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# From Wastes to High Value Added Products: Novel Aspects of SSF in the Production of Enzymes

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**Abstract**

Solid state fermentation (SSF), a process that occurs in the absence or near absence of water, has been used for the production of various high value added products such as enzymes and other organic components. This paper reviews the recent studies reported on the use of SSF for the production of enzymes; lipases, proteases, cellulases, hemicellulases, ligninases, glucoamylases, pectinases and inulinases. The microorganisms used for fermentation are mostly fungi and substrates are waste materials from the agriculture and food industry. This shows the advantages of SSF from an economical and environmental viewpoint. The paper provides an update on several issues, *viz*. wastes, microorganisms and scale-up and control of the process of fermentation in solid-state.

**Keywords:** Cellulases, enzymes, lipases, proteases, process scale-up, solid state fermentation.

## 1. Introduction

Solid-state fermentation (SSF) is a process of fermentation performed on non-soluble materials, namely the substrate, in the absence or near absence of water (Salihu et al., 2012). The substrate acts mainly as source of nutrients for the microorganisms responsible for the fermentation. There are various groups of microorganisms used in SSF depending on the final product considered necessary to be obtained from the fermentation. Among these, filamentous fungi, like species of *Aspergillus* and *Rhizopus* are the best adapted microbial species reported in most recent studies (Belmessikh et al., 2013; Cunha et al., 2012; Dhillon et al., 2011a,b; Thanapimmeth et al., 2012). The SSF process has been extensively used for the production of high value added products such as enzymes, biofuel, biosurfactants and biopesticides (Singhania et al., 2009).

In particular, for the production of enzymes, the fermentation is commonly conducted in a liquid medium containing the required dissolved nutrients (Colla et al., 2010). This process of fermentation is known as submerged fermentation (SmF), which presents the benefit of homogeneity of the culture media used and possibility of controling the parameters like temperature and pH. However, there are several advantages of SSF over the use of SmF (Mitchel et al., 2006). A comparison between SSF and SmF is presented in Table 1. SSF allows for the production of enzymes with higher activity and stability with lower water and energy demands. Additionaly, from the environmental and economical perspectives, the main advantage of SSF is related to lower volume of effluent produced, compared to SmF, and the possibility of carrying out the process under non-sterile conditions (Subramaniyam & Vimala, 2012). SSF uses low-cost waste products mainly from the sector of agriculture and food industries, such as wheat bran and peels of fruits and vegetables. These wastes can be used as ideal substrates for the microbial fermentation due to their rich contents of organic

components, which are considered as essential sources for carbon, nitrogen and many micronutrients that are important for the production of metabolites. Despite these advantages of SSF, the use of this type of fermentation in industrial processes is not widely applied due to challenges and limitations concerning monitoring, controlling and scaling-up of the process (Salihu et al., 2012; Sukumaran et al., 2010). For example, one of the critical issues regarding the latter is the inability to remove the heat excess generated by microbial metabolism during the fermentation. Another important disadvantage of SSF is the handling of solids on large scales, as 200 kg is reported to be the maximum weight of solids to be used in the fermentation on industrial scales. Both are considered being the main disadvantages of SSF for industrial applications.

The objective of this paper is to review the recent studies reported on the use of SSF of waste materials for the production of enzymes, as high value products. The paper provides an update on various aspects: wastes, microorganisms and SSF process scale up and control.

## 2. Wastes used in SSF

The wastes used in the processes of SSF for the production of enzymes are mainly of animal and plant origin from food industry. Table 2 outlines the wastes and microorganisms used in the processes of SSF and also the enzymes produced. In this section, a detailed description of the wastes of animal and plant origin used is also presented.

### 2.1. Wastes of animal origin

The waste materials of animal origin, including tannery solid wastes and cow dung, chicken feather and fish flour, have been mainly used in the production of only

proteases through SSF processes (Table 2).

Tannery solid wastes, in the form of raw hide trimmings and splits, limed and green animal fleshings, chrome shavings and hair wastes, are produced with huge quantities during leather manufacturing and are not usually used or under-used (Nalawade et al., 2009). Therefore, these waste materials are creating a solid waste disposal problem in tanneries. Kumar et al. (2009) studied the use of animal fleshings, the proteinaceous part of tannery solid wastes, as substrate for the production of aspartic protease by a *Synergistes* sp. It was suggested that there is a possibility to produce this enzyme by SSF using a cheap substrate and moreover, the enzyme obtained exhibited high stability in various organic solvents. Hair wastes have been also used in SSF for the production of proteases (Abraham et al., 2014). This waste mixed with raw sludge from wastewater treatment has been valorised by SSF without the inoculation with a pure microorganism. Alkaline protease was produced as a consequence of the degradation of hair by the microbial populations developed. Stabilized compost was another by-product of the process.

Cow dung, as an inexpensive waste material, has been evaluated as a substrate for the production of protease by *Halomonas* sp. through SSF (Vijayaraghavan & Vincent, 2012). A high production of halo-tolerant alkaline protease was obtained when compared with a substrate of wheat bran under the same process conditions. Accordingly, cow dung, which is characterised by its increased availability and low costs, might be used in future research as a key substrate in the production of protease enzymes. Keratin wastes such as chicken feather has been utilised in SSF by a feather degradating strain of *Bacillus subtilis* (Rai et al., 2009). The process conditions were optimised in order to maximise the yield of β-keratinase, which is a type of protease. This was one of the important studies that shows the successful use of a keratin waste

material in the production of enzymes. Fish flour, a fish processing by-product, mixed with polyurethane foam has been used by *Aspergillus oryzae* for the production of a proteolytic extract (Garcia-Gomez et al., 2009). This extract showed a higher enzymatic activity, i.e. a higher degree of protein hydrolysis, when tested on fish muscle compared to a commercially available enzyme. Therefore, it was concluded from the results of this research that it was highly feasible to use fish flour as a substrate in the production of proteolytic enzymes.

### 2.2. Wastes of plant origin and food industry

There are various types of wastes of plant origin and also of food industry that have been used in processes of SSF for the production of enzymes (Table 2). These waste materials include wastes of wheat and rice, such as wheat bran and rice husk, peels and pomace of fruits and vegetables, sugarcane bagasse, soy and cotton wastes, waste bread and brewery spent grain. Therefore, by using such a wide range of waste materials, it was possible to obtain through the process of fermentation several types of enzymes, i.e. lipases, proteases, cellulases, xylanases, pectinases, amylases and inulinases.

#### 2.2.1. Wastes of vegetable oil

Wastes of vegetable oil (oil cakes) have been used for the production of lipases, proteases and xylanases through SSF. Lipase has been produced when cakes of edible oil have been used as substrate. For instance, Colla et al. (2010) have been used soybean oil cake as a substrate after adding about 10% rice husk for increasing the porosity of the media that allows for oxygen transfer, in the presence of *Aspergillus* spp. as inoculum. In comparison to SmF, SSF led to higher enzymatic activity of lipase. This

was due to the fact that in solid substrates the nutrients are more concentrate than in liquid medium. This resulted in excellent cell-to-substrate interaction that consequently led to a higher enzyme production. Another oil cake used for the production of this enzyme was ground nut oil cake (Chaturvedi et al., 2010). It was shown the enzyme production through the fermentation by *Bacillus subtilis* was highly affected by various process conditions such as pH and moisture levels. It was found that the maximum yield of lipase was at a moisture of 70% and pH of 8.0. Interestingly, the oil cake of *Jatropha curcas*, a major energy crop in Thailand, has been used for the production of several enzymes through SSF as mentioned in recent literature (Mahanta et al., 2008; Joshi & Khare, 2011; Ncube et al., 2012). The outcome of the research performed was desirable, as it was possible to obtain enzymes such as proteases, lipases and xylanases by using various microorganisms of *Pseudomonas*, *Scytalidium* and *Aspergillus*. It is of high importance to establish a beneficial disposal of this waste material, as it is characterised by high contents of toxic compounds such as antineutrinos and phorbol esters (Ahmed & Salimon, 2009).

### 2.2.2. Wastes of wheat, rice, sugarcane and palm trunk

Wastes of wheat and rice, sugarcane bagasse and oil palm trunk have been mainly used as substrates for the production of cellulases through SFF. Therefore, it can be observed that there is a view to developing low cost production systems for cellulase enzymes.

Wheat bran materials, as a lignocellulosic material, among various kitchen and agro-industrial wastes, such as corn cobs, peelings of fruits and sawdust, appeared to be the best suited substrate producing appreciable yields of cellulase enzyme, in the presence of an inoculum of *Aspergillus niger* and *Trichoderma reesei* (Bansal et al.,

2012; Dhillon et al., 2011). Interestingly, there was no need for a supplementation of exogenous nutrients and therefore this research highlights the potential of wheat bran as possible raw material for the enzyme production. Wheat bran and *A. niger* were also evaluated for the production of cellulase and xylanase under SSF by Dhillon et al. (2011), and enzyme yields were compared with SSF where the inoculation and substrate were mixed with *Trichoderma reseei* and rice husk with a ratio of 1:1 and 2:3, respectively. In this case, it was reported that mixed microbial cultures and waste materials led to the production of higher amounts of enzymes. ~~than the use of a single microbial strain and wheat bran as a sole substrate. Mixed culture combinations have the ability to utilize the substrate, especially if there is more than one substrate, as energy sources are better used than in pure single strain cultures.~~ In addition, the inclusion of rice straw provided an additional source for the carbon required by the microorganisms used. This is in agreement with another research work that optimised the production of cellulase by *Aspergillus funigatus* under SSF (Soni et al., 2010). Moreover, wheat bran has been also used as a supplement to soybean hulls for the production of cellulase using a mixed microbial culture of *Trichoderma reesei* and *Aspergillus oryzae* (Brijwani et al., 2010). Mixed cultures clearly showed their compatibility for hyper enzyme production.

Sugarcane bagasse, a waste product that is generated from the sugarcane industry in huge amounts, has been used evaluated as a substrate for the production of cellulase through SFF. Mekala et al. (2008) addressed the optimisation of environmental parameters and media for the fermentation by using *Trichoderma reesei* for enhancing the yield of the enzyme. A suitable SSF process has been developed for cellulase production with this cheap biomass resource as substrate. In addition, Cunha et al. (2012) have evaluated sugarcane bagasse as a substrate for the production of cellulase

through SSF and SmF. The fungus *Aspergillus niger* has been used as an inoculum in both methods. It was shown that SSF was superior compared to SmF, as in this first case the cellulase production was 3-fold higher. This was due to the fact that in SSF, the nutrients are more concentrate than in liquid media used in SmF, as previously explained (section 2.2.2.1). On the other hand cellulase production is controlled by feedback, i.e. the more substrate available the higher the enzyme yield. The advantage of SSF of this waste material is: first, this method is an economical process for the use of a lignocellulosic waste that exhibits a long-standing difficulty in the costs associated with the enzymatic hydrolysis of this material by other methods and second, SSF developed can go a long way in bringing down the cost of cellulases, which will eventually help to develop economical processes for bio-fuel production.

Direct utilization of complex untreated oil palm trunk, a cheap and abundant material, for cellulases and xylanase production by lignocellulosic degrading fungi such as *Aspergillus fumigatus* was evaluated under SSF (Ang et al., 2013). The palm trunk, which was isolated from cow dung, was used as sole carbon source for the fungus during the fermentation process. The ability to produce xylanases with high levels of cellulases was also shown. However, in future studies, there is still a need for statistical optimisation of all the parameters involved in the fermentation process.

### 2.2.3. Wastes of fruit and vegetable industries

The industry of fruits and vegetables is producing a high amount of wastes and therefore, it is interesting to use these materials in processes of SSF. Peels and pomace have been used for the production of enzymes. These enzymes include mainly cellulases, xylanases, pectinases and proteases.

Potato peels have been determined as one of the best substrates among various

agro-industrial wastes for the production of alkaline proteases by *Bacillus subtilis* (Mukherjee et al., 2008). Interestingly, citrus peels were chosen as a substrate for the production of enzymes because it is an important agroindustrial by-product that offers several carbon sources required for the growth of microorganisms and for the production of phytases, pectinases and xylanases (Mamma et al., 2008). Microorganisms readily use this waste in fermentations due to its rich composition, especially due to its high content of organic matter, which is about 80%, being total dietary fibres (above 50%) free sugars and pectin the main compounds. This composition justifies the use of citrus peels as inducing substrate for the production of multienzyme complexes, without the need for the addition of pectic materials as inducers to the media used in the fermentation (Kang et al., 2004).

In addition, citrus peel is the major solid waste that is generated by the citrus processing industry, which represents approximately more than the half of the fresh fruit weight. Accordingly, the disposal of this by-product poses a big challenge to the fruit industry, where this waste is mostly pelletised and employed as animal feed or pectin precursor. This waste has been successfully used by Rodriguez-Fernandez et al. (2011) for producing pectinase and xylanase by *Aspergillus niger* through SSF. The kinetics of microbial growth related to the synthesis of the enzymes has been determined. Moreover, citrus waste has been also utilised for the production of phytase by the same fungus and a scale-up process was achieved (Rodriguez-Fernandez et al., 2012, 2013).

Pomace of fruits and vegetables has been recently used as substrate for the production of protease and cellulase through the process of SSF. Apple pomace was the substrate for obtaining cellulase through the fermentation by *Aspergillus niger* (Dhillon et al., 2012a,b). Results showed a rapid bioproduction of fungal cellulase using this low cost waste material especially with a supplementation of inducers such as lactose.

Tomato pomace has been also used as a substrate in SSF for the production of protease by the same genus of fungi (Belmessikh et al., 2013). The use of this tomato waste constitutes an efficient and inexpensive substrate for the enzyme production and a suitable mean for the waste valorisation towards an attempt for reducing the ecological impact.

## 3. Microorganisms used in SSF

This section deals with the research work performed on the microorganisms used in the processes of SSF and the substrates and end-products obtained (Table 2). The microorganisms used in the SSF processes for the production of enzymes are fungi and bacteria, mainly *Aspergillus* spp. and *Bacillus* spp., respectively, which will be discussed in detail.

### 3.1. Fungi

Fungi are the best adapted microbial species reported in most recent studies for the production of enzymes through SSF. This is due to the ability of these microorganisms to grow on surfaces of solid wastes and penetrate into the inter-particle spaces of the substrates. The fungal hyphae can also penetrate some solid structure of the matrix.The fungal genera used are *Aspergillus, Penicillium* and *Rhizopus* (Table 2). The fungal genus *Aspergillus* has a broad range of species that have been used in the processes of SSF. These species include *A. niger*, *A. oryzae*, *A. terreus*, *A. fumigatus*, *A. foetidus*, *A. sojae* and *A. candidus*, where the most frequently used fungus is SSF is *A. niger*, a filamentous mesophilic fungus. This fungus was used to produce a multi-enzyme preparation containing pectinolytic, cellulolytic, and xylanolytic enzymes under SSF process on citrus peels (Mamma et al., 2008). This process was enhanced by the

optimization of initial pH of the culture medium and moisture levels. Most importantly is the water activity, which limits the microbial growth. After the SSF process, the fermented substrate was either directly exposed to auto hydrolysis or new materials were added, and the in situ produced multi-enzyme systems were successfully used for the partial degradation of orange peel polysaccharides. Fermentable sugars were liberated, which could be converted to bioethanol. In a more recent study on SSF using the same substrate and fungus, the production of these enzymes were optimised based on aeration conditions to allow for a sufficient amount of oxygen that is required for the growth of the microorganism and the removal of $CO_2$ and metabolic heat (Rodriguez-Fernandez et al., 2011). In addition, a mathematical model was applied to determine the different kinetic parameters related to SSF.

*Aspergillus niger* was used in SSF for the production of citric acid and cellulase enzyme, by using apple pomace and apple pomace ultrafiltration sludge, which are by-products from the apple processing industry (Dhillon et al., 2011; Dhillon et al., 2012a). The addition of 3-4% of ethanol and methanol to the apple pomace substantially increased the values of the citric acid attained. The cellulase obtained, after its recovery being optimized using various extraction solvents, was used for the saccharification of apple pomace and brewer's spent grain. Sugarcane bagasse was also used for the production of this enzyme by SSF, where a combination of SSF and submerged fermentation was shown to be superior to the conventional submerged method due to the improved assimilation of sugarcane bagasse and fungal growth morphology (Cunha et al., 2012). The concentration of the substrate was fundamental in the comparison. The germination of the fungi on a solid-state medium allowed for the development of a dispersed filamentous form, which resulted in superior cell-to-substrate interaction and accordingly a higher production of the enzyme. In addition, cellulase was produced by

using SSF of various wastes, agricultural and kitchen wastes such as corn cobs, carrot peelings, composite, grass, leaves, fruit peels, rice husk, sugarcane bagasse, saw dust, wheat bran and wheat straw (Bansal et al., 2012). Of all the substrates tested, it was found that wheat bran is the most suited substrate for a high production of cellulase. *A. niger* was also used in the production of proteases and lipases (Paranthaman et al., 2009; Colla et al., 2010; Edwinoliver et al., 2010). For instance, Paranthaman et al. (2009) studied the production of protease using rice brokens and rice mill wastes as substrates in SSF. The protease obtained could be commercially used in detergents and leather industry.

*A. oryzae* was used in SSF for the production of cellulase, proteases and xylanases. Thanapimmeth et al. (2012) showed that it is feasible to use deoiled *Jatropha curcas*, a major energy crop in Thailand used for biodiesel, seed cake as a substrate in the process of SSF after the optimisation of the conditions of moisture, inoculum and temperature. Recently, Pirota et al. (2013) used a new strain of *Aspergillus oryzae* isolated from the Amazon rain forest in SSF processes in the production of xylanases. The substate used was wheat bran and the production of xylanase was on a lab scale with a possibility of scaling up of the process. *Aspegillus oryzae* was also used in mixed cultures in the production of enzymes by SSF. This fungus was used with *Aspergillus giganteus*, *Phanerochaete chrysosporium* and *Trichoderma virens* in SSF on cotton seed-coat fragment waste as substrate (Csizar et al., 2007). The enzyme complexes produced were composed of hydrolytic and oxidative enzymes, such as cellulases and xylanases. *Aspergillus oryzae* was also used with *Aspergillus awamori* or *Trichoderma reesei* in the production of glucoamylase and protease or cellulase enzymes via SSF, using wheat bran which is a waste product of the wheat milling industry, or soybean hulls as substrate, respectively (Du et al., 2008; Brijwani et al., 2010).

### 3.2 Bacteria

In general, bacteria are not widely used in the production of enzymes through SSF. The bacteria are mainly of the genus *Bacillus* (Table 2), specifically its species *subtilis*, *licheniformis*, *pumilus* and *firmus*, which have been used in the production of amylases, proteases, lichenases and xylanases (Mukherjee et al., 2008, 2009; Nimkar et al., 2010; Kapilan & Arasaratnam, 2011; Chaari et al., 2012). The waste materials used as substrate in the SSF were mainly agrowastes, such as potato peel and pea pomace, and chicken feather that is considered an animal waste by-product. *B. subtilis* was most often used in SSF processes. This bacterial species was used in SSF for the production of proteases and α-amylases.

Amylase was successfully produced using wheat and rice bran as substrate materials for the SSF process after optimisation of the various parameters such as pH and temperature (Nimkar et al., 2010). In addition, Mukherjee et al. (2009) found that potato peel, which is considered as a novel inexpensive substrate, was the best waste material among agro-industrial waste residues to be used for the production of amylase due to its high starch contents and the absence of mono-saccharides. This waste material was combined with other agrowastes such as grass and protein sources, which allowed for the production of protease by using the same species of *Bacillus* (Mukherjee et al., 2008). Recently, potato peel was also utilised by the bacterium *Bacillus firmus*, isolated from marine sediment of Parangipettai coast, to produce thermostable alkaline amylase by SSF process at optimised process conditions (Elayaraja et al., 2011).

## 4. Enzymes produced by SSF, their process conditions and applications

In general enzymes have been extensively produced by submerged fermentation

(SmF) and have been commercially available since many decades (Anwar & Saleemuddin, 1998; Pandey et al. 2003; Queiroga et al., 2012). Recently, the production of enzymes on solid state fermentation has been implemented in order to reduce the costs involved, especially if residues are used as substrates, and enhance the field of application (Sandhya et al., 2005; Kumar et al., 2009). However, there is indeed few research work developed on bench scale solid substrate fermentation, as the majority of this research was conducted on few grams of substrate materials, i.e. on a lab-scale. In addition, some research work was performed in media with high moisture contents of more than 70%, which might be due to some solid substrates that are able to retain high moisture levels, whereas SSF is defined as fermentation being performed in the absence or nearly absence of free water (Pandey et al. 2003).

This section is discussing in detail the following enzymes; lipases, proteases, cellulases and xylanases and other enzymes, such as fucoidanase and pectinases that are obtained through the process of SSF. In addition, there will be a detailed description on the conditions of the fermentation process and the various applications of lipases, proteases, cellulases and xylanases.

## 4.1. Enzymes

### 4.1.1. Lipases

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are enzymes capable of catalyzing the hydrolysis of triacylglycerols to glycerol and fatty acids at an oil-water interface and reactions of esterification, transesterification and interesterification of lipids (Sharma et al., 2001). In recent years, there has been an increasing interest in the study of lipases mainly due to their potential applications to a wide range of industrial sectors (Hasan et al., 2006; 2009). In the chemistry and pharmaceutical industries,

lipases are used in the production of surfactants, detergents and antibiotics; whereas in the food industry these enzymes are used to synthesize emulsifiers and develop flavours. Commercially useful lipases are typically obtained as microbial extracellular enzymes. However, since lipases are products of industrial interest, their production must be coupled with low cost processes. These enzymes would be economically manufactured in processes of SSF that utilize residues as substrates and that give high yields

The substrates used, which varied in amount from few grams up to 3 kg, in the processes of SSF for the production of lipases are oil wastes, wheat bran and sludge (Table 2). The activity of the lipase varies to a very high extent; the range of this activity is from 4.5 to 120,000 U/g. Wastes of oil, such as solid wastes from the production of vegetable oils (oil cakes), are one of the common waste materials used as substrates for the production of lipase by SSF. Oil cakes are good supports for microbial growth necessary during the process of SSF, because this waste has excellent sources of proteinaceous nutrients needed for the microbial fermentation, i.e. requiring low or no supplementation (Ramachandran et al., 2006). This waste material has also another advantage. It is inexpensive and available in high amounts from the oil industry.

Oil cakes of coconut, ground nut, mustard, linseed and neem has been used as substrates in SSF in the presence of *B. subtilis* for the production of extracellular lipase on a lab-scale using 10 g of waste materials (Chaturvedi et al., 2010). It was observed that the nature of the substrate significantly influenced the impact of initial moisture content and therefore affected the process of SSF. The physical nature and water holding capacity of the substrate are important criteria for its use in SSF process because the moisture content is an important factor that determines the microbial growth and activity of the enzyme. In another study of Edwinoliver et al. (2010), coconut oil

388 cake was also used and mixed with wheat bran and rawa for the production of lipase
389 through SSF in the presence of *A. niger*. The scaling up of the process was possible
390 from lab-scale to bench scale using up to 3 kg of wastes as substrates. The SSF process
391 led to a maximum activity of the lipase of 745.7 U/g. In addition, babassu cake
392 supplemented with sugar cane molasses as a substrate and the fungus *Penicllium*
393 *simplicissimum* were used for the production of lipase, on a lab-scale, with a maximum
394 activity of 314 U/g through SSF (Guturra et al., 2009). This fungus has been also used
395 in SSF for the production of lipase, but by using other oil wastes such as soybean cake
396 and castor bean waste showing an enzymatic activity of 317 and 80.24 U/g respectively
397 (Rigo et al., 2009; Godoy et al., 2011).

398 The oil cake of biodiesel crops, which contain about 50% oil called biocrude that
399 can be converted into biodiesel by esterification, are also used as substrates for the
400 production of lipase enzymes through SSF. Lipase was produced by using Niger oil
401 cake, as it is rich in various nutrients such as fatty acids and sugars, through SSF on a
402 lab-scale, where 5 to 10 g of wastes were used as substrate (Imandi et al., 2010). The
403 marine yeast *Yarrowia lipolytica* was used as an inoculum for the fermentation. There
404 was a low enzyme activity obtained, with a maximum of 26.42 U/g. Another biodiesel
405 crop called *Jatropha curcas*, a major energy crop in Thailand, was used by Mahanta et
406 al. (2008) for the production of lipase through SSF by *Pseudomonas aeruginosa*. The
407 seed cake supported good bacterial growth and enzyme production of an activity of 625
408 U/g, due to the composition of this cake that contains a high content of fat and fibres.

409 Winterisation residue from oil refinery and raw sludge were used as solid
410 matrices for the processes of SSF for lipase production on a bench scale using 2.5 kg of
411 waste materials, where the fermentation was dependent on the microbial consortium
412 present (Santis-Navarro et al., 2011). Winterisation residue was used a source of fat and

413 the sludge was added as co-substrate and inoculum. It was reported that the lipolytic
414 activity of the enzyme obtained reached a maximum of 120,000 U/g in the fermented
415 solid, which is substantially higher than activities reported in other research on SSF.
416 This highlights the possibility to work with solid wastes as effective biocatalysts, a topic
417 that has been scarcely treated in SSF literature.
418
419 **4.1.2. Proteases**
420 Proteases (EC 3.4.21-24), which are hydrolases that catalyze the cleavage of
421 peptide bonds in proteins, are a highly complex group of enzymes that differ in their
422 substrate specificity and catalytic mechanism (Sumantha et al., 2006; Turk, 2006).
423 These enzymes are classified into three main categories; alkaline, neutral and acid
424 proteases on the basis of pH range in which their activities are optimal. Therefore,
425 proteases are the most important industrial enzymes that account for about 60% of the
426 world market of industrial enzymes. The importance of these enzymes is reflected in
427 their tremendous applications in both physiological and commercial fields, for example
428 in detergent formulations, textile, food, and pharmaceutical industries (Queiroga et al.,
429 2012).
430 The preferred source of proteases is microorganisms, rather than plant and
431 animal tissues, to their broad biochemical diversity and their susceptibility to genetic
432 manipulation (Ellaiah et al., 2002). Among microbes, fungi as enzyme producers have
433 many advantages, since they could be mostly GRAS (generally regarded as safe) strains
434 and the produced enzymes are extracellular, which makes easy its recuperation from the
435 fermentation broth. Accordingly, the overall cost of the production of a complex group
436 of enzymes is very high, mainly due to low yield of enzymes because most of the costs
437 are related to the recovery and purification. Additionally, there are other high costs

associated with the substrates, i.e. the commercial media required (Singhania et al., 2009). Therefore, development of novel processes to increase the yield of proteases coupled with lowering down these costs are highly appreciable. Furthermore, proteases produced by using commercial media possess undesirable flavours, which are unsuitable for applications in food and pharmaceutical industries. Therefore, during the recent years, efforts have been directed to explore the means to reduce the protease production costs through improving the yield and the use of cost-free or low-cost substrates such as agricultural waste materials in processes of SSF for the production of proteases.

The waste materials used as substrates, which highly varied from 5 g to 1.4 kg, in the processes of SSF for the production of proteases are mainly of plant origin, such as potato peel, soy fibres, tomato pomace and wheat bran or of animal origin like tannery solid wastes, chicken feather and cow dung (Table 2). The activity of the enzyme obtained through SSF also highly varied from around 20 to more than 50,000 U/g.

Several studies on the utilisation of residues of plant origin in the production of proteases through SSF were carried out on wheat bran. Merheb-Dini et al. (2010) used the microorganism *Thermomucor indicae-seudaticae* in the presence of wheat bran and wheat bran mixed with casein at a ratio of 80:20 respectively for the production of protease with an enzymatic activity of 168 U/g. In addition, wheat bran has been used as substrate in the production of protease with a maximum activity of 5-20 U/g by a fungal strain of *Schizophyllum commune* and *Myceliophthora* sp. (Boyce & Walsh, 2012; Zanphorlin et al., 2011). Mukherjee et al. (2008) screened various agro-industrial and kitchen waste materials of plant origin, such as oil cake, wheat and rice bran, grass, banana leaves, potato peels and used tea leaves, for the use as substrate for protease

production through SSF by *Bacillus subtilis*. It was found that the substrates of potato peel and grass led to the production of proteases with the highest protease activity of up to 2,383 U/g. In another study by Abraham et al. (2013), the effect of three agro-industrial residues was examined; hair waste, coffee husk and soy fibre. Soy fibre presented the highest yield for protease production showing an enzymatic activity of 47,331 U/g. Recently, tomato pomace was used in a comparative study of protease production by cultivating *Aspergillus oryzae* in SSF and submerged fermentation (Belmessikh et al., 2013). The results obtained showed a highest enzymatic activity of 21,309 U/g in case of the process of SSF. There were recent few studies on the utilisation of residues of *Jatropha curcas* (oil cake), which is a major energy crop that cannot be used in nutrition or animal feed due to its toxicity. Mahanta et al. (2008) and Thanapimmetha et al. (2012) investigated the potential ultilisation of this oil cake as substrate for protease production by *Pseudomonas aeruginosa* and *Aspergillus oryzae*, respectively. The results demonstrated that the utilisation of this waste material for the enzyme production was a viable approach, with an activity of about 2,000 up to 14,000 U/g. Moreover, Chutmanop et al. (2008) compared the use of *Jatropha* oil cake with wheat and rice bran as substrates in SSF for the production of proteases under the same fermentation conditions and by using the same inoculum of *Aspergillus oryzae*. Interestingly, it was found that the protease activity produced by the oil cake was 30-40% higher than that of wheat and rice bran, due to the fact that this cake has a very high protein content that can be utilised by the microorganism for the production of the enzyme.

Residues of animal origin, tannery waste and cow dung, have been utilised in SSF process in the research work of Kumar et al. (2009) and Vijayaraghavan et al. (2012), respectively. Tannery solid wastes, which consist of hide trimmings and limed

488 animal fleshing, was considered as a proteinaceous substrate for the production of
489 proteases, with activities up to 755 U/g, under SSF by using *Synergistes* sp. Similarly,
490 hair waste from the tanning industry mixed with raw sludge from waste water treatment,
491 without the need for inoculations of pure microorganisms, were valorised for the
492 production of protease, where a maximum enzymatic activity of 56,270 U/g was
493 reached (Abraham et al., 2014). Cow dung was used in the presence of an inoculum of
494 *Halomonas* sp. leading to the production of proteases of a relatively high activity of
495 1,351 U/g, which was substantially higher compared to other waste materials of plant
496 origin that have been used under the same process conditions.
497
498 **4.1.3. Cellulases and Xylanases**
499 Cellulose and xylan are the first two most abundant natural biopolymers, which
500 are most dominating agricultural wastes (Zhang, 2008). The lignocellulosic biomass of
501 most plants consist of mainly cellulose (a homologous polymer of glucose linked by β-
502 1-4 glycosidic bonds); lesser hemicelluloses (a heterologous polymer of 5- and 6-carbon
503 sugars with sugar acids) that contains principally xylan; and finally lignin (a complex
504 aromatic polymer). Cellulose, only its amorphous form, is synergistically hydrolysed by
505 a complex enzyme system named as cellulases; such as cellobiohydrolase or
506 exoglucanase, carboxymethylcellulase or endoglucanase and cellobiase or β-glucosidase
507 (EC 3.2.1.91, 3.2.1.4 and 3.2.1.21 respectively), while the degradation of xylan requires
508 various enzymes; essentially endo-1-4,-β-xylanase (EC 3.2.1.8) and to some extent β-
509 xylosidase, α-glucuronidase, α-L-arabinofuranosidase and acetylxylan esterases (Maki
510 et al., 2009; Van Dyk & Pletschke, 2012). The lignocellulosic biomass, as it can be
511 degraded, it is a renewable and abundant resource in agricultural industry, with an
512 appropriate treatment, with great potential for bioconversion to value-added

513 bioproducts. Therefore, cellulases and xylanasse are now considered as a major group of
514 industrial enzymes that have various industrial applications.

515 Techniques of fermentation, due to their economic and environmental
516 advantages, have been widely used for a feasible production of cellulases and xylanase
517 (Subramaniyam & Vimala, 2012). The most frequently used techniques are SmF and
518 SSF, where the latter being the most beneficial due to the use or recycle of wastes that
519 are cheap and highly available.

520 As previously mentioned, cellulose and xylan are present in plants and therefore
521 the substrates used for the production of the enzymes of cellulases and xylan are only of
522 plant origin (Table 5). These substrates are wastes of soybean, wheat, rice, corn, cotton,
523 sugarcane bagasse and fruits such as apple, as well as residues from wood industries.
524 The yield of the cellulases represented for 3 enzymes as activities of filter paper (FPase)
525 for cellobiohydrolase or exoglucanase, carboxy methylcellulase (CMCase) for
526 carboxymethylcellulase or endoglucanase and β-glucosidase (BGase) for cellobiase or
527 β-glucosidase. The yield for xylanase is shown for the activity of endo-1-4,-β-xylanase.

528 Soybean hulls have been used as a substrate for the production of cellulases and
529 xylanase through SSF by a mixed culture of *A. oryzae* and *Trichoderma reesei*
530 (Brijwani et al., 2010, 2011). The maximum enzymatic activity obtained was 101 and
531 505 U/g for the carboxymethyl cellulase and xylanase, respectively. Results revealed
532 that the additional use of wheat bran in the substrate positively affected the enzymatic
533 activities obtained through the fermentation process. The SSF process was proven to be
534 a valuable technique for producing a system of cellulases and xylanase enzymes with
535 balanced activities, which were able to efficiently saccharify lignocellulosic biomass.
536 Wheat bran, untreated and without any supplements, as a sole substrate has been also
537 evaluated for the production of cellulolytic enzymes through SSF by using the same

538 microbial culture as inoculum. For instance, Bansal et al. (2012) and Dhillon et al.
539 (2011) achieved an enzymatic activity for carboxymethyl cellulase and xylanase up to
540 about 300 and 2,700 U/g.

541 Wastes of rice, such as the straw and husk, have been recently utilised as
542 substrate materials during the fermentation by *A. oryzae* and *Trichoderma reesei* for the
543 production of enzymes. Rice straw supplemented with wheat bran in the ratio of 3:2
544 resulted in the highest enzymatic activity of up to 132 U/g for carboxymethyl cellulase,
545 whereas the xylanase reached a very high activity of 3,106 U/g (Dhillon et al., 2011). In
546 similar studies, the fungus *Aspergillus fumigatus* has been used as inoculum for the
547 process of fermentation, where a cellulolytic activity of up to 251 U/g for β-glucosidase
548 enzyme and 2,782 U/g for xylanase have been reported (Soni et al., 2010).

549 The feasibility of using apple pomace for cellulase production under SSF was
550 evaluated. The fermentation by *Trichoderma* sp. and a supplement of lactose and corn-
551 steep solid allowed for obtaining of an enzyme activity with a maximum of 7.6 U/g for
552 the exoglucanase (Sun et al., 2010). This activity substantially increased to above 130
553 U/g and an activity of carboxymethyle cellulase of about 150-170 U/g was also
554 reported, using an inoculum of *Aspergillus niger* and especially when lactoserum, which
555 is a source of lactose, was added as a moistening medium (Dhillon et al., 2012 a,b).
556 There was also a high activity of xylanase of 2,619 U/g obtained.

557

558 **4.1.4. Other Enzymes**

559 In addition to the enzymes of lipase, protease, cellulases and xylanase that were
560 discussed in detail in previous sections (4.1-4.3), there are other enzymes obtained
561 through SSF processes. These enzymes include mostly glucoamylase, pectinase and
562 inulinase, which will be discussed in this section. There has been also few research

studies performed on the production of certain proteolytic enzyme with a mycotoxin hydrolytic activity, named as ochratoxin A (OTA)-hydrolysing enzyme, and fucoidanase (Abrunhosa et al., 2011; Rodriguez-Jasso et al., 2013, respectively). The latter is able to hydrolyse marine hetero-polysaccharides, called as fucoidans, that have a wide range of biological activity, e.g. anticoagulant, antithrombotic and antiproliferative activities.

#### 4.1.4.1. Glucoamylase

Glucoamylase belongs to the amylases enzymes that hydrolyse polysaccharides, such as starch and its degradation products, into molecules of glucose, maltose and dextrin. Amylases are one of the important enzymes in the industry due to their diverse applications, e.g. in the food (bakery products), paper, textiles, pharmaceutical and detergents industries (Botella et al., 2009). These enzymes are classified into α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3), which is known as amyloglucosidase or γ-amylase (Norouzian et al., 2006). This enzyme, which hydrolyses α-1,4- and α-1,6-glucosidic linkages at the non-reducing ends of polysaccharides, has been recently produced through SSF by using species of the fungal genus *Aspergillus*. Melikoglu et al. (2013) utilised waste bread as a substrate for the production of this enzyme. At optimum process conditions, such as a moisture of 60% and an incubation period of 144 h, it was possible to obtain an activity of glucoamylase of up to 114 U/g. Moreover, protease enzyme was also obtained through the process of fermentation. Accordingly, this study shows that waste bread could be successfully used as a primary substrate for obtaining enzymes. In another study, the production of glucoamylase was presented by using substrates of agro-residues of rice wastes, wheat bran, cotton seeds, corn steep solids, sugarcane bagasse and edible oil cakes (Zambare,

588 2010). The optimisation of the SSF process showed that the highest enzyme activity
589 obtained of ~ 2,000 U/g was with a substrate of wheat bran at a moisture content of 50%
590 and pH of 6, after an incubation of 120 h.
591
592 **4.1.4.2. Pectinases**
593 Pectinases consist of endo- and exo-polygalacturonases (EC 3.2.1.15 and
594 EC 3.2.1.67/82, respectively) and are enzymes that degrade pectin, a complex
595 heteropolysaccharide containing galacturonic acid residues that is a principal
596 component of the middle lamella and primary cell wall of higher plants (El-Sheekh et
597 al., 2009). These enzymes are therefore of great importance to the food industry as they
598 are predominantly used in the clarification of juices, as well as to textile and plant fibre
599 processing industries. In addition, pectinases are applied as food additive for
600 monogastric animals, such as food for pets.
601 An economical and feasible alternative for the production of pectinases is SSF,
602 where it has been found that species of the fungus *Aspergillus* are one of the
603 microorganisms that are able to produce these enzymes during the fermentation. Demir
604 and Tari (2014) found that wheat bran, among various agro industrial wastes, was the
605 most suitable substrate for the production of polygalacturonase using *Aspergillus sojae*.
606 The optimum process conditions that favoured the enzyme production were 4 days of
607 fermentation time at a temperature of 37°C and initial moisture of 62% which resulted
608 in an enzyme activity of up to 536 U/g. In addition, waste products of citrus fruits were
609 used as substrates for the production of pectinases by *Aspergillus niger*. The feasibility
610 of using citrus peels was evaluated in a bench-scale bioreactor (Rodriguez-Fernandez et
611 al., 2011). A mathematical model was applied to determine the different kinetic
612 parameters related to the enzyme production through SSF. The best conditions for

pectinase production were at 60% initial moisture and a pH of 5.0 at and 30°C. The maximum activity of pectinase of up to 265 U/g was produced after a fermentation time of 3 days. Ruiz et al. (2012) utilised lemon peel pomace as substrate in a laboratory scale bioreactor at the same condition but with a moisture content of 70%. Results showed that high levels of pectinase activities were obtained, up to a maximum of more than 2,000 U/g, which suggested this process as very promising for pectinase production.

**4.1.4.3. Inulinase**

Inulinases, most commonly known as endo-inulinase (EC 3.2.1.7), are enzymes that hydrolyse inulin into fructose (Chi et al., 2009). The application of these enzymes are in the production of high fructose syrups and fructoligosaccharides, which are compounds with high nutritional values and therefore can be used in low-calorie diets and as a source of dietary fibres in food preparations. Although the inulinases could be obtained from vegetable and animal sources, microorganisms such as *Aspergillus*, *Kluyveromyces* and *Staphylococcus* are the best sources for the commercial production of inulinases. This is due to their easy production and high yields obtained. SSF could be one of the useful approaches for the production of these enzymes. *Kluyveromyces marxianus* was utilised as inoculum for the fermentation in recent studies. Dilipkumar et al. (2013) obtained inulinase using pressmud as substrate, where parameters like air flow rate and particle size were optimised, leading to a maximum enzyme activity of ~ 300 U/g. Sugarcane bagasse was also used as a substrate for the production of the enzyme with a maximum activity of 590 U/g (Astolfi et al., 2011). The optimised temperature and moisture was 30C and 65% respectively, at a fermentation time of 24 h. The study showed the technical feasibility of the process of production of inulinase

through SSF.

### 4.2. Process conditions

The production of enzymes through SSF required the study of process conditions suitable for the enzyme production, such as temperature, incubation time, pH, moisture content, and types and inoculum levels of pure strain bacteria or fungi added or other sources of microorganisms, e.g. raw sludge.

#### 4.2.1. Lipase

These conditions for the production of lipase were at temperatures of about 20-30 or higher than 45°C, neutral pH and moisture levels of 50 to 75% (Table 3). The microorganisms used as inoculum required for the fermentation are fungi such as *Aspergillus* and *Penicillium*, *Yarrowia* yeast and *Bacillus* and *Pseudomonas* bacteria. Depending on the scale, i.e. amount of substrate used, the optimum temperature for the fermentation was 20 up to 45°C for an incubation period of 2 to 20 days, at pH values of 4.5-8 and moisture levels of 50-70%.

The optimisation of the production of lipase through SSF has been studied, mainly on a lab-scale, using experimental designs such as Plackett-Burman and central composite designs. Rigo et al (2009) studied the lipase production through SSF by using *Penicillium* sp. and soybean meal as substrate. Initially, the effect of different carbon-to-nitrogen ratios (C/N ratios) on lipase production was evaluated and it was considered 6.11 as optimum. Furthermore, the optimum conditions found were at a temperature of 20°C for a fermentation period of 5 days. Higher lipase activities were found in a wide range of pH from 4.0 to 9.0, with a pH of 7 as optimum by using the substrate of soybean and *Penicillium* sp. as inoculum. The moisture level used during SSF was 75%. In another study by Mahanta el al. (2008), the production of lipase through SSF by

using oil cake and *Pseudomonas aeruginosa* has been optimised for moisture content (29 - 80%), incubation time (24 - 144 h) and pH (6.0 - 8.5). It was observed that the optimum moisture level was at 50%. A higher level of moisture content causes a decrease in porosity, development of stickiness and an increase in the chances of contamination and, accordingly, a decrease in the gas exchange occurs. A lower level of moisture led to sub-optimal microbial growth during the fermentation and a lower degree of the swelling of substrate. The highest yield of the enzyme production was at 120 hours of incubation and there was no significant effect of pH on the lipase production. Imanti et al. (2010) have used *Yarrowia* yeast in the fermentation process and reported that the moisture content was optimised at a level of 60%. The incubation time at which the highest lipase production was obtained was 96 hours as longer periods led to the depletion of nutrients, accumulation of toxic end products, and the change in pH or loss of moisture and shorter incubation times were not sufficient for the microbial growth and hence the lipase production. The effect of pH on the lipase production in the presence of *Bacillus subtilis* as inoculum was studied by Chaturvedi et al. (2010). The lipase activity increased when increasing the pH from 6 to 8 and on further increase of pH to 9 and 10, the lipase activity decreased. This shows that the optimum pH for the lipase production was around a pH of 8.

In another approach of optimisation, Garlapati et al. (2010) have used modelling combined with optimization as two vital steps for maximizing the efficacy of SSF. Response Surface Methodology (RSM), a statistical technique which generates a mathematical model, coupled with Differential Evolution, which is an optimization technique, have been used. This approach has been used to maximise the lipolytic activity by *Rhizopus oryzae* through SSF. The maximum lipase activity was observed at 35°C, 5.28, 60% and 116h for temperature, pH, moisture and incubation time,

689 respectively. These obtained results of optimization were experimentally validated and
690 it was suggested that the developed model and optimization appear to be useful for the
691 design and control of the extracellular lipase production through SSF by using this
692 microorganism.

693 **4.2.2. Protease**

694 The process conditions for the production of proteases were at mesophilic
695 (30°C) up to thermophilic temperatures (50°C), pH levels of 6-8.5 and moisture levels
696 of about 50% (Table 4). Fungi, such as species of *Aspergillus*, and bacteria, mainly
697 *Bacillus subtilis* were the most predominant microorganisms used for the production of
698 proteases. Concerning the scale of the production of the proteases, most of research
699 work was done on a lab scale by using a maximum of 25 g of substrate, where an
700 Erlenmyer falsk was used as a reactor (Merheb-Dini et al., 2010; Zanphorlin et al.,
701 2012; Boyce & Walsh, 2012; Vijayaraghavan et al., 2012). However, in recent research
702 work by Abraham et al. (2013, 2014), a 4.5 l air tight reactor was used, working under
703 near-adiabatic conditions, allowing for the use of 1.25 kg of solid substrate.

704 The initial moisture content required may vary depending upon the type of
705 substrates and microorganisms used. However, it has to be considered that the keystone
706 in SSF is to remove the metabolic heat produced during the fermentation in order to
707 maintain constant moisture levels during the process, when saturated air is used for the
708 cooling. The optimisation of the moisture content has been studied in the processes of
709 SSF for the production of enzymes. For example, Mukherjee et al. (2008) found that
710 50% initial moisture contents of the substrates of potato peels and grass were optimum
711 for the production of protease by *B. subtilis*, whereas the optimum moisture in the case
712 of wheat bran was 30%. Moreover, when *Jatropha* seed cake was used as substrate in
713 the fermentation by *A. oryzae* at different levels of moisture of 45 to 55%, an optimum

714 moisture content of 45% was reported (Thanapimmetha et al., 2012).

715 There has been an optimisation for the source of carbon and nitrogen required by
716 the microorganisms used as inoculum for the production of proteases during the process
717 of SSF. In most processes of SSF, maltose and xylose were the optimum sources for
718 carbon and yeast and beef extract, sodium nitrate and peptone for nitrogen, respectively.
719 Mukherjee et al. (2008) tested several sources of carbon and nitrogen required for the
720 growth of *B. subtilis*. The carbon sources were glucose, fructose, galactose, maltose,
721 sucrose and lactose, being maltose the best source for obtaining the maximum enzyme
722 activity of ~ 1,100 U/g, whereas the activities were sequentially 50 and 400 for
723 galactose and lactose and glucose, fructose and sucrose. Additionally, it was found that
724 beef extract, followed by yeast extract, rather than ammonium salts and casein, and
725 served as the best nitrogen sources producing enzyme activities of ~ 1,400, 1,000, 420
726 and 400 U/g, respectively. In a study by Mahanta et al. (2008) using *Jatropha* seed cake
727 as substrate and *Pseuodomanas aeruginosa* as inoculum, it was also found that the
728 enrichment with maltose compared to other sugars led to an increase in the production
729 of protease. The best nitrogen source was peptone, where ammonium chloride and
730 sodium nitrate were also tested. Recently, carbon sources such as glucose, lactose,
731 trehalose, maltose, xylose and starch, and nitrogen sources such as gelatin, ammonium
732 nitrate, peptone, yeast extract, urea and casein were evaluated for the fermentation by
733 *Halomonas* sp. when a substrate of cow dung was used (Vijayaraghavan et al., 2012).
734 The optimum enzyme production was achieved with a combination of xylose and yeast
735 extract.

736 There have been different statistical methods used for the optimisation of various
737 parameters, rather than individual optimisation, in the processes of SSF for the
738 production of proteases. By adjusting the conditions to optimum levels, the protease

739 production increased up to 5 times compared to non-optimised experiments. Belmessikh
740 et al. (2013) used the experimental designs of Plackett Burman and the Central
741 Composite design for the study of the effect of five enrichment factors (wheat bran,
742 casein, ammonium nitrate, sodium chloride and zinc sulphate) on the enzyme
743 production with a substrate of tomato pomace by *A. oryzae*. It was reported that only
744 two factors, casein and sodium chloride, had a significant effect on the production. This
745 was due to the fact that during the fermentation process, casein could provide intact
746 peptides that were necessary in the induction, whereas sodium chloride might have had
747 a role in the protection of the enzyme from denaturation. In addition, the fermentation
748 time was also optimised to 96 hours for the optimum production of protease. The
749 optimised SSF led to a higher production of protease by about 1.5 times than non-
750 optimised processes. Furthermore, optimization via Taguchi method was performed to
751 evaluate the effect of five factors on the protease production by *A. oryzae*
752 (Thanapimmetha et al., 2012). The effect of three different levels of five factors,
753 including initial moisture content of the substrate used (*Jatropha* seed cake), inoculum
754 size, temperature, type of porous substrate and fermentation time, were examined.
755 These levels were as the following; moisture content (45%, 50% and 55%), inoculums
756 size (1%, 5%, 10%), temperature (25°C, 30°C, 35°C), porous substrate (*Jatropha* oil
757 cake, *Jatropha* oil cake mixed with coconut cake and *Jatropha* oil cake mixed with
758 cassava bagasse, both mixtures with a ratio of 4:1), and time (84, 96 and 108 h). The
759 optimum conditions for the protease production of up to a maximum of 14,273 U/g
760 were 45% moisture content, 10% inoculum size, 30°C incubation temperature, *Jatropha*
761 cake mixed with cassava bagasse as porous substrate at 84 h of fermentation time. This
762 statistical approach provided a satisfactory outcome in defining the optimal conditions,
763 as the optimised process led to an increase of 4.6 times in the protease yield. Rai et al.

(2009) reported the application of RSM for the optimization of the media composition for β-keratinase production by *Bacillus subtilis* using chicken-feather as substrate. The factors studied were the fermentation time (24 h, 48 h, 72 h, 96 h and 120 h), initial moisture content of the substrate (33%, 43%, 50%, 60%, 67% and 75%), supplementation with co-carbon sources (glucose, fructose, galactose, maltose, sucrose, lactose and starch at 10%) and co-nitrogen sources ($NH_4Cl$, $NaNO_3$, yeast extract, beef extract, casein and peptone at 1%) were studied. The optimized culture conditions were at a time of 71h, 50% moisture and with maltose and sodium nitrate as the best co-carbon and co-nitrogen sources, respectively. The results showed that the optimisation led to a 5-fold increase in the enzyme obtained, up to 95.3 U/g, compared to non-optimized conditions.

**4.2.3. Cellulases and Xylanases**

The temperatures, pH and moisture used for the fermentation were mostly mesophilic (30°C) or slightly thermophilic (45 or 50°C), 4 to 7 and 50 to 80%, respectively (Table 5). The process conditions of pH and moisture were not controlled in a lot of studies. The enzymes are produced by a variety of microorganisms including bacteria, actinomycetes and fungi. However, in recent research works, fungi of the generea *Trichoderma* and *Aspergillus* have been reported as the most important microorganisms used as inocula for the process of SSF. Moreover, the effect of pre-treatment of the substrate used on the production of enzymes was studied.

Bansal et al. (2012) evaluated various process parameters during the fermentation by *Aspergillus niger* of agriculture and kitchen waste residues for the production of cellulase complex. The effect of acid and alkali pre-treatment of substrates used was studied. The alkali treatment led to increased yield of enzymes when the wastes, especially potato peels, were utilised as substrate compared to

789 untreated waste materials. This was mainly due to the fact that alkaline pre-treatment
790 dissolve lignin present in the lignocellulosic waste and expose the cellulose and
791 hemicellulose fractions for enzyme and microbial actions. Moreover, untreated
792 substrates contain a variety of nutrients may probably have an inhibitory effect on the
793 fermentation process and thus leading to a lesser production of enzymes. However, in
794 the case of using wheat bran as substrate, the untreated waste induced the highest
795 production of enzyme components. In addition, it was demonstrated that appreciable
796 levels of enzymes could be produced over a wide range of temperatures (20-50°C) and
797 pH (3.0-8.0), with an optimum of 30°C and 6.5 respectively, at initial moisture contents
798 of 60%. These results were in agreement with other research work performed with the
799 aim of producing cellulase. The same optimum pH of 6.5 was found to be the best pH
800 for the enzyme production by the *A. niger* and when using municipal solid wastes as
801 substrate (Gautam et al., 2011). The temperature of 30°C was found to be optimum for
802 incubation of the fungus used as inoculum for the production of enzymes. Brijwani et
803 al. (2010) reported 30°C as the optimum temperature during the fermentation using
804 soybean hulls and wheat bran as substrates by *Aspergillus oryzae* and *Trichoderma*
805 *reesei*, for the production of cellulase and β-glucosidase. This temperature, together
806 with the optimised moisture and pH of 70% and 5 respectively, was used for scale-up
807 processes and a further experimental analysis using novel bioreactor for the production
808 of cellulase complex enzymes (Brijwani et al., 2011). In addition, Sun et al. (2010)
809 found 32°C as the optimum temperature when evaluating the feasibility of using apple
810 pomace as a substrate for cellulase production by *Trichoderma* sp.
811 Thermophilic temperatures of about 50°C were also found to be optimum in the process
812 of SSF for the production of cellulase complex enzymes by *Aspergillus fumigatus*,
813 where lignocellulosic wastes were used as substrate. For instance, Liu et al. (2011)

814 optimised the cultivation conditions and results showed that for cellulases, both endo-
815 and exoglucanase; the best conditions were at a temperature of 50°C, in the presence of
816 an initial moisture of 80% and a pH of 4.0. Soni et al. (2010) reported the optimisation
817 of cellulase production at 45°C, where the culture produced maximal levels of enzyme
818 activity on a medium containing rice straw and beef extract as carbon and nitrogen
819 source, respectively. It was also concluded that optimisation of the process of
820 fermentation by mixing different substrates is a strategy for improvement of the
821 production of cellulase enzymes.
822
823 **4.3. Applications**
824 **4.3.1. Lipase**
825 Lipase enzyme produced by SSF has great biotechnological potential
826 applications, mainly due to the thermophilic and thermostable properties. The enzyme
827 has various applications in oil, pharmaceutical, food and chemical industries (Sharma &
828 Hasan, 2006; Salihu et al., 2012). Recently, lipase produced by SSF was used in
829 synthesis reactions, food applications and treatment of waste water (Table 6).
830 In synthesis reactions, lipases have an important application in the field of
831 bioenergy, especially for the production of biodiesel which is currently an expanding
832 sector in research and on industrial level. Lipase obtained through SSF of sugarcane
833 bagasse and sunflower oil cake by *Burkholderia cepacia* was used to catalyse the
834 synthesis of biodiesel in a fixed-bed reactor (Salum et al., 2010, Liu et al., 2013). This
835 synthesis was through the ethanolysis of soybean oil in a medium free. It was possible
836 to achieve a biodiesel yield of about 90% after 46 hours of reaction. Compared with
837 some commercial lipases, this process avoids the need for expensive processing steps
838 such as enzyme recuperation and immobilization and co-solvent separation and

839 therefore has potential to decrease the costs associated with enzyme-catalyzed synthesis
840 of biodiesel. In addition, lipase produced by SSF, using *Rhizopus* sp. as a thermotolerant
841 fungus, was used as a catalyst for the enzymatic esterification of oleic acid and ethanol
842 (Martinez-Ruiz et al., 2008). Olive oil and perlite were used as an inducer and inert
843 support, respectively. The results demonstrated that the lipase can be successfully used
844 for the synthesis of ethyl oleate, with high etherification rates and substrate conversion,
845 over short reaction periods under conditions when ethanol is in excess. Similarly,
846 Hernandez-Rodriguez et al. (2009) showed that in addition to the lipase produced by
847 *Rhizopus* sp., the enzyme produced by the thermophilic fungus *Rhizomucor* sp. through
848 SSF can be used in the ethyl oleate synthesis reaction.

849 In food applications, lipase produced in SSF by *Rhizopus oryzae* and *Rhizopus*
850 *microsporus*, on a mixture of sugarcane bagasse and sunflower seed meal, was used in
851 interesterification processes of oils to produce fat products with desirable properties
852 (Rasera et al., 2012). This enzyme was able to catalyze the interesterification of a
853 mixture of palm stearin, palm kernel oil and a concentrate of triacylglycerols enriched
854 with omega-3 polyunsaturated fatty acids. This application could be suitable for the
855 production of edible fat products such as margarines and shortenings with low
856 production costs. Another application of the lipase produced by SSF was in the
857 bioremediation of the waste cooking oil (Kumar et al., 2012). The enzyme was
858 produced by *Penicillium chrysogenum* in the presence of wheat bran and waste grease.
859 The results showed that the enzyme could be employed for the bioremediation of used
860 cooking oil such as soya, canola, sunflower and corn oil that contain polyunsaturated
861 oils, which degrade to toxic compounds upon heating.

862 Wastewater has been treated by a lipase enzymatic preparation, with 0.1% (w/v)
863 of solid enzymatic preparation at 30ºC for 24 h, produced by *Penicillium* sp. during

solid-state in an anaerobic digester (Rosa et al., 2009). The waste water that was from the dairy industry contained 1200 mg oil and grease per litre. The oil and grease hydrolysis resulted in a final free acid concentration eight times higher than the initial value. This approach showed the importance of the application of enzymatic preparations obtained by SSF in the treatment of fatty wastewater, with high efficiencies, using anaerobic reactors. In addition, Damasceno et al. (2012) used of a lipase produced by SSF with *Penicillium simplicissimum* using babassu cake as substrate. This enzyme, with a concentration of 0.19% (w/v), was combined with a lipid biosurfactant of 114 mg/L, at 33ºC, produced from *Pseudomonas aeruginosa* and used for the methane production by anaerobic treatment of a wastewater with a high fat content from a poultry processing plant. These results showed the synergistic effect of these two bio-products on the hydrolysis of fats from the effluent, with the potential to treat a poultry processing effluent rich in oils and greases, without using a flotation step. Thus, this approach allowed for the elimination of the problem of generating solid waste and enhancing the production of methane.

**4.3.2. Protease**

Alkaline protease produced by SSF processes has been used as an inclusion in detergent formulations, where the suitability of such an enzyme in this application depends on certain factors such as enzyme stability and compatibility with detergent components (Venugopal et al., 2006). In addition, the enzyme should be thermostable and it is preferred to have the ability to act as a detergent component at different temperatures, including room temperature. Another application of this enzyme was in the process of dehairing of goat and cow hides. This enzymatic process of dehairing could lead to the consumption of less water and harmful chemical reagents used in traditional methods. Therefore the alkaline protease produced through SSF could have

potential applications in detergent formulations as well as in the leather processing industry.

Mukherjee et al. (2008) applied the produced alkaline protease by *Bacillus subtilis* through SSF process, as an additive in laundry detergents. The protease showed the ability to function in a broad range of temperatures, i.e. high thermal resistance and remained active at room temperature, high stability and compatibility with commercial detergents. It was observed that the enzyme retained 33-90% of its original activity at 37°C in the presence of commercial detergents. In addition, it was observed that the enzyme obtained was free of any undesirable flavour, which could be advantageous for further applications in food and pharmaceutical industries. Vijayaraghavan et al. (2012) evaluated the effect of alkaline protease obtained through SSF, by *Halomonas* sp., on surfactants, detergents, solvents and goat hide. The enzyme was remarkably stable on surfactants, such as Tween-20, Triton X-100 and Brij-35 displaying 112%, 202% and 178% activity respectively. There was also a high stability observed on various commercial detergents and organic solvents, such as ethanol, acetone and methanol, with an activity range from 61 to 224% and 49 to 263%, respectively. In addition, the protease effectively dehaired goat hides. This property of the enzyme found as highly significant since most of commercial dehairing proteases are produced by *Bacillus* bacteria (Subba et al., 2009). Recently, Abraham et al. (2014) have also shown that the protease produced through SSF by the microbial populations developed on the hair solid wastes biodegradation process can be used as a satisfactory alternative for the dehairing of cow hides.

Acid proteases produced by SSF processes have applications in the sector of food science and technology, where recently these enzymes have been used in the field of milk and dairy industry. Merheb-Dini et al. (2010) reported the application of an acid

protease, produced from a new and local strain of *Thermomucor* and using only wheat bran as substrate, in the hydrolysis of bovine casein of milk and the investigation of its peptide profile obtained for a better understanding of the proteolytic activity of the enzyme. Results revealed that the acid protease exhibited high milk-clotting activity and low proteolytic activity. These properties might encourage future experiments by using this microbial enzyme on cheese production where the enzyme could be used as a substitute for animal rennin. The advantages of using such a microbial protease are mainly related to the low cost production of such an enzyme since in industrial applications the minimisation of costs is of a crucial importance. Another application of the protease in the field of dairy industry was investigated by Boyce and Walsh (2012). The enzyme produced by *Schizopyllum commune* was used to remove an industrial-like milk fouling deposit (containing about 35% minerals) from stainless steel. This experiment imitated the cleaning-in-place (CIP) operations that use acidic and alkaline solutions in cleaning of various equipments used in the dairy industry, especially heat transfer surfaces used during thermal treatments of milk where milk deposits are continuously formed. The results of this research work showed that suitable cleaning was achieved using this enzymatic cleaning procedure without the use of environmentally harmful and corrosive chemicals.

**4.3.3. Cellulases and xylanase**

Cellulases and xylanase have major and numerous industrial applications, such as in pulp and paper manufacture as well as in the textile industry for polishing of fabrics and laundry detergents for improving fabric softness. For example, Das et al. (2013) used these enzymes, which were produced through optimised processes of SSF, for the deinking of waste pulp of laser printed paper, i.e. mainly the removal of chromophores and hydrophobic compounds. In addition, cellulase enzymes are used in

939 the extraction process of fruit and vegetable juices, starch processing and formulations
940 used for animal feeds (Dhillon et al., 2012a,b; Singhania, et al., 2009). Cellulases have
941 found promising applications for non-specific hydrolysis of chitosan to produce
942 chitooligosaccharides with low molecular weight, which showed high antibacterial
943 activity (Xia et al., 2008).

944 From biotechnological perspectives, the most important and recent application
945 of cellulases and xylanase produced through SSF is in the generation of potentially
946 sustainable energy sources such as sugars and biofuels or, specifically, bio-ethanol.
947 These enzymes are used to hydrolyse cellulosic waste materials to sugars that can be
948 fermented, usually by yeasts, to bioethanol and/or biofuel compounds. It is shown that
949 there is a wide potential to develop a simple biological process to produce ethanol from
950 a variety of lignocellulosic substrates, i.e. by hydrolyzing and fermenting carbohydrates,
951 which are considered as waste materials produced in huge amounts especially in the
952 agro-industrial sector. Liu et al. (2011) directly applied the cellullase enzymes, in their
953 crude form, obtained through SSF processes in the hydrolysis of corn stover. The
954 hydrolysates, reducing sugars obtained, were further used as a substrate for the
955 production of ethanol through the fermentation by *Saccharomyces cerevisiae*. The same
956 biofuel was produced through SSF by sequential saccharification of corn fibre where
957 fermentation by the yeast was allowed leading to the production of ethanol (Rasmussen
958 et al., 2010). SSF followed by buffered anaerobic incubation converted a substantial
959 fraction of corn fibre into harvestable reducing sugars, through the action of cellulases
960 and xylanase obtained from the process of fermentation. The sugars released were
961 fermented with or without the yeast to yield bio-ethanol, in the presence of the
962 cellulolytic fungi used for SSF, where the highest yield was obtained in case of utilising
963 yeast in the process. Several improvements to the production of ethanol were suggested,

964 i.e. optimising the growing conditions such as moisture, pH, temperature and inoculum
965 used. Similarly, in a study by Sukumaran et al. (2009), it was shown that ethanol can be
966 produced using the saccharification of three different feed stock; rice straw, sugarcane
967 bagasse and water hyacinth biomass, followed by the yeast fermentation. It was reported
968 that the highest sugar yield and subsequent ethanol production was in the case of using
969 rice straw. Interestingly, crude unprocessed cellulase obtained, which was not high in its
970 yield, was sufficient to produce ethanol from wheat straw in simultaneous
971 saccharification and fermentation by the yeast (Lever et al., 2013). Therefore, the
972 findings of this research could suggest that using SSF of lignocellulosic wastes may be
973 employed instead of commercial enzyme manufacture, which has usually the
974 disadvantage of a production that is associated with high costs.
975
976 **5. Process Scale-up and Control of SSF**
977 Most research work performed reporting the production of enzymes through
978 SSF use production on a laboratory scale, i.e. batch mode in shaken flasks where few
979 grams of substrate is added. There are technological and operational constrains that
980 limit the scaling-up of the process of fermentation. These constrains are mainly related
981 to the removal of the excess heat formed and the temperature control during
982 fermentation, and also the agitation of solids and handling techniques required for solid
983 substrates (Singhania et al., 2009). Table 7 summarises various aspects of lab scale vs
984 large scale SSF processes. Therefore, large-scale production of enzymes has not yet
985 been proven feasible. However, there are a considerable number of studies focusing on
986 the use of bioreactors in SSF studies at pilot scale for the production of protease and
987 lipase enzymes (Edwinoliver et al., 2010; Santis-Navarro et al., 2011; Abraham et al.,
988 2014). According to a recent review by Thomas et al. (2013), few SSF processes have

been developed at industrial scale: delignification of biomass, dyes bioremediation or *Jatropha* cake detoxification. All these processes have a common objective of enhancing enzymes production, although the enzymes are not targeted as a product, but their effect on the biomass is sought.

In general, there are some basic steps required to scale-up the production of enzymes through SSF (Salihu et al., 2012). Firstly, there is a need to choose suitable microorganisms and substrates, which have been reviewed in the current paper (sections 2 and 3). Secondly, it is required to study various process parameters, e.g. optimisation of moisture, pH and inoculum used. These were discussed in detail concerning the production of lipase, protease and cellulases (sections 4.1-4.3). Thirdly, the scale-up process is performed, which depends mainly on operating conditions (aerations, mass and heat transfer) and process control (Singhania et al., 2009; Li et al., 2013) ~~and optimisation studies~~. A last step might be the study of the technical, environmental and economical viabilities of the process developed.

### 5.1. Challenges of Process Scale-up

The main aspects of scaling-up the production of enzymes through SSF include challenges and recent advances. SSF is difficult to scale-up due to the three-phase heterogeneous nature of the substrate and the existing gradients inside the reactor in temperature, pH, moisture, oxygen and inoculum (Rodriguez et al., 2010, Salihu et al., 2012). In addition, the absence of free water during the fermentation leads to poor heat removal and accessibility of nutrients resulting in slow microbial growth which might lead low or no production of enzymes obtained at the end of the fermentation process. On the other hand, difficult agitation of solid substrates might occur which leads to physical and chemical heterogeneous distribution. Moreover, the heat generated due to

the metabolic activities of microorganisms is in most cases an inconvenient for biotechnological processes especially when the optimum growth of microorganisms is affected and a large part of the enzymes produced during SSF can be heat-denatured. Another important challenge of scale-up processes is the control of pH within the system during the fermentation, as this control is required to manage the growth of microorganisms and the subsequent production of enzymes. Therefore, the control of heat transfer is one of the major crucial issues in the design and operation of large-scale SSF fermenters. There is also a need to firstly, monitor on-line the parameters throughout the process, such as temperature and pH (Ali & Zulkali, 2011). Most importantly, oxygen consumption and the carbon dioxide evolution are important measurements because they represent the best way of monitoring the growth of microorganisms inside the reactor. As a more sophisticated and no-invasive proposal, Jiang et al. (2012) successfully monitored physical and chemical changes at a 100L pilot bioreactor using FT-NIR spectroscopy coupled with vector data description, thus avoiding chemical analysis Secondly, it is needed to adequately mix the substrates within the fermenter without negatively affecting the growth of microorganisms as well as the substrates used.

### 5.2. Recent Advances on Process Scale-up

There are a number of bioreactors that have been designed to overcome the problems of scale up. Commonly used SSF bioreactors are classified into four types based on the pattern of aeration and/or agitation system employed (Mitchel et al., 2006). These types are tray, packed-bed, rotating and stirred-drum and forcefully aerated agitated reactors. Each of these types have their own advantages and disadvantages, which promoted the necessity to develop novel bioreactors with better design in order to

solve major problems related to the scale-up processes for the production of enzymes through SSF. There are few recent studies on the overcoming of some of the disadvantages and limitations regarding the scaling-up processes. An extensive analysis on the design and operation of bioreactors in SSF has been published in the recent review by Thomas et al., (2013). Also Yoon et al. (2014) describe in detail the use of bioreactors for cellulase production.

Scaling up of the process of SSF for the production of enzymes from waste material was shown to be successful. Edwinoliver et al. (2010) scaled up the process of lipase production from 10 g up to a level of 3 kg, using *A. niger* as inoculum. The strategy of scaling-up included the transfer of the optimised process conditions developed at laboratory level to pilot production level, where temperature and moisture were online monitored and controlled during the process of fermentation. Another strategy for the scaling-up was mainly depending on control of air flow intensity as a key factor during the production of phytase (Rodriguez-Fernandez et al., 2012). At a 10-fold scale-up, from 2 to 20 kg drum bioreactors with a paddle agitation, the control of the air flow intensity was required to maintain the temperature constant during the fermentation, as well as to cool the fermenter at late stages or to allow for the removal of metabolic heat generated. In addition, the air flow was able to provide oxygen that is considered as a crucial factor for the growth of microorganisms.

Recently, there were studies on the scaling up of SSF process for the production of ethanol. Soni et al. (2013) demonstrated that a rotary drum reactor can be directly scaled up to a larger capacity up to 100 L, by using SSF optimised operating conditions obtained at laboratory levels, using flask batch modes. The results showed that the scale-up process is feasible and has commercial potential, especially when the substrate used, which was sugarcane bagasse, was pre-treated with alkali prior to the process of

fermentation. Interestingly, Li et al. (2013) reported an advanced SSF technology, which is capable of overcoming most problems associated with the scale-up and large-scale fermentation processes. An efficient system for the control of mass and heat was connected to a continuous solid-state rotary drum fermentation reactor, developed by the research group, where a newly developed microbial strain was used that allowed for the shortening of the time of fermentation.

Different approaches for an easily scalable process have been reported by Santis-Navarro et al., (2011) and Abraham et al. (2014). In these approaches, the temperature was not controlled and enzymes were produced in a batch fermentation process similar to the composting. The temperature rose to thermophilic values due to heat released and decreased to ambient values during the fermentation of the readily biodegradable matter, which has been consumed.

Recently, in other SSF applications, there were also studies on the scaling up of SSF process for the production of bioethanol. Lin et al. (2013) demonstrated that a rotary drum reactor can be directly scaled up to a larger capacity up to 100 l, by using SSF optimized operating conditions obtained at laboratory levels, using flask batch modes and the thermotolerant yeast *Kluyveromyces marxianus* as an inoculum for the fermentation. The results showed that the scale-up process is feasible and has commercial potential, especially when the substrate used, which was sugarcane bagasse, was pretreated with alkali prior to the process of fermentation. The alkali pretreatment of this substrate allows for a direct carbon source for the growth of microorganisms in the SSF system (Chandel et al., 2012). Interestingly, Li et al. (2013) reported an advanced SSF technology, which is capable of overcoming most problems associated with the scale-up and large-scale fermentation processes. An efficient system for the control of mass and heat was connected to a continuous solid-state rotary drum

fermentation reactor, developed by the research group. A newly developed microbial strain of *Saccharomyces cerevisiae* was used that allowed for the shortening of the time of fermentation of a substrate of sweet sorghum stems.

## 6. Final Remarks

In summary, this review provides an update on recent studies that are dealing with the use of SSF for the production of enzymes, and it especially covers issues related to wastes, microorganisms and scale-up and control of the process of fermentation. The main focus was on the production of lipases, proteases, cellulases, xylanases, glucoamylases, pectinases and inulinases. For the process of fermentation, the inocula used were mostly fungi, like various species of *Aspergillus*, and substrates were waste materials obtained from the sector of agriculture and food industry. The use or recycle of these wastes, which are very cheap and highly available in big amounts, shows a high benefit of SSF from the economical and environmental perspectives.

Accordingly, SSF presents a substantial advantage over SmF, which has been extensively used for the production of commercially available enzymes since many decades. Nevertheless, the majority of research conducted on SSF was on lab-scale, whereas the large scale/commercial production of enzymes is still not developed because of constraints related to the scaling-up of the process. For instance, the absence of free water during the process leads to poor mixing and heat removal that results in slow microbial growth and a subsequent low or no production of enzymes. Recent developments are based on transfer of the optimised process conditions developed at laboratory level to larger scales, control of heat and mass transfer and on-line monitoring of the parameters, such as temperature and pH, where air flow has been shown to be crucial for the maintenance of constant temperatures and microbial growth.

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**Tables**

Table 1. A comparison between SSF and SmF for the production of enzymes, showing the main advantages and disadvantages

| Factor | SSF | SmF |
|---|---|---|
| Substrate | No-cost materials, e.g. waste products | Very expensive media ingredients |
| Inoculum | Not necessary | Essential |
| Aseptic conditions | Not needed | Essential |
| Moisture | No free water | Liquid media required |
| Agitation | Very difficult | Easy |
| Process Control (T, pH) | Difficult | Easy |
| Contamination | Less chance | High risk |
| Enzyme Yield | Very high | Low |
| Downstream processing | Easy, cheap, not time consuming | Very difficult, very expensive |
| Liquid waste | Not produced | High quantities |
| Scale up | Difficult, new design equipments needed | Easy, industrial equipments available |
| Volume and costs of equipments | Small reactors can be used, low costs | Large-scale reactors required, very high costs |

Table 2. Types of various waste materials used in the SSF processes for the production of enzymes, including the microorganisms if present in these processes.

| Category of waste | Types of waste | Microorganisms | Enzymes | References |
|---|---|---|---|---|
| Wastes of animal origin | Fish flour | *Aspergillus niger* | Proteases | Garcia-Gomez et al., 2009 |
| | Chicken feather | *Bacillus subtilis* | | Rai et al., 2009 |
| | Cow dung | *Halomonas* sp. | | Vijayaraghavan et al., 2012 |
| | Hair waste | nc | | Abraham et al., 2013, 2014 |
| | Tannery solid waste | *Synerggistes* sp. | | Kumar et al., 2009 |
| Wastes of plant origin and food industry | Oil wastes | *Aspergillus niger*, *Bacillus subitilis*, *Penicillium* spp. | Lipases, Amylase | Colla et al., 2010; Chaturvedi et al., 2010; Edwinoliver et al., 2010, Godoy et al., 2011 |
| | Wheat wastes | *Aspergillus oryzae* | Protease | Thanapimmeth et al., 2012 |
| | | *Rhizopus oryzae* | Lipases | Garlapati & Banerjee, 2010 |
| | | *Apergillus* spp., *Trichoderma reesei* | Cellulases, Xylanase | Dhillon et al., 2011; Bansal et al., 2012 ; Farinas et al., 2011 ; Pirota et al., 2013 |
| | | *Aspergillus sojae* | Pectinase | Demir & Tari, 2014 |
| | | *Apergillus* spp. | Cellulase | Soni et al., 2010; Dhillon et al., 2011 |
| | | *Aspergillus niger* | Proteases | Paranthaman et al., 2009 |
| | | *Aergillus fumigatus* | Cellulase | Liu et al., 2011 |
| | Rice wastes | *Aspergillus terreus* | Cellulase | Narra et al., 2012 |
| | | *Bacillus pumilus* | Xylanase | Kapilan & Arasaratnam, 2011 |
| | | *Aspegillus niger*, *Penicillium* sp. | Lipases | Rigo et al., 2009; Colla et al., 2010 |
| | Soy wastes | nc | Proteases | Abraham et al., 2013 |
| | | *Aspergillus oryzae*, *Trichoderma reesei* | Cellulases, Xylanase | Brijwani et al., 2010, 2011 |
| | Peels of fruits and vegetables | *Aspergillus niger* | Cellulase, xylanase, pectinase, phytase | Rodriguez-Fernandez , et al., 2011, 2012, 2013, Mamma et al., 2008 |

| | | | |
|---|---|---|---|
| | *Bacillus subtilis* | Proteases | Mukherjee et al., 2008, 2009 |
| | *Bacillus firmus* | Amylase | Elayaraja et al., 2011 |
| | *Aspergillus niger* | Cellulases | Cunha et al., 2012, Mekala et al., 2008 |
| Sugarcane bagasse | | | |
| | *Kluvyromyces marxianus* | Inulinases | Astolfi et al., 2011 |
| Cotton wastes | *Aspergillus* spp. | Hydrolytic & oxidative enzymes | Csiszar et al., 2007; Liu et al., 2011 |
| Pomace of fruits and vegetables | *Aspergillus* spp. | Proteases, cellulases | Belmessikh et al., 2013; Dhillon et al., 2012a,b |
| | *Bacillus licheniformis* | Lichenase | Chaari et al., 2012 |
| Grape waste | *Pleurotus eryngii* | Lignolytic enzymes | Akpinar et al, 2012 |
| Oil palm trunk | *Aspergillus fumigatus* | Cellulases, Xylanses | Ang et al., 2013 |
| Waste bread | *Aspergillus awamori* | Amylases, proteases | Melikoglu et al., 2013 |
| Agrowastes | *Bacillus subtilis* | Amylase | Mukherjee et al., 2009; Nimkar et al., 2010 |
| Winterisation residues, sludge | nc | Lipases | Santis-Navarro et al., 2011 |

nc: not controlled

Table 3. Substrate, process conditions, microorganisms and enzyme activity of lipase produced by SSF

| Substrate | | Process conditions | | | Microorganisms | Enzyme Activity (U/g) | Reference |
|---|---|---|---|---|---|---|---|
| Type | Amount (g) | T (°C) | pH | Moisture (%)* | | | |
| Coconut oil cake, wheat bran, wheat rawa | Up to 3000 | 30 | nc | 60 | *Apergillus niger* | 745.7 | Edwinoliver et al., 2010 |
| Mix of oil cakes | 10 | 30 | 8 | 70 | *Bacillus subtilis* | 4.5 | Chaturvedi et al., 2010 |
| Niger seed oil cake | 10 | 30 | 6.4-6.8 | 60 | *Yarrowia lipolytica* | 26.42 | Imandi et al., 2010 |
| Babassu cake | 10 | 30 | nc | 70 | *Penicillium simplicissimum* | 314 | Gutarra et al., 2009 |
| Castor bean waste | 20 | 30 | nc | nc | *Penicillium simplicissimum* | 80.24 | Godoy et al., 2011 |
| Soybean meal | 10 | 20 | 7 | 75 | *Peniciullium* sp. | 317 | Rigo et al., 2009 |
| Soybean meal, rice husk | 50 | 30 | 4.5 | 60 | *Apergillus niger* | 25.22 | Colla et al., 2010 |
| Jatropha curcas seed cake | 5 | 30 | 7 | 50 | *Pseudomonas aeruginosa* | 312.5 | Mahanta et al., 2008 |
| Wheat bran | 4 | 35 | 5.28 | 60 | *Rhizopus oryzae* | 96.52 | Garlapati & Banerjee, 2010 |
| Winterisation residue, sludge | 2,500 | Higher than 45 | 7 | 50 | nc | 120,000 | Santis-Navarro et al., 2011 |

nc: not controlled
* Initial moisture content of the substrate(s) used

Table 4. Substrate, process conditions, microorganisms and enzyme activity of protease produced by SSF.

| Substrate | | Process conditions | | | Microorganisms | Max enzyme activity (U/g) | Reference |
|---|---|---|---|---|---|---|---|
| Type | Amount (g) | T (ºC ) | pH | Moisture (%)* | | | |
| Potato peel, grass | 100 | 50 | 8 | 50 | *Bacillus subtilis* | 2,383 | Mukherjee et al., 2008 |
| Fish flour, polyurethane foam | 30 | 30 | nc | 50 | *Aspergillus niger* | 120.78 | Garcia-Gomez et al., 2009 |
| Soy fiber residues | 1250 | nc | 8.5 | 40-60 | Nc | 47,331 | Abraham et al., 2013 |
| Tomato pomace | 10 | 30 | 6.8 | 60 | *Aspergillus oryzae* | 21,309 | Belmessikh et al., 2013 |
| Jatropha seed cake | 5 | 30 | 6 | 50 | *Pseudomonas aeruginosa* | 1818 | Mahanta et al., 2008 |
| | 25 | 30 | nc | 45 | *Aspergillus oryzae* | 14,273 | Thanapimmetha et al., 2012 |
| Wheat bran, casein | 5 | 45 | nc | 60 | *Thermomucor indicae-seudaticae* | 167.6 | Merheb-Dini et al., 2010 |
| Wheat bran | 5 | nc | nc | nc | *Myceloiphthora* sp. | 19.8 | Zanphorlin et al., 2011 |
| | 10 | 24-40 | nc | 50 | 14 Fungal strains | 5.05 | Boyce & Walsh, 2012 |
| | few grams | 27 | acidic | 60 | *Aspergillus oryzae* | $8.3 \times 10^3$ | Vishwanatha et al., 2009 |
| Chicken feather | 5 | 50 | 8 | 50 | *Bacillus subtilis* | 95.3 | Rai et al., 2009 |
| Cow dung | 5 | 37 | 8 | 50 | *Halomonas* sp. | 1,351 | Vijayaraghavan et al., 2012 |
| Tannery solid waste | 5 | 37 | 6 | 50 | *Synergistes* sp. | 755 | Kumar et al., 2009 |
| Hair wastes | 1400 | nc | 8.5 | 40-60 | nc | 56,270 | Abraham et al., 2014 |

nc: not controlled
* Initial moisture content of the substrate(s) used

Table 5. Substrate, process conditions, microorganisms and enzyme activity of cellulases and xylanase produced by SSF.

| Substrate | | Process conditions | | | Microorganisms | Enzymes | | Reference |
|---|---|---|---|---|---|---|---|---|
| Type | Amount (g) | T (°C) | pH | Moisture (%)* | | Type | Max activity (U/g) | |
| Soybean hulls | 10,240 | 30 | 5 | 70 | *Aspergillus oryzae*, *Trichoderma reesei* | FPase<br>CMCase<br>Bgase<br>Xylanase | 5.39<br>58.57<br>18.36<br>242 | Brijwani et al., 2011 |
| Soybean hulls, wheat bran | 100 | 30 | 5 | 70 | *Aspergillus oryzae*, *Trichoderma reesei* | FPase<br>CMCase<br>Bgase<br>Xylanase | 10.78<br>100.67<br>10.71<br>504.9 | Brijwani et al., 2010 |
| Rice straw | 5 | 45 | 7 | 75 | *Aspergillus fumigatus* | FPase<br>CMCase<br>Bgase<br>Xylanase | 3.37<br>98.5<br>250.9<br>2,782 | Soni et al., 2010 |
| Rice straw, wheat bran | 10 | 30 | nc | nc | *Aspergillus oryzae*, *Trichoderma reesei* | FPase<br>CMCase<br>Bgase<br>Xylanase | 35.8<br>132.34<br>33.71<br>3,106 | Dhillon et al., 2011 |
| Apple pomace | 10 | 30 | nc | 70 | *Trichoderma* sp. | FPase | 7.6 | Sun, 2010 |
| Apple pomace, rice husk | 40 | 30 | nc | 75 | *Aspergillus oryzae* | FPase<br>CMCase | 133.68<br>172.3 | Dhillon et al., 2012b |
| Wheat bran | 5 | 30 | nc | 40-57 | *Trichoderma reesei* | FPase<br>CMCase<br>Bgase | 4.55<br>135.44<br>21.49 | Sukumaran et al., 2009 |
| Rice husk, wheat bran | 30 | 30 | nc | nc | nc | FPase<br>CMCase | 6.3<br>26 | Hu et al., 2011 |
| Apple pomace, lactoserum | 40 | 30 | nc | nc | *Aspergillus niger* | FPase | 130.4 | Dhillon et al., 2012a |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Rice husk, wheat bran & straw, corncob, kitchen wastes | 5 | 30 | 6.5 | 60 | *Aspergillus oryzae*, *Trichoderma reesei* | CMCase<br>Bgase<br>Xylanase<br>FPase<br>CMCase<br>Bgase | 148.9<br>90.1<br>2,619<br>15.9<br>297<br>33.2 | Bansal et al., 2012 |
| Rice, wheat & cotton straw, corncob | 20 | 50 | 4 | 80 | *Aspergillus fumigatus* | FPase<br>CMCase | 144.6<br>526.3 | Liu et al, 2011 |

FPase: Filter paper activity for cellulase
CMCase: Carboxy methyle cellulase
BGase: β-glucosidase

nc: not controlled
* Initial moisture content of the substrate(s) used

Table 6. Applications of lipase, protease and cellulases and xylanase enzymes

| Enzyme | Applications | References |
|---|---|---|
| Lipase | Oil, pharmaceutical, food and chemical industries: synthesis reactions (biodiesel production), food applications (interesterification of oils) and treatment of waste water | Sharma & Hasan, 2006; Salihu et al., 2012; Damasceno et al., 2012 ; Raser et al., 2012 |
| Protease | Detergent formulations, dehairing (leather industry), dairy industry | Venugopal et al., 2006; Abraham et al., 2014 ; Merheb-Dini et al., 2010 |
| Cellulases and Xylanase | Paper manufacture, textile industry, bioethanol production | Das et al. (2013); Liu et al. (2011) ; Lever et al. (2013) |

Table 7. Conditions needed for lab scale vs. large or commercial scale SSF

| Condition | Lab scale | Large Scale |
|---|---|---|
| pH control | Possible through pH adjustment | Not possible |
| T control, Heat Removal | Easy, Possible through controlled temperature water bath | Possible by managing aeration, costly, possible presence of T gradients along the solid matrix in bed reactors. |
| Handling of solid substrates | Very easy | Very difficult |
| Inoculation | Easy, not expensive | Very high costs, difficult homogenization. |
| Agitation | Very easy | Possible in some reactors configurations such as rotatory drums, high energy cost. |
| Aeration | Sufficient | Moderate-high, high energy costs |