abundance of these species were 12.3 and 6.1% for SB50 and 6.1 and 2.6% for SB90 respectively. *P. taiwanensis* has been

widely related to cellulose degradation systems, for its β-glucosidase production and the potential enhancement of the growth of other cellulolytic bacterias (Eichorst et al., 2013). On the other hand, *S. composti* has been related to lignin degradation and the production of acids in aerobic conditions (Karadag et al., 2013). The presence of these two species confirmed the results obtained in both sequential batch operations, which presented significant lignin and cellulose degradation. Furthermore, from *Paenibacillaceae* family *Paenibacillus* and *Thermobacillus* genera were the most abundant. Several species from both genera have been widely reported as strong cellulose degraders such as *P.chitinolyticus* (Mihajlovski et al., 2015), which was found on SB50 and SB90 in near 2% of relative abundance. Also *T.composti* has been found in 4 and 2.5% in SB50 and SB90 respectively. This specie is associated with cellobiase and xylanolytic activity during thermophilic stage in composting processes (de Gannes et al., 2013). Lately it has been characterized as halotolerant, which is of great interest for its bioethanol potential using ionic liquids (Watanabe et al., 2007).

Fungi and yeasts

A total of 12 families were detected for C, CH, SB50 and SB90 as presented in Figure 5.7. First of all, it is possible to notice that in all samples there is a great amount of non-identified DNA, described only as Eukariota, representing 25.7, 5.5, 44.8 and 5.8% of mycobiota from C, CH, SB50 and SB90 respectively. This relatively high rate of unidentified fungi may be consequence of the still scarce and sometimes confusing information available on data base concerning molecular characterization of fungi, especially in comparison with bacteria, which now limits the potential for elucidating the structure of the mycobiota associated to different environments (Langarica-Fuentes et al., 2014).

Remarkably, most of the fungi and yeasts in SB50 and SB90 come from CH where four families account for 94.4% of all mycobiota. This similarity was particularly notable for SB50, with remarkable differences as expressed by the values of the Sorensen´s-Dice index associated to both comparisons, SB50 *v*. C and SB50 *v*. CH (Figure 6). Fungal biodiversity increased in the final materials compared to CH as the Shannon indices obtained were 1.63, 0.98, 1.39 and 1.33 for C, CH, SB50 and SB90. Predominant families were *Phaffomycetaceae*, *Dipodascaceae*, and two unidentified families of the class of *Tramellomycetes*. Distribution in SB50 and SB90 was similar in content but not in proportion, resulting in higher qualitative homology in contrast to quantitative. *Phaffomycetaceae* family was present in CH in 7.8% and in negligible amounts in C. This family thrived on both reactors, achieving a relative abundance of 21.9 and 43.2% in SB50 and SB90 respectively. In this family two yeasts species were identified.

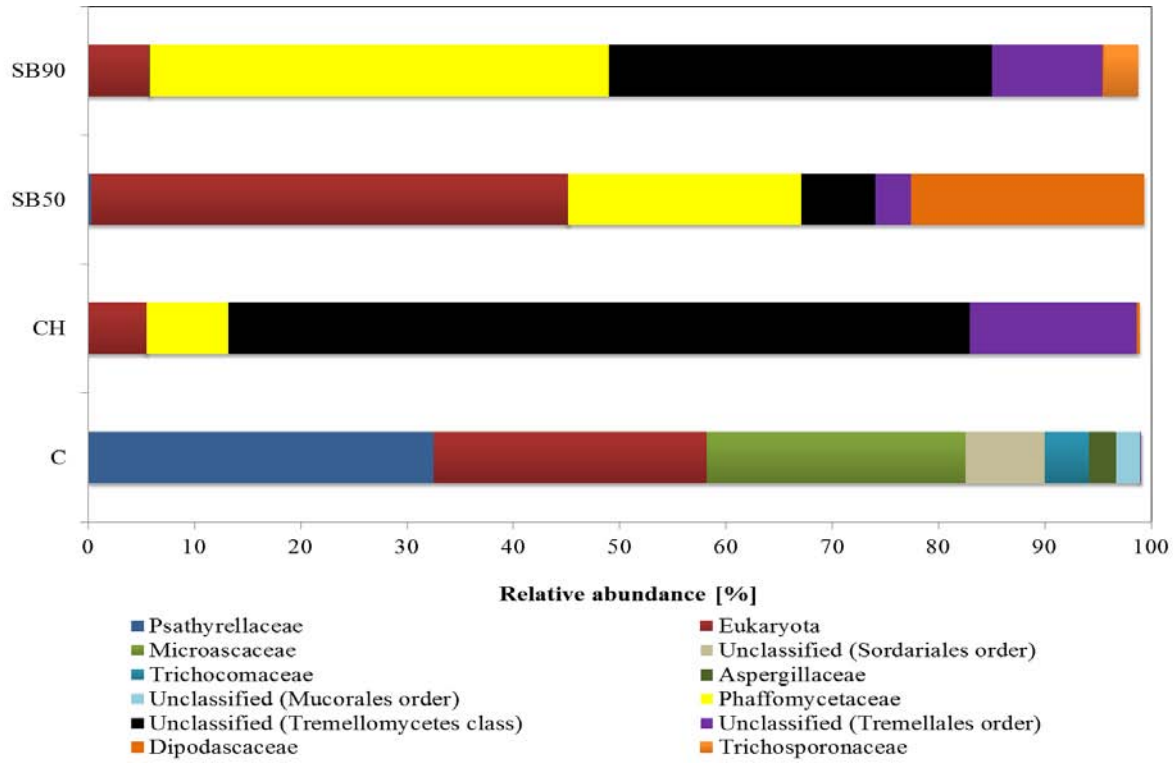

Figure 5.7. Identified fungal and yeasts families in Compost (C), Coffee Husk (CH) and the final product of reactors SB50 and SB90.

The most abundant specie was identified as *Cyberlindnera jardinii*,(17.8 and 34.0% in SB50 and SB90 respectively), which is able to metabolize pentoses and tolerates lignin by-products, which makes it a suitable specie for the treatment of lignocellulosic wastes (Nordberg et al., 2014, Lopez-Gonzalez et al., 2015b). The second specie was *Barnettozyma californica* (4.1 and 9.1% in SB50 and SB90 respectively), a xylose-fermenting and xylanase producer yeast (Morais et al., 2013). In this sense, in SB90, most of fungal/yeast population is focused on hemicellulases metabolism which, in a way, could increase cellulose availability by relaxing the rigid lignocellulosic matrix, which would result in an easier accessibility to this macropolymer, and an increase in the cellulases production. Another difference between both final fermented solids is the high abundance of *Dipodascaceae* family only in SB50, with a 21.9%. in comparison with 0.15% of SB90. *Dipodascus australiensis* and *Galactomyces* sp. are the most representative yeasts of this family and has been proven to be xylanase producer and the latter, cellulose degrader with mild halotolerant characteristics (He et al., 2016). Finally in *Tremellomycetes* class, *Tremellales* order, 3 different genera were detected as *Trichosporonales* sp. LM659, uncultured *Tremellales* and uncultured *Trichosporon* which all together account as 85.5, 10.3 and 46.5% of CH, SB50 and SB90 respectively. No species were identified in this order, however some reports on different species of this order has been proven to be unconventional due to absence of diauxic effect, i.ef ermenting hexoses and pentoses while also

effectively utilizes xylose and *N*-acetylglucosamine, which are building blocks of lignocellulosic materials (Kourist et al., 2015).

Overall performance of the process and the microbial communities involved

In general, solids obtained at the end of SB50 and SB90 fermentations, provided a strongly specialized mixed inoculum for lignocellulosic materials degradation. Cellulose degrading activity was mainly provided by bacterial populations; however it is important to remark that cellulolytic bacteria do not always express the full cellulose degradation system (Behera et al., 2016). On the other hand, hemicellulose degradation potential was provided by fungal/yeasts species. In spite of the abundance of hemicellulose degraders, hemicellulose degradation was negligible (Table 1). It is possible that this biomass was not active during the process, or the activity was low in comparison with cellulose or even lignin degraders, or that the hemicellulolytic microorganisms act partially on the heteropolymer, promoting relaxation but not degradation in an extensive rate.

Considering these results it is likely that synergistic or complementary actions take place in the total microbiota of the reactors. Degradation of cellulose does not occur in isolation. As a constituent of the lignocellulosic matrix, the action of cellulases is not the only event needed for the transformation of this glucose polymer (Kanokratana et al., 2015). Thus, the biodegradation process is the results of different cooperative actions that first promote the relaxation of the enmeshed structure that characterize lignocellulose. This initial event allows the access of enzymes to the site of action and, consequently, is one of the key steps in order to achieve the depolymerisation, not just of cellulose but of all macropolymers in lignocellulose too (Duarte et al., 2012). From a microbial point of view, the complexity of the process acts as a pressure factor that promotes the selection for a microbiota metabolically adapted to the nutritional demands associated to lignocellulosic environments. This is particularly true for artificial habitats, as in the case here described, in which microbiome shifts from a metabolically diverse community to a specialized microbiota (López-González et al., 2014). In this sense, it would be highly improbable that lignocellulosic and lignocellulosic-inhibiting populations coexist, after the occurrence of such selective process. Nevertheless, it cannot be excluded some other inhibitory effects associated to the products resulting from the action of cellulases and other lignocellulosic enzymes, as glucose, cellobiose or phenolic compounds, although microorganisms that consume these inhibitory compounds use to be members of the lignocellulosic community (Wongwilaiwalin et al., 2013).

In light of the experimental data presented in this work, it is possible to remark that the use of fermented solids as inoculum in a sequential batch operation is very successful. The proposed operation offers a way to reduce costs in inoculum requirements and potentially to the reduction of

solid wastes generation as suggested by Farinas (2015). The studies on microbial diversity on non-sterile SSF processes are scarce. In this process a specialized biomass provides the generation of an enzymatic pool, containing different enzymatic activities related to lignocellulose degradation. The positive effects of using a multi-enzymatic preparation have been reported by Melikoglu et al., (2013). These authors were able to improve bioethanol production by producing several enzymes by SSF. Furthermore, it has been proven that the use of these enzymatic pools without any additional processing steps provides better results than commercial preparations, reducing operational costs (Lever et al., 2005). In summary, the proposed process presents the following economic advantages: the use of organic wastes instead of pure substrates; saving the investment and the operating costs related to sterilization; saving the costs of producing inoculum for each batch, since fermented solids from one batch are re-used to inoculate the following batch; the potential use of the multienzymatic extracts without additional purification steps. Further economic assessment should confirm the cost effectiveness of the process.

5.1.4 Conclusion

Operation of SSF of coffee husk in sequential batches has been proven as a suitable strategy for cellulases production using a mixed inoculum. SB50 and SB90 strategies provided a sustained fermentation for 18 and 12 days respectively and cellulase production stabilised at around 10 FPU g^{-1}DM. 48h batches in the strategy SB90 provided a more consistent operation while SB50 required more time to reach a pseudo-steady state. Both strategies obtained cellulase activity in the reported range of production. The sequential process allowed the enrichment of cellulose and hemicellulose degraders, eliminating the requirements of fresh inoculum for each batch. Bacterial communities obtained at the end of both processes came from compost with great cellulose degradation potential. Fungal and yeasts communities came mostly from coffee husk, with high hemicellulose degradation potential. The development of these operational strategies and further biological characterization of the end product could eventually benefit the process economics by providing a standard and specialized inoculum for a continuous solid state fermentation for cellulases production.

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CHAPTER 6. REPRODUCIBILITY ASSESSMENT OF CELLULASE PRODUCTION THROUGH SOLID-STATE FERMENTATION

Part of this chapter is submitted for publication to Biotechnology for Biofuels. "Cellulase and xylanase production at pilot scale by solid-state fermentation from coffee husk using specialized biomass: Process operation and microbial communities consistency."

Cerda, A., Mejias, L., Gea, T., Sánchez, A.

Summary

This chapter summarizes and discusses the reproducibility and of three SSF, performed in 50-L reactors using coffee husk as the substrate and a previously propagated specialized inoculum.

This study has two main sections, one is the study of the three different fermentations carried out in 4.5L reactors. These fermentations performed as propagation for the specialized inoculum that was used for the pilot fermentations. The other section is the complete study of the pilot fermentations, in terms of biological activity parameters, enzymatic activity, gaseous emissions and microbial communities monitoring. As mentioned in other chapters, solid wastes mixtures are heterogeneous in comparison to liquid mediums used in conventional fermentations; in addition, these processed were inoculated with a fermented solid coming from another dynamic and complex process. Considering these aspects and looking for an standard and consistent SSF, the objective of this compilation is to know how this heterogeneity influences the productive processes and if these processes are environmentally friendly. There is a great lack of information regarding SSF using non sterile substrate, for this reason, the conclusions of this work will provide useful information for further SSF standardization. This work was carried out in collaboration with Laura Mejías.

6.1 Process operation and microbial communities consistency in a solid state fermentation for cellulase and xylanase production.

6.1.1 Materials

Coffee husk (CH) was kindly provided by Marcilla S.A (Montcada, Barcelona, Spain) and stored frozen at -20ºC until use. CH presented a general characterization of: moisture content of 60.8±0.4% (wet basis), pH 6.5±0.1 and C/N ratio of 13.3±0.5. Fiber content in CH was 25.7±0.2, 14.6±0.1 and 17.6±0.5% (dry basis) of cellulose, hemicellulose and lignin, respectively.

The inoculum used in this work consisted of a specialized consortium of microorganisms able to produce cellulases using CH as sole substrate. This was previously obtained operating SSF by sequential batch (SB) using compost as starting inoculum. Main identified species were the bacteria *Pseudoxanthonomas taiwanensis* and *Sphingobacterium composti* and the yeasts *Cyberlindnera jardinii* and *Barnettozyma californica*, all of them previously reported as lignocellulose degraders. The procedure to obtain this inoculum is described in Chapter 5. Briefly, compost was added in a 10% (w/w) ratio to a mixture of CH and wood chips as bulking agent in a 1:1 (v/v) ratio to provide the proper porosity to the mixture (Ruggieri et al., 2009). During the entire process there was no evidence

suggesting wood chips degradation due to the short time of each cycle. Sequential batch operation was performed in cycles until stable cellulase production of 10 FPU g^{-1} DM (filter paper units per grams of dry matter). SB operation started with a 48h fermentation stage followed by a substrate exchange of 90% of volume every 48h. The process was operated for several cycles until stable operation was achieved and the final fermented solids were stored frozen for further use as specialized inoculum. A full characterization of the specialized consortium was performed by DNA sequencing and the complete characterization is presented in Chapter 5 section 5.1.4d. The specialized inoculum presented a general characterization of: moisture content of 55.0±3.1% (wet basis), pH 9.1±0.1 and C/N ratio of 13.1±0.6. Fiber content in the specialized inoculum was 23.5±0.5, 13.3±0.5 and 18.3±0.6% (dry basis) of cellulose, hemicellulose and lignin, respectively.

6.1.2 Experimental procedure

SSF was performed in 50 L pilot bioreactors in triplicate batches (R1, R2 and R3), the complete system is described in chapter 3 section 3.2.3. Each batch was inoculated with fermented solids from a 4.5 L propagation reactor (P1, P2 and P3, respectively) inoculated with the thawed specialized consortium. The experimental set up used for the propagation reactors is describes in chapter 3 section 3.2.2. Both propagation and pilot operations were carried out under near adiabatic conditions, using thermally isolated reactors in all cases. Full description is presented below

a) Inoculum preparation in propagation reactors

A propagation reactor was required to provide the proper amount of biomass to inoculate the pilot reactors. Inoculum was obtained by using an initial mixture containing CH and 10% of the mixed specialized inoculum with wood chips, which was used in a ratio of 1:1 (v/v), giving a total weight of 1.2 kg per batch. The entire process was carried out for 48h in 4.5 L air-tight reactors, working under near adiabatic conditions and oxygen controlled aeration (120-240 mL min^{-1}, oxygen setpoint 11.5% in air) as described in chapter 3 section 3.4.1.

b) Pilot reactor operation

Three batches were performed in 50 L closed reactors. A schematic diagram of the pilot reactor and a detailed description can be found in chapter 3 section 3.2.3 (Puyuelo et al., 2010). Temperature, exhaust gas oxygen concentration and inlet airflow were monitored during the trials. The experiments were performed with forced aeration and airflow was manually adjusted to ensure that the oxygen content in the reactor remained above 10%, in order to provide full aerobic conditions (Puyuelo et al., 2010).

The mixtures were prepared by mixing CH and the specialized inoculum obtained from the propagation reactor in a 90:10 (w/w) ratio respectively. Wood chips were added as bulking agent in a volume ratio of 1:1 (v/v). The final weight of the mixture was 15.2 kg for each reactor. The first batch (R1) was performed to obtain a full profile of enzymatic activity production, collecting gaseous and solid samples at 0, 8, 16, 24, 35, 48, 58, 72 and 134 h, according to the methods described in section 3.3.5. In order to assess the reproducibility of the process, two additional batches (R2 and R3) were performed until the moment of maximum cellulase activity.

c) Sampling

Gaseous samples were collected in 1-L Tedlar® bags for ammonia (NH_3), volatile organic compounds (VOC), nitrous oxide (N_2O) and methane (CH_4) content determination before opening of the reactor, according to the methods described in chapter 3 section 3.3.5. Once the reactor was opened and after homogenization, solid samples were collected for the determination of cellulase and xylanase activities. Filter paper activity (FPase), carboxymethylcellulase (CMCase) and β-glucosidase (BGase) activities were measured for cellulase production. Xylanase (Xyl) production was followed for hemicellulase production.

In addition to the enzymatic measurements, the solid samples were analyzed in order to determine the neutral detergent fiber, acid detergent fiber and lignin content. These analysis were performed according to the methods presented in section 3.4. Degradation percentage of cellulose, hemicellulose and lignin were calculated according to a mass balance and considering the weight evolution throughout the process.

6.1.3. Results

a) Specialized inoculum preparation

Figure 6.1 presents the sOUR and the temperature profiles of the three propagation reactors (P1, P2 and P3) for inoculum preparation. It is possible to observe that a similar trend is presented in all three propagation reactors. However, the heterogeneity of the materials led to a series of small differences during the processes despite of using the same specialized consortium.

P2 and P3 started at temperatures of 21 and 25ºC respectively, which was notably higher than initial P1 temperature (12ºC, Figure 6.1b). This difference was attributed to the preparation process of the

substrate, which includes a defrosting stage. Major differences were found during transition to thermophilic stage after 24h of operation. After reaching 45ºC the automatic control started to act

according to the oxygen requirements of the fermentation, which can be clearly observed in the oscillation of sOUR profiles of P2 and P3. Control actuation reflects high biological activity in these two reactors in detriment to P1, where sOUR only started to oscillate at the end of the fermentation. In this sense, at the end of the fermentation sOUR obtained for P1, P2 and P3 were 2.6, 3.0 and 3.1 mg O_2 g^{-1} DM h^{-1} for P1, P2 and P3, respectively.

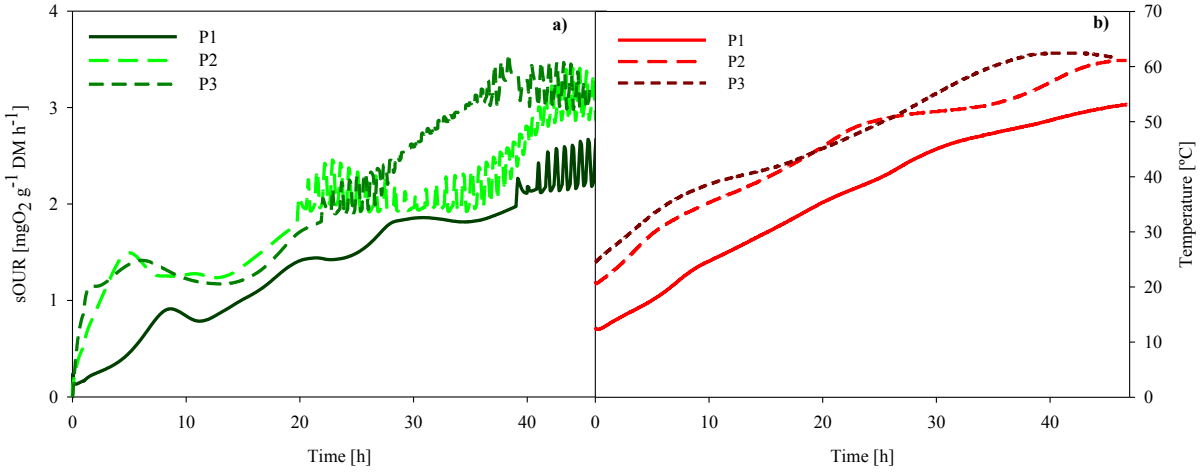

Figure 6.1. Operation profile of a) sOUR and b) temperature of the propagation reactors P1, P2 and P3 (4.5L) of a specialized inoculum using coffee husk as substrate.

Additionally, COC resulted in very similar with values of 86.1 and 94.8 mg O_2 g^{-1} DM for P2 and P3 respectively. In contrast, P1 presented a COC of 65.3 mg O_2 g^{-1} DM, which represents around 70% of the COC of P2 and P3. It is highly possible that initial temperature affected the performance of P1, in detriment of biological activity (Sundh & Rönn, 2002). It is for this reason that, in order to improve reproducibility of the process, in terms of biological and enzymatic activity, the initial temperature of the process must be set on proper values to reduce the lag phase of microbial communities that will grow on the reactors.

Cellulase activity produced in the three reactors was 8.1±0.2, 4.6±0.1 and 8.4±0.2 FPU g^{-1} DM for P1, P2 and P3, respectively. This specialized inoculum was obtained in a SSF process performed by sequential batch operation reaching consistent cellulases production within 8-9 FPU g^{-1} DM (see chapter 5, section 5.1.3b). In this sense, the enzymatic activity observed in P2 is an unexpected value. Further research should focus on finding correlations between specific operational parameters and enzymatic activity to predict it.

b) Pilot reactor operation

Figure 6.2 presents the fermentation profile for a 134 h operation using a previously propagated inoculum. The fermentation presented a quick start-up with no lag phase as observed in the sOUR and temperature thermophilic profiles (Figure 6.2a). This was probably due to the initial conditions of the inoculum, which was in its highest biological and enzymatic activity.

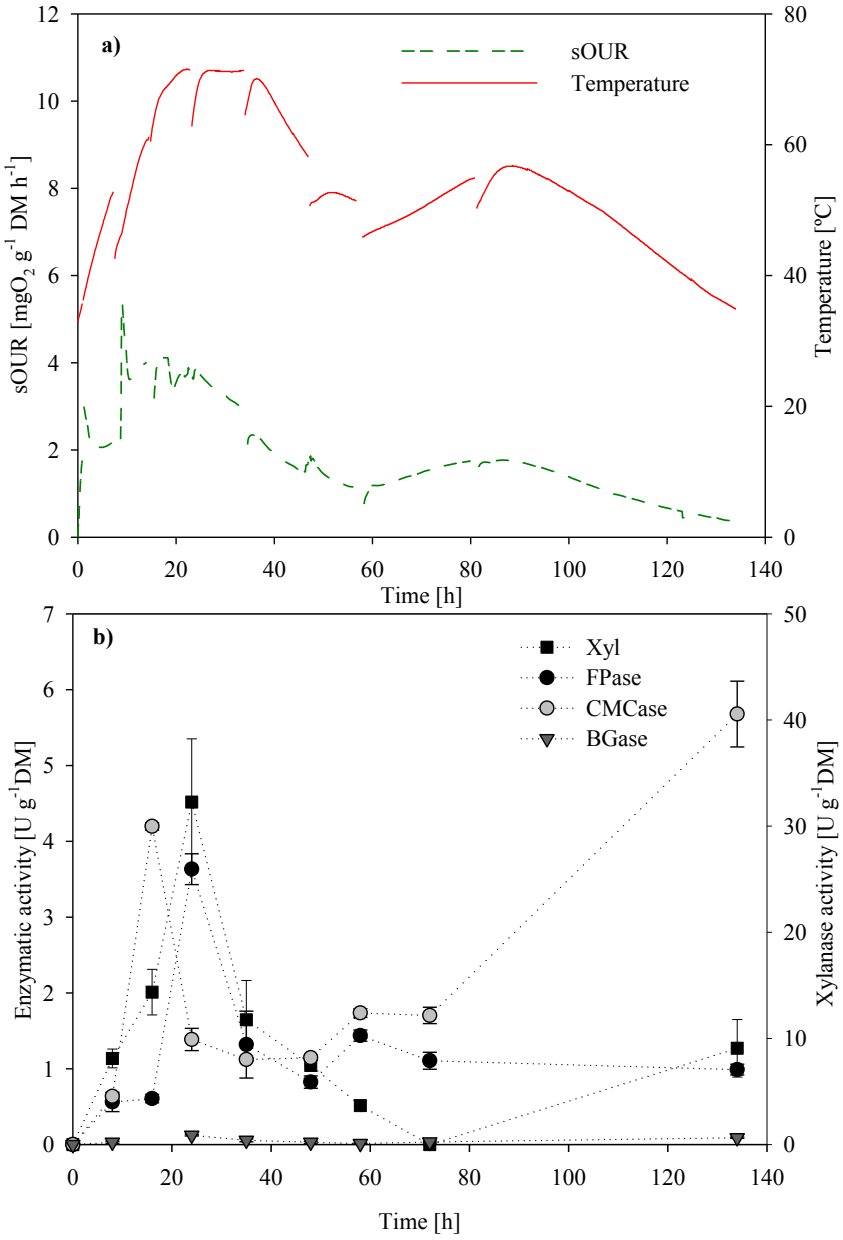

Figure 6.2. Operation profile of a) sOUR and Temperature and b) FPase, CMCase, BGase and Xyl production profile in a pilot reactor (50L) using a propagated specialized inoculum and coffee husk as

substrate, during 134 h. All enzymatic activities were expressed as U g^{-1}DM with exception of FPase, which is expressed as FPU g^{-1}DM.

Biological activity was found at its maximum during the first 30h of fermentation in a full thermophilic stage. Maximum sOUR and temperature were 4.1 mg O_2 g^{-1} DM h^{-1} and 71ºC, respectively, at 24h. Oxygen requirements reached a total COC of 243.2 mg O_2 g^{-1} DM. COC found at 24h of fermentation, at its highest sOUR, was 80.2 mg O_2 g^{-1} DM, which roughly represents 33% of the total oxygen consumption. It is important to highlight the importance of choosing the exact moment to have the maximum enzymatic activity, which in occasions, can match with the maximum biological activity, reflected as sOUR (Abraham et al., 2013) or not (Cerda et al., 2016).

In addition, pH reached an alkaline value of 9.22±0.01 and a C/N ratio of 12.99±0.15, which reflected organic matter degradation. These conditions remained until the end of the fermentation, achieving final values of 9.34±0.01 for pH and a C/N ratio of 11.43±1.03.

Cellulolytic and hemicellulolytic activities also showed their maximum during the most active stage of the fermentation (Figure 6.2b). FPase, BGase and Xyl reached their maximum production in the first 24h of operation with values of 3.6±0.2 FPU g^{-1} DM, 0.12±0.01 U g^{-1} DM and 32±5 U g^{-1} DM, respectively. FPase and BGase presented a local maximum, decreasing after this moment to negligible values. This can be attributed to several factors such as nutrient depletion or the inhibition of the enzymatic system due to the formation of by-products (Brijwani & Vadlani, 2011; Salgado et al., 2015) or non productive adsorption to lignin hydrolyzates (Gao et al., 2014). On the contrary, Xyl activity presented a dramatic decrease to almost zero value at 72h of fermentation and increased to 9±2 U g^{-1} DM by the end of the fermentation.

CMCase profile resulted in a completely different trend. Maximum CMCase activity was obtained at the end of the fermentation, with a value of 5.67±0.43 U g^{-1} DM. Also, the second highest peak was obtained at 16h of fermentation, just prior to maximum FPase, Xyl and BGase production, reaching a value of 4.19±0.05 U g^{-1} DM. CMCase activity is related to the random degradation of the amorphous sites of the cellulose chain, i.e, they are mainly responsible for the reduction of the polymerization degree of cellulosic substrates to provide a more available substrate for exoglucanase action (Kostylev & Wilson, 2012). Considering metabolic economics seems likely that CMCase would be produced only when required for further hydrolysis.

The measurements for cellulase and xylanase activity are currently performed using several different methods, some of them with many concerns on its reproducibility (Coward-Kelly et al., 2003). It is

for this reason that the comparison between researches is complex; however, a summary of several enzyme production processes are presented in Table 6.1.

Table 6.1. Summary of maximum cellulase and xylanase activities production (FPase, CMCase, BGase and Xyl) obtained by SSF.n.r: not reported, m.r: mixed inoculum. SH: soybean hulls, RH: rice husk, OP: orange peels, WS: wheat straw, RS: Rice straw, WB: wheat bran, POW: palm oil waste, SB: soybean, SCB sugarcane bagasse and CH: coffee husk.

				Enzymatic activity (U g^{-1}DM)				
Substrate	**Amount (g)**	**Inoculum**	**SSF time (h)**	**FPase**	**CMCase**	**BGase**	**Xyl**	**References**
SH:WB	100	*Aspergillus oryzae*	96	10.8	100.7	10.7	504.9	(Brijwani & Vadlani, 2011)
RH	5	*Aspergillus oryzae*	96	3.1	14.1	10.6	n.r	(Bansal et al., 2012)
OP	5	*Aspergillus oryzae*	96	1.9	1.0	3.0	n.r	(Bansal et al., 2012
WS	5	*Aspergillus níger*	96	13.6	11.2	5.5	n.r	(Bansal et al., 2012
RS	10	*Aspergillus níger*	96	9.0	30.6	21.2	936.1	(Dhillon et al., 2011)
WB	10	*Aspergillus níger*	96	13.6	48.2	21.7	2601	(Dhillon et al., 2011)
WS	25	*Trichoderma reesei*	240	1.2	n.r	n.r	n.r	(Lever et al., 2010)
POW	1	*Aspergilllus turbingensis*	120	2.4	n.r	n.r	11.8	(Lever et al., 2010)
WB:SB	5	*Aspergillis fumigatus*	120	5.0	56	105.8	10.6	(Delabona et al., 2013)
SCB	5	*Pleurotus ostreatus*	72-120	0.25	0.13	n.r	11.0	(Membrillo et al., 2011)
CH	1,200	*m.i*	48	8-9	n.r	n.r	n.r	Chapter 5
CH	15,200	*m.i*	24	3.08	2.51	0.13	44.51	This work

All enzymatic activities are produced in a wide range of values, probably due to the variety of inocula and substrates used in those researches. FPase, CMCase and BGase production in this work was found

in the reported range of production, as seen in Table 6.1. As for xylanase production, the reported range of production (10.6 to 2601 U g^{-1} DM, according to Table 6.1) is wider than the reported for cellulase. In spite of the low production values obtained in this work when comparing to literature, it has to be stated that some authors have found even lower levels of enzymatic activities production (Mansour et al., 2016).

Considering cellulase and xylanase production, it is remarkable the fact that most of the production systems shown in Table 6.1 are carried out using small amounts of substrates, using pure strains and long fermentation times. In this sense, the results obtained in this work are promising, due to the significant cellulase and hemicellulase production in a short fermentation time, therefore allowing the faster valorization of organic wastes and improving process economics.

Regarding fiber degradation, results are presented in Figure 6.3. Cellulose, hemicellulose and lignin degradation started early with a 1.1, 12.4 and 7.0% degradation respectively in the first 8 h of operation. During the most active stage of the process at 24 h of operation, partial degradations of cellulose, hemicellulose and lignin were of 4.9, 13.4 and 4.1%, which are in accordance with reported literature (Salgado et al., 2015). At the end of the fermentation a final cellulose degradation was 24.1%. In addition, final lignin hydrolysis was 11.25%, which is higher than the observed by other researches in short solid-state fermentations (Umasaravanan et al., 2011). The most interesting result was obtained for hemicellulose hydrolysis. A final degradation of 34.9% was achieved in spite of the relatively low Xyl values as compared with literature (Table 6.1).

This process was performed using a non-sterile substrate, which provides a complex dynamic process, involving different metabolisms. For this reason, enzymatic (or not enzymatic) products cannot be properly correlated to operational parameters, but only expressed as the net result of these different metabolisms. This might be the reason for not finding correlations among cellulases enzymatic complex and xylanase activities production with their respective substrates. Even more, in all cases, no correlation has been found using any of the parameters followed during the process. This is probably due to the fact that parameters like sOUR or temperature, even though useful, provide an extremely simplified overview of the process. For this reason, there is the need to seek for different parameters or techniques to properly correlate the enzymatic production profile. In this sense, the identification of microbial communities appears as an attractive alternative that enable the potential direct correlation among substrate consumption and enzyme production.

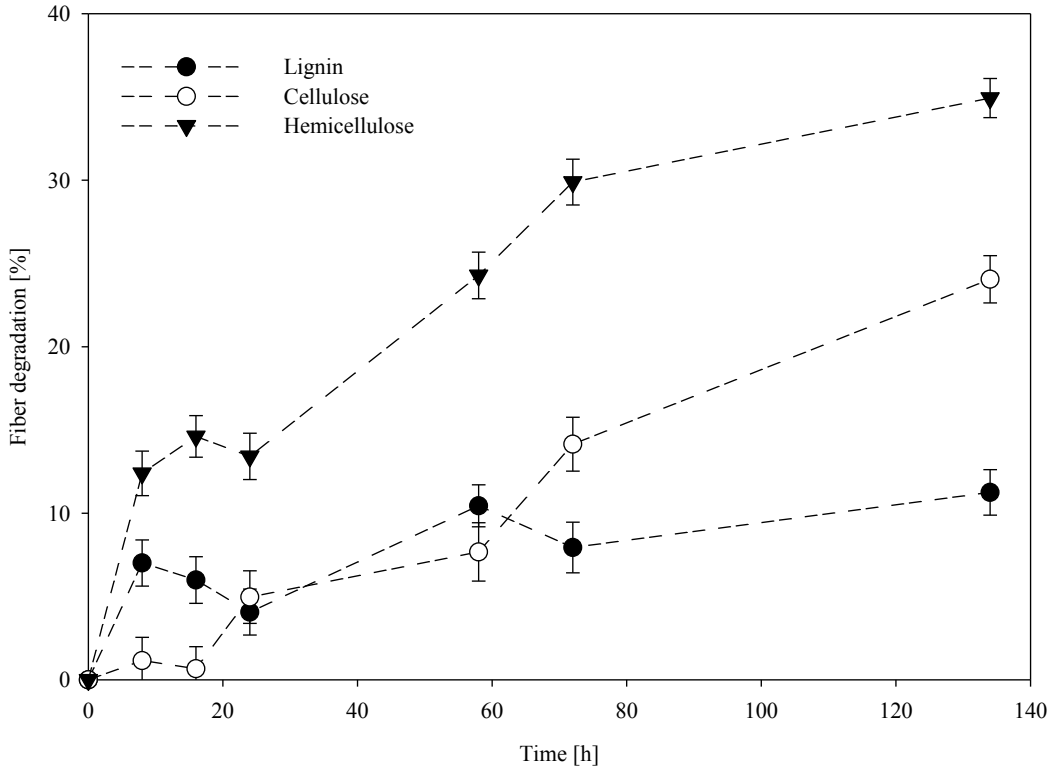

Figure 6.3. Cellulose, hemicellulose and lignin degradation profile obtained during pilot scale solid-state fermentation using a specialized inoculum and coffee husk as substrate, during 134 h. Degradation is expressed as mass percentage obtained from a mass balance.

c) Gaseous emissions

Figure 6.4 presents the evolution of the cumulative emissions of CH_4, N_2O, VOCs and NH_3 throughout the SSF process carried our during the first pilot batch (R1).

Ammonia was emitted in higher amounts than the rest of the gases analyzed, and was mainly released after the thermophilic stage at the highest biological activity condition as reported by other authors (Maulini-Duran et al., 2015). Ammonia emission factor was $3.5 \cdot 10^{-2}$ kg Mg^{-1} CH, obtained at 134 h. Ammonia emission is mainly related to nitrogen compounds content such as ammonium from proteins, which can be stripped out as ammonia through the exhausted gas outlet. VOCs, on the other hand, were also found as one of the main contributors of emissions throughout the process with a total emission factor of $1.1 \cdot 10^{-2}$ kg Mg^{-1} CH at 134 h of fermentation. In spite of this value, when it is considered a productive process for cellulase and xylanase, the SSF should take only 24 h, where the emission factor was $2.6 \cdot 10^{-3}$ kg Mg^{-1} CH.

Previous studies reported VOC emission factors 1000-fold higher than the obtained in this work (Maulini-Duran et al., 2015), using orange peels (rich in limonene and other volatile compounds) as substrate for cellulase production in a 5 day fermentation. VOCs emitted during the current process presented an almost linear trend, indicating a nearly continuous degradation of the carbonaceous material as suggested in other studies (Pagans et al., 2006b).

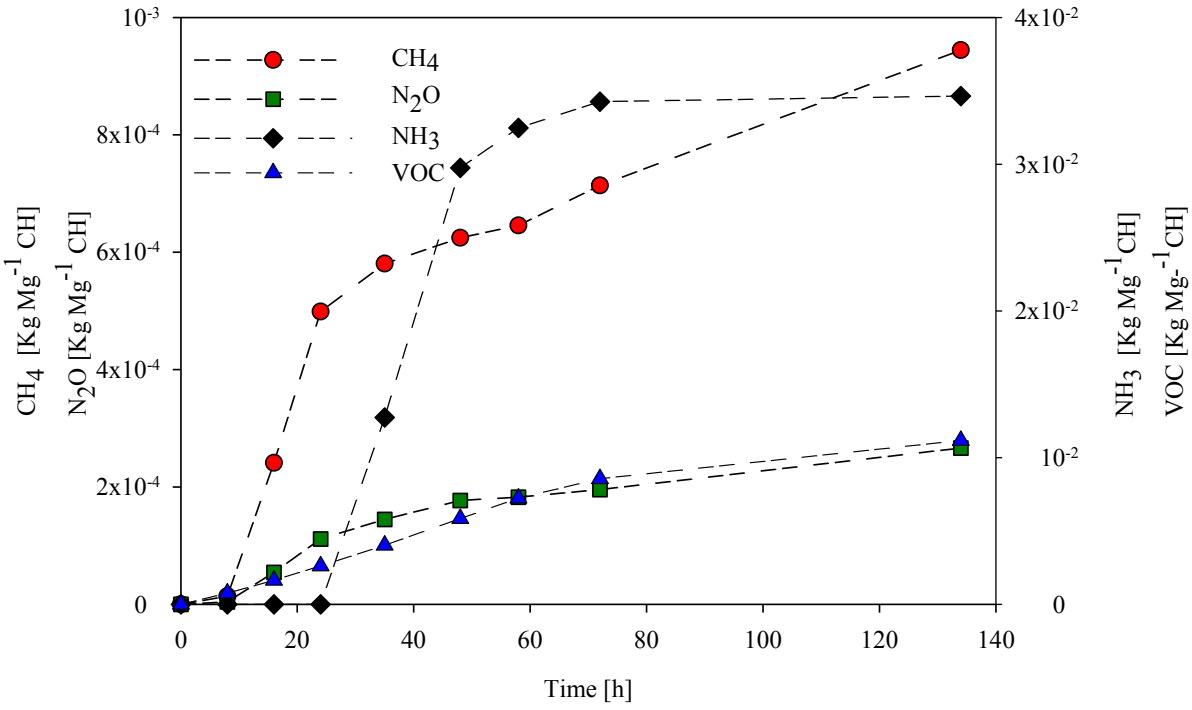

Figure 6.4. Cummulative gaseous emissions generated during pilot scale solid-state fermentation using a specialized inoculum and coffee husk as substrate.

In contrast to NH_3 and VOC emissions, CH_4 and N_2O are not directly related to composition but to the appearance of anaerobic zones in the solid matrix of the reactor, especially at larger scales than those reported in SSF literature. In the case of, N_2O emissions, these are reported to be associated either to anaerobic or anoxic conditions by means of the heterotrophic denitrification and nitrifier denitrification processes during ammonia degradation as reported by some authors (Wunderlin et al., 2013). In this study, CH_4 and N_2O emissions were only observed in very low amounts mainly during the most active stage of the fermentation. In this context, the entire 134 h process presented total cummulative emission factors of $9.4 \cdot 10^{-4}$ and $2.6 \cdot 10^{-4}$ kg Mg^{-1} CH for CH_4 and N_2O, respectively.

Even more, when considering the 24 h productive process, these factors even lower, achieving values of $4.9 \cdot 10^{-4}$ and $1.1 \cdot 10^{-4}$ kg Mg^{-1} CH for CH_4 and N_2O, respectively. Other studies presented emissions of these pollutants obtained during the highest biological activity in similar processes (Maulini-Duran et al., 2015), which are in agreement with this work. However, the obtained emission factors were 10-fold higher than those observed in that process. The absence of published data on the emissions of pollutant gases from SSF unable the comparison of the obtained values, although in any case they are lower than those of similar processes, such as composting (Pagans et al., 2006a; Pagans et al., 2006b).

The complete emission profile was performed in order to fully assess the potential environmental impact and its correlation with enzyme production. However, if it is considered the period of time when maximum enzymatic production is achieved, only VOC emissions are relevant due to NH_3 it is only produced after 32h of fermentation. This is of great importance, when considering a potential gas treatment.

The operational measurements presented in Figures 6.2, 6.3 and 6.4 were performed in order to fully understand the process dynamics and environmental impact, however, in terms of cellulase and xylanase production, the process should be stopped at 24 h.

d) Replicates and consistency

In Figure 6.5a it is presented the first 24 h of operation of the previous SSF (see Figure 2 for the complete profile) where maximum cellulase and xylanase activities were determined. Two replicates of this 24 h fermentation are presented in Figure 5b and 5c.

Average of the initial pH and C/N ratio for the triplicates were 8.32±0.01 and 13.32±0.46 respectively. Initial temperature in the three pilot reactors were 27.8, 27.8 and 29.2ºC for fermentations R1, R2 and R3, respectively. The optimal initial conditions of the inoculum along with these temperature values allowed a rapid start-up of the fermentation, obtaining a very similar profile during the first 8h. It is during this period that the thermophilic stage started in all replicates with an average temperature of 47-48ºC. After this period differences on the sOUR profile appeared in R1 (Figure 5a) in comparison with R2 and R3. It is possible that the initial conditions of the inoculum could have affected the performance of the pilot fermentations. Final sOUR and COC obtained at 24 h of fermentation for the three processes were 3.6 mg O_2 g^{-1} DM h^{-1} and 77.6 mg O_2 g^{-1} DM for R1, 3.9 mg O_2 g^{-1} DM h^{-1} and 65.5 mg O_2 g^{-1} DM for R2 and 4.9 mg O_2 g^{-1} DM h^{-1} and 71.8 mg O_2 g^{-1} DM for R3, respectively.

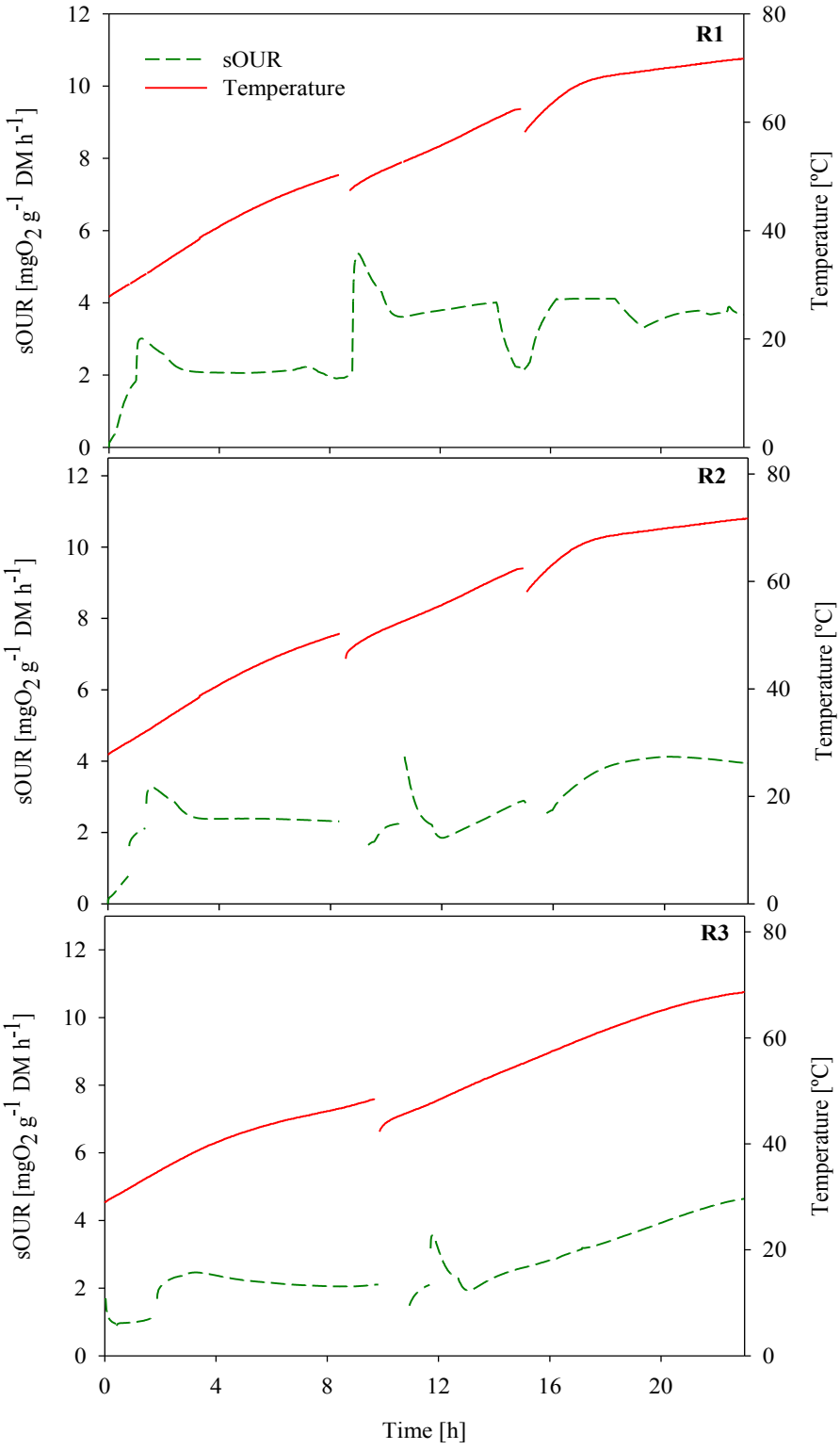

Figure 6.5. Operation profile of OUR and temperature of the three replicates of the pilot solid-state fermentation R1, R2 and R3 (50L) using a propagated specialized inoculum with coffee husk as substrate.

Enzymatic activities obtained at 24 h of fermentation are presented in Table 2. ANOVA analysis showed that only BGase production presented no significant differences among the three fermentations. This enzyme is crucial during cellulose degradation, due to its role on cellobiose degradation. BGase relieves the cellulase system for cellobiose accumulation and therefore, endo- and exo-glucanases inhibition. BGase controls endo and exoglucanase action, which can act with a synergetic effect, depending on the characteristics of the substrate (Kuhad et al., 2016; Rabinovich et al., 2002).

FPase activity obtained in R2 and R3 presented no significant differences, as well as Xyl activity for R1 and R3. Differences among the enzymatic yields can be attributed to the complexity of the substrate and the nature of cellulolytic enzymes. Endoglucanases, reflected as CMCase, bound randomly into any section of cellulose structure. This action directly affects all the following hydrolysis steps with synergetic and repressive actions, which will affect the entire biodegradation process (Kostylev & Wilson, 2012).

Additionally, in Table 6.2 is presented the average of enzymatic activities of the three fermentations with their respective variation coefficient.

Table 6.2.Cellulase and xylanase yields obtained in the three replicates of the pilot solid-state fermentation. Values of each enzymatic activity that do not share a letter are significantly different. n.m not measured.

Replicate	FPase ($FPU\ g^{-1}DM$)	CMCase ($U\ g^{-1}DM$)	BGase ($U\ g^{-1}DM$)	Xyl ($U\ g^{-1}DM$)
R1	3.63±0.20(A)	1.38±0.14(C)	0.12±0.00(E)	42.71±2.62(F)
R2	2.86±0.25(B)	(n.m)	0.12±0.00(E)	(n.m)
R3	2.91±0.37(B)	3.62±0.72(D)	0.15±0.06(E)	47.71±4.53(F)
Average ± s.d	3.08±0.49	2.51±1.31	0.13±0.04	44.51±8.30
Deviation coefficient (%)	16	52	27	19

FPase, BGase and Xyl production were 3.08±0.49 FPU g^{-1} DM, 0.13±0.04 U g^{-1} DM and 44.51±8.3 U g^{-1} DM with a variation coefficient of 16, 27 and 19%, respectively. These variation coefficients can be considered relatively high; however, when working with organic wastes the situation is different. Variation coefficients only from proximal composition of the solid wastes can range between 10 to 50% (Leroy et al., 1992), therefore it is likely that any process that includes solid wastes has an intrinsic high variation. Moreover, using mixed enzymatic compounds obtained by SSF in highly controlled processes can achieve variation coefficients of nearly 10% (Martínez-Ruiz et al., 2008). In this sense, the variation coefficients obtained in this work can be considered as acceptable, taking into account that it is a complex process using non sterile material in a pilot scale.

As for CMCase, the average production was 2.51±1.31 U g^{-1} DM with a variation coefficient of 52%. The high variation of the production of CMCase can be expected due to their random action into any section of the cellulose structure and the impossibility to control its action, as mentioned before.

In light of these results combined with the differences among propagation reactors, it seems of great importance to regulate or control the initial temperature of the fermentations, in order to reduce possible variations in the fermentations. Initial differences determine the performance of the pilot fermentations with a major influence on the development of microbial communities.

e) Microbial characterization

Bacteria

A total of 30 bacterial families were identified in the final products of propagation reactors (P1, P2 and P3) and the pilot fermentations (R1, R2 and R3). The full composition is presented in Figure 6.6.

As seen in Figure 6, there are three main dominant families in all assessed samples: *Paenibacillaceae*, *Xanthomonadaceae* and *Sphingobacteriaceae;* these are also the main families identified in the original inoculum obtained in Chapter 5, Figure 5.5. The sum of these families account for a 45.6, 47.9 and 43.4% for P1, P2 and P3 and 40.5, 40.9 and 39.4% for R1, R2 and R3, respectively. In this context, the addition of the specialized inoculum to a propagation reactor and then as inoculum for a pilot SSF generated great similarities among the bacterial diversity at family level. Regarding the samples obtained from the propagation reactors, P1 and P2 presented similar relative abundance of the microbial populations; however, P3 showed slight differences. For instance, P1 and P2 showed low presence of the family *Flavobacteriaceae* (2.1 and 2.4% for P1 and P2), while P3 showed significant abundance this family (13.4%). The main specie found in this family was *Flavobacterium anatoliense,* which is a strict aerobic bacteria isolated from several environments that has been

reported as unable to grow on cellulose and, even more, it has been reported not to be able to hydrolyze complex polysaccharides (Kacagan et al., 2013). In spite of the differences, the predominant specie found in all propagation reactor samples was the same: *Pseudoxanthomonas taiwanensis*. This bacterium was found predominant in the original specialized inoculum (Cerda et al., 2017) and it was able to survive and colonize the propagation reactors, achieving a relative abundance of 14.7, 14,9 and 9.6% in P1, P2 and P3 respectively. *P. taiwanensis* has been widely related to cellulose degradation systems, for its β-glucosidase production and the potential enhancement of the growth of other cellulolytic bacteria (Eichorst et al., 2013).

The fermented solids obtained from the propagation reactors were used as inoculum for the pilot SSF. The final product of the latter was analyzed for bacteria identification, which results are presented in Figure 6.6.

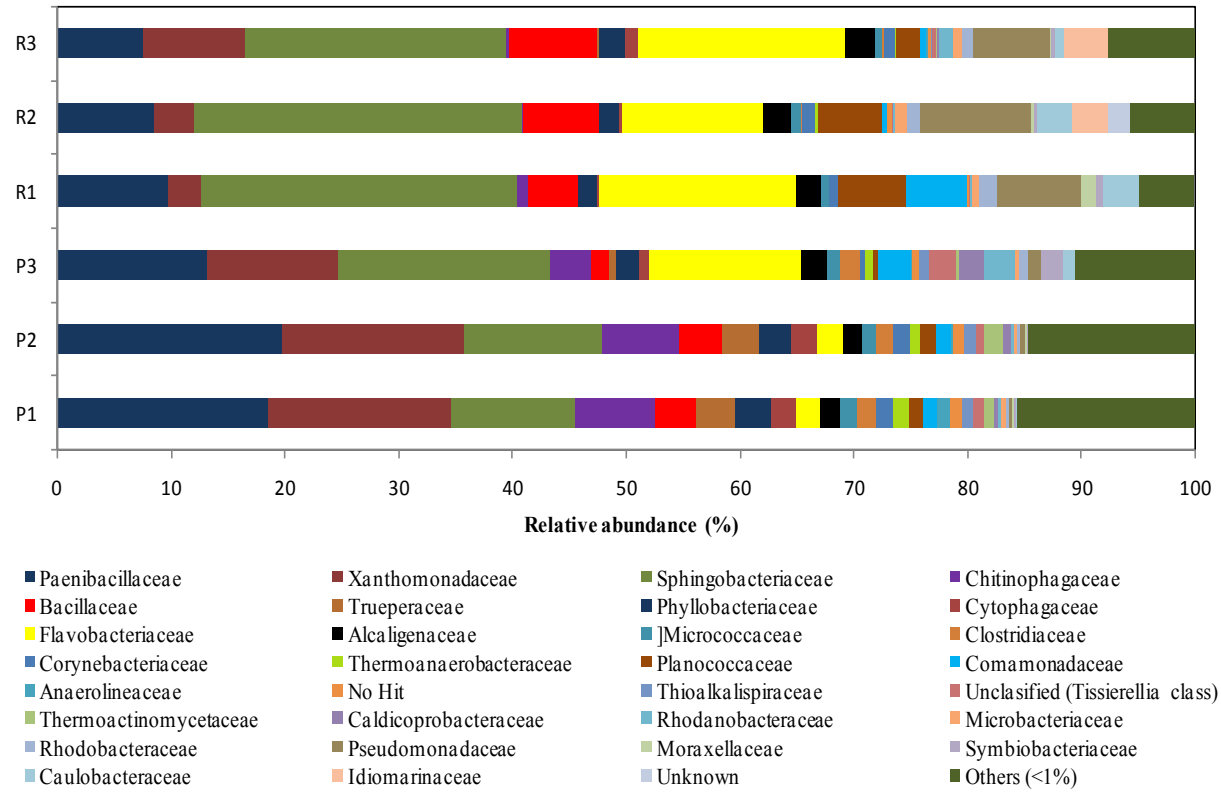

Figure 6.6. Bacterial distributions at the family level according to the16s sequencing for the final products of propagation reactors (P1, P2 and P3) and pilot fermentations at highest cellulase activity (R1, R2 and R3). Only families with a relative abundance >1% in all samples are depicted. Families detected with a relative abundance < 1% in these samples are grouped as "others".

The results showed that the same families predominant on propagation reactors were predominant on the pilot SSF. In spite of the great resemblance, there is a shift on the predominant specie when comparing with propagation reactor. In this sense, *Sphingobacteriaceae* family became more relevant when compared with the propagation reactors, with a 27.9, 28.9 and 22.9% of relative abundance for R1, R2 and R3, and 10.9, 12.2 and 18.8 for P1, P2 and P3, respectively. The harsh conditions generated during pilot SSF could have affected the development of the different microorganisms in the solid matrix. In this context, strong thermophillic and alkaline environments favored the growth of bacteria able to thrive at these conditions, among them *Sphingobacterium thermophillum* (15.8, 11.1 and 9.7% for R1, R2 and R3) and *Sphingobacterium arenae* (3.6, 7.8 and 6.8% for R1, R2 and R3). The new dominant species relegate *P.taiwanensis* to a specie with a minor presence in the solid matrix, with a relative abundance of 2.4, 2.7 and 8.2% for R1, R2 and R3, respectively. *S. thermophilum* and *S. arenae* have been reported to be present during the thermophillic stage of composting processes with a potential for β-glucosidase production (Yabe et al., 2013). In addition, it has been found that several cellulase obtained under these conditions can be halotolerant (Gladden et al., 2014), which is of great interest when a it is considered to use in a potential use in bioethanol production.

In general, the relative abundance at family level presented high similarities in propagation reactors and the pilot SSF triplicates. Also, it is interesting to highlight the fact that families with relative abundance below 1% (registered in Figure 6.6 as "others") are higher in the propagation reactors than in the pilot SSF. This indicates a further reduction on biological diversity probably associated with the pilot SSF conditions as confirmed by the Shannon diversity index for propagation reactors P1, P2 and P3 were 2.6, 2.4 and 2.2 and for pilot SSF were 1.8, 1.7 and 1.9 for R1, R2 and R3 respectively.

Fungi and yeasts

A total number of 21 fungal families have been identified in the final products of propagation reactors (P1, P2 and P3) and the pilot fermentations (R1, R2 and R3). The full composition is presented in Figure 6.7. As seen in this figure, *Phaffomycetaceae* was found as the dominant family in all the assessed samples. This family accounted for a total of 74.4, 64.5 and 48.2% for P1, P2 and P3, and 79.7, 61.9 and 77.8% for R1, R2 and R3, which represented most of the biological diversity of the process. This family was also found as predominant in the initial inoculum, with a relative abundance of 43.2% (Chapter 5, Figure 5.6).

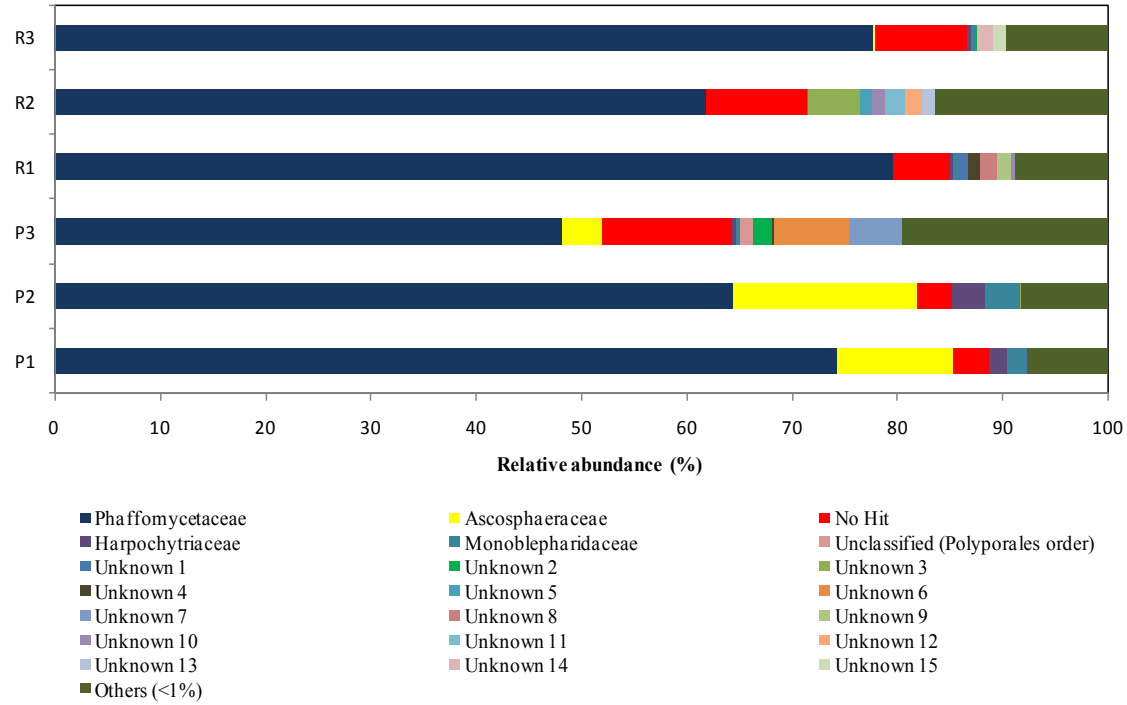

Figure 6.7. Fungal distributions at the family level according to the ITS sequencing for the final products of propagation reactors (P1, P2 and P3) and pilot fermentations at highest cellulase activity (R1, R2 and R3). Only families with a relative abundance >1% in all samples are depicted. Families detected with a relative abundance < 1% in these samples are grouped as "others".

These results proved, at family level, a consistent propagation and colonization of the specialized mycobiota present in the initial inoculum. Even more, when performing the pilot SSF, the biological diversity was further reduced, maintaining *Phaffomycetaceae* as the predominant family and increasing its relative abundance. Two species of this family appear as dominant in propagation reactors: *Cyberlidnera jardinii* with 64.3, 55.1 and 3.5% for P1, P2 and P3 and *Barnettozyma californica* with a 10.1, 9.4 and 43.6% for P1, P2 and P3 respectively. These two species were also found as dominant in the initial inoculum (Chapter 5, Figure 5.6) with a relative abundance of 34 and 9.1% for *C.jardinii* and *B. californica*, respectively. There is a clear difference on the relative abundance of these species in P3 when compared with P1 and P2. Both of these species are related to hemicellulose degradation with potential tolerance to grow in the presence of lignin hydrolyzates, which is of great interest for a potential use on bioethanol production (Morais et al., 2013; Nordberg et al., 2014).

Identified families from the fermented solids from the pilot SSF are presented in Figure 6.7. These results presented great similarities, at family level, with propagation reactors, although a major shift occurred at specie level. In pilot SSF the dominant specie was *B. californica* with a 72.6, 59.7 and 72.8% of relative abundance, leaving *C. jardinii* as a minor component of the mycobiota with an abundance below 3% in all cases. *B. californica*, as mentioned before, is related to hemicellulose degradation and an important xylanase producer; however, it has been also reported as an enzyme producer for lignin degradation (Martorell et al., 2012). This specie is a producer of tyrosinase, which is an enzyme able to oxidize phenolic compounds that can potentially enhance lignin hydrolizates degradation. The great abundance of this specie in the pilot SSF can, in a way, explain the high degradation of lignin in the reactors (above 11% of degradation).

In general, in the pilot SSF, most of the identified mycobiota presented high hemicellulose and lignin degradation potential. Improvements of degradation on these structures can make cellulose structure more accessible for the enzymes to act and, therefore, increase cellulase production. In addition, biological diversity obtained in the pilot reactors was reduced when compared with the propagation reactors. Shannon index for R1, R2 and R2 were 0.44, 0.62 and 0.50 and 1.02, 0.99 and 0.97 for P1, P2 and P3, respectively.

The most important result on these experiments relies on the fact that when the operational conditions of the reactors are similar, the biomass that is able to colonize the reactors is the same, even when the fermentation is carried out using non sterile substrates. On the contrary, when the scale or operational conditions such as temperature changed, there is a shift in microbial communities affecting slightly cellulase and xylanase production.

As a final remark, it has to be stated that there is no previous research performed on the reproducibility of pilot scale SSF. Many aspects considered as SSF drawbacks have been undertaken, resulting in the development of a more robust and sustainable process. The use of a standard inoculum using wastes as substrate makes this technology more consistent for its application at larger scales, with the additional economical benefit of using organic wastes as raw material.

6.4. Conclusion

The use of a specialized inoculum on a pilot scale SSF presented very promising results. Cellulase and xylanase production at pilot scale was successful, achieving values in accordance with the literature in a short period of time. The gaseous emissions detected showed that the process generates low amounts of pollutants, below the reported emissions in SSF or similar processes. Only VOCs were

emitted in significant amounts, obtaining almost negligible emission values of NH_3, N_2O and CH_4. The triplicates carried out for the pilot SSF presented small differences in enzymatic production; however, in general, the values remained in the expected range of production. The strong conditions of temperature and pH provided in the pilot SSF affected the productive process and also the developing of the microbial diversity. Biological diversity reduction, reflected on Shannon index, was observed for both bacteria and fungi when the process was scaled up. Considering the possibility of a more efficient waste management with the production of a value-added product, the proposed process can be described as a sustainable, robust and environmentally friendly process.

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CHAPTER 7. INOCULATION STRATEGIES FOR AMYLASE PRODUCTION IN A SEQUENTIAL BATCH OPERATION

This chapter has been published in Journal of Environmental Chemical Engineering. "Long term enhanced solid-state fermentation: Inoculation strategies for amylase production from soy and bread wastes by *Thermomyces* sp. in a sequential batch operation".

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Summary

In parallel to the experiments for cellulase production explained in detailed in chapter 5, other experiments were carried out for the production of amylase using soy fiber and bread waste as the substrate. Amylase production was assessed due to the fact that during this period of time a postdoctoral researcher: Dr. Mamdouh El-Bakry, was carrying out lab scale SSF using thermophilic bacteria as inoculum, with positive results. In light of the success on preeliminar tests using 4.5 L reactors, it was decided to develop different reinoculation strategies, in order to reach a continuous operation. The full description of the experimental methodology and the obtained results are detailed in the following section. In addition, a brief introduction to amylase production "state of the art" is presented.

7.1 Reinoculation strategies to enhance amylase production in solid-state fermentation using *Thermomyces sp*. as inoculum.

7.1.1 Background

In the last decade solid-state fermentation (SSF) has received increased attention from researchers as a bio-based process. This technology allows for the valorisation of wastes for the production of valuable commodities such as enzymes (Saxena et al., 2011; El-Bakry et al., 2015; Pitol et al., 2016).

SSF presents some advantages in comparison with conventional fermentations such as higher productivity or reduced energy requirements, and low wastewater output (Pitol et al., 2016). However, important drawbacks are described concerning the SSF scale up largely due to heat transfer, sterilization costs and culture heterogeneity (Mitchell et al., 2006; Casciatori et al., 2016). Abraham et al. (2013) proposed to perform SSF as a thermophilic batch process under near-adiabatic conditions, similar to the composting process which is a robust, well known, easily scalable and low-cost process. This self-heating process does not involve heating/cooling costs since the temperature evolve due to the heat released in the biodegradation process. Based on this proposal, El-Bakry et al. (2016) proved that thermophilic strains inoculated on non-sterile wastes were able to grow and compete with autochthonous microbiota and significantly increase the protease production. Still the setting up of a long term operation in continuous or semi-continuous regime remains a challenge and very few attempts have been reported (Alkasrawi et al., 2013; Cheirsilp and Kitcha, 2015; Farinas, 2015).

Enzymatic products such as amylases have great potential in different biotechnological applications such as food, fermentation, detergent, textile and paper industries (Farinas, 2015). Some thermophilic amylase-producers microorganisms have been found during the highest bio-oxidative activity stage

during composting processes(Zeng et al., 2005; López-Gonzalez et al., 2015a; López-Gonzalez et al., 2015b). In addition, it has been well established that these microorganisms are able to produce substantial amounts of amylase in SSF systems (Babu and Satyanarayana, 2005; Kunamneni et al., 2005; Saxena et al., 2011).

Several attempts for the optimization of enzymatic production have been reported, such as nutritional supplementation for microorganism growth (Petrova et al., 2000; Mukherjee et al., 2009; Hashemi et al., 2013), use of engineered strains (Petrova et al., 2000; Rajagopalan and Krishna, 2010) or operational strategies such as batch or fed-batch (Tao et al., 1998; Astolfi et al., 2011; Cheirsilp and Kitcha, 2015). However, most SSF for the production of enzymatic compounds have been tested at lab scale and under sterile substrate conditions (Farinas, 2015).

The first aim of this work was to develop a SSF process for the production of amylases using a thermophilic strain growing on non-sterile wastes in the lab scale (500-mL reactors) and bring it to the bench scale (10-L reactors), based on the proposal of Abraham et al. (2013) and El-Bakry et al. (2016). Second goal was to perform a first approach to the assessment of the effect of inoculation strategies in sequential batch operation in order to develop a long term operation process in a solid-state fermentation configuration at the bench scale. SSF of organic wastes operated in sequential batches was evaluated for the production of amylases using thermophilic and amylase producer microorganisms (*Thermomyces* sp. and *Geobacillus* sp.).

7.1.2 Materials

Soy fibre residues (SF) were provided by Soy Nature (Barcelona, Spain). Bread waste (BW, expired pre-cooked baguettes) was procured from a local market. Both wastes were obtained in a sufficient amount to do the entire experiments with the same material as substrate. Materials were used as received in 500 mL reactors and in the case of the inoculation strategies experiments, aliquots were frozen separately prior to their use. Characterization of both substrates is presented in Table 7.1. Moisture was initially adjusted to 70% using tap water in all the experiments.

In these experiments, the inoculum was selected among two thermophilic and amylase producers: *Geobacillus* sp. ATCC-31198 and *Thermomyces* sp. ATCC-200065. These microorganisms were incubated in Petri dishes on supplier´s recommended agar media: ATCC® Media 1207 and ATCC® Media 350 respectively.

Table 7.1. Characterization of raw materials used on SSF (average ± standard deviation) * all percentages in dry basis except for dry mass, in wet basis.

Parameter*	Soy fiber (SF)	Bread waste (BW)
Dry mass (%)	17.7±1.3	62.8±0.8
Organic matter (%)	96.8±1.3	96.95±0.2
C (%)	67.5±1.8	48.1±2.1
N (%)	4.4±0.7	2.5±0.2
pH	7.0±0.7	6.1±0.1
Starch (%)	3.1±0.1	75.7±0.1

Both strains were grown in a liquid medium containing the same formulation than that used for Petri dishes. Final pH was adjusted to 7.0 ± 0.2 and 100 mL were incubated in a 500 mL Erlenmeyer flask at 150 rpm and 55ºC for 16h. Final culture broth was used as inoculum in all lab scale fermentations and in the propagation reactor for sequential batch experiments in a 10% w/w ratio.

7.1.3 Experimental procedure

a) Selection of inoculum type and size.

Solid-state fermentations were carried out at 55°C for 7 days in 500 mL Erlenmeyer flasks with 100 g of wet substrate and at fixed airflow of 20 mL min^{-1}. Also, 10 g of wood sticks were added to the substrate as a bulking agent to provide porosity. Oxygen content was measured continuously in the reactors gas output. Specific oxygen uptake rate (sOUR) was calculated with equation 1. These experiments were performed using the set up described in chapter 3, section 3.1.4

Samples were taken daily for amylase activity determination. Firstly, *Geobacillus* sp. and *Thermomyces* sp. were evaluated in the solid-state fermentation of SF and BW for the production of amylases and the best inoculum was selected for further experiments. Secondly, mixtures of different SF:BW ratios (0:100, 10:90, 50:50, 90:10 and 100:0 (w/w) were evaluated and the best mixture selected for following experiments in reactors. Experiments were carried out in triplicates.

b) Scaling-up of amylase production

The scaling-up effect on the process defined at lab scale was assessed by monitoring two 4.5 L reactors (described in chapter 3, section 3.1.5) for 14 days in order to have a complete profile of all relevant parameters of fermentation. One reactor was operating without inoculation as control. A second reactor was inoculated directly with liquid *Thermomyces* sp. culture. Amylase activity was determined at days 0, 4, 7, 10 and 14 of operation.

c) Inoculation strategies for operation in sequential batches

Experiments were performed using the same system that in section 7.1.3.b.

One first batch was performed as a propagation reactor (PR) and it was inoculated directly with *Thermomyces sp*. liquid culture in a 10% w/w ratio according to El-Bakry et al. (2016). Samples were taken from this reactor in two different moments to act as inoculum for the following batches. Three sequential batches (SB) were performed and each batch was inoculated with solids from the previous batch. Inoculation size was 10% w/w (fermented solids / fresh solids in the reactor) in all cases. The three strategies assayed are described below:

- Strategy MOUR: Using as inoculum the fermented solids obtained at the moment of maximum sOUR, that is, solids with Thermomyces sp. at maximum growth rate.
- Strategy MAA: Using as inoculum the fermented solids obtained at the end of the process, that is, solids obtained at the moment of maximum amylase activity (AA).
- Strategy MAAE: Using as inoculum the solids obtained after amylase recovery when its maximum activity was reached at the end of the process.

A scheme of the inoculation strategies is presented in Figure 7.1

Sampling was performed on a composite sample of the reactor, after homogenizing all the reactor material at the time of substrate exchange. These samples were analyze on its amylase activity production and starch content. In addition, routine analysis described in chapter 3, section 3.3 were performed to the solid samples.

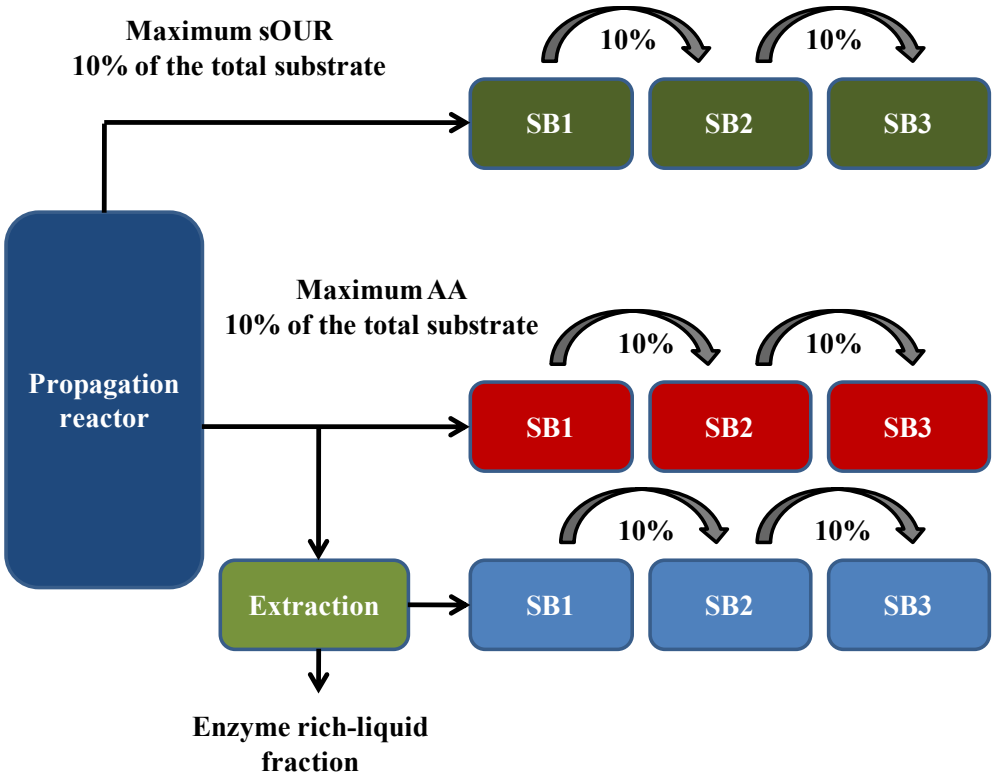

Figure 7.1. Scheme of the reinoculation strategies developed to enhance amylase production.

7.1.4 Results

a) Selection of inoculum type and size.

Fig. 7.2 shows sOUR and amylase activity profiles for experiments in 500 mL reactors for inoculum selection using SF (Fig. 7.2a and b) and BW (Fig. 7.2and d) as substrate. As observed in Fig. 7.2a and 1c, higher sOUR values were obtained in reactors inoculated with *Thermomyces* sp. and *Geobacillus* sp. strains than in non-inoculated reactors, when using SF and BW as sole substrate. This increase was more obvious in the case of soy fibre, which presented a higher sOUR than bread waste confirming a higher biodegradability. sOUR values were maximum at around 24h in all the cases and slowly decreased after that. Similar results were obtained for amylase activity: inoculation showed a positive effect on amylase production for both substrates, and higher enzymatic yields were obtained for SF than for BW. Maximum activity was obtained 48 h after reaching the maximum value of sOUR and slightly decreased after that. For both substrates, reactors inoculated with *Thermomyces* sp. presented higher values of sOUR and AA than reactors with *Geobacillus* sp. Best process performance was obtained using SF and *Thermomyces* sp. with a maximum sOUR of 11.8 mgO_2 g^{-1}DM h^{-1} and a

maximum amylase production of $34 \cdot 10^3$ U g^{-1}DS. Fungi were expected to be better inoculum due to their ability to produce all required enzymes to fully degrade polymeric compounds and also to be able to survive in extreme conditions (De Gannes et al., 2013). Also, fungi play a key role in solid fermentations such as composting processes, where *Thermomyces* sp. has been detected during thermophilic and highest amylolytic activity production stage (Zhang et al., 2014; López-Gonzalez et al., 2015). These authors reported the predominance of *Thermomyces* sp. in temperatures ranging 50-60ºC and alkaline pH, which is also the range of its optimum growth regarding temperature and pH.

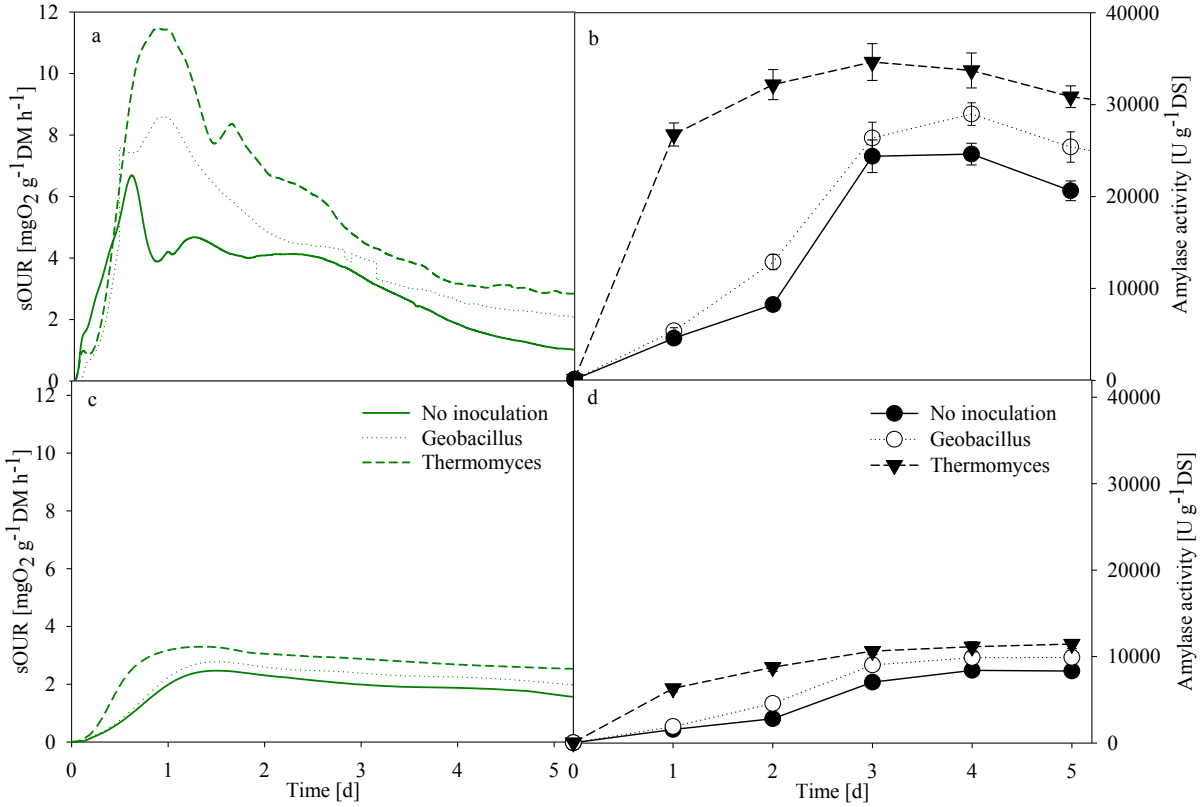

Figure 7.2. Solid-state fermentation at 500 mL scale using *Thermomyces* sp. and *Geobacillus* sp. as inoculums. Profiles of sOUR and amylase activity are presented using soy fiber a) and b) and bread wastes c) and d) respectively.

In consequence, *Thermomyces* sp. was selected as the best strain. De Castro et al (2013) reported the synergistic effect of different wastes used as substrates for amylase production by SSF. Accordingly, further experiments were performed with different mixtures of SF and BW to assess this potential synergistic effect. Table 7.2 summarizes maximum sOUR and amylase activity for all evaluated mixtures using different ratios of SF:BW. It was found that sOUR and amylase production were improved at high SF content due to its higher content and availability of easily biodegradable organic matter. Also the mineral content of SF is higher than BW content, especially in Mg, P, and K which

are required for the growth of amylase producing microorganisms (Tuncel et al., 2014; Kim et al., 2015). Starch contained on BW most probably acted as inducer for amylase production as reported by some researchers (Tao et al., 1998; Mukherjee et al., 2009) and nutrients present on SF provided proper conditions for the proliferation of microorganisms. It has been reported that several cations present in great amounts on SF such as Ca^{2+}, acted as stabilizing agent or co-factors for amylases (Petrova et al., 2003; Mukherjee et al., 2009), generating an amylase activity increase, which is in accordance with obtained results.

All the mixtures showed a maximum amylase production on day 4 of process. Maximum amylase activity was obtained at a 90:10 SF:BW ratio, with a value over 39.9 $\cdot 10^3$ U g^{-1}DS and maximum sOUR of 11.5 mgO_2 g^{-1}DM h^{-1}. Lowest amylase production was obtained with BW as sole substrate, with a maximum activity of $10.9 \cdot 10^3$ U g^{-1}DS. As it is possible to observe in all enzymatic profiles, amylase production is not directly related to maximum biological activity or growth. This fact was expected as amylase production is regulated by starch inducible system and repressed in presence of glucose or mannose isomers (Song, 1990). Maximum amylase was observed 48h after maximum sOUR as also reported by Kunamneni et al. (2005) and Petrova et al. (2003).

Table 7.2. Maximum sOUR and amylase activity obtained in SSF using different SF:BW ratios.

SF:BW (%w/w)	Maximum sOUR (mgO_2 g^{-1}DM h^{-1})	Maximum amylase activity (10^3 U g^{-1}DS)
0:100	3.4	10.9 ± 0.5
10:90	4.1	14.7 ± 0.7
50:50	5.8	18.9 ± 0.9
90:10	11.5	39.9 ± 0.9
100:00	11	34.6 ± 1.2

b) Scale-up effect on amylase production.

The selected process (substrates mixture 90:10 w/w and *Thermomyces* sp) was evaluated in 4.5 L reactors and results are presented in Figure 7.3. Maximum sOUR and temperature were obtained at day 2 of operation in both control and inoculated reactors. As observed at lab scale, maximum biological activity did not match with amylase maximum production, which was obtained 48h after the maximum sOUR (day 4) in both cases. There was a clear effect of inoculation in terms of biological activity and amylase activity production (El-Bakry et al., 2016). *Thermomyces* sp. inoculated reactor presented a temperature of 65ºC and a maximum sOUR of 11.1 mgO_2 g^{-1}DM h^{-1} which represented an 8 and 47% increase respectively in comparison with non-inoculated reactors. Also, using *Thermomyces* sp. as inoculum presented a positive effect on amylase activity with a 36% increment in comparison with control reactor, reaching a maximum of $41.03 \cdot 10^3$ U g^{-1}DS.

Similar results were obtained at 4.5 L and 500 mL scales for maximum sOUR and amylase activity. This confirms the easy scalability of the self-heating SSF and its suitability to work with thermophilic strains with non-sterile substrates.

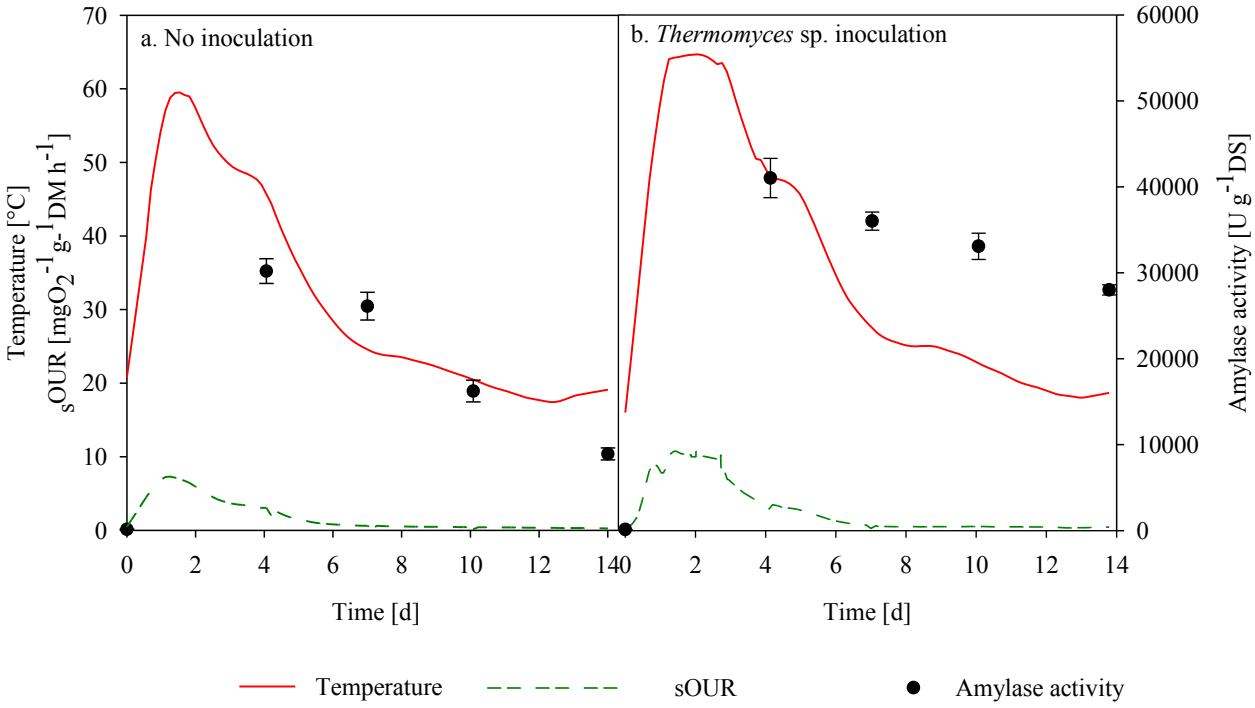

Figure 7.3. Solid-state fermentation at 4.5 L scale using SF:BW 90:10 ratio as substrate. Profiles of sOUR, temperature and amylase activity are presented using a) no inoculation, b) *Themomyces* sp. inoculation.

c) Inoculation strategies for operation in sequential batches.

Process profile for the propagation reactor is presented in Figure. 7.4a, 7.5a and 7.6a. A lag phase of nearly 2 days was observed. This was due to the use of previously frozen substrates. As reported by Pognani et al. (2012), freezing for less than one year does not affect the biodegradable organic matter content or the biological activity measured as sOUR, but a lag phase may appear. Thermophilic conditions were reached after that and remained nearly for 48h, achieving maximum sOUR of 23 mgO_2 g^{-1}DM h^{-1} after sampling the reactor. Sampling sOUR was 17 mgO_2 g^{-1}DM h^{-1} and obtained amylase activity of $31.7 \cdot 10^3$ U g^{-1}DS. Total oxygen consumed was 725 mgO_2 g^{-1}DM in 5 days. At the end of the fermentation (48h after maximum sOUR) amylase activity was 15% higher with a value of $36.6 \cdot 10^3$ U g^{-1}DS.

- Strategy MOUR

Figure 7.4 presents the process profiles for PR plus three sequential batches (SB) performed with inoculation using solids obtained at the moment of maximum sOUR (gap in temperature curve of SB2 was due to data acquisition failure). In all four cases, maximum sOUR was achieved after sampling, which could be of importance considering the quality of the inoculums for the sequential batches (El-Bakry et al., 2016).

The three sequential batches presented maximum temperatures ranging between 53-61°C similar to PR. As for sOUR profile, maximum values were obtained also after sampling, reaching values of 21, 23, 19 and 19 mgO_2 g^{-1}DM h^{-1} for PR and SB 1, 2 and 3 respectively (Figure 7.4).

Amylase activity obtained during maximum sOUR were 31.7, 21.9, 7.1 and $43.0 \cdot 10^3$ U g^{-1}DS and during maximum AA 36.6, 39.1, 47.2 and $29.3 \cdot 10^3$ U g^{-1}DS for PR and SB1, 2 and 3 respectively.

In general terms, sOUR increase and lag phase reduction was only obtained during SB 1. Latter inoculations resulted in lower or equal sOUR, obtained only after homogenization of reactors in the sampling process. In addition, each sequential inoculation produced an increase of 0.8, 0.9 and 15.1% in total oxygen consumption in comparison with propagation reactor.

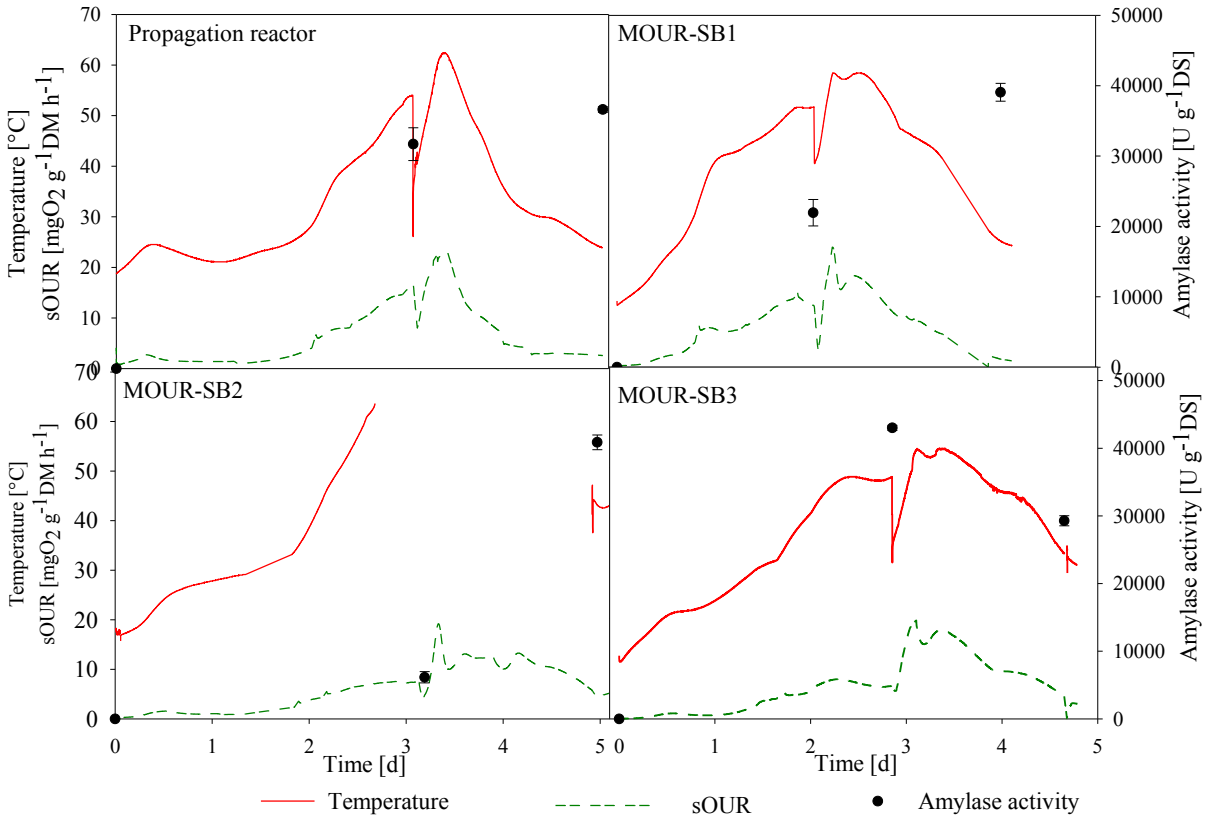

Figure 7.4. MOUR strategy in 4.5 L reactors using SF:BW 90:10 ratio as substrate. Profiles of sOUR, temperature and amylase activity are presented for PR and three sequential batches MOUR-SB1, MOUR-SB2 and MOUR-SB3.

Inoculation with solids from maximum sOUR period from PR was expected to provide a reduction in lag phase and an increase in sOUR. This is based on the fact that fungi from the class Eurotiomycetes, such as *Thermomyces* sp. have been found predominant in thermophillic stage during composting processes (Ugwuanyi et al., 2004; López-Gonzalez et al., 2015a; López-Gonzalez et al., 2015b), indicating that a suitable environment for inoculated *Thermomyces* sp. is provided. This fact was observed through all sequential batches, where a slight increase in sOUR and total oxygen consumption was obtained.

- Strategies MAA and MAAE

Figure 7.5 presents process profiles for PR and SB 1, 2 and 3 for MAA strategy. Temperature profile was similar in all four processes, reaching values near 60°C. Maximum sOUR values were in the range of 21-25 mgO_2 g^{-1}DM h^{-1} except for the first sequential batch (Figure 7.5b). However, total oxygen consumption presented a sustained increase of 1.9, 54.2 and 76.6% in the SB 1, 2 and 3 respectively in comparison with the PR. Also lag phase was gradually reduced in each SB, and sOUR peaked in the mesophilic phase in SB 2 and 3 with values 8-9 mgO_2 g^{-1}DM h^{-1}.

During thermophilic period (temperature >45ºC), high amylase activity was produced in all batches. Highest amylase production of $228.9 \cdot 10^3$ U g^{-1}DS was obtained at the end of the MAA-SB2, which represents a 500% increase in comparison with the propagation reactor. In the MAA-SB3, amylase activity was $156.9 \cdot 10^3$ U g^{-1}DS, 329% higher than PR.

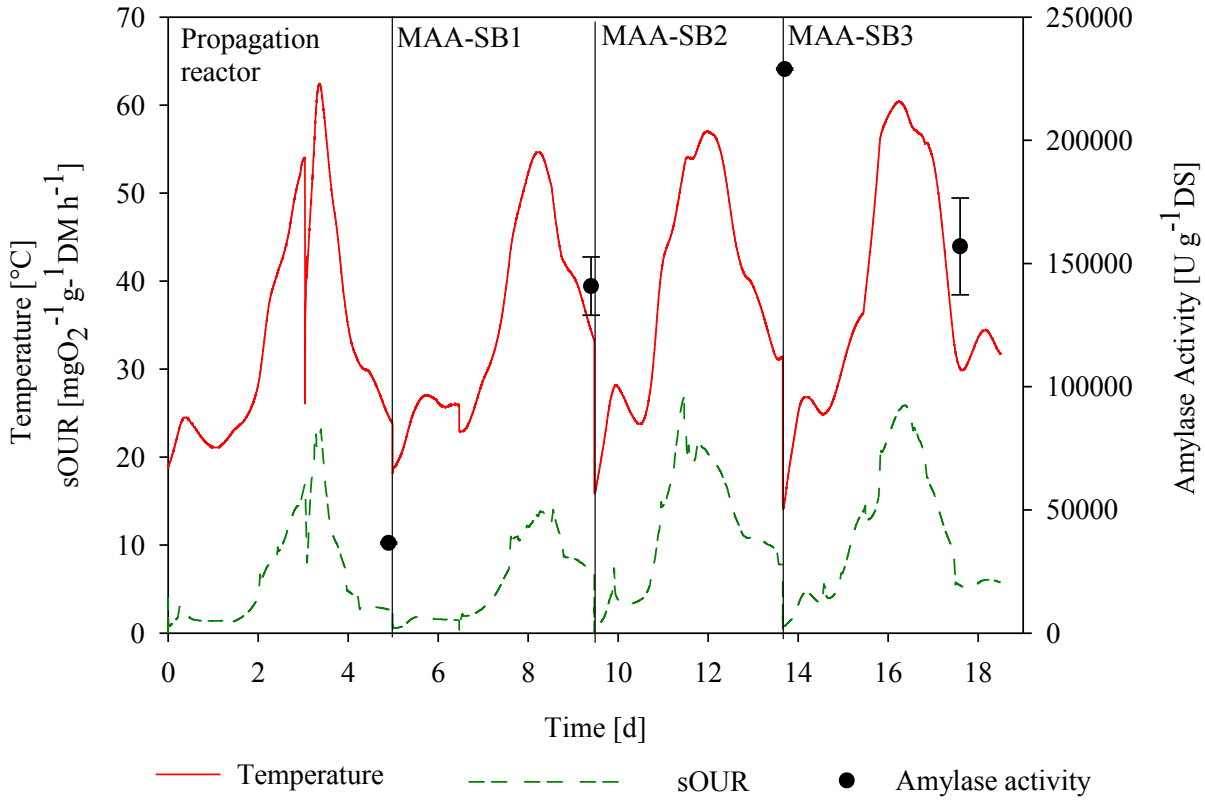

Figure 7.5. MAA strategy in 4.5 L reactors using SF:BW 90:10 ratio as substrate. Profiles of sOUR, temperature and amylase activity are presented for PR and three sequential batches MAA-SB1, MAA-SB2 and MAA-SB3.

Figure 7.6 shows the process profile for PR and the three sequential batches, using final solids after the extraction of soluble and enzymatic components as inoculums. This solid material would be the actual final output of the process after amylase recovery as targeted product.

Maximum sOUR in the three SB were lower than in the PR. A 16.6 % decrease in the total oxygen consumption on first SB was obtained but increased by 1.1 and 23.2 % in SB 2 and SB 3. Thermophilic phase was longer in the three SB than in PR. Amylase activity was higher in the three SB than in PR, with an increase of 27, 98, 56% in SB 1, 2 and 3 respectively. Maximum activity of the sequential experiments was obtained at the end of batch 2, achieving a value of $72.4 \cdot 10^3$ U g^{-1}DS.

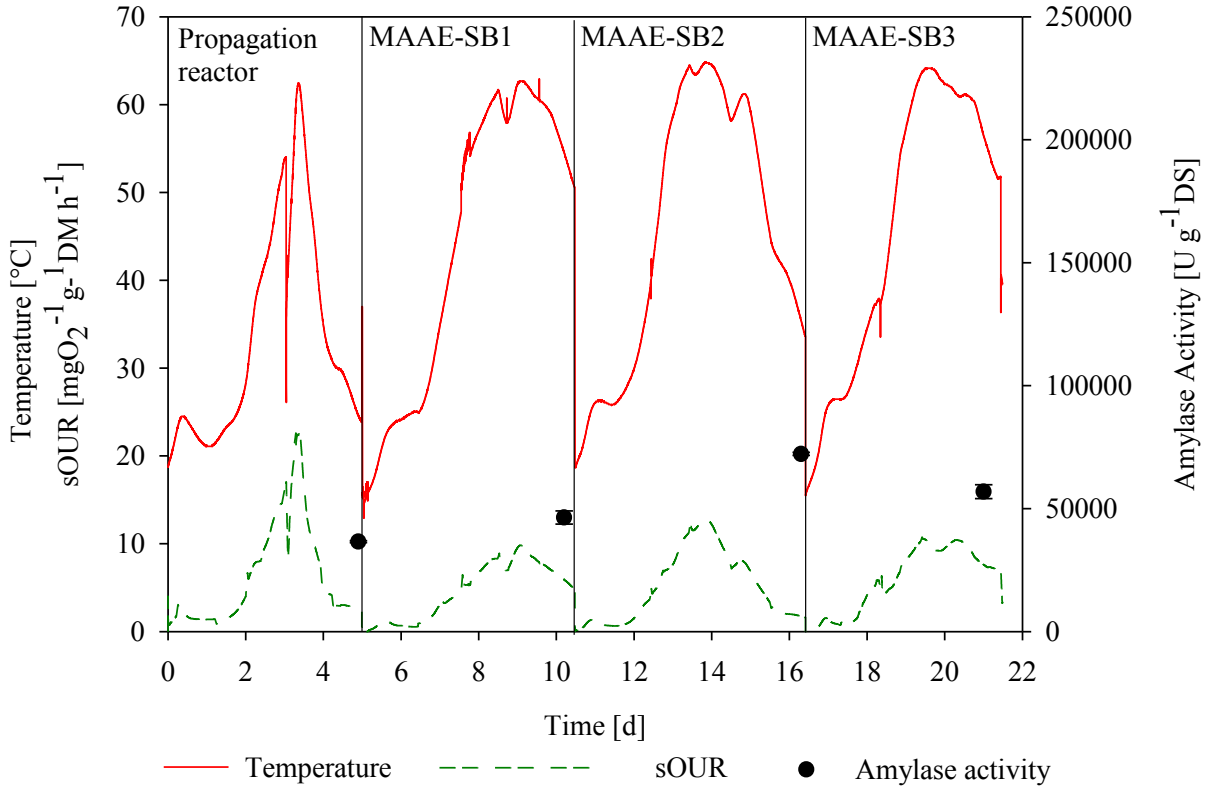

Figure 7.6. MAAE strategy in 4.5 L reactors using SF:BW 90:10 ratio as substrate. Profiles of sOUR, temperature and amylase activity are presented for PR and three sequential batches MAAE-SB1, MAAE-SB2 and MAAE-SB3.

Regarding MAA and MAAE strategies, temperature profiles and total oxygen consumption presented a different trend than PR. Temperature is known as a crucial parameter to describe microbial activity during aerobic fermentation of solid heterogeneous material. It has been stated that inoculation with different microorganisms to a solid state fermentation could lead to a rapid rate of temperature elevation and for a more prolonged time of high-temperature process (Jurado et al., 2015).

In MAA strategy, inoculation with fermented solid inoculum with high enzymatic and soluble content in sequential batches may have generated a fast hydrolysis of available substrate for the consumption of all present microorganisms. On the other hand, an extraction of soluble and enzymatic compounds in the inoculums could have generated a reduction in sOUR values and in the oxygen consumption rate, due to a lack of hydrolytic capacity in comparison with propagation reactor and the other evaluated batches with different inoculation strategies.

A reduction in amylase activity in the SB3 in both MAA and MAAE strategies can be attributed to several factors such as the possible repression on enzymatic synthesis (Babu and Satyanarayana., 1995; Mukherjee et al., 2009), presence of hydrolytic enzymes such as proteases (Abraham et al., 2013), to a depletion of the amorphous form of the solid substrate (Tao et al., 1998) or even more, to a change in the production profile of amylases.

It is a fact that sugars and low polymerization degree polysaccharides are normally fast consumed by microflora present during fermentation. It is reasonable to assume that with high production of amylase activity, high amounts of sugars are released which could cause catabolic repression of amylase synthesis. On the other hand, researchers have presented that catabolic repression at high reducing sugars content occurs even in metabolically engineered de-repressed strains (Rajagopalan and Krishnan, 2010).

The lack of literature about amylase production by inoculation strategies in sequential solid state fermentations and the many different methods of amylase activity determination made difficult to compare the obtained results with other reported researches. However, other enzymes have been produced in fed batch strategy (Cheirsilp and Kitcha, 2015), with results found in accordance to this study. In any case it is proved that the operation strategy is a key factor for the implementation of solid state fermentation at the commercial level. Further research shall focus on determining the number of sequential batches that can be performed following this strategy. Nevertheless, both MAA and MAAE strategies allowed a remarkable enhancement on amylase activity. In our opinion, both strategies are suitable for industrial operation: in strategy MAA, 10% of the solids would be diverted to inoculate the following batch prior to enzyme extraction; in strategy MAAE, 10% of the solids after extraction would be recycled to inoculate the next batch. To finally decide which the best strategy is, it would be necessary to perform a complete economical and environmental assessment.

7.1.4 Conclusion

Operation of SSF of soy and bread wastes in sequential batches has been proven as a suitable strategy for amylase production with *Thermomyces* sp. Additionally, it opens a new valorisation alternative for these wastes. A SSF process was operated for 19 days and the enhancement of amylase production was accomplished. In terms of amylase yield, the most suitable inoculation strategy was using fermented solids at the end of the batch to inoculate the following batch, obtaining a 500% increase in productivity and eliminating the need of producing fresh inoculum for each batch. Nonetheless this, the final strategy selection should consider economical and environmental aspects. The development

of these operational strategies could benefit process economics and provides the possibility of a continuous operation for amylases and potentially for other enzymes.

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APPENDIX

Appendix I. Calibration curve for FPase determination.

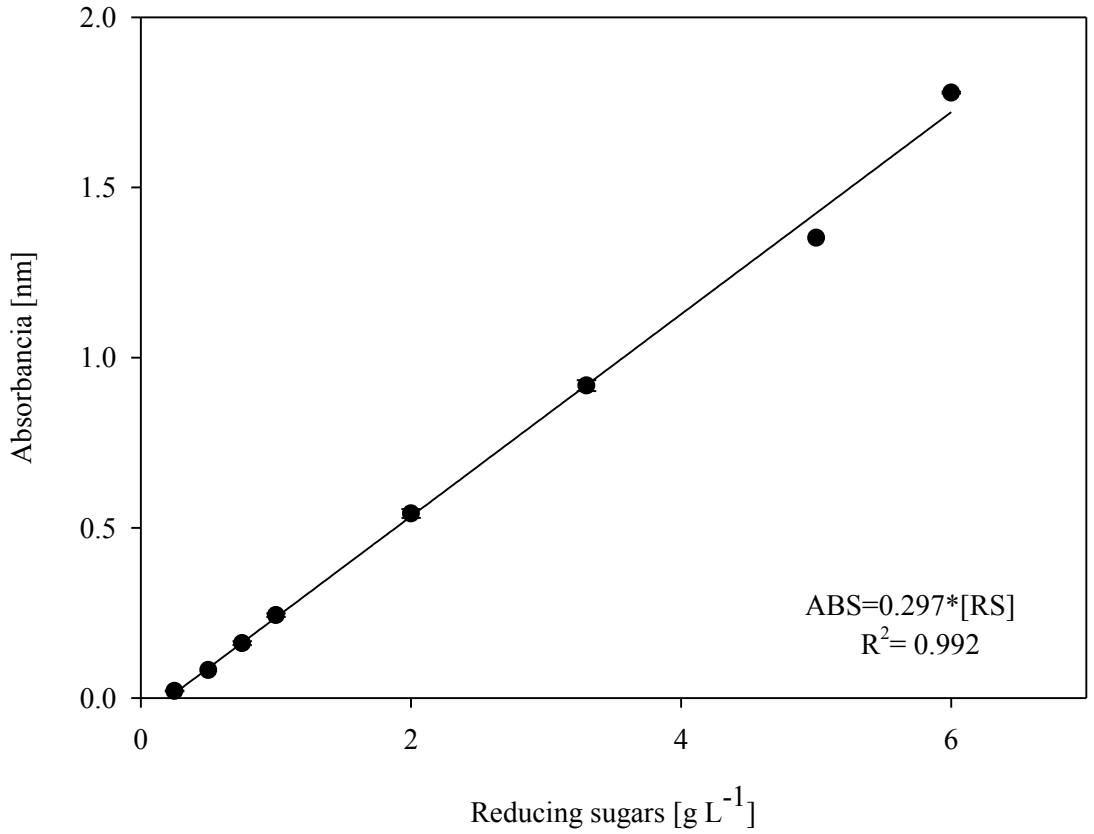


Figure I.

Appendix II. Calibration curve for CMCase determination.

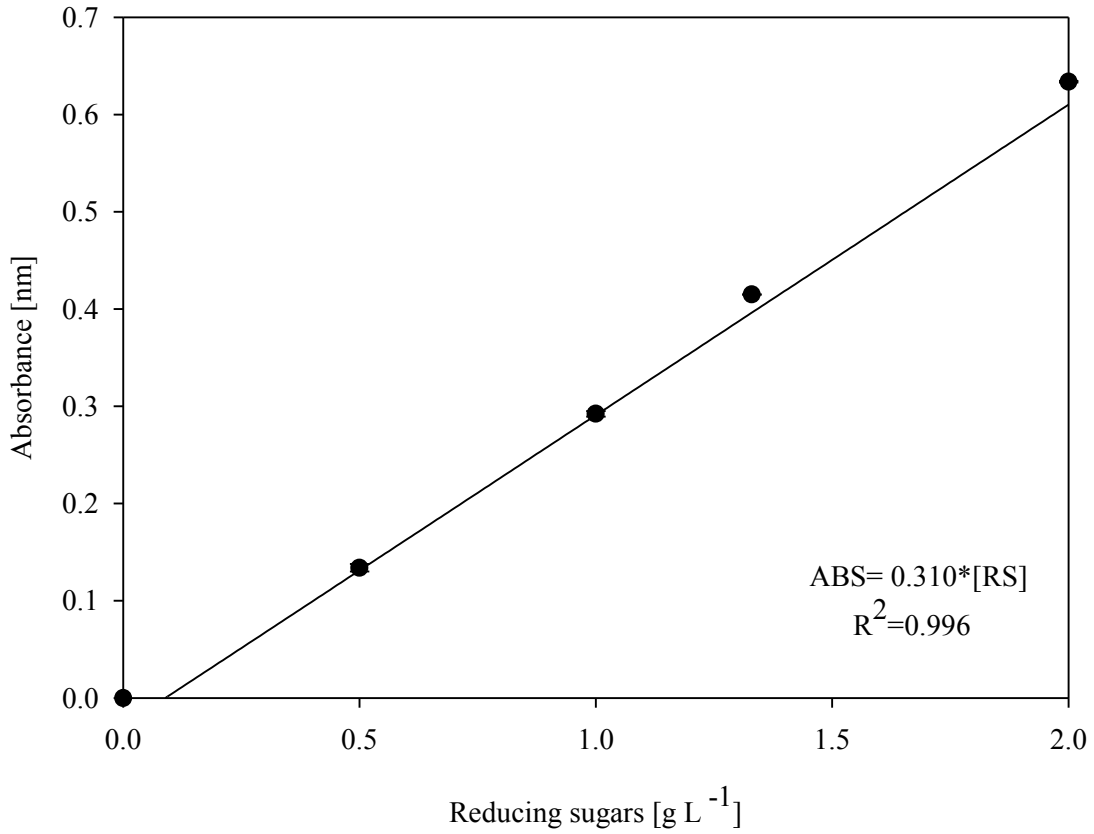
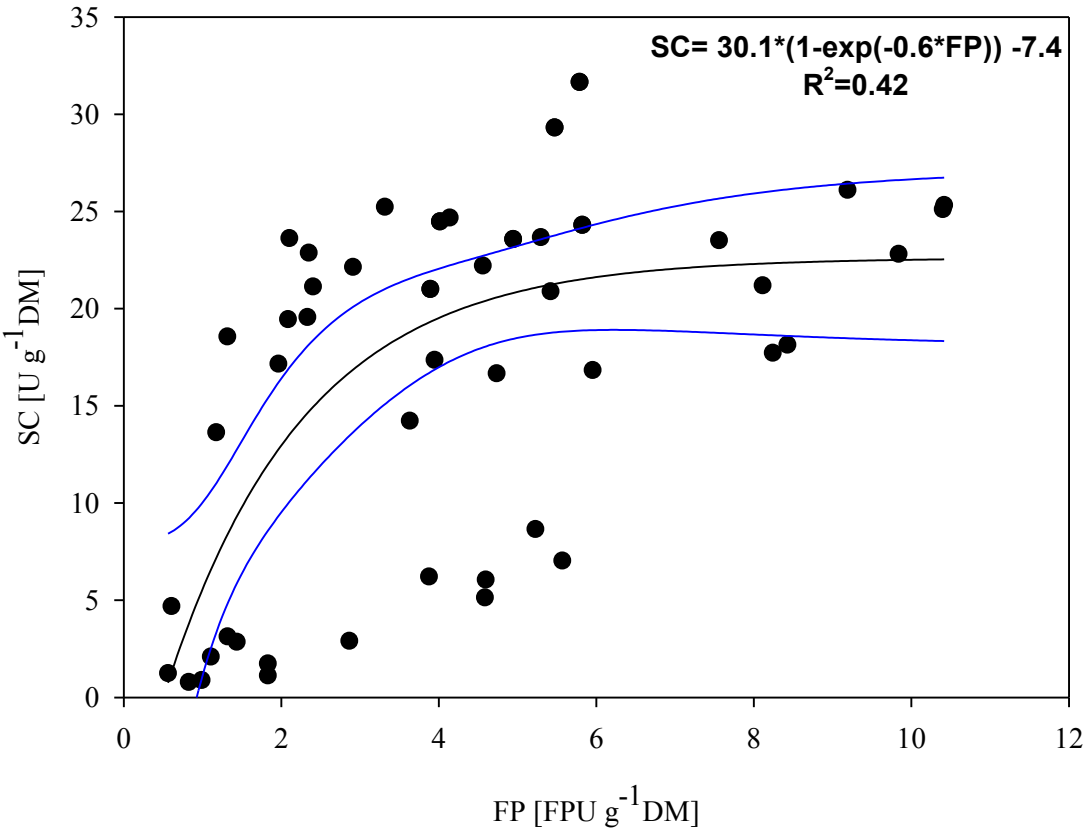


Figure II.

Appendix III. Comparative between substrates in cellulase determination.

In order to partially assess the difficulties to work with filter paper as a substrate for cellulase determination, microcrystalline cellulose was evaluated as a potential substrate in all experiments carried out in chapters 5 and 6. Figure III shows the correlation obtained using all the experimental data.



There is no evident correlation among the two substrates, which indicates that the effect of the enzymatic pool obtained in each fermentation affects differently the two substrates. In first place, it can be observed that on one hand, cellulase activity using microcrystalline cellulose (SC) increases quickly and reaches a *plateau*, while using filter paper as the substrate (FP) the increment is gradual.

In second place, the obtained values of cellulose activity using SC substrate were substantially higher than using filter paper. Maximum cellulase activity using microcrystalline cellulose as substrate resulted in values 2 or even 3-fold higher than when filter paper was used as substrate.

This difference can be somehow explained by considering the following aspects: heterogeneity of insoluble cellulose, unclear dynamic interactions between insoluble substrate and cellulase components, and the complex competitive and/or synergic relationships among all three enzymes that

are involved in overall cellulase effects. All these aspects could limit the setup of proper strategies, depending on activity screening approaches.

According to some authors, SC could become a preferred substrate to replace filter paper because it is a more recalcitrant substrate, yielding a more stringent substrate for total cellulase activity than filter paper does; and activities measured on microcrystalline cellulose could more accurately represent hydrolysis ability on pretreated lignocellulose, because its characteristics are closer to those of pretreated lignocelluloses, based on cellulose accessibility to cellulase and the degree of polymerization (Zhang and Lynd, 2004).

Microcrystalline cellulose has two characteristics that could explain the differences of cellulose activity measurement with two different substrates. In first place, microcrystalline cellulose has almost one third of the glycosidic bonds content of filter paper. This is important because endocellulase acts upon these bonds, which would imply a better performance using filter paper as substrate. However, another important characteristic of microcrystalline cellulose corresponds to its polymerization degree. This parameter is very low (150-500) in microcrystalline cellulose in comparison with filter paper (100-2800), which could imply a higher accessibility of exocellulases for the product released for the action of endocellulase (Percival Zhang et al., 2006). In this sense, even if endocellulases could perform better on filter paper, exocellulases are reported to be responsible for most of the product released.

In addition, it is important to take into account that sugar quantification by DNS considers reducing sugars (with reducing ends), which implies several considerations. One of the most important parameter is the reducing ends content in the different substrates, which for SC and FP are 0.67 and 0.13% respectively (Percival Zhang et al., 2006). In this sense, hydrolysis of microcrystalline cellulose can release much more reducing ends than filter paper as in these experiments, but not necessarily would consider total cellulose activity, which could be the possible explanation for the differences obtained by using two different substrates.

Furthermore, in both cases, the results may suffer from an underestimation of cellulase activity when glucose is used as the standard and β-glucosidase is not in excess. Due to the relatively low BGase values obtained in all cases, it is highly possible that both activity measurements are underestimated.