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REVIEW PAPER

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

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

Keywords Lignocellulose · Enzyme · Solid state fermentation · Optimization · Large scale · Environmental applications

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1 Introduction

The field of industrial biotechnology has evolved rapidly in recent years as a combined result of international political desire and important progress in molecular biology research and tools (OECD 2011). Enzyme production is one important subject of this field. Its traditional application markets reside in the food, paper, textiles, pharmaceutical and chemical (mainly detergents) industries (van Beilen and Li 2002). However, more recently new applications have emerged aiming environmental applications mainly those of waste treatment such as anaerobic digestion. In

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fact, two of today's major problems are the increasing generation of waste with its consequent environmental problems and the search for new sources of energy due to the increasing demand on energy sources and the concern about the remaining amount of fossil resources. One important feedstock that could be used as a renewable energy source is lignocellulose which has recently attracted significant attention.

Regardless of their source lignocellulosic materials consist of three main polymers: cellulose, a homopolymer of β-1,4 linked glucose units; hemicellulose, a heteropolymer of pentoses and hexoses with a backbone built up by sugar monomers like xylose; and lignin, a complex aromatic polymer made of different types of phenylpropane units namely syringyl and guaiacyl units (Acharya et al. 2010; Deswal et al. 2011). Cellulose, the main component of lignocellulose, is the most abundant organic compound. It is the major constituent of all plant materials and forms about one-third to half of plant tissues and is constantly replenished by photosynthesis (Pandey et al. 2000). Mandels et al. (1974) indicated that lignocellulose represents 40–60 % of municipal solid waste, and is also abundant in waste from forest products, agriculture and fruit and vegetable processing. And up until today, Hoornweg and Bhada-Tata (2012) report that the paper fraction, mainly lignocellulose, varies in the MSW composition between 2 and 68 % according to the region of the world; that is without taking into account the lignocellulosic fractions of the remaining MSW components. It is therefore a huge organic reservoir on earth and a major renewable source of energy. In fact according to the world energy outlook (IEA 2013), biomass represented 7 % of the overall energy commodities produced in 2013. Cellulose and hemicellulose are respectively totally and partially biodegradable but remain protected and unavailable inside the lignocellulosic structure. Therefore a huge energetic potential is confined within lignocellulosic matrices and its expression depends on the disruption of the structure. Therefore overcoming the recalcitrance of natural lignocellulosic materials, which need to be hydrolyzed to produce fermentable sugars, is a major technological challenge (Kiranmayi et al. 2011). A pretreatment step is thus required in order to tap into this energetic pool. Physical and chemical pretreatments have shown positive results, however their corresponding economic, energetic and operational costs present major drawbacks. Production of inhibitors has also been identified as another important drawback as it is the case when applying, for example, the widely used steam explosion pretreatment (Alvira et al. 2010; Horn et al. 2011). Biological pretreatment, using enzymes, has also shown interesting results but the cost is prohibitive for environmental applications. In fact, current large scale production of enzymes is mainly achieved under submerged fermentation (SmF) conditions and aimed at the production of high value-added products of the pharmaceutical and food industries (Jegannathan and Nielsen 2013).

In this context, solid state fermentation (SSF) emerges as an interesting alternative for the in situ production of endogenous enzymes that will be used to enhance the hydrolysis of lignocellulosic matrices. Although, in certain conditions, SSF was considered as an "in vivo" pretreatment method to enhance enzymatic saccharification of lignocellulosic biomass in ethanol production processes (Alvira et al. 2010). For the purpose of this review, SFF corresponds to the enzyme producing stage. Figure 1 illustrates how the SSF process can be integrated in a waste treatment system with downstream anaerobic digestion.

The paper thus reviews the experimental research on the cellulolytic enzyme production through SSF. Going by theoretical classification based on water activity, only fungi and yeasts are termed as suitable microorganisms for SSF. Nevertheless, experience has shown that some bacterial cultures can be well managed and manipulated for SSF processes (Pandey 2003). The reviewed literature thus comprises three categories based on the type of inoculum and the temperature of the culture medium: using bacterial species, using fungal species under thermophilic conditions and finally the most widely spread category of using fungal species under mesophilic conditions. The remaining sections discuss the main challenges and scientific locks that inhibit the application of SSF in the environmental field at the industrial scale.

2 Review of SSF experiments using bacterial species

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

Krishna (1999) used the bacterium *Bacillus subtilis CBTK106*, isolated from banana waste, to produce cellulase under both SSF and SmF conditions. Several

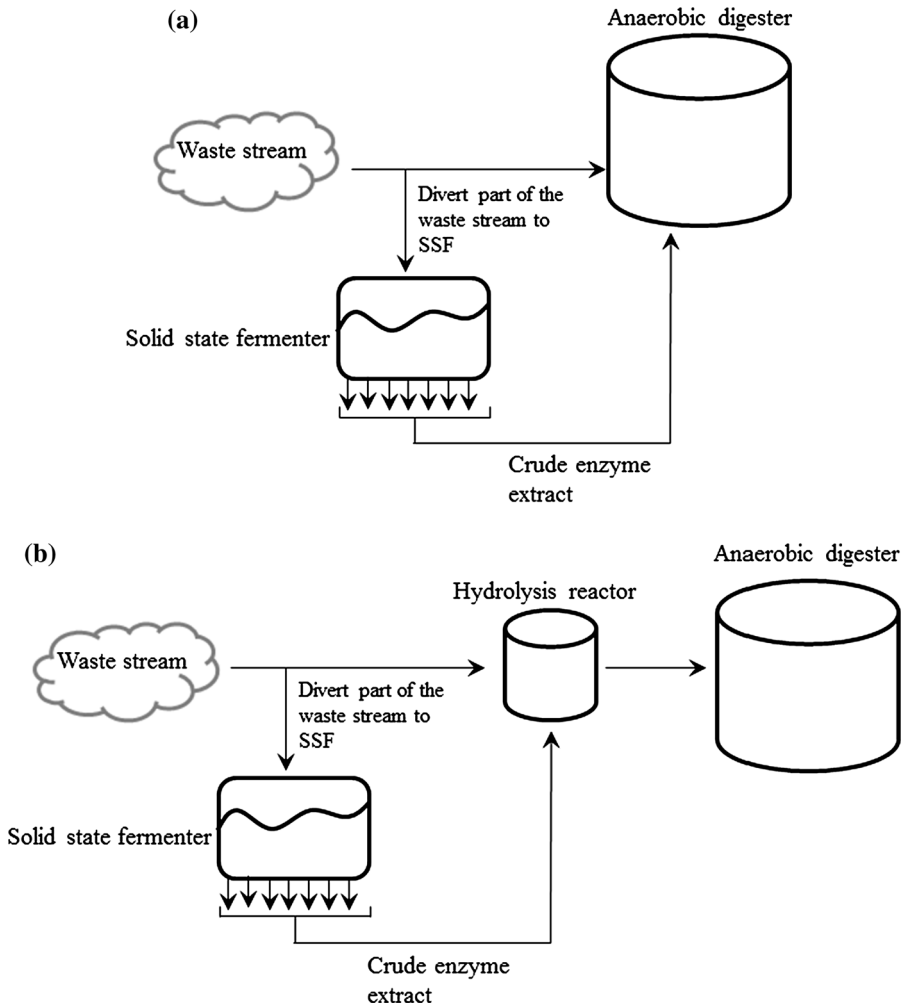


Fig. 1 Schematic diagram presenting the integration of the SSF process in **a** single-stage AD system and **b** two-stage AD system

parameters were considered in the experimental setup to optimize exoglucanase (FPase), endoglucanase (CMCase) and β-glucosidase (cellobiase) production. Unfortunately although many factors were studied, they were optimized one factor at a time (OFAT); this type of experimental approach is very criticized since it can easily lead to biased results and interpretations. Results showed that cellulase production was not affected by neither acidic nor basic pretreatment. Autoclaving was more interesting. As mentioned by Camassola and Dillon (2009), pressure cooking at a controlled pH of plant materials results in greater susceptibility to enzyme hydrolysis. It is also believed to avoid the formation of monosaccharaide degradation products such as furfural and hydroxymethyl furfural which can interfere with subsequent cellulose hydrolysis. Optimal levels of enzyme production were thus found at 70 % moisture level, 400 μm particle size, 7.0 pH, 15 % inoculum level size (w/v), 35 °C and 72 h fermentation time. Inorganic nitrogen sources were more effective than organic sources and there was no significant impact of additional carbon source. Interestingly, optimal reported pH values usually vary between 5.5 and 6.0 when using fungi in SSF. Hideno et al. (2011) reports optimal pH as low as 4.0 using the filamentous fungus *Acremonium cellulolyticus* on rice straw. But as it will be shown later, in SSF processes using bacterial species optimal pH values are more alkaline. Another point to mention is that the author (Krishna 1999) believed that since there was no pH control in the system, after 72 h the enzymes would be denatured and thus the activity

decreased. This hypothesis is questionable since in later reviewed work, the fermentation process extends longer than the reported 72 h. A very important finding of this work is the fact that cellulase titer was 12-folds higher under SSF than SmF.

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

Finally, Asha Poorna and Prema (2007) used *Bacillus pumilus* for the production of endoxylanase under SSF conditions. The screening of different substrates revealed that wheat bran is the one with the highest potential. Ten grams of wheat bran were thus mixed and inoculated. Using an OFAT approach as well, optimal operational parameters were 0.5 mm for particle size, 72 % moisture, 35 °C, 10 % inoculum size (v/w) and pH 9. Maximum enzyme titer was recorded at 72 h during for a total incubation period of 120 h. It is obvious that only *Bacillus* species were used as bacterial inoculum for SSF. In all reviewed work (Table 1), maximum enzyme activities were obtained after 72 h of fermentation with fine particle size and at narrow ranges of temperature (35–37 °C) and moisture (70–75 %). As shown in Table 2, comparable CMCase values were reported in both Krishna (1999) and Gessesse and Mamo (1999). Nevertheless, reported xylanase activity in Asha Poorna and Prema (2007) were at least 30 times higher than those reported by Gessesse and Mamo (1999) although the experimental conditions were very comparable and all authors used wheat bran as the substrate. This might be partly explained by a higher efficiency of the subspecies that was used. However discrepancies in the analytical protocols used to measure the enzymatic activity could be a more plausible explanation to this significant difference in the results.

3 Review of SSF experiments using fungal species under thermophilic conditions

Fungal species are the most widely used inoculum in SSF along with mesophilic conditions: mostly 25–30 °C and in few cases 40 °C. Nevertheless, this section deals with experiments run by Kalogeris et al. (2003) and Dave et al. (2012) who used the same species *Thermoascus aurantiacus* under thermophilic conditions (50–60 °C) to produce cellulolytic multienzyme complexes.

In Kalogeris et al. (2003), SSF was carried with 2.5 g carbon source mixed with of a mineral solution at 80 % humidity level, for 7 days under static conditions. At the end of the process, enzymes were extracted with distilled water by shaking at 50 °C. This temperature is high since usually this step is done at room temperature (RT) or at 4 °C but it may have been used since the SSF itself was carried out under thermophilic conditions. The authors used the OFAT approach to optimize enzyme production of both CMCase and β-glucosidase. Several types of carbon were tested and untreated wheat straw proved to be the most efficient. As shown in Krishna (1999), enzyme activities were much higher with inorganic nitrogen sources. Optimum temperature was found at 50 °C and optimal pH at 4.0; in fact filamentous fungi prefer acidic environments with optimal pH between 3.8 and 5.0.

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

Table 1 Summary table of all operational parameters used in the reviewed literature

References	Substrate	Inoculum	Pretreatment	Autoclaving	SSF duration	pH	T (°C)	Particle size	Moisture (%)
Chahal (1985)	Wheat straw, aspen pulp	*T. reesei*	NaOH at 121 °C	121 °C for 20 min	22 days		30		80
Deschamps et al. (1985)	Straw + wheat bran	*Trichoderma harzanium*	N/A	120 °C for 120 min	66 h	5.8	30	<0.5 mm	74
Krishna et al. (1995)	Soyhull	*P. chrysosporium*	N/A	Sterilization	6 days	4	25		67
Gessesse and Mamo (1999)	Wheat bran	*Bacillus* sp.	Add Na_2CO_3	Yes	72 h		37		75
Krishna (1999)	Dry banana fruit stalk	*B. subtilis*	N/A	121 °C for 60 min	72 h	7	35	0.4 mm	70
Kalogeris et al. (2003)	Wheat straw	*T. aurantiacus*	N/A	121 °C for 20 min	7 days	4	50		80
Lu et al. (2003)	Wheat bran	*A. sulphureus*	N/A	121 °C for 30 min	72 h	6.6–6.8	30–33		40–50
Reddy et al. (2003)	Banana waste	*P. ostreatus*, *P. sajor-caju*	N/A	121 °C for 120 min	10 days		25		75
Kang et al. (2004)	Rice straw + wheat bran	*A. niger*	N/A	121 °C for 30 min	5–6 days	7	28		65
Asha Poorna and Prema (2007)	Wheat bran	*B. pumilus*	N/A	121 °C for 20 min	72 h	9	35	0.5 mm	72
Membrillo et al. (2008)	Sugarcane bagasse	*P. ostreatus*	N/A	121 °C for 15 min	8 days		29.5	1.68 mm	80
Acharya et al. (2010)	Distillery spent wash	*A. ellipticus*	N/A	121 °C for 15 min	7 days	5	30		83
Chen et al. (2011)	Corn stover	*T. reesei*	Steam explosion	121 °C for 20 min	7 days	4.8	30		72
Deswal et al. (2011)	Wheat bran	*Fomitopsis* sp.	N/A	121 °C	16 days	5.5	30		77.8
Guowei et al. (2011)	Corn stalk and wheat bran	*T. reesei*	N/A	121 °C for 20 min	5 days	5	30		50
Jabasingh and Nachiyar (2011)	Coir pith	*A. nidulans*	NaOH at 0.8 %	121 °C for 30 min	11 days	5	40		70
Rodriguez-Fernandez et al. (2011)	Citrus peel	*A. niger*	N/A	121 °C for 20 min	96 h	5	30	0.8–3 mm	60
Dave et al. (2012)	Deoiled Jatropha seedcake	*T. aurantiacus*	N/A	121 °C for 15 min	6 days	4–5	50		67

Table 1 continued

References	Substrate	Inoculum	Pretreatment	Autoclaving	SSF duration	pH	T (°C)	Particle size	Moisture (%)
Kim and Kim (2012)	Empty palm fruit bunch fiber	*Penicillium verruscolum*	1 M NaOH at 20 % (w/v)	121 °C for 60 min	6 days	6	30	2–4 mm	
Qian et al. (2012)	Wheat bran + ground corncob	*A. niger*	N/A	121 °C for 30 min	72 h	6	28		70

recorded at middle inoculum size. This observation is important since depending upon the target enzyme to be produced the inoculum level could be modified. This parameter will depend on the downstream use of the enzyme mixture produced.

In terms of operational parameters, a higher moisture level was used in Kalogeris et al. (2003) as shown in Table 1. However, regarding the enzymatic activities, the FPase level reported by Dave et al. (2012) were low and CMCase and β-glucosidase were lower than those of Kalogeris et al. (2003). In fact if the carbon content of wheat straw accounts for 50 % of the substrate, CMCase levels would be six times higher and β-glucosidase twice as high when using wheat straw. This means that while using the same species and with optimal conditions, the type of substrate has a significant impact on enzyme production levels.

4 Review of SSF experiments using fungal species under mesophilic conditions

As indicated earlier, most of the SSF processes are carried out under mesophilic conditions. For this reason, this section is divided into two major subsections based on whether the substrate undergoes or not a pretreatment step. Autoclaving of the solid matrix is nevertheless not considered as a pretreatment. In fact unless stated differently all reviewed work includes an autoclaving step of the substrate before the inoculation is performed.

4.1 Without substrate pretreatment

Given the various results found in this category, this latter will be divided into different sub-categories based on the type of inoculum used.

4.1.1 Aspergillus species

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

Lu et al. (2003) studied the impact of temperature of the koji and water activity on the xylanase production using *Aspergillus sulphureus* as the inoculum at both lab (18 g of wheat bran) and pilot scales (500 kg of substrate). The total incubation time was set at 72 h. Parameters were optimized consequently. At lab-

Table 2 Maximum enzymatic activities (FPase, CMcase, β-glucosidase and xylanase) recorded in the reviewed works

References	FPase	CMCase	β-glucosidase	Xylanase
Chahal (1985)	326.8 IU/g dry substrate		402.8 IU/g dry substrate	10,260 IU/g dry substrate
Deschamps et al. (1985)	18 IU/g dry solid	198 IU/g dry solid		
Krishna et al. (1995)	29.1 U/g dry solid	74.8 U/g dry solid		
Gessesse and Mamo (1999)		12.5 IU/g dry substrate		700 IU/g dry substrate
Krishna (1999)	2.8 IU/g dry substrate	9.6 IU/g dry substrate	4.5 IU/g dry substrate	
Kalogeris et al. (2003)		1572 U/g carbon	101.6 U/g carbon	
Lu et al. (2003)				1200 IU/g dry koji
Reddy et al. (2003)	Very low	Very low		0.1411 U/mg protein
Kang et al. (2004)	34.2 IU/g substrate	130 IU/g substrate	107 IU/g substrate	14,196 IU/g substrate
Asha Poorna and Prema (2007)				21,431 IU/g dry substrate
Membrillo et al. (2008)	0.18 *I*U/g dry weight			7.59 IU/g dry weight
Acharya et al. (2010)	13.38 IU/g substrate	130.92 IU/g substrate	26.68 IU/g substrate	
Chen et al. (2011)	194.18 IU/g dry substrate		155.8 IU/g dry substrate	
Deswal et al. (2011)	3.492 IU/g substrate	71.699 IU/g substrate	53.679 IU/g substrate	
Guowei et al. (2011)	92.16 U/g koji	377.02 U/g koji		
Jabasingh and Nachiyar (2011)	816.5 IU/g dry substrate	637.25 IU/g dry substrate		
Rodriguez-Fernandez et al. (2011)				65.38 U/g dry solid
Dave et al. (2012)	4.87 U/g substrate	124.44 U/g substrate	28.52 U/g substrate	
Kim and Kim (2012)		6.5 U/g solid		8.8 U/g solid
Qian et al. (2012)				508 U/g

scale, the optimal temperature was found to be 30–33 °C given a 50 % moisture and 0.5–0.6 cm depth of the substrate. It was shown that growth was low when the temperature was below 23 °C and burn out took place at 43 °C. Then by fixing the temperature, optimal moisture content was found between 40 and 50 %. At pilot-scale, a reactor made up of wooden trays with 15 cm gap in between was used. When tests were run naturally, implying no action except ventilation, the temperature at the substrate surface was at 31–32 °C, while it reached 45 °C at 1.0–1.2 cm depth. But when the temperature and moisture were regulated, maximum xylanase activity was 1.5-folds higher in 64 h of fermentation. These results are one of the few references of SSF experiments using an important amount of waste and the closest to upscaling tests.

Kang et al. (2004) investigated the ability of *Aspergillus niger* KK2 to produce cellulases and hemicellulases in SSF by using rice straw and wheat bran. Five grams of substrate were incubated at 65 % moisture at 28 °C. The enzyme extract was assayed for endoglucanase, filter paper (FPase), β-glucosidase, β-xylosidase and xylanase activities. Kang et al. (2004) considered that the production of cellulases and hemicellulases were substrate-dependent. Therefore, the choice of the appropriate inducing substrate is essential for enzyme production. In this perspective, different rice straw to wheat bran ratios were tested to check the impact on enzyme production. Maximum FPase and xylanase were recorded on solely rice straw and maximum β-xylosidase under 1:4 ratio. Interestingly CMCase and β-glucosidase activities were similar irrespectively of the tested ratio. Those findings agree with those of Dave et al. (2012). Maximum enzyme activities were generally obtained after 5–6 days of incubation.

In the work of Acharya et al. (2010), cellulase production was carried out using 5 g wheat straw as the substrate, an anaerobic treated distillery spent

wash as the medium and *Aspergillus ellipticus* as the inoculum. Tween 80 was also added with the inoculum and incubation took place at 30 °C for 7 d. Crude enzyme mixture was analyzed for FPase, CMCase and β-glucosidase activities. Using the RSM method, four factors at three levels each were tested: initial pH of the effluent, moisture ratio, effluent concentration in % v/v and inoculum size. Results showed that maximum enzyme activities occurred at 1.4×10^8 spores inoculum level but varying pH and effluent concentrations: maximum FPase occurred at pH 5 and 60 % effluent concentration, maximum β-glucosidase at pH 3 and 40 % effluent concentration and CMCase at pH 5 and 40 % effluent concentration.

Rodriguez-Fernandez et al. (2011) focused mainly on optimizing aeration conditions for pectinase and xylanase production via SSF using *A. niger* F3. For that purpose, a horizontal drum bioreactor was used. It was filled with nutrient supplemented 2 kg citrus peel substrate at 60 % initial moisture and pH 5. Saturated air was supplied into the bioreactor via a compressor and the drum was connected to a system with sensors to analyze O_2 and CO_2 concentration of the gas outlet. SSF was run at 30 °C. The fermentation time was fixed at 96 h and aeration intensity was tested at four levels 0.50, 0.75, 1.00 and 1.25 V kg M (volumetric air flow L air/kg medium/min). First results showed that maximum synthesis of pectinases and xylanases took place at 1.0 V kg M. It is important to note that only 0.2 V kg M is needed to oxidize the substrate; this amount is calculated by considering the oxygen required to carry out the total oxidation of the organic materials present in the medium. But since air plays an essential role in the removal of the heat generated during the oxidation, an additional intensity is required for the SSF (Gervais and Molin 2003). Under optimal conditions, the rate of synthesis of the two enzymes was different. Pectinase production was at its highest at 72 h of fermentation while that of xylanase increased after 72 h.

Finally, Qian et al. (2012) tested the impact of operational conditions of different carbon sources on β-glucosidase production and fermentation productivity (FP), defined as the percentage of dry weight of fermented product to the dry weight of the initial solid substrate. Experiments were conducted using 10 g of solid medium, supplemented nutrients and inoculated with *A. niger*. Five parameters were optimized using the OFAT approach in the following order: initial moisture content (IMC), inoculum level, initial pH, incubation temperature and finally fermentation period. Results showed that maximum growth of the mycelium does not necessarily coincide with maximum enzyme production. Optimal pH, temperature and fermentation time were 6, 28 °C and 72 h, respectively. The two last results were rather expected given the OFAT approach used. Qian et al. (2012) also studied the effect of the ratio of wheat bran/ground corncob and the type of nitrogen source on enzyme activity and FP. They found that those latter were highest at 80/20 ratio. This reflects the impact of the solid support. Wheat bran loosens the solid medium and overcomes the agglomeration of the substrate, thus helping in air diffusion and better oxygen supply for the growth of *A. niger* and easier removal of CO_2 and heat generated during SSF. However, at very high levels, the substrate increases water loss due to quick volatilization during fermentation and hence inhibits the growth of microorganism, subsequently declining the enzyme production. For the nitrogen sources, unlike Krishna (1999) and Membrillo et al. (2008), there was no significant difference between inorganic and organic nitrogen sources.

In terms of operational conditions with the different experiments using *Aspergillus*, most differences can be noted with the moisture content which varies between 40 and 70 % while using wheat bran as the substrate (Table 1). In addition to that, both Lu et al. (2003) and Kang et al. (2004) used relatively high pH values (neutral pH) which are not common using filamentous fungi. Most authors focused on xylanase activity while using *Aspergillus*; recorded activity values vary very significantly eve using the same substrates. Xylanase titers recorded in Table 2 can be 270 times higher (Kang et al. 2004); these differences cannot actually be explained by the differences in experimental conditions.

4.1.2 *Pleurotus species*

Reddy et al. (2003) tested the growth of *Pleurotus ostreatus* and *Pleurotus sajor-caju* on leaf biomass and pseudostems of banana waste. The objective was to produce lignolytic and cellulolytic enzymes such as laccase, FPase and CMCase. The authors were mostly interested in laccase, a lignin modifying extracellular oxidoreductase, which activity appears to be regulated by morphogenesis (Reddy et al. 2003). For the SSF process, 25 g of each substrate incubated at 25 °C.

Samples were collected from day 10 to day 40 at 5-day intervals. This is a wide range of follow-up period although it is missing the first period during which most of the other works in the literature were based. Both *P. ostreatus* and *P. sajor-caju*, grown on either type of tested biomass, produced significant titers of laccase activity, but very low CMCase and FPase activities. The production of laccase on leaf biomass was also twice as high as that on pseudostems. Nevertheless, the maximum laccase activity was 16 times lower than that obtained by other researchers with *P. sajor-caju* grown on rubber tree sawdust (Tan and Wahab 1997). FPase activity is actually essential to degrade high ordered or crystalline forms of cellulose acting synergistically with CMCase active enzymes. Given the low values produced of FPase and CMCase and high titers of laccase, the authors noted that with this process lignin can be used while leaving intact the cellulose, making the banana waste a good source of animal feed (Reddy et al. 2003). These results actually imply that the experimental conditions are not favorable for enzyme production; in fact this latter actually occurs during the first days of the process.

Another work was conducted by Membrillo et al. (2008) using sugar cane bagasse for the production of protein and lignocellulolytic enzymes with two *P. ostreatus* strains (CP-50 and IE-8). The authors focused on the impact of the inorganic nitrogen source and substrate particle size on enzyme production. Five grams of substrate at 80 % moisture were incubated at 29.5 °C for 8 days under static conditions. *P. ostreatus* IE-8 showed differences in mycelium growth due to the N source while the strain CP-50 did not show any significant difference. Results revealed important differences in the inherent characteristics of every strain which make generalization difficult even within the same species of microorganisms.

In general, as shown in Table 2, the poorest performance in terms of enzymatic activities were recorded with *Pleurotus* species.

4.1.3 *Trichoderma species*

Deschamps et al. (1985) compared the solid state cultivation of *Trichoderma harzianum* on 80:20 mixture of straw and wheat bran under both static and mixed non-aseptic conditions. For all experiments, pH was controlled and cellulase yield and endoglucanase activity were measured. For the static SSF, optimal conditions were found at 30 °C, pH 5.8, 74 % initial moisture content and aeration rate of 6–8 L/h. For mixed SSF assays, the optimal incubation temperature was also 30 °C, with 65 mL/h spray water rate. Optimal moisture was found reduced to 69 % most probably due to the mixing. The aeration rate was increased progressively from 4 to 40 L/min. During the experiment, the pH decreased from 5.7 to 5.2 and then increased to 6.3 at 66 h of incubation. The authors consider that the decrease in pH may be due to the ammonium uptake during the growth phase while the pH increase results from the loss of protein due to mycelium autolysis. Overall results show that during the upscaling test, cellulase yield was reduced by almost 40 % and endoglucanase activity by 25 %. These are important data points to be taken into account for upscaling. Operational parameters should therefore be modified in order to reduce enzyme activity losses during scale changes. Enzyme activities found in this work were among the highest reported in the literature (Table 1).

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

While using comparable matrices (Table 1), enzyme activity results were more important for Guowei et al. (2011) as shown in Table 2. This could be mainly due to the lowest moisture content and the higher follow-up period: 74 and 50 % moisture and 66 h and 5 days for Deschamps et al. (1985) and Guowei et al. (2011) respectively.

4.1.4 *Other species*

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

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

And Deswal et al. (2011) carried out SSF using the brown-rot fungus *Fomitopsis* sp. RCK2010. Five grams of wheat bran were incubated at 30 °C. Taking one factor at a time, Deswal et al. (2011) optimized the following parameters: pH, substrate to moisture ratio, N source, amino acid, vitamins and surfactant addition. Optimal conditions were found at pH 5.5 and 30 °C for FPase, CMCase and β-glucosidase after 16 days of incubation.

Given the different operational conditions (Table 1) for this section, enzyme activities reported in Table 2 were comparable.

4.2 With substrate pretreatment

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

Chahal (1985) was the first author to use a pretreated substrate in a SSF process. Two mutant strains of *T. reesei* (QMY-1 and Rut-C30) were tested on two types of substrates: wheat straw (WS) and aspen pulp prepared by chemical-thermomechanical process (CTMP). The substrates were pretreated using sodium hydroxide at 121 °C. Hemicelluloses and lignins were thus solubilized and retained in the medium. Chahal (1985) believed that the lignin and hemicellulose that were kept in the system played an important role in the increase of cellulase yield. After pretreatment, the pH was adjusted to 5.8. For the production of cellulase, 5 g of substrate were autoclaved and incubated at 30 °C with 80 % final moisture. Cellulase, β-glucosidase, and xylanase titres were measured on the enzyme extracts. Half the concentration of nutrients was sufficient to reach the optimum cellulase titre as well as cellulase yield. Pretreatment was also important since the two mutants showed a significant decrease in enzyme production when the untreated WS was used.

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

Another reference with alkaline pretreatment is that of Kim and Kim (2012). The authors used empty palm fruit bunch fiber (EPFBF) as the substrate for solid state bioconversion and production of cellulase enzymes using *Penicillium verruculosum*. Characterization of the substrate showed that due to the pretreatment the percentage of cellulose increased by 18 % and that of hemicellulose decreased by 13 %. Results showed that avicelase, CMCase and xylanase activities reached the maximum at day 6 of the SSF process.

Unlike previous references, Chen et al. (2011) used *T. reesei* YG3 to study the biodegradation of fractioned steam exploded corn stover in solid-state fermentation. Corn stover samples were manually separated as leaf, shell and core. Each fraction was then separated into miscellaneous cells (MC) and fascicule (FC). SSF was carried out in a Petri dish which was loaded with 10 g dry weight substrate at 30 °C for 7 days. In general, leaf was the best fermentation substrate with highest cellulase yield as well as filter paper, CMCase and β-glucosidase activities. Chen et al. (2011) argued that cellulase is an induced enzyme that is when the substrate is not easily hydrolyzed, the fungus is prone to produce cellulase continuously and massively. Both cellulose and hemicellulose are inducers of cellulase, but the former is degraded more slowly; interestingly leaf contained the highest amount of hemicellulose.

As shown in Table 1, the substrates used in this section are varied and certain experimental conditions are unique: the longest follow-up period is found for Chahal (1985) and a fermentation temperature of 40 °C is recorded for Jabasingh and Nachiyar (2011). However in terms of enzymatic activities (Table 2),

the highest values were recorded in this section. This could be explained by the pretreatment of the substrate which provides the advantage of increasing the availability of both cellulose and hemicellulose. This does not however apply to empty fruit bunch fiber (Kim and Kim 2012) which led to the lowest enzyme activities. In this case, it is mainly the type of the substrate that is not adequate for the enzyme production.

5 Challenges of SSF process for environmental large scale applications

5.1 Enzyme cost

The major challenge of solid state fermentation applied to environmental processes is the economical context. Until recently the application of SSF has been restricted to high value products that do not include for example anaerobic treatment of waste to produce methane. Although it has been previously demonstrated that the enzymatic pretreatment of solid waste improves the efficiency of its subsequent anaerobic digestion (Berlin et al. 2005; Yang and Wyman 2006; Gusakov et al. 2007; Lee et al. 2008), the cost of commercial enzymes is currently too high to consider their use as a feasible waste pretreatment for large-scale production. The cost of enzymes is a major contributing factor in the cost of pretreatment. Tu et al. (2007) indicate that the cost of cellulase and β-glucosidase has been estimated to account for approximately 50 % of the cost of the hydrolysis process for the production of ethanol from softwood for example. The high enzyme loadings currently needed to achieve reasonable rates and yields are other contributing factors in the high cost (Yang and Wyman 2006). Parmar et al. (2001) approximated the overall treatment of 1 dry ton of sewage sludge at US100$. As for Jordan and Muller (2007), initial estimates of the cost of enzymes used to treat 1ton of spent mushroom compost in a liquid–solid system is 803.9€ excluding any mechanical or operational expenses. Berlin et al. (2005) indicated that the cost of cellulase for hydrolysis of pretreated corn stover was reduced by 20–30-fold from 2001 to 2005 due to lowered production costs and increases in enzyme production. Nevertheless, at least another threefold reduction is needed for full scale process commercialization (Berlin et al. 2005).

In this context, the in situ production of lignocellulolytic enzymes using SSF processes could overcome this drawback, but first the SSF process should be adapted to reconcile appropriate productivity requirements for enzyme activities and lower economic revenues. Given current treatment costs and valorization prices of methane, the cost of enzymes should be below 10€/t of waste treated for the enzymatic treatment to be economically feasible.

5.2 Optimal operational parameters

To properly run a solid state fermentation process the following parameters should be considered: These include substrate type and particle size, substrate pretreatment, inoculum, nutrient supplementation, moisture content, pH, aeration, temperature and mixing (Lynd et al. 2002; Pandey et al. 2008b). In the experimental work reviewed earlier, researchers considered one or more of those factors to optimize enzyme production. Table 1 summarizes the different experimental conditions used.

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

5.3 Reactor design

Industrial equipment is currently available for SmF but there is an important need for engineering and new equipment design under solid state. SSF is an aerobic process in which oxygen requirement is supplied by the oxygen present in the gaseous air and the one

present in dissolved form in the water associated with the solids (Muniswaran et al. 2002). In the SSF bed, the oxygen diffusion rate depends upon the transport properties of the bed which shrinks due to mycelial growth. This latter changes the bed porosity and hence the effective diffusivity: the carbon dioxide traveling in the opposite direction hampers the oxygen transport into the bed (Muniswaran et al. 2002). At lab-scale, SSF systems are usually flushed with oxygen at different rates but at the industrial scale, aeration systems vary according to the substrate type and also the bioreactor design. Aeration plays in fact also an important role in heat dissipation and regulation of the moisture level. In practice, only air is used for temperature control in SSF which requires a large quantity of air, exceeding the amount necessary for microbial respiration (Gervais and Molin 2003; Rodriguez-Fernandez et al. 2011). The SSF process is also highly exothermic mainly due to the production of CO_2 and consumption of O_2; each mole of CO_2 produced during the oxidation of carbohydrates releases 673 kcal (Raimbault 1997). Pandey (2003) confirms that sometimes the accumulation of heat could lead to temperatures in some locations of the bed 20 °C higher than the incubation temperature. For this reason, temperature is considered a crucial parameter that contributes to the success of the SSF process.

Therefore, the suitable bioreactor design should overcome heat and mass transfer effects and allow easy diffusion and extraction of metabolites. SSF fermenters could be either static, intermittently mixed or continuously mixed, with and without forced aeration through the biomass.

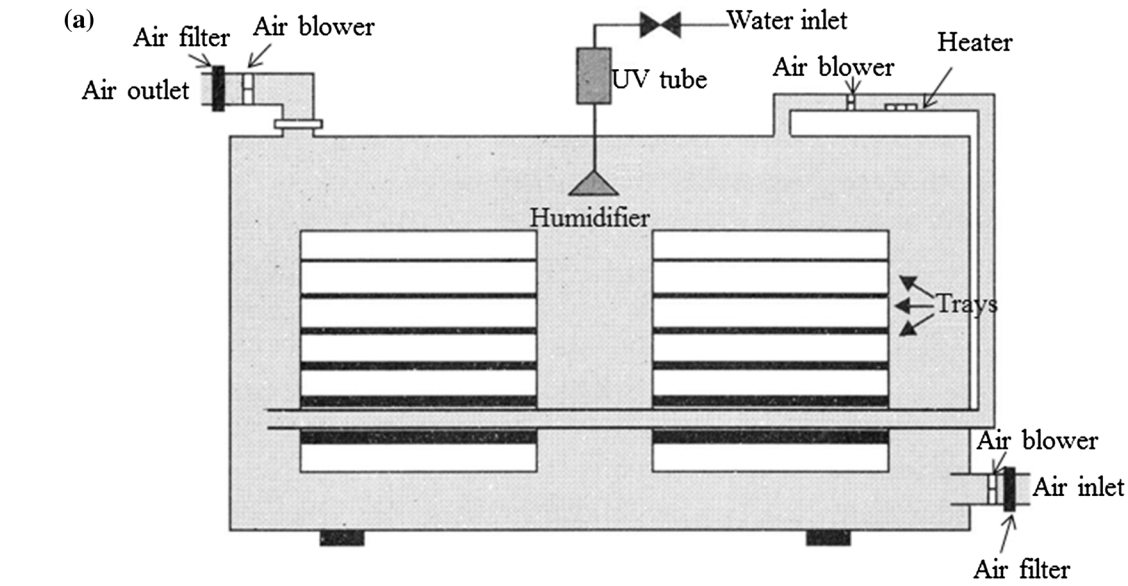
According to Pandey et al. (2008a), there are generally two main types, the tray type and the rotating drum type which could be run with and without mixing (Fig. 2). In addition to those, column and deep trough type of bioreactors have recently emerged but their scaling poses serious problems (Pandey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

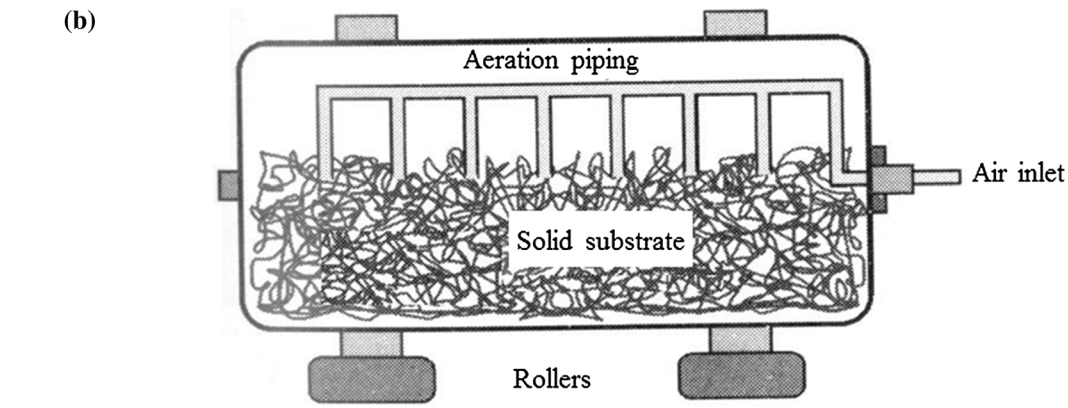
Fig. 2 Schematic diagrams of different types of SSF reactors ▶ **a** koji-type tray reactor, **b** rotating drum bioreactor and **c** packed column bioreactor system (from Sermanni and Tiso 2008)

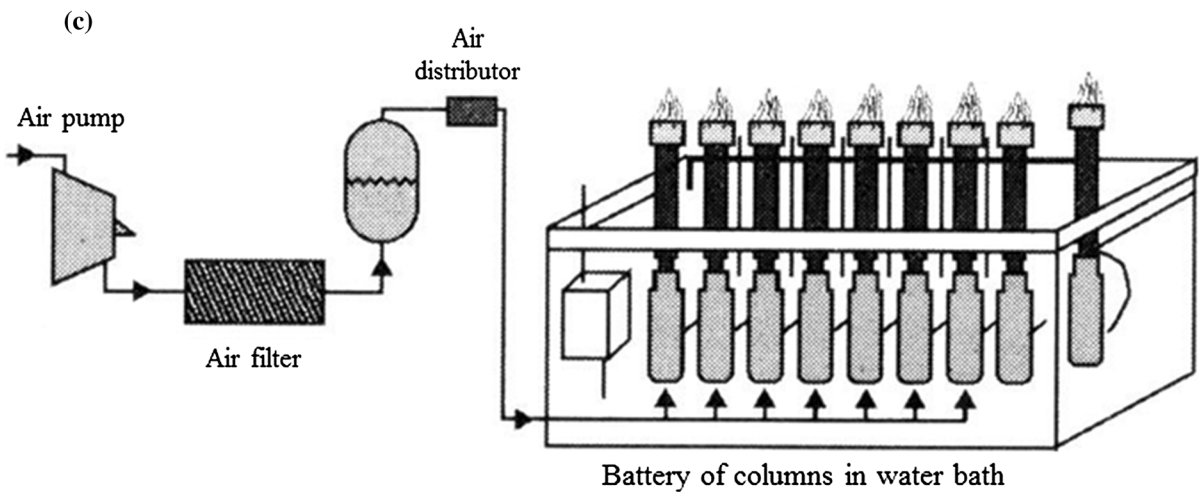
5.4 Composition and stability of the enzymatic mixture

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

This brings the attention to the point concerning the efficiency of the produced enzymes. The objective behind enzyme production is their downstream use in the biodegradation of lignocellulosic matrices. In the reviewed work, only few authors evaluated this efficiency through saccharification potential tests. Chahal (1985) tested the hydrolytic potential of the cellulase system produced at pH 4.8 and 6.7; they found that 65 % of the hydrolysis occurred during the first 20 h for both pH levels. After 96 h of hydrolysis,







almost 90 % of the saccharification potential was expressed. In Kalogeris et al. (2003), an enzyme saccharification test of cellulose was also carried out but interestingly at 60 °C; after 48 h only 29 % cellulose hydrolysis took place. Another low 20 % saccharification level of wheat bran was recorded by Acharya et al. (2010) after 8 h of incubation. Deswal et al. (2011) compared the enzymatic hydrolysis of untreated and alkali pretreated rice straw and wheat straw matrices using crude enzyme extracts. They observed that the alkali treatment releases lignin moieties and thus increases the accessibility to cellulose by the enzymes. Finally, Dave et al. (2012) ran some saccharification tests at 60 °C for wheat straw, rice straw and sugarcane bagasse. Results showed that the concentrations of reducing sugars increased up to 48 h for wheat straw and rice straw and only till 36 h for sugarcane bagasse. Correlation data between added enzymatic activities, sugar release or hydrolysis and the different operational parameters should be studied. This will be used to define appropriate hydrolysis conditions depending upon the type of matrix used.

Finally, in commercial enzymatic solutions chemical stabilizers are added to maintain the quality of the product. But when considering SSF crude enzyme mixtures, their tolerance for mainly pH and temperature variations dictates or at least limits the options of their downstream use. For this purpose, some authors have studied pH stability as well as the thermostability of their produced crude enzyme extracts. In this context, Kalogeris et al. (2003) determined the stability profile of the enzyme mixture in a temperature range of 50–90 °C and pH range of 3–11. Results showed that the entire enzymatic activity was retained at pH values of 5–8. Focusing mainly at endoglucanase and β-glucosidase, it was found that at 70 °C their half-lives were respectively 2.5 and 1 d, but decreased to 42 and 18 min at 80 °C. Acharya et al. (2010) also tested the activities of the same enzymes at temperature varying between 30 and 80 °C for 60 min and pH of 3–10. The residual activities of both enzymes remained very high until 60 °C but dropped to 50 % at 80 °C. In addition to that, optimal pH was 4, while 80 % of relative activity was observed at pH 7 and only 30 % at pH 10. Finally, Dave et al. (2012) tested enzyme thermostability (from 30 to 100 °C) and pH stability (from 3 to 10) and found that relative activities at 30 °C for endo-β-1,4-glucanase and β-glucosidase were 35 and 10 %, respectively, increased up to 100 % at 70 °C and then dropped to 60 and 45 % at 100 °C. In general, endo-β-1,4-glucanase was highly stable in the pH range of 3–6 (relative activities higher or equal to 85 %) and β-glucosidase showed high stability in the pH range of 3–8 (relative activities higher or equal to 90 %). Maximum activities for both enzymes were measured at pH 4.

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

6 Conclusions

The main driving force behind the development of SSF processes for environmental applications, more specifically anaerobic digestion of lignocellulose-rich substrates, is a serious need for low cost large-scale production of enzymes. Both purchase and production costs of commercial cellulases contribute to a large proportion of the total costs of bioenergy production. Taking this into account, on-site cellulase production under SSF from lignocellulosic biomass could be considered as cost-effective strategy (Hideno et al. 2011). Within this context, research has been conducted to find optimal operational conditions for enzyme production at lab-scale. Nevertheless, major challenges remain for large scale development in environmental applications. Operational parameters, mainly substrate autoclaving and inoculum, are developed without taking into account the economics of the change in scale. Very few authors looked into the efficiency of the crude enzyme mixtures and their stability in the process. Large scale equipment is available for submerged fermentation but not for the solid state. For SSF to be up to the challenge of the new energy era; future research should be reoriented to answer both technical and economic feasibilities of large scale applications.

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