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MINI-REVIEW

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Biotechnological advantages of laboratory-scale solid-state fermentation with fungi

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Abstract Despite the increasing number of publications dealing with solid-state (substrate) fermentation (SSF) it is very difficult to draw general conclusion from the data presented. This is due to the lack of proper standardisation that would allow objective comparison with other processes. Research work has so far focused on the general applicability of SSF for the production of enzymes, metabolites and spores, in that many different solid substrates (agricultural waste) have been combined with many different fungi and the productivity of each fermentation reported. On a gram bench-scale SSF appears to be superior to submerged fermentation technology (SmF) in several aspects. However, SSF up-scaling, necessary for use on an industrial scale, raises severe engineering problems due to the build-up of temperature, pH, O_2, substrate and moisture gradients. Hence, most published reviews also focus on progress towards industrial engineering. The role of the physiological and genetic properties of the microorganisms used during growth on solid substrates compared with aqueous solutions has so far been all but neglected, despite the fact that it may be the microbiology that makes SSF advantageous against the SmF biotechnology. This review will focus on research work allowing comparison of the specific biological particulars of enzyme, metabolite and/or spore production in SSF and in SmF. In these respects, SSF appears to possess several biotechnological advantages, though at present on a laboratory scale only, such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of microorganisms specialized for water-insoluble substrates or mixed cultivation of various fungi, and last but not least, lower demand on sterility due to the low water activity used in SSF.

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Introduction

Free water does not appear to be the natural milieu for the majority of microorganisms. Not even marine microorganisms prefer swimming in free seawater since more than 98% of isolates from the marine environment have been obtained from the underwater surfaces of solid substrates, and less than 1% of all known fungi have been found in marine habitats (Carlile and Watkinson 1994; Kelecom 2002). The evolution of higher fungi took place on solid growth substrates. Ascomycetes and Basidiomycetes spent their evolutionary history as terrestrials, with only some species adapting to water later in their evolution. Fungal products of biotechnological interest, i.e. enzymes, secondary metabolites and spores, were developed for use in moist solid substrates but not in liquids. Consequently, the cultivation of microorganisms in aqueous suspension may rather impair their metabolic efficiency. In this respect, submerged fermentation technology (SmF) may be considered a kind of violation of the natural habitats of wild-type microorganisms. However, solid state (substrate) fermentation (SSF) is currently used only to a small extent for enzyme and secondary metabolite production because of severe process engineering problems. On the other hand, very efficient microbial strains, well adapted to submerged fermentation by genetic engineering are available for enzyme production on an industrial scale.

SSF is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and energy source. The fermentation takes place in the absence or near absence of free water, thus being close to the natural environment to which microorganisms are adapted (Pandey et al. 2000). More generally, SSF can be

understood as any process in which substrates in a solid particulate state are utilized (Mitchell et al. 2000b).

The aim of SSF is to bring the cultivated fungi or bacteria into tight contact with the insoluble substrate and thus to achieve the highest substrate concentrations for fermentation. This technology results, although so far only on a small scale, in several processing advantages of significant potential economic and ecological importance as compared with SmF (Table 1). However, there are also several disadvantages of SSF, which have discouraged use of this technique for industrial production. The main obstructions are due mainly to the build-up of gradients—of temperature, pH, moisture, substrate concentration or pO_2—during cultivation, which are difficult to control under limited water availability.

A considerable amount of work has been done in recent years to understand the biochemical engineering aspects of SSF processing (Mitchell et al. 2000a, 2000b; Pandey 2003). It is rather surprising that the technical problems of SSF have not yet been solved, as SSF is one of the oldest biotechnological processes known. Already 5,000 years ago fungi were cultivated in SSF for the production of food, e.g. probably the oldest known fermentation of rice by *Aspergillus oryzae* was used to initiate the koji process; the mould *Penicillium roquefortii* has been used for cheese production for 4,000 years, and soja sauce has been produced in Asia and bread in Egypt since 3,000 years ago (Pandey et al. 2001).

Biotechnological applications of SSF are widespread (for reviews see Raimbault 1998; Pandey et al. 2000, 2001). Reviews concerning the production of secondary metabolites (Balakrishnan and Pandey 1996; Robinson et al. 2001), aflatoxins (Barrios-Gonzalez and Tomasini 1996), technical enzymes (Pandey et al. 1999), bacterial enzymes (Babu and Satyanarayana 1996), starch saccharifying enzymes (Selvakumar et al. 1998), cellulase (Cen and Xia 1999), cellulolytic enzymes (Nigam and Singh 1996b), Chinese food (Han et al. 2001), the bioconversion of lignocellulose (Tengerdy and Szakacs 2003), mushroom cultivation and natural flavours (Wang 1999), and protein-enriched food (Nigam and Singh 1996a) have also been published.

This review will focus on recently published research work allowing direct comparison of biotechnological production of enzymes, metabolites and spores in SSF and SmF. In addition to the above-mentioned processing advantages on a laboratory scale, SSF also possesses several biological advantages when compared with submerged fermentations. Such advantages include higher fermentation productivity, less catabolic repression, low water demand and hence, lower sterility demand due to the low water activity, cultivation of microorganisms requiring a solid support, and mixed cultivation of various fungi (Table 1).

Production of enzymes

Approximately 90% of all industrial enzymes are produced in SmF, frequently using specifically optimized, genetically manipulated microorganisms. In this respect SmF processing offers an insurmountable advantage over SSF. On the other hand, almost all these enzymes could also be produced in SSF using wild-type microorganisms (Filer 2001; Pandey et al. 2001). Interestingly, fungi, yeasts and bacteria that were tested in SSF in recent decades exhibited different metabolic strategies under conditions of solid state and submerged fermentation.

Table 1 Biotechnological advantages of solid state fermentation (SSF) against submerged technology (SmF)

Advantages	Consequences	Problems to be solved
Biological advantages		
Low water demand	Less waste water	Building of moisture gradients
High concentration of the endproduct	Lower downstream costs	
Catabolite repression significantly lower or missing	Fermentation in the presence of glucose	
Utilisation of solid substrate	High concentration of the growth substrates	Building of substrate gradients
		Building of pH gradients
Lower sterility demands	Mixed cultures of fermenting microorganisms	
Solid support for microorganism		
Simulation of the natural environment	Better performance of cultivated microorganisms	
Fermentation of water-insoluble solid substrates		
Mixed culture of microorganisms	Synergism of metabolic performance	
Processing advantages		
High-volume productivity	Smaller fermenter volumes	
Low energy demand for heating		Building of temperature gradients
Easy aeration		Building of oxygen gradients on a large scale
Utilisation of otherwise unusable carbon sources	Cheap and abundant carbon sources	
No anti-foam chemicals	No lost of microorganisms during fermentation	

Direct comparison of various parameters such as growth rate, productivity or volume activity favoured SSF in the majority of cases. It has also become clear (as mentioned in nearly every review cited) that the cost-factor for the production of "bulk-ware" enzymes in most cases favours SSF over SmF. Tengerdy (1996) estimated fermentation costs of cellulase production at US \$0.2 kg^{-1} in an in situ SSF, in contrast to US \$20 kg^{-1} in a stirred tank reactor.

The low estimated costs of SSF are due to the rather traditional preferential claim of SSF, viz. SSF utilises complex, heterogenous agricultural wastes as substrates and uses low-cost technology regarding sterility and regulation demands. However, attempts to reduce costs by using cheap substrates have hampered biotechnological progress in SSF because of the strongly increased diversity in SSF research. There is no consensus on either the methods, the microorganisms or the substrates used, that would allow comparison with other cultivation technologies. The broad spectrum of substrates used represents an especially severe problem. As already mentioned, one great advantage of SSF has always been the possibility of using substrates that are abundant, cheap, and not applicable to SmF. However, regardless of the differences in process up-scaling, the scientific and technological impact of research data is difficult to compare when results are obtained with different microorganisms producing different products and using such a vast variety of substrates as pineapple, mixed fruit, maosmi waste, wheat rawa with raspberry seed powder, broiler matter, corn stover, almond meal, apple pomace, corncob, barley husk, banana waste, soybean cake, cacao jelly, sweet lime rind, cassava, soybean, amaranth grain, eucalyptus kraft pulp, coffee residues, hardened chickpeas, lignite, rubber or orange bagasse (see Table 2). To facilitate comparison of results, the use of inert substrates as solid supports is becoming increasingly important (Gautam et al. 2002; for review, see Ooijkaas et al. 2000).

Surprisingly, biological parameters, such as the stability of the produced enzymes at high temperature or extreme pH, have also been reported to be better in SSF (Deschamps and Huet 1985; Acuna-Arguelles et al. 1995). Catabolite repression or protein degradation by proteases—severe problems in SmF—were often reduced or absent in SSF (Solis-Pereira et al. 1993; Aguilar et al. 2001). In contrast, much less research has been carried out to evaluate the metabolic differences of microorganisms when cultivated in SSF or SmF.

Aspergillus sp. as a model system

Since 1917, when Currie described the production of citric acids by *Aspergillus niger* in surface cultures on a solid support (cited in Rehm 1967), this fungus has become a model organism for solid-state biotechnology. In recent years scientists have concentrated on enzyme production by *Aspergillus* sp. to clarify the biological background for the observed differences displayed by the microorganisms tested under conditions of the two fermentation techniques (Table 3). One important biological factor in favour of SSF was the low catabolite repression, which appeared to be limiting enzyme production by *Aspergillus niger* in SmF (Nandakumar et al. 1999). The authors investigated the efficiency of *A. niger* CFTRI 1105 in the production of α-amylase and amyloglucosidase. The production of the two enzymes was reduced at glucose concentrations higher than 10 mg ml^{-1} in SmF, whereas in SSF no catabolite repression was observed at glucose concentrations as high as 150 mg ml^{-1} when added to wheat bran as solid substrate. The lack of catabolite repression allowed for fast

Table 2 Variety of some substrates, products and microorganisms involved in solid state fermentation

Substrates	Product	Microorganism	Reference
Almond meal	Lipases	*Rhizopus oligosporus*	Ul-Haq et al. (2002)
Apple pomace, corncob, barley husk	Dye degradation	White-rot fungi	Robinson et al. (2002)
Banana waste	Ligninolytic enzymes	*Pleurotus* sp.	Reddy et al. (2003)
Broiler matter	Biocontrol agent	*Bacillus thuringiensis*	Adams et al. (2002)
Cacao jelly	Endo-polygalacturonase	*Peacilomyces clavisporus*	Souza et al. (2003)
Cassava, soyabean, amaranth grain	Aroma	*Rhizopus oryzae*	Christen et al. (2000)
Coconut cake	Lipases	*Candida rugosa*	Benjamin and Pandey (1997)
Coffee residues	Edible mushroom	*Pleurotus ostreatus*	Fan et al. (2000)
Corn stover	Cellulolytic enzymes	*Fusarium oxysporum*	Panagiotou et al. (2003)
Eucalyptus kraft pulp	Xylanase	*Streptomyces* sp.	Beg et al. (2000)
Hardened chickpeas	Tempeh	*Aspergillus* sp.	Reyes-Moreno et al. (2000)
Lignite	Solubilised coal	*Trichoderma atroviride*	Hölker and Höfer (2002)
Orange bagasse	Pectinase	*Thermoascus aurantiacus*	Martins et al. (2002)
Pineapple, mixed fruit, maosmi waste	Citric acid	*Aspergillus niger*	Kumar et al. (2003)
Rubber	Recycling	*Gordonia* sp.	Arenskötter et al. (2003)
Soybean cake	Proteases	*Penicillium* sp.	Germano et al. (2003)
Tahiti lime	Pectinases	*Aspergillus* sp.	Dartora et al. (2002)
Wheat rawa with rashberry seed powder	Neomycin	*Streptomyces marinensis*	Ellaiah et al. (2003)

growth of the fungus in the presence of high sugar concentrations (Favela-Torres et al. 1998).

Viniegra-Gonzalez and coworkers (2003) demonstrated, using logistic and Luedekind-Piret equations, that the higher productivity of invertase, pectinase and tannase in SSF was due to better growth of *A. niger* in SSF, resulting in higher biomass production, and more efficient biosynthesis of enzymes under conditions without catabolite repression. Moreover, the breakdown of enzymes by contaminating proteases was eight times higher in SmF than in SSF. This is in accordance with previously published results (Acuna-Arguelles et al. 1995) demonstrating up to 50 times higher production of exo-pectinase by *A. niger* CH4 in SSF as compared to SmF. In addition, the exo-pectinase produced in SSF was more heat- and pH-stable although its K_m for pectin as substrate was higher than that of the enzyme from SmF. Diaz-Godinez and coworkers (2001) found in the same system, using polyurethane as an inert carrier, that catabolite repression by sucrose occurred only in SmF; in SSF sucrose addition enhanced the enzyme activity. Also, unwanted protease activity was very low, whereas the growth rate, and consequently the final biomass, was higher due to a better oxygen supply in SSF.

The lack of catabolite repression in SSF was also reported by Solis-Pereira and coworkers (1993). These authors found that more exo-pectinase per gram biomass was, indeed, produced in SmF than in SSF; however, the enzyme obtained from SSF exhibited maximal values of exo-pectinase activity because of the low protease level. Maldonado and Strasser de Saad (1998) obtained comparable results with pectin as sole carbon source. SSF cultures of *A. niger* produced higher amounts of pectin esterase and polygalacturonase and required a shorter time for enzyme secretion. A shorter cultivation time to produce α-L-rhamnosidase and a better activity: inducer ratio was shown for *Aspergillus terreus* in SSF by Elinbaum et al. (2002). Aguilar and coworkers (2001) described the advantages of tannase production by *A. niger* in SSF. The yield of enzyme and its catalytic activity were higher than in SmF. Further advantages of SSF technology were the usage of high concentration of tannic acid leading to a concomitant increase in enzyme activity (as compared with SmF) and significantly reduced catabolite repression by glucose (50 g l^{-1}).

The lack of catabolite repression is not a general rule, but depends on the synthetic pathways of individual enzymes. Blandino and coworkers (2002) demonstrated in *Aspergillus awamori* that exo-polygalacturonase was repressed by glucose released from starch, whereas endo-polygalacturonase was not. Asther and coworkers (2002) showed different enzymatic profiles during the cultivation of *A. niger* using sugar beet pulp both as solid support and as carbon source in SSF and SmF. Two additional esterases hydrolysing methyl caffeate and methyl *p*-coumarate were found only in SSF cultures. Northern blot analysis demonstrated the expression of feruloyl and cinnamoyl esterases under both fermentation conditions; however, the catalytic activities were significantly higher in SSF than in SmF.

Recently, optimisation of enzyme production by *A. niger* in SSF [e.g. phytase (Mandviwala and Khire 2000) and xylanase (Park et al. 2002)] was achieved using statistical response surface methodologies. Phytase—hydrolysing phytic acid to inositol and phosphoric acid—has been used to reduce the environmental loading by phosphorus from animal production facilities. The enzyme activity was optimized up to 884 U g^{-1} substrate after 144 h of fermentation in SSF, a value compatible with SmF (Krishna and Nokes 2001). Phytase production by *A. niger* seems to be correlated with fungus morphology. Phytase production was similar in SSF and SmF during substrate-dependent growth as filamentous mycelia or in small pellets, but was higher compared with growth in the large pellets that occurred predominantly in SmF (Papagianni et al. 1999).

The molecular and physiological reasons underlying the different behaviour of fungi in SSF and SmF are presently best understood in *Aspergillus oryzae* (Biesebeke et al. 2002). An interdisciplinary research group in The Netherlands demonstrated that heat production and oxygen uptake resulted mainly from the aerial growth of fungal mycelium. At low water activity, *A. oryzae* accumulated glycerol, erythriol and arabitol. Three different proteins (35, 28 and 20 kDa) secreted by the fungus were identified and found only during the SSF process. Northern analysis showed that at least two protease genes were expressed in SSF but not in SmF. Molecular differences in gene expression depending on the fermentation technique have been described by Ishida and coworkers (2000). These authors found that of the two genes *gla*A and *gla*B (both coding for glucoamylase), the latter was expressed significantly only under SSF conditions. Promoter motifs were identified that indicated that *gla*B was induced by starch, high temperature (42°C), low water activity and physical barriers to hyphal extension.

Production of enzymes by other microorganisms in SSF

SSF possesses advantages for enzyme production also by other fungi (Table 4). Higher enzyme production in SSF as compared with SmF has been described for xylanase by *Melanocarpus albomyces* IIS-68 (Jain 1995), endopolygalacturonase by *Peacilomyces clavisporus* 2A.UMIDA.1 (Souza et al. 2003), and β-galactosidase by *Klyveromyces lactis* (Becerra and Gonzalez Siso 1996). The lack of catabolite repression in SSF technology, as demonstrated for *Aspergillus* sp., was an important finding also with other microorganisms, both fungal and bacterial. The production of xylanase by *Penicillium canescence* 10-10c in SSF was, in contrast to SmF, not repressed by high glucose or xylose concentration (Bakri et al. 2003). For *Rhizopus oryzae*, however, catabolic repression of tannase was observed. After 70 h cultivation, tannase activity decreased rapidly. Nevertheless, the effect might also be

Table 3 Enzyme production by *Aspergillus niger* in SSF and SmF

Product	Microorganism	Parameter compared	SSF	SmF	Reference
Alpha-amylase	*Aspergillus niger* CFTRI 1105	Catabolite repression	Low	High	Nandakumar et al. (1999)
Amyloglucosidase	*A. niger* CFTRI 1105	Catabolite repression	Low	High	Nandakumar et al. (1999)
Amyloglucosidase	*A.* sp GP-21	Production	Higher	Lower	Mamo and Gessesse (1999)
Beta-fructofuranosidase	*A. niger*	Productivity (U l^{-1} h^{-1})	149.1	58.3	Ashokkumar et al. (2001)
Beta-fructofuranosidase	*A. niger* (mutant)	Productivity (U l^{-1} h^{-1})	322	154.2	Ashokkumar and Gunasekaran (2002)
		Catabolite repression	No	Yes	
Endo-pectinase	*A. niger* CH4	Productivity (U ml^{-1} h^{-1})	0.06	0.01	Acuna-Arguelles et al. (1995)
		pH-stability	Higher	Lower	
		Heat stability	Higher	Lower	
		Substrate inhibition	No	Yes	
Esterase	*A. niger* I-1472	Production (nkat/mg dw vs nkat/ml)	20	0.4	Asther et al. (2002)
		Biomass (ergosterol content mg^{-1} g dw^{-1})	2	1	
Exo-pectinase	*A. niger* C28B25	Activity (IU l^{-1})	7,150	1,714	Diaz-Godinez et al. (2001)
		Catabolite repression	No	Yes	
Exo-pectinase	*A. niger* CH4	Productivity (U ml^{-1} h^{-1})	0.14	0.0002	Acuna-Arguelles et al. (1995)
		pH stability	Higher	Lower	
		Heat stability	Higher	Lower	
		Substrate inhibition	No	Yes	
Invertase	*A. niger* (mutants)	Production	Higher	Lower	Montiel-Gonzales et al. (2002)
Invertase	*A. niger* (C28B25/N-402/Aa20)	Maximal activity (U l^{-1})	3,663	1,180	Romero-Gomez et al. (2000)
		Productivity (U l^{-1} h^{-1})	87	20	
Lipase (acidic)	*A. niger NCIM 1207*	Production (IU/g dw vs IU/ml)	630	18	Mahadik et al. (2002)
Pectin-esterase	*A. niger*	Production in 24 h	4	1	Maldonado and Strasser de Saad (1998)
		Fermentation time	Shorter	Longer	
		Catabolite repression	Low	High	
		Productivity (U l^{-1})	3.16	1.14	
Pectin lyase	*A. niger* 148	Production	3	1	Taragano and Pilosofa (1999)
Pectin lyase	*A. niger* CH4	Productivity (U ml^{-1} h^{-1})	0.008	0.0002	Acuna-Arguelles et al. (1995)
		pH-stability	Higher	Lower	
		Heat stability	Higher	Lower	
		Substrate inhibition	No	Yes	
Phytase	*A. niger*	Production	884 U g^{-1}	Comparable	Krishna and Nokes (2001)
Polygalacturonase	*A. niger*	Production in 24 h	6	1	Maldonado and Strasser de Saad (1998)
		Fermentation time	Shorter	Longer	
		Catabolite repression	Low	High	
		Productivity (U l^{-1})	2.28	0.48	
Rhamnosidase	*Aspergillus terreus*	Cultivation time	Shorter	Longer	Elinbaum et al. (2002)
Tannase	*A. niger* Aa-20	Activity (IU l^{-1})	12,000	2,500	Aguilar et al. (2001)

Table 3 (continued)

Product	Microorganism	Parameter compared	SSF	SmF	Reference
		Productivity (IU h^{-1} l^{-1})	2,560	816	
		Catabolite repression	No	Yes	
		Enzyme degradation	No	Yes	

dw dry weight

due to the appearance of toxic substances or to down-regulation by the end product, gallic acid (Kar et al. 1999).

The advantages of SSF apply also to bacteria. Kapoor and Kuhad (2002) investigated the production of alkaline polygalacturonase by *Bacillus* sp. MG- cp-2 under different growth conditions and found maximal catalytic activities of 342 U (ml culture suspension)$^{-1}$ in SmF and 23,076 U (g bulk substrate)$^{-1}$ in SSF. However, these results are not directly comparable since values per volume units (in SmF) are compared with those per weight units (in SSF). Thus, not all results concerning enzyme production by bacteria are consistent. Dey and Agarwal (1999) described 3–4 times higher productivity of a heat stable α-amylase by *Streptomyces megasporus*, and Beg and coworkers (2000) found up to 2.5 times higher productivity of a heat-stable xylanase by *Streptomyces* sp. QG-11–3 when both bacteria were cultivated in SSF. Similarly, *Bacillus subtilis* produced about 12 times more cellulase (Krishna 1999) and several times more pectinase (Kashyap et al. 2003) when cultivated in SSF as compared with SmF. Cultivation of bacteria in SSF, e.g. for enzyme production by *Bacillus thuringiensis*, was successfully scaled up into a 70 m^3 bioreactor, thus reaching a significant industrial scale (Hongzhang et al. 2002).

Mixed culture cultivation for production of enzymes in SSF

A unique, and inimitable, advantage of SSF in cultivation of microorganisms is the possibility to use mixed cultures and thus to exploit metabolic synergisms among various fungi. In natural habitats, fungi typically grow in symbiotic associations on solid substrates such as soils or plant material. Biodegradation or mineralisation of these complex substrates requires participation of a broad spectrum of different enzymes produced by different microorganisms (Gupte and Madamwar 1997; Koroleva et al. 2002; Stepanova et al. 2003). However, results of investigations are difficult to interpret in such cases. Gutierrez-Correa and Tengerdy (1997) investigated growth parameters and enzyme production by either *Trichoderma reesei* LM-UC4 or its mutant LM-CU41 grown on sugar cane bagasse in mixed cultures with *Aspergillus phoenicis* QM329. Biomass, as well as the level of cellulase, endoglucanase and β-glucosidase activities, increased synergistically when the wild strains were cultivated together. This effect was absent when *T. reesei* mutant LM-CU41 was substituted for the wild strain. The authors concluded that the mutant had lost the ability for complex interactions with other fungi. However, the same mutant in a mixed culture with *A. niger* (ATCC 10864) exhibited higher xylanase (Gutierrez-Correa and Tengerdy 1998) and cellulase (Castillo et al. 1994; Gutierrez-Correa et al. 1999) activities than were reached by the individual wild strains. Massadeh et al. (2001) compared mixed cultures of *T. reesei* QM9414 and *Aspergillus terreus* SUK-1 cultivated with sugar cane bagasse as substrate with single cultures

Table 4 Effectiveness of enzyme production by microorganisms in SSF and SmF

Product	Microorganism	Parameter	SSF	SmF	Reference
Alpha-amylase	*Streptomyces megasporus*	Productivity (U min^{-1} mg $protein^{-1}$)	206	643–804	Dey and Agarwal (1999)
Cellulase	*Bacillus subtilis*	Total enzyme production (relative)	12	1	Krishna (1999)
Cellulase	*Trichoderma* sp.	Costs (US $/kg)	0.2	20	Tengerdy (1996)
Laccase	*Pleurotus ostreatus*	Variability of activity	Low	High	Baldrian and Gabriel (2002)
Laccase	*Panus tigrinus*	Total enzyme activity	2.5	1	Fenice et al. (2003)
Ligninase	*Phanerochaete chrysosporium*	Activity	6	1	Fujian et al (2001)
Manganese peroxidase	*Phanerochaete chrysosporium*	Activity	10	1	Fujian et al (2001)
Manganese peroxidase	*Panus tigrinus*	Total enzyme activity	7	1	Fenice et al. (2003)
Polygalacturonase	*Bacillus* sp MG-cp-2	Production U/g vs U/ml	23,706	360	Kapoor and Kuhad (2002)
Tannase	*Rhizopus oryzae*	Activity (U/l)	32.76	23.86	Kar and Banerjee (2000)
Xylanase	*Streptomyces* sp. QG-11-3	Production (U/ml)	203	81	Beg et al. (2000)
Xylanase	*Penicillium canescens* 10-10c	Catabolite repression	No	Yes	Bakri et al (2003)

of the two fungi. These authors indeed found higher concentrations of reducing sugars and higher cellulase activity in the mixed culture, but not the expected synergism to degrade the substrate.

Production of secondary metabolites

The production of secondary metabolites represents another aspect of SSF application which has gained in importance in recent years. Various biologically active secondary metabolites have been produced in SSF (Balakrishnan and Pandey 1996; Barrios-Gonzalez and Mejea 1996; Robinson et al. 2001). The production of secondary metabolites is often coupled with the stationary growth phase of the microorganism used, and depends on N or P limitation together with excess carbon and energy source. In SSF, secondary metabolite production also appears to be triggered by reduced water and nutrient supply. Furthermore, it often depends on the association of microbial mycelia with the solid substrate or with an inert support. Several fungi need a solid substrate as an anchor for optimal growth and productivity. Therefore, genetically modified organisms, which were optimised for liquid cultivation conditions, have often been used in SmF whereas natural isolates have played a major role in SSF.

Red rice, a traditional Asian food, has for thousand of years been prepared by fermentation of steamed rice by *Monascus purpureus* in SSF. The fungus produces six different polyketide pigments coloured from bright yellow to deep red, which have found applications both as food additives and pharmaceuticals (Johns and Stuart 1991; Juzlova et al. 1996). The attempt to cultivate *M. purpureus* in SmF resulted in much lower pigment production (Hsu et al. 2002). SSF has played a key role, particularly in the production of Asian foods, since it operates under semi-sterile fermentation conditions (Han et al. 2001; Su et al. 2003). A mixed-culture of fungi and yeasts, which cannot be established in SmF, can produce, in a synergistic way, various aroma-active components (Nout and Aidoo 2002). Fu and coworkers (2002) identified 70 volatile compounds, 29 of which had aroma activity, when bamboo shoots were fermented in SSF. All of these together were necessary for the traditional food flavour, and could not be produced in that combination by fermentation in SmF.

Another problem that could not be satisfactorily solved in SmF were changes in oxygen supply due to changes in the growth medium during the course of fermentation. In many cases fungi need a highly viscous medium for secretion of the required metabolites. This viscosity is achieved through secretion of polymeric substances during fungal growth. In such cases, SSF appears to be the better alternative since stirrer speed and oxygen supply play no role (Elibol and Muvituna 1997).

Several bioactive secondary metabolites such as antibiotics, mycotoxins (reviewed by Barrios-Gonzalez and Tomasini, 1996), bacterial endotoxins, alkaloids, or plant growth factors, can be obtained in SSF with significantly higher yield than in SmF (Table 5). Especially in the

cultivation of *Streptomycetes*, SSF is a true alternative, because the production of secondary metabolite is coupled to the formation of spores formed by the aerial mycelia of the bacteria. Thus, antibiotics such as penicillin (Barrios-Gonzalez et al. 1993), cephalosporin (Jermini and Demain 1989), cyclosporin A (Balakrishnan and Pandey 1996; Ramana Murthy et al. 1999), tetracycline (Yang and Ling 1989), oxytetracyclines (Yang and Wang 1996), rifamycin (Krishna et al. 2003) and iturin (Ohno et al. 1993) have been fabricated with higher productivity, defined as product concentration per volume, in SSF than in SmF.

The production of iturin by *Bacillus subtilis* in SSF was investigated by Ohno et al. (1993, 1996); iturin production was found to be up to 20 times higher in a 3-kg SSF bioreactor [0.55–0.8 mg (g wet culture)$^{-1}$ day^{-1}] as compared with a 5 l SmF bioreactor [0.0032–0.044 mg (g wet culture)$^{-1}$ day^{-1}]. In addition, iturin from the SSF bioreactor exhibited higher anti-fungal activity, presumably due to a longer side chain (Table 5).

Giberellic acid (GA_3) is a plant growth hormone. It is produced by *Giberella fujikuroi* or *Fusarium moniliforme* as a secondary metabolite in the stationary growth phase. In SSF, GA_3 was produced in higher quantities and shorter fermentation time than in SmF: 240 mg GA_3 kg^{-1} dry material of cassava was harvested after 36 h SSF whereas only 23 mg GA_3 l^{-1} culture medium was produced within 120 h in SmF (Tomasini et al. 1997). In an optimised system, Machado et al. (2002) obtained 492.5 mg GA_3 kg^{-1} coffee husk used as solid substrate. This was 6.1 times higher than the productivity of SmF technology.

Ergot alkaloids, such as lysergic acid diethylamide, are used as pharmaceutical drugs. Hernández and coworkers (1993) demonstrated a 3.9 times higher yield of the alkaloids when *Claviceps fusiformis* was cultivated in SSF as compared with SmF. Differential cultivation of *Claviceps purpurea* in SSF and SmF revealed no difference in the amount of produced ergot alkaloids; however, the spectrum of secondary metabolites differed. This was very likely due to the sensitivity of the fungus to anti-foam substances, the high oxygen demand and/or to the end product inhibition of enzymes synthesizing ergot alkaloids (Balakrishnan and Pandey 1996).

The production of high amounts of mycotoxins, such as ochratoxin A (OTA) and OTB, in shaken solid state fermentations was reported by Harris and Mantle (2001). The final concentration obtained in SSF (up to 10 mg OTA g^{-1} substrate) was significantly higher than that from comparable submerged cultures. Mycophenolic acid was produced by *Penicillium brevi-compactum* in SSF in concentrations of up to 425 mg kg^{-1} wheat bran (Sadhukhan et al. 1999). For both processes the scale-up appears to be problematic. The SSF scale was 40 g and 10 g wheat bran material, respectively.

Despite growing well in SmF, some fungi produce secondary metabolites only in the late stationary phase when cultivated on solid surfaces. The coprophilic fungus *Coniochaeta ellipsoida* produces the tetramic acid antibiotic coniosetin only in SSF (Segeth et al. 2003). In a new type of SSF bioreactors constructed so as to allow several experiments to be run in parallel under almost identical

Table 5 Production of secondary metabolites by various microorganisms in SSF as compared with SmF

Product	Microorganism	Parameter	SSF	SmF	Reference
6-Pentyl-alpha-pyrone	*Trichoderma harzianum*	Productivity	17	1	Sarhy-Bagnon et al. (2000)
Bafilomyycin B1 + C1	*Streptomyces halstedii* K122	Production	Yes (cultivated in thin layers)	No (submerged)	Frandberg et al. (2000)
Benzoic acid	*Bjerkandera adusta*	Production	3.5	1	Lapadatescu and Bonnarme (1999)
Benzyl alcohol	*Bjerkandera adusta*	Production	10	1	Lapadatescu and Bonnarme (1999)
Cephamycin C	*Streptomyces clavuligerus*	Stability	Higher	Lower	Kota and Sridhar (1998)
Coconut aroma	*Trichoderma* sp.	Production	Higher	Lower	de Alberto et al. (2002)
Ergot alcaloids	*Claviceps fusiformis*	Production	3.9	1	Hernández et al. 1993
Giberellic acid GA3	*Giberella fujikuroi*	Production (mg/kg vs mg/l)	492	80	Machado et al. (2002)
Giberellic acid GA_3	*Giberella fujikuroi*	Production (mg/kg vs mg/l)	240	23	Tomasini et al. (1997)
Giberellic acid GA_3	*G. fujikuroi*	Productivity (accumulation)	3.5	1	Balakrishnan and Pandey (1996)
Iturin	*Bacillus subtilis*	Productivity (mg g wet culture^{-1} day^{-1})	0.55–0.8	0.032–0.044	Ohno et al. (1993)
Ochratoxin	*Aspergillus ochraceus*	Yield	Higher	Lower	Harris and Mantle (2001)
Oxytetracycline	*Streptomyces rimossus*	Storage stability (over 6 months)	No loss of activity	Loss of activity	Yang and Wang (1996)
Penicillin	*Penicillium chrysogenum*	Production (mg/l)	13	9,8	Barrios-Gonzalez et al. (1993)
Rifamycin-B	*Amycolatopsis mediterranei*	Production	16	1	Venkateswarlu et al. (2000)
Tetracycline	*Streptomyces viridifaciens*	Stability of production	Higher	Lower	Yang and Ling (1989)

conditions (Hölker 2002), optimisation of culture composition was carried out on a 1 kg solid substrate scale. The results unambiguously indicated rye and oat bran as the best substrates for cultivation of *C. ellipsoida* in SSF. The process was scaled up to 5 kg solid substrate volume using a sterile combined trickle film/fluidised bed bioreactor (Hölker 2003a), yielding a maximal coniosetin concentration of 1.4 mg/g freeze dried substrate (unpublished results, Bioreact, Bonn, Germany/Aventis Pharma Deutschland, Frankfurt, Germany).

Production of spores

SSF is currently the best method of obtaining fungal spores by aerial hyphae. The properties of spores produced in SSF differ distinctly from those obtained in SmF. Fungal spores used as biocontrol agents against fungal plant pathogens, e.g. *Botrytis cinera*, *Sclerotinia sclerotiorum* or white-rot fungi, are produced preferentially in SSF because the spores obtained are of higher quality. They are more resistant to desiccation and are more stable in dry state. To obtain high numbers of spores, a combination of SmF (for biomass production in a first step) and SSF (for subsequent spore production) proved to be successful (Deshpande 1999; Tengerdy and Szakacs 2003). *Penicillium oxalicum* spores obtained by SSF were shown to have a higher surface hydrophobicity, a better rate of survival after 27 weeks of storage and were less damaged by freeze-drying. In addition, the SSF-produced spores have a higher biocontrol activity against *Fusarium oxysporum* f. sp. *lycopersici* (Pascual et al. 2000). Spores produced in SSF display morphological, functional and biochemical differences compared to those produced in submerged process, enabling the former to persist longer under natural environmental conditions.

Trichoderma harzianum, potentially active against various plant pathogens, e.g. *B. cinera*, forms smaller spores with a thicker cell wall and higher resistance against UV radiation. Munoz and coworkers (1995) found that the observed higher hydrophobicity of SSF-produced spores is due to an increased concentration of a large (14 kDa) hydrophobin-like protein excreted to the surface of aerial spores. Because of the easier handling and up-scaling of SmF cultures, attempts were undertaken to produce spores of *Ulocladium atrum*, which was also active against *B. cinera*, in submerged fermentation with good results. However, in contrast to SSF-produced spores, the ability of submerged spores to germinate decreased rapidly after 6 months (Frey and Magan 2001). *Coniothyrium minitans* represents another important biocontrol agent that is active against the plant pathogenic fungus *Sclerotinia sclerotiorum*. Its spore production was possible only at the surface of not-agitated liquid cultures or in SSF and has not so far been achieved in submerged culture (Oostra et al. 2000).

Spores for applications in the food industry have been often produced in SSF. *Penicillium roquefortii*, *P. camemberti* and *P. nalgoviensis* used as starter culture in the production of blue cheese and salami have been produced predominantly in SSF because of better yields of homogenous and pure spores (Larroche and Gros 1989). Attempts to produce *P. camemberti* spores in submerged batch cultivation resulted in sufficient maximum spore counts of 1.6×10^{8} ml^{-1} culture medium. However, the quality of these spores was not evaluated and they sporulated only when glucose repression was suppressed by the addition of calcium. Moreover, the treatment with Ca^{2+} was not successful for all tested strains (Bockelmann et al. 1999). Industrial spore production of *P. nalgiovensis* was performed in 100 g SSF-batches with bread as solid substrate resulting in spore counts of $1–2\times10^{9}$ g^{-1} solid substrate after 18 days of cultivation. Because of the metabolic heat and the high sterility requirements in food applications, up-scaling was not feasible. In a new type of bioreactor (Hölker 2000) spores were produced on a 5 kg-scale, resulting in spore counts of 1.6×10^{9} g^{-1} substrate in a 14 day cultivation (Hölker 2003b, and unpublished results from Bioreact).

Concluding remarks

Results discussed in this review clearly demonstrate that SSF, as long as the cultivation volume is kept to a litre scale, represents the superior technology regarding process productivity, product quality and processing costs. However, SSF is difficult to scale up because of the build-up of gradients in temperature, pH, moisture, oxygen, substrate and inoculum. Balakrishnan and Pandey (1996a) concluded in their review that the production of enzymes and secondary metabolites on an industrial scale in SSF is currently hampered only by the unavailability of suitable bioreactors. Barrios-Gonzales and Mejiea (1996) and Robinson et al. (2001) also recognized the potential importance of SSF, which, in their opinion, will meet the requirements of the increasing worldwide demand for secondary metabolites.

Although a direct comparison between SSF and SmF is very difficult due to the different consistencies of the microbial cultures used in the two technologies, microorganisms involved in SSF have a higher metabolic potential since they proliferate in an almost natural environment, i.e. under conditions of limited free water and with a solid support for growth. Whereas there has been significant development in SSF processing, regarding both biochemical engineering (Pandey 2003) and reactor design with the goal of scaling up the process, very little if any work has been done as yet to elucidate the molecular and physiological background of the different behaviour of individual microorganisms when cultivated on solids or in liquids. The physiological and molecular biological aspects of microbial cultivation can thus be regarded as the current “black box” of SFF biotechnology (Biesebeke et al. 2002). This may, and will, be changed by more focused consideration of the biological parameters applicable to SSF and SmF. Thus, the perspective that SSF will gain in prevailing significance in the industrial production of

enzymes, secondary metabolites and spores by wild type microorganisms is warranted since, compared with SmF, it is more effective in several aspects including lower energy and sterility demands as well as higher stability of products and variability of microorganisms used, especially the use of mixed cultures.

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