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Lipase Production in Solid-State Fermentation (SSF): Recent Developments and Biotechnological Applications

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ABSTRACT

Lipases are the most widely used biocatalysts, because they can catalyze several unnatural and remarkable reactions in non-aqueous media, such as bio-fuel production, production of value-added products such as esters, organic acids, food, beverage, cosmetics and pharmaceutical materials. Solid-state fermentation (SSF) represents an interesting alternative to produce industrial enzymes at lower costs due to the possibility of using inexpensive agro-industrial residues as culture media. This review aims to explore various agriculture by-products like husk, straw, agricultural raw materials, waste of the oil industry, among others that are locally available and are also cost-effective requiring low nutrient supplementation to produce microbial lipase(s) in SSF. Enzyme production is associated with the growth of the bacterial culture. The physico-chemical fermentation parameters such as pH of the medium, moisture content, particle-size, nature of particles and microbial inoculum level play crucial role(s) in lipase production. SSF has gained renewed interest and fresh attention of researchers to develop processes to achieve large-scale enzyme production by solid waste treatment and in its application in the industry to synthesize the products of commercial value.

Keywords: agricultural raw material, biofuel production, lipases, solid-state fermentation
Abbreviations: ANN, ANOVA; **aw**, water activity; **DAG**, diacyl glycerol; **EVOP**, Evolutionary operation; **MAG**, monoacyl glycerol; **RSM**, response surface methodology; **SmF**, submerged state fermentation; **SSF**, solid state fermentation

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INTRODUCTION

Solid-state fermentation (SSF) is a valuable technique for utilization of agro-industrial by-products to produce value added product(s) of commercial interest. SSF is defined as any fermentation process performed on a non-soluble material that acts both as physical support as well as source of nutrients in absence of free flowing liquid (Pandey 1992). The technique involves inoculation and growth of microbes on porous particulate solid substrate maintaining low moisture content. The water content and nutrients present in the substrate support the growth of microorganisms and the organisms secrete useful enzymes while growing on solid substrate (Pandey *et al.* 2003). Lipases an interesting class of acyl hydrolases (E.C. 3.1.1.3) has been in the centre stage of bio-catalytic reactions as they are naturally endowed with the potential to retain bio-catalytic activity in both aqueous as well as organic media. Lipases are ubiquitous in nature and are produced by various animals and most of the microorganisms. Lipases of microbial origin, mainly bacterial and fungal represent the most widely used class of enzymes in biotechnological applications and organic chemistry. A large number of lipases have been screened for their use as food additives (flavour modifying enzymes), industrial reagents (glyceride hydrolyzing enzyme), stain removers (detergent additives), digestive drugs, diagnostic enzymes in medical applications, neutraceuticals, surfactants and additives in cosmetics (Verma and Kanwar 2010). The conventional reactions performed by lipases in aqueous media are often referred as hydrolysis; release of alcohol and corresponding fatty acid(s) molecules during enzymatic action on substrates such as glycerol or similar esters (**Fig. 1**). These reactions are indispensable for the bioconversion of lipids (triacylglycerol) and usually proceed with higher regio- and/or enantio-selectivity. However, the reverse reaction referred as esterification that could be efficiently achieved in organic media/water restricted conditions (organic solvents or non-ionic fluids) involves the formation of an ester along with water molecules as by-product of such reactions. Microbial lipases show a broad spectrum of industrial applications due to their greater stability, substrate specificity and lower production cost when compared to other sources. Additionally, an enormous biodiversity of microorganisms improves alternative biotechnological processes and justifies the search for new lipases. Filamentous fungi are often recognized as the best lipase

Received: 4 May, 2011. Accepted: 7 February, 2012.

Invited Review

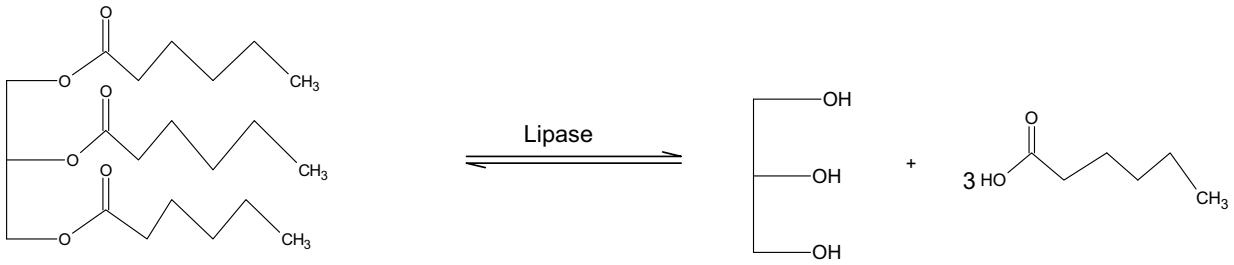

Fig. 1 Lipase-catalyzed hydrolytic reaction.

producers and are currently the preferred sources since they produce extracellular lipases (Carvalho *et al.* 2005). Most lipase-producing fungal species belong to the genera *Rhizopus*, *Mucor*, *Geotrichum*, *Penicillum* and *Aspergillus* (Carvalho *et al.* 2005; Hita *et al.* 2009). Moreover, fungi are considered as the most efficient source of lipases and other enzymes involving the process of SSF. The use of directed evolution can be very helpful to optimize existing lipases with respect to desired properties (Reetz *et al.* 2002). SSF has also been used to produce lipases from various by-products of agriculture and industrial origin that have little commercial value (Mala *et al.* 2007). Heat transfer capacity of the solid substrate is very low so this inherent property can be exploited for the growth of lipase producing-thermophilic bacteria that need high temperature for their growth.

Temperature of the substrate in SSF critically affects the growth of microorganisms, spore formation, their germination and product formation. Thus it may be advantageous for growing thermophiles to produce thermophilic enzymes at low cost. However, the traditional static SSF has difficulties in mass- and heat-transfer (Raghavarao *et al.* 2003). The enzymes produced by SSF in the form of solids can be used as naturally immobilized biocatalysts or dried form (Hellner *et al.* 2010) for synthetic reactions (Hernández-Rodríguez *et al.* 2009). Though some new strategies have been devised by some of the research workers like air pressure pulsation and agitation in which rotation was carried out to solve the problem of heterogeneous substrate utilization and product accumulation. The shearing forces have proved to improve the enzyme yield effectively in the SSF production of cellulose (Sun *et al.* 1999; Chen *et al.* 2002, 2005). The effect of shear force shall depend upon the nature and particle size of the substrate. Therefore, the selection of appropriate raw materials and the development of safe and environment friendly strategies to support sustainable processes are also essential.

In India, cereals (wheat, maize, sugarcane baggase, rice bran and straw) provide potential renewable raw materials for use in SSF (Pandey *et al*. 2000). Although, wheat contains all the nutrients generally required for most bacterial fermentations for the production of platform chemicals, these are not in directly accessible form(s). Therefore, the pretreatment/ processing is required in order to release these nutrients from the complex substrates. Complete starch and protein hydrolysis would create the potential to formulate media for a range of fermentations (Koutinas *et al.* 2007). Lipases, which display maximum activity toward water-insoluble long-chain acylglycerides, are mainly sourced from fungi and are exploited as valuable biocatalysts that retain catalytic functions in both aqueous and organic media. Although submerged fermentation (SmF) is preferred option for lipase production by most bacteria (Demir and Tukel 2010; Ji *et al.* 2010) yet SSF has proved to be a better approach for the production of lipases of fungal origin as compared to other genera. In a statistical comparison of the production of lipase in SmF and SSF systems the yield of lipase was quite high in solid-state cultivation than in submerged fermentation (Colla *et al.* 2010). The physico-chemical parameters influence the yield of lipase thus these must be optimized for optimum yield of enzymes through efficient optimization technique. The statistical techniques such as RSM, ANN (ANOVA), and EVOP (evolutionary operation) have been investigated and proved to be a better approach to achieve cost effective production and high yield of lipases (Contesini *et al.* 2009; Acıkel *et al.* 2010). It has also been reported that SSF results in more stable products, higher productivities with lower water, energy and sterility demands. In contrast to SmF, smaller fermenters and easier downstream processing measures are required in SSF (Robinson *et al.* 2001). In present review the authors have made an effort to discuss the prospects and challenges for lipase production through SSF and the potential applications of this process in the field of biotechnology.

Brief history of SSF

SSF has been known since ancient times in Asian countries. However, in western countries SSF was nearly ignored after 1940 due to the fact that SmF had become a model technology for production of any compound by fermentation including production of penicillin (Murado *et al.* 1998). In the last two decades, SSF has attracted renewed attention for the production of enzymes, metabolites and other valuable products due to several biotechnological advantages such as higher fermentation productivity, higher end-product concentration, higher product stability, and lower catabolic repression (Holker *et al.* 2004). SSF closely resembles the natural habitat of microorganisms on moist solids/ soils and is thought to be responsible for the beginning of fermentation techniques in ancient time (Mitchell and Lonsane 1990). SSF involves the growth of microorganisms mostly on surface of solid materials or substrate, which can absorb or retain water both in the presence or absence of soluble nutrients (Viniegra González *et al.* 1998). Current developments in SSF biotechnology are yielding new applications for lipases in medical as well as in industrial sectors. SSF holds tremendous potential for the production of enzymes of industrial importance. In a previous study, 38 filamentous fungi cultivated under SSF were screened for lipase activity and enantioselectivity in kinetic resolutions of racemic secondary alcohols by acetylation with vinyl acetate performed in organic solvents. The enzymes were not extracted and the SSF preparations were used as the source of lipase (Ibrahim 2008). It is envisaged that in some cases microbes growing during SSF may utilize and reduce the undesirable antinutrients/toxicants present in the substrates (Reddy and Pierson 1994). The approach has been proved successful in reducing gossypol content by 78% in cotton seed meal by SSF using *Geotrichum candidum* (Sun *et al.* 2008). Also in a recent study the production of lipase has been carried out by SSF from a mixture of wastewater sludges in a medium containing solid industrial wastes rich in fats, at 45°C in 20 days in 4.5-L reactors. The lipases produced were most active at 61-65°C and at pH 7.7–9 (Navarro *et al.* 2011). The SSF preparation represents inexpensive, naturally immobilized biocatalysts that can be successfully applied in preparative kinetic resolutions of racemic compounds. It can be of special interest in those processes where the crude fermented products may be used directly as an enzyme source (Pandey *et al*. 1998). The renewed interest in the production of lipase by SSF emerges because of the fact that enzyme obtained through SSF seems to be

more thermostable, economical with various advantages and the process scale-up is also easy to obtain lipase in bulk for commercial use.

ADVANTAGES OF SOLID STATE FERMENTATION

Eco-friendly process that avoids environment pollution and agriculture waste or by-product accumulation

There has been considerable interest to produce enzymes in SSF processes (Shankar and Mulimani 2007; Mahanta *et al.* 2008; Ruchi *et al.* 2008). Agricultural waste is one of the most renewable biomaterial available in agrarian states. Out of the total available agricultural waste only a fraction is used as animal feed while the remaining is disposed of by burning which ultimately causes a highly detrimental impact on the environment as reflected in an increase in the level of green house gases. SSF provides an environment friendly approach with many valuable advantages. SSF has probably a firm solution in order to obtain higher enzyme yields, as most of our enzymes are inducible including lipases. The addition of inducer further increases the rate of utilization of nitrogen and carbon sources present in the agro-industrial wastes.

Economic and low capital investment

SSF has built substantial credibility in the recent years for the production of microbial products including enzymes through inexpensive media and it seems to be an appropriate process for developing countries (Singhania *et al*. 2009). Large-scale production of enzymes like lipases and their applications to produce commercial products in the developing countries is of significant value because it requires low investment and low energy input with expected higher yield of product(s). In a previous study the production cost and energy demand in bioethnol production by SSF were studied and recorded data showed that bioethanol production by SSF is a less energy demanding process and dry matter used reduces the capital cost with higher co-product credit and therefore has a significant effect on the overall process economy (Sassner *et al.* 2006). Most of the commercial enzymes used in the developing countries for industrial purposes are imported either from Denmark or Belgium. Solid state fermentation processes are therefore of special economic interest for countries with an abundance of biomass and agro-industrial residues, as these can be used as cheap raw materials. One aspect regarding use of enzymes as diet additives is the commercial cost, because the fermentation medium is one of the important components that determine the final price of the product, and thus the low cost substrates are most preferred substrates.

Economic analysis of the production of lipase by *Penicillium restrictum* in SSF and SmF cultures showed that the investment necessary for SmF was 78% higher than that required for SSF; consequently, the price of the SSF product was 47% lower. The investment returns from the SSF process often would reach 68% within next couple of years (Castilho *et al.* 1999, 2000). In SSF the selected fermenter configuration for each process, influences the production scale, total capital investment, unitary product cost as well as profitability indicators. In a previous study, for the production of lipase a base-case lipase plant was designed to produce 100 m^3 of lipase concentrate per year (Durand *et al*. 1996). The solid state fermentation plant was designed to operate 330 days per year (7920 h/year) and the turnaround time was estimated as 20% of the fermentation time, *i.e*. 4.8 h for SSF and 12.8 h for SmF, resulting in total batch times of 28.8 and 76.8 h, respectively. The separation batch time was 12 h in both the cases. The economic analysis suggested that SSF was certainly a good way of utilizing nutrient-rich solid wastes as a substrate. Both food and agricultural wastes are produced in huge amounts and since they are rich in carbohydrates and other nutrients, they can serve as a substrate for the production of bulk lipase using SSF technique. Thus, overall, the process carried out for the lipase production in this way could be considered as quite economical and beneficial for commercial use.

Equipment specifications and space requirement

A process will probably be most economical when all the equipments are operating at an optimal utilization rate. In the lipase SSF process, downstream processing equipments account for a significant part of the project cost (Castillho *et al.* 2000). In biotechnological batch processes, long fermentation times are observed in large fermenter that leads to big downstream processing equipments with large idle intervals. Therefore, the number of fermenter units that minimizes total capital investment corresponds to the quantity that optimizes the utilization of separation equipments. Consequently, the optimal number of fermenter has a close relation to the ratio of culture batch time (fermentation plus turnaround time) and separation batch time (Luccioa *et al.* 2002; Raghavrao *et al.* 2002). Since the costs of separation equipments are significant in SSF, a great increase in total capital investment is observed every time a new set of separation equipments are added to the existing process.

Manual labour cost and payback time

In SSF the low energy input, simple design of the bioreactor, easy product recovery and high productivity per unit volume/unit mass obviously minimizes the input cost. The use of complex substrate/agriculture wastes also discourages the growth of contaminating organisms because of associated low moisture content. When lipase production in *Penicillium restrictum* by SmF and SSF was analysed, the total cost of the product obtained was 47% lower than the selling price, payback time was 1.5 years, return on investment was 68% and internal return rate was 62% for a 5-year-project life. Furthermore, the profitability of the process remain high including labour cost and all administrative charges, distribution and selling cost and general expenses with eventual increases of 40% in product concentration or total capital investment, or decreases of 20% in product price (Castillho *et al.* 2000). The use of cheap and easily available raw material is the basis of lower production cost in SSF. The high yield of the product or enzyme under optimized SSF parameters reduces the time of product formation. However, to maintain a uniform temperature, optimal agitation rate and homogeneity of the carbon/energy source in the SSF substrate may be some of the challenging aspects to run the SSF processes in a continuous mode.

Increased thermo-stability of lipases

The differences between the kinetic properties of the lipases isolated from *Rhizopus* spp. suggest that when they were tested, one or both fungal lipases have been associated with non-proteic compounds originating from the culture medium. As previously reported, lipase from a thermotolerant fungus *Rhizopus homothallicus* was more thermostable using SSF fermentation than liquid fermentation procedures. The specific activity on trioctanoin were 8600 U/mg with SmF and 10,700 U/mg with SSF, and the temperature at which maximum activity occurs was 30°C with SmF and 40°C with SSF with corresponding half-lives at 50°C of 0.44 h with SmF and 0.72 h with SSF (Robinson *et al.* 2001; Mateos *et al.* 2006). The two enzymes were monomers having identical protein structure, since the N-terminal sequences and peptide maps were identical. Proteins produced under both cell culture conditions showed some unexpected differences in several respects, such as their specific activity, heat stability and fatty acid specificity. Since the proteins are identical, these differences must have been due to the effects of small molecules contacted by the lipase during the production and purification processes. One possibility on these lines might be the presence of olive oil (or

its degradation products), tightly bound to the lipase. Thus from the above study it was clear that the properties of the lipases might depend on the enzyme production and purification conditions. Experiments are in progress to check the above two possibilities. The heat generated by the metabolic activities results in a rise in the temperature of reaction system that must preferentially produce thermostable enzymes by solid state fermentation because of the adaptation of the microorganisms at raised temperature existing *in situ* in the SSF substrate.

CLASSIFICATION OF SUBSTRATES FOR SSF

The solid material generally is a natural compound consisting of agricultural and agro-industrial by-products and residues, urban residues, or a synthetic material (Pandey *et al.* 2003). SSF holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as an enzyme source. The selection of a suitable solid substrate for the fermentation process is a critical factor and involves the screening of a number of agro-industrial materials for microbial growth and product formation. It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using in SSF processes (e.g., ligno-cellulose), to make them more amenable for microbial growth.

Agricultural raw materials and waste products

SSF plays an important role, and has a great perspective for the bioconversion of plant biomass. Lignocellulose may be a good feedstock for the production of bio-fuels, enzymes and other biochemical products by SSF. Crop residues (straw, corn by-products, baggase, etc.) are particularly suitable for this purpose, since they are available in large quantities in processing facilities. The major substrates used in SSF by various researchers are wheat straw (Dinis *et al.* 2009), rice straw (Huang *et al.* 2009; Yu *et al.* 2009), soyabean bran (Wolski *et al.* 2009), seseame oil cake (Singh and Satyanarayana 2008), sweat sorghum (Yu *et al.* 2008), Mahua flowers (Mohanty *et al.* 2009), corn stover (Zhao and Xia 2009), *Jatropha curcas* seed cake (Mahanta *et al.* 2008), creosote bush leaves, lemon peel (Orzua *et al.* 2009), wheat bran, rice husk, paddy straw dust, sugarcane baggase, corncob (Nagar *et al.* 2011), among other examples. Wheat bran however, holds the key, and has most commonly been used, in various processes (Pandey *et al.* 1994). Lignocellulosic material of agricultural origin that comprises more than 60% of plant biomass may be a suitable substrate for the production of value added products. To fully utilize the potential of lingo-cellulose, it has to be converted by chemical and/or biological processes in to simple sugar molecules. There has been an increased exploitation of organic residues of natural agricultural products as well as agro-industrial waste over the past few decades. The *Jatropha curcas* seeds are used as a substrate for lipase production in India and various Asian countries (Liang *et al.* 2010; Saetae and Suntornsuk 2010). The composition of deoiled seed cake is protein, 60%; fat, 0.6%; ash, 9%; fibre, 4% and carbohydrates, 26% (Rakshit *et al.* 2008) that can be used as feedstock after detoxification of phenolic compounds. Crop residues such as bran, husk, baggase and fruit seeds are utilized as a potential raw material in SSF as they provide an excellent substratum for the growth of microorganism supplying the essential nutrients to them and thus producing value-added products by this technique (Pandey 1992; Pandey and Soccol 1998, 2000; Pandey *et al.* 1999a, 2000a, 2000b, 2000c, 2000d).

Industrial wastes

Changing feeding habits of human beings and all the excess tallow produced are not used in soap industry (Bhatti *et al.* 2008). Hence, it is economical to consider tallow, a low cost feedstock, in oleo-chemical industries for the production of fatty acids and their corresponding esters using lipases because they make the process energy efficient than the conventional thermal fat-splitting process, which requires operations at elevated temperature and pressure (Edwinoliver *et al*. 2009; Bajaj *et al.* 2010). Recently, grease waste supplemented with wheat bran has been used as a substrate for the production of lipase (Kumar *et al.* 2011). Also the production of lipase has been reported from a mixture of wastewater sludge in a medium containing solid industrial wastes rich in fats, under thermophilic conditions for 20 days in 4.5-L reactors. These lipases produced by SSF were most active (120,000 UA/g) at 61–65°C (Santis-Navarro *et al.* 2011). Industrial residues, molasses, wastewaters from dairies, olive mill solid waste and slaughter houses are rich in biodegradable organic molecules and nutrients that contain high levels of fats and proteins and that have a low biodegradability coefficient. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes. Utilization of industrial residues as support-substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilized residues. The fungal genus *Penicillium* is also a good producer of extracellular enzymes such as lipases, proteases, cellulases and xylanases (Hamlyn *et al.* 1987). In the organic waste polar groups, such as carboxylate, amide or amine, which would be heavily hydrated in an aqueous environment are not tolerated and, if they are required, they should be protected with a lipophilic unit. The alkyl chain of the acid moiety should be preferably of straight chain nature possessing at least 3-4 carbon atoms. Reaction rates may be improved by using 'activated' esters bearing halo alkyl groups, the remaining hydrogen atom in both substrate types must not be replaced by a substituent, since esters of tertiary alcohols and tri-substituted carboxylates are usually not accepted by lipases. The stereo-chemical preference of the most commonly used microbial lipases is well known (e.g. from *Pseudomonas* sp. and *Candida* sp.).

Synthetic materials

The inert synthetic material of household and society origin acts as an attachment place for the microorganisms, in addition to organic material, which also functions as a source of nutrients, due to which it is called support-substrate. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. The solid support along with organic material trimmings, dust and saw dust containing important nutrients to the microorganisms growing in it should be considered as an ideal substrate. The synthetic materials containing glyceride moieties are most useful substrates for lipases; they possess a chiral alcohol moiety. It was understood that lipases were particularly useful for the resolution or asymmetric esters bearing a chiral alcohol moiety. The polymeric material containing secondary alcoholic groups are usually more selectively transformed than the primary alcohols. Generally, hydrolytic enzymes produced by the fungi and bacteria for the hydrolysis of synthetic material including lipases, cellulases and pectinases are produced in nature by fungi to sustain their growth. The major species of fungi involving the production of lipases by the hydrolysis of glycerides and polyalcoholic groups in the synthetic wastes are *Trichoderma* spp. and *Aspergillus* spp.

METHODS OF PRODUCTION OF LIPASE ON SOLID SUBSTRATUM

The solid media used in SSF contain less water but an important gas phase exists between the particles. This feature has an enormous importance, since the thermal conductivity of the air is very poor as compared to the water. In addition, SSF employs a great variety of matrices, which vary in composition, mechanical resistance, porosity and water holding capacity. Generally several types of reactors are able to

Table 1 Major genera of bacteria and fungi employed in lipase SSF.

Microorganism used	Substrate used	Reference
Bacillus thermoleovocan, *Bacillus* sp., *Pseudomonas* sp., *Serratia* sp., *Streptococcus* sp.	Agricultural waste, wheat bran and straw	Raimbault 1998; Mohan *et al.* 2008
Penicillium abeanum, *P. aurantiogriseum*, *P. cyclopium*, *P. avellaneum*, *P. brevicompactum*, *P. camembertii*, *P. caseicolum P. candidum*, *P. charlesii*, *P. chrysogenum*, *P. citrinum*, *P. corylophilum*, *P. corymbiferum*, *P. crustosum*, *P. duclauxii*, *P. egyptiacum*, *P. expansum*, *Penicillium P74*	Soybean meal, soybean oil, sugarcane molasses, steep liquor, yeast hydrolysate, and pure soybean meal	Benjamin 1998; Chahinian *et al.* 2000; Sharma *et al.* 2001; Jaeger *et al.* 2002; Cavalcanti *et al* 2005; Fernández-Lafuente *et al.* 2010
Rhizopus rhizopodiformis, *R. pusillus*, *Rhizopus* sp., *R. oryzae*, *R. arrhizus*, *R. chinensis*	Bagasse, olive oil-cake, cassava, sweet potato, pineapple waste, carrot-processing waste, okara (soy-residues), carob pod, wheat bran, wheat straw and wheat floor	Cordova *et al.* 1997; Yang *et al*. 2004; Shukla and Gupta 2007
Aspergillus niger, *A. foetida*, *Colletotrichum gloeosporioides*, *Yerwinia lipolytica*	Cottonseed, groundnut and rice bran oil cake, sugarcane bagasse, castor-bean, coffee husk, corncobs, polyurethane, cassava, sugarcane bagasse, sugarcane press mud and coffee husk	Lima *et al.* 1995; Kamini *et al.* 1998; Hang *et al.* 1998; Leangon *et al.* 1999; Roukas *et al.* 1999; Falony *et al.* 2006; Balaji and Ebenezer 2008
Yeast, *Endomicopsis burtonii*, *Saccharomyces cerevisiae*, *Schwanniomyces castelli*	Tape cassava, rice bran and food waste	Raimbault 1998; Babu and Rao 2007; Rigo *et al.* 2010

Table 2 Various advantages of SSF processes used for lipase production.

Benefits of lipase in SSF	Reference(s)
A credible process for the production of microbial products including enzymes through inexpensive media and it is an appropriate process for developing countries.	Pandey *et al.* 1994; Singhania *et al*. 2009
The high yield of the product or enzyme under optimized SSF parameters reduces the time of product formation.	Durand *et al*. 1996; Ramachandran *et al.* 2007
Requirement of simple fermentation equipments account for a significant part of the project cost. Long fermentation times are observed in large fermenter, and at the same time leads to large downstream processing equipments with large idle intervals**.**	Raghavrao *et al.* 2002
Fungal spores produced by a SSF culture are more stable, more resistant to dehydration, had rigid cell wall and smaller volume of conidiophores.	Muñoz *et al.* 1995
A thermotolerant fungus *Rhizopus homothallicus* was more thermostable using solid state fermentation than liquid fermentation procedures.	Mateos *et al.* 2006
Low effluent generation during solid state fermentation also account for ease of handling of process and on the other side helps to reduce environmental pollution.	Yang *et al*. 2001; Brand *et al*. 2000; Kumar *et al.* 2011
The fermented product of solid state fermentation can be directly used for animal feed also it provides more energy and rich in nutrients.	Yang *et al.* 2001; Brand *et al.* 2001
Bioeconomics of solid state fermentation is proved to be advantageous, the obtained by products with higher added value, like fructose, ethanol, biodiesel and aminoacyl esters may represent an alternative to attain better process economics.	Luccioa *et al.* 2002; Lohith and Divakar 2007

run at laboratory-scale with small quantities of medium but intense heat generation and heterogeneity complicate scale-up in the system (Lonsane *et al.* 1992). Various bioreactor types that have been used in SSF processes mainly includes packed beds, rotating drums, gas-solid fluidized beds and other stirred bioreactors (Mitchell *et al*. 2000). The type of aeration or the mixed system employed can distinguish the bioreactors. The fermenter types used in the SSF include mainly tray fermenter, covered pan fermenter, drum fermenter, column fermenter, packed bed fermenter, intermittently stirred bed fermenter, wooden cell fermenter, vertical incubation cell fermenter, conveyor fermenter, butler type corn storage bin fermenter, and other miscellaneous designs. The microorganisms belonging to various genera have been used for lipase production by SSF (**Table 1**).

Selection of substrate for SSF

The selection of a suitable solid substrate for the fermentation process is a critical factor and involves the screening of a number of agro-industrial materials for optimal microbial growth and product formation. The nature of the solid substrate (screening) employed is the most important factor affecting SSF processes, and its selection depends upon many factors mainly related with cost and availability of several agro-industrial residues. Among various factors, which are important for microbial growth and activity in a particular substrate, particle size and moisture level/ water activity are the most important (Echevarria *et al.* 1991; Barrios-Gonzalez *et al.* 1993; Pandey *et al.* 1994; Liu and Tzeng 1999). Generally, smaller substrate particles provide a larger surface area for microbial attack but if they are too small they might result in substrate agglomeration as well as poor growth of the seeded microorganisms (Sarrette *et al.* 1992; Roussos *et al.* 1993; Pastrana *et al.* 1995; Smail *et al.* 1995; Zadrazil and Punia 1995). It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using in SSF processes thereby making them more easily accessible for microbial growth. In contrast, larger particles provide better aeration but a limited surface for microbial attack. Therefore, a compromised particle size must be selected for each particular process. Research on the selection of suitable substrates for SSF has mainly been centered on agro-industrial residues due to their potential advantages for filamentous fungi, which are capable of penetrating into the hardest of these solid substrates, aided by the presence of turgor pressure at the tip of the mycelium (Ramachandran *et al.* 2004).

There are several reports dealing with extracellular lipase production by fungi such as *Rizhopus*, *Aspergillus* and *Penicillium* spp. on different solid substrates (Christen *et al.* 1995; Cordova *et al.* 1998; Kamini *et al.* 1998; Gombert *et al.* 1999; Miranda *et al*. 1999) under SSF (**Table 2**). However, a few studies have investigated the synthesis of lipase by yeasts using SSF technique. The C/N ratio of the medium is an important parameter for lipase production by the yeast *Candida rugosa* (Rao *et al.* 1993). In some instances SmF and SSF systems have been compared for lipase production and it has been found that enzyme yields were higher and stable in the latter (Rivera-Muñoz *et al.* 1991; Ohnishi *et al.* 1994; Christen *et al.* 1995; Benjamin *et al.* 1996a, 1996b, 1997a, 1997b). The other factors that can affect microbial extracellular lipase production are pH, temperature, aeration and medium composition.

Types of bioreactors

1. Immersed bioreactor

It is a cylindrical glass vessel consisting of a jacket with a round bottom. Inoculated microorganisms like fungi or bacteria colonize a wire network filled with a support placed into the bioreactor vessel. They move upwards and downwards by means of a pneumatic system, remaining more time outside than inside the medium. Difficulties in controlling important culture parameters, such as mass-transfer and heat removal, have not been completely overcome (Cohen *et al.* 2002) as previously reported and the production of *Rhizopus oligosporous* lipase in a simple Erlenmeyer flask could be performed at a small scale (Huq *et al.* 2001). Wheat bran was placed in 250-ml cotton plugged Erlenmeyer flask and moistened with phosphate buffer. After sterilization, the flasks were inoculated with fungal inoculum and incubated at 30°C for 72 h. After incubation the substrate-free enzyme extract showed good lipase activity (30 ± 2.1 U/g substrate). In another report, the production of acidic thermophilic lipase was carried out in SSF on a solid residue of babassu cake, which is a by-product of babassu oil industry. Fermentations were carried out in lab-scale tray-type bioreactors, containing 10 g cake forming a 1 cm deep layer. The babassu cake was supplemented with sugar cane molasses (6.25% w/w, dry basis), moistened to 70% (w/w) and sterilized by autoclaving at 121°C, 15 psi for 20 min (Gutarra *et al.* 2005). Afterwards, solid-liquid separation was done by pressing followed by centrifugation at 2000 × *g* for 2 min (Gombert *et al.* 1999). Also the production of solvent-tolerant lipase from *Pseudomonas aeruginosa* was carried out on *Jatropha curcus* seed cake (Mahanta *et al.* 2009). SSF was carried out by inoculating deoiled *J. curcas* seed cake treated with inoculum followed by incubation at 30°C. The samples were aseptically withdrawn at various time intervals and the production of lipase was studied.

2. Packed-bed bioreactor

This reactor is composed of a jacketed glass column filled with the bio-particle system which supports the organism (fungus) used for SSF. Humidified air is supplied in a continuous way. A divided and humidified solid (organic material) acts as both support and nutrient source and the process essentially occurs in the absence of free water. In a second mode, a nutritionally inert solid (synthetic material), which exclusively acts as a support, is soaked in a nutrient solution. This technique reproduces the natural microbiological processes like composting and ensiling. However, the available information does not indicate an ideal bioreactor for solid-state processes for lipase production. Accurate models describing the biological phenomena and mass/heat transfer phenomena are necessary to optimize SSF bioreactor operations (Gelmi *et al.* 2000).

3. Rotary drum-type bioreactor

It comprises a wire mesh cylinder, which rotates slowly inside a cylindrical glass vessel containing the culture medium at its lower part. The wire mesh cylinder contains the support together with the fungus. When the wire mesh cylinder rotates, both the carrier and the fungus are impregnated with the culture medium and, at the same time, they are in contact with the air of the upper part of the vessel, permitting a suitable oxygen transfer. Despite the potential of SSF for the production of commercially valuable products, this technique is at present under-utilized. One of the major drawbacks in commercial SSF applications is the limitation in the design and operation of large-scale bioreactors (Ashley *et al*. 1999). However, they are difficult to construct given the complexity of bioreactor behavior and the scarcity of process measurement (Pena-Lillo *et al.* 2000). In addition, evolutionary operation (EVOP) has also been used as the important tool for the optimization of enzyme production in a complex system like SSF, which is also a critical step (Banarjee and Bhattacharyya 2003).

4. Tray-type bioreactor

It consists of flat trays, where bio-particle system is placed forming a layer of about 1.5 or 2 cm of thickness. The bioreactor may be kept in a chamber at constant temperature with passive aeration (Pandey *et al.* 1999). The SSF fermenters were used for various purposes, incorporating several modifications for improved operation and performance. Among these several types of SSF reactors, rotary drum bioreactors (RDBs) provided relatively gentle and uniform mixing by improving baffle design, since there was no agitator within the substrate bed. Rotating drums have been used as bioreactors for SSF since the 1930s and are already tested to make many products. The problems with this bioreactor are particle agglomeration over time; shear sensitive mycelia with high rotation speed and difficulties in controlling heat and mass transfer inside the bed. As most of the rotary type drum reactors are mainly used for aerobic SSF. To design an industrial-scale RDB, temperature distribution in the radial direction of the substrate bed is a very important factor. Lipase extraction from the SSF medium is performed in the absence of free liquid, and recovery of the fermentation product requires its extraction from the solid fermented medium. Intracellular proteins contaminated the enzyme solution obtained by pressing mechanically fermented solids. In order to avoid this contamination the lipase extraction was studied using different solutions to get the most convenient extract during purification.

FACTORS AFFECTING THE PRODUCTION OF LIPASE IN SSF

Fungi have been considered to be the organisms most easily adapted to SSF because their hyphae can grow on particle surfaces and penetrate into the inter-particle spaces and thereby colonizing solid substrates (Santos *et al.* 2004). However, several studies have reported satisfactory results in terms of obtaining different products by SSF using bacterial cultures (Kapoor *et al*. 2000; Kashyap *et al.* 2003; Virupakshi *et al*. 2005; Sabu *et al.* 2006). The enzyme production by either SmF or SSF process is highly affected by numerous factors with large variations in terms requirement of medium composition and cultural conditions (Ibrahim *et al*. 1987, 1991; Lim and Ibrahim 1993). Various factors affecting the production of lipase in SSF have been described in detail (**Table 3**).

Effect of substrate particle size

Smaller substrate particles provide larger surface area for microbial attack and, thus, its optimal size is a desirable factor. However, too small a substrate particle may result in substrate accumulation, which may interfere with microbial respiration/aeration/exhaust of built up gases, and therefore result in poor growth (Pandey *et al.* 1999; Sabu *et al.* 2006). In contrast, larger particles provide better respiration/ aeration efficiency (due to increased inter-particle space), but provide limited surface for microbial attack. This necessitates a compromised particle size for a particular process. Smaller size particles result in compact substrate, which hinders mycelia penetration and gaseous exchange. However, too small a substrate particle may results in substrate accumulation, which may interfere with microbial respiration/aeration, and therefore causes a poor growth. In contrast, larger particles provide better respiration/aeration efficiency (due to increased inter-particle space), but provide limited surface for microbial attack. This necessitates a compromised particle size for a particular process.

Table 3 Important factors affecting the production of lipase in SSF.

Parameter	Modulation in the SSF bioprocess	Reference(s)
Substrate size	Smaller size particles resulted in compact substrate which hindered mycelia penetration and gaseous exchange**.** Generally, smaller substrate particles provide larger surface area for microbial attack and, that is a desirable characteristic of the particles. However, too small substrate particles may result in product accumulation, which may interfere with microbial respiration/ aeration, gaseous and liquid mass transfer and therefore results in poor growth of microbes.	Kapoor *et al*. 2000; Sabu *et al.* 2006; Nigam *et al*. 2009
Moisture content	Moisture content in the SSF can vary due to evaporation of the existing water through metabolic heat evolution, water consumption and liberation through fungal metabolism and also due to environmental factors. The moisture content in the substrate also depends on the types of microorganisms and the optimum water content with respect to a particular substrate appears important as it determines the productivity of SSF process.	Lonsane *et al.* 1985; Kashyap *et al.* 2003; Nigam 2009; Nagar *et al.* 2011
Inoculum size	Generally high inoculum size reduces the lag phase of fungal growth and therefore the maximum lipase production can be achieved in a shorter time. Increasing inoculum size in case of *Aspergillus flavus* using rice husks and wood substrates resulted in a rapid initial growth, although the enzyme production did not increase significantly.	Raimbault and Alazard 1980; Adinarayana *et al.* 2003; Nigam and Pandey 2009
Cultivation temperature	Temperature is related directly to *a*w and aeration. One limitation of SSF is ability to remove excess heat generated by metabolism by microorganism due to the low thermal conductivity of the solid medium. SSF requires more aeration for quick heat dissipation than as a source of oxygen. Increased bioreactor temperature causes denaturation of products, especially thermo-labile substances.	Santos *et al*. 2004; Guimarães *et al.* 2009
Heat conductivity of the substrate particle	The poor heat conductivity of the material and its low water content markedly reduced heat transfer that also depended on the size of the particles of the solid layer. Increase in temperature due to the release of metabolic heat can reach up to 3000 kcal/kg of assimilated substrate at the centre of the reactor with a radial gradient of 58^{o}C. During composting, temperature may reach 80^{o}C and becomes a limiting factor causing drying out of the medium and a decline in water activity and nutrient availability.	Lonsane *et al.* 1985; Nigam *et al.* 2009
Lipid material/ inducer and different oils	Lipid materials such as fats and oils, triacylglycerol, fatty acids, glycerol, surface active compounds, lipid derivatives, substrate analogs and other lipidic compounds can be the substrates of lipolytic enzymes. Sesame oil was found to give the highest lipase production. The lipase was specific towards lipid fatty acids with longer chain length, which are present in oil such as sesame oil, olive oil, and corn oil.	Mahadik *et al.* 2002; Guimarães *et al.* 2009
Oligo-elements additional supplements	Supplementation of the solid substrates with additional carbon and nitrogen source(s) result in small changes in C/N leading to great variations in enzyme activities and rapid initial growth. The necessary moisture in SSF exists in a complex form which is absorbed within the solid matrix and that was considered advantageous for the growth because of the possible efficient oxygen transfer process.	Raghavarao *et al.* 2002; Pérez-Guerra *et al*. 2003; Zhang *et al.* 2008
Nitrogen source	Most N-sources tested (except urea) reduced the production of lipase activity compared to the initial medium containing yeast extract. With urea, the activity was around six times higher than with yeast extract. A similar observation has been reported using *Penicillium restrictum* in SSF. However, lipase production in *Penicillium aurantiogriseum* was stimulated using ammonium sulphate.	Freire *et al.*1997; Sztajer and Maliszewska 1989; Gombert *et al.* 1999; Lima *et al.* 2003; Guimarães *et al.* 2009
Surfactants	Increase the secretion of proteins by increasing the permeability of the cell membrane. Added to improve the production of extracellular enzymes [Polyoxyethylene sorbitan monoleate (Tween 80) and polyoxyethylene sorbitan monolaurate (Tween 20) and sodium dodecyl sulfate (SDS); 0.1% (w/w)].	Grbavcic *et al.* 2011
Spore formation	Fungal spores produced by a SSF culture are more stable, more resistant to dehydration and have a higher germination rate after freezing than spores obtained by SmF. This has been attributed to the higher hydrophobicity, more rigid cell wall, and smaller volume of conidiophores obtained with SSF cultures.	Muñoz *et al.* 1995; Hölker and Lenz 2005

Effect of moisture content

Water is present in very limited amount in the SSF system and is thus optimum water content is important as it determines the productivity of SSF process (Lonsane *et al.* 1985). Moisture content in the SSF can vary due to evaporation of the existing water through metabolic heat evolution, water consumption and liberation through fungal metabolism and also due to environmental factors. The moisture content in the substrate also depends on the types of microorganisms and the substrate used in the SSF. At the same time the amount of moisture content also varies depending on the water-binding characteristics of the substrate. Considering the water loss based upon its overall consumption and production during the fermentation, the actual water required by the system is normally not known since it exists in a complex form within the solid matrix. However, it is important for the fungal growth as it allows efficient oxygen transfer (Raimbault *et al.* 1998). The results suggested that the water content determines the effect of organic solvent on reaction rate. Water content of the reaction system can also be fixed using saturated salt solution having different *aw* (Water activity of a liquid may be defined as the vapor pressure of a liquid divided by that of pure water at the same temperature). It was observed that the enzyme exhibited satisfactory stability throughout the pre-equilibration procedure when either KCl or K_2SO_4 was used. A rapid drop in the activity of the lipase was observed as the initial water content that was bound to the biocatalysts was removed gradually by $MgCl_2$ or molecular sieves. Saturated solutions of $MgCl_2$ and molecular sieves might remove the essential water, which is responsible for the bio-catalytic activity of the enzyme. A similar study carried out using the lipase of *Candida cylindracea* based on the esterification of oleic acid and butanol in organic solvents (Ibrahim *et al.* 1998) showed that the activity depends on the availability of water content in the reaction system. In order to achieve the optimum water content, salt hydrates and saturated salt solutions were also used in adjusting the overall water content in the system. The redistribution of water also depends on the support materials for lipase immobilization in which the support forms a strong bonding with water around the biocatalyst particles. Thus, the removal of water resulted in the collapse of these bonds, which ultimately deactivated the enzyme's bio-catalytic activity.

Effect of inoculum size

High inoculum size is expected to reduce the lag phase of fungal growth and therefore the maximum lipase production may be achieved in a shorter time. On increasing inoculum size in case of *Aspergillus flavus* using rice husks and wood substrates resulted in a rapid initial growth, although the enzyme production did not increase significantly. In some cases the inoculum did not affect the production but only affected the growth of microorganism (Raimbault and Alazard 1980). The decline in enzyme production with high inoculum size was related to the high biomass, which ap-

peared to be responsible for the reduction in enzyme biosynthesis under nutrient limited conditions in fermentation mashes.

Effect of cultivation temperature

The effect of fermentation temperature in the range of 28-45°C has been found suitable for the production of lipase (Navarro *et al.* 2011). Temperature plays an important role in SSF process as it significantly affects the germination of spores. However, as the spores germinate the optimum temperature for mycelia propagation may change. The temperature shift is made more complex considering SSF generated substantial amount of heat throughout the process, which will affect the water content in the SSF system. Higher temperature resulted in a lower lipase activity that might be related to low enzyme stability at raised temperature. Temperature is related directly to *a*w and aeration. One limitation of SSF is ability to remove excess heat generated by metabolism by microorganism due to low thermal conductivity of the solid medium. In practice, SSF requires aeration more for heat dissipation than as a source of oxygen. Increased bioreactor temperature causes denaturation of products, especially thermo-labile substances (Santos *et al.* 2004). The amount of specific enzymes produced by SSF is often greater than obtained by SmF (Aguilar *et al.* 2004). For example, beside lipase, the production of polygalacturonase and pectin lyase by *A. niger* was 5 and 1.3 times higher in solid-state culture than in submerged culture, respectively (Solis-Pereyra *et al.* 1993; Tagarano and Pilosof 1999) when the production of endo-polygalacturonase and exo-polygalacturonase by *A. niger* between SSF and SmF was compared. Production of both enzymes was greater in SSF cultures. Further, the time required for synthesis was shorter in SSF. Thus, in the light of the above study, a similar practice can be carried out for the production of lipases by lipase producing genera of bacteria and fungi. Enzyme synthesis was stimulated when the substrate contained higher sugar concentrations, in SSF, while in SmF, production decreased reflecting catabolite repression in SmF, but not in SSF.

Heat conductivity associated with substrate particles

The poor heat conductivity of the material and its low water content markedly reduce heat transfer that also depends on the size of the particles of the solid layer. Increase in temperature due to the release of metabolic heat can reach up to 3000 kcal from 1 kg of assimilated substrate at the centre of the reactor with a radial gradient of 58°C. During composting, temperature may reach 80°C and becomes a limiting factor causing drying out of the medium and a drop in *aw* and nutrient availability. Temperature is usually measured in the solid layer and in the gas flow at the entry and the exit of the bioreactor. Thermosensors/thermistors or metallic probes 'Pt 100' (Durand *et al.* 1993; Durand *et al.* 1996; Zadrazi *et al.* 1996) are most often used to measure temperature. Temperature sensors are generally inserted radially at various distances from the centre of the fermenter and linked to control system with various levels of sophistication. The use of pure soybean meal and babassu cake (Gutarra *et al.* 2007) showed that lipase activities of 21.0 and 30.0 U/g could be obtained at 48 and 36 h of fermentation, respectively. The use of wheat meal as substrate and *Y. lipolytica* as microorganism yielded lipase activities of 69 U/g (Domínguez *et al.* 2003). In a previous study, the use of soybean oil could not be considered an important inductor for lipases production, which was in disagreement with some works presented in the literature that affirmed that fats and oils are promising inductors for hydrolytic enzymes production (Hasan *et al*. 2006; Rodríguez *et al.* 2006; Azevedo *et al.* 2007). Production of thermo-stable lipases by *Rhizopus rhizopodiformis* and *Rhizomucor pusillus*, using sugarcane baggase supplemented with olive oil could yield 79.6 and 20.2 U/g lipase after optimization with suitable inducers (Cordova *et al*. 1998; Rodríguez *et al.* 2006). An improvement in lipase yield by fermentation of sugarcane baggase by *R. homothallicus* caused by the addition of olive oil and urea could be achieved. However, medium supplementation may not always improve lipase production. For instance, no improvement in supplementation of sesame seed cake and soybean cake could be observed in the production of lipases by SSF using *Aspergillus niger* MTCC 2594 and *Penicillium simplicissimum* (Kamini *et al*. 1998).

Effect of lipidic material and different oils as inducer(s) for lipase production

Lipids are generally essential inducers to lipase production. Numerous researches have shown that microbial lipases are highly inducible in the presence of lipid materials such as fats, oils, triacylglycerol, fatty acids, glycerol, surface-active compounds and other lipid compounds added in the production medium (Wolski *et al.* 2009). The lipid materials serve as the substrates of lipolytic enzymes, hydrolytic products of lipids, lipid derivatives and the substrate analogs. Sesame oil was found to give the highest lipase production. The higher microbial growth compared to the system in the absence of lipid material indicated that the lipid material can also act as substrate for the fungal growth. Generally, most lipases are specific towards lipid fatty acids with longer chain length, which are present in oil such as sesame oil, olive oil, and corn oil. Triglycerides with shorter chain length such as tributyrin showed much lower lipase production. Higher concentration of oil results in the formation of a biphasic system, which prevented oxygen transfer and nutrient assimilation by the fungus from the substrate.

It had been proposed that the fatty acids had important effects on lipase production (Mahadik *et al.* 2002). The different effects of oils on the lipase production were caused by the fatty acids from the metabolism of oils, and better lipase production appeared to be correlated with a higher content of oleic acid in oil. All the oil-related substrates can be used as carbon sources that could improve the cell growth to different extent, and oleic acid and linoleic acid were the best ones for growth, except olive oil. The synthetic activity of membrane-bound lipase was enhanced by oleic acid and palmitic acid, and the induction of oleic acid was close to that of olive oil, which resulted in a synthetic activity of 2.8 times than the control. Linoleic acid exhibited a negative effect on the lipase synthetic activity. These results could be helpful to explain why soybean oil and sunflower oil did not induce the lipase production with synthetic activity. As a hydrolysis product from triglyceride, the presence of glycerol inhibited the production of cell-bound lipase with only half of synthetic activity, whereas the mixture of oleic acid and glycerol was able to improve the lipase activity effectively. This showed that oleic acid indeed was an important factor for the induction of the cell-bound lipase. However, this mixture was similar to the main components of olive oil, but the obtained lipase activity did not reach the level seen in olive oil. On the other hand, the use of triolein gave lipase production comparable to that seen in olive oil.

Effect of additional supplementation of oligo-elements

The nutrients available in an SSF system are derived completely from the degradation of organic compounds present in solid substrates. Low production of the degrading enzymes may often result in poor growth of microbes due to limited availability of the nutrients in the substrate. Therefore, it is important to enhance the microbial growth mainly during the initial stage of the fermentation with concomitant production of degrading enzymes. Therefore, the supplementation of a solid substrate with an additional carbon and/or nitrogen source results in small changes in C/N leading to great variations in enzyme activities and rapid

initial growth. The necessary moisture in SSF exists in a complex form within/associated with the solid matrix that is advantageous for the growth because of the possible efficient oxygen transfer process. The water content is quite low and the microorganism is in contact with gaseous oxygen in the air (Raghavarao *et al.* 2002). This natural process has been utilized in industrial applications in a controlled way to produce a desired product (Pérez-Guerra *et al*. 2003). A solution of micronutrients was incorporated in the above medium, containing olive oil and lactose as energy and carbon sources, in order to observe its effect on lipase production. Lipase production, using this medium was 40 times lower than that obtained by the improved medium, suggesting a repressive effect of glucose on lipase biosynthesis. This final improved medium contained (g/l): olive oil, 40; urea, 4; lactose, 5; K_2HPO_4, 5; $MgSO_4$, 1; polyvinyl alcohol 1.6 and 4 ml of a solution of oligo-elements.

Effect of nitrogen source

The high concentration of nitrogen sources in the medium is effective in enhancing the production of lipases by most of the bacterial genera. During lipase SSF, a study on the kinetics of lipase production and pH changes was performed. Visible growth, lipase activity and pH changes occurred between 5 and 10 h and the observed cultural parameters remained more or less constant until 20 h. All the N-sources tested (except urea) reduced the production of lipase activity compared to the initial medium containing yeast extract. With urea, the activity was around six times higher than that obtained with yeast extract. A similar observation was reported using *Penicillium restrictum* in SSF (Gombert *et al.* 1999). Further, it was also observed that lipase production in *Penicillium aurantiogriseum* was stimulated using ammonium sulphate (Lima *et al.* 2003). In a few other studies, peptone gave the best results (Sztajer and Maliszewska 1989; Freire *et al.* 1997). Possibly, the peptone contained certain co-factors and amino acids, which fulfilled the physiological requirements of *Penicillium* for lipase biosynthesis (Sztajer and Maliszewska 1989). In contrast, *Rhodococcus homothallicus* IRD 13a strain did not require any growth factor. It was worth noting that fungal lipases are usually produced in SmF processes, using complex culture medium containing yeast extract, peptone, soy meal or corn steep liquor (Pimentel *et al.* 1994; Hatzinikolaou *et al.* 1996; Berto *et al.* 1997; Fadiloglu *et al.* 1997; Chahinian *et al*. 2000; Yang *et al.* 2005; Sharma *et al.* 2006) as compared to SSF.

Effect of surfactants

The presence of surfactants in the particulate substrate often tends to increase the secretion of proteins by increasing the permeability of the microbial cell membrane (Silva *et al.* 2005). The Tween 80 and Tween 20 that were used as non-ionic surfactants and SDS that was used as an anionic surfactant at 0.1% (w/w) acted on the surface of cell membrane and resulted in the enhancement of lipase production. An indigenous *Pseudomonas aeruginosa* strain producing lipase was studied to assess its compatibility with several surfactants, oxidizing agents and commercial detergents. The lipolytic activity increased in the presence of Triton X-100 (Grbavcic *et al.* 2011).

Effect of spore formation

Fungal spores produced by a SSF culture are more stable, more resistant to dehydration and have a higher germination rate than spores obtained by SmF (Hölker and Lenz 2005). This characteristic was attributed to the higher hydrophobicity, more rigid cell wall, and smaller volume of conidiophores obtained with SSF cultures (Munoz *et al.* 1995). Fungi grown in SSF culture at low *a*w tend to accumulate polyols such as glycerol, mannitol, erythrol and arabitol in their cells. The composition of the polyol pool depends on growth conditions and represents an adaptation to the low humidity condition in SSF needed to maintain the turgor pressure of the cells. These compounds are secreted by the mycelium and correspond to metabolites present in the fermented material (Holker *et al.* 2004; Ruijter *et al.* 2004).

Influence of SSF on microorganism physiology and product extraction

SSF is a process that occurs in the absence or near absence of any fluid in the space between particles (Lonsane *et al.* 1985). In this system, water is present in the solid substrate whose capacity for liquid retention varies with the type of material use in SSF. In contrast, in SmF the nutrients and microorganisms are both submerged in water. Microbial growth and metabolism nearly always occur in an aqueous phase as do diffusion of solutes and/or substrates. CO_2/O_2 exchange, on the other hand, can occur both in liquid and in the gas phase. The esterification of glycerol with conjugated linoleic acid (CLA) and long-chain fatty acids from menhaden oil catalyzed by the lipases has been reported (Torres *et al.* 2001). As compared with those from *Pseudomonas cepacia*, *Candida antarctica*, and *Mucor miehei*, lipase from *P. camembertii* gave a much slower reaction rate and a poor yield. A two-step successive enzymatic reaction for the synthesis of monoacyl glycerides (MAG) of CLA was also attempted (Watanabe *et al.* 2002). The first involved *P. camembertii* lipase-catalyzed esterification between CLA and glycerol for 10 h at 30°C, affording a CAL conversion of 84%. The reaction was continually conducted at 5 mmHg for dehydration, furnishing an increasing conversion of 95% after another 24 h. The content of MAG increased to 88.6 wt% in the mixture after 15 days. In order to reduce the long reaction time of this process the same research group improved it by combining low temperature with low vacuum (Watanabe *et al.* 2004). Enzymatic esterification with the selected molar ratio (1/5) of CLA to glycerol for 20 h at 5°C (80.8% conversion) was followed by dehydration at 5 mmHg for another 16 h. The final conversion arrived at 94.5%. And in the reaction mixture, the contents of MAG and diacylglycerides (DAG) were 92.7 and 2.9 wt%, respectively. Also, they proposed that there was a critical temperature for the production of MAG under which MAG was solidified and excluded from the solution, thus failing to be further bio-transformed to DAG. They investigated the influence of temperature on the synthesis of MAG of C10–C18 free fatty acids, and determined the critical temperatures for each MAG. It provided a convenient approach to predict the optimal temperature for the synthesis of MAG. *P. abeanum* lipase has been employed to enrich docosahexaenoic acid in tuna oil, and in order to improve lipophilicity, synthesis of kojic acid esters via lipase-catalyzed esterification was attempted (Liu *et al.* 1998). Lipase from *P. camembertii* proved to be the best catalyst for the production of kojic acid monooleate. The effects of several variables such as organic solvents, acyl donors and metal salts on the enzymatic reaction were explored which acted as a cosmetic whitening agent, and an anti-tussive, an agent used for lowering blood sugar. However, this compound suffered from low bioavailability due to its poor membrane penetration. In addition, the lipase was specific toward the medium-chain fatty acid vinyl esters, particularly vinyl octanoate.

BIOTECHNOLOGICAL APPLICATIONS

As an alternative, SSF has been developed and proved to be an economical asset to produce various enzymes including lipases and esterases. Interest in lipases has greatly increased in recent years due to their diverse applications (**Table 4**) in foods, detergent, cosmetic, organic synthesis, and pharmaceutical industries (Edwinoliver *et al.* 2009; Shua *et al.* 2010; Treichel *et al.* 2010). Microorganisms are potent lipase producers and moulds are widely recognized for higher enzyme production due to their ability to utilize various substrates with vigorous growth and sporulation on the

Table 4 Broader applications of lipase(s).

Source of lipase	Application(s)	References(s)
Bacillus cereus MTCC 8372 and *Pseudomonas aeruginosa* MTCC 4714	Synthesis of flavour esters, geranyl butyrate and geranyl acetate	Kanwar *et al.* 2008; Verma and Kanwar 2008
Commercial lipase 'Steapsin' immobilized onto silica	Synthesis of ethyl ferulate, butyl ferulate and isopropyl ferulate	Chandel *et al.* 2011; Kumar and Kanwar 2011a, 2011b
Pseudomonas aeruginosa MTCC-4713	Synthesis of methyl acrylate	Kanwar *et al*. 2006
Penicillium expensum and *Burkholderia cepacia* LTEB11	Synthesis of biodiesel	Kaieda *et al.* 2001; Ramachandran *et al.* 2007; Singhania *et al.* 2007; Saluma *et al*. 2010; Yang *et al.* 2010
Rhizopus chinensis	Synthesis of ethyl caprylate and ethyl oleate	Xu *et al*. 2003
Penicillium camembertii; *P. roqueforti*	Resolution of recemic mixture, production of biologically active secondary metabolites, antibiotics, alkaloids, plant growth factors and organic acids	Van der Deen *et al.* 1994; Johns *et al.* 2006; Ramachandran *et al.* 2007
Aspergillus spp.; *Candida* spp.	Production of monoacyl glycerols	Nagy *et al.* 2006
Candida rugosa	Preparation of L-prolyl, L-phenylalanyl, L-tryptophanyl and L-histidyl esters of carbohydrates	Lohith and Divakar 2007
A thermotolerant yeast strain	Production of ethanol	Yu *et al.* 2008

substrate matrix. SSF reproduces the natural microbiological processes like composting and ensiling. In industrial applications this natural process can be utilized in a controlled way to produce a desired product. Hydrolytic activity and synthetic activity are often used to characterize a lipase catalytic ability, and the hydrolytic activity was mostly preferred. Generally, one lipase can catalyze its reaction in both directions, but some of enzymes can exhibit only one catalytic activity, while others show both under certain conditions. A problem therefore occurs that synthetic activities of the enzymes in organic solvents intriguingly do not correspond with the hydrolytic activities observed in aqueous solutions (Teng *et al*. 2007).

In order to investigate or improve the synthetic activity for lipase, studies for lipase production need to be performed based on synthetic activity. However, scanty literature is available to investigate the lipase production in light of the synthetic activity. The low moisture content means that fermentation can only be carried out by a limited number of microorganisms, mainly yeasts and fungi, although some bacteria have also been used (Pandey *et al.* 2000a). SSF offers inherent advantages for the production of bulk chemicals and enzymes (Pandey *et al.* 1999a). Thirty-eight filamentous fungi cultivated under SSF were screened for lipase activity and enantioselectivity in kinetic resolutions of racemic secondary alcohols by acetylation with vinyl acetate performed in organic solvents. The enzymes were not extracted and the SSF preparations were used as the source of lipase. The SSF preparation represents inexpensive, naturally immobilized biocatalysts, which can be successfully applied in preparative kinetic resolutions of racemic compounds. Another lipase application is in the synthesis of acetone glycerol acyl esters using the immobilized lipase of *Mucor miehei* and *Pseudomonas* sp. (Ibrahim *et al.* 1989). Previously, in several studies the synthesis of enzymes including lipases (Pandey *et al.* 1999a), flavours (Ferron *et al.* 1996), colourants (Johns and Stuart 1991) and other substances of interest to the food industry has shown that SSF can give higher yields (Tsuchiya *et al.* 1994) or better product characteristics than SmF. In addition, costs are much lower due to the efficient utilization and value-addition of wastes (Castilho *et al.* 2000; Robinson and Nigam 2003) as noticed in the production of *Penicillium restrictum* lipase in both SmF and SSF. Both food and agricultural wastes are produced in huge amounts and since they are rich in carbohydrates and other nutrients, they can easily serve as a substrate for the production of bulk chemicals and enzymes using SSF technique. In contrast, larger particles provide better aeration but a limited surface for microbial attack. Therefore, a compromised particle size must be selected for each particular process (Pandey *et al.* 1999a). There are several reports dealing with extracellular lipase production by fungii such as *Rizhopus* sp., *Aspergillus* sp. *and Penicillium* sp. on different solid substrates (Christen *et al.* 1995; Cordova *et al.* 1998; Kamini *et al.* 1998; Gombert *et al.* 1999; Miranda *et al.* 1999) under SmF. However, few researchers have investigated the synthesis of lipase by yeasts using SSF technique. The C/N ratio of the medium is an important parameter for lipase production by the yeast *Candida rugosa* (Rao *et al.* 1993). A comparison was made between SmF and SSF systems for lipase production (Rivera-Muñoz *et al.* 1991; Ohnishi *et al.* 1994; Christen *et al.* 1995; Benjamin and Pandey 1996a, 1996b, 1997a, 1997b) and it was noticed that the enzyme yields were higher and stable in SSF. Several factors can affect extracellular lipase production such as pH, temperature, aeration and medium composition. Furthermore, the presence of triglycerides or fatty acids has been reported to increase lipolytic enzyme secretion by a certain number of microorganisms (Marek and Bednarski 1996). Therefore, in SSF the type of substrate could be used to enhance the production of enzymes, as several food and agro-industrial wastes are rich in fatty acids, triglycerides and/or sugars. Previously it has been reported that the great potential of food-agro-industrial wastes (ground nut and barley bran) as support-substrates for lipase production in solid state cultures of the yeast *Y. lipolytica*, since they led to much higher activities than those found using an inert support (Dominguez *et al.* 2003). Some of the applications which are extensively studied included the use of lipase in oleo-chemicals and fine chemical synthesis (Ibrahim and Tan 1991; Ibrahim 1992; Ibrahim and Tan 1994; Ibrahim and Chin 1996; Nagy *et al.* 2006), detergency, protease in detergency and degradation of allergenic proteins in rubber latex (Tham and Ibrahim 1993).

Synthesis of medically important esters and flavors

The lipase showed lower activities toward ester bonds of polyunsaturated fatty acid esters compared with those of other fatty acid esters. After the enzymatic reaction, the content of docosahexaenoic acid in Tuna oil increased by more than 2-fold. Our research group described the immobilization of *Bacillus cereus* lipase and other commercially available lipases on to various matrices and their applications in the synthesis of esters such as geranyl butyrate, ethyl ferulate and isopropyl ferulate (Kanwar *et al.* 2008; Kumar and Kanwar 2011a, 2011b). We also performed repetitive esterification catalyzed by immobilized lipase and efforts are continued in this direction to achieve the potential biocatalyst with improved catalytic activity in repetitive bio-transformations (Kumar and Kanwar 2011a, 2011b). The synthesis of geranyl acetate by *Bacillus cereus* MTCC 8372 lipase immobilized on to a hydrogel, poly (methacrylic acid-*co*-dodecylmethacrylate-*cl*-*N*-*N*-methylene bisacrylamide) was carried out with 82.8 mM yield (Verma and Kanwar 2008) and the synthesis of isopropyl myrsitate by the same biocatalyst gave a yield of 66.0 mM

(Verma *et al.* 2008). Lipase of *Pseudomonas aeruginosa* MTCC-4713 was bound onto a poly (AAc-*co*-HPMA-*cl*-EGDMA) hydrogel that was employed to synthesize methyl acrylate with a yield of 84.9 mM (Kanwar *et al*. 2006).

Silica gel was demonstrated to be the adsorbents permitting optimal immobilization of microbial lipase. After 7 h, a methyl ester yield of 92.8% was afforded through a similar three-step methanolysis approach. Recently, the production of biodiesel from corn oil mediated by crude lipase from *P. expansum*-mediated *trans*-esterification in [BMIm]PF6 has been reported (Yang *et al.* 2010). The enzymatic trans-esterification was enhanced greatly using [BMIm]PF6. A much higher methyl ester yield (69.7%) was furnished in the ionic liquid after 25 h, as compared to the yields of 19.4, 14.0, and 1.0% obtained in *tert*-butanol, solvent-free system, and hexane, respectively. In the modification of biologically active compounds kojic acid, 5-hydroxy-2 (hydroxymethyl)-1, 4-pyrone, possessed inhibitory activities against mushroom and plant polyphenol oxidases and tyrosinases (Chen *et al.* 1991).

Lipase catalyses in organic solvents

Numerous lipase-catalyzed reactions have been carried out in a variety of organic solvents, because of the significant advantages of the organic solvent system (Zaks *et al.* 1988). However, some of the limitations of the solvent dependent systems include; the solvent needs to be separated and regenerated, residual traces may remain in final product, the cost of the solvent itself, the increase in plant costs and the cost of solvent recycling can lead to economic limits for the enzyme-catalyzed process. Therefore, the solvent-free systems were developed as "more natural" processes. In order to test the catalytic ability of mycelium-bound lipase with high synthetic activity from *R. chinensis* in a solvent free system, synthesis of ethylcaprylate and ethyloleate was performed by lyophilized mycelium. Reactions were carried out with 80 g/L mycelium-bound lipase, starting from an equimolar amounts ethanol and acid without organic solvent at 40°C (Xu *et al*. 2003). The results showed that, the mycelium-bound lipase was a strong catalyst for the esterification between the ethanol and long chain fatty acid. After 5 h, the recorded conversions for ethylcaprylate and ethyloleate were more than 90%, even though the corresponding concentration of ethanol was 5.0 and 2.6 M.

The use of non-conventional media has become an interesting area of research particularly in the synthetic reactions (Gupta *et al.* 1992; Ibrahim *et al.* 1997). Most enzymes are stable in organic solvents, although the stability depends on a large number of parameters. It is therefore important to control all the governing parameters in order to obtain a constant maximum initial velocity of the reaction. One of the main parameters for a successful bio-catalytic activity in organic synthesis is the water concentration in the reaction system that reflected the level of enzyme hydration. However, it is not a question of whether water is necessary or not for the reaction, it's the extent of *aw* that is appropriately describes the role of water in organic solvent reaction (Zaks and Klibanov 1988). The *aw* available in the reaction system is difficult to assay for the given bio-catalytic reaction. At the same time, the available water is not only essential for bio-catalysis but water also plays an active role in determining the stability of the biocatalyst particles in the organic environment. Stability of biocatalysts particles will therefore determine the rate of enzyme catalyzed reactions. The amount of water required to maintain the protein stability varied depending on the types of protein, varying from a few molecules to several hundreds of water molecules per molecules of enzyme (Zaks and Klibanov 1988).

Resolution of recemic mixture

In the resolution of the racemic mixtures, lipases have been demonstrated to be one of the most useful catalysts for the resolution of the racemic alcohols, amines and acids due to exquisite enantioselectivity (Gotor-Fernández *et al.* 2006). For example, the preparation of (S)-2-ethylhexyl *p*-methoxycinnamate (S)-7 via lipase-catalyzed sequential kinetic resolution was achieved (Majeric *et al*. 1996). In the first step, *P. camembertii* lipase was used for the resolution of racemic 2-ethylhexanol (R,S)-5 with vinyl acetate, in which (R)-5 was acylated preferentially, leaving (S)-enantiomer untouched. Kellogg and co-workers reported the successful resolution of the racemic mixture (±)-8 with *n*-butanol in the presence of *P. roqueforti* lipase (Van der Deen *et al.* 1994). After 24 h of incubation, conversion reached 51% and (+)-8 was obtained with 98% ee (E > 130). The products include fatty acids from hydrolysis of lipids, esters or glycerides via esterification reactions and modified lipids and structural triglycerides via the trans-esterification reactions, namely acidolysis, alcoholysis and inter-esterification (Ibrahim *et al.* 1997). The SSF materials can also be used as a source of naturally immobilized lipase preparation for enantiomeric reaction (Nagy *et al.* 2006). These esters are used as intermediates for the production of MAG via the mild acidic hydrolysis. Monoacyl glycerols are widely used as emulsifiers mainly in food, cosmetics and pharmaceutical industries. Monoacyl glycerols can be synthesized chemically either via glycerolysis reaction or hydrolysis of fats and oils at high temperature using inorganic catalysts.

Bioprocess development in industry

In industrial applications SSF can be utilized in a controlled way to produce the desired product. Development of bioprocesses, such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, bioconversion of biomass, biotransformation of crop-residues for nutritional enrichment, biopulping, and production of value-added products, such as biologically active secondary metabolites, including antibiotics, alkaloids, plant growth factors, enzymes, organic acids, biopesticides, including mycopesticides, bioherbicides, biosurfactants, biofuel, aroma compounds, etc. have witnessed unprecedented growth of SSF technology (Pandey *et al.* 2000). Enzymes such as, lipase, tannase, laccase, protease, alpha-galactosidase lipase organic acid, such as, lactic acid, citric acid and bio-ethanol, antibiotics such as cephamycin and other metabolites as polyunsaturated acid, iturin A, pigment, hexyl laureate, palatinose, and also spores, have been successfully produced employing SSF (Johns *et al.* 2006; Ramachandran *et al.* 2007).

SSF in bio-refineries

Bio-refineries have added more value to SSF, as biomass is the only better source of energy to meet needs of the future generation, which adds to the importance of agro-residual waste (Ramachandran *et al.* 2007; Singhania *et al.* 2007). Lipase production by SSF using agro-industrial residues for enzymatic applications is in great demand at present time. Employing packed bed, anaerobic packed bed and fluidized bioreactor, esters, organic acids, ethanol and bio-diesel has been successfully used (Wu *et al.* 2007).

Detoxification of pollutants

SSF has been employed to detoxify hazardous chemicals produced by the industries as major pollutants of the present environment. Thousands of tons of lipidic material thrown away by the oil mills and sugar mills pollute our environment badly. Thus the lipase produced by the bacterial and fungal genera may prove to be beneficial for the removal of such hazardous material from the environment.

CONCLUSION

The present review highlights the present status of SSF of lipases, and its bioeconomics in comparison to SmF. Whe-

ther we intend to employ SSF or SmF, the main purpose of any of these two approaches is to obtain an enhanced yield of the enzyme, optimal utilization of complex substrate, low supplementation of carbon and/ or nitrogen source and good productivity in a short period of time. There are some obvious differences among the lipase-producing microbial genera in several respects, such as their specific activity, heat stability, and fatty acid specificity and thermostability. As the lipase produced by SSF is found to be more stable at ambient temperature and wider pH ranges thus it can be a better alternative for SmF. Further the SSF, in our opinion, is environmental friendly, requires less labour and less lab equipments and thus it can be adapted for production of lipases as well as other commercially important enzymes in developing countries. The exploitation of waste material for the production of value aided products like lipases in a developing country not only represents great advantage to researchers but also to the local industries in supporting the commercialization activities. Industrial biotechnology based on the production and application of enzymes has become the national priority due to the fact that enzymes are powerful biocatalysts, which can be used in wide applications ranging from new products synthesis to process development, environmental management and services. This means that SSF of lipases can be exploited towards the creation of wealth from biotechnology.

ACKNOWLEDGEMENTS

This work has been funded under a Major Research Project F. No. 34-269/2008 (SR) dated Dec. 30, 2008 awarded to one of the authors [SSK] by University Grants Commission, New Delhi, India; and partial financial assistance provided by Department of Biotechnology, Ministry of Science & Technology, Government of India to Department of Biotechnology, Himachal Pradesh University, Shimla, India. Finally, the authors thank Dr. Jaime A. Teixeira da Silva for completing a thorough revision of the grammar.

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