Universidad de Valladolid

ESCUELA DE INGENIERÍAS INDUSTRIALES

DEPARTAMENTO DE INGENIERÍA QUÍMICA Y
TECNOLOGÍA DEL MEDIO AMBIENTE

TESIS DOCTORAL:

IN SITU LIGNOCELLULOLYTIC ENZYME PRODUCTION BY SOLID STATE FERMENTATION

Presentada por Alicia A. Mansour para optar al grado de doctora por la Universidad de Valladolid

Dirigida por:

María Fdz-Polanco Iñiguez de la Torre
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Certifican que:

Alicia A. Mansour ha realizado bajo su dirección el trabajo "In situ lignocellulolytic enzyme production by solid state fermentation", en el Departamento de Ingeniería Química y Tecnología del Medio Ambiente de la Escuela de Ingenierías Industriales de la Universidad de Valladolid. Considerando que dicho trabajo reúne los requisitos para ser presentado como Tesis Doctoral expresan su conformidad con dicha presentación.

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ABSTRACT

Experimental plans were designed in order to develop solid state fermentation (SSF) for in situ lignocellulolytic enzyme production. The enzyme extract will be used to increase biodegradation and methane production in downstream anaerobic digestion (AD). In that perspective, four lignocellulolytic enzymes were identified of interest: total cellulase (FPase), carboxymethylcellulase (CMCase), β-glucosidase and xylanase. Their corresponding concentrations or activities were monitored all along the conducted research.

Following an extensive literature review, substrate type, autoclaving, inoculum type, pH, moisture and nutrient addition were identified as the factors that impact enzyme production under SSF conditions at the laboratory scale. A scale-down of the enzyme activities analytical methods to 96-well plate test format was however first proven necessary. Analysis had to adapt to large factorial experiments.

Two consecutive sets of experiments were run focusing on the development of the SSF process using three substrates: municipal solid waste (MW), paper/cardboard (PC) and brewer's spent grain (SG). First the factors impacting the SSF process and their corresponding ranges were narrowed down using a D-optimal design experimental plan over a 5-day fermentation period. A full design experimental plan later optimized the experimental conditions. Humidity level and pH were the most significant impacting factors. Their effect was non-linear over the tested range. Since the optimal enzyme mixture could not be defined, all three substrates were kept in the different experiments.

Using the identified optimal conditions, a follow-up over two weeks of triplicates of the different substrates identified the production profile of the four enzymes. The above defined

fermentation time falls well within the optimal range. These assays also put forward the importance of the indigenous microflora which could substitute external inoculum. Finally, brewer's spent grain was used as a model substrate to study the impact of two enzyme samples, one commercial as a reference and the enzyme extract obtained from SSF of the three substrates, on methane production in three lab-scale AD systems: single-stage, two-stage and a percolation system coupled with AD. Hydrolysis results were encouraging but the impact on methane production using the experimental setup was shown on the third tested system. At least a 30% increase of methane production can be achieved using enzymes.

RESUMEN

Un proceso de “Solid State Fermentation” (SSF, Fermentación en Estado Sólido) de residuos orgánicos para la producción de enzimas lignocellulosicas y la posterior utilización del extracto enzimático para aumentar la biodegradabilidad y la producción de metano, mediante digestión anaerobia, de los residuos tratados fue desarrollado a escala laboratorio. Un extenso análisis de la bibliografía existente permitió la identificación del tipo de substrato, del autoclavado, del tipo de inoculo, del pH, de la humedad y de la adición de nutrientes como los factores clave del proceso que determinan la producción de enzimas mediante SSF. La gran cantidad de parámetros de proceso hizo necesario el diseño y aplicación de planes de experiencia específicamente diseñados para jerarquizar el impacto de cada uno de los parámetros y sus posibles interacciones.

Una de las repuestas más importantes para evaluar la eficacia del proceso es la concentración o actividad de las enzimas, medida a lo largo de toda la investigación. Se identificaron cuatro enzimas de interés: Total Cellulase (FPase), Carboxymethylcellulase (CMCase), β-glucosidase and Xylanase. Para adaptar los métodos analíticos a los amplios experimentos factoriales se demostró primordial su “scale-down” al formato de placa de 96 pozos.

Dos planes de experiencia consecutivos fueron realizados sobre tres substratos diferentes: Residuo Solido Urbano (RSU), la fracción papel y cartón del RSU y residuos sólidos de la producción de cerveza (Brewery Spent Grains). El primer plan experimental, diseño D-Optimal, permitió afinar los factores y sus rangos de valores durante un proceso de fermentación de 5 días. El segundo, un Full-Design plan experimental, optimizó las condiciones experimentales. Humedad y pH fueron identificados como los parámetros más significativos, con un impacto no linear en los rangos estudiados.

Las condiciones óptimas identificadas fueron utilizadas para realizar un experimento, en triplicado, para definir los perfiles de producción de las cuatro enzimas a partir de los tres residuos y por un periodo de dos semanas. Los resultados pusieron de manifiesto que por una parte el tiempo de 5 días utilizado previamente estaba dentro del rango óptimo y por otra parte la importancia de la microflora indígena capaz de substituir la inoculación externa. Finalmente, el proceso global fue evaluado utilizando el residuo de la producción de cerveza como substrato modelo. Dos complejos enzimáticos, uno comercial como referencia y el extracto enzimático obtenido por SSF de nuestros residuos, fueron utilizados para estudiar el impacto en la hidrólisis y en la producción de metano en tres configuraciones de digestión anaerobia: single-stage (una sola etapa), two-stage (doble etapa, hidrólisis + digestión anaerobia) y percolación combinada con digestión anaerobia. En términos de hidrolisis, los resultados obtenidos son alentadores. Sin embargo, en términos de producción de metano, y teniendo en cuenta las condiciones experimentales, solo la configuración percolación combinada con digestión anaerobia demostró que la utilización de enzimas incrementa en al menos un 30% la producción de metano.

CHAPTER 1

OVERVIEW

1.1. Introduction

1.1.1. Context

Society is facing a number of key socio-economic and environmental challenges which require innovative solutions (Berka and Cherry, 2006). One major challenge is to re-evaluate efficient energy utilization or to find alternative uses for natural and renewable resources, using clean technologies (Iqbal et al., 2013). In fact, a large fraction of the world's total energy demands is supported by non-renewable fossils resources (EIA, 2013). But with the increasing energy demand and environmental concern there is an increasing search for energy production alternatives such as anaerobic digestion (AD). In this process, different types of biodegradable feedstocks have been used but the attention has lately shifted towards a huge reservoir of energy which is the lignocellulosic feedstock. This biomass can be a perfect candidate for producing bioenergy and biobased products just only considering the part of the feedstock that does not directly compete with food or feed production (Sawatdeenarunat et al., 2015). It is an abundantly available bioresource with a global yield of over 200 billion dry metric tons per year (Kumar et al., 2008). Anaerobic digestion of lignocellulosic biomass can therefore provide an excellent opportunity to convert these substrates into renewable energy (Sawatdeenarunat et al., 2015).

Regardless of their source, lignocellulosic materials consist of three main polymers: cellulose, hemicellulose and lignin (Acharya et al., 2010; Deswal et al., 2011; Montoya et al.,

2012). Cellulose is a linear homopolymer of glucose units; the chains of cellulose tend to forms microfibrils with alternating crystalline and amorphous regions (Lynd et al., 2002; Jabasingh and Nachiyar, 2011). Hemicelluloses are polymers composed of monomeric components mainly xylose, mannose, galactose, arabinose and methylglucuronic acid (Santoni et al., 2015). Finally, lignin is mainly made up of guaiacyl and syringyl units to which phenylpropanoid units can also be cross-linked (Santoni et al., 2015). Therefore for the complete valorization of this lignocellulosic feedstock, the bottleneck resides in its complex chemical structure which is resistant to degradation mainly under anaerobic processes. The best option to overcome the limitation is pretreatment.

1.1.2. Pretreatment processes

Pretreatment becomes then a crucial process step for the biochemical conversion of lignocellulosic biomass. It is required to alter the structure of cellulosic biomass and make cellulose more accessible for biodegradation (Mosier et al., 2005). It has been recognized as one of the most expensive processing steps in cellulosic biomass-to-fermentable sugars conversion (Alvira et al. 2009; Carvalheiro et al., 2008; Hendriks and Zeeman, 2008; Taherzadeh and Karimi, 2008). There are 5 main categories of pretreatment as identified by Harmsen et al. (2010): mechanical, chemical, thermal, combined chemical and mechanical and finally biological.

Mechanical pretreatment consists mainly of milling or the reduction of particle size to make material handling easier and to increase surface/volume ratio. There is also ultrasonication but it has been mostly investigated at the laboratory scale (Harmsen et al., 2010). In most cases, mechanical pretreatment is most often followed by another processing step.

Several sub-categories are found under **chemical pretreatment**:

- Acid catalyzed hydrolysis consists of two types. On one hand, dilute acid treatment is one of the most effective pretreatment methods for lignocellulosic biomass (Harmsen et al., 2010). There are two types of weak acid: high temperature and continuous flow process for low-solids loading (T> 160 °C, 5-10 wt% substrate concentration) and low temperature and batch process for high-solids loading (T≤160°C, 10-40% substrate concentration) (Harmsen et al., 2010). This type of pretreatment has proven its efficiency but its major drawback is the degradation of hemicellulose sugars into furfural and hydroxymethyl furfural, strong inhibitors to microbial fermentation (Harmsen et al., 2010). On the other hand, there is strong acid hydrolysis. It is very expensive and causes significant operational problems (Galbe and Zacchi, 2002; Sun and Cheng, 2002) mainly equipment corrosion problems. It allows operation and low/medium temperature and pressure but neutralization costs hamper its general use (Carvalheiro et al., 2008).
- Alkaline hydrolysis is divided into two major groups: pretreatments that use sodium, potassium, or calcium hydroxide and others that use ammonia. In general, alkaline pretreatment increases cellulose digestibility through the removal of lignin from the biomass (Harmsen et al., 2010). It also removes acetyl and various uronic acid substitutions on hemicellulose that lower the accessibility of the enzyme to the hemicellulose and cellulose surface (Chang and Holtzapple, 2000).
- Organosolv processes use an organic solvent or mixtures of organic solvents with water for removal of lignin before enzymatic hydrolysis of the cellulose fraction (Harmsen et al., 2010). Common solvents for the process include ethanol, methanol, acetone, and ethylene glycol. Temperatures used for the process can be as high as 200°C although lower temperatures can be sufficient depending on the type of biomass and the use of a catalyst (Ghose et al., 1983). Possible catalysts include inorganic or organic acids (Sun and Cheng,

2002). The solvent itself can be an inhibitor for the enzymatic hydrolysis and fermentation step therefore it must be removed prior to fermentation.

- Oxidative delignification could be achieved using hydrogen peroxide, lignin polymers are converted into acids among other types carboxylic acids which act as inhibitors in the fermentation step. Therefore they have to be neutralized or removed. It also affects the oxidation of hemicellulose which will not be further used in the sugar production. Ozone is another option and focuses on lignin degradation as well (Sun and Cheng, 2002).Wet oxidation however with oxygen or air in combination with water at elevated temperature and pressure (Mcginnis et al., 1983).
- Room Temperature Ionic Liquids (RTIL) are salts that are in the liquid phase at temperature as low as room temperature. They are usually comprised of an inorganic anion and an organic cation of very heterogeneous molecular structure (Van Rantwijk, 2003). At the time being, there is no industrial application employing RTIL.

Thermal pretreatment includes on one hand liquid hot water processes. These are biomass pretreatments with water at high temperature and pressure. For temperatures below 100°C, no hydrolytic effect is expected on the substrate (Abatzoglu et al., 1992), whereas above 220°C some cellulose degradation can take place (Bouchard et al., 1990; Torres et al., 1986). On the other hand, there is steam explosion (uncatalyzed or catalyzed). It is considered as one of the most applied pretreatment processes in which high-pressure saturated steam is injected into a batch or continuous reactor filled with biomass. Limitations of steam explosion include the formation of degradation products that may inhibit downstream processes (Garcia-Aparicio et al., 2006).

Chemical and mechanical combined pretreatment includes the following:

- In ammonia fibre explosion (AFEX), biomass is treated with liquid ammonia at high temperature and pressure (Teymouri et al., 2005). AFEX reduces the lignin content and

removes some hemicellulose while decrystallizing cellulose. The cost of ammonia and especially of ammonia recovery drives the cost of the pre-treatment, although ammonia is easily recovered due to its volatility (Holtzapple et al., 1994).

- In CO_2 explosion, high pressure CO_2 is injected into the batch reactor and then liberated by an explosive decompression.
- Combined mechanical/alkaline pretreatment consists of a continuous mechanical pretreatment (e.g. milling, extrusion, refining) of lignocellulosic biomass with the aid of an alkali. The resulting fractions consist of a soluble fraction (containing lignin, hemicellulose and inorganic components) and a cellulose-enriched solid fraction. The combination of alkaline pretreatment with mechanical action increases the efficiency of the pretreatment compared to alkaline pretreatment, but the use of expensive chemicals remain necessary, and recycling and waste treatment is an important issue.

And finally there is the **biological pretreatment** which involves the use of enzymes. The main advantage of using those protein catalysts is to better use the raw materials, save water and energy, and most importantly replace toxic chemical processes (Berka and Cherry, 2006). Enzyme-catalyzed reactions take place under relatively mild and ecologically friendly conditions, are highly specific and greatly accelerate the rates of the reactions in which they participate (Berka and Cherry, 2006). Therefore this type of pretreatment can actually overcome high energy demands and most importantly inhibitor formation resulting from chemical pretreatment processes. In large scale environmental applications, enzyme hydrolysis is mainly associated to bioethanol production. Few processes have emerged lately for waste treatment such as REnescience and Fiberight processes.

1.1.3. Enzymes and solid state fermentation

The fact of having large scale processes using enzymes implies that these latter have proven their efficiency in degrading lignocellulose although their use has been limited to industries

with high value-added products. Their current commercial production takes place mostly under submerged fermentation (SmF) conditions which present high operating and capital costs. SmF occurs in large liquid fermenters, that need to be sterile and with a complete control of the growth factors. In addition to that, extraction and refining operations such as cell disruption, precipitation and solid-liquid separation that lead to the final product add to the already high production costs.

This is where solid state fermentation (SSF) emerges as an interesting alternative for the *in situ* production of endogenous enzymes that will be used to enhance the hydrolysis of lignocellulosic matrices in environmental applications. SSF is defined as the aerobic microbial transformation of solid materials. It is the process during which microbial growth occurs on moist solid particles without the presence of free water (Pandey et al. 2008a). The water thus exists in a complex form within the solid matrix or as a thin layer either absorbed to the surface of the particles or less tightly bound within the capillary regions of the solid (Raimbault 1997, Roussos and Pyle 1997).

For the environmental applications, the main driving force behind the development of SSF processes is a serious need for large-scale low cost production of enzymes. On-site cellulase production under SSF from lignocellulosic biomass could therefore be a cost-effective strategy (Hideno et al., 2011). Cellulose-based strategies can make the biorefinery processing more economical by increasing commercial enzyme volumetric productivity, producing enzymes using cheaper substrates, producing enzyme preparations with greater stability for specific processes and producing cellulases with higher specific activity on solid substrates (Kiranmayi et al., 2011). Under SSF conditions polymer insoluble substrates (Raimbault, 1997) and agro-industrial wastes could be used; this is an interesting alternative that helps in value-addition of those wastes solving also their disposal problem (Krishna et al., 1995; Roussos, 1997). SSF operates under high cellulose concentration which leads to

higher enzyme yields. Other advantages of SSF include the limited consumption of water and the absence or near absence of effluent production (Krishna, 1999; Pandey et al., 2008b). And given the nature of the microorganisms used in the SSF process, static conditions are preferred in order to keep intact the mycelia growth of the fungi. The aeration through the spaces between the substrate particles allow the agitation to be discontinued when necessary (Roussos and Pyle, 1997). SSF is therefore less energy consuming and requires low volumes of the equipment.

SSF process has also its limitations. The main disadvantages are the risk of high temperature and the removal of excess heat due essentially to the low thermal conductivity of the solid substrate (Gervais and Molin, 2003). The difficulty of parameter control mainly the control of the pH, and the critical roles of water content and water activity and sometimes the need for pretreatment of the solid material add to those limitations (Roussos and Pyle, 1997; Pandey et al., 2008a).

1.2. Research objectives

The purpose of this work is the use of in situ produced enzymes under SSF conditions to increase biodegradation and methane production in downstream anaerobic digestion.

Various research works have been conducted on enzyme production at the lab-scale focusing mainly on substrates from the food industry. The current study includes new substrates of interest based on their representativity, abundance and/or environmental interest. In addition to that, experimental conditions were defined taking into account both economic and technical limitations of the scaling up process. This is why the main objective of this research is to define optimal operational conditions for maximal lignocellulolytic enzyme production under SSF conditions considering the above constraints or aspects.

Three lignocellulose rich substrates were studied. A paper/cardboard mixture is considered as a standard sample of the lignocellulosic complex. The remaining matrices are

municipal solid waste and brewer's spent grain, very important feedstock. The worldwide annual production of MSW goes up to 1.3 billion tons (Hoornweg and Bhada-Tata, 2012) and that of spent grain up to 38.6 million tons (Mussatto, 2014).

Additionally to evaluate enzyme production, an important objective was to study possible correlations that could be established between enzyme activities used and reducing sugars produced during the enzymatic hydrolysis of the different matrices. And finally, brewer's spent grain was considered as a model matrix to have a first- hand overall approach on the use of enzymes in three different anaerobic treatment configurations.

1.3. Dissertation layout

This dissertation is divided into a total of eight chapters with an overview in chapter 1 and conclusions and perspectives in chapter 8.

Chapter 2 reviews operational parameters that impact the solid state fermentation (SSF) process. These include substrate type and particle size, substrate pretreatment, inoculum, nutrient supplementation, moisture content, water activity, pH, aeration, temperature and mixing. The different research efforts conducted at the lab-scale are presented according to the inoculum source. The objective is to bring forward the main challenges that hinder large scale application of the SSF process.

Chapter 3 focuses on the analytical methods used in enzyme activity measurements. Existing reference methods are chemicals and labor intensive and unsuitable for large factorial experiments. Based on an extensive literature review and on experimental results, reference and microplate adapted methods were compared to define the most adequate 96-well plate adapted filter paper, carboxymethylcellulase, β-glucosidase and xylanase activity tests.

The following chapters focus on the lab-scale development of the SSF process on municipal solid waste, brewer's spent grain and paper/cardboard fractions using

commercially available inoculum. Chapter 4 presents the screening of the different operational conditions. Substrate type, autoclaving, inoculum type, pH, moisture and nutrient addition were considered in a 46-assay D-optimal design experimental plan. After 5 days of incubation at 30°C, enzymatic activities were analyzed and the significance of each tested parameter measured. Results were used to define the 30-assay full design optimizing experimental plan presented in chapter 5.

Under optimal operational conditions, chapter 6 presents the follow-up in time of the enzyme production on the three matrices. Both fermentation time and the importance of the inoculum in enzyme production were identified.

Finally, chapter 7 presents the overall approach to study the impact of using enzymes on brewer's spent grain in three lab scale AD systems: single-stage, two-stage and a percolation system coupled with AD. For that purpose, one crude enzyme mixture produced from a SSF process and a commercial enzyme mixture were used to pretreat the substrate.

This work can be the basis for a technical and economic feasibility study of using enzymes in waste treatment process.

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CHAPTER 2

Review of solid state fermentation for lignocellulolytic enzyme production: challenges for environmental applications

ABSTRACT

Within the context of increasing environmental concern, energy production from lignocellulosic substrates is gaining great interest. Enzymes have proven their efficiency in the degradation of the lignocellulosic complex but their use remains limited in environmental applications mainly due to their prohibitive cost. Therefore, solid state fermentation (SSF) emerges as an interesting alternative for the *in situ* production of lignocellulolytic enzymes. This work first reviews operational parameters that impact the SSF process. These include substrate type and particle size, substrate pretreatment, inoculum, nutrient supplementation, moisture content, water activity, pH, aeration, temperature and mixing. Various research efforts on the lab scale optimization of SSF are discussed. They are presented according to the type of inoculum used in the process: bacterial and fungal species under both mesophilic and thermophilic conditions. Using different substrates, authors aim at maximizing enzyme production taking into account one to several operational parameters. Reviewed research puts forward major challenges for the scale up phase. Adaptation of the operational parameters, enzyme production cost and loading, enzyme mixture quality and efficiency and finally reactor design remain the main challenges for large-scale application.

Keywords: lignocellulose, enzyme, solid state fermentation, optimization, large scale, environmental

2.1. Introduction

The field of industrial biotechnology has evolved rapidly in recent years as a combined result of international political desire and important progress in molecular biology research and tools (OECD 2011). Enzyme production is one important subject of this field. Its traditional application markets reside in the food, paper, textiles, pharmaceutical and chemical (mainly detergents) industries (van Beilen and Li 2002). However, more recently new applications have emerged aiming environmental applications mainly those of biofuel production and waste treatment. In fact, two of today's major problems are the increasing generation of waste with its consequent environmental problems and the search for new sources of energy due to the increasing demand on energy sources and the concern about the remaining amount of fossil resources. It is within this context that the use of lignocellulosic residues has attracted significant attention.

Regardless of their source lignocellulosic materials consist of three main polymers: cellulose, a homopolymer of β-1,4 linked glucose units; hemicellulose, a heteropolymer of pentoses and hexoses with a backbone built up by sugar monomers like xylose; and lignin, an amorphous polymer of phenyl propanoid units (Acharya et al. 2010, Deswal et al. 2011). Cellulose, the main component of lignocellulose, is the most abundant organic compound. It is the major constituent of all plant materials and forms about one-third to half of plant tissues and is constantly replenished by photosynthesis (Pandey et al., 2000). It represents 40-60% of municipal solid waste, and is also abundant in waste from forest products, agriculture and fruit and vegetable processing (Mandels et al. 1974). It is therefore a huge organic reservoir on earth and a major renewable source of energy. Cellulose and hemicellulose are respectively totally and partially biodegradable but remain protected and unavailable inside the lignocellulosic structure. Therefore a huge energetic potential is confined within lignocellulosic matrices and its expression depends on the disruption of the structure.

Therefore overcoming the recalcitrance of natural lignocellulosic materials, which need to be hydrolyzed to produce fermentable sugars, is a major technological challenge (Kiranmayi et al. 2011). A pretreatment step is thus required in order to tap into this energetic pool. Physical and chemical pretreatments have shown positive results however their corresponding economic, energetic and operational costs present major drawbacks. Production of inhibitors has also been identified as another important drawback as it is the case when applying, for example, the widely used steam explosion pretreatment (Alvira et al. 2010, Horn et al. 2011). Biological pretreatment, using enzymes, has also shown interesting results but the cost is prohibitive for environmental applications. In fact, current large scale production of enzymes is mainly realized under submerged fermentation (SmF) conditions and aimed at the production of high value-added products in the pharmaceutical and food industries.

Solid state fermentation (SSF) emerges as an interesting alternative for the *in situ* production of endogenous enzymes that will be used to enhance the hydrolysis of lignocellulosic matrices in environmental applications. Although, in certain conditions SSF was considered as an “in vivo” pretreatment method to enhance enzymatic saccharification of lignocellulosic biomass in ethanol production processes (Alvira et al. 2010).

This paper reviews the principles and operational parameters of the SSF process. It then presents a wide review of experimental results about the production of cellulolytic enzymes using SSF. Finally, the main challenges and scientific locks that inhibit the application of SSF in the environmental field at the industrial scale are discussed.

2.2. Operational parameters of the SSF process

Solid state fermentation could be defined as the aerobic microbial transformation of solid materials. It is the process during which microbial growth occurs on moist solid particles without the presence of free water (Pandey et al. 2008a). The water thus exists in a complex form within the solid matrix or as a thin layer either absorbed to the surface of the particles or

less tightly bound within the capillary regions of the solid (Raimbault 1997, Roussos and Pyle 1997).

This section reviews the important parameters that need to be considered in order to properly run a solid state fermentation process. These include substrate type and particle size, substrate pretreatment, inoculum, nutrient supplementation, moisture content, water activity (a_w), pH, aeration, temperature and mixing (Lynd et al. 2002, Pandey et al. 2008b).

2.2.1. Type and particle size of the substrate

The substrate used in the SSF process should be porous with large surface area per unit volume to allow microbial growth and proper air flow (Raimbault 1997). It can be either an inert material (synthetic or natural) within which the carbon and energy source are adsorbed or could itself provide these sources (Raimbault 1997, Pandey et al. 2008b). Its composition impacts the concentrations and proportions of the different enzymes produced (Pandey et al. 2008b, Camassola and Dillon 2009). It is also the major contributor to the cellulase production cost (Guowei et al. 2011); therefore cheap naturally occurring lignocellulose could be used instead.

Not only the substrate type but its particle size is also essential. In general, small particle size provides larger surface area for microbial attack but too small a size leads to agglomeration and interferes with microbial respiration/aeration (Zadrazil and Puniya 1995, Pandey et al. 2008b). In parallel, large particle size provides better respiration/aeration efficiency but limited surface for microbial growth (Pandey et al. 2008b). It would also absorb less moisture, swell less and thus by drying rapidly support only a sub-optimal growth of fungi (Zadrazil and Puniya 1995).

2.2.2. Substrate pretreatment

Substrate pretreatment can be applied upstream some SSF processes in order to reduce the recalcitrant nature of lignocellulose. Pretreatment is mostly expected to reduce the

crystallinity of the lignocellulosic complex (Mandels et al. 1974, Chen et al. 2011, Jabasingh and Nachiyar 2011) thus increasing the accessibility of the surface area of the cellulose. It is also thought to affect the pH profile during the SSF process (Roussos 1997). Pretreatment generally includes size reduction (grinding, chopping, milling, cryo-milling…), enzymatic hydrolysis, dry-pretreatment, thermochemical pretreatment, use of alkaline hydrogen peroxide, vapor treatment, steam explosion, microwave irradiation…(Raimbault 1997, Roussos 1997, Pandey et al. 2000, Chen et al. 2011).

2.2.3. Inoculum

During the SSF process, the inoculum develops and produces the desired end-product although other associated enzyme activities could always be found in trace amounts (Pandey et al. 2008b). Bacteria and yeast can be grown on solid substrates but filamentous fungi remain the most adapted microorganisms for SSF (Pandey et al. 2000). The hyphal mode of growth of fungi as well as their high tolerance for low water activity (aw) and high osmotic pressure makes them competitive for the bioconversion of solid substrates (Gervais and Molin 2003). Cellulolytic enzymes are known to be produced mostly by both soft rot and white rot fungi such as Trichoderma, Phanerochaete, Aspergillus, Pleurotus and Penicillium (Gokhale et al. 1991, Reddy et al. 2003, Deswal et al. 2011, Qian et al. 2012, Kiranmayi et al. 2011).

2.2.4. Nutrients

Nutrient addition is a parameter that mostly applies to lab-scale experiments. At this level, it is generally preferred to add nutrients in order to discard any nutrient deficiency. Several recipes exist but the elemental composition comprises besides the carbon source: major nutrients such as K, P, Mg, S and Ca and trace elements such Fe, Co, Mn, Zn, Cu. For example, Deshpande et al. (2008) observed that cellulase complex and xylanase production by T. reesei was enhanced 2-3 fold by supplementing the solid substrate with a synthetic

medium with whey (40%) and peptone (0.15%). In some cases, vitamins and surfactants like Tween 20 or Tween 80 are added. The surfactant is supposed to affect the permeability of microbial cell membrane and lead to increasing extracellular enzyme production (Guowei et al. 2011).

2.2.5. Moisture and water activity

As in every biological process, moisture is an essential parameter in SSF. Under solid state conditions, the substrate should possess enough moisture to support growth and metabolism of microorganism (Pandey 2003). On one hand, low water content could help avoid bacterial contamination since bacteria compete less at lower moisture content. But it can also lead to poor accessibility of nutrients and reduce the solubility of the lignin and the swelling capacity of substrate. This eventually causes high water stress and consequently a decrease in growth and enzyme production (Acharya et al. 2010). On the other hand, high moisture might result in decreased substrate porosity which prevents oxygen penetration (Pandey 2003). In all cases, if no moisture control is applied, water content could significantly decrease during the SSF process (Roussos and Pyle 1997).

Besides moisture, water activity (aw) is a parameter that some researchers consider. aw is defined as the ratio of the vapor pressure of the water in the substrate to the vapor pressure in pure water (Pandey et al. 2008b). For pure water aw= 0 and it decreases as the concentration of the solutes increases. In general bacteria require higher values of water activity compared to fungi. Some fungi can grow at aw values as low as 0.62 while others require a minimum of 0.8 or 0.9 (Raimbault 1997). Unfortunately, there is no direct measurement of aw. Therefore in process control, it is mostly moisture that is taken into account.

2.2.6. pH

The correct pH is critical for the success of solid state fermentation (Raimbault and Alazard 1980) since along with temperature it influences the transport of enzyme across the

membrane. In addition to that, extracellular enzymes are stable only at a particular pH and may be denatured rapidly at lower or higher values. The pH must then be low enough at the beginning of the process to avoid bacterial growth (Raimbault 1997). But it decreases gradually during the initial stages of the fermentation due to mycelial growth and then increases in the later stages (Roussos 1997). In general, the production of most fungal cellulases falls in the pH range of 4.5-5.0 (Acharya et al. 2010).

2.2.7. Aeration and temperature control

SSF is an aerobic process in which oxygen requirement is supplied by the oxygen present in the gaseous air and the one present in dissolved form in the water associated with the solids (Muniswaran et al. 2002). In the SSF bed, the oxygen diffusion rate depends upon the transport properties of the bed which shrinks due to mycelial growth. This latter changes the bed porosity and hence the effective diffusivity: the carbon dioxide traveling in the opposite direction hampers the oxygen transport into the bed (Muniswaran et al. 2002). At lab-scale, SSF systems are usually flushed with oxygen at different rates but at the industrial scale, aeration systems vary according to the substrate type and also the bioreactor design. Aeration plays in fact also an important role in heat dissipation and regulation of the moisture level. In practice, only air is used for temperature control in SSF which requires a large quantity of air, exceeding the amount necessary for microbial respiration (Gervais and Molin 2003, Rodriguez-Fernandez et al. 2011).

The SSF process is highly exothermic mainly due to the production of CO_2 and consumption of O_2; each mole of CO_2 produced during the oxidation of carbohydrates releases 673 kcal (Raimbault 1997). Pandey (2003) confirms that sometimes the accumulation of heat could lead to temperatures in some locations of the bed 20°C higher than the incubation temperature. For this reason, temperature is considered a crucial parameter that contributes to the success of the SSF process.

2.2.8. Mixing

SSF is characterized by a reduced liquid phase (approximately the water holding capacity of the substrate in the case of the water-liquid phase) and thus presents high viscosity. Therefore, mixing such media leads to great shear forces that drastically injure the microbial cells (Gervais and Molin 2003). It should thus be used if it does not adversely affect the microorganism. But the main advantage of mixing is dissipating the metabolic heat that is produced by distributing the cool material throughout the reactor (Ashley et al. 1999). In fact, if tolerated by the microorganisms, 10-60 mixing events per hour or a low rotation speed mixing (1-15 rpm), can decrease the maximum temperature attained in a packed bed (Ashley et al. 1999, Pandey et al. 2008a).

2.3. Review of SSF experiments

This section will provide specific examples of the influence of selecting the SSF parameters described earlier on the production of lignocellulolytic enzymes. Going by theoretical classification based on water activity, only fungi and yeasts are termed as suitable microorganisms for SSF. Nevertheless experience has shown that some bacterial cultures can be well managed and manipulated for SSF processes (Pandey 2003). The reviewed literature below thus comprises two main categories based on the type of inoculum used: bacterial species and fungal species. The latter section is then divided into additional two sub-categories based on the temperature at which the SSF process is run: thermophilic and the most widely spread mesophilic conditions. All reviewed results are presented in chronological order. Table 1 summarizes maximum enzymatic activities of the reviewed work below.

Table 1. Maximum enzymatic activities (FPase, CMcase, β-glucosidase and xylanase) recorded in the reviewed works

Reference	FPase	CMCase	β-glucosidase	Xylanase
Chahal, 1985	326.8 IU/ g dry substrate		402.8 IU/g dry substrate	10260 IU/g dry substrate
Deschamps et al., 1985	18 IU/g dry solid	198 IU/g dry solid		
Krishna et al., 1995	29.1 U/g dry solid	74.8 U/g dry solid		
Gessesse and Mamo, 1999		12.5 IU/g dry substrate		700 IU/g dry substrate
Krishna, 1999	2.8 IU/g dry substrate	9.6 IU/g dry substrate	4.5 IU/g dry substrate	
Kalogeris et al., 2003		1572 U/g carbon	101.6 U/g carbon	
Lu et al., 2003				1200 IU/g dry koji
Reddy et al., 2003	very low	very low		0.1411 U/mg protein
Kang et al., 2004	34.2 IU/g substrate	130 IU/g substrate	107 IU/g substrate	14196 IU/g substrate
Poorna and Prema, 2006				21431 IU/g dry substrate
Membrillo et al., 2008	0.18 *I*U/g dry weight			7.59 IU/g dry weight
Acharya et al., 2010	13.38 IU/g substrate	130.92 IU/g substrate	26.68 IU/g substrate	
Chen et al., 2011	194.18 IU/g dry substrate		155.8 IU/g dry substrate	
Deswal et al., 2011	3.492 IU/ g substrate	71.699 IU/g substrate	53.679 IU/g substrate	
Guowei et al., 2011	92.16 U/g koji	377.02 U/g koji		
Jabasign and Nachiyar, 2011	816.5 IU/ g dry substrate	637.25 IU/ g dry substrate		
Rodriguez-Fernandez et al., 2011				65.38 U/g dry solid
Dave et al., 2012	4.87 U/g substrate	124.44 U/g substrate	28.52 U/g substrate	
Kim and Kim, 2012		6.5 U/g solid		8.8 U/g solid
Qian et al., 2012				508 U/g

2.3.1. Bacterial species

SSF technique is believed to be unsuitable for bacterial cultures although some successful fermentation experiments have been achieved.

Krishna (1999) used the bacterium *Bacillus subtilis CBTK106,* isolated from banana waste, to produce cellulase under both SSF and SmF conditions. Several parameters were considered in the experimental setup to optimize enzyme production: chemical pretreatment (hydrolysis with varying acid and base concentrations at 30°C for soaking periods of 6 h to 24 h), autoclaving (at 121°C for 30 or 60 min), moisture level (55% to 75%), particle size of the banana fruit stalk (200- 2400 µm), pH, temperature, nitrogen addition, additional carbon source (glucose, lactose, sucrose and cellulose), level of added inoculum and fermentation time (0 to 72 hours). For the SSF experimental setup, 10 g of dry banana fruit stalk were moistened with a phosphate, potassium and magnesium based nutrient solution in 250-mL Erlenmeyer flasks. The substrate was later autoclaved, cooled to about 30°C and inoculated before incubation. At the end of the fermentation, the enzyme from the fermented banana fruit stalk medium was extracted, centrifuged and analyzed for exoglucanase (FPase), endoglucanase (CMCase) and β-glucosidase (cellobiase) activities. Unfortunately although many factors were studied, they were optimized one factor at a time (OFAT); this experimental approach is very criticized since it easily leads to biased results and interpretations.

Cellulase production was not affected by either acidic or basic pretreatment. However autoclaving was more interesting. As mentioned by Camassola and Dillon (2009), pressure cooking at a controlled pH of plant materials results in greater susceptibility to enzyme hydrolysis. It is also believed to avoid the formation of monosaccharaide degradation products such as furfural and hydroxymethyl furfural which can interfere with subsequent cellulose hydrolysis. Optimal levels of enzyme production were found at 70% moisture level,

400 µm particle size, 7.0 pH and 35°C. Interestingly, optimal reported pH values vary between 5.5 and 6.0 when using fungi in SSF. Hideno et al. (2011) reports optimal pH as low as 4.0 using the filamentous fungus *Acremonium cellulolyticus* on rice straw. But as it will be shown later, in SSF processes using bacterial species optimal pH values are more alkaline. Krishna (1999) also found that inorganic nitrogen sources are more effective that organic ones and that additional carbon sources did not show a significant difference with the control. Finally, maximal cellulase production occurred at 15% inoculum size (v/w) and highest enzyme titres were recorded after 72 h of fermentation. In fact; since there was no pH control in the system, the authors believed that after 72 h the enzymes were denatured and thus the activity decreased. This hypothesis could be questioned since in later reviewed work, the fermentation process extends longer than the reported 72 hours. An interesting finding of this work is the fact that cellulase titre was 12 folds higher under SSF than SmF.

Gessesse and Mamo (1999) used *Bacillus* sp. AR-009 for the production of xylanase. For the experiments, 10 g of wheat bran were placed in 250-mL Erlenmeyer flasks. They were mixed with a mineral solution before sterilization by autoclaving, cooling down and then inoculation. Varying levels of moisture content (33% to 82%) and source addition of different carbohydrate and nitrogen compounds were tested. Maximum production of xylanase activity was observed after 72 h for a total SSF process of 108 h and optimal moisture level at 75%.

Finally, Asha Poorna and Prema (2007) used *Bacillus pumilus* for the production of endoxylanase under SSF conditions. The screening of different substrates revealed that wheat bran is the one with the highest potential. Ten grams of wheat bran were thus mixed and inoculated in 250-mL Erlenmeyer flasks. Using an OFAT approach, particle size, moisture level, pH, temperature and inoculum size were optimized. Optimal parameter values were 0.5 mm for particle size, 72% moisture, 35°C, 10% inoculum size (v/w) and pH 9. Maximum

enzyme titer was recorded at 72 h during for a total incubation period of 120he medium retained 93% of its enzymatic activity at 96 h and 83% at 120 h.

2.3.2. Fungal species

2.3.2.1. Under thermophilic conditions

Other outliers in the literature review correspond to the experiments run by Kalogeris et al. (2003) and Dave et al. (2012). The authors used the same species *Thermoascus aurantiacus* under thermophilic conditions (50-60°C) to produce cellulolytic multienzyme complexes. As mentioned earlier, most reviewed authors work under mesophilic conditions: mostly 25-30°C and in few cases 40°C.

In Kalogeris et al. (2003), different carbon sources were supplemented with a mineral solution prior to heat sterilization at 121°C for 20 min. The SSF was carried out in 100 mL Erlenmeyer flasks containing 2.5 g of dry carbon source mixed with 10 ml of a mineral solution. After inoculation, incubation was carried for 7 days under static conditions. At the end of the process, enzymes were extracted with distilled water by shaking at 50°C. This temperature is high since usually this step is done at room temperature (RT) or at 4°C but it may have been used since the SSF itself was carried out under thermophilic conditions. After centrifugation, the clarified supernatant was used for measuring endoglucanase (CMCase) and β-glucosidase activities. Kalogeris et al. (2003) also used the OFAT approach to optimize enzyme production. Several types of carbon source (wheat straw, rice straw, corn cobs, wheat bran, oat bran…) were tested and untreated wheat straw proved to be the most efficient. As shown in Krishna (1999), enzyme activities were much higher with inorganic nitrogen sources; ammonium sulphate at 1% w/v gave the highest activity values. Optimum temperature was found at 50°C and optimal pH at 4.0; in fact filamentous fungi prefer acidic environments with optimal at pH 3.8-5.0.

In Dave et al. (2012), *Thermoascus aurantiacus* was equally used on deoiled Jatropha seed cake. SSF was carried out in 250- mL Erlenmeyer flasks with 5 g of Jatropha seed cake as the carbon source in addition to 5 ml of a nutrient mineral solution. Both medium and substrate were sterilized at and then inoculated. The flasks were later incubated at 50°C under stationary conditions for 6 days. The enzyme mixture was harvested, filtered and then centrifuged. The clear supernatant was purified and analyzed for filter paper activity (FPase), endo-β-1,4-glucanase (CMCase) and β-glucosidase. Using the response surface methodology (RSM) and Box-Behnken design (BBD), four parameters were studied at three different levels each: initial pH (4, 5 and 6), moisture ratio (liquid to solid ratio of 1:0.5, 1:1 and 1:1.5), ammonium sulfate concentration (0.5%, 1.0% and 1.5%) and inoculum size (1, 3 and 5 plugs). Results showed that most of the fungal cellulases were produced at pH range of 4.0-5.0. In general, enzyme production was significantly affected by the moisture ratio. Maximum enzyme production was obtained at the lowest moisture content. Dave et al. (2012) believe that the reduction in enzyme production at high moisture may be due to the reduction in substrate porosity, changes in the structure of the substrate particles, reduction in gas volume and decrease in microbial growth. Ammonium sulfate concentration was not significantly different for the different enzymes. And the effect of inoculum size was enzyme-type dependent: maximum filter paper activity was obtained at highest inoculum size while highest β-glucosidase and endo-β-1,4-glucanase were recorded at middle inoculum size.

2.3.2.2. Under mesophilic conditions

Most of the SSF processes are carried out under mesophilic conditions. For this reason, this section is divided into two major subsections based on whether the substrate undergoes or not a pretreatment step. Autoclaving of the solid matrix is nevertheless not considered as a pretreatment.

2.3.2.2.1. Without substrate pretreatment

2.3.2.2.1.1. Aspergillus sp.

Aspergillus is the most widely used microorganism for the inoculation of the SSF process.

Lu et al. (2003) studied the impact of temperature of the koji and water activity on the xylanase production using *Aspergillus sulphureus* as the inoculum at both lab and pilot scales. At lab-scale, 18g of wheat bran were mixed with 20 ml mineral salt solution in 500-mL Erlenmeyer flasks. The mixture was autoclaved, cooled, inoculated and then incubated for 72 hours. During the fermentation, both temperature and humidity were controlled automatically. At pilot-scale, a reactor made up of wooden trays with 15 cm gap in between was used. The total mixture had a weight of 500 kg. The medium was autoclaved and then cooled down to 32°C using microbe-free air. The moisture medium was later adjusted by spraying sterilized water. The parameters were optimized consequently. Therefore, at lab-scale, the optimal temperature was found to be 30-33°C given a 50% moisture and 0.5-0.6 cm depth of the substrate. It was shown that growth was low when the temperature was below 23°C and burn out took place at 43°C. Then by fixing the temperature, the moisture content was varied and it was found that in fact 40-50% moisture is optimal for *A. sulphureus*; this moisture corresponds to a_w of 0.93-0.96.At pilot-scale when tests were run naturally, implying no action except ventilation, the temperature at the substrate surface was at 31-32°C, while it reached 45°C at 1.0-1.2 cm depth. But when the temperature and moisture were regulated, maximum xylanase activity was 1.5 folds higher in 64 h of fermentation. In fact, under controlled conditions relative humidity of the air was maintained at 90% by spraying cooled steam water and turning over the medium at an interval of 4h after 24h cultivation.

Kang et al. (2004) investigated the ability of *Aspergillus niger* KK2 to produce cellulases and hemicellulases in SSF by using rice straw and wheat bran. For the experiment, 5 g of

substrate were mixed with a basal medium in 250-mL Erlenmeyer flasks and the initial moisture was adjusted to 65% with distilled water. The mixture was then autoclaved, cooled and inoculated before running the incubation at 28°C. The enzyme extract was assayed for endoglucanase, filter paper (FPase), β- glucosidase, β- xylosidase and xylanase activities. Kang et al. (2004) considered that the production of cellulases and hemicellulases are substrate-dependent. Therefore, the choice of the appropriate inducing substrate is essential for enzyme production. In this perspective, ratios of 1:4 to 5:0 of rice straw to wheat bran were tested to check the impact on enzyme production. Maximum FPase and xylanase were recorded on rice straw and maximum β-xylosidase under the 1:4 ratio. Interestingly CMCase and β-glucosidase activities were similar irrespective of the tested ratio. Maximum enzyme activities were generally obtained after 5-6 days of incubation.

In the work of Acharya et al. (2010), no synthetic nutrient solution was added during the SSF process. They used anaerobically treated distillery spent wash for the production of cellulases by *Aspergillus ellipticus*. Cellulase production was carried out in 250- ml Erlenmeyer flasks containing 5 g of wheat straw as the substrate and the distillery spent wash (25 ml) as the medium. The mixture was sterilized and then inoculated; Tween 80 was added with the inoculum. Incubation took place at 30°C for 7 d. Crude enzyme mixture was centrifuged and the clear supernatant was analyzed for FPase, CMCase and β-glucosidase activities. Using the RSM method, four factors at three levels each were tested: initial pH of the effluent (3, 5 and 7), moisture ratio (1:2, 1:4 and 1:6), effluent concentration in % v/v (20, 40 and 60) and inoculum size ($2.8x10^6$, $0.7x10^7$ and $1.4x10^8$ spores). Results showed that maximum enzyme activities occurred at $1.4x10^8$ spores inoculum level but varying pH and effluent concentrations: maximum FPase occurred at pH = 5 and 60% effluent concentration, maximum β-glucosidase at pH = 3 and 40% effluent concentration and CMCase at pH = 5 and 40% effluent concentration.

Rodriguez-Fernandez et al. (2011) focused mainly on optimizing aeration conditions for pectinase and xylanase production via SSF using *Aspergillus niger* F3. For that purpose, a horizontal drum bioreactor was used. It was filled with nutrient supplemented 2 kg citrus peel substrate at 60% initial moisture and pH 5. Saturated air was supplied into the bioreactor via a compressor and the drum was connected to a system with sensors to analyze O_2 and CO_2 concentration of the gas outlet. SSF was run at 30°C. The fermentation time was fixed at 96 h and aeration intensity was tested at four levels 0.5, 0.75, 1 and 1.25 V kg M (volumetric air flow L air/kg medium/min). First results showed that maximum synthesis of pectinases and xylanases took place at 1 V kg M. It is important to note that only 0.2 V kg M is needed to oxidize the substrate; this amount is calculated by considering the oxygen required to carry out the total oxidation of the organic materials present in the medium. But since air plays an essential role in the removal of the heat generated during the oxidation, an additional intensity is required for the SSF (Gervais and Molin 2003). Under optimal conditions, the rate of synthesis of the two enzymes was different. Pectinase production was at its highest at 72 h of fermentation while that of xylanase increased after 72 h. This could be most likely driven by the reduction of pectin available as the carbon source in the medium. It also shows that xylan is a poorer source of carbon for energy and growth of *A. niger*.

Finally, Qian et al. (2012) tested the impact of operational conditions of different carbon sources on β-glucosidase production and fermentation productivity (FP), defined as the percentage of dry weight of fermented product to the dry weight of the initial solid substrate. Experiments were conducted in 250-ml Erlenmeyer flasks containing 10 g of solid medium with supplemented nutrients. *Aspergillus niger* was added after sterilization and cooling of the mixture to RT. Five parameters were optimized using the OFAT approach in the following order: initial moisture content (IMC) at 55, 60, 65, 70 and 75% tested at 28°C for 72 h, inoculum level at 10^3, 10^5, 10^7 and 10^8 spores/g at 28°C, for 72 h at optimal IMC, initial

pH at 2, 3, 4, 5, 5.5, 6 and 7 at optimal IMC at 28°C for 72 h, incubation temperature (24, 28, 30, 32 and 36°C) for 72 h and finally fermentation period (12, 24, 36, 48, 60 and 72 h). Optimal IMC was 70%. Highest FP occurred at inoculum level 10^3 spores/g while highest β-glucosidase activity was observed at 10^7 spores/g. This implies that maximum growth of the mycelium does not necessarily coincide with maximum enzyme production. Optimal pH, temperature and fermentation time were 6, 28°C and 72 h, respectively. The two last results are rather expected since all the optimization was based on these two assumptions. Qian et al. (2012) also studied the effect of the ratio of wheat bran/ground corncob and the type of nitrogen source on enzyme activity and FP. They found that those latter were highest at 80/20 ratio. This reflects the impact of the solid support. Wheat bran loosens the solid medium and overcomes the agglomeration of the substrate, thus helping in air diffusion and better oxygen supply for the growth of *A. niger* and easier removal of CO_2 and heat generated during SSF. However, at very high levels, the substrate increases water loss due to quick volatilization during fermentation and hence inhibits the growth of microorganism, subsequently declining the enzyme production. For the nitrogen sources, unlike Krishna (1999) and Membrillo et al. (2008), there was no significant difference between inorganic and organic nitrogen sources. Qian et al. (2012) added also an interesting observation regarding the changes in microbial growth of *A. niger* and β-glucosidase production with time during the incubation. The authors divided the fermentation period into: early-stationary phase, logarithmic growth phase, steady phase and decline phase according to FP and enzyme production. For the beginning of the fermentation (0-12 h), the germinating spores resulted in near 100% FP due to changes in dry matter weight loss and no enzyme activities. During 12-36 h, *A. niger* grew very fast resulting in a rapid increase of dry matter weight loss that is a decrease in FP. With *A. niger* growing rapidly and FP falling rapidly, β-glucosidase production was biosynthesized fast.

2.3.2.2.1.2. Pleurotus sp.

Reddy et al. (2003) tested the growth of *Pleurotus ostreatus* and *Pleurotus sajor-caju* on leaf biomass and pseudostems of banana waste. The objective was to produce lignolytic and cellulolytic enzymes such as laccase, FPase and CMCase. The authors were mostly interested in laccase, a lignin modifying extracellular oxidoreductase, which activity appears to be regulated by morphogenesis (Reddy et al., 2003). For the SSF process, 25 g of each substrate were placed in 1 L conical flask and moistened with 75 ml distilled water. The flasks were autoclaved, inoculated and incubated at 25°C. Samples were collected from day 10 to day 40 at 5-day intervals. This is a wide range of follow-up period although it is missing the first period during which most of the other works in the literature are based. Both *P. ostreatus* and *P. sajor-caju* grown on either type of tested biomass produced significant titers of laccase activity, but very low CMCase and FPase activities. The production of laccase on leaf biomass was also twice as high as that on pseudostems. Nevertheless, the maximum laccase activity was 16 times lower than that obtained by other researchers with *P. sajor-caju* grown on rubber tree sawdust (Tan et al. 1997). FPase activity is actually essential to degrade high ordered or crystalline forms of cellulose acting synergistically with CMCase active enzymes. Given the low values produced of FPase and CMCase and high titers of laccase, the authors noted that with this process lignin can be used while leaving intact the cellulose, making the banana waste a good source of animal feed (Reddy et al. 2003).

Another work was conducted by Membrillo et al. (2008) using sugar cane bagasse for the production of protein and lignocellulolytic enzymes with two *P. ostreatus* strains (CP-50 and IE-8). The authors focused on the impact of the inorganic nitrogen source and substrate particle size on enzyme production. For the experiments, 250-mL Erlenmeyer flasks were used with 5 g of the substrate at 80% moisture. The mixture was autoclaved, inoculated and incubated at 29.5°C for 8 d under static conditions. *P. ostreatus* IE-8 showed differences in mycelium growth due to the N source: the highest growth was observed for wheat straw

extract, followed by ammonium sulphate and finally urea. The strain CP-50 did not show any significant difference. For the total protein production, the media supplemented with ammonium sulphate showed the highest levels at 1.68 mm particle size. Surprisingly, there was no impact of the nitrogen source at particle size lower than 0.92 mm. For the IE-8 strain, maximum xylanase and laccase activities were obtained with ammonium sulphate addition while maximal CMCase and FPase were obtained without it. In general strain CP-50 was less sensitive and showed less enzyme production. These results reveal important differences in the inherent characteristics of every strain which make generalization difficult even within the same species of microorganisms.

2.3.2.2.1.3. Trichoderma sp.

Deschamps et al. (1985) compared the solid state cultivation of *Trichoderma harzianum* on 80:20 mixture of straw and wheat bran under both static and mixed non-aseptic conditions. The mixture was milled, mixed with a nutrient solution and autoclaved. The static incubators were aerated with water saturated air. The stirred reactor tanks were commercial bread making blender of 65 L capacity. These reactors were mixed occasionally and water was sprayed to remove excess heat. The pH was controlled by adding correcting solution to the water supplied during the cooling cycles. For all experiments, cellulase yield and endoglucanase activity were measured. For the static SSF, optimal conditions were found at 30°C, pH 5.8, 74% initial moisture content and aeration rate of 6-8 L/h. For mixed SSF assays, the optimal incubation temperature was also 30°C, with 65 mL/h spray water rate. Optimal moisture was found reduced to 69% most probably due to the mixing. The aeration rate was increased progressively from 4 to 40 L/min. During the experiment the pH decreased from 5.7 to 5.2 and then increased to 6.3 at 66 h of incubation. The authors consider that the decrease in pH may be due to the ammonium uptake during the growth phase while the pH increase results from the loss of protein due to mycelium autolysis. Overall results show that

during the upscaling test, cellulase yield was reduced by almost 40% and endoglucanase activity by 25%.

Using *Trichoderma reesei* HY07, Guowei et al. (2011) evaluated the impact of ammonium sulphate , Tween 80, inoculum and temperature on the production of FPase and CMCase. Enzyme activities were optimized separately. In the cultivation medium, 4 g of corn stalk and 6 g of wheat bran were mixed with a nutrient solution in a 250 -mL Erlenmeyer flask. The pH was adjusted to 5 before autoclaving, cooling, inoculating and then incubating for 5 days. For both enzyme activities, optimal temperature was 30°C and the addition of Tween 80 showed positive results. In fact, this surfactant affects the permeability of microbial cell membrane contributing to an increase in enzyme production (Guowei et al. 2011). Nevertheless, optimal addition level of AS was 0.5 and 1.5% for FPase and CMCase, respectively. Maximal production of CMCase was reached using an inoculum addition 60% lower than that found optimal for FPase.

2.3.2.2.1.4. Other species

This part presents the work of researchers that used less widely spread fungal species for the production lignocellulolytic enzymes through SSF.

Krishna et al. (1995) used *Phanerochaete chrysosporium* on soyhull for cellulase production. In the SSF experiment, 20 g of freshly collected soyhull were mixed with 40 mL distilled water in 250-mL Erlenmeyer flask. After sterilization, pH was adjusted, nutrients added and the inoculated substrate incubated for 6 days. Maximum FPase activity was reached at pH 4 and 25°C. The authors also studied the effect of the type of nitrogen source. Contrary to the results of Gokhale et al. (1991), urea (2% w/w dry soyhull) gave the best results with an increase of 2.5 folds in the activity.

Anisha et al. (2010) used *Streptomyces griseoloalbus* to study the impact of aeration conditions on the production of α-galactosidase on soybean flour. For the SSF, 50g of

soybean flour, moistened with a mineral salt solution at pH 7, were autoclaved before inoculation. Unlike most authors the moisture content was low, around 40%. In fact besides this work only Lu et al. (2003) used a moisture level as low as 40-50% and 50% with Guowei et al. (2011). The authors used a packed bed reactor with heat-sterilized vertical glass column aseptically filled with pre-inoculated soybean flour. Water saturated air was supplied through the bottom of the column. Aeration rates were varied from 1 to 3 vessel volume/min (vvm) and fermentation was carried out for 120 h at 30°C.Two blanks were run, one in a similar column but without aeration, and another one in a 250 mL Erlenmeyer flask containing 10 g of inoculated substrate. The highest α-galactosidase activity was reached after 96 h using force aeration of 2 vvm, which corresponded to twice the yield obtained in flasks. Lower enzyme yields were observed when the aeration was lower than 2 vvm due to inadequate oxygen supply. At higher rates of aeration, the reduction in α-galactosidase yield could be due to reduction in water content of the fermented matter below the critical level, which adversely affects the growth and microbial activity.

Finally, Deswal et al. (2011) carried out SSF using the brown-rot fungus *Fomitopsis sp.* RCK2010. Five grams of wheat bran were moistened with mineral salt solution (1:3.5 substrate to moisture ratio) at pH 5. Flasks were later sterilized, cooled to RT and inoculated before incubation at 30°C. Taking one factor at a time, Deswal et al. (2011) optimized the following parameters: pH, substrate to moisture ratio, N source, amino acid, vitamins and surfactant addition. Optimal conditions were found at pH 5.5 and 30°C for FPase, CMCase and β-glucosidase after 16 days of incubation.

2.3.2.2.1. With substrate pretreatment

The principal pretreatment used for the substrate is the alkaline pretreatment. But as presented in Krishna (1999), both acidic pretreatment and autoclaving have also been used.

Chahal (1985) was the first author to use a pretreated substrate in a SSF process. Two mutant strains of *Trichoderma reesei* (QMY-1 and Rut-C30) were tested on two types of substrates: wheat straw (WS) and aspen pulp prepared by chemical-thermomechanical process (CMTP). The substrates were pretreated using sodium hydroxide at 121°C. The author specified that this pretreatment was mild as it did not delignify the substrates; hemicelluloses and lignins were thus solubilized and retained in the medium. Chahal (1985) believed that the lignin and hemicellulose that were kept in the system played an important role in the increase of cellulase yield. After pretreatment, the pH was adjusted to 5.8. For the production of cellulase, 5 g of substrate were mixed with 5 ml nutrient solution, autoclaved and incubated at 30°C with 80% final moisture. Cellulase, β-glucosidase, and xylanase titres were measured on the enzyme extracts. Half the concentration of nutrients was sufficient to reach the optimum cellulase titre as well as cellulase yield. Pretreatment was also important since the two mutants showed a significant decrease in enzyme production when the untreated WS was used.

Jabasingh and Nachiyar (2011) also used an alkaline pretreatment but on coir pith at different time intervals. Unlike Chahal (1985), the purpose of the pretreatment was to delignify the substrate. For the SSF experiment, the substrate was mixed with a nutrient solution, sterilized and inoculated with *Aspergillus nidulans* before incubation for 11 days. Four independent variables were studied for both untreated and NaOH treated coir pith: amount of coir pith (from 6 to 10 g), moisture content (between 60 and 70%), pH (from 3 to 7) and temperature (from 20 to 60°C). Results showed that the use of the pretreatment was significant; the best results were obtained at 0.8% NaOH since at this concentration the lignin content was found to be minimal. All parameters were significant and maximum cellulose activity and cellulose yield were obtained at coir pith of 8 g, 70% moisture content, pH 5 and 40°C. To better represent the changes to which the substrate is subjected, the authors used

scanning electron microscopy (SEM) to study the surface morphology, X-ray diffraction (XRD) to examine the structural properties of substrates and FTIR spectrophotometry between 400 and 4000 cm^{-1}. XRD showed that the crystallinity of the samples decreased from α-cellulose standard, untreated coir pith, pretreated coir pith and finally hydrolyzed pretreated coir pith. FTIR band intensities of lignin peaks (1238 cm^{-1}) were predominant in the untreated coir pith. This latter peak shifted after the alkali pretreatment due to the delignification effect of the NaOH pretreatment and it totally disappeared in the samples after hydrolysis. Qualitative confirmation in the change of morphology was provided by SEM.

Another reference with alkaline pretreatment is that of Kim and Kim (2012). The authors used empty palm fruit bunch fiber (EPFBF) as the substrate for solid state bioconversion and production of cellulase enzymes using *Penicillium verruculosum*. Characterization of the substrate showed that due to the pretreatment the percentage of cellulose increased by 18% and that of hemicellulose decreased by 13%. SSF was performed in 2-L tissue culture roller bottles using 150 g of the alkali pretreated EPFBF and 0.3L minimal medium at 30°C for 8 days. Results showed that avicelase, CMCase and xylanase activities reached the maximum at day 6.

Unlike previous references, Chen et al. (2011) used *Trichoderma reesei* YG3 to study the biodegradation of fractioned steam exploded corn stover in solid-state fermentation. Corn stover samples were manually separated as leaf, shell and core. Each fraction was then separated into miscellaneous cells (MC) and fascicule (FC). SSF was carried out in a Petri dish which was loaded with 10 g dry weight substrate containing 80% steam exploded fraction, 20% wheat bran and 25 mL inorganic salt solution. The pH of the solution was adjusted to 4.8 and then the medium was autoclaved, seeded aseptically and well mixed before incubation at 30°C for 7 days. In general, leaf was the best fermentation substrate with highest cellulase yield as well as filter paper, CMCase and β-glucosidase activities. Chen et

al. (2011) argued that cellulase is an induced enzyme that is when the substrate is not easily hydrolyzed, the fungus is prone to produce cellulase continuously and massively. Both cellulose and hemicellulose are inducers of cellulase, but the former is degraded more slowly; interestingly leaf contained the highest amount of hemicellulose. Regarding weight loss profile, Chen et al. (2011) indicated that at the beginning of the process only a small amount of dry matter weight was lost. During 3-5 d of incubation, *T. reesei* YG3 grew fast along with an increase in cellulase activity and an important weight loss. The highest dry material weight loss was observed in MC tissue of the leaf and reached around 29%.

2.4. Challenges of SSF process for environmental large scale applications

The major challenge of solid state fermentation applied to environmental processes is the economical context. Until recently the application of SSF has been restricted to high value products that do not include for example anaerobic treatment of waste to produce methane. Although it has been previously demonstrated that the enzymatic pretreatment of solid waste improves the efficiency of its subsequent anaerobic digestion (Berlin et al. 2005, Yang and Wyman 2006, Gusakov et al. 2007, Lee et al. 2008), the cost of commercial enzymes is currently too high to consider their use as a feasible waste pretreatment for large-scale production. The cost of enzymes is a major contributing factor in the cost of pretreatment. Tu et al. (2007) indicate that the cost of cellulase and β-glucosidase has been estimated to account for approximately 50% of the cost of the hydrolysis process for the production of ethanol from softwood for example. The high enzyme loadings currently needed to achieve reasonable rates and yields are other contributing factors in the high cost (Yang and Wyman 2006). Berlin et al. (2005) indicated that the cost of cellulase for hydrolysis of pretreated corn stover was reduced by 20 to 30- fold from 2001 to 2005 due to lowered production costs and increases in enzyme production. Nevertheless, at least another 3-fold reduction is needed for full scale process commercialization (Berlin et al. 2005).

In this context, the *in situ* production of lignocellulolytic enzymes using SSF processes could overcome this drawback, but first the SSF process should be adapted to reconcile appropriate productivity requirements for enzyme activities and lower economic revenues.

2.4.1. Optimal operational parameters

Operational parameters and reactor design are the first locks to deal with for large-scale processes. On one hand, parameters such as pH, temperature and humidity have been optimized, however autoclaving and inoculum addition remain as major limitations for the process scale-up. It has been noted that for all the reviewed work, the substrate is autoclaved in order to create a sterile medium in which no other microorganism can compete with the desired mycelial growth. However, when SSF needs to be coupled with an anaerobic digester (as shown in Figure 1) the overall process becomes economically unfeasible. Moreover, the type, cost and amount of the inoculum to be added is to be taken into account. Asha Poorna and Prema (2007) for example found that the optimal addition of the inoculum is at 10% (v/w); this addition level is impossible for a large scale production. The inoculum, if necessary, should be carefully chosen based mainly on its market availability.

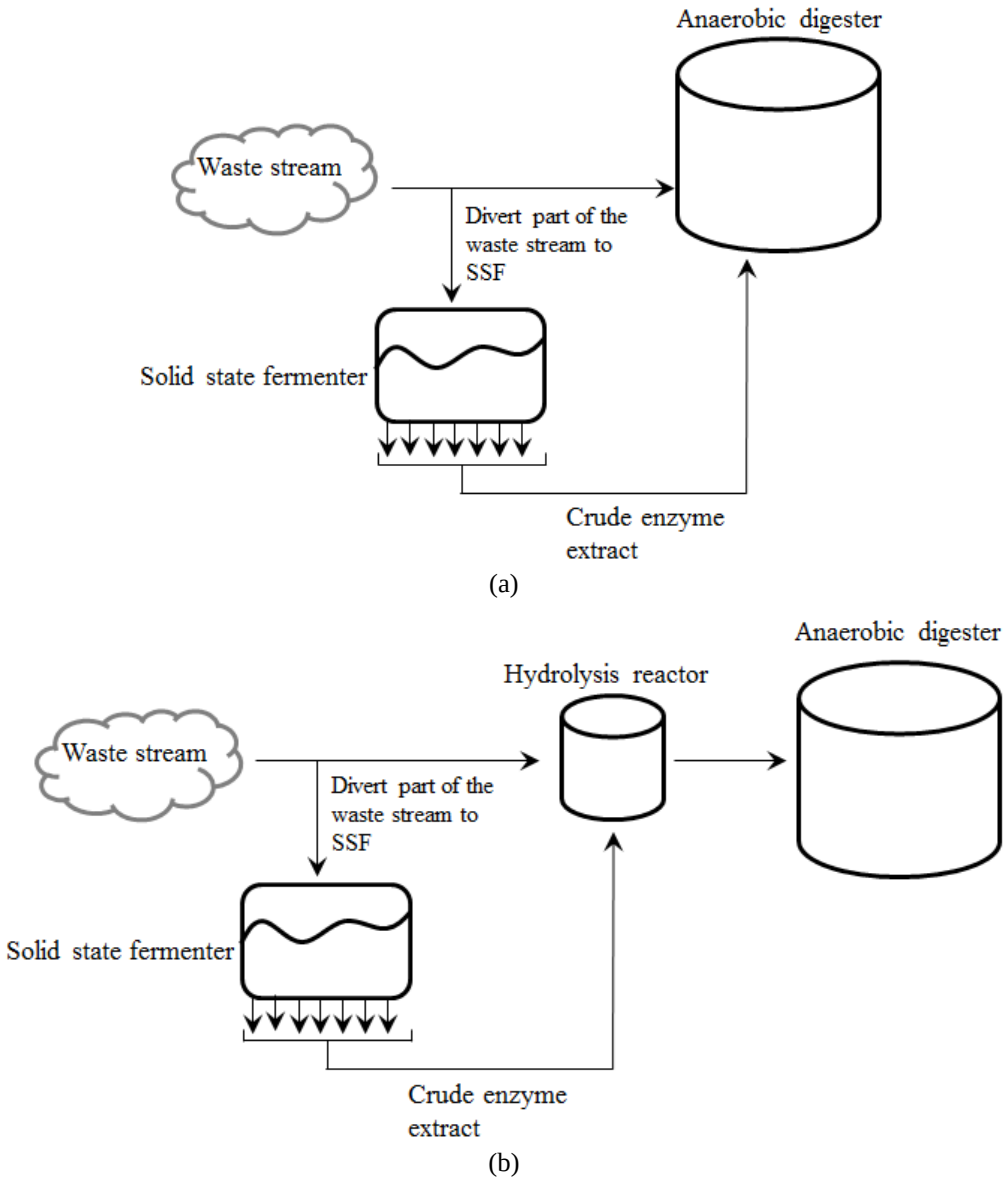

Figure 1. Schematic diagram presenting the integration of the SSF process in (a) single-stage AD system and (b) two-stage AD system

2.4.2. Reactor design

Industrial equipment is currently available for SmF but there is an important need for engineering and new equipment design under solid state. The SSF process is mostly aerobic in nature. The suitable bioreactor design should overcome heat and mass transfer effects and allow easy diffusion and extraction of metabolites. SSF fermenters could be either static, intermittently mixed or continuously mixed, with and without forced aeration through the biomass.

According to Pandey et al. (2008a), there are generally two main types, the tray type and the rotating drum type which could be run with and without mixing (Figure 2). In addition to those, column and deep trough type of bioreactors have recently emerged but their scaling poses serious problems (Pandey et al. 2008a). The tray type is most widely represented in the koji process. Several authors such as Kotwal et al. (1998) and Wang et al. (2004) have used it. The major problems in the tray type are the risks of high temperature and lack of oxygen in the center that usually arises since the design lacks forced aeration. Packed bed reactors (Figure 2) on the other hand involve a static bed aerated from the bottom throughout the fermentation (Ashley et al. 1999, Anisha et al. 2010). It is thought that they could provide better process economics and a great deal of handling ease (Pandey 2003). But Ashley et al. (1999) indicate that this static configuration could lead to axial temperature profiles with the highest temperature, sometimes over 20°C higher than the inlet air temperature, occurring at the top of the bed (Pandey 2003). In order to prevent temperature reaching undesirable levels, two strategies based on an axial heat transfer model, namely periodic reversing of the direction of air flow and periodic mixing were proposed (Ashley et al. 1999). Despite this limitation, packed bed bioreactors remain more adequate than tray fermenters since forced aeration may partially allow overcoming the temperature control problems although high temperatures can still be reached next to the air outlet. It can therefore be noted that some researchers have started to look into the design constraints but, additional effort remain to be invested.

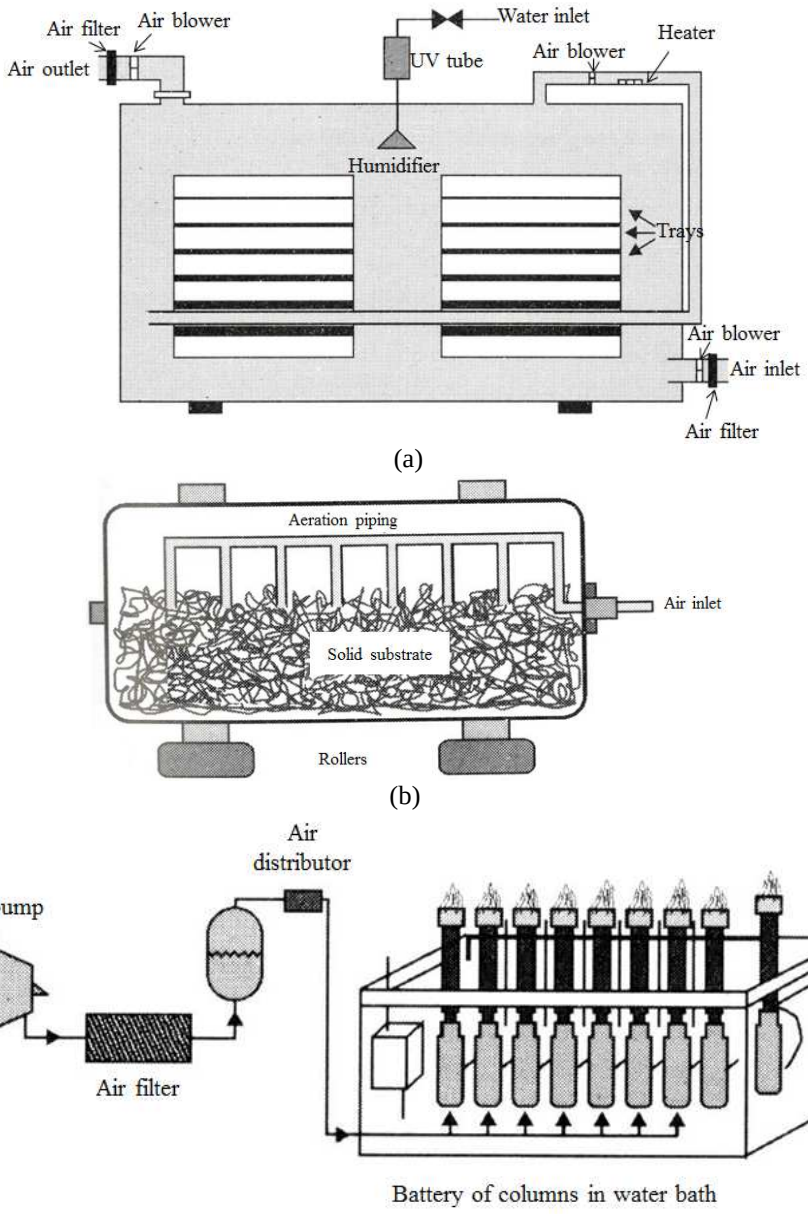

Figure 2. Schematic diagrams of different types of SSF reactors (a) koji-type tray reactor, (b) rotating drum bioreactor and (c) packed column bioreactor system (from Sermanni and Tiso 2008).

2.4.3. Composition and stability of the enzymatic mixture

When using commercial enzymes, specific formulas based mainly on the ratio of FPase to β-glucosidase activities have been studied. Most used ratios vary from 1:1.75 to 1:2 since it is hypothesized that β-glucosidase improves cellulose hydrolysis by reducing end product inhibition by cellobiose (Berlin et al. 2005, Yang and Wyman 2006, Chen et al. 2007, Rosgaard et al. 2007). However, the enzyme activities found in a crude enzyme mixture can only be manipulated through operational conditions although the exact results cannot be

completely guaranteed. As shown in Table 1, even when using the same inoculum, enzymatic yields can very due to small changes in the operational conditions.

This brings the attention to the point concerning the efficiency of the produced enzymes. The objective behind enzyme production is their downstream use in the biodegradation of lignocellulosic matrices. In the reviewed work, only few authors evaluated this efficiency through saccharification potential tests. Chahal (1985) tested the hydrolytic potential of the cellulase system produced at pH 4.8 and 6.7; they found that 65% of the hydrolysis occurred during the first 20 h for both pH levels. After 96 h of hydrolysis, almost 90% of the saccharification potential was expressed. In Kalogeris et al. (2003), an enzyme saccharification test of cellulose was also carried out but interestingly at 60°C; after 48 h only 29% cellulose hydrolysis took place. Another 20% saccharification was also reported by Acharya et al. (2010) after 8 h of incubation. Deswal et al. (2011) compared the enzymatic hydrolysis of untreated and alkali pretreated rice straw and wheat straw matrices using crude enzyme extracts. They observed that the alkali treatment releases lignin moieties and thus increases the accessibility to cellulose by the enzymes. Finally, Dave et al. (2012) ran some saccharification tests at 60°C for wheat straw, rice straw and sugarcane bagasse. Results showed that the concentrations of reducing sugars increased up to 48 h for wheat straw and rice straw and only till 36h for sugarcane bagasse.

Finally, in commercial enzymatic solutions chemical stabilizers are added to maintain the quality of the product. But when considering SSF crude enzyme mixtures, their tolerance for mainly pH and temperature variations dictates or at least limits the options of their downstream use. For this purpose, some authors have studied pH stability as well as the thermostability of their produced crude enzyme extracts. In this context, Kalogeris et al. (2003) determined the stability profile of the enzyme mixture in a temperature range of 50 to 90°C and pH range of 3 to 11. Results showed that the entire enzymatic activity was retained

at pH values of 5 to 8. Focusing mainly at endoglucanase and β-glucosidase, it was found that at 70°C their half-lives were respectively 2.5 and 1 d, but decreased to 42 and 18 min at 80°C. Acharya et al (2010) also tested the activities of the same enzymes at temperature varying between 30 and 80°C for 60 min and pH of 3 to 10. The residual activities of both enzymes remained very high until 60°C but dropped to 50% at 80°C. In addition to that, optimal pH was 4, while 80% of relative activity was observed at pH 7 and only 30% at pH 10. Finally, Dave et al. (2012) tested enzyme thermostability (from 30 to 100°C) and pH stability (from 3 to 10) and found that relative activities at 30°C for endo-β-1,4-glucanase and β-glucosidase were 35% and 10%, respectively, increased up to 100% at 70°C and then dropped to 60% and 45% at 100°C. In general, endo-β-1,4-glucanase was highly stable in the pH range of 3 to 6 (relative activities higher or equal to 85%) and β-glucosidase showed high stability in the pH range of 3. to 8 (relative activities higher or equal to 90%). Maximum activity for both enzymes was measured at pH 4.

All these results should be gathered and confirmed for given operational conditions in order to define how the SSF process can be properly integrated in an overall waste valorization process.

2.5. Conclusions

The main driving force behind the development of SSF processes for environmental applications is a serious need for low cost large-scale production of enzymes. Both purchase and production costs of commercial cellulases contribute to a large proportion of the total costs of bioenergy production. Taking this into account, on-site cellulase production under SSF from lignocellulosic biomass could be considered as cost-effective strategy (Hideno et al. 2011). Within this context, research has been conducted to find optimal operational conditions for enzyme production at lab-scale. Nevertheless, major challenges remain for large scale development in environmental applications. Operational parameters, mainly

substrate autoclaving and inoculum, are developed without taking into account the economics of the change in scale. Very few authors looked into the efficiency of the crude enzyme mixtures and their stability in the process. Large scale equipment is available for submerged fermentation but not for the solid state. For SSF to be up to the challenge of the new energy era; future research should be reoriented to answer both technical and economic feasibilities of large scale applications.

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CHAPTER 3

Scale down of lignocellulolytic enzyme activities analysis: developing microplate-based assays

ABSTRACT

With the increasing use of enzymes in environmental applications, there is a need for analytical methods adapted to large factorial experiments. Existing reference methods are chemical and labor intensive and unsuitable to analyze in parallel a large number of samples. Based on an extensive literature review and on experimental results, this work compares reference and microplate adapted methods to define the most adequate filter paper, carboxymethylcellulase, β-glucosidase and xylanase activity tests. In the adapted methods, the total volume of the enzymatic reaction was reduced from 1-1.5 mL to 60-120 µL. Statistical analysis of the activities measured on enzyme mixtures by applying the 96-well plate reduced methods showed that they were not significantly different to the activities obtained with reference tests.

Keywords: 96-well plate, filter paper activity, carboxymethylcellulase, β-glucosidase, xylanase.

3.1. Introduction

Emerging applications in industrial biotechnology are multiplying mainly those using enzymes in biofuel production and waste treatment of lignocellulosic matrices. But the three structural polymers of lignocellulose (cellulose, hemicellulose and lignin) present a complex configuration as shown in Figure 1.

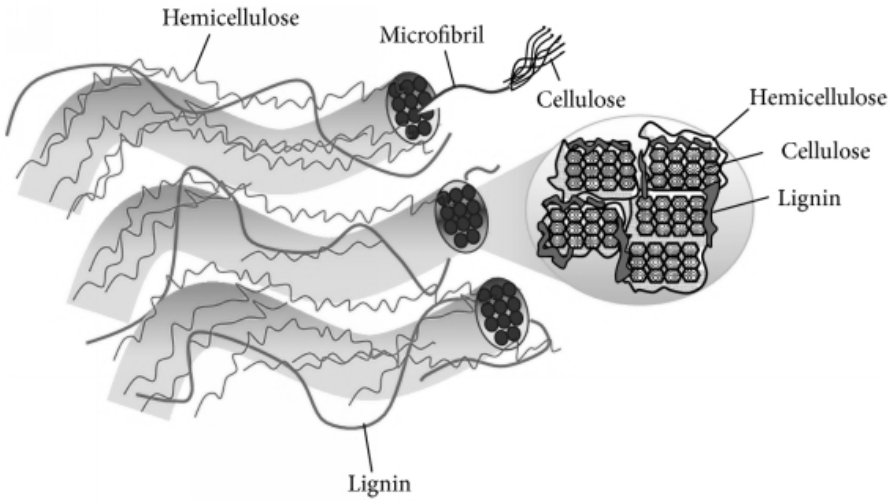

Figure 1. Schematic diagram of the three components of lignocellulose: cellulose, lignin and hemicellulose (Lee et al., 2014).

Cellulose is a linear homopolymer of glucose units (Figure 2); the chains of cellulose tend to forms microfibrils with alternating crystalline and amorphous regions. It is hydrolysed by cellulase, a complex of at least 3 groups of enzymes (Deswal et al., 2011; Jabasingh and Nachiyar, 2011): endoglucanase (endo-1,4-β-D-glucanase) which acts randomly on soluble and insoluble cellulose chains, exoglucanase (Exo-1,4-β-D-glucanase, cellobiohydrolase) which liberates cellobiose from the reducing and non-reducing ends of cellulose chains and β-glucosidase (cellobiase) which liberates glucose from cellobiose. Hemicelluloses are polymers composed of monomeric components mainly xylose, mannose, galactose, arabinose and methylglucuronic acid (Figure 2). Xylanases are involved in the degradation of hemicellulose. Similar to cellulases, they can act synergistically to achieve hydrolysis. Predominant enzymes within this system are endoxylanases which attack the polysaccharide backbone and β-xylosidases which hydrolyze short xylo-oligosaccharides to xylose (Kim and

Kim, 2012; Montoya et al., 2012). Finally, the chemical structure of lignin is also complex: it is mainly made up of guaiacyl and syringyl units (Figure 2) to which phenylpropanoid units can also be cross-linked. Ligninase is a generic name for a group of isozymes that catalyze the oxidative depolymerization of lignin. The most widely known enzymes to degrade lignin are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Montoya et al., 2012).

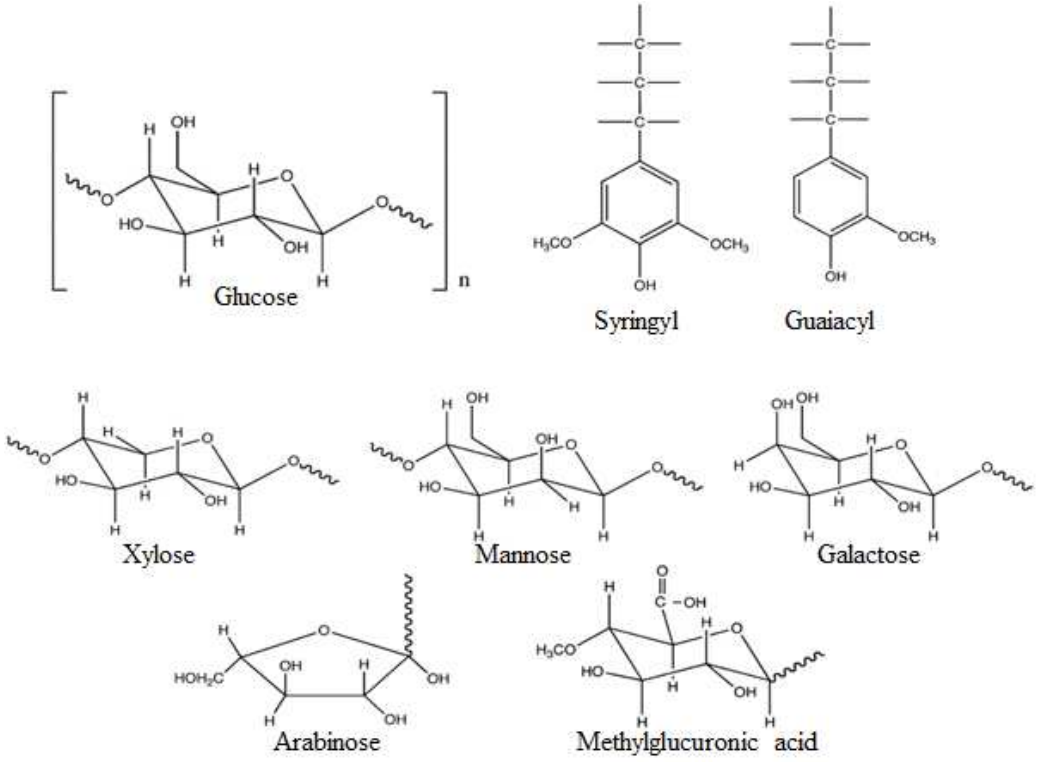

Figure 2. Chemical structures of the different monomeric components: glucose of cellulose, syringyl and guaiacyl of lignin, xylose, mannose, galactose, arabinose and methylglucuronic acid of hemicellulose (Santoni et al., 2015).

Besides the diversity of enzymes that can be involved, both operating and capital costs of using enzymes in environmental applications for bioethanol or biogas production are very high. It becomes then essential to follow-up the fate of enzymes in the process in terms of their corresponding enzymatic activity. For that purpose, reference methods exist but they are labor-intensive, time consuming, chemical intensive and most importantly not adapted to large factorial experiments. Although some reduced protocols have been reported in the literature, they were not necessarily compared to the reference method.

The main objective of this work is to review reference and other existing protocols for four enzymes: total cellulase or filter paper activity (FPase), carboxymemthylcellulase (CMCase), β-glucosidase and xylanase. Using enzyme solutions, chosen methods are experimentally compared. New microplate-based methods are proposed when existing reduced methods do not compare well with the reference.

3.2. Materials and Methods

3.2.1. Analytical methods for measuring enzyme activity

3.2.1.1. Filter paper activity (FPase, total cellulase, filter paper cellulase, FP cellulase, exo-1,4-β-D-glucanase, exoglucanase)

One Filter Paper Unit (FPU) is defined as the amount of enzyme that releases one µmol of glucose per minute in the assay reaction. FPase estimates the total cellulase activity in a medium. It is generally assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman No.1 filter paper as substrate in 50 mM sodium citrate buffer (pH 4.8) at 50°C for up to 60 min. Using three commercial enzymes (FP1, FP2 and FP3), three methods were compared. The reference method is indicated by the International Union of Pure and Applied Chemistry (IUPAC) and described in Ghose (Ghose, 1987). It is based on using 50 mg of substrate with a final total reaction volume of 24.5 ml. A 96-well plate adapted method using 3.4 mg substrate presented in Xiao et al. (Xiao et al., 2004) was also tested. It follows the same steps described in the reference method but at liquid/solid ratio of 28.24; compared to 30 in the reference. Finally, a new method (FPase.mod) is proposed based on the IUPAC reference with a 1/20 reduction in volume.

Enzyme mixtures were diluted at different concentrations. Table 1 summarizes the different steps of the three analytical methods for FPase analysis.

Table 1. Summary table of the three compared methods for FPase analysis with Ghose (1987) as standard IUPAC, Xiao et al. (2004) and FPase mod.

	Ghose (1987) – reference method	Xiao et al. (2004)	FPase mod.
Substrate		Whatman n°1 paper	
Amount of substrate	50 mg	3.4 mg	2.4 mg
Buffer type		50 mM sodium citrate buffer (pH 4.8)	
Buffer volume	1 ml	0.064 ml	0.048 ml
Sample volume	0.5 ml	0.032 ml	0.024 ml
Liquid/solid ratio	30	28.24	30
Total reaction volume	1.5 ml	0.096 ml	0.072 ml
Incubation at 50°C for 60 min	Test tubes in water bath	96-well microplate in dry bath	96-well microplate in dry bath
Volume of DNS	3 ml	0.1 ml	0.144 ml
Color development	100°C for 5 min	95°C for 5 min	100°C for 5 min
Dilution	Add 20 ml H_2O	Mix 0.16 ml H_2O to 0.036 ml color developed mixture	Mix 0.196 ml H_2O to 0.044 ml color developed mixture
Reading wavelength	545 nm	540 nm	545 nm

3.2.1.2. Carboxymethylcellulase (CMCase, Endo-1,4-β-D-glucanase, EC 3.2.1.4)

One unit (U) of CMCase activity is defined as the amount of enzyme that liberates 1 µmol of reducing sugar as glucose per minute under the analysis conditions.

The IUPAC reference method for CMCase was also described by Ghose (1987). Xiao et al. (2005) presented a protocol adapted to 96-well plates, in which 0.03 ml sample and 0.03 ml of 2% CMC are mixed and incubated for 30 min at 50°C. After incubation, 0.06 ml DNS are added and the mixture is heated at 95 C for 5 min. Later, 0.01 ml of color developed mixture is transferred for absorbance reading at 540 nm. In addition to the two above mentioned methods; eight different combinations of a modified method (CMCase.mod) were tested with varying substrate concentrations and substrate to sample ratios. All methods were

compared using two enzyme mixtures CMC1 and CMC2. Table 2 summarizes the different analytical methods used to measure CMCase.

Table 2. Summary table of the ten compared methods for CMCase analysis with Ghose (1987) as reference, Xiao et al. (2005) and FPase mod. (eight different combinations).

	Ghose (1987) – reference method	Xiao et al. (2005)	CMCase.mod (8 different combinations)
Substrate	Carboxymethylcellulose salt (CMC salt)		
Buffer	50 mM sodium citrate buffer (pH 4.8)		
Substrate concentration	2% CMC in buffer	2% CMC in buffer	2% or 4% CMC in buffer
Substrate volume	0.5 ml	0.03 ml	0.03 ml
Sample volume	0.5 ml	0.03 ml	0.03 ml or 0.06 ml
Total reaction volume	1 ml	0.06 ml	0.06 ml or 0.09 ml
Incubation at 50°C for 30 min	Test tubes in water bath	96-well microplate in dry bath	96-well microplate in dry bath
Volume of DNS	3 ml	0.06 ml	0.15 ml and 0.18 ml
Color development	100°C for 5 min	95°C for 5 min	95°C for 5 min
Cooling	N/A	N/A	N/A
Dilution	Add 20 ml H_20	N/A	Wihout dilution or Mix 0.2 ml H_2O with 0.04 ml color developed mixture
Reading wavelength	540 nm	540 nm	540 nm

3.2.1.3. β-glucosidase (β-D-gluside glucohydrolase, EC 3.2.1.21)

Unlike all other three enzymes studied in this work, β-glucosidase test does not require the use of a dinitrosalicylic acid (DNS) solution. One unit (U) of β-glucosidase activity is defined as the amount of enzyme which produces 1 µmol of p-nitrophenol (pNP) per minute from 4-nitrophenyl α-D-glucopyranoside (pNPG).

The reference method for the enzyme activity was described by Kubicek and Pitt (López Abelairas, 2010). A reaction mixture containing 1 ml of pNPG (1 mM), 1.8 ml of acetate buffer (pH 4.8) and 0.2 ml of sample was incubated at 50°C for 30 min. The absorbance was read at 412 nm. A reduced volume protocol was presented by López Abelairas (2010) although not adapted for 96-well plates. A reaction mixture 0.1 ml of 1 mg/ml pNPG and

0.1 ml sample was incubated at 50°C for 30 min and then the reaction was stopped by adding 0.5 ml of 2% Na_2CO_3. Absorbance was measured at 410 nm. Therefore for the purpose of this work, three modified methods based on the reference (BGase.mod1) and another modified (BGase.mod2) based on López Abelairas (2010) were tested. Results were compared using two enzyme mixtures BG1 and BG2. Table 3 summarizes the different analytical methods used to measure β-glucosidase.

Table 3. Summary table of the five compared methods for β-glucosidase with Kubicek and Pitt (1982) as reference, BGase.mod1 (three different combinations) and BGase.mod2

	Kubicek and Pitt (1982)-reference method	BGase.mod1 (3 combinations)	BGase.mod2
Substrate	pNPG		
Buffer	50 mM sodium citrate buffer (pH 4.8)		
Substrate concentration	10 mM pNPG	10 mM pNPG	1 mg/ml pNPG
Substrate volume	1 ml	0.1 ml	0.1 ml
Sample volume	0.2 ml	0.02 ml	0.1 ml
Total reaction volume	1.2 ml	0.12 ml	0.2 ml
Incubation	40°C for 10 min	40°C for 10 min or 50°C for 10 min	50°C for 30 min
Color development/ Reaction stopping	1 ml of 1 M Na_2CO_3 (followed by centrifugation)	0.1 ml of 1 M Na_2CO_3 or Mix 0.06 ml sample with 0.15 ml of 2% Na_2CO_3	86 µL of color developed reaction mixture mixed with 214 µL of 2% Na_2CO_3
Absorbance reading	412 nm	412 nm	410 nm

3.2.1.4. Xylanase (1,4-β-D-xylan xylanohydrolase EC 3.2.1.8)

One unit (U) of xylanase is defined as the amount of enzyme, which produces 1 µmol reducing sugar as xylose per min in the reaction mixture under the specified conditions.

The reference method was reported by Ghose and Bisaria (1987). For the assay, 0.5 ml sample and 0.5 ml of 1% beechwood xylan prepared in 50 mM sodium citrate buffer (pH 4.8) were mixed and incubated at 50°C for 30 min. After incubation, 3 ml DNS were added and

the mixture was put in the boiling water bath for 5 min for color development. The reaction was then diluted with 20 ml of H_2O and the absorbance was determined at 540 nm. The method presented in Bailey et al. (1992) was also tested since it is the result of an interlaboratory study. A mixture of 1.8 ml of 1% xylan and 0.2 ml sample were incubated at 50°C for 5 min. Three ml of DNS were then added and the mixture was boiled for 15 min before a final addition of 10 ml of water. Absorbance was measured at 540 nm. The protocol of Cianchetta et al. (2012) was added to the comparison study since it is the only 96-well adapted protocol found in the literature. The authors applied a 25-fold reduction of the reference method. In addition to the methods presented above, 12 different combinations of a modified method (Xase.mod1) based on the reference method and 2 combinations of another modified method (Xase.mod2) based on that of Bailey et al. (1992) are proposed.

The different analytical protocols were compared using two enzyme mixtures X1 and X2. Table 4 summarizes the 17 different analytical methods tested to measure xylanase activities.

Table 4. Summary table of the 17 compared methods for β-glucosidase with Ghose (1987) as reference, Bailey et al. (1992), Cianchetta et al. (2012), Xase.mod1 (12 different combinations) and Xase.mod2 (two different combinations).

	Ghose and Bisaria (1987) – reference method	Bailey et al. (1992)	Cianchetta et al. (2012)	Xase.mod1 (12 combinations)	Xase.mod2 (2 combinations)
Substrate	1% beechwood xylan in buffer				
Buffer	50 mM sodium citrate buffer (pH 4.8)				
Substrate volume	0.5 ml	1.8 ml	0.02 ml	0.02 ml to 0.09 ml	0.09 ml
Sample volume	0.5 ml	0.2 ml	0.02 ml	0.01 ml to 0.03 ml	0.01 ml
Total reaction volume	1 ml	2 ml	0.04 ml	0.06 ml to 0.1 ml	0.1 ml
Incubation	50°C for 30 min	50°C for 30 min	50°C for 20 min	50°C for 30 min	50°C for 30 min
Volume of DNS	3 ml	3 ml	0.12 ml	0.18 ml DNS to 0.06 ml mixture	0.15 ml
Color development	Boil in water bath for 5 min	Boil for 15 min	Boil for 15 min	Boil for 5 to 15 min	Boil for 15 min
Dilution	20 ml H_2O	Add 10 ml H_2O	0.04 ml of color developed reaction mixture mixed with 0.2 ml of H_2O	0.04 ml of color developed reaction mixture mixed with 0.2 ml of H_2O or without dilution	0.04 ml of color developed reaction mixture mixed with 0.2 ml of H_2O or without dilution
Absorbance reading	540 nm	540 nm	540 nm	540 nm	540 nm

3.2.2. Protocol for measuring reducing sugars

The 2-hydroxy-3,5-dinitrobenzoic acid most commonly known as the dinitrosalicylic acid (DNS) is very often used for the analysis of reducing sugars released during the enzymatic activity tests. For the DNS solution, 10 g of DNS were dissolved in 500 ml of distilled water at 50°C. The mixture was then cooled down to room temperature (RT) before adding 20 ml of 2 N sodium hydroxide solution and 300 g of Rochelle salts (potassium sodium tartrate

tetrahydrate). The volume was finally brought to 1 L with distilled water. The solution was kept at RT in the dark.

Reducing sugars were analyzed according to Navarro et al. (2010). A mixture of 60 µL DNS and 60 µL sample was incubated for 10 min at 94°C and then cooled down for 5 min at room temperature. Later 100 µL were transferred to a reading plate for absorbance measurement at 540 nm.

3.2.3. Reading absorbance

In non-reduced analytical protocols, an Uvi Light XTD 2 spectrophotometer (Secomam, ALES cedex, France) was used to measure absorbance. For 96-well plate adapted tests, a PowerWave XS2 microplate spectrophotometer (BioTek Instruments, Inc., Vermont, USA) with Gen5^{TM} software was used.

It is important to note that in reduced protocols, given the small volumes used in the analysis, the plates were always covered with adhesive seals to reduce evaporative losses.

3.2.4. Calculating enzyme activities

Defining the critical value is the first step before carrying out any unit calculation of the enzymatic activity. This term was first introduced in Ghose (1987) as the middle point in the calibration range. The origin of the middle point goes back to the works of Working and Hotelling (1929), in which this point was defined as that with the highest probability in a trend. Therefore, for the different enzymatic activity calculations, the critical value should be first defined based on a specific calibration range. Then, the corresponding enzyme activity unit equation was derived. Note that enzyme samples were diluted at different concentrations and at least two dilutions were taken into account to derive the enzyme activity; the corresponding concentration to the critical value should fall between the two chosen enzyme concentrations.

3.2.4.1. FPase

A linear glucose standard curve was constructed using absolute glucose amounts in mg plotted against absorbance. Absorbance values of the different dilutions of the enzyme mixtures (after subtraction of the enzyme blanks) were thus translated into glucose. The critical value is defined as F_c (mg glucose); by plotting glucose against enzyme concentration (= 1/dilution), the critical concentration releasing F_c was defined.

One FPU is 1 µmol of glucose/min which corresponds to 0.18 mg glucose/min. Therefore, F_c is produced by a given sample volume (ml) in a given time (min). This leads to defining the FPU units as follows:

$$FPU = \frac{\frac{Fc}{0.18 * sample\ volume * incubation\ time}}{critical\ concentration}\ \mu mol/(min.ml) \vee units/ml$$

3.2.4.2. CMCase

The calculations are identical to the FPase units considering a critical value C_c. The enzymatic activity is thus expressed as:

$$CMC = \frac{\frac{Cc}{0.18 * sample\ volume * incubation\ time}}{critical\ concentration}\ \mu mol/(min.ml) \vee units/m\,l$$

3.2.4.3. β-glucosidase

A linear pNP standard curve was constructed using absolute pNP amounts in µmol plotted against absorbance. Absorbance values of the different dilutions of the enzyme mixtures (after subtraction of the enzyme blanks) are thus translated into pNP. The critical value is defined as B_c (µmol pNP); by plotting pNP against enzyme concentration, the critical concentration releasing B_c was defined.

One unit (U) of β-glucosidase activity is defined as 1 µmol pNP/min. Therefore, B_c is produced by a given sample volume (ml) in a given time (min). This leads to the following equation:

$$\beta - glucosidase = \frac{\frac{Bc}{sample\ volume * incubation\ time}}{critical\ concentration}\ \mu mol/(min.ml) \vee units/ml$$

3.2.4.4. Xylanase

A linear xylose standard curve was constructed using absolute xylose amounts in µmol plotted against absorbance. Absorbance values were then translated into xylose. The critical value is defined as X_c (µmol xylose); by plotting xylose against enzyme concentration, the critical concentration releasing X_c was defined.

One unit (U) of xylanase activity is defined as 1 µmol xylose/min. Therefore, X_c is produced by a given sample volume (ml) in a given time (min). This leads to the following equation:

$$Xylanase = \frac{\frac{Xc}{sample\ volume * incubation\ time}}{critical\ concentration}\ \mu mol/(min.ml) \vee units/ml$$

3.2.5. Statistical analysis

For each enzyme activity method, enzyme mixtures were diluted at least at 5 different levels and three replicates were carried out for every dilution. All data was then analyzed using the statistical package XLSTAT (version 2012.6.06). An analysis of variance (ANOVA) followed by a Tukey HSD (Honest Significant Difference) at 95% confidence level was run to determine which test was least significantly different from the chosen reference protocol.

3.3. Results and Discussion

In each of the following sections, an overview of the existing analytical methods is first presented to justify the choice of the tested protocols. Calibration curves and statistical results are shown followed by a detailed description of the best 96-well adapted enzyme activity test.

3.3.1. FPase

Although the IUPAC method is described in Ghose (1987), the oldest referenced method for FPase is that of Mandels et al. (1976). This latter was used by many researchers such as Chahal (1985) and Krishna (1999). In fact the IUPAC method is based on that of Mandels et al. (1976) but differs with the amount of water added (20 ml instead of 16 ml) and the

wavelength at which the absorbance is read (540 nm instead of 550 nm). It was widely used by many researchers such as Camassola and Dillon (2009), Deswal et al. (2011) and Hideno et al. (2011). Krishna et al. (1995) and Guowei et al. (2011) used it as well; however in the former the Na-citrate buffer is at 100 mM (pH = 5) and in the latter the incubation takes place for only 30 min. More recently, Adney and Baker (1996) proposed a modified version of the same method, in which the dilution before reading the absorbance was increased from a water to color developed mixture ratio of 4.44:1 to 12.5:1.

For the reduced protocols, Decker et al. (2003) proposed a method with a total reaction volume of 0.08 ml using 2.67 mg of cellulose powder in 50 mM citrate buffer. Plates were incubated at 50°C in custom modified microtiter plate incubators for 60 min. Later 150 µL DNS reagent were added before incubation at 98°C for 10 min for color development. After this stage, 200 µL deionized water were mixed with 10 µL color developed mixture before reading the absorbance at 540 nm. This protocol was however not tested since it is based on powdered cellulose as the substrate as compared to Whatman n°1 paper used in all other reviewed work. Two additional reduced methods were reported by Xiao et al. (2004). In the first, the enzymatic reaction volume was reduced to 60 µL (40 µL buffer and 20 µL sample) and in the second to 96 µL (64 µL buffer and 32 µL sample). According to the authors, statistical analyses of the cellulase activities showed no significant difference between the different types of tests. For the comparison study, the protocol based on the larger sample volume was considered. Finally, more recently Camassola and Dillon (2012) described another protocol based on a 1/10 volume reduction of that used by Mandels (1976). The total reaction volume was thus reduced to 150 µL with 0.3 ml DNS addition and then a 2:1 dilution ratio of water to color developed mixture. The authors reported that according to the their protocol the results were statistically similar to those of Mandels et al. (1976), when using the method of Decker et al. (2003) the data variability was important and the 60-µL

protocol of Xiao et al. (2004) gave significantly higher values. This method was not included in the comparison but the FPase.mod method is based on the same approach of volume reduction.

Statistical analysis shows that the proposed method FPase. mod provides results that are not significantly different from the reference (Table 5). Results based on the Xiao et al. (2004) method are lower than those obtained by the reference. This might be explained by the fact that the ratio of enzyme to substrate is lower when compared to the other methods; the amount of enzyme is thus limiting. These interpretations apply to the three tested enzyme mixtures. Figure 3 shows the calibration lines for the reference method and that of FPase.mod. Table 5 summarizes the average results of the different tested protocols with R^2 value of 0.81.

Table 5. Statistical analysis results for FPase (R^2 = 0.81)

Tested method	Average (FPU/mL)	Groups
Ghose (1987)	*59*	*A*
FPase.mod	54	A
Xiao et al. (2004)	43	B

*Groups followed by the same letter are not significantly different (P≤ 0.05)

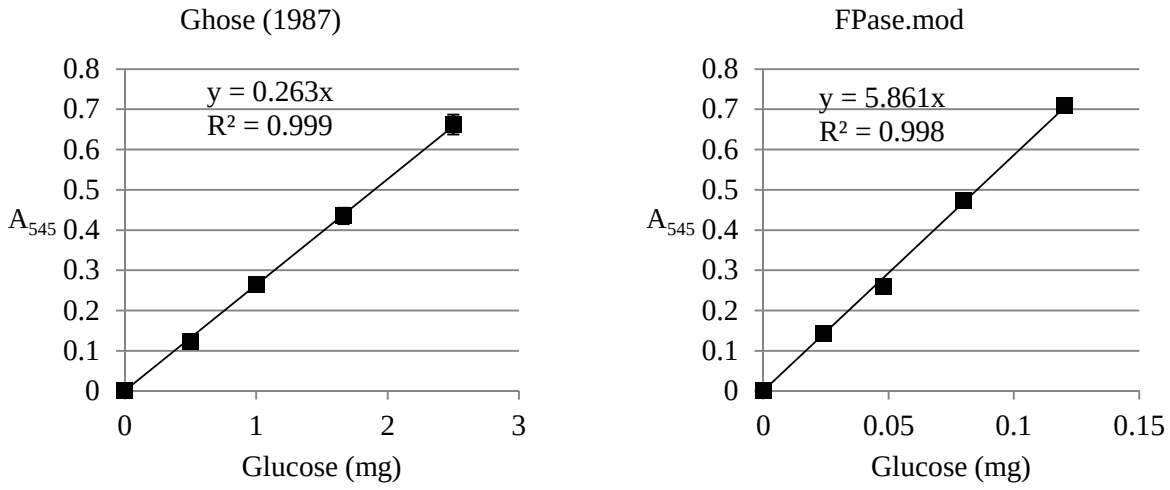

Figure 3. Calibration curves of glucose in the analysis of FPase activity for the reference method (Ghose, 1987) and best 96-well plate adapted method (FPase.mod).

3.3.2. CMCase

The IUPAC reference (Ghose, 1987) has been used by many authors such as Gokhale et al. (1991), Krishna et al. (1995), Krishna (1999), Camassola and Dillon (2009) and Acharya et al. (2010). And many other authors have also modified it. For example, Kalogeris et al. (2003) used 4% CMC while Membrillo et al. (2008) and Guowei et al. (2011) used 1% CMC. Deswal et al. (2011) used the buffer at pH 5.5, Hideno et al. (2011) ran the incubation at 45°C and Kiranmayi et al. (2011) used 1.5 ml of buffer at pH 5.0 and ran the incubation at 30°C. Finally, Kim and Kim (2012) performed the reactions at 30°C for 60 min in 50 mM Tris-HCl buffer (pH 7.2). A reduced method was described by Konig et al. (2002). A mixture of 0.03 ml sample was placed in 2-ml Eppendorf tubes and pre-incubated for 5 min at 40°C in a water bath. Later 0.3 ml of 4% CMC was added and the mixture was incubated at 40°C for 20 min. This was followed by an addition of 0.15 ml DNS solution and incubation in boiling water bath for 10 min. Finally 1.5 ml water were added before reading the absorbance at 530 nm. However, the only 96-well adapted method is that of Xiao et al. (2005). For comparison purposes, the reference method and that of Xiao et al. (2005) were tested. For the latter, the authors indicated that their results are highly reproducible and accurately measured the endoglucanase activity compared with the IUPAC standard method. The eight different combinations proposed in Table 2 take into account the variations proposed by the other authors.

Given the results of the statistical analysis shown in Table 6, the protocol of Xiao et al. (2005) underestimates the enzymatic activities. Several scenarios of the CMCase.mod protocol did not significantly differ from the reference method. However, the best method is CMCase.mod (#6), which is the least significant different compared to the reference. Figure 4 shows the calibration curves of both the reference and chosen methods. The chosen protocol corresponds to a total reaction mixture of 0.06 ml in which 0.03 ml of 4% CMC in citrate

buffer and 0.03 ml of sample were mixed. After incubation at 50°C for 30 min, 0.18 ml DNS was added and placed at 100°C for 15 min. Before reading the absorbance at 540 nm, 40 µL color developed mixture were mixed with 200 µL distilled water.

Table 6. Statistical analysis results for CMCase (R^2 = 0.897)

Tested method	Average (U/mL)	Groups			
Ghose (1987)	*3494*	*A*			
CMCase.mod (#6)	3249	A			
CMCase.mod (#8)	2885	A	B		
CMCase.mod (#5)	2532	A	B	C	
CMCase.mod (#7)	2447	A	B	C	
CMCase.mod (#1)	2098		B	C	
Xiao et al. (2005)	2051		B	C	
CMCase.mod (#2)	1659			C	
CMCase.mod (#3)	334				D
CMCase.mod (#4)	289				D

*Groups followed by the same letter are not significantly different (P≤ 0.05)

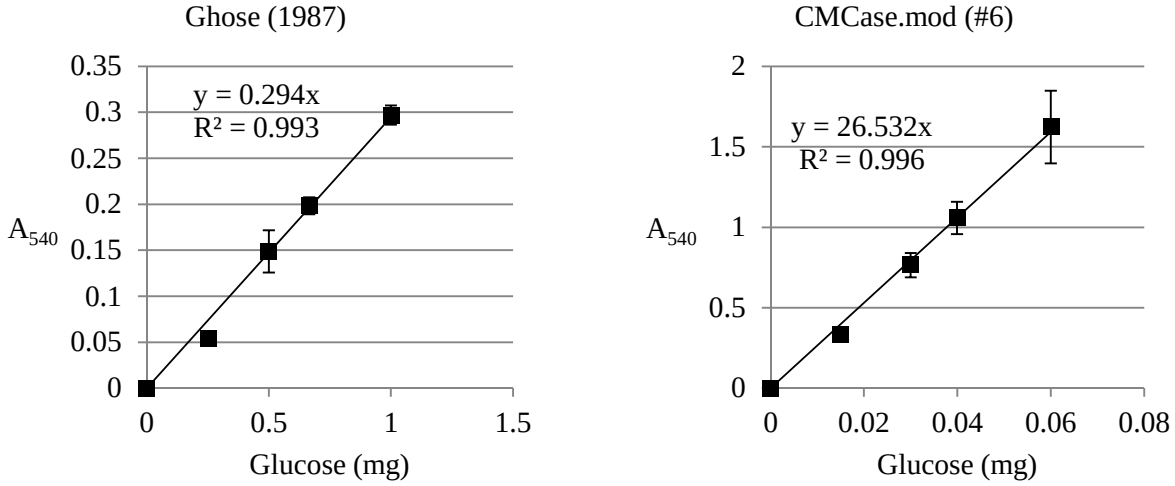

Figure 4. Calibration curves of glucose in the analysis of CMCase activity for the reference method (Ghose, 1987) and best 96-well plate adapted method (CMCase.mod #6).

3.3.3. β-glucosidase

For β-glucosidase, the IUPAC method was described by Wood and Bhat (1988) using cellobiose as substrate. At the exception of Chahal (1985) who uses a 1% salicine solution, all other reviewed works refer to pNPG as substrate. For this reason the most widely used method, Kubicek and Pitt (1982) was considered as the reference method; it was cited in Wood and Bhat (1988), Christakopoulos et al. (1994), Acharya et al. (2010) and Deswal et al.

(2011). Some reduced methods are reported but they are not adapted to 96-well plates. Gokhale et al. (1991) reduced the reaction mixture to 1 ml with 0.9 ml of pNPG (1 mg/ml) and 0.1 ml sample. The mixture was incubated at 65°C for 30 min. Later 2 ml of 2% sodium carbonate were added for color development and absorbance was measured at 410 nm. With also 1 ml reaction mixture, Hideno et al. (2011) mixed 0.1 ml of 10 mM pNPG, 0.05 ml sample, 0.2 ml 50 mM acetic acid buffer (pH 4.8) and 0.65 ml distilled water. The mixture was incubated at 45°C for 10 min and the reaction was terminated by adding 0.5 ml of 1 M Na_2CO_3 Absorbance was measured at 420 nm. Finally in Qian et al. (2012), the reaction mixture consisted of 0.4 ml of pNPG (8 mM), 0.4 ml sample and 1.2 ml citrate phosphate buffer. The mixture was incubated at 45°C for 30 min and the reaction was stopped by adding 2 ml of cold 0.5 M sodium carbonate buffer. The absorbance of the liberated p-NP was read at 400 nm.

More recently, López-Abelairas (2010) used in the reaction mixture 0.1 ml of 1 mg/ml pNPG and 0.1 ml sample. This mixture was incubated at 50°C for 30 min and then the reaction was stopped by adding 0.5 ml of 2% Na_2CO_3. Absorbance was measured at 410 nm. For this protocol, the reaction mixture can fit in the well of a plate but the dilution step was modified to adapt the final volume. It is important to note that Kim and Kim (2012) developed a 96-well adapted method, in which the enzyme activity was measured by incubating enzyme samples with 10mM pNPG in 50mM Tris-HCl buffer at a final volume of 0.2 ml for 60 min at 30°C. The amount of pNP liberated was determined spectrophotometrically at 420 nm. Unfortunately no specific details of the method were published and therefore, it was not possible to include it in the comparison study.

As presented in Table 3, the modified methods BGase.mod1 and BGase.mod2 take into account the different variations tested in the literature. Figure 5 shows the calibration curves of both reference and chosen methods. As shown in Table 7, BGase.mod2 overestimates the

enzymatic activities. Only one modified method based on Kubicek and Pitt (1982) compares best to the reference method (BGase.mod1 (#3). In this latter, a reaction mixture of 0.1 mL 10 mM pNPG and 0.02 mL sample was incubated at 50°C for 10 min. Later 0.06 ml sample was mixed with 0.15 ml of 2%Na_2CO_3 before reading absorbance at 412 nm.

Table 7. Statistical analysis results for β-glucosidase (R^2 = 0.990)

Tested method	Average (U/mL)	Groups			
BGase.mod1 (#2)	766	A			
BGase.mod2	432		B		
Kubicek and Pitt (1982)	*237*			*C*	
BGase.mod1 (#3)	214			C	
BGase.mod1 (#1)	149				D

*Groups followed by the same letter are not significantly different (P≤ 0.05)

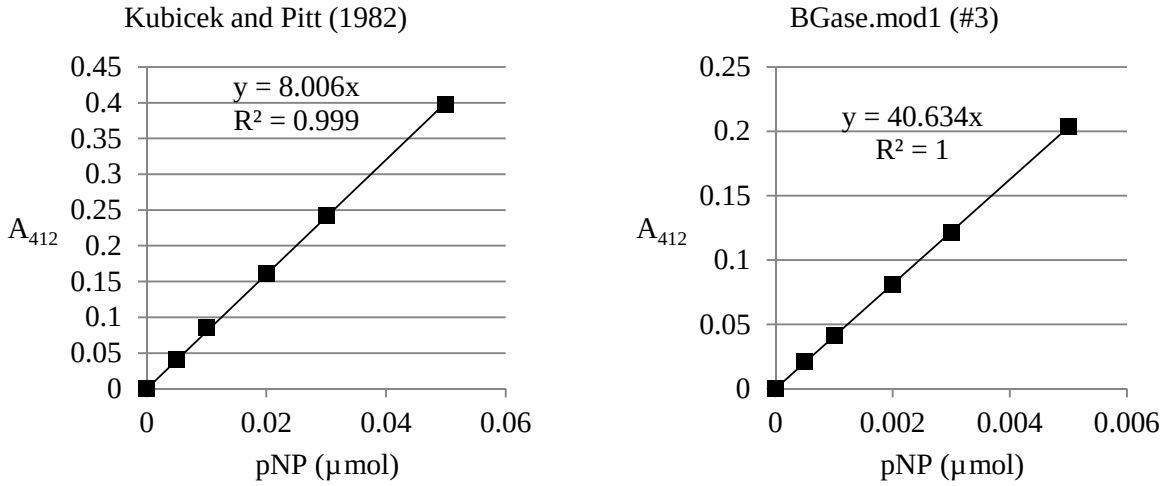

Figure 5. Calibration curves of pNP in the analysis of β-glucosidase activity for the reference method (Kubicek and Pitt, 1982) and best 96-well plate adapted method (BGase.mod #3).

3.3.4. Xylanase

For xylanase, the reference method is described in Ghose and Bisaria (1987) and used also in Gokhale et al. (1991). The protocol proposed by Bailey et al.(1992) was also referenced by Loera and Cordova (2003), Membrillo et al. (2008) and Camassola and Dillon (2009).

Besides the two above methods; some reduced protocols have been also referenced. In Konig et al. (2002), 0.03 ml sample was pre-incubated for 5 min at 40°C and then added to 0.3 ml of 1.5% (w/v) xylan equilibrated at 40°C. The mixture was incubated at 40°C for 20 min. Later,

0.15 ml DNS were added and the new mixture was boiled in a water bath for 5 min. Before measuring the absorbance at 530 nm, 1.5 ml of water were added. In Saha (2002), xylanase activity was assayed in a reaction mixture of 0.5 ml containing boiled oat spelt xylan (1% w/v), 50 mM acetate buffer and the sample. After 30 min incubation at 50°C, reducing sugars were measured according to Miller (1959). In Lu et al. (2003), the same approach was followed by on a reaction mixture of 2 ml of 0.8% (w/v) oat xylan solution and 2 ml sample incubated at 40°C for 30 min. It is also the case in Rodriguez-Fernandez et al. (2011) where 0.5 ml of 1.0% (w/v) birchwood xylan and 0.5 ml sample were incubated at 50°C for 10 min before adding 1 ml of DNS. After heating for 5 min in boiling water bath, 5 ml of water were added and absorbance was read at 540 nm. None of the above mentioned protocols were adapted for the 96-well plate, except that reported by Cianchetta et al. (2012). For this reason this latter was considered for comparison protocols. The other proposed protocols in the comparison study took into account the variations found in the other reviewed methods.

Out of all tested methods, statistical analysis (Table 8) shows that both Xase.mod1 (#12) and Xase.mod1 (#8) are not significantly different from the reference. The former is chosen because the percent difference is less important (7% vs. 12%). The method proposed by Bailey et al. (1992) largely overestimates the enzymatic activities and that of Cianchetta et al. (2012) largely underestimates them.

Table 8. Statistical analysis results for xylanase (R^2 = 0.970)

Tested method	Average (U/mL)	Groups								
Bailey et al. (1992)	15447	A								
Xase.mod2 (#2)	9954		B							
Xase.mod1 (#6)	9647		B							
Xase.mod2 (#1)	9543		B	C						
Xase.mod1 (#5)	9329		B	C						
Xase.mod1 (#11)	9081		B	C						
Xase.mod1 (#12)	8638			C	D					
Ghose	*8094*				*D*	*E*				
Xase.mod1 (#8)	7143					E	F			
Xase.mod1 (#7)	6662						F			
Xase.mod1 (#10)	6361						F	G		
Xase.mod1 (#9)	6198						F	G		
Cianchetta	5590							G		
Xase.mod1 (#2)	4366								H	
Xase.mod1 (#1)	4067								H	
Xase.mod1 (#4)	2865									I
Xase.mod1 (#3)	2828									I

In the chosen protocol, 0.08 ml 1% beechwood xylan in citrate buffer and 0.02 ml sample were incubated at 50°C for 30 min. Later 0.18 ml of DNS was added before boiling for 5 min. Dilution of 0.04 ml color developed mixture in 0.2 ml water was performed before measuring the absorbance at 540 nm. Figure 6 shows the calibration curves of both the reference and chosen methods.

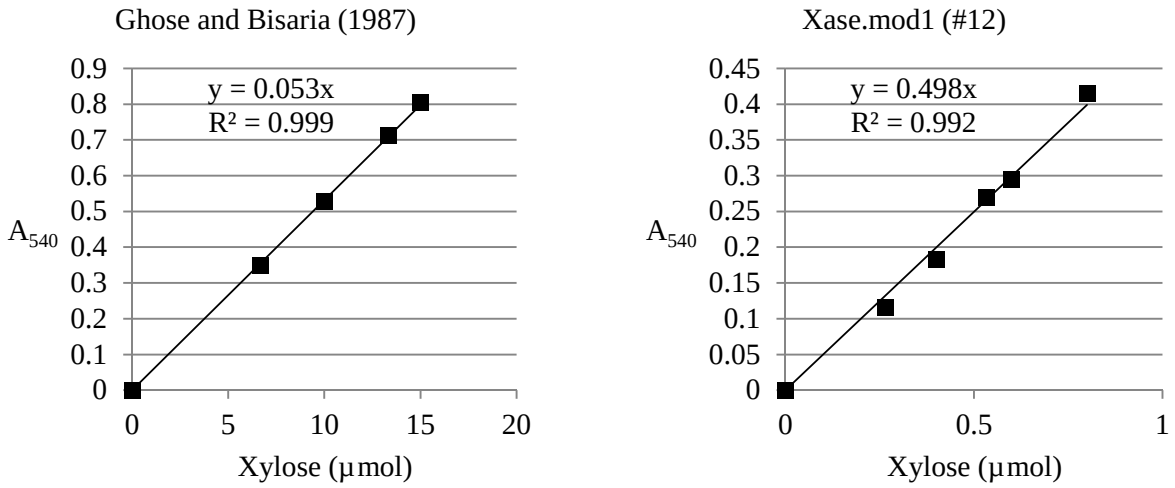

Figure 6. Calibration curves of xylose in the analysis of xylanase activity for the reference method (Ghose and Bisaria, 1987) and best 96-well plate adapted method (Xase.mod #12).

3.4. Conclusions

Four enzymes, FPase, CMCase, β-glucosidase and xylanase, were identified of interest to degrade the lignocellulosic complex under anaerobic conditions. In this context, large factorial experiments need to be conducted but most existing measurement protocols of those enzymes are not adapted. This work identifies through extensive and systematic analytical work microplate-based protocols for these enzymes of interest: 0.072 mL reaction volume with Whatman n°1 paper as substrate for FPase, 0.06 mL reaction volume with 4% CMC as substrate for CMCase, 0.12 ml reaction mixture with 10 mM pNPG as substrate for β-glucosidase and 0.1 mL mixture with 1% beechwood xylan as substrate for xylanase.

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CHAPTER 4

Screening of operational conditions of solid state fermentation for the in situ production of lignocellulolytic enzymes on different solid matrices

ABSTRACT

Solid state fermentation emerges as an interesting alternative for the in situ production of lignocellulolytic enzymes. This work aims at screening different operational conditions to develop the process on complex matrices such as municipal solid waste, brewer's spent grain and paper/cardboard fractions using commercially available inoculum. Substrate type, autoclaving, inoculum type (three Pleurotus ostreatus strains), pH (4-6), moisture (60-80%) and nutrient addition are the factors incorporated in a 46-assay D-optimal design. After 5 days of incubation at 30°C, cellulase, carboxymethylcellulase, β-glucosidase and xylanase activities are measured. For maximum enzyme production, substrate and inoculum types and tested pH range did not impact significantly the results. Autoclaving and increase in moisture content had negative effects while nutrient addition a positive effect. Maximum enzyme activities of 0.35, 2.48, 4.58 and 4.70 U/g DM were recorded for the tested enzymes respectively.

Keywords lignocellulose, solid state fermentation, D-optimal design, municipal solid waste, brewer's spent grains, paper

4.1. Introduction

Anaerobic digestion (AD) is an attractive waste treatment process. It is an environmentally friendly technology to reduce organic waste and a renewable source of energy (EU directive 2001/77/CE). Thus it responds to today's problem of increasing generation of waste and search for new sources of energy.

Lignocellulose, the major constituent of vegetable organic matter, is mainly composed of cellulose, hemicellulose and lignin intimately linked in a semi-crystalline structure. Cellulose is the most abundant organic compound. It is found in plant materials, wastes from forest products, agriculture and fruit and vegetable processing (Mandels et al., 1974). According to Barlaz *et al.* (1990), municipal solid waste (MSW) for example is made up mainly of 51% of cellulose, 15% of lignin, 12% of hemicellulose, and 4% of proteins. Interestingly, 73% of the methane potential in MSW is found in the cellulose followed by 17% in the hemicellulose. Cellulose and hemicellulose are respectively totally and partially biodegradable but remain protected inside the lignocellulosic structure during the AD process. Therefore despite the diverse composition of substrates, lignocellulose could probably be responsible for limiting degradation (Vargas-Garcia *et al.*, 2007). A huge energetic potential is therefore confined within lignocellulosic matrices and its expression depends on the disruption of the structure. The most important technological challenge is thus overcoming the recalcitrance of natural lignocellulosic materials (Kiranmayi et al., 2011). In this perspective a pretreatment step should be incorporated otherwise the energetic pool remains unavailable for anaerobic digestion. Physical and chemical pretreatments have shown positive results however their corresponding economic, energetic and operational costs present major drawbacks. Enzymatic pretreatment has shown promising results but the major inconvenient is the cost of the enzymes.

So far the cost of adding commercial enzymes and that of a well-controlled process takes over the added value of the increased methane production and the possibility of reducing waste treatment time. Few studies have actually estimated the total cost of this treatment. Parmar et al. (2001) approximated the overall treatment of 1 dry ton of sewage sludge at US 100$. As for Jordan and Muller (2007), initial estimates of the cost of enzymes used to treat 1 ton of spent mushroom compost in a liquid-solid system is 803.9€ excluding any mechanical or operational expenses. The cost of cellulase and β-glucosidase has been estimated to account for approximately 50% of the cost of the hydrolysis process for the production of ethanol from softwood (Tu et al., 2007). Although the cost of cellulase was reduced over the last decade, current cost enzyme-catalyzed hydrolysis continues to deter widespread commercialization. In this context solid state fermentation (SSF) could be the best alternative for *in situ* production of enzymes that could be incorporated in the waste treatment system.

The key objective of this work is screening operational conditions for the in situ production of endogenous enzymes through SSF. Produced enzymes through this process will be used to enhance the hydrolysis of lignocellulosic matrices. This concept aims at significantly increasing the anaerobic biodegradability of lignocellulosic matrices and the gain in energy production in the anaerobic treatment step. The novelty of this work resides in: the use of both more complex substrates such as municipal solid waste for example and the use of commercially available inoculum sources. The main concern behind this research is to participate in the development of the SSF process keeping in mind economic constraints for a future successful scale up process.

4.2. Materials and Methods

4.2.1. Matrices of interest

For enzyme production under solid state fermentation, three types of matrices are tested: municipal solid waste (MW fraction), paper/cardboard (PC fraction) mixture recovered from

a sorting facility and brewers' spent grain (SG fraction). Table 1 summarizes the lignocellulosic composition of the tested matrices based on their corresponding Van Soest fractions: soluble (SOL), hemicellulose-like (HEM), cellulose-like (CEL) and lignin and cutin-like (LIC). All three matrices were dried at 80°C until stabilization of the weight and then grinded using a 1 cm mesh.

Table 1. Van Soest characterization (%) of the solid matrices MW, PC and SG in terms of soluble (SOL), hemicellulose-like (HEM), cellulose-like (CEL) and LIC (lignin and cutin-like) fractions.

	%			
Matrix	SOL	HEM	CEL	LIC
MW	25.2	14.9	31.9	28
PC	24.6	13.9	40.3	21.2
SG	40.8	36.9	19.1	3.3

4.2.2. Sources and types of inoculum

Three types of inoculum of pure mycelial species grown on millet are provided by Sylvan Inc.: *Pleurotus ostreatus* var. *florida* (ETNA), *Pleurotus ostreatus* KRYOS and *Pleurotus ostreatus* SPOPPO (sporeless species protected by CPV rights). Each inoculum is added at 20% w/w to each substrate before the SSF experiment.

4.2.3. Lignocellulolytic enzyme production under solid state fermentation

Solid state fermentation is carried out in 250 mL Erlenmeyer flasks sterilized by autoclaving at 121°C for 15 min and later cooled to room temperature. Ten grams of dry substrate are then moistened with 10 mL of the following mineral solution (g/L): NH_4Cl, 15.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.5; $CoCl_2$, 0.45; $MnSO_4.H_2O$, 0.1; $ZnSO_4.H_2O$, 0.1; $CaCl_2$, 0.5 and yeast extract, 5.0. The inoculum is added and mixed with the substrate using sterilized rods. After recording the total weightof each flask, they are incubated at 30°C for 5 d. At the end of the fermentation process, the total weight is again recorded to assess the loss in weight during the SSF process.

For the extraction of the produced enzymes, 100 mL of tap water are added. The mixture is shaken for 1 hr at 180 rpm and then filtered under vacuum through Whatman GF/A type microfiber filters (pore size 1.6 µm). On one hand, the substrate is dried at 105°C until weight stabilization. The dried weight is recorded to assess the dry matter loss through the SSF process. It is later submitted to a characterization of the lignocellulosic fractions. On the other hand, the filtrate volume is recorded to assess water loss. It is later concentrated using Vivaspin^{TM} 20 (GE Healthcare) sample concentrators with molecular weight cutoff (MWCO) of 10 KD. The concentrated enzyme solutions are assayed for the following enzymatic activities: filter paper cellulase (FPase), carboxymethylcellulase (CMCase), β-glucosidase and xylanase.

4.2.4. D-optimal design experimental plan

For screening purposes, substrate type (MW, PC and SG), substrate pretreatment (with and without autoclaving), inoculum type (ETNA, KRYOS and SPOPPO), pH of the medium (4 and 6), moisture (60% and 80%) and nutrient addition (with and without addition) are identified as important factors that impact the performance of the SSF process. To take into account those multiple qualitative and quantitative factors with their different corresponding levels and assess interactions among them, a D-optimal design (Modde 9.0, Umetrics) experimental plan is run. The 46-assay matrix with 43 assays and 3 central points allows running less replicates than a classical design. The full matrix of the experimental plan is presented in Table 2.

Table 2. 46-assay matrix of the D-optimal design (assays 44 to 46 correspond to the triplicate of the central point).

#	Substrate	Inoculum	Autoclaving	pH	Moisture (%)	Nutrient
1	PC	SPOPPO	Yes	4	60	with
2	MW	SPOPPO	Yes	4	60	with
3	SG	KRYOS	Yes	4	60	with
4	MW	KRYOS	No	4	60	with
5	PC	ETNA	No	4	60	with
6	SG	ETNA	No	4	60	with

7	PC	KRYOS	Yes	6	60	with
8	SG	ETNA	Yes	6	60	with
9	SG	SPOPPO	No	6	60	with
10	MW	ETNA	No	6	60	with
11	SG	SPOPPO	Yes	4	80	with
12	PC	KRYOS	Yes	4	80	with
13	PC	ETNA	Yes	4	80	with
14	PC	SPOPPO	No	4	80	with
15	MW	ETNA	No	4	80	with
16	PC	SPOPPO	Yes	6	80	with
17	MW	KRYOS	Yes	6	80	with
18	SG	KRYOS	Yes	6	80	with
19	MW	SPOPPO	No	6	80	with
20	PC	KRYOS	No	6	80	with
21	PC	ETNA	No	6	80	with
22	SG	ETNA	No	6	80	with
23	SG	KRYOS	Yes	4	60	without
24	MW	ETNA	Yes	4	60	without
25	MW	SPOPPO	No	4	60	without
26	SG	SPOPPO	No	4	60	without
27	PC	KRYOS	No	4	60	without
28	MW	SPOPPO	Yes	6	60	without
29	SG	SPOPPO	Yes	6	60	without
30	MW	KRYOS	Yes	6	60	without
31	PC	ETNA	Yes	6	60	without
32	PC	SPOPPO	No	6	60	without
33	SG	KRYOS	No	6	60	without
34	SG	ETNA	No	6	60	without
35	PC	SPOPPO	Yes	4	80	without
36	MW	SPOPPO	Yes	4	80	without
37	MW	KRYOS	Yes	4	80	without
38	SG	ETNA	Yes	4	80	without
39	SG	KRYOS	No	4	80	without
40	PC	ETNA	No	4	80	without
41	PC	KRYOS	Yes	6	80	without
42	MW	ETNA	Yes	6	80	without
43	SG	SPOPPO	No	6	80	without
44-46	SG	SPOPPO	Yes	5	70	without

4.2.5. Analytical methods

4.2.5.1. Solid content

Total solids (TS) and volatile solids (VS) are measured on the solid substrate before and after SSF. TS correspond to the amount of dry substrate after drying the fresh matter at 105°C till weight stabilization and VS to the amount of substrate lost after calcination of a dry substrate at 550°C for 5 h.

4.2.5.2. Characterization of the lignocellulosic complex

The lignocellulosic complex is characterized in each substrate, before and after SSF, according to Van Soest et al. (1991) using FIWE Raw Fiber Extractor from VELP Scientifica. The protocol identifies four fractions: SOL, HEM, CEL and LIC. One gram of substrate is extracted with 100 mL hot water for 30 min followed by extraction with neutral detergent for 60 min (extraction of the SOL fraction, neutral detergent fiber (NDF) residue), hot acid detergent for 60 min (extraction of the HEM fraction, acid detergent fiber (ADF) residue) and for 180 min in cold, 72% sulfuric acid (extraction of the CEL fraction, acid detergent lignin (ADL) residue). The residual material corresponds to the LIC fraction and may contain lignin and cutin. After each extraction step, the VS content is determined in the residues. All fractions are expressed as a percentage of total VS according to the following equations: SOL = 100 – NDF; HEM = NDF – ADF; CEL = ADF - ADL and LIC = ADL.

4.2.5.3. Enzyme activities

For the measurement of the enzymatic activities, 96-well plate adapted analytical protocols developed in the labs of Veolia Research and Innovation were followed. Absorbance were read using PowerWave XS2 (BioTek Instruments, Inc., Vermont, USA) with Gen5^{TM} software.

One filter paper unit (U) is defined as the amount of enzyme that releases one µmol of glucose per minute in the assay reaction. FPase is assayed by measuring the release of glucose from a mixture containing 2.4 mg Whatman n°1 paper as substrate in 48 µL 50 mM sodium citrate buffer and 24 µL of enzyme sample. The mixture is incubated at 50°C for

60 min. Dinitrosalicylic (DNS) solution is used for color development and absorbance is read at 545 nm.

One unit of CMCase activity is defined as the amount of enzyme that liberates 1 µmol of reducing sugar as glucose under the analysis conditions. A mixture of 30µL of 4% CMC solution and 30 µL of enzyme sample are incubated at 50°C for 30 min. Later 180 µL DNS are added, followed by dilution with distilled water before absorbance reading at 540 nm.

One unit of β-glucosidase activity is defined as the amount of enzyme which produces 1 µmol of p-nitrophenol (pNP) from 4-nitrophenyl α-D-glucopyranoside (pNPG). For the analysis, 100 µL pNPG are mixed with 20 µL enzyme sample and incubated at 50°C for 10 min. The reaction is stopped using 1 M sodium carbonate solution and absorbance is read at 412 nm.

Finally one unit of xylanase is defined as the amount of enzyme, which produces 1 µmol reducing sugar as xylose per min in the reaction mixture under the specified conditions. A mixture of 80µL of 1% beechwood xylan solution and 20 µl enzyme sample is incubated at 50°C for 30 min. Later 180 µL of DNS are added followed by dilution with distilled water. Absorbance is read at 540 nm.

4.2.5.6. Data analysis

All data are statistically analyzed using Modde 9 and SIMCA from Umetrics.

4.3. Results and Discussion

4.3.1. Defining experimental conditions

An extensive literature review has helped defining the several operational parameters of the experimental plan. This applies mainly for the substrate pretreatment with autoclaving, nutrient addition, pH and moisture ranges. However, technical and economic concerns dictated the choice of the substrate and inoculum types as well as the fermentation time.

In order to produce lignocellulolytic enzymes, the substrate should be rich with this complex. But since the application should be foreseen for large-scale applications, the substrate should also be an important feedstock. For this work, paper/cardboard fractions might at a first glance not be interesting mainly because the elimination processes of this feedstock lies mainly in the recycling. But it can be considered as a representative sample of lignocellulosic complex. This is not the case for MSW and brewer's spent grain. According to Hoornweg and Bhada-Tata (2012), the worldwide annual production of MSW goes up to 1.3 billion tons per year and it will double by 2025. The annual production of spent grain is also important; 38.6 million tons per year according to Mussatto (2014). Until now spent grain was mainly used as animal feed but efforts are increasing to divert its use towards energy production, chemical and biotechnological processes (Mussatto, 2014). In addition to that, Table 3 shows a list of substrates used to produce lignocellullolytic enzymes by SSF. The substrates tested in this work do not appear in the literature review; this brings an additional interest to study their potential.

Table 3. List of substrates used in solid state fermentation for the production of lignocellulolytic enzymes and their corresponding references.

Substrate	References
Aspen pulp	Chahal, 1985
Banana fruit stalk	Krishna, 1999
Banana waste	Reddy et al., 2003
Citrus peel	Rodriguez-Fernandez et al., 2011
Coir pith	Jabasingh and Nachiyar, 2011
Corncob	Qian et al., 2012
Corn stalk	Guowei et al., 2011
Corn stover	Chen et al., 2011
Deoiled Jatropha seedcake	Dave et al., 2012
Distillery spent wash	Acharya et al., 2010
Empty palm fruit bunch fiber	Kim and Kim, 2012
Rice straw	Kang et al., 2004
Soyhull	Krishna et al., 1995
Sugarcane bagasse	Membrillo et al., 2008

Wheat bran	Deschamps et al., 1985; Gessesse and Mamo, 1999; Lu et al., 2003; Asha Poorna and Prema, 2006; Deswal et al., 2011; Guowei et al., 2011; Qian et al., 2012
Wheat straw	Deschamps et al., 1985; Kalogeris et al., 2003; Kang et al., 2004

Research studies have shown consistently that inoculum addition is essential to produce enzymes. Different microorganisms have been reported mainly *Aspergillus* (Kang et al., 2004; Acharya et al., 2010; Kiranmayi et al., 2011), *Trichoderma* (Chahal, 1985; Deschamps et al., 1985) and *Pleurotus* species (Membrillo et al., 2008). At lab-scale the inoculum cost is not discussed. But in this work, the solid state fermentation process is proposed upstream other environmental applications such as anaerobic digestion. Therefore, the inoculum is carefully chosen to meet the following three requirements: commercially available in large scale production, easy to handle and rapid mycelial growth. In this context, fungal mycelia produced commercially by Sylvan Inc. were used. These products target food grade mushroom production. This implies that they are safe and will not present any health or environmental risk.

Finally, the SSF process is run for 5 d although literature data shows fermentation times ranging from 66 h (Deschamps et al., 1985) to 22 d (Chahal, 1985). But going back to the large scale application, the SSF process should be included in an overall waste treatment process. It is thus more comparable to a pretreatment step; this is why a short process time of 5 d was considered.

4.3.2. Physical and chemical changes through SSF

The substrate in the SSF process is the source of carbon that is essential for enzyme production. Following the changes that it undergoes during SSF is important to better control the process. Not only that but once the enzymes are produced, this substrate should be treated and thus its characterization is important for its downstream disposal.

The distribution of the collected data on %TS loss, %VS loss and % water loss is shown in Figure 1. During the 5-day SSF process, TS and VS losses for all tested assays were 10.3 and 16.4% on average respectively. During SSF, the substrate is partially degraded leading to loss in the solid matter but the growing mycelium participates in the formation of new matter. However, since the mycelium is intimately attached to the substrate the actual matter loss of the substrate cannot be determined. But it can only be assumed that the degradation of the initial substrate is more important than the measured values. In all cases, the VS loss is important and impacts subsequent biological degradation. For example if the substrate is to be degraded anaerobically, the methane potential would be less important.

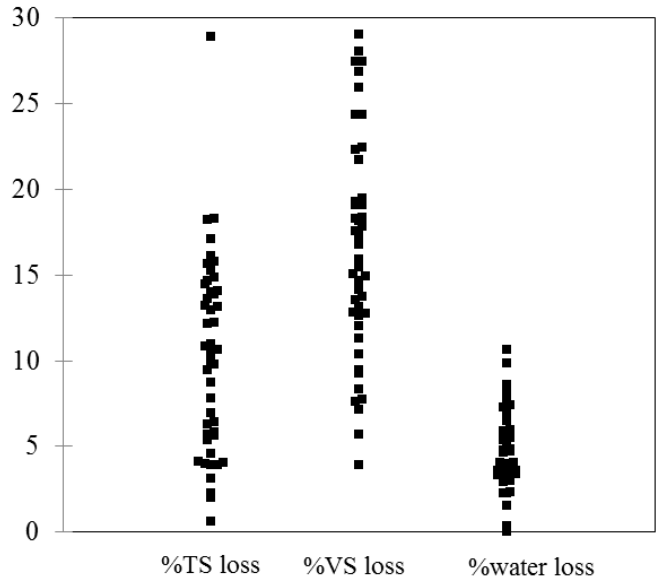

Figure 1. Scattergrams for %TS loss, %OM loss and % water loss for all tested matrices in the 46 assays during the SSF process.

Water loss was measured at 4.6% on average. This is a relatively low value which if translated at an upper scale implies that there is no need for humidity control during the short SSF period Interestingly the scattering of the water loss data is much less important than those of %TS and VS losses.

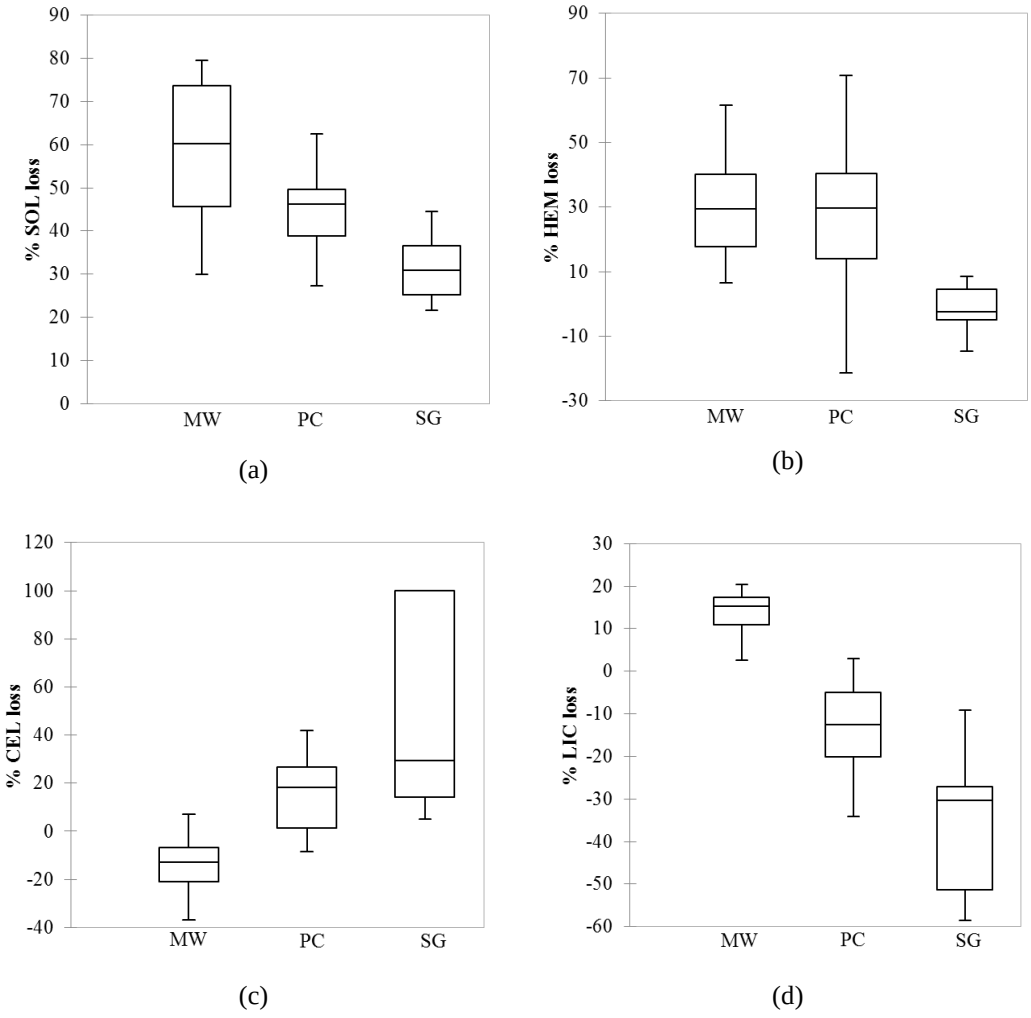

Figure 2. Box plots of the % loss in the four van Soest fractions of the matrices MW, PC and SG: (a) soluble fraction (SOL), (b) hemicellulose-like fraction (HEM), (c) cellulose-like fraction (CEL) and (d) lignin and cutin-like fraction (LIC).

Box plot representations of the % loss in the absolute content of the different Van Soest fractions are presented in Figure 2. In general, the highest losses correspond to the SOL fraction: 59, 47 and 30% on average for MW, PC and SG respectively. These results are expected since what is soluble and most readily available should be used by the growing microorganisms in the medium. For HEM loss, 29, 26 and -2% are recorded for MW, PC and SG respectively. Interestingly SG is very rich in hemicellulose (Table 1) but during the SSF this fraction remains almost intact while its CEL fraction is mostly degraded (48% loss). The LIC loss for SG is reported at - 35%; as shown in Table 1; lignin content in SG is very low (3.3%) therefore the error margin could explain the above result. Finally, MW and PC have

opposite trends regarding CEL and LIC losses. These trends cannot be explained at this level because in the experimental plan format no replicates are run besides the central points.

4.3.3. Physical and chemical changes through SSF

The main objective of this work is to achieve a primary screening of the operational parameters that control SSF processes to produce lignocellulolytic enzymes.

4.3.3.1. Enzyme activities produced

Four types of enzymes are targeted in the SSF process: FPase, CMCase, β-glucosidase and xylanase. Figure 3 presents the Box plot distribution of the enzyme activities measured in the D-optimal design. An average production of 0.08 U/g dry matter (DM) is recorded for FPase with a maximum of 0.35 U/ g DM on SG. This value is actually twice the activity reported by Membrillo et al. (2008) using *Pleurotus ostreatus* on sugarcane bagasse. For CMCase, average production is calculated at 0.65 U/g DM with a maximum at 2.48 U/g DM on MSW although other comparable results were also obtained on PC. These levels could not be compared to literature data since using *Pleurotus ostreatus* and *Pleurotus sajor-caju*, Reddy et al. (2003) only indicated that very low levels of CMCase were produced using banana waste. Unfortunately, for β-glucosidase no literature data was reported using neither the substrates tested nor *Pleurotus* species. Average enzyme production was 0.62 U/g DM with a maximum recorded at 4.58 U/g DM on MSW. Finally, average xylanase was found at 1.1 U/g DM with a maximum at 4.70 U/g DM on PC. Reddy et al. (2003) reported 0.14 U/mg protein on banana waste and Membrillo et al. (2008) a maximum of 7.59 U/g DM on sugarcane bagasse.

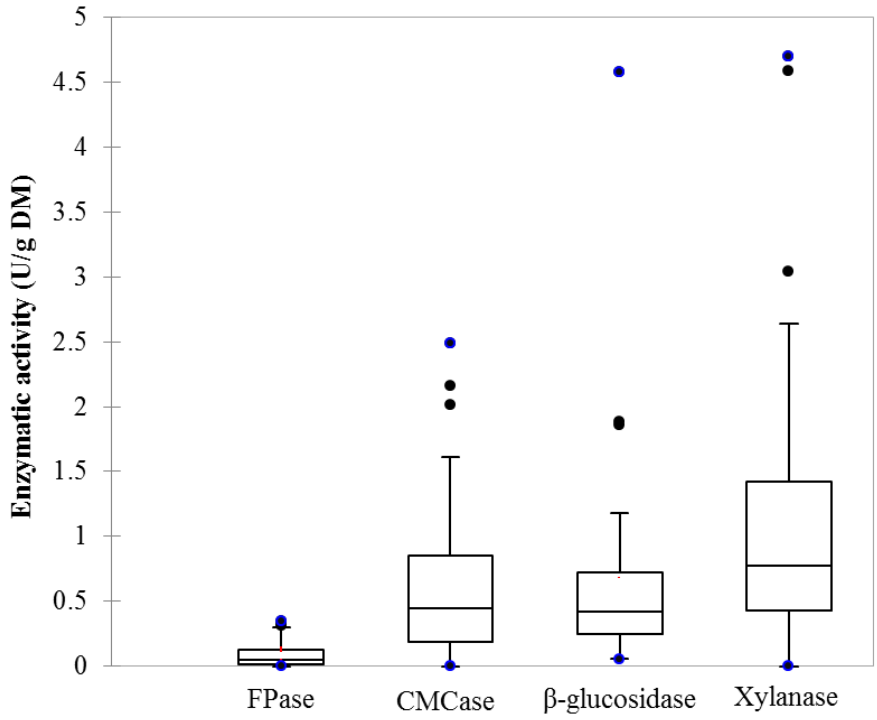

Figure 3. Box plots of the measured FPase, CMCase, β-glucosidase and xylanase activities in the different assays of the D-optimal design plan.

Figure 4 shows scattergrams of the different FPase, CMCase, β-glucosidase and xylanase activities reported in the literature on different substrates. Average values of 128, 161, 110 and 5375 U/g DM were recorded for the four enzymes of interest respectively. But what is important to note is that the range of values for each enzyme is very important: 0.2 to 817, 7 to 637, 5 to 402 and 8 to 21430 U/g DM for FPase, CMCase, β-glucosidase and xylanase respectively. These important variations are mainly due to the different analytical methods used to measure the corresponding enzymatic activity and would actually explain the very high enzymatic activities reported by certain authors. Another reason could be due to the different matrix used to produce the enzymes. In fact the carbon source is known to affect the concentrations and proportions of different enzymes in a mixture that is produced (Pandey et al., 2008b). In all cases, as shown earlier, enzyme activities reported in this work compare well with literature data in which experimental conditions were more strictly controlled mainly in terms of inoculum.

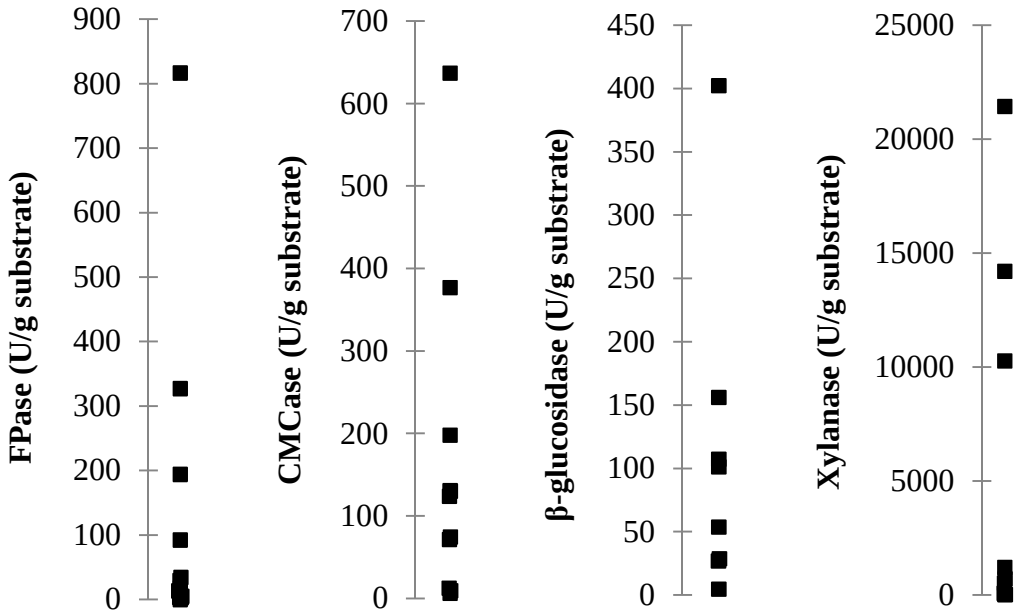

Figure 4. Scattergrams of reported literature data of FPase, CMCase, β-glucosidase and xylanase activities

4.3.3.2. Significance of operational parameters on enzyme production

The main objective of the experimental plan is to define the significance of the different tested factors. Correlation studies assess the level of accuracy at which the equation proposed by the regression analysis can predict the observed data points; it is defined by the coefficient of determination R^2. As shown in Figure 5, R^2 values of 0.516, 0.855, 0.861 and 0.760 were found for FPase, CMCase, β-glucosidase and xylanase respectively. Although only 51.6% of the data could be explained by the model for FPase; at least 76% is predicted for the other three enzymes.

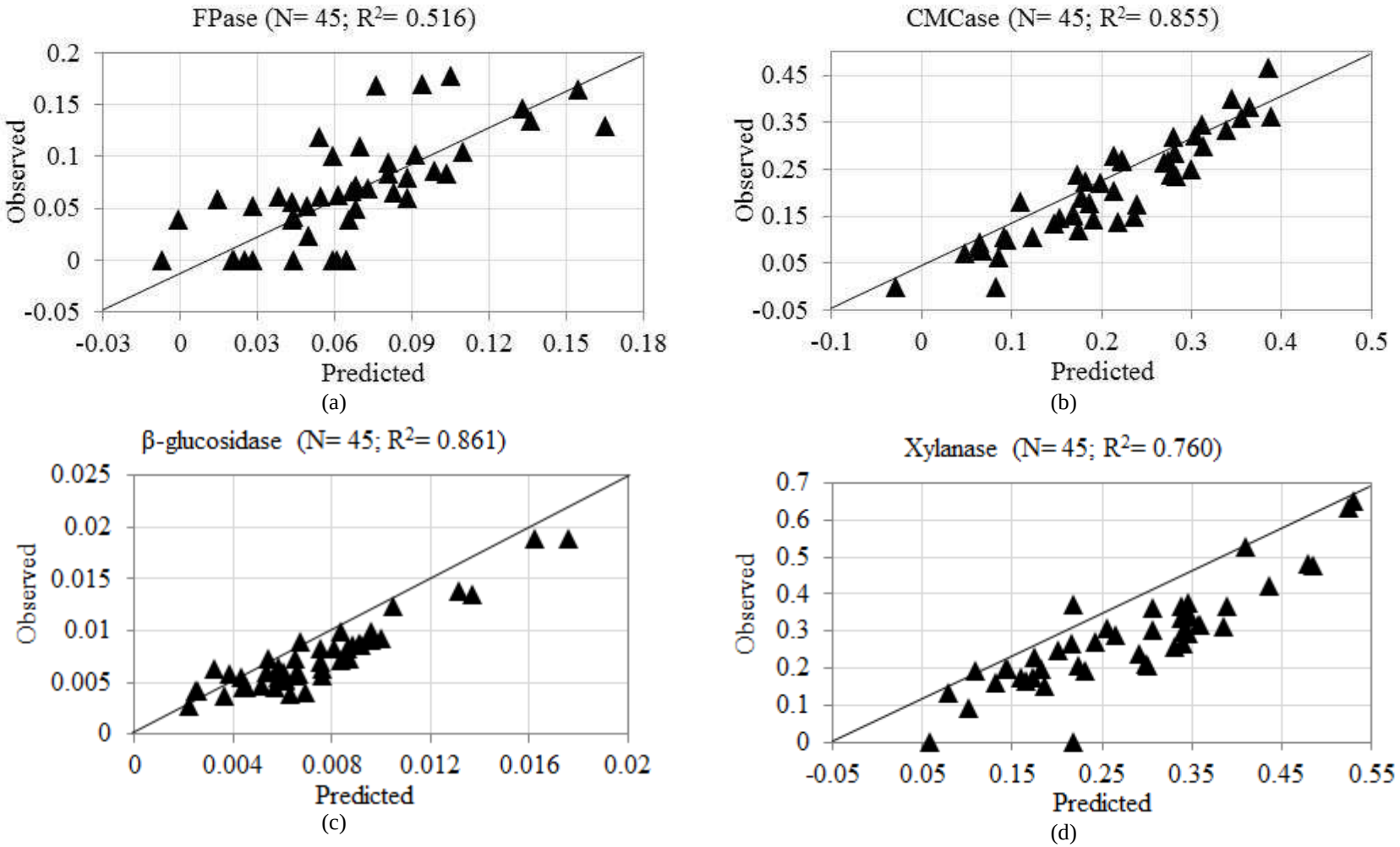

Fig. 5. Correlation results with observed versus predicted values for FPase (a), CMCase (b), β-glucosidase (c) and xylanase (d)

Since R^2 values are encouraging the second step is to look into the impact of each factor on the specific enzyme production. All results are shown in Figure 6. Each graph shows the significant factors per enzyme in decreasing order of importance. In certain cases, some interactions are identified; an interaction means that the effect of one independent variable or factor depends on the value of another independent variable. Going by the order of factors identified earlier, the substrate MW showed a positive impact for β-glucosidase production (Fig. 6c). SG showed a negative effect for both β-glucosidase and CMCase, negative interactions with nutrient addition for β-glucosidase and xylanase, another negative interaction with inoculum ETNA also for β-glucosidase and finally a positive interaction with autoclaving for CMCase.

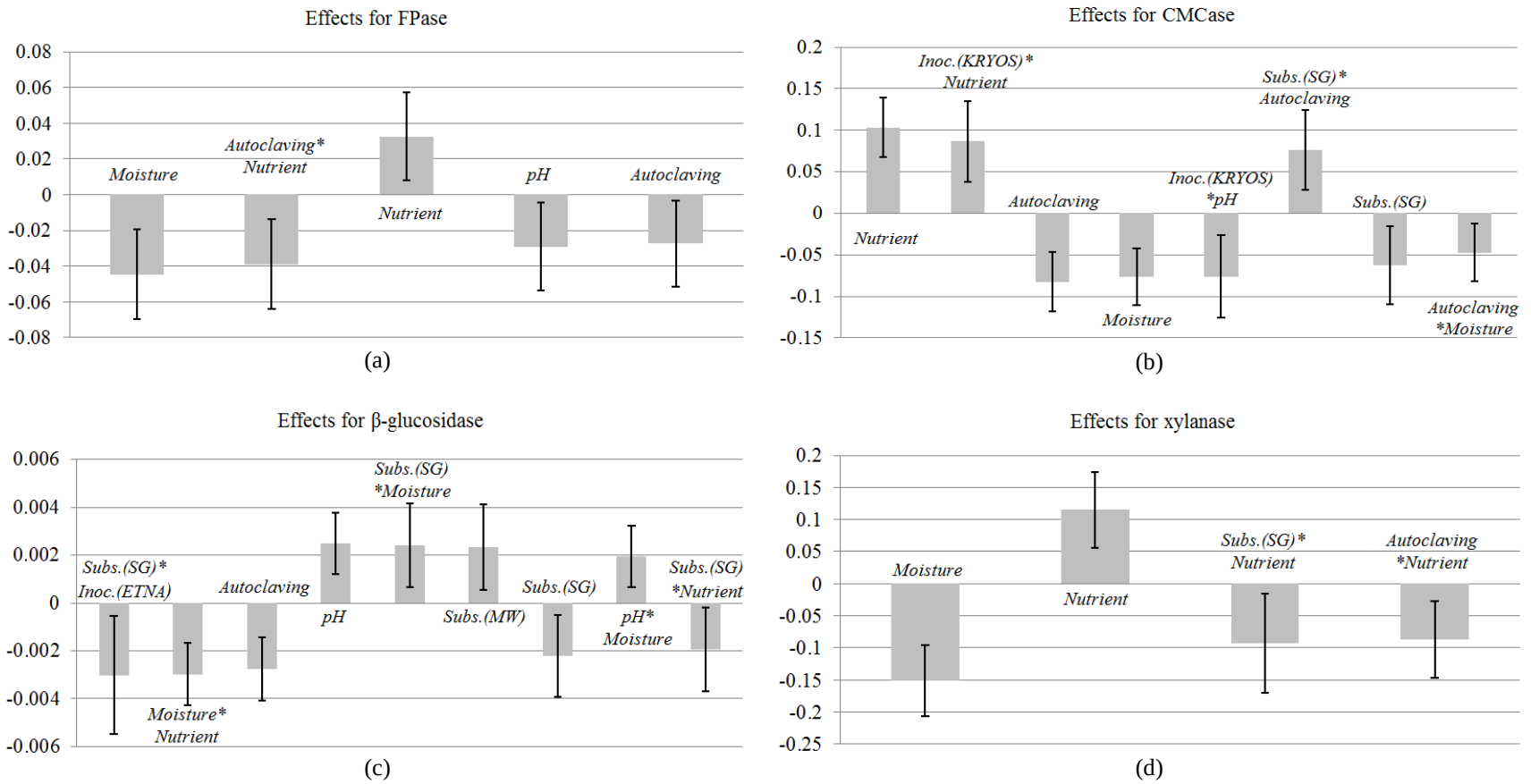

Figure 6. Statistical analysis results of the different factors in the D-optimal design presented per enzyme type: FPase (a), CMCase (b), β-glucosidase (c) and xylanase (d).

Autoclaving the matrix has a negative impact on FPase, CMCase and β-glucosidase production. Negative interaction is found between autoclaving and nutrient addition for both FPase and xylanase productions.

There was no significant impact of the inoculum type on any of the enzyme productions; besides the negative interaction of ETNA with SG, a positive interaction of KRYOS with nutrient addition is shown for CMCase (Fig.6b).

The correct pH is critical for the success of solid state fermentation (Raimbault and Alazard, 1980) since it impacts the transport of enzyme across the membrane. It also affects the stability of extracellular enzymes. It must be low enough at the beginning of the process to avoid bacterial growth (Raimbault, 1997). According to Roussos (1997), pH decreases gradually during the initial stages of the fermentation due to mycelial growth and then increases in the later stages. In general, the production of most fungal cellulases falls in the pH range of 4.5-5.0 (Acharya et al., 2010; Kim and Kim, 2012; Qian et al., 2012). This is why at this level of the work the tested range (4 to 6) did not show very clear answers: a negative effect on FPase, a positive effect on β-glucosidase and two interactions shown in Fig.6b and 6c were only measured.

For the moisture range, some very low levels of 40-50% were used in Lu et al. (2003) and Guowei et al. (2011) but most other SSF experiments were run at higher moisture levels that reach up to 83% (Acharya et al., 2010). This factor is actually very substrate dependent but since process development is aimed for larger scale applications, then factor values should be found as average values that could apply to wide substrate types. Therefore, increasing moisture from 60 to 80% has shown clear negative impacts on FPase, CMCase and xylanase productions. Two negative interactions are shown in Fig. 6b and 6c and two positive ones is Fig. 6c.

Finally nutrients were added at the lab-scale to discard any nutrient deficiency especially that the matrices used for enzyme production were dried and grinded in order to make sure that the samples are homogeneous and representative of the actual matrix. Results showed that adding the nutrient solution has a positive impact in all enzymes except β-glucosidase for which the factor was not significant. Several positive and negative interactions were recorded in Fig. 6a to 6d.

Based on the data discussed above, it can be concluded that for further process optimization of the SSF process, nutrient addition should be included, no autoclaving is necessary and any inoculum from the three tested types could be used for mycelial growth and further enzyme production Nevertheless, the pH range could not be further narrowed at this level of experimental work. It is also the case for the substrate type because as mentioned earlier only the spent grain substrate had some negative impacts while the two others did not show clear significant effects. In addition to that it is essential to note that when judging the impact of an enzyme mixture, all enzymes should be taken into account. Since at this level the quality of the best enzyme mixture is not identified, if a factor does not present systematic impact, it cannot be taken out from further research. Finally, it would also be interesting to decrease the range of the moisture levels.

4.4. Conclusions

FPase, CMCase, β-glucosidase and xylanase enzymes were identified of interest for lignocellulose anaerobic degradation. Their production under SSF conditions was studied taking into account six factors with a 46-assay D-optimal design: substrate type and autoclaving, inoculum, pH, moisture and nutrient addition. Screening results showed that nutrients are necessary but not autoclaving. No significance difference was noted among the three inocula although SPOPPO showed the best results. For further process optimization, pH

range of 4-6 should be kept along with the three substrates MW, PC and SG. Finally, moisture range could be shifted to lower values (50-70%).

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CHAPTER 5

Optimizing lignocellulolytic enzyme production through solid state fermentation for downstream hydrolysis of different solid matrices

ABSTRACT

This work aims at optimizing operational conditions to develop solid state fermentation on municipal solid waste, brewer's spent grain and paper/cardboard matrices. Substrate type, pH (4-6) and moisture (50-70%) were taken into account in a 30-assay full design. Pleurotus ostreatus SPOPPO from a commercially available source was used as inoculum. After 5 days of incubation at 30°C, cellulase, carboxymethylcellulase, β-glucosidase and xylanase activities were measured. For maximum enzyme production, pH and moisture showed non-linear impacts. Maximum enzyme activities were 0.99, 5.50, 12.00 and 15.20 U/g DM for the tested enzymes respectively. Enzyme efficiency was assayed by using all crude enzyme extracts to hydrolyze the three matrices at 5% (w/v) substrate load at 35°C. Maximum glucose concentrations of 37.6, 7.6 and 89.9 mg/g dry substrate were measured for municipal waste, spent grain and paper/cardboard respectively. Finally, high correlation coefficients were found between applied enzyme load and released glucose.

Keywords: solid state fermentation, lignocellulolytic enzyme, full design, hydrolysis, municipal solid waste, brewer's spent grains, paper.

5.1. Introduction

Lignocellulose is mainly composed of cellulose, hemicellulose and lignin. All fractions are intimately linked in a semi-crystalline structure which limits its degradation in anaerobic digestion processes. Due to this structure, an important energetic potential remains locked within lignocellulosic matrices. Its expression is in fact the most important technological challenge (Kiranmayi et al., 2011). In this perspective enzymatic hydrolysis of lignocellulose has shown very promising results although the major limitation is its corresponding high cost. Recent studies have shown that enzyme cost can account for 50% of the total cost of hydrolysis in certain ethanol production systems (Tu et al., 2007).

Within this context, solid state fermentation (SSF) emerges as an alternative for *in situ* production of lignocellulolytic enzymes in order to overcome the cost of commercial enzymes; these latter targeting essentially high value added products such as pharmaceutical industries. It would then be possible to integrate the SSF process within a waste treatment facility to improve biodegradation of the recalcitrant lignocellulose and increase biogas and subsequently methane production. It is the increasing environmental concern and the search for new sources of renewable energy that render methane an interesting target.

In this perspective, previous work was conducted to screen the impact of different factors on the enzyme production: substrate type, substrate autoclaving, inoculum type (three *Pleurotus ostreatus* strains), pH (4-6), moisture (60-80%) and nutrient addition. Using a D-optimal experimental design plan, results showed that for maximum enzyme production, substrate and inoculum types and tested pH range did not have significant impact. In addition to that, negative effects were found for autoclaving and increasing moisture content and a positive effect for nutrient addition.

The key objective of this work is therefore optimizing operational conditions for the in situ production of endogenous enzymes through SSF taking into account the results of the

screening tests. Produced crude enzyme extracts will be used on the different matrices through hydrolysis or saccharification tests. Besides using novel and complex substrates such as municipal solid waste and commercially available inoculum, this works aims at looking into the efficiency of the SSF enzymes. It sheds the light on the load and composition of hydrolytic enzymes which are most often left out in the literature. The final goal of this research is increasing the anaerobic biodegradability of lignocellulosic matrices while taking into account technical and economic factors of future scale up.

5.2. Materials and Methods

5.2.1. Matrices of interest

Three matrices were used for enzyme production under solid state fermentation conditions: municipal solid waste (MW fraction), paper/cardboard (PC fraction) from a sorting facility and brewery's spent grain (SG fraction). Table 1 summarizes the composition based on the Van Soest characterization: soluble (SOL), hemicellulose-like (HEM), cellulose-like (CEL) and lignin and cutin-like (LIC) fractions. For the purpose of this work, all tested matrices were dried at 80°C until stabilization of the weight and then ground using a 1 cm mesh.

Table 1. Van Soest characterization (%) of the tested solid matrices municipal waste (MW), paper and cardboard fraction (PC) and spent grain (SG) in terms of soluble (SOL), hemicellulose-like (HEM), cellulose-like (CEL) and LIC (lignin and cutin-like) fractions.

	%			
Matrix	SOL	HEM	CEL	LIC
MW	25.2	14.9	31.9	28
PC	24.6	13.9	40.3	21.2
SG	40.8	36.9	19.1	3.3

5.2.2. Source and type of inoculum

Pleurotus ostreatus SPOPPO (sporeless species protected by CPV rights), was provided by Sylvan Inc. as mycelium grown on millet. It was added at 20% w/w to each substrate.

5.2.3. Lignocellulolytic enzyme production under solid state fermentation

Solid state fermentation was carried out in 250 mL Erlenmeyer flasks sterilized by autoclaving at 121°C for 15 min and later cooled to room temperature. Ten grams of dry substrate were moistened with the following nutrient solution (g/L): NH_4Cl, 15.0; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.5; $CoCl_2$, 0.45; $MnSO_4.H_2O$, 0.1; $ZnSO_4.H_2O$, 0.1; $CaCl_2$, 0.5 and yeast extract, 5.0. The inoculum was added and mixed with the substrate using sterilized rods. The total weight of each flask was recorded and then flasks, closed using cotton plugs, were incubated at 30°C for 5 d. At the end of the fermentation process, the total weight was again recorded to assess weight loss during the SSF process.

For the extraction of the produced enzymes, 100 mL of tap water were added. The mixture was shaken for 1 hr at 180 rpm and then filtered under vacuum through Whatman GF/A type microfiber filters (pore size 1.6 µm). The solid residue was dried at 105°C until weight stabilization to assess dry matter loss through the SSF process. It was later characterized through Van Soest fractionation. The filtrate volume was recorded to assess water loss. It was later concentrated using Vivaspin™ 20 (GE Healthcare) sample concentrators with molecular weight cutoff (MWCO) of 10 kD. Concentrated enzyme solutions were assayed for the following enzymatic activities: filter paper cellulase (FPase), carboxymethylcellulase (CMCase), β-glucosidase and xylanase.

5.2.4. Full design experimental plan

Substrate type (MW, PC and SG), pH (4, 5 and 6) and moisture (50, 60 and 70%) were considered as significant factors that impact the SSF process. To define the optimal conditions and assess the significance of all factors and possible interactions, a 30-assay full design (Modde 9.0, Umetrics) experimental plan was run. Detailed description of all tested conditions is presented in Table 2.

Table 2. Matrix of the 30-assay full design experimental plan for the three tested factors: substrate type, pH and moisture level.

#	Substrate	pH	Moisture level (%)
1	PC	4	50
2	MW	4	50
3	SG	4	50
4	PC	5	50
5	MW	5	50
6	SG	5	50
7	PC	6	50
8	MW	6	50
9	SG	6	50
10	PC	4	60
11	MW	4	60
12	SG	4	60
13	PC	5	60
14	MW	5	60
15	SG	5	60
16	PC	6	60
17	MW	6	60
18	SG	6	60
19	PC	4	70
20	MW	4	70
21	SG	4	70
22	PC	5	70
23	MW	5	70
24	SG	5	70
25	PC	6	70
26	MW	6	70
27	SG	6	70
28	SG	5	60
29	SG	5	60
30	MW	5	60

5.2.5. Saccharification tests

To test the efficiency of the produced enzymes, saccharification tests were run. In 15-mL glass tubes were mixed 0.5 g of solid substrate, 150 µL of the crude enzyme mixture and 10 mL of citrate buffer. All tests were run in triplicates along with blanks at 35°C for 24 h. At the end of the tests, supernatants were recovered and filtered at 0.45 µm to measure reducing sugars.

5.2.6. Analytical methods

5.2.6.1. Solid content

Total solids (TS) and volatile solids (VS) were measured before and after SSF. TS correspond to the amount of dry substrate after drying the fresh matter at 105°C till weight stabilization and VS to the amount of substrate lost after calcination of a dry substrate at 550°C for 5 h.

5.2.6.2. Characterization of the lignocellulosic complex

Each substrate was characterized, before and after SSF, according to Van Soest et al. (1991) using FIWE Raw Fiber Extractor from VELP Scientifica. The protocol identifies four fractions: SOL, HEM, CEL and LIC. One gram of substrate is extracted with 100 mL hot water for 30 min followed by extraction with neutral detergent for 60 min (extraction of the SOL fraction, neutral detergent fiber (NDF) residue), hot acid detergent for 60 min (extraction of the HEM fraction, acid detergent fiber (ADF) residue) and for 180 min in cold, 72% sulfuric acid (extraction of the CEL fraction, acid detergent lignin (ADL) residue). The residual material corresponds to the LIC fraction and may contain lignin and cutin. After each extraction step, the VS content was determined in the residues. All fractions were expressed as a percentage of total VS according to the following equations: SOL = 100 – NDF; HEM = NDF – ADF; CEL = ADF - ADL and LIC = ADL.

5.2.6.3. Enzymatic activities

For the measurement of the enzymatic activities, 96-well plate adapted analytical protocols, developed in the labs of Veolia Research and Innovation, were followed. Absorbance was read using PowerWave XS2 (BioTek Instruments, Inc., Vermont, USA) with Gen5^{TM} software.

One filter paper unit (FPU) is defined as the amount of enzyme that releases one µmol of glucose per minute in the assay reaction. FPase was assayed by measuring the release of glucose from a mixture containing 2.4 mg Whatman n°1 paper as substrate in 48 µL 50 mM sodium citrate buffer and 24 µL of enzyme sample. The mixture was incubated at 50°C for

60 min. Dinitrosalicylic (DNS) solution was used for color development and absorbance is read at 545 nm.

One unit of CMCase activity is defined as the amount of enzyme that liberates 1 µmol of reducing sugar as glucose under the analysis conditions. A mixture of 30µL of 4% CMC solution and 30 µL of enzyme sample was incubated at 50°C for 30 min. Later 180 µL DNS were added, followed by dilution with distilled water before absorbance reading at 540 nm.

One unit of β-glucosidase activity is defined as the amount of enzyme which produces 1 µmol of p-nitrophenol (pNP) from 4-nitrophenyl α-D-glucopyranoside (pNPG). For the analysis, 100 µL pNPG were mixed with 20 µL enzymesample and incubated at 50°C for 10 min. The reaction was stopped using 1 M sodium carbonate solution and absorbance was read at 412 nm.

Finally, one unit of xylanase is defined as the amount of enzyme, which produces 1 µmol reducing sugar as xylose per min in the reaction mixture under the specified conditions. A mixture of 80µL of 1% beechwood xylan solution and 20 µL of enzyme sample was incubated at 50°C for 30 min. Later 180 µL of DNS were added followed by dilution with distilled water. Absorbance was read at 540 nm.

5.2.6.3. Reducing sugar analysis

Reducing sugars were analyzed according to Navarro et al. (2010). For that purpose, 60 µL DNS and 60 µL sample were mixed and incubated for 10 min at 94°C. The mixture was then cooled down for 5 min at room temperature. Later 100 µL were transferred to a reading plate for absorbance measurement at 540 nm.

5.2.6. Data analysis

All data are statistically analyzed using Modde 9 and SIMCA from Umetrics.

5.3. Results and Discussion

5.3.1. Defining experimental conditions

Based on previously conducted work, only substrate type, moisture and pH were taken into account as impacting factors of the SSF process through the full design experimental plan detailed in Table 2. The remaining experimental conditions were set taking into account the limitations of a probable scale-up process. Therefore, MSW and spent grains, important worldwide feedstock, were tested (Hoornweg and Bhada-Tata, 2012; Mussatto, 2014). The paper/cardboard fraction was considered as a model matrix of lignocellulose. The inoculum, *Pleurotus ostreatus* SPOPPO, was chosen based on its commercial availability, easiness of handling and rapid growth. It is also of food grade quality without any identified health or environmental risks. Finally, the fermentation time was set at 5 d to enable the integration of the process in existing waste treatment facilities.

5.3.2. Physical and biochemical changes through SSF

During the SSF process, the carbon in the substrate is used to enable mycelial growth. The process is maintained until the carbon is depleted or when the enzyme production is low and does not compensate engaged operational costs. Nevertheless, this substrate should be treated. Therefore, following the changes that it undergoes will shed more light on understanding the process and the substrate's downstream treatment.

During the 5-day SSF process, TS and VS losses for all tested assays were 17.2 and 23.4% on average respectively. These values are in fact 67.8 and 42.7% higher than those obtained during the screening tests (10.3 and 16.4% respectively). This implies that substrate degradation and mycelial growth are more important under these factors; observations that are rather expected under narrower tested ranges. Interestingly, water loss was on average 2.7%, 40% lower than under screening conditions. This could probably be due to the fact that the tested moisture range is lower. Nevertheless both recorded values, under screening and optimizing conditions, remain low and do not imply drying of the substrate.

Box plot representations of the % loss in the absolute content of the different Van Soest fractions are presented in Figure 1. In general, the highest losses correspond to the SOL fraction: 28, 45 and 48% on average for MW, PC and SG respectively. For HEM loss, 35, 19 and 18% are recorded for MW, PC and SG respectively. Although under screening conditions SG showed almost no loss in the hemicellulose fraction, under the new experimental conditions an interesting level of hemicellulose loss is measured. This implies that the tested conditions are more favorable for this fraction's degradation. The CEL fraction evolution does not however show the same trend: for both MW and PC an important increase in the degradation level (13 and 47% respectively) is measured as opposed to a drop from 48 to 19% for SG. Finally, LIC fraction losses were also difficult to interpret: 20%, -13 and -246% for MW, PC and SG respectively. The low LIC content of SG could partly explain the results however the corresponding PC loss could be due to a limitation of the Van Soest test itself when applied to these types of matrices. For future tests, it would be interesting to test the NREL method for the determination of structural carbohydrates and lignin in biomass (Sluiter et al., 2012).

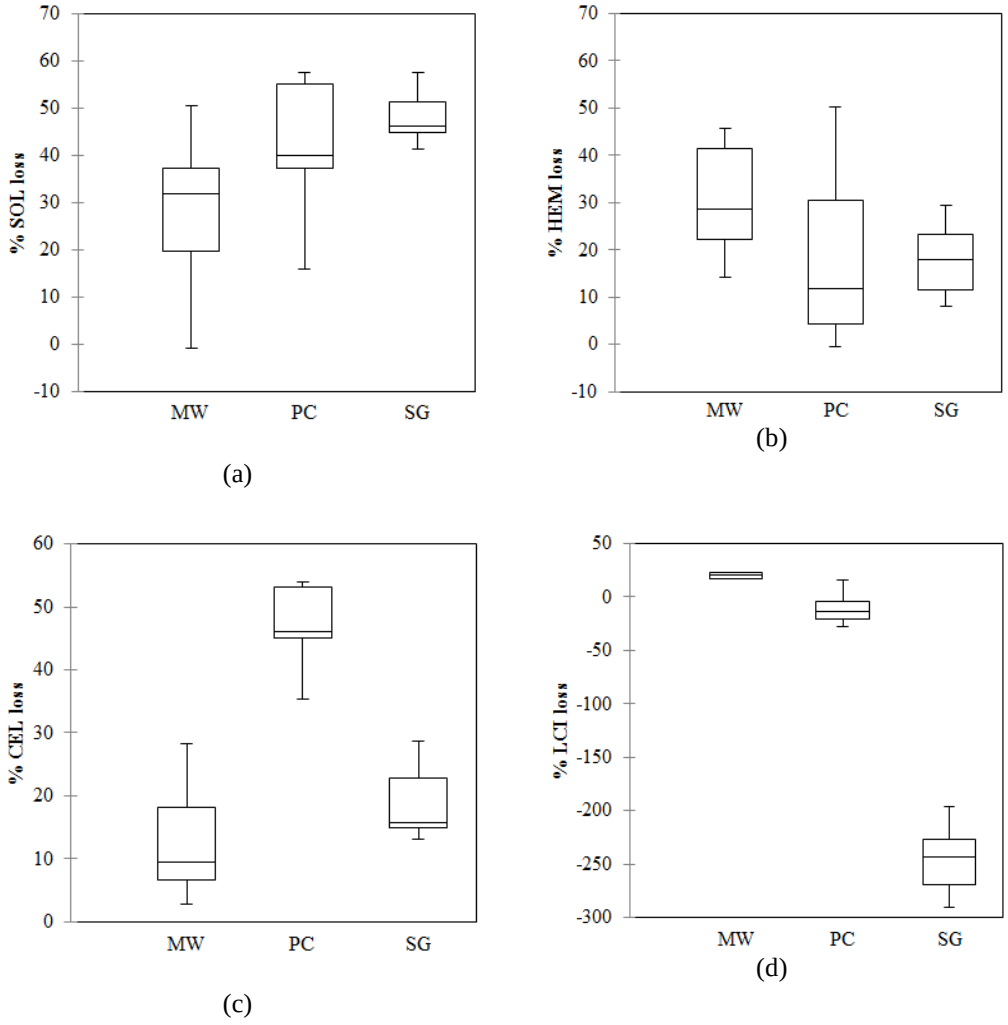


Fig. 1. Box plots of the % loss in the four van Soest fractions of the matrices MW, PC and SG : (a) soluble fraction (SOL), (b) hemicellulose-like fraction (HEM), (c) cellulose-like fraction (CEL) and (d) lignin and cutin-like fraction (LIC).

5.3.3. Enzyme production through SSF

The main objective of this work is to define average optimal conditions to produce lignocellulolytic enzymes under solid state conditions from three different substrates.

5.3.3.1. Enzyme activities produced

Four types of enzymes are targeted in the SSF process: FPase, CMCase, β-glucosidase and xylanase. Figure 2 presents scattergram distributions of the enzyme activities measured in the full experimental design and Table 3 includes average and maximum activity values reported in both D-optimal (screening conditions) and full design (optimization conditions) tests. An average production of 0.34 U/g dry matter (DM) is recorded for FPase with a maximum of

0.99 U/ g DM on SG. Interestingly the average value reported in this work was the highest found during the screening tests. And the maximum value is 5.5 times higher than that indicated by Membrillo et al. (2008) using *Pleurotus ostreatus* on sugarcane bagasse. For CMCase, average production is calculated at 2.89 U/g DM with a maximum at 5.50 U/g DM on PC although other interesting results were obtained on MW. For β-glucosidase, average enzyme production was 4.77 U/g DM with a maximum recorded at 12.00 U/g DM on MW. Finally, average xylanase was found at 7.63 U/g DM with a maximum at 15.20 U/g DM on SG with other interesting, but lower, results on both MW and PC. Unfortunately, no results were found in the literature to compare both CMCase and β-glucosidase data, but maximum xylanase was twice as that reported by Membrillo et al. (2008) on sugarcane bagasse.

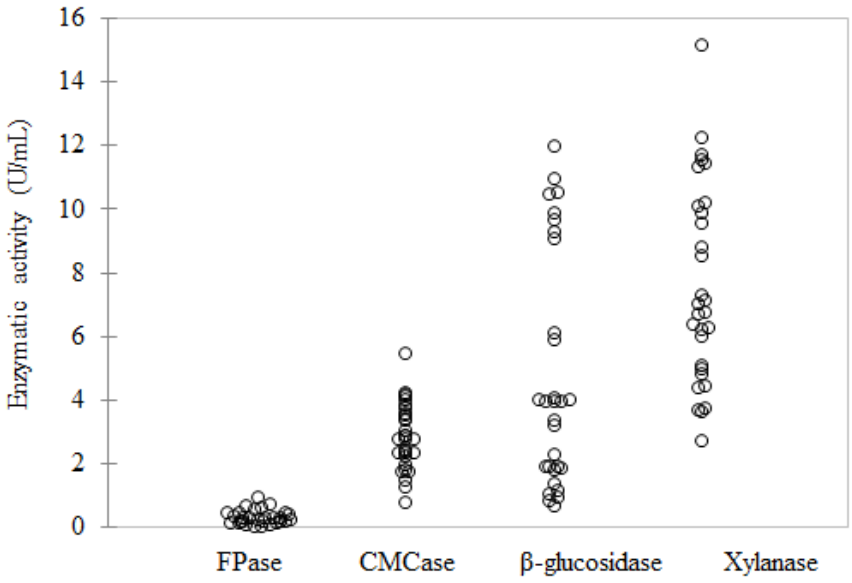


Fig. 2. Scattergrmas of the measured FPase, CMCase, β-glucosidase and xylanase activities in U/mL for the different assays of the full design plan

Table 3. Average and maximum enzyme activities for FPase, CMCase, β-glucosidase and xylanase in U/g DM reported under both screening and optimization tests.

	U/g DM							
	FPase		CMCase		β-glucosidase		Xylanase	
	Average	Max	Average	Max	Average	Max	Average	Max
Screening	0.08	0.35	0.65	2.48	0.62	4.58	1.10	4.70
Optimization	0.34	0.99	2.89	5.50	4.77	12.00	7.63	15.20

As shown in Table 3, average enzyme activities obtained during optimization tests are 4.3, 4.4, 7.7 and 6.9 times higher for FPase, CMCase, β-glucosidase and xylanase than those of the screening tests. Maximum values are only 2.8, 2.2, 2.6 and 3.2 times higher for the same enzymes. This is encouraging since the objective of this work is to define better operational conditions. But it was also interesting to check the evolution of the same experimental points over time. Unfortunately only 5 points coincided between the two series of tests. While looking into the amount of enzymes produced, all data did not match. Some points differed significantly in certain enzymes. This could be actually explained by the fact that the comparison is done between single data points and that no replicates were run. The tested biological system is based on the development of mycelial growth of its inherent microflora. Since no control system was imposed on the process, differences can exist among different points in time. It would be important to compare the evolution of average values.

5.3.3.2. Impact of operational conditions on enzyme production

Before looking into the impact of each tested factor on enzyme production, statistical data analysis provides regression equations that predict observed data. In that perspective a coefficient of determination (R^2) is proposed: when all data points could be predicted by the model then $R^2=1$. R^2 values of 0.495, 0.567, 0.931 and 0.676 were found for FPase, CMCase, β-glucosidase and xylanase respectively (Figure 3). Compared to the screening tests, slight decrease is noted for both FPase and xylanase (4 and 10% respectively) and a slight increase in accuracy for β-glucosidase (8%). But the 34% decrease in accuracy for CMCase could not be explained. These coefficient values, under the experimental conditions and for biological systems, are satisfactory enough to develop the statistical analysis.

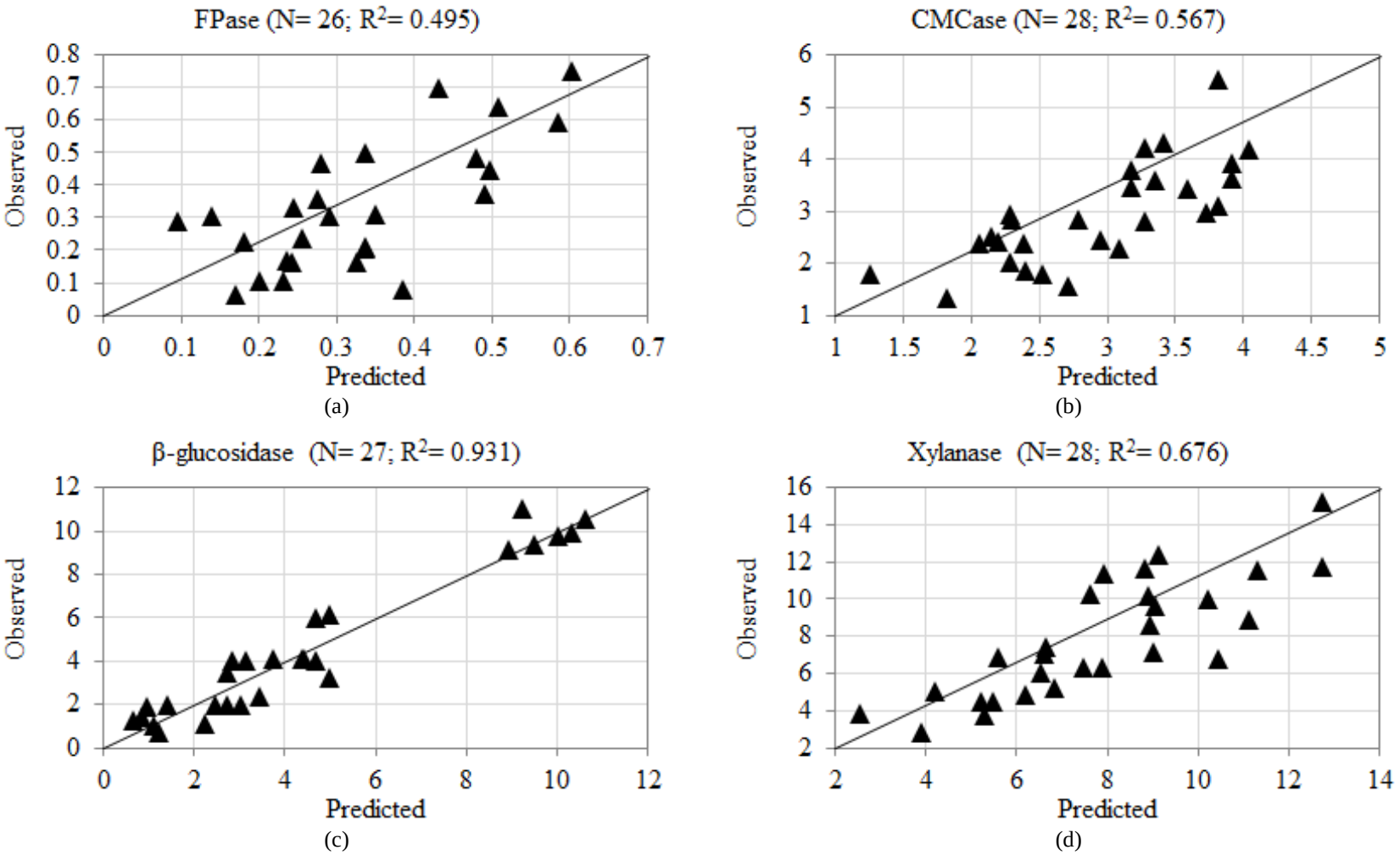


Figure 3. Correlation results with observed versus predicted values for FPase (a), CMCase (b), β-glucosidase (c) and xylanase (d)

Figure 4 shows the statistical analysis results of the three different tested factors for the four enzymes. Each graph shows the significant factors per enzyme in decreasing order of importance. Significant effects are shown in black whereas non-significant effects in grey. In certain cases, some interactions are identified; an interaction means that the effect of one independent variable or factor depends on the value of another independent variable.

Interestingly, although maximum FPase and xylanase activities were produced on SG sample, results show that SG has a positive impact only on xylanase production (Fig. 4d) and negative effects on all remaining three enzymes. In addition to that, the effect of the SG substrate is the only significant factor on both FPase and CMCase productions (Fig. 4a and 4b). Substrate MW has also a negative effect on the production of β-glucosidase (Fig. 4c).

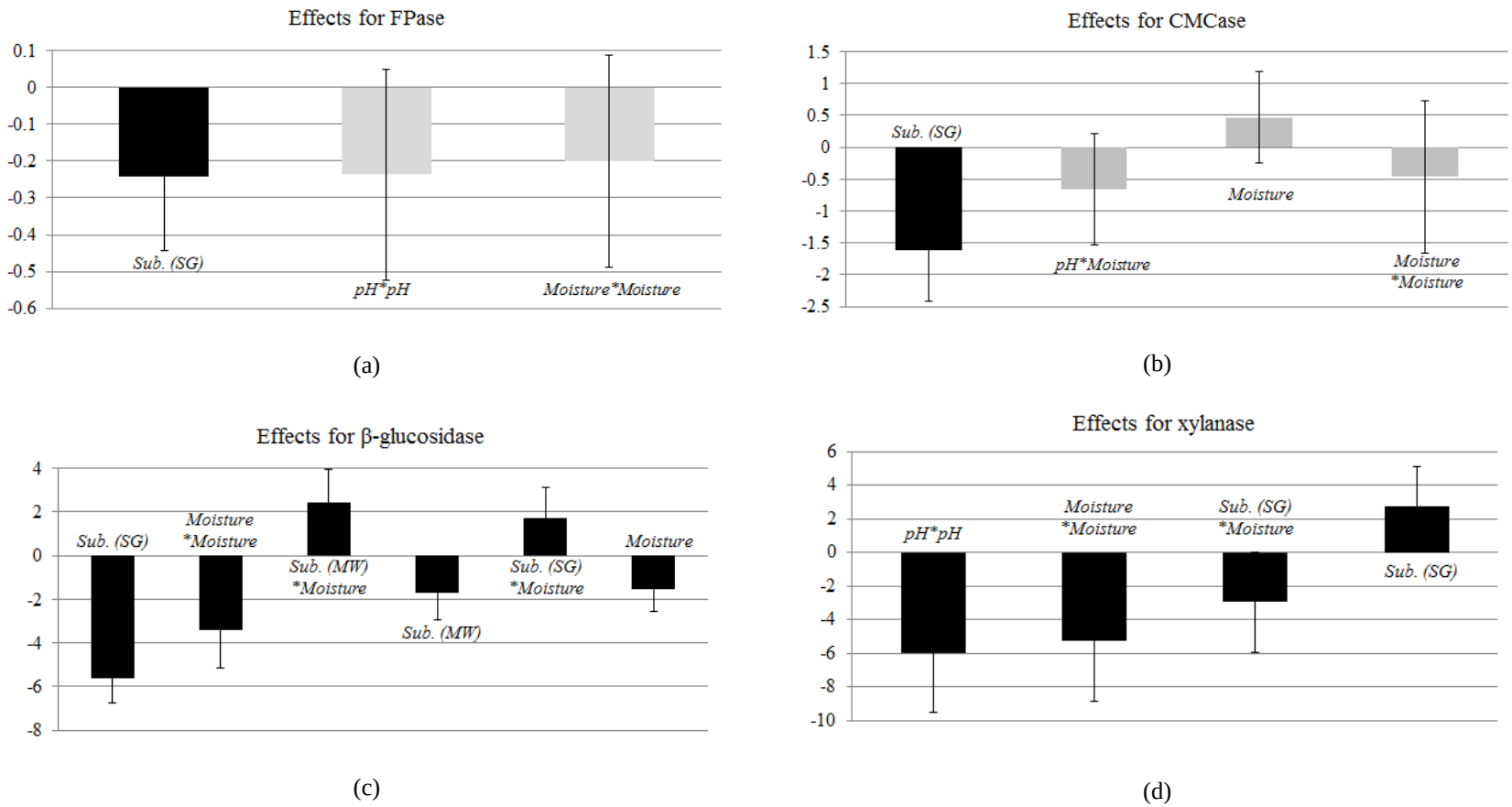


Figure 4. Statistical analysis results of the different factors in the full design presented per enzyme type: FPase (a), CMCase (b), β-glucosidase (c) and xylanase (d). Significant results are in black and non-significant results in grey color.

Increasing moisture from 50 to 70% has a negative impact on β-glucosidase production. Its interactions with SG and MW are both positive on the same enzyme production but all the remaining significant interactions are negative. The interaction moisture*moisture is negative and significant for β-glucosidase and xylanase and not significant for FPase and CMCase . This type of result implies a non-linear impact of the tested factor on enzyme production: at 50% moisture the enzyme production is low, it increases at 60% and decreases again at 70%. Figure 5 shows an illustration of the non-linear effect of moisture on all four enzyme activities for the three tested matrices. In fact, 60% moisture is relatively low since average values in the literature are around 70% moisture except 40-50% reported in Lu et al. (2003) and Guowei et al. (2011) as well as 60% reported in Rodriguez-Fernandez et al. (2011).

Increasing pH from 4 to 6 shows a significant negative interaction on xylanase and a non-significant one on FPase production. As it is the case for moisture, this implies a non-linear impact is illustrated in Figure 6 for the three matrices. A pH value of 5 is thus the optimal reference. This agrees with the findings of several authors that recommend a range of 4.5 to 5.0 for optimal enzyme production (Kim and Kim, 2012; Qian et al., 2012).

Based on the data discussed above, it can be concluded that spent grain might not be an interesting substrate for enzyme production. For the different substrates, incubation at 60% moisture and pH 5 along with nutrient addition for 5 d are the optimal conditions for lignocellulolytic enzyme production under SSF processes.

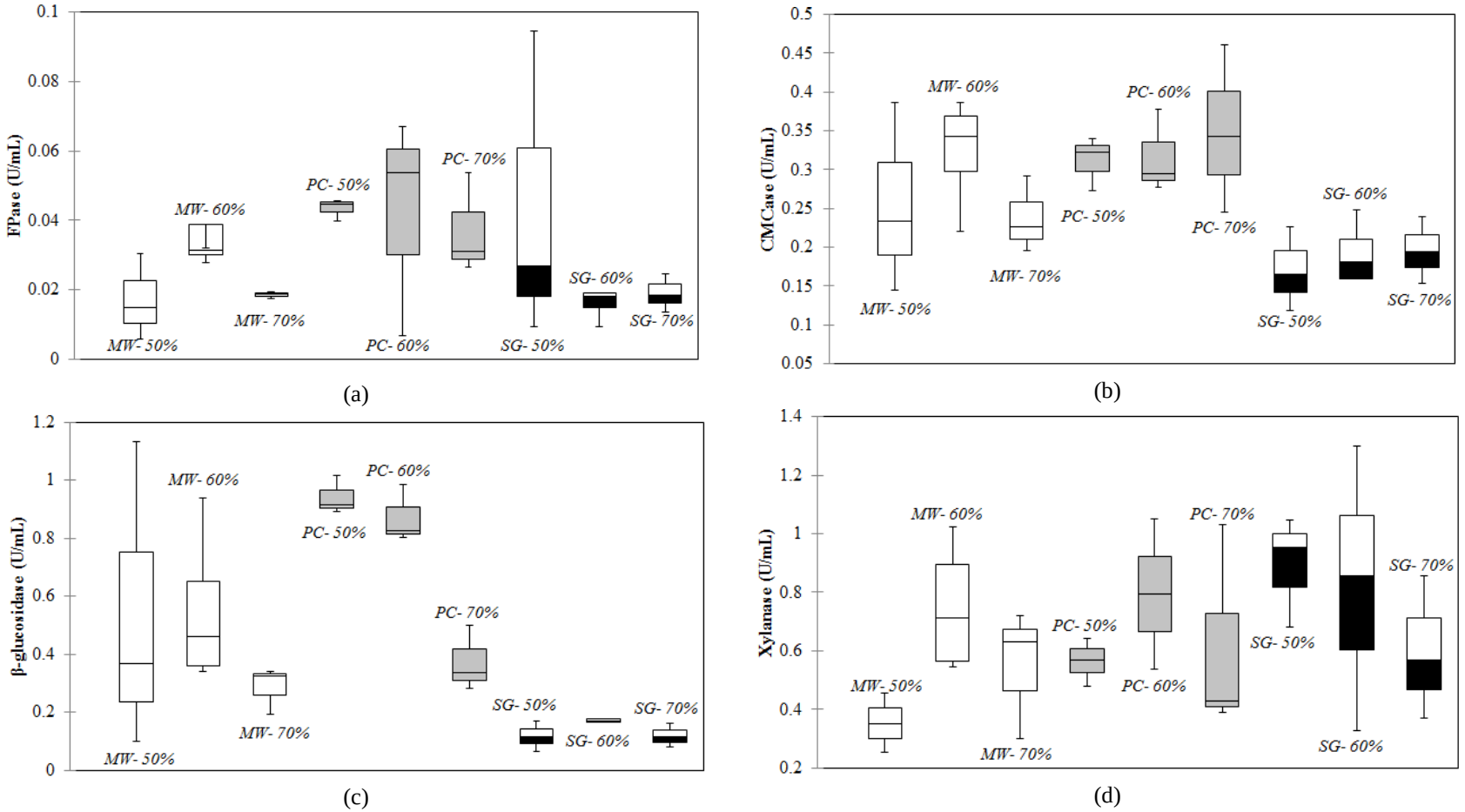


Figure 5. Box plots illustration of the non-linear effect of moisture level for the three matrices on FPase (a), CMCase (b), β-glucosidase (c) and xylanase (d) activities in U/mL.

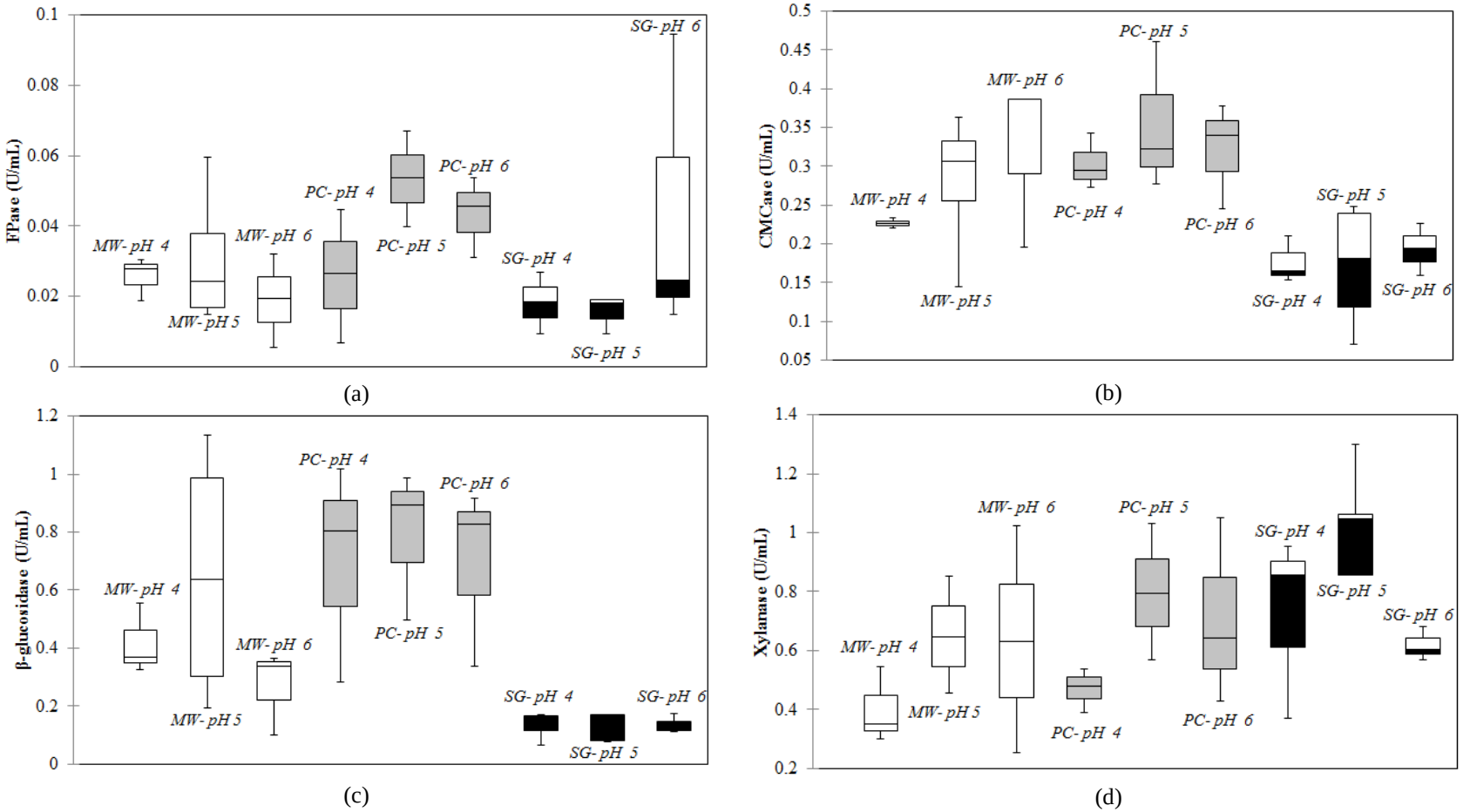


Figure 6. Box plots illustration of the non-linear effect of pH level for the three matrices on FPase (a), CMCase (b), β-glucosidase (c) and xylanase (d) activities in U/mL.

5.3.4. Efficiency of produced enzymes

Crude enzyme extracts produced are applied in the downstream biodegradation of lignocellulosic matrices. In the current work, the 30 different enzyme extracts from the full design plan were used to degrade the three tested matrices. Therefore, a total of 270 saccharification tests (all tests were run in triplicates) were performed. Results (Figure 7) show that the spent grain matrix is more prone to enzymatic degradation when compared to municipal solid waste and paper/cardboard fractions. This could be explained by its low lignin content (3.3%) as compared to the other two matrices (Table 1). These findings agree with those of Mussatto et al. (2008) in which higher glucose concentrations were found for delignified brewer's spent grain (BSG) after enzymatic hydrolysis as compared to the control. With the removal of both lignin and hemicellulose, the cellulose crystallinity is reduced and the material porosity and the accessible surface area are increased favoring the enzyme attack (Mussatto et al., 2008). On average 9.4, 3.3 and 30 mg glucose /g DM were detected in the medium for MW, PC and SG respectively.

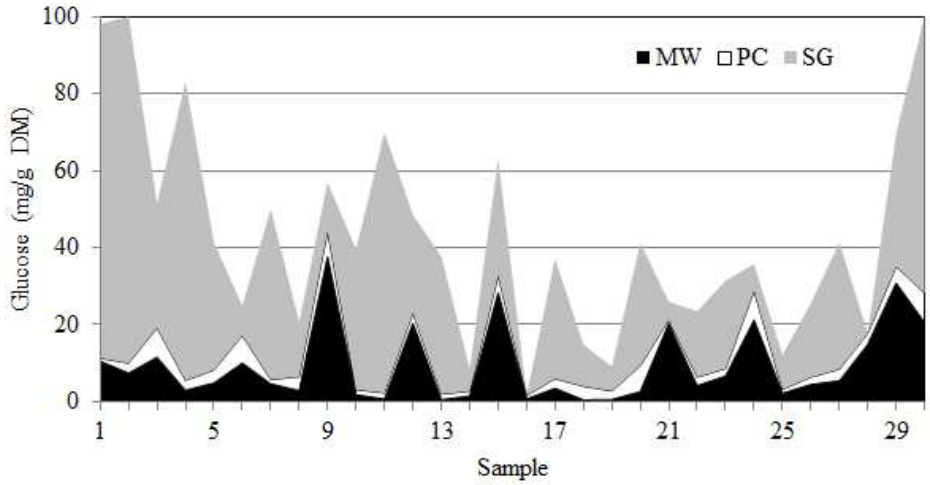


Figure 7. Stacked area chart for glucose produced (mg/g DM) during the hydrolysis tests on the three matrices MW, PC and SG.

In fact; to assess the efficiency of the above mentioned results, Table 4 summarizes the findings of several authors using different enzyme sources for the hydrolysis of various solid matrices. Some experimental conditions of the hydrolysis differ significantly from the

conducted research. First, in Table 4, it is found that the temperature at which hydrolysis takes place varies between 45 and 60°C, while that of the current work is 35°C. In fact, enzymes will be used downstream in the anaerobic digestion which can be either mesophilic (at 35°C) or thermophilic (at 50°). But since the former is more widely applied, it was decided to select mesophilic conditions although it is thought that lignocellulolytic enzymes can be more efficient at 50°C. Second, the duration of the hydrolysis varies between 8 and 96 h; therefore the 24 h contact time used in this work is to the lower end of that range. Nevertheless, Chahal (1985) indicated that up to 66.5% of the hydrolysis occurred in the first 20 h; in his corresponding work hydrolysis was run for a total of 96 h. Finally, the substrate load varied from 2 to 15% (w/v) as compared to 5% in this research. This substrate level was considered as it compares with conditions found in wet anaerobic digesters. It is however important to mention that lower substrate ratios will enable a better contact between the enzymes in the liquid phase and the solid substrate.

Table 4. Summary of different enzymatic hydrolysis experiments in terms of substrate type, enzyme source and load, experimental conditions and hydrolytic efficiency.

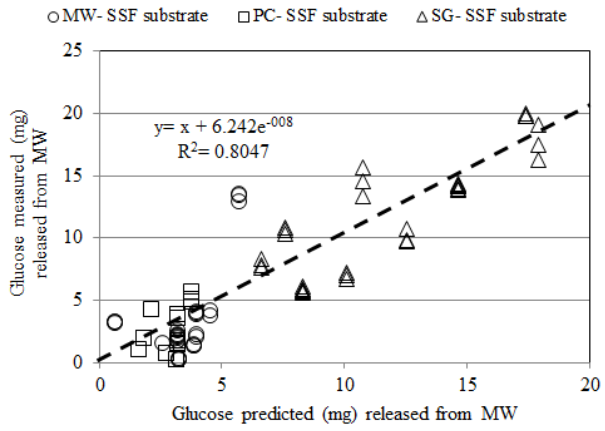
Reference	Matrix	Enzyme source	Enzyme load	Hydrolysis experimental conditions	Results
Chahal (1985)	Delignified wheat straw	Crude enzyme extract	20 FPU/g substrate	96 h	Total sugars (g/L) of 99.75 at pH 6.7 and 86.14 at pH 4.8
Kalogeris et al. (2003)	Cellulose	Crude enzyme extract	60 endoglucanase units/g susbstrate	15% substrate (w/v) in citrate phosphate buffer incubated at 60°C, 200 rpm for 48h	29% hydrolysis with 3% (w/v) glucose and 4.3% TRS (total reducing sugars)
Petersson et al (2007)	Pretreated winter rye, oilseed rape and faba bean straw	Cellubrix L. (commercial)	30 FPU/ g DM	2% substrate at 50°C and pH 4.8	Sugar yield of 49% for winter rye, 58% for oilseed rap and 43% for faba bean straw
Chen et al. (2008)	Delignified maize straw	cellulase from *T. reesei* and cellobiase from *A. niger*	20 FPU and 10 β-glucosidase units/g substrate	100 ml mixture with citrate buffer (pH 4.8) incubated at 50°C and 150 rpm for 48h	81.2% hydrolysis yield with 64.1 g glucose/L
				2L reactor (110 g/L final substrate concentration at pH 4.8) at 50°C for 72 h.	83.3% hydrolysis yield with 89.5 g TRS/L and 56.7 g/L glucose
Mussatto et al. (2008)	Brewer's spent grain (BSG): untreated, cellulignin and cellulose pulp	Celluclast 1.5L (commercial)	45 FPU/g substrate	2% substrate (w/v) incubated in buffer at 45°C, 100 rpm for 96 h	Glucose concentrations (g/L) of 0.84 for untreated BSG, 5.65 for cellulignin and 17.21 for cellulose pulp
Acharya et al. (2010)	Wheat bran	Crude enzyme extract	15 FPU/ g substrate	4% substrate (w/v) in citrate buffer	20% saccharification after 8h (25.52 mg sugar/g of substrate)
Deswal et al. (2011)	Alkali pretreated wheat straw and rice straw	Crude enzyme extract	25 FPU/ g substrate	2% substrate w/v in citrate buffer at 50°C and 150 rpm for 24 h	TRS mg/g substrate of 214.044 for pretreated wheat straw and 157.16 for rice straw

Dave et al. (2012)	Alkali pretreated sugarcane bagasse, wheat straw and rice straw	Crude enzyme extract	20 FPU/g	6.7% substrate (w/v) in sodium acetate buffer incubated at 60°C for 48h	Reducing sugar (mg/g susbtrate) of 291 for wheat straw, 294 for rice straw and 301 for sugarcane bagasse

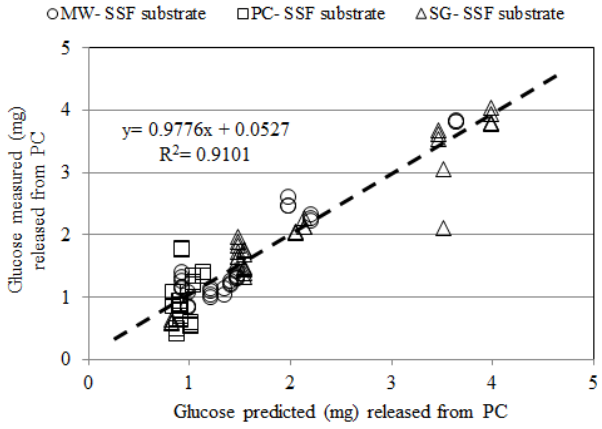
Enzyme load is an important criterion for comparison. It reflects the efficiency of the enzyme system that is applied and determines its corresponding cost. Unfortunately, most of the authors focus only on the expression of the total cellulase or filter paper units (FPU) to express the enzyme load. Table 4 shows an enzyme dosage varying between 15 to 25 FPU per gram of substrate. Only Kalogeris et al. (2003) presented the endoglucanase units (CMCase units) and Chen et al. (2008) supplemented its FPU with β-glucosidase units. It is indicated that β-glucosidase improves cellulose hydrolysis by reducing end product inhibition by cellobiose, while xylanase activity increases the accessibility of cellulose to cellulases (Berlin et al., 2005). In this work, enzyme loads in terms of FPU were much lower than seen in the literature: maximum of 0.10, 0.10 and 0.13 FPU/g DM were applied for MW, PC and SG respectively. Higher β-glucosidase loads were applied: 0.27, 0.27 and 2.86 U/g DM for the three matrices.

But the enzyme load cannot be interpreted without taking into account the corresponding released glucose. In fact, highest recorded glucose concentrations were 37.6, 7.6 and 89.9 mg glucose/g DM or 1.9, 0.4 and 4.5 g glucose/L on MW, PC and SG respectively. These values are very encouraging because in the first place the enzyme load used is 100 to 250 times lower than the literature. And if only untreated substrates are considered from Table 4, Acharya et al. (2010) reported a sugar concentration of 25.52 mg/g substrate and Mussatto et al. (2008) 0.84 g glucose/L. Therefore, despite higher hydrolysis temperature, higher contact time and higher enzyme loads, the results of this work are more interesting. Therefore, the enzymatic hydrolysis rate of a substrate is not necessarily directly proportional to the enzyme load. As mentioned earlier, some authors such as Chen et al. (2008) have emphasized the role of other enzymes such as β-glucosidase. Nevertheless CMCase and xylanase along with other accessory enzymes could play an important role in the degradation and the definition of proper hydrolytic enzyme dosage.

Finally, data on total glucose released in the saccharification tests and the corresponding amounts of enzymes added were combined to study whether a possible correlation can be found. No correlation was found when all data points were combined however when the database was split based on the type of substrate used in the hydrolysis, interesting correlation data was found. Figures 8a through 8c show the correlation results with observed versus predicted total glucose per type of matrix. Illustrations within each graph are also split based on the type of substrate used to produce the enzyme in the SSF process. Results show that important correlations can be identified and few data transformations were necessary. For MW, enzyme activities were transformed into corresponding logarithmic values while glucose remained in mg; an R^2 value of 0.8047 was obtained. For PC, enzyme activities were transformed into their corresponding logarithmic values and glucose into its square root; R^2 was found at 0.9101. Finally data transformation for SG was identical to that of MW but with the highest recorded R^2 of 0.9187. In addition to the coefficients it is interesting to note that enzymes produced on SG induced the highest glucose concentrations on both MW (Figure 6a) and PC (Figure 6b) while enzymes produced on MW and PC induced highest glucose concentrations on SG (Figure 6c). This is in fact the first step towards identifying proper enzyme formulations for the optimal hydrolysis of a given substrate.



(a)



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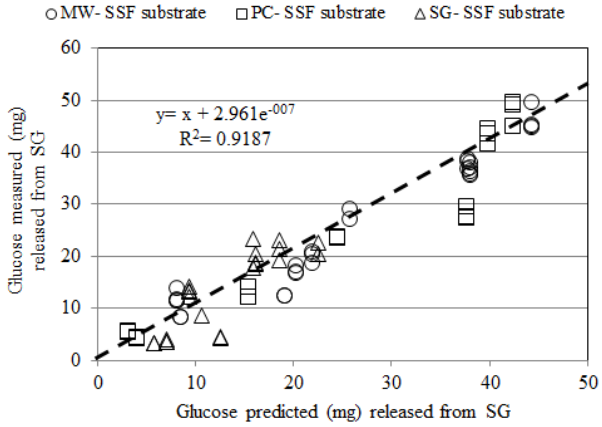

(c)

Figure 8. Correlation results with observed versus measured total glucose (mg) per type of hydrolyzed matrix: MW (a), PC (b) and SG (c).

5.4. Conclusions

FPase, CMCase, β-glucosidase and xylanase are enzymes of interest for lignocellulose anaerobic degradation of MW, PC and SG matrices. Their production under SSF conditions was optimized in a 30-assay full design taking into account three factors: substrate type, pH and moisture. Results showed that both pH and moisture have non-linear effect on enzyme production and spent grain was the least advantageous substrate. Produced crude enzyme extracts were used to hydrolyze the three matrices. Very interesting glucose concentrations were obtained given the low enzyme loads applied. High correlation coefficients were finally found between enzyme load composition and released glucose.

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CHAPTER 6

Effect of treatment time and addition of inoculum on lignocellulolytic enzyme production under solid state fermentation

ABSTRACT

Solid state fermentation is an interesting alternative for in situ enzyme production, especially when using lignocellulosic substrates. Three matrices, municipal solid waste, brewery's spent grain and paper, were tested at 30°C and 60% humidity using Pleurotus ostreatus. For total cellulase, carboxymethylcellulase, β-glucosidase and xylanase production, maximum enzyme activity values of 2.3, 12.5, 33.0 and 67.3 U/g DM were recorded. Interestingly control tests, without inoculum, showed higher enzyme production (2.8, 11.9, 14.6 and 86.3 U/g DM for the above mentioned enzymes respectively) and validation tests over time confirmed those observations. However, the main limitation of SSF without inoculum is its lower reproducibility.

Keywords: solid state fermentation, time, lignocellulolytic enzymes, inoculum, reducing sugars.

6.1. Introduction

Enzyme production is an important subject of industrial biotechnology that, in recent years, has experienced a shift towards environmental applications such as biofuel production. The main focus has been regarding the valorization of lignocellulose, a major worldwide feedstock with a complex chemical structure. The major challenge in this process is mostly the need for a large-scale low cost production of enzymes since this latter contributes to a large proportion of the total cost of bioenergy production (Jordan and Muller, 2007; Tu et al., 2007). Therefore enzyme production under solid state fermentation (SSF) from lignocellulosic biomass could be a cost effective strategy (Hideno et al., 2011). In fact, enzymes are currently produced under submerged fermentation (SmF) conditions in which substrates are soluble (Raimbault et al., 1997). The cost of the chemicals used in SmF is an important drawback of the process (Chahal, 1985) whereas agro-industrial wastes could be an interesting alternative in SSF. This approach could help in value-addition of those agro-industrial wastes solving also their disposal problem (Krishna et al., 1995; Roussos, 1997). In general, cellulose-based strategies can make the biorefinery processing more economical by increasing commercial enzyme volumetric productivity, producing enzymes using cheaper substrates, producing enzyme preparations with greater stability for specific processes and higher specific activity on solid substrates (Kiranmayi et al., 2011).**Table 1: Reported literature data based on substrate type, SSF range and optimal duration and maximum enzyme activities (FPase, CMCase, β-glucosidase and xylanase)**

Reference	Substrate	SSF duration		Maximum enzyme activities			
		Range	Optimal	FPase	CMCase	β-glucosidase	Xylanase
Chahal, 1985	Wheat straw, aspen pulp	5 to 30d	22d	326.8 IU/ g DM	-	402.8 IU/g DM	10260 IU/g DM
Deschamps et al., 1985	Straw, wheat bran	0 to 66h	66h	18 IU/g DM	198 IU/g DM	-	-
Gessesse and Mamo, 1999	Wheat bran	0 to 108h	72h	-	12.5 IU/g DM	-	700 IU/g DM
Lu et al., 2003	Wheat bran	0 to 72h	72h	-	-	-	1200 IU/g dry koji
Reddy et al., 2003	Banana waste	0 to 40d	10d	very low	very low	-	0.1411 U/mg protein
Kang et al., 2004	Rice straw, wheat bran	0 to 7d	5-6d	34.2 IU/g substrate	130 IU/g substrate	107 IU/g substrate	14196 IU/g substrate
Asha Poorna and Prema, 2006	Wheat bran	0 to 120h	72h		-		21431 IU/g DM

Chen et al., 2011	Corn stover	0 to 7 d	7d	194.18 IU/g DM	-	155.8 IU/g DM	-
Deswal et al., 2011	Wheat bran	0 to 17d	16d	3.492 IU/ g substrate	71.699 IU/g substrate	53.679 IU/g substrate	-
Rodriguez-Fernandez et al., 2011	Citrus peel	0 to 120h	96h	-	-	-	65.38 U/g DM
Kim and Kim, 2012	Empty palm fruit bunch fiber	0 to 8d	6d	-	6.5 U/g solid	-	8.8 U/g solid
Qian et al., 2012	Wheat bran, ground corncob	0 to 96h	72h	-	-	-	508 U/g

Various research works have been developed at the lab-scale with the objective of producing the maximal amount of enzyme. For that, it is essential to identify the enzyme production profile over time; this is first objective of this work. This will allow help in identifying the optimal fermentation time. As shown in Table 1, this parameter is substrate and operational parameters dependent. It is thus essential to identify it especially when with the current research focusing on new matrices rich in lignocellulose: municipal solid waste (MW) and brewer's spent grain (SG) in addition to paper/cardboard (PC) fractions. Besides their novelty, the worldwide annual production of these matrices is very important: 1.3 billion tons per year for MW (Hoornweg and Bhada-Tata, 2012) and 38.6 million tons for spent grain (Mussatto, 2014). Therefore, studying new valorization methods can lead to significant environmental and economic impacts in waste treatment facilities.

In order to improve Keeping in mind the concern of the process scale-up, it was also decided to look into the role of the indigenous microflora in the enzyme production. In general, bacteria and yeast can be grown on solid substrates but filamentous fungi remain the most adapted for SSF and more specifically basidiomycetes, the preferred choice for enzyme production and protein enrichment (Pandey et al., 2000). In general, cellulolytic enzymes are known to be produced by both soft rot and white rot fungi such as Trichoderma, Phanerochaete, Aspergillus, Pleurotus and Penicillium (Reddy et al., 2003; Deswal et al., 2011; Kiranmayi et al., 2011). Therefore, an inoculum was always added to the SSF system and almost no reference can be found in, what can be defined by Pandey et al. (2008b) as, natural indigenous SSF. The current work tries then to assess the impact of not using an external inoculum as it is the case for example in composting and ensiling.

The work will be thus divided into the follow-up with time of enzyme production, comparison with control tests without inoculation and validation tests for the impact of the inoculum. Various research works have been developed at the lab-scale with the objective of

producing the maximal amount of enzyme. For that, it is essential to identify the enzyme production profile over time; this is first objective of this work. This will allow help in identifying the optimal fermentation time. As shown in Table 1, this parameter is substrate and operational parameters dependent. It is thus essential to identify it especially when with the current research focusing on new matrices rich in lignocellulose: municipal solid waste (MW) and brewer's spent grain (SG) in addition to paper/cardboard (PC) fractions. Besides their novelty, the worldwide annual production of these matrices is very important: 1.3 billion tons per year for MW (Hoornweg and Bhada-Tata, 2012) and 38.6 million tons for spent grain (Mussatto, 2014). Therefore, studying new valorization methods can lead to significant environmental and economic impacts in waste treatment facilities.

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The work will be thus divided into the follow-up with time of enzyme production, comparison with control tests without inoculation and validation tests for the impact of the inoculum.

6.2. Materials and Methods

6.2.1. Matrices

For enzyme production under solid state fermentation, three types of matrices were tested: municipal solid waste (MW fraction), paper/cardboard (PC fraction) mixture recovered from a sorting facility and brewery's spent grain (SG fraction). Figure 1 presents the volatile solid (VS) content of each substrate in addition to their corresponding lignocellulosic composition based on the Van Soest fractions: soluble (SOL), hemicellulose-like (HEM), cellulose-like (CEL) and lignin and cutin-like (LIC). All three matrices were dried at 80°C until stabilization of the weight and then grinded through a 1 cm mesh.

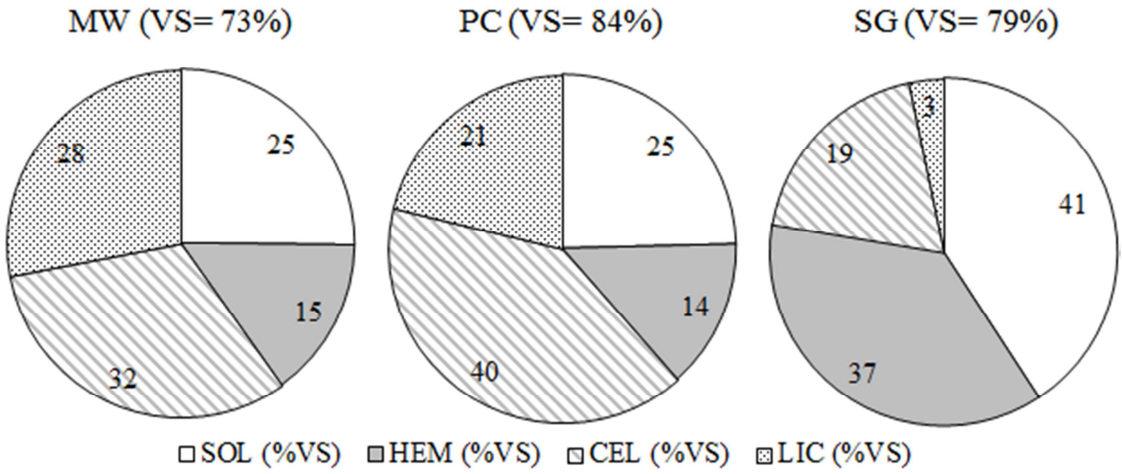


Figure 1. Pie chart representation of the Van Soest characterization (%VS) of the solid matrices MW, PC and SG in terms of soluble (SOL), hemicellulose-like (HEM), cellulose-like (CEL) and LIC (lignin and cutin-like) fractions.

6.2.2. Lignocellulolytic enzyme production through solid state fermentation

Solid state fermentation was carried out in 250 mL Erlenmeyer flasks sterilized by autoclaving at 121°C for 15 min and later cooled to room temperature. Ten grams of dry substrate were moistened with 15 mL of the following nutrient solution (g/L) in which the pH is adjusted to 5.0: NH_4Cl, 10.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.33; $CoCl_2$, 0.3; $MnSO_4.H_2O$, 0.07; $ZnSO_4.H_2O$, 0.07; $CaCl_2$, 0.33 and yeast extract, 3.3. *Pleurotus ostreatus* SPOPO (sporeless species from Sylvan Inc. grown on millet) was added at 20% (w/w) and mixed

with the substrate using sterilized rods. The total weight of each flask was recorded and then flasks, closed using cotton plugs, were incubated at 30°C.

6.2.3. Crude enzyme extraction

For the extraction of the produced enzymes, 100 mL of tap water were added. The mixture was shaken for 1 hr at 180 rpm and then filtered under vacuum through Whatman GF/A type microfiber filters (pore size 1.6 µm). The solid residue was dried at 105°C until weight stabilization to assess dry matter loss through the SSF process. The filtrate volume was recorded to assess water loss. It was later concentrated using VivaspinTM 20 (GE Healthcare) sample concentrators with molecular weight cutoff (MWCO) of 10 KD. Concentrated enzyme solutions were assayed for the following enzymatic activities: filter paper cellulase (FPase), carboxymethylcellulase (CMCase), β-glucosidase and xylanase.

6.2.4. Experimental setups

Two sets of experiments were run. To study the profile of enzyme production over time, SSF was started using the three inoculated matrices (MW, PC and SG) in triplicates. Sampling points were collected at days 3, 4, 5, 7, 11 and 14. Control points, without inoculum, were also run in triplicates with sampling events only at days 5 and 14.

To assess the impacts of both inoculum and indigenous microflora, a second setup of 90 tests was run: triplicates of non-inoculated MW, PC and SG matrices with and without substrate autoclaving. Sampling events were fixed at 0, 3, 4, 5 and 14 days.

6.2.5. Measuring total and volatile solids

Total solids (TS) were measured before and after SSF. TS correspond to the amount of dry substrate after drying the fresh matter at 105°C till weight stabilization. Volatile solids (VS) were measured in order to run the Van Soest fractionation. VS correspond to the amount of substrate lost after calcination of a dry substrate at 550°C for 5 h.

6.2.6. Van Soest fractionation

Lignocellulosic characterization was run according to Van Soest et al. (1991) using FIWE Raw Fiber Extractor from VELP Scientifica. The protocol identifies four fractions: SOL, HEM, CEL and LIC. One gram of dry substrate was extracted with 100 mL hot water for 30 min followed by extraction with neutral detergent for 60 min (extraction of the SOL fraction, neutral detergent fiber (NDF) residue), hot acid detergent for 60 min (extraction of the HEM fraction, acid detergent fiber (ADF) residue) and for 180 min in cold, 72% sulfuric acid (extraction of the CEL fraction, acid detergent lignin (ADL) residue). Therefore the residual material corresponds to the LIC fraction and may contain lignin and cutin. After each extraction step, the VS content was determined in the residues. All fractions were expressed as a percentage of total VS according to the following equations: SOL = 100 – NDF; HEM = NDF – ADF; CEL = ADF - ADL and LIC = ADL.

6.2.7. Measuring enzyme activities

For the measurement of the enzymatic activities, 96-well plate adapted analytical protocols, were followed (Mansour et al., 2015 submitted to Analytical Biochemistry). Absorbance was measured using PowerWave XS2 (BioTek Instruments, Inc., Vermont, USA) with Gen5TM software.

One filter paper unit (FPU) is defined as the amount of enzyme that releases one µmol of glucose per minute in the assay reaction. FPase was assayed by measuring the release of glucose from a mixture containing 2.4 mg Whatman n°1 paper as substrate in 48 µL 50 mM sodium citrate buffer and 24 µL of enzyme sample. The mixture was incubated at 50°C for 60 min. Dinitrosalicylic (DNS) solution was used for color development and absorbance is read at 545 nm.

One unit of CMCase activity is defined as the amount of enzyme that liberates 1 µmol of reducing sugar as glucose under the analysis conditions. A mixture of 30µL of 4% CMC

solution and 30 µL of enzyme sample was incubated at 50°C for 30 min. Later 180 µL DNS were added, followed by dilution with distilled water before absorbance reading at 540 nm. One unit of β-glucosidase activity is defined as the amount of enzyme which produces 1 µmol of p-nitrophenol (pNP) from 4-nitrophenyl α-D-glucopyranoside (pNPG). For the analysis, 100 µL pNPG were mixed with 20 µL enzyme sample and incubated at 50°C for 10 min. The reaction was stopped using 1 M sodium carbonate solution and absorbance was read at 412 nm.

Finally, one unit of xylanase is defined as the amount of enzyme, which produces 1 µmol reducing sugar as xylose per min in the reaction mixture under the specified conditions. A mixture of 80µL of 1% beechwood xylan solution and 20 µl of enzyme sample was incubated at 50°C for 30 min. Later 180 µL of DNS were added followed by dilution with distilled water. Absorbance was read at 540 nm.

6.2.8. Measuring reducing sugars

Reducing sugars were analyzed according to Navarro et al. (2010). A mixture of 60 µL dinotrosalicylic acid solution (DNS) and 60 µL sample was incubated for 10 min at 94°C and then cooled down for 5 min at room temperature. Later 100 µL were transferred to a reading plate for absorbance measurement at 540 nm.

6.3. Results and Discussion

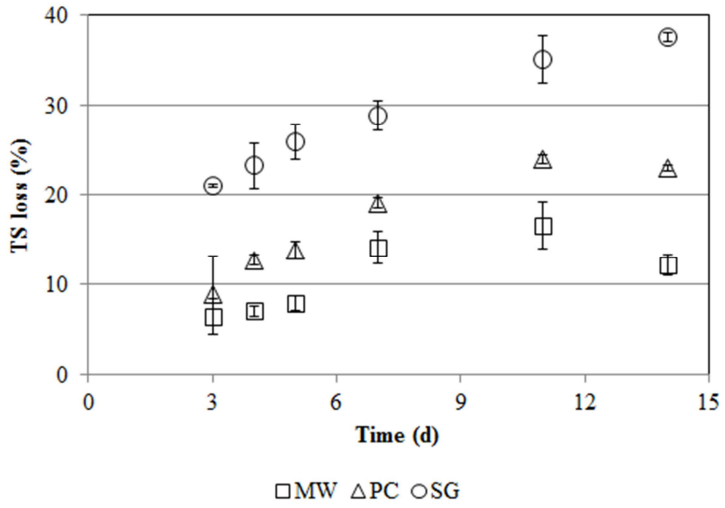
6.3.1. SSF follow-up with time for inoculated matrices

6.3.1.1. Total solid and water content losses

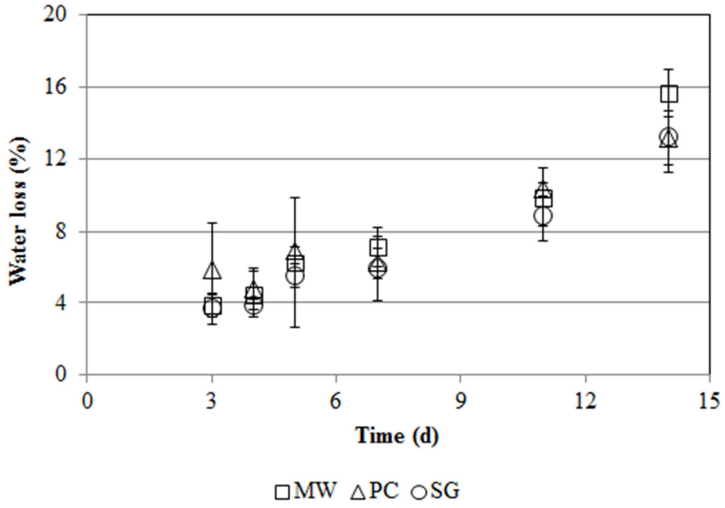
SSF could be defined as the aerobic microbial transformation of solid materials. It is the process during which microbial growth occurs on moist solid particles without presence of free water (Pandey et al., 2008a). Water exists within the solid matrix or as a thin layer either absorbed onto the surface of the particles or less tightly bound within the capillary regions of the solid (Raimbault, 1997; Roussos and Pyle, 1997). Therefore it was decided to look first at

the evolution of both total solids and water content of the different matrices during the process.

Ten grams of dry substrate were added to each flask in addition to two grams of inoculum. On one hand, it is important to note that the mycelium of P. ostreatus is grown directly on the millet and could not be separated from the support; therefore the exact amount of mycelium added was not determined. Some techniques were reported in the literature such as the measurement of ergosterol content, which is a component of fungal cells (Regner et al., 1994). However, its determination is sometimes difficult because other sterols from the vegetal biomass can interfere in its determination. If the fungal growth is significant, another parameter that could indicate fungal growth is the increment of protein content (Pena et al., 2012). On the other hand, during SSF the mycelium will further grow on the substrate: there is an increase in the solid content due to the mycelial growth and a decrease of the solid mass due to the degradation of the substrate itself. In fact an important limitation of SSF is the lack of reliable methods for growth characterization studies (Favela-Torres et al., 1998). The direct determination of biomass growth is very difficult since the fungal hyphae penetrate into and bind the mycelium tightly to the substrate (Raimbault, 1997). The heterogeneity and complicated nature of the materials used as surfaces of growth interfere with the accurate determination of process parameters. This is why the TS loss expressed in his work corresponds to an overall weight loss. Figure 2a shows the evolution with time of the %TS: most of the solid loss occurs within 5 to 7 days of incubation and then the rate of degradation decreases. After 7 d of incubation, TS losses of 14, 19 and 29% are recorded for MW, PC and SG as compared to 12, 23 and 38% after 14 d. The highest %TS loss is thus measured for SG and the lowest for MW. Chen et al. (2011) reported % weight loss varying from 16 to 28% after 7 d for different corn stover fractions used as substrate to produce cellulase under SSF.Therefore the values reported in this work do compare with literature data.



(a)



(b)

Figure 2. % total solids (TS) (a) and moisture losses (b) over time for the matrices MW, PC and SG inoculated with *P. ostreatus*

In biological systems, water plays two fundamental functions (Gervais and Molin, 2003). It has a solvent function at the level of the organism as well as the cell; it provides nutrients and scavenges wastes or metabolites under the dissolved form. It also provides a structural function implicated in the stability and function of the biological structures organized at the molecular and cellular levels. Under SSF, water content could decrease due to this biological activity. Moisture control might become essential in order to keep the substrate at the adequate moisture content that can support growth and metabolism of the microorganisms (Pandey, 2003). At this scale of work, no moisture control was applied and as shown in

Figure 2b until day 7 of incubation, water loss reached 7.1, 6.2 and 5.9% for MW, PC and SG respectively. Highest values were obviously measured after 14 d: 15.6, 13.2 and 13.2%. In Lu et al. (2003), after 68 h incubation moisture level dropped from 60 to 25% for SSF carried out on pilot scale without any control and from 55 to almost 30% when temperature and humidity levels were adjusted. The data of this research is lower than the reported literature since the scale is smaller and therefore the heat generated is much less important. With 10 g of substrate in 250 mL flasks aeration is enough not to allow an important increase in temperature whereas Lu et al. (2003) reported temperature increasing from 20 to 60°C.

6.3.1.2. Lignocellulolytic enzyme production

Regardless of their source lignocellulosic materials consist of three main polymers: cellulose, hemicellulose and lignin (Acharya et al., 2010; Deswal et al., 2011; Montoya et al., 2012). Cellulose is a linear homopolymer of glucose units; the chains of cellulose tend to forms microfibrils with alternating crystalline and amorphous regions. It is hydrolyzed by cellulases, a complex of at least 3 groups of enzymes: endoglucanase, exoglucanase (CMCase) and β-glucosidase (Lynd et al., 2002; Jabasingh and Nachiyar, 2011). Their overall activity is expressed as filter paper activity or FPase. Hemicelluloses are polymers composed of monomeric components mainly xylose, mannose, galactose, arabinose and methylglucuronic acid (Santoni et al., 2015). Xylanases are involved in the degradation of hemicellulose. Finally, the chemical structure of lignin is also complex: it is mainly made up of guaiacyl and syringyl units to which phenylpropanoid units can also be cross-linked (Santoni et al., 2015). The most widely known enzymes to degrade lignin are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Montoya et al., 2012).
In this work, four enzymes will be measured over time: FPase, CMCase, β-glucosidase and xylanase.

The profile of enzymes activities should follow that of the mycelial growth. An interesting description was provided by Qian et al. (2012) regarding the changes in microbial growth of *A. niger* and β-glucosidase production with time during a 96-h incubation. The authors divided the fermentation period into: early-stationary phase, logarithmic growth phase, steady phase and decline phase according to fermentation productivity (FP) which corresponds to % of the dry weight of the fermented product to the dry weight of the initial substrate and enzyme production. For the beginning of the fermentation (0-12h), the spores that were germinating resulted in near 100% FP due to changes in dry matter weight loss and no enzymes activities. During 12-36h, *A. niger* grew very fast resulting in a rapid increase of dry matter weight loss that is a decrease in FP. With *A. niger* growing rapidly and FP falling rapidly, β-glucosidase production was biosynthesized fast to reach its peak value. This was also reported earlier in Reddy et al. (2003) who followed different enzyme activities for over 40 d of incubation. In that perspective, Figure 3 shows the evolution of the four enzymes over time on the three tested matrices. The trend described earlier could actually be found for MW and PC however it is much less obvious for SG except for its corresponding β-glucosidase production (Figure 3c). This difference could be attributed to the relatively different lignocellulosic composition of spent grains: higher SOL fraction and very low LIC fractions as compared to MW and PC (Figure 1). Therefore in terms of rate of mycelial growth, brewery's spent grains are less advantageous than municipal waste and paper/cardboard fractions.

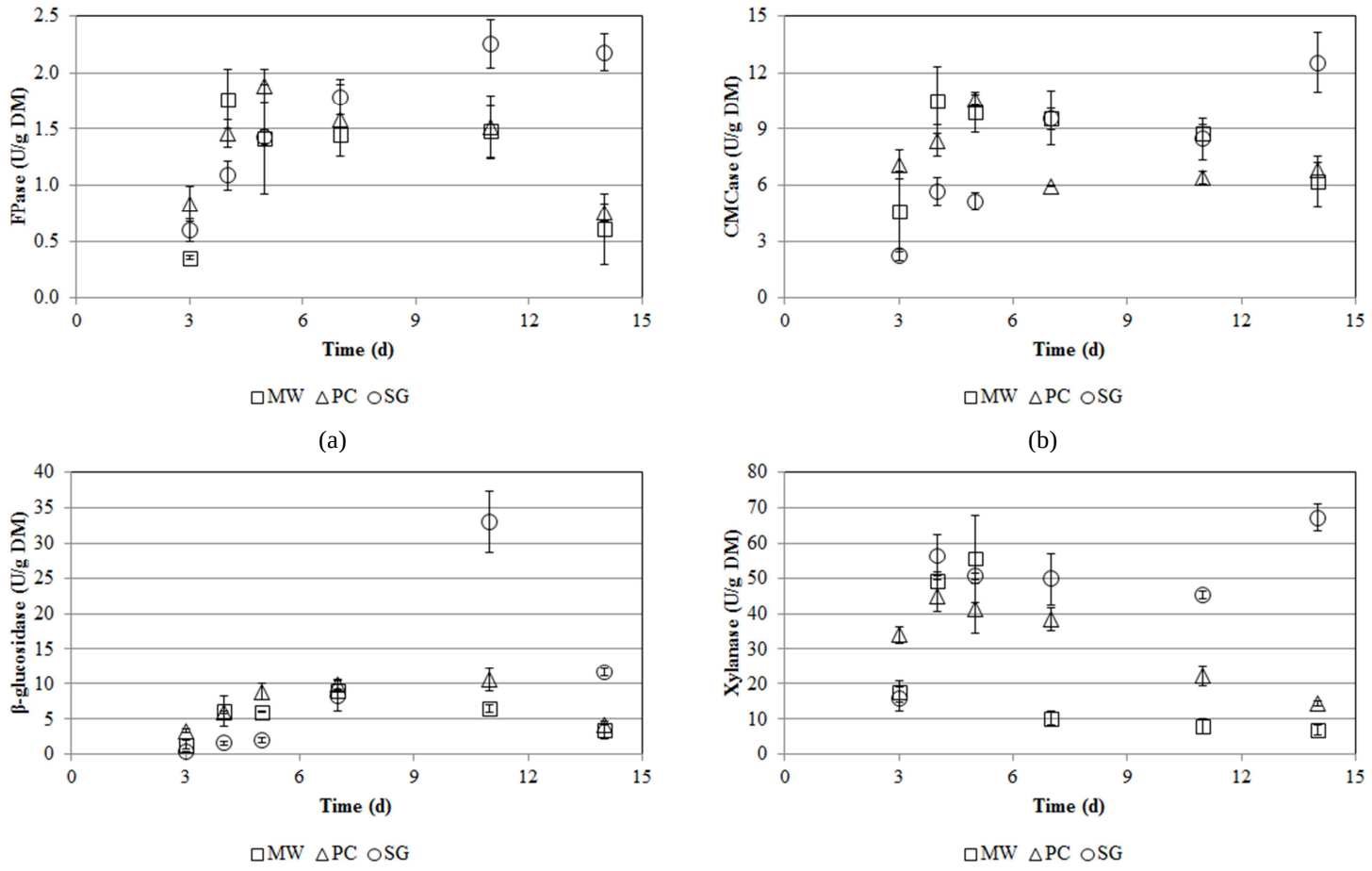


Figure 3. Evolution over time of enzyme activities FPase (a), CMcase (b), β-glucosidase (c) and xylanase (d) for inoculated matrices MW, PC and SG

The observation above is also reflected into the time at which maximum enzyme activity is measured. For MW, maximum activities are recorded at day 4 for FPase and CMCase, day 5 for xylanase and day 7 for β-glucosidase. For PC, maximum values correspond to days 5 for FPase and CMCase, 4 for xylanase and 7 for β-glucosidase. For SG however, maximum reported values within the experimental period correspond to day 11 for FPase and β-glucosidase and day 14 for the remaining two enzymes. In fact, literature data regarding SSF optimal duration (Table 1) is mostly around an average of 4 d despite some extreme values such as 10 d for Reddy et al. (2003), 16 d for Deswal et al. (2011) and 22 d for Chahal (1985). Therefore, MW and PC would correspond more to what can be found on average in the literature.

To assess the efficiency of the SSF process, it is most important to look at the amount of enzymes produced. Maximum activities of 1.8, 1.9 and 2.3 FPase U/g DM, 10.5, 10.6 and 12.5 CMCase U/g DM, 8.9, 10.6 and 33 β-glucosidase U/g DM and finally 50.5, 45 and 67.3 xylanase U/g DM were recorded for MW, PC and SG respectively. As compared to the data presented in Table 1, these efficiencies are low but they do compare with some findings in which the substrate was autoclaved before inoculation in order to limit any competition with indigenous flora. Therefore in this research, it can be concluded that the production is very important given the experimental conditions. It is also important to add that for enzyme activity measurement, the analysis protocol may vary making it difficult to compare certain results.

6.3.2. SSF follow-up with time for control assays in first experimental plan

In the first experimental setup, control assays were sampled only at days 5 and 14. A total of 24 points can be compared to see the impact of inoculum addition. Interestingly for only 5 data points (CMCase for MW and PC at day 5 and for SG at day 14 and xylanase for SG at days 5 and 14) enzyme activity is higher for the samples with inoculum. Detailed results for

these points are shown in Table 2. These findings were never reported in the literature. They actually imply that the indigenous microflora in the different substrates can, under proper operational conditions, allow a competitive enzyme production. Therefore in scale-up tests, if this result can be validated, an important operational cost, that of the inoculum, could be cut out.

6.3.3. Impact of the substrate's indigenous microflora

Given the above results, it was decided to run a new experimental setup with 90 assays. Half of the flasks were used with autoclaved substrates in order to validate the importance of the native microorganisms. Sampling events were set only at days 0, 3, 4, 5 and 14. In fact to integrate this SSF process in a treatment facility it is more interesting to have a short fermentation period.

6.3.3.1. Comparing results over time for assays without inoculum

Sampling points at days 5 and 14 were repeated over time (first and second experimental plans) and thus will be used to assess the reproducibility of the tests. Table 2 shows the results with their corresponding standard deviations. Lower losses for both TS and water occur in the validation tests and this applies to all data points. Regarding enzyme activities, xylanase data are systematically higher for the validation tests for all three matrices although for the remaining enzymes most of the results in the first experimental tests are higher. Interestingly enough, regardless of the experimental test, assays without inoculum present higher enzymatic activities. Therefore, the observations made earlier can be validated; however the reproducibility of the tests cannot be confirmed. This would be mainly due to the fact that the enzyme production system depends, when no autoclaving and no inoculum, on a dynamic system of native species. Therefore there is always a risk in assessing the exact amount of enzyme produced but a proper control of the operational conditions might help in

defining a range of production. This should be taken into account when assessing technical and economic feasibilities of the process.

Table 2: Control points data for MW, PC and SG after 5 and 14 days incubation in terms of % TS loss, % water loss and enzyme activities in U/g DM (FPase, CMCase, β-glucosidase and xylanase): firt experimental plan (#1) and second experimental plan (#2)

		MW		PC		SG	
		Day 5	Day 14	Day 5	Day 14	Day 5	Day 14
#1	% TS loss	16.7 ± 0.5	23.9 ± 1.7	23.2 ± 1.9	31.9 ± 1.2	35.2 ± 0.9	42.7 ± 0.1
	% water loss	8.5 ± 0.3	13.6 ± 1.8	7.5 ± 0.7	12.8 ± 1.1	6.2 ± 1.1	13.1 ± 1.0
	FPase (U/g DM)	2.1 ± 0.3	2.0 ± 0.1	2.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.2	2.6 ± 0.2
	CMCase (U/g DM)	6.8 ± 0.0	11.3 ± 1.5	9.3 ± 0.7	11.9 ± 0.8	6.0 ± 0.6	10.8 ± 1.3
	β-glucosidase (U/g DM)	14 ± 3.2	3.6 ± 1.0	14.6 ± 0.4	12.4 ± 0.3	5.7 ± 1.0	14.1 ± 0.2
	Xylanase (U/g DM)	68.4 ± 2.0	31.4 ± 1.5	86.3 ± 3.1	40.2 ± 4.4	45.8 ± 1.1	66.9 ± 2.0
#2	% TS loss	10.2 ± 1.3	16.8 ± 4.4	17.0 ± 2.8	25.8 ± 1.0	23.6 ± 0.2	38.2 ± 1.6
	% water loss	4.7 ± 0.5	9.5 ± 1.3	5.9 ± 0.1	8.6 ± 0.1	4.4 ± 0.1	6.7 ± 1.3
	FPase (U/g DM)	2.2 ± 0.3	1.7 ± 0.4	2.6 ± 0.3	1.7 ± 0.3	1.4 ± 0.2	2.1 ± 0.2
	CMCase (U/g DM)	13.5 ± 0.9	9.8 ± 1.1	16.5 ± 2.8	7.5 ± 1.5	8.0 ± 0.6	5.5 ± 0.4
	β-glucosidase (U/g DM)	5.0 ± 0.9	8.7 ± 0.4	6.7 ± 2.6	9.1 ± 0.1	1.9 ± 0.2	10.2 ± 3.0
	Xylanase (U/g DM)	179.6 ± 9.3	86.0 ± 11.1	225.9 ± 9.8	61.8 ± 9.9	191.8 ± 11.4	153.1 ± 1.0

6.3.3.2. Impact of autoclaving on TS and water losses in second experimental plan

Figure 4 presents the % weight and water losses for the control assays with and without substrate autoclaving. Results show that total solid losses are relatively higher for the substrate without autoclaving. This can be explained by a higher biological activity through substrate degradation and mycelial growth. The profiles of the curves vary also between the substrates in Figure 4a whereas data points for MW and PC overlap when autoclaved. In Figure 4b, data points for water loss are comparable. At this scale of work, humidity change could be mainly due to evaporative losses.

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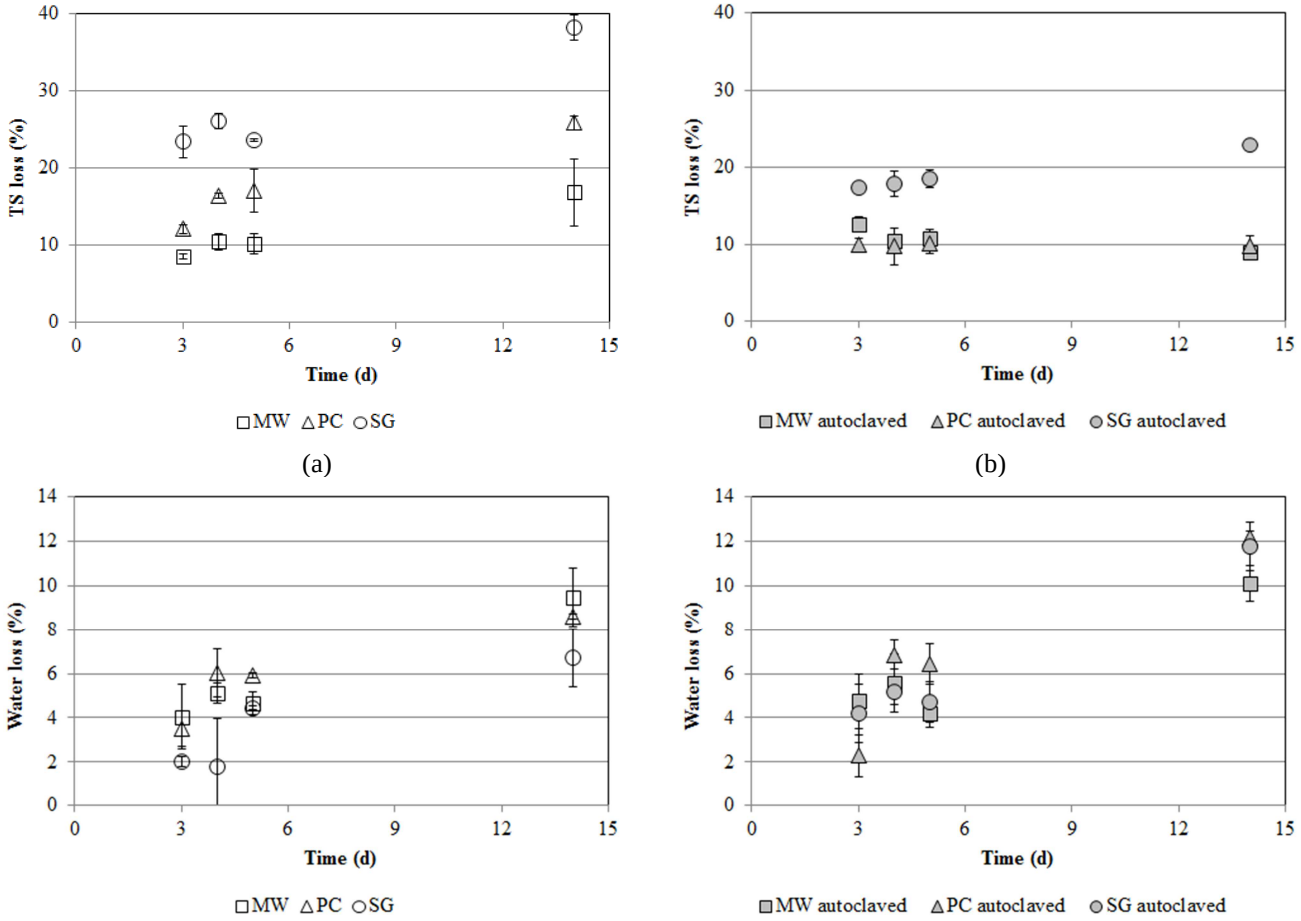


Figure 4. Evolution over time of % TS and water losses during SSF of matrices MW, PC and SG without autoclaving (a and c) and with autoclaving (b and d).

6.3.3.3. Lignocellulolytic enzyme production with and without inoculum

Evolution of the different enzyme profiles are shown in Figure 5. No enzyme activities were recorded for most of the assays in which the substrate is autoclaved; therefore all presented data correspond only to assays without substrate autoclaving. In fact for most data points, enzyme activities are higher without inoculum as compared to inoculated tests. A non-sufficient inoculum level might partially explain these observations however the indigenous microflora of the substrates plays also an important role. In fact, given the results, it will be most probable that inoculation has a negative effect on enzyme production. The inoculum creates a competition in the medium that adversely impacts the SSF process.

Interestingly peak enzyme activities for MW and PC compare well with and without inoculum mainly for FPase, CMCase and xylanase. For β-glucosidase since the data point at day 7 is missing, the enzyme profile cannot be predicted. This is also the case for SG for both enzymes FPase and β-glucosidase in which data point at day 11 is missing. For the remaining enzymes, CMCase and xylanase, peak values for SG appear at day 5 as compared to day 14 with inoculum.

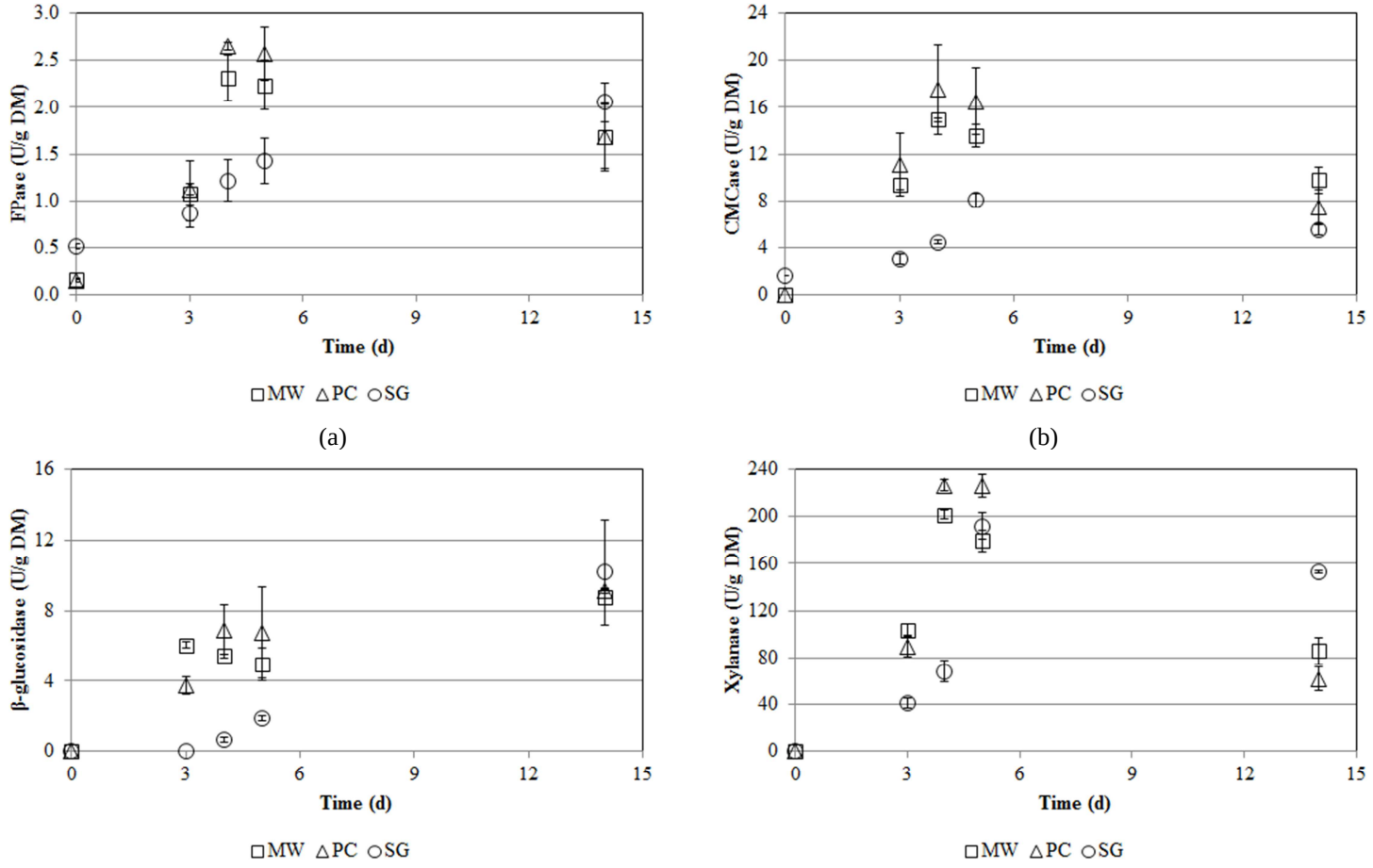
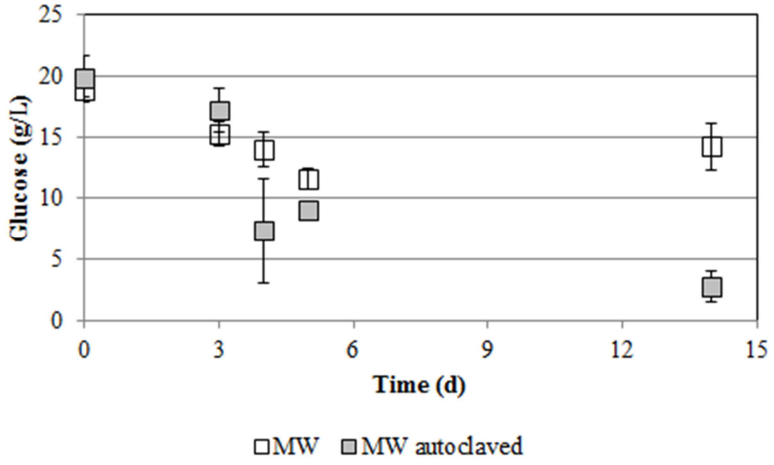


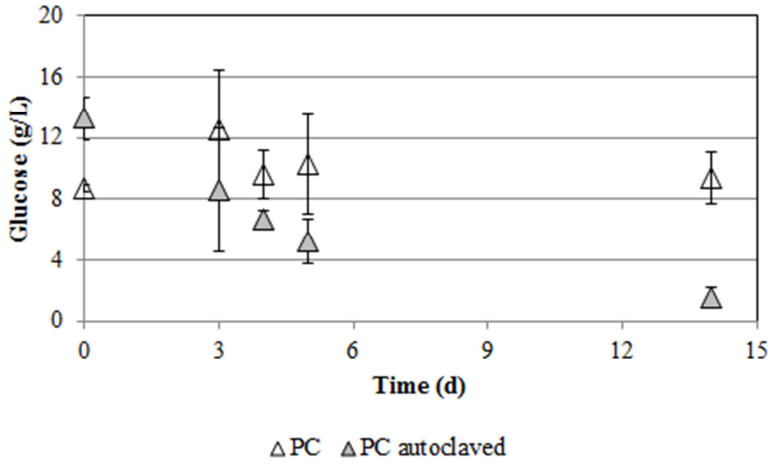
Figure 5. Evolution over time of enzyme activities FPase (a), CMcase (b), β-glucosidase (c) and xylanase (d) for control matrices MW, PC and SG.

6.3.4. Reducing sugar release in crude enzyme extracts in second experimental plan

SSF is not only a way to produce enzyme but it is also a pretreatment process for lignocellulosic substrates. Maximum enzyme activities were defined to help stop the process at the proper time otherwise the enzymes will be consumed in situ. This is why reducing sugars were measured in the different enzyme extracts of the validation tests. Figure 6 shows the evolution of the glucose concentration over time for MW (Figure 6a), PC (Figure 6b) and SG (Figure 6c). Regardless of the SSF substrate, reducing sugar concentration profiles are similar. For autoclaved matrices, at varying concentrations at day 0 (13, 20 and 80 g/L for MW, PC and SG respectively), reducing sugars decrease with time until they reach almost zero. A biological activity must therefore be ongoing for the sugar to be consumed. This is possible since despite autoclaving the substrate, tests were not run under aseptic conditions which do not exclude a risk of contamination. However, without autoclaving, sugar concentration decreases but then gets to an inflexion point given the high concentration measured at day 14. According to Qian et al. (2012), this should correspond to the stage where the mycelium has stopped growing and enzymes are used to degrade the organic matter.



(a)



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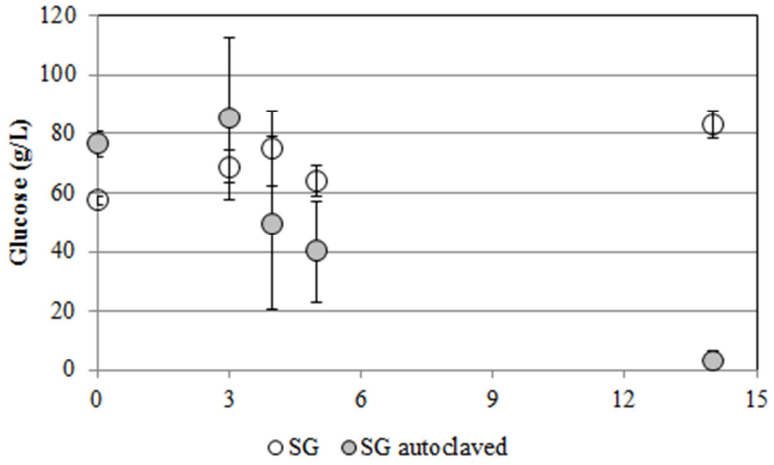

(c)

Figure 6. Evolution of glucose concentrations (g/L) over time in crude enzyme extracted from control tests of MW (a), PC (b) and SG (c) with and without substrate autoclaving

6.4. Conclusions

At 30°C and 60% humidity, highest lignocellulolytic enzyme activities were mostly found between 4 and 5 d of incubation under solid state fermentation conditions. Interestingly indigenous microflora can have a very interesting impact on the enzyme production under appropriate operational conditions. No autoclaving and no inoculum addition are necessary for the substrate to properly run the SSF process. However, the main limitation is the reproducibility of the data. At a higher scale, biochemical changes may induce waste settling which alters aeration and thermal exchange and depending upon the source of the substrate the indigenous flora differs. All these parameters should be taken into account while up-scaling the process.

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CHAPTER 7

Evaluating the impact of enzyme addition in three anaerobic digestion systems treating brewer's spent grain

ABSTRACT

Anaerobic digestion (AD) is an excellent treatment alternative to convert brewer's spent grain (BSG) into methane, a renewable energy. But this lignocellulosic substrate requires pretreatment to improve its biodegradation. One crude enzyme mixture from a solid state fermentation (SSF) process and a commercial enzyme mixture were used to pretreat BSG. Their impact is studied on three lab scale AD systems: single-stage, two-stage and a percolation system coupled with AD. There was no impact on methane production in neither single nor two-stage AD. But in the latter, up to 56, 148 and 116% increases in soluble COD, glucose and acetate levels were observed respectively in the hydrolysis step. Nevertheless, 30% increase in soluble COD was observed during the percolation phase and up to 65% increase in methane production due to the use of SSF enzymes.

Keywords: anaerobic digestion, brewer's spent grain, single-stage, two-stage, percolation, enzyme, hydrolysis.

7.1. Introduction

Brewer's spent grain (BSG) is a by-product of the beer brewing process. It is very rich in sugars and proteins therefore its main and quickest alternative for elimination has been animal feed (Mussatto et al., 2006; Xiros et al., 2008). More recently interest has grown in this substrate due to its low price, large availability throughout the year and valuable chemical composition[3]. Its annual production is estimated at 38.6 million tons according to Mussatto (2014). It is a lignocellulosic biomass that does not directly compete with food production. Therefore within the context of increasing environmental concern and economic incentives, efforts of diverting its use towards bioenergy and bioethanol processes (Beldman et al., 1987; Bochmann, 2007) have multiplied in the last decade.

Anaerobic digestion (AD) of lignocellulosic biomass provides an excellent opportunity to convert abundant bioresources into renewable energy (Sawatdeernarunat et al., 2015). But where cellulose is a major constituent of the feedstock, its hydrolysis is rate-limiting in overall AD (Noike et al., 1985). According to van Lier et al. (2008) this is mainly due to the lack of sufficient and specific extracellular enzymes. Hydrolysis becomes therefore the bottleneck of the bioconversion process (Xiros and Christakopoulos, 2009).

Hydrolysis can actually be achieved through physical and chemical pretreatments. This work however focuses on the use of biological enzymatic pretreatment. In fact, emerging applications in industrial biotechnology, mainly those using enzymes, are multiplying in biofuel production and waste treatment of lignocellulosic matrices. Two different sources of enzymes are tested: a crude enzyme extract produced from a lab-scale solid state fermentation (SSF) system and a commercial enzyme. The main objective is to compare the impact of using those enzymes throughout three lab-scale anaerobic digestion systems: traditional single-stage mesophilic AD, two-stage mesophilic AD and a percolation system coupled with AD system.

7.2. Materials and Methods

7.2.1. Matrix characterization

Fresh BSG were recovered from a local brewery in France. Wet BSG had an initial moisture content of 24.3% and a volatile solids (VS) content of 95.7% of TS. It was dried at 80°C until weight stabilization and stored in sealed containers. Lignocellulosic fraction was characterized according to Van Soest et al.(1991) using FIWE Raw Fiber Extractor from VELP Scientifica. The protocol identifies four fractions: soluble (SOL), hemicellulose-like (HEM), cellulose-like (CEL) and lignin and cutin-like (LIC) expressed as % VS. SOL, HEM, CEL and LIC fractions were measured at 28, 44, 19 and 9%VS respectively.

7.2.2. Enzyme sources

Solid state fermentation was carried out in 250 mL Erlenmeyer flasks. Ten grams of dry substrate were moistened with 15 mL of the following nutrient solution (g/L) in which the pH was adjusted to 5.0: NH_4Cl, 10.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 0.33; $CoCl_2$, 0.3; $MnSO_4.H_2O$, 0.07; $ZnSO_4.H_2O$, 0.07; $CaCl_2$, 0.33 and yeast extract, 3.3. *Pleurotus ostreatus* SPOPO (sporeless species from Sylvan *Inc*. grown on millet) was added at 20% (w/w) and mixed with the substrate. After 5 d of incubation at 30°C, 100 mL of tap water were added and the mixture shaken for 1 h at 180 rpm. The crude enzyme was obtained after filtration under vacuum through Whatman GF/A type microfiber filters (pore size 1.6 µm).

For the purpose of this work, three crude enzyme solutions obtained from SSF using municipal solid waste, paper/cardboard fractions and BSG were mixed. They were concentrated using VivaspinTM 20 (GE Healthcare) sample concentrators with molecular weight cutoff (MWCO) of 10 kD. The final product will be referred to as SSF enzyme. It presented the following enzymatic activities: 0.2, 3.1, 4.5 and 19.7 U/L for total cellulase or filter paper activity (FPase), β-glucosidase, carboxymethylcellulase (CMCase) and xylanase respectively.

The commercial enzyme source presented the following activities: 188.2, 75.0, 524.2 and 700.4 U/mL for the same enzymes above.

7.2.3. Experimental setups

Three different AD systems were assessed and all tests were run in triplicates. For the single-stage mesophilic AD, an automatic methane potential test system (AMPTS) from Bioprocess control was used to assess the biochemical methane potential (BMP). A total volume of 400 mL mixture was prepared using 1 g VS of BSG. The inoculum used was a mixture of digestates from a municipal wastewater treatment plant and a co-digestion plant treating agro-industrial substrates. Mineral and oligo-element solutions were added into the reaction mixture and the inoculum to substrate ration (I/S) was fixed at 2 (Angelidaki et al., 2009). All bottles were placed in a 35°C water bath with continuous agitation. The AMPTS system registers biogas production online. Only methane is recorded since the gas flux is passed through a 3 M sodium hydroxide solution that acts as a carbon dioxide trap. For tests with enzyme addition, 200 µL SSF enzymes or 100 µL commercial enzymes were added at the beginning of the tests. These enzyme solution volumes correspond to 0.04, 0.62, 0.89 and 3.94 U/g DM for FPase, β-glucosidase, CMCase and xylanase activities respectively for SSF enzymes and 19, 98, 52 and 70 U/g DM for commercial enzyme system. Follow-up was kept until stabilization of methane production.

For the two-stage AD system, 1 g VS of BSG were placed in 25 mL glass and mixed with 20 mL 0.05 M sodium acetate buffer (pH 4.8). Tubes were kept at 35°C in an incubator with agitation at 180 rpm. After 24 h, some samples were collected to measure soluble COD, volatile fatty acids (VFA), reducing sugar concentrations and enzyme activities. To assess the methane potential in the anaerobic stage, the content of other tubes were transferred to the AMPTS system. For this configuration enzymes were added in the hydrolysis stage at the same concentrations as in single-stage AD.

For the last configuration, only SSF enzymes were used in triplicate besides the triplicate controls. For the percolation system, 1.5 kg fresh BSG were placed in 5 L pilots (Figure 1) at room temperature. A total of 8 L sodium carbonate buffer (with or without added enzymes) was used for organic matter extraction at a rate of 4 L/h. SSF enzymes were added at a rate of 2, 26, 37 and 165 U/g DM for FPase, β-glucosidase, CMCase and xylanase activities respectively. During 7 d, a follow-up of pH, total alkalinity (TA), CODs, VFA, reducing sugars and enzyme activities was achieved. BSG's lignocellulosic fraction was also characterized at the end of the experiment. To evaluate methane production, 100 mL of percolate were transferred to the AMPTS system.

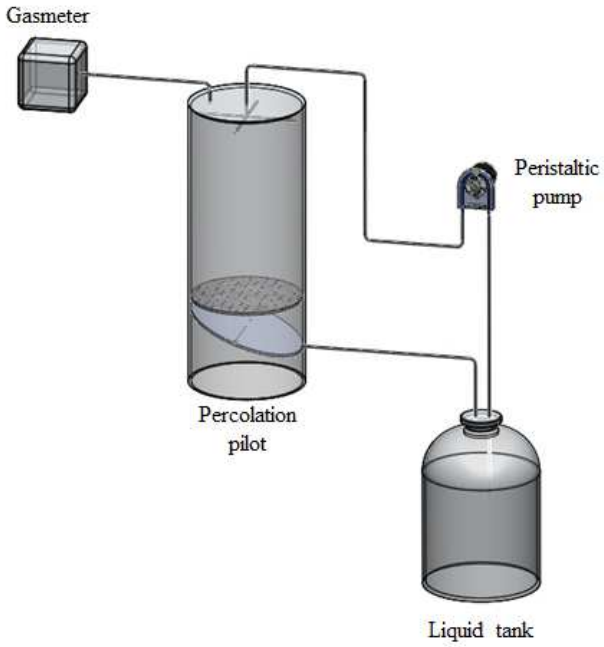


Figure 1. Schematic diagram of the 5 L percolation pilot.

7.2.4. Enzyme activities

For the measurement of the enzymatic activities, 96-well plate adapted analytical protocols, developed in the labs of Veolia Research and Innovation, were followed. Absorbance was measured using PowerWave XS2 (BioTek Instruments, Inc., Vermont, USA) with Gen5TM software.

One filter paper unit (FPU) is defined as the amount of enzyme that releases 1 µmol of glucose per minute from Whatman n°1 paper as substrate. One unit of CMCase activity is defined as the amount of enzyme that liberates 1 µmol of reducing sugar as glucose with 4% CMC solution. One unit of β-glucosidase activity is defined as the amount of enzyme which produces 1 µmol of p-nitrophenol (pNP) from 4-nitrophenyl α-D-glucopyranoside (pNPG). And one unit of xylanase is defined as the amount of enzyme, which produces 1 µmol reducing sugar as xylose per min from 1% beechwood xylan.

7.2.5. Analysis

All liquid samples were filtered before analysis through 0.2 µm fiber glass filters. COD and TA were measured using corresponding testing kits from Hach Lange. The speciation and concentration of volatile fatty acids (VFA) were measured using a GC with flame ionization detector 7890A (Agilent) with hydrogen as carrier gas and crotonic acid as internal standard. Finally reducing sugar analysis was prepared according to Navarro et al. (2010). For that purpose, a mixture of 60 µL dinitrosalicylic acid solution (DNS) and 60 µL sample was incubated for 10 min at 94°C and then cooled down for 5 min at room temperature. Later 100 µL were transferred to a 96-well reading plate for absorbance measurement at 540 nm.

7.3. Results and Discussion

7.3.1. Enzyme activities in the medium

Lignocellulosic substrates consist of three main polymers: cellulose, hemicellulose and lignin (Acharya et al., 2010, Deswal e al, 2011; Montoya et al., 2012). Cellulose is a linear chain of glucose units which forms microfibrils with alternating crystalline and amorphous regions. It is hydrolyzed by cellulases, a complex of at least 3 groups of enzymes: endoglucanase, CMCase and β-glucosidase (Lynd et al., 2002). Their overall activity is expressed as filter paper activity or FPase. Hemicelluloses are polymers composed of monomeric components mainly xylose, mannose, galactose, arabinose and methylglucuronic acid (Santoni et al.,

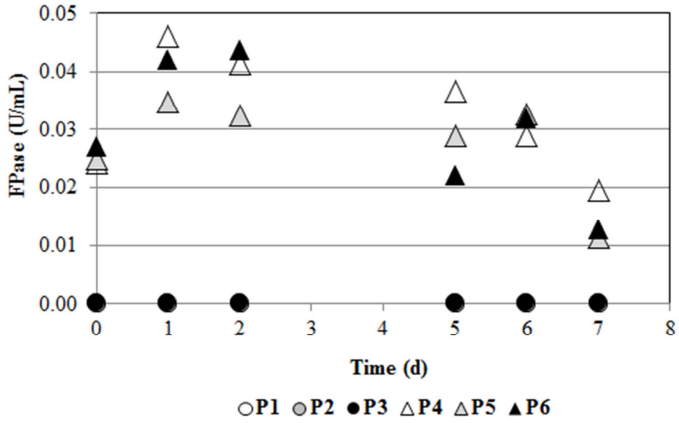
2015). They are degraded by xylanases. Finally, lignin has a complex chemical structure mainly made up of guaiacyl and syringyl units to which phenylpropanoid units can also be cross-linked (Santoni et al., 2015). The most widely known enzymes to degrade lignin are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Montoya et al., 2012). Only FPase, CMCase, β-glucosidase and xylanase are measured in this work.

When added to any biological system, enzyme activities are expected to decrease and this is translated by a decreased rate of hydrolysis. This observation could be attributed not to a change in substrate reactivity but rather structural obstacles retarding the activity of the enzyme or non- productive adsorption of cellulases to lignin via hydrophobic bonding (Rosgaard et al., 2006). Other mechanisms such as sheering and thermal inactivation of the enzymes and end product inhibition of the cellulase complex were also reported (Mussatto et al., 2008). Most research has focused on reducing the non-specific binding phenomenon. Yang and Wyman (2006) were successfully able to reduce both cellulase and β-glucosidase binding on lignin by adding bovine serum albumin (BSA) prior to introducing the enzymes. And Chen et al.(2008) added the non-ionic surfactant Tween 80 to decrease this unproductive adsorption of the enzyme to the lignin part of the substrate.

In the current work, enzyme activities were not measured in the single-stage AD because the AMPTS setup does not allow sampling without perturbing the biological system. At the end of the hydrolysis step of the two-stage AD, enzyme activities were measured. Given the original enzyme concentrations added, 100, 52 and 11% losses were calculated for FPase, β-glucosidase and xylanase activities respectively for the SSF enzymes. No loss was found for the CMCase. Higher losses were recorded for the systems with commercial enzymes: average losses of 84, 75, 87 and 86% for FPase, β-glucosidase, CMCase and xylanase respectively. These results are interesting since commercial enzyme mixtures are usually chemically stabilized, it would be expected that they be more resistant in the biological medium. But

commercial enzyme to SSF enzyme ratios decreased from 24 to 430 before hydrolysis to 7 to 9 after hydrolysis. This implies a certain advantage to enzymes produced under SSF conditions and a confirmation that when enzymes are used, they should be added on a regular basis to take into account the losses.

In the percolation stage, samples were collected from the buffer tank on days 0, 1, 2, 5, 6 and 7. In all the following results, pilots P1 through P3 correspond to control pilots and pilots P4 through P6 are pilots with added SSF enzymes. Figure 2 presents the different enzymatic activity profiles over time. Data showed that once the crude enzyme mixture was added to the buffer tank (day 0), there was an important loss in enzymatic activities: 69, 91, 92 and 89% loss for FPase, CMCase, β-glucosidase and xylanase respectively. In fact the pH increase for the enzymes from 5 (in the crude enzyme mixture) to 8 upon mixing with the sodium carbonate may have led to inactivation of the proteins. This can also explain why at day 1 the enzyme activities are higher than at day 0. But enzyme activities are relatively stable over the first 3 sampling days; xylanase activities remain stable for over 6 days of percolation (Figure 2d). After 7 days, 40, 49, 45 and 33% enzyme activity losses are calculated for FPase, CMCase, β-glucosidase and xylanase respectively from day 0. These values are very encouraging especially when compared to the losses recorded during the hydrolysis system earlier.



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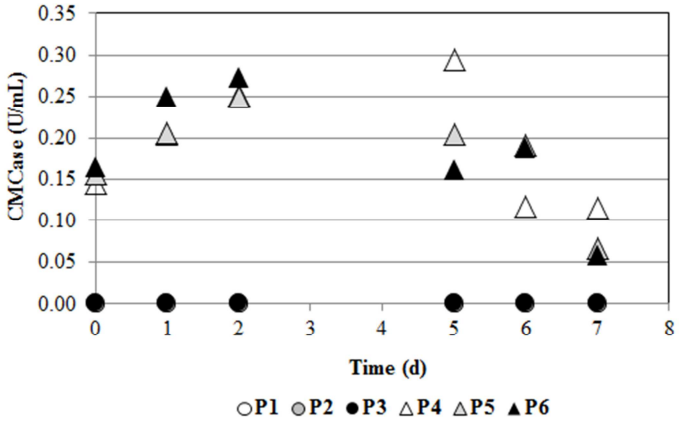

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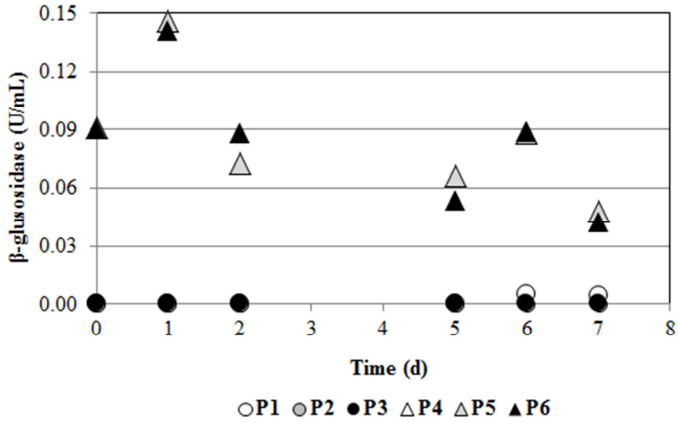

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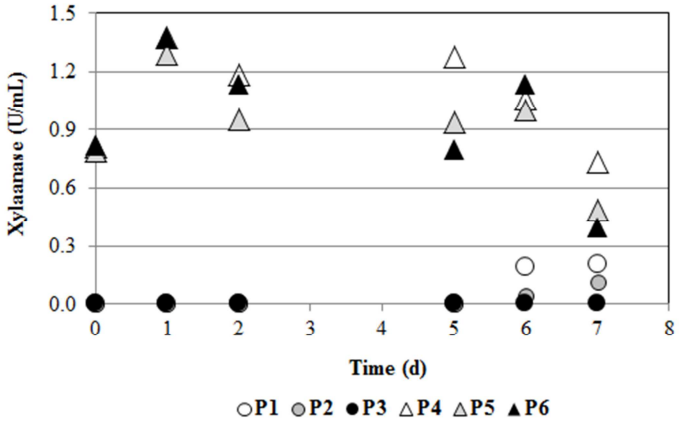


(d)

Figure 2. Evolution of enzyme activities in U/mL with time during the percolation phase for FPase (a), CMCase (b), β-glucosidase (c) and xylanase (d).

7.3.2. Impact of enzymes on the hydrolysis step

The hydrolysis step is identified in the two-stage AD and the percolation phase. Several parameters were measured in this work but interestingly most literature data focuses mainly on the amount of sugars released during hydrolysis. To our knowledge, no data were found on soluble COD or VFA levels. Going by chronological order, Beldman et al. (1987) used several commercial enzyme preparations to hydrolyze BSG at 10% (w/w) suspension. Incubation took place in succinate buffer (pH 4.8), 40°C for 70 h. To inhibit microbial growth, 0.01% sodium azide and 0.2% sodium sulfite were added. Results showed that reducing sugar concentrations varied between 0.4 and 14.6 g/100 g BSG after alkaline or acidic pretreatment and 8.4 and 13.7 g/100 BSG after enzymatic pretreatment. When both pretreatments were combined, reducing sugar concentrations were reported between 12.4 and 34.2 g/100g BSG. More recently, three different forms of BSG, original (untreated), pretreated by dilute acid (cellulignin), and pretreated by a sequence of dilute acid and dilute alkali (cellulose pulp), were hydrolyzed using cellulase (Mussatto et al., 2008). Substrate at 2% (w/v) concentration was mixed with sodium citrate buffer and the 25-mL reaction mixture was supplemented with sodium azide to inhibit microbial contamination. Incubation took place in a rotary shaker at 100 rpm, 45°C for 96 h. Glucose concentrations of 1, 5.5 and 17 g/L were recorded for untreated, cellulignin and cellulose pulp respectively. And finally in White et al. (2008), BSG was first autoclaved at 121°C for 15 min before undergoing enzymatic pretreatment. Hydrolysis took place in 40 mL reaction volume at 10% (w/v) substrate concentration, pH between 5 and 6, 50°C with agitation at 130 rpm for 18 h. Sugar concentrations were recorded at 9.2 g/100 g BSG for the control and 29.2 to 39.4 g/100 g BSG after different acid pretreatments.

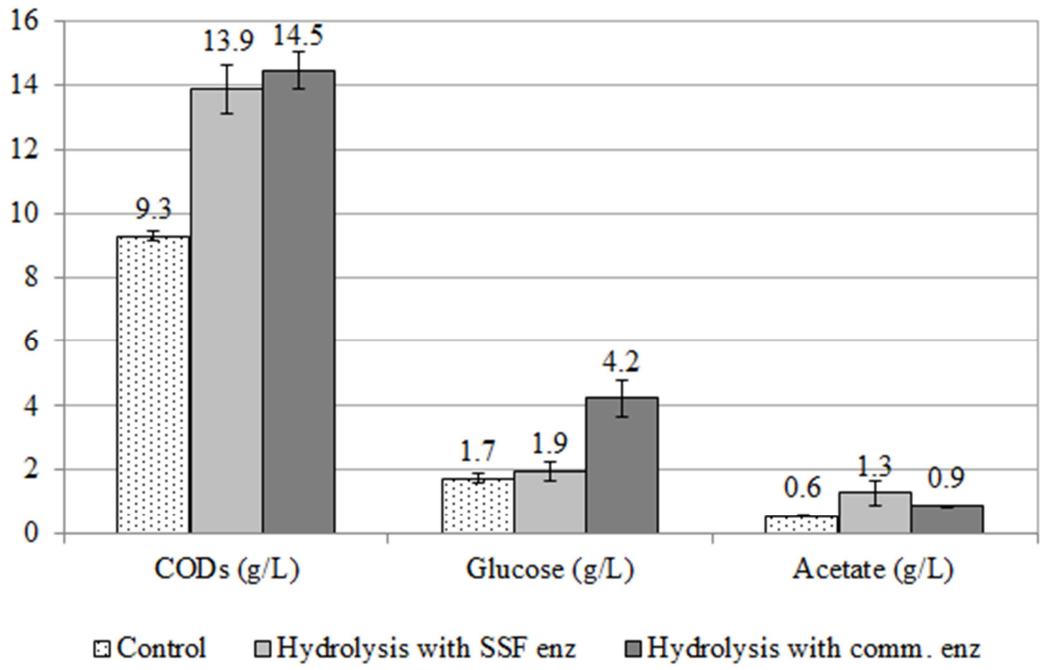


Figure 3. Soluble COD (g/L), glucose (g/L) and acetate (g/L) levels measured at the end of the 24-h hydrolysis step in the two-stage AD for BSG control, hydrolysis with SSF enzymes and hydrolysis with commercial enzymes.

For two-stage AD, Figure 3 summarizes the results for CODs, glucose and acetate concentration only since no other VFA was detected in the supernatant. Enzymatic pretreatment increases CODs by 1.5 and 1.6 times and acetate by 2.2 and 1.5 times for SSF and commercial enzymes respectively when compared to the control. Glucose concentrations of 1.7, 1.9 and 4.2 g glucose/L were recorded for the control, BSG with SSF enzymes and BSG with commercial enzymes respectively. These values correspond to 3.4, 3.8 and 8.4 g glucose/100 g BSG. When compared to the literature these results are very encouraging since comparable data points would correspond to 8.4-13.7 g /100 g BSG in Beldman et al. (1987), 1 g/L in Mussatto et al. (2008) and 9.2 g/100 g BSG in White et al. (2008). In fact higher glucose levels were recorded when another type of pretreatment is combined to the enzyme addition. Acid pretreatment was most advantageous, although other authors such as Xiros et al. (2008) recommended alkali pretreatment and Niemi et al. (2012) milling to increase carbohydrate solubilization. In addition to that, the applied pretreatment is very mild compared to what has been reported elsewhere. In Wang et al. (2015) for example,

thermochemical pretreatment was applied at 90°C and pH 10.7 followed by three different enzymatic treatment with varying temperatures (50-60°C) and decreasing pH (8 to 4.5) for a total of 28 h. Higher initial solids concentration would also produce hydrolysate with higher sugar concentration (White et al, 2008) and the current work is operated at 5% TS content as compared to 15-20% in White et al. (2008). Regarding enzyme levels in the hydrolysis Beldman et al. (1987) used 1 to 2% (w/w) enzyme concentration, Mussatto et al.(2008) 45 FPase units/g substrate and White et al. (2008) 700, 250 and 500 U/g substrate for total cellulase, β-glucosidase and xylanase respectively. These concentrations are significantly much higher. In addition to that, hydrolysis temperatures were higher (40-50°C) and in White et al. (2008) BSG was autoclaved before enzyme addition. These reasons combined could actually explain the differences observed.

During the 7-day percolation stage, a follow-up over time was achieved for pH, TA, glucose, CODs and VFA. All samples were collected from the buffer tank. The sodium carbonate buffer was at average pH value of 8 at day 0 for all pilots. With time, pH decreased more importantly in the first 3 d for pilots P4 through P6 but settled at an average of 6.6 for all reactors at day 7. The same trend was observed for TA: it decreased from 4.3 g $CaCO_3$/L to an average of 1.93 g $CaCO_3$/L at day 7. BSG is rich in sugars, therefore a release in glucose is measured at day 1 even in P1 to P3 (1.8 g/L glucose on average). At day 5, glucose levels are null for P1 to P3 and settle around 0.05 g/L for P4 to P6 until day 7. Figure 4 presents the evolution of soluble COD in g O_2/L over time for the six percolation tests. Presented data of pilots P4 to P6 are corrected since the crude enzyme mixtures contained additional COD that was most probably released during the extraction phase after SSF. Nevertheless, the difference between the two tested configurations is significant. In fact, 82 and 81% of the CODs was released from controls and pilots with added SSD enzymes in the first 2 days of percolation. However the most important difference between the two setups

was observed at day 5 with 43% difference. This value decreased to reach 30% at day 7. This implies that when using enzymes in a percolation system, the recirculation period could be significantly decreased with an increase in the COD extraction from the system given that the collected percolate is sent to the anaerobic treatment system.

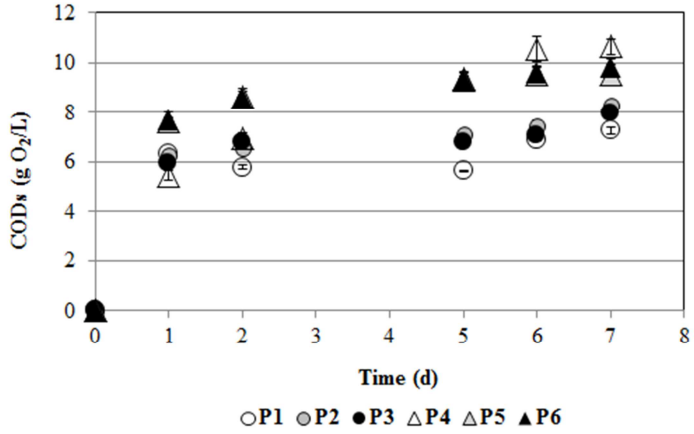
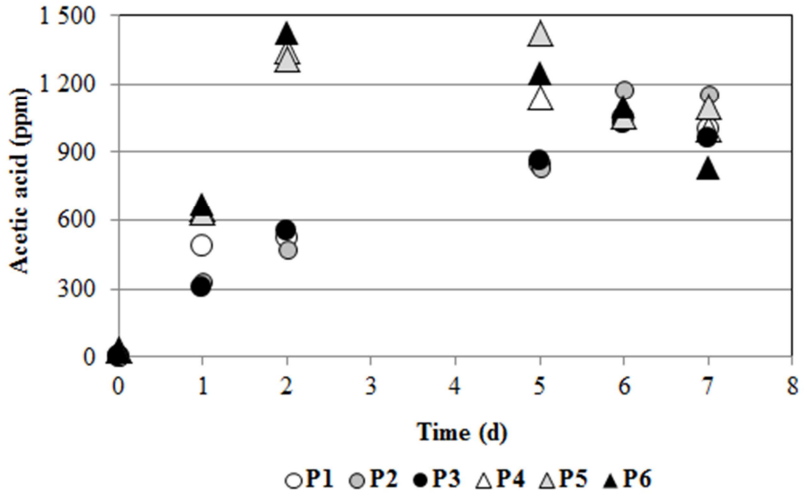


Figure 4. Evolution in time of the soluble COD (gO_2/L) for the six percolation pilots during the 7-d recirculation (P1 to P3 are control pilots and P4 to P6 are pilots with added SSF enzymes).

The impact of enzymes is also reflected in the VFA analysis. Only acetate and butyrate were found in the different percolates. Figure 5 shows the evolution of these two VFA over time. For acetic acid, the concentration increased significantly from 0 to 1360 ppm on day 2 for P4 to P6 and then decreased slightly to reach an average of 980 ppm on day 7. For P1 to P3 the rate of increase was slower: 515 ppm on average on day 2 and 1039 ppm on day 7. In all cases, the concentration of acetate remains higher for enzymatically treated BSG as shown in Bochman et al. (2007). No butyric acid was detected in any pilot in the first 2 days however starting day 5, 435 and 1129 ppm were measured for control and enzyme pilots respectively. It is for these latter that the concentration was highest at day 7: 3398 ppm versus 1638 ppm for the controls.



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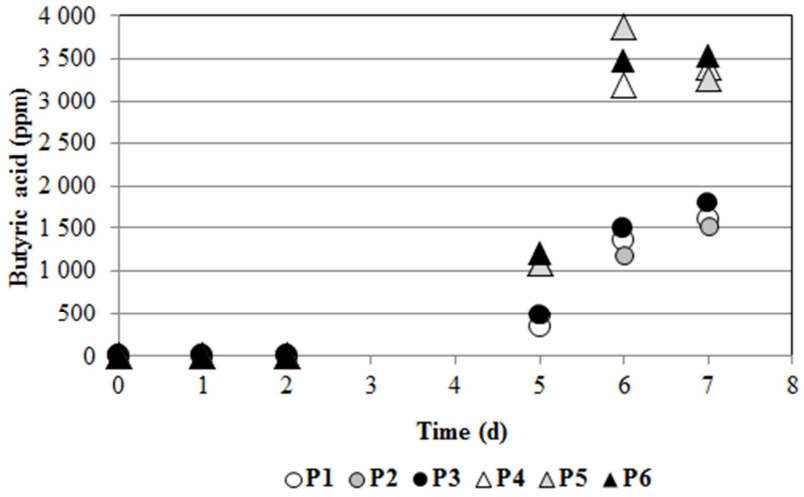
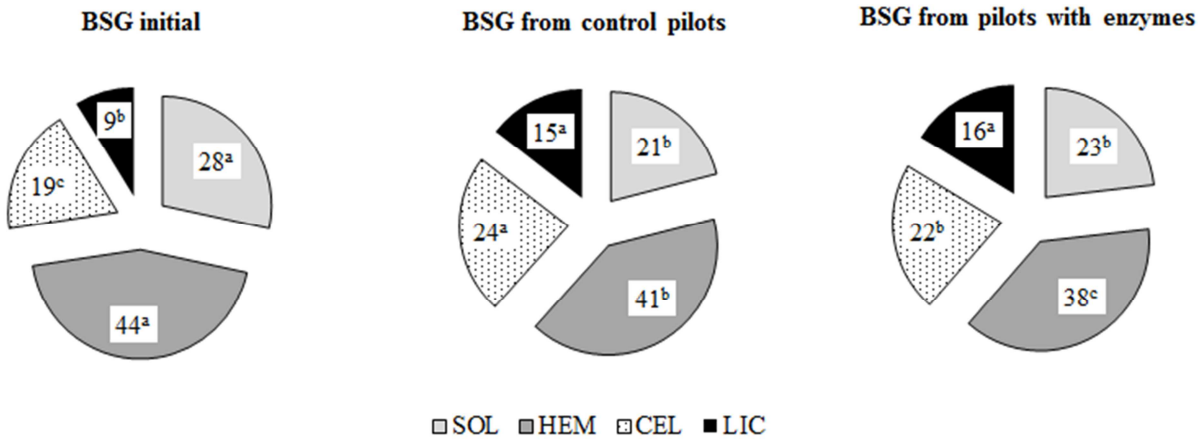


(b)

Figure 5. Evolution in time in ppm of acetic acid (a) and butyric acid (b) for the six percolation pilots during the 7-d recirculation (P1 to P3 are control pilots and P4 to P6 are pilots with added SSF enzymes).

Finally at the end of the percolation phase, BSG was recovered from the different pilots. Most hydrolysis parameters were measured in the liquid phase so it was decided to look at the changes in the solid matrix due to liquid recirculation. Figure 6 shows the Van Soest fractionation of the lignocellulosic fraction of BSG; all fractions are expressed in %VS. The composition of BSG agrees in fact with reported data by Xiros et al. (2008). When comparing control and enzyme pilots, soluble and lignin fractions do not significantly differ but this is not the case for HEM and CEL fractions. Enzymes in the recirculation system did, as expected, degrade some of the hemicellulose and cellulose fractions present in the BSG matrix.

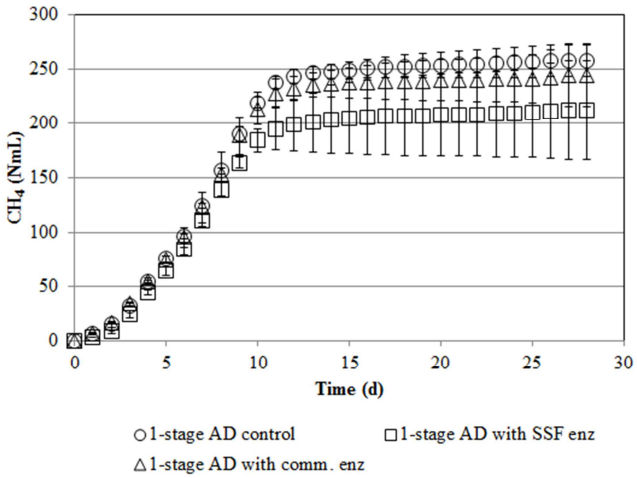


(a) (b) (c)

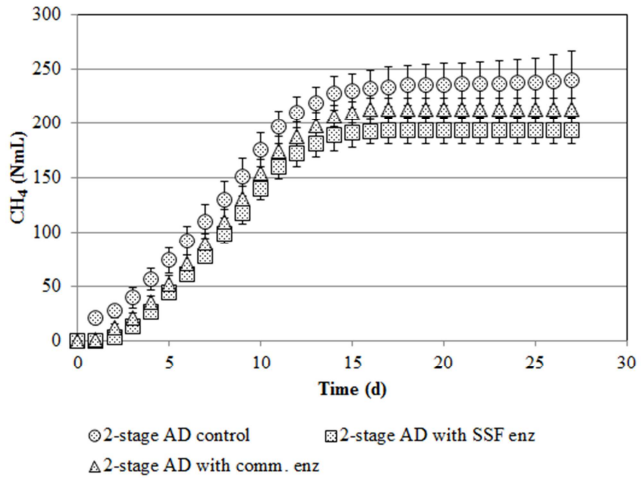
Figure 6. Pie chart representation of the Van Soest characterization (%VS) of initial BSG sample (a), BSG after percolation in control pilots (b) and BSG after percolation using SSF enzymes (c) in terms of soluble (SOL), hemicellulose-like (HEM), cellulose-like (CEL) and LIC (lignin and cutin-like) fractions. Values with the same superscript are not significantly different at 95% confidence level.

7.3.3. Impact on methane production

Methane production in AMPTS system was followed until stabilization. Figure 7 shows the total methane produced for the single-stage AD, two-stage AD and the percolation system coupled with AD. The methane potential for untreated BSG is on average 237 L CH_4/kg VS which is slightly higher than the 205 L CH_4/kg VS reported by Wang et al. (2015). Although the latter value is obtained from a continuous operation of a 5-L continuously stirred tank reactor (CSTR). Bochman et al. (2015) reported 409.5 L CH_4/kg VS potential. These differences result from the differences in composition that exist among different sources of BSG. In fact, ranges in g/100 dry BSG of 9.0-25.4 for cellulose, 19.0-40.2 for hemicellulose and 11.5-27.8 for lignin have been reported in the literature (Xiros et al., 2008).



(a)



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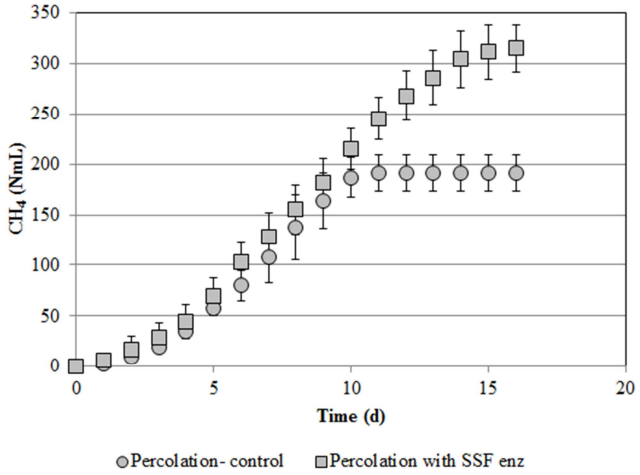

(c)

Figure 7. Methane production in mL for one-stage AD (a), two-stage AD (b) and percolation associated with AD (c) for control and enzyme configurations.

Figures 7a and 7b show that there are no significant differences between control and the two enzyme pretreated configurations. Important differences were reported in the hydrolysis step: up to 56% increase in CODs, 148% increase in glucose concentration and 116% increase in acetate concentration. Nevertheless, there was no impact on the methane production. This could be due to the experimental setup itself. The difference in COD concentration in the hydrolysis step is on average of 4.9 g/L. But the added volume to the AMPTS is only 20 ml which corresponds to a final concentration difference of 0.24 g/L. Given that the residual CODs in the system at the end of BMP test is 0.5 g/L. This implies that the amount of COD added initially is very low and its impact on the methane production would thus be negligible. The contact time in the hydrolysis step could also be increased: 24 h at 35°C are the mildest conditions observed. In fact, Bochman et al. (2007) reported that methane production was only slightly higher for enzymatically treated BSG as compared to the control. In general, for the single-stage AD enzyme addition should be increased in terms of frequency but here again the conditions of the test did not allow it.

Results were different for the percolation-AD system from which 100 mL percolate were added for the measurement of the methane potential. After 16 d, 191 and 315 mL CH_4 were produced for the control and enzyme added system respectively. This corresponds to 65% increase in methane production. This difference is very important but does not take into account the methane potential due to the CODs brought in by the crude enzyme extract. To estimate the difference between the two configurations, calculation can be made based on the CODs difference at day 7. Ifthe 2.2 g CODs/L were converted to methane, this would correspond to 77 77 and not 124 mL CH_4. The increase in methane production would then become 40%. The use of enzymes in a percolation system associated with a downstream AD of the percolate remains very interesting. It is also important to note that although the methane equivalent of the enzyme extract is subtracted to see only the effect of enzyme

addition; on an actual large-scale plant with SSF integrated system, this methane potential is an actual increase that cannot be dissociated from the overall energy production balance.

7.4. Conclusions

Enzymes are efficient in the hydrolysis of brewer's spent grain in two-stage AD and percolation system associated downstream with AD system for the percolate. The methane potential test did not show the impact of enzyme for neither single nor two-stage systems. However, for the percolation system an increase of 65% methane was measured. This is a very encouraging result especially that most conducted research processes remain economically unattractive. Breakthroughs are still awaited for and they include among others effective pretreatment, operation at high solids loading under non-aseptic conditions and efficient fermentation (Weimer et al., 2009). This work makes a first step towards a simpler pretreatment that can make the economics of an integrated AD process less questionable.

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CHAPTER 8

Conclusions and Perspectives

The data herein reported sustain that solid state fermentation can be operated as a biostimulation concept. This implies that indigenous microorganisms are diverse enough to achieve lignocellulolytic enzyme production on complex matrices such as municipal solid waste, brewer's spent grain and paper/cardboard. The applied experimental conditions were simpler than those generally reported in the literature, mainly no substrate pretreatment and no inoculum added. Nevertheless when compared to other research works, the performance of the process was as efficient.

The scale down of the analytical methods of the enzymes of interest, FPase, CMCase, β-glucosidase and xylanase, was successfully compared with reference methods and facilitated the analytical follow-up in the different experimental plans. These adapted methods are now available to be used elsewhere to make the comparison of different research works possible.

This work also shed the light on the behavior of the enzymes in both their culture SSF medium and their reaction medium. Although the work has been conducted at a laboratory scale, the collected data clearly point out the trend that can occur at the industrial-scale. The fate of enzymatic activity in the system can define the rate and frequency of application of those catalysts in order to achieve higher yields.

The correlation study between enzyme activities and released reducing sugars in the hydrolysis system was not complete since it did not allow us to draw general conclusions regarding the "adequate enzyme recipe". The correlation however within the optimization plan yielded high correlation factors, indicating an interesting

research perspective. In addition to that, data has shown that the impact of adding enzymes is not linear.

Finally the application of enzymes in the different AD systems proved its success at the hydrolysis level where high levels of organic matter solubilization were observed. In terms of methane production, only the experimental setup with percolation associated with AD showed a highly significant positive impact with more than 30% increase on methane production. This implies two very important conclusions, the AD system configuration is a key parameter for enzyme application and enzymatic pretreatment of lignocellulosic feedstock enhances energetic valorization of this type biomass.

The main remaining challenge of the SSF process is its scale-up. The data obtained in this work could be used as base for a feasibility study that will provide reliable economics of using enzymes with an integrated AD process.