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Fungal Lipase Production by Solid State Fermentation-An Overview

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Abstract

Importance of enzymes is ever-growing specifically microbial lipases which are of great industrial significance because of their applications in detergent, food, pharmaceutical, chemical and leather industry. Solid state fermentation (SSF) is an economical alternative for large scale production of enzymes that are produced by fungi. Therefore, production of lipases by solid state fermentation is a good and preferred option than submerged fermentation (SmF). The important factors in fermentation are carbon concentration, nitrogen concentration, pH, growth temperature, fermentation time and moisture content. This review mainly focuses on production of fungal lipase by solid state fermentation using various fungal strains, substrates and fermentation conditions. Enzyme characteristics, industrial application and assay methods of lipase, biomass estimation, enzyme extraction methods and engineering aspects of fermentation are also dealt with briefly. The main aim of the review is to give an overview of advancements in solid state fermentation for production of fungal lipase hitherto.

Keywords: Lipase; Solid State Fermentation (SSF); Submerged Fermentation (SmF); Fungal *Sp*; Agricultural Substrates

Introduction

Solid state fermentation

(SSF) is an economical alternative technique to submerged fermentation (SmF) for large scale production of industrial enzymes, where raw materials and processing is cheap with reduced chemical cost and less processing risk. It is defined as 'the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free flowing water' [1]. This technique uses agricultural substrates with various carbohydrates, nitrogen sources, salts as well as nutrients like starch, cellulose, pectin, lignin, fibers and minerals. SSF technique has the potential to produce desired microbial products more efficiently than submerged fermentation (SmF) and has practical and economical advantages over SmF [2]. It has been reported that SSF with fungal strains results in greater productivity than submerged fermentation [3,4]. Substrates that have been traditionally fermented by solid state fermentation for enzyme production include agricultural wastes such as rice husk, wheat bran, beans, corn steep dry, sugar cane bagasse, coffee pulp, lemon peel and apple pomace [5-11]. Currently, major countries in the world are implementing this technique for large scale production of enzymes due to its higher titer values [12]. The substrates used for SSF can be classified as (a) agricultural raw materials and waste products (b) industrial wastes and (c) synthetic materials [13].

Lipase

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) (Figure 1) are a group of enzymes which catalyze the hydrolysis of triglycerides to diglycerides, and monoglycerides which are further hydrolyzed to glycerol and free fatty acids (Figure 2). Although there is lack of knowledge of the intrinsic kinetics of microbial growth, lipases are assumed to interact at oil-water interface. However, the kinetics regarding the adsorption processes is known [14,15]. Lipases occur widely in bacteria [16-18], yeasts [19,20] and fungi [21,22]. These enzymes also display catalytic activity towards a large variety of alcohols and acids in ester synthesis provided that the water activity is low. They can hydrolyze the ester bonds, trans-esterified triglycerides, resolve raceme mixture and are used in the synthesis of ester bonds in non-aqueous media [23-25]. Fungi are widely recognized as the best lipase sources and used preferably for industrial applications. A few of the general fungal genus include *Rhizopus, Aspergillus, Pencillium, Mucor, Ashbya, Geotrichum, Beauveria, Hunicola, Rhizomucor, Fusarium, Acremonium, Alternaria, Eurotrium* and *Ophiostoma*. Fungal species which produces lipases are *Candida rugosa, Candida Antarctica, T. lanuginosus, R. miehei, Pseudomonas, Mucor* and *Geotrichu*. Fungal lipases are often reported as *Aspergillussp, Candida rugosa, Candida antarctica, Thermomyceslanuginosus, Rhizomucormiehei, Mucor* and *Geotrichum* [26-28]. Lipases play an important role in lipid metabolism in eukaryotes at various stages like fat digestion, reconstitution, adsorption, and lipoprotein metabolism. Lipases are also found in energy tissues of plants and the mechanism of their interactions with lipids at the interface is still a subject of investigation [29]. Lipases provide improved worldwide attention due to their diverse industrial applications and versatility in nature. The world's enzyme demand is met by 12 major producers and 400 minor suppliers across the globe, 60% of the world's supply of industrial enzymes is from Europe alone and strikingly 75% of these industrial enzymes include lipases [26,30]. The importance of lipase as biocatalyst is well understood. It is used to catalyze several unnatural and remarkable reactions in non-aqueous media that include bio-fuel production, production of value added products like esters, organic acids, food, beverage, cosmetics, and pharmaceutical materials [13].
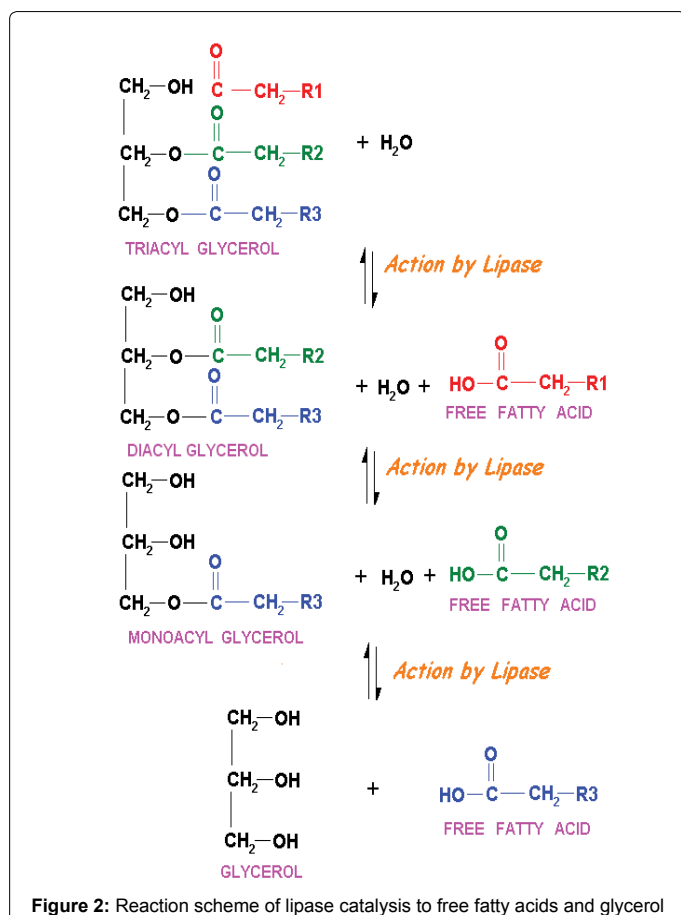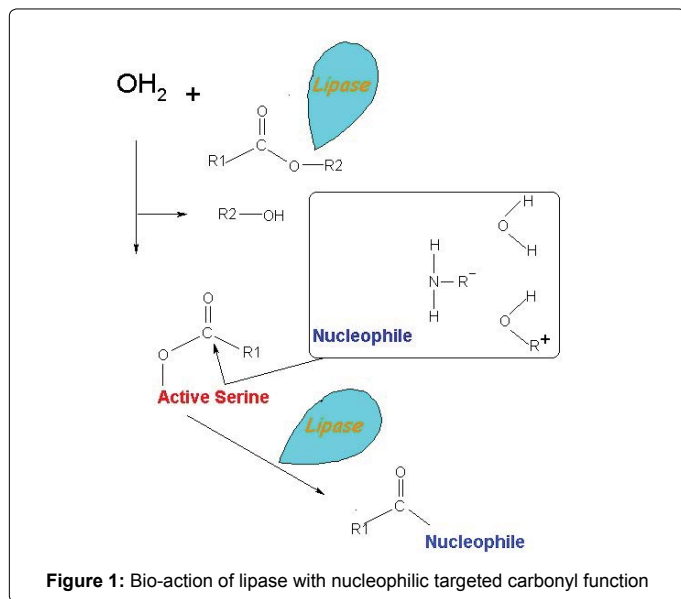
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Figure 1: Bio-action of lipase with nucleophilic targeted carbonyl function

Figure 2: Reaction scheme of lipase catalysis to free fatty acids and glycerol

Fungal Strains Producing Lipase

Several lipase producing fungal strains have been used for lipase production by SSF which include *Rhizopus rhizopodiformis* A13 and *Rhizomucor pusillus* A16 obtained from ORSTOM culture collection [31]. Other important fungi used are *Aspergillus niger* MTCC2594 [32], *Penicillium restrictum* [33], *Rhizopus oligosporous* [34], *A.niger*

NCIM 1207 [35], *Rhizopus delemer* [36] and *Aspergillus oryzae* [37], Among these strains, *Aspergillus niger* is found to produce significant quantities of enzyme and is regarded as GRAS by Food and Drug Administration (FDA). *A. niger* MTCC 2594 cultured on gingelly oil cake has a lipase activity of 363.6 µ/g dry substrate [32], whereas maximum lipase activity of 630 IU/g dry solid substrate was observed using *A.niger* NCIM 1207 [35]. Fungal strains producing lipases by SSF are tabulated in detail in Table 1.

Substrates used for lipase

Different types of agricultural wastes and oily substrates are used for the lipase because of their lipolytic nature. This includes rice bran, wheat bran, gingelly oil cake, almond meal, mustard oil cake, neem oil cake, groundnut oil cake, gingerly seed and groundnut kernel that are used routinely for lipase production by SSF. Lipase production from *Aspergillus niger* by SSF using gingelly oil cake as substrate is reported [32]. The feasibility of obtaining lipase with *Rhizopus delemar* growing on a polymeric resin has also been investigated [36]. Babassu oil cake is used as a substrate for the lipase production by *Penicillium restrictum* in SSF [33]. Among these substrates, almond meal is found to be the potential solid substrate [34], whereas wheat bran with olive oil as lipid substrates is suitable for the production of acidic lipase in SSF with maximum productivity [35].

Fermentation Conditions for Lipase Production in SSF

There have been numerous reports on the optimization of culture medium, agricultural substrates and nutrition factors for lipase production in SSF [38]. Production of lipases depends on different parameters like carbon and nitrogen concentrations, pH of the medium, temperature growth, moisture content and fermentation time required [39].

Substrates used for lipase production

Rice bran: In the case of *Candida rugosa*, addition of urea to rice bran enhanced lipase production during optimization [40]. Although addition of carbohydrates increase the production, but it causes contamination and this leads to overall reduction in the efficiency [32]. It has been reported that using maltose had only a marginal effect on lipase production with rice bran as substrate [41]. Fermentation time of 48 h at 30°C with an inoculum concentration of 1ml/g bran conducted by rice bran with a particle size of less than 250 µm results in a higher lipase production [42].

Wheat bran: Fungal *Mucor javanicus* IAM 6089 with wheat bran as substrate showed an activity of about 52 U/g dry bran after 6 days [43]. In the N-rich medium, lipase activity was increased by 4.3 times when it is compared to the basal medium [37]. This study further suggested that enriching the substrates had significant influence on lipase production compared to C/N ratio.

Almond meal: Ikram ul-Haq et al. [34] screened ten moulds to observe maximum lipase activity. Maximum activity of 48.0 ± 2.1 U/g was shown with almond meal, which contains required amount of carbon, nitrogen, sucrose, gum and proteins. In addition, a combination of 50% olive oil cake and sugarcane bagasse as substrate also showed improved activity. *Rhizopus rhizopodiformis* demonstrated a high activity of 79.6 U/g dry matter (DM) equivalent to 43.04 U/ml whereas, *Rhizomucor pusillus* showed an activity of 20.24 U/g DM equivalent to 10.83 U/ml [34].

Olive oil: Venkat et al. [40] showed that oil content significantly affects the lipase yield. It has been demonstrated that 2% of olive oil

Microorganisms	Solid Substrate	Inducers	Fermentation conditions (hours)	Lipase Activity Units	References
Fusarium oxysporum	Wheat bran	Cetyl trimethylammonium bromide	96 h, pH-8.5, 40°C, high MC	111.48 U/ml	[66]
Aspergillus flavus	Wheat bran and Castor oil cake	-	96 h, pH-7.0, 30°C, MC 64% w/w	121.35 U/gds	[60]
Aspergillus niger	Rice bran	-	pH-6.87, 30.3°C	121.53 (U/g dss)	[62]
Colletotrichum gloeosporioides	Sunflower oil	Triton X-100	pH-6.5, 25°C	2560 U/g DM	[63]
Yarrowia lipolytica NCIM 3589	Palm Kernal cake	-	MC 70% v/w, 96 h	18.58 U/ gds	[64]
Aspergillus niger J-1	Olive oil and Glucose	-	pH-7.0, 30°C for 7 days	9.14 IU/g dss	[61]
Aspergillus niger MTCC 2594	Wheat bran, Coconut oil cake and Wheat rawa	Olive oil, Sesame oil and Sunflower oil	72h, 30°C, pH-7.0, MC 60% v/w	745.7 ± 11 U/gds	[65]
Rhizomucar rhizopodiformis	Sugar cane bagasse and Olive oil cake	-	20 h-24 h	79.60 U/gds	[31]
Aspergillus niger MTCC 2594	Gingelly oil cake	-	250 ml EF, 30°C, 120 h, pH-7.0, MC 60%	363.6 /gds	[32]
Penicillium restrictum	Babassu oil cake	Peptone, Oliveoil and Starch	EF, 30°C, 15-65 h,	30.3 U/g	[33]
Rhizopus oligosporous NCIM 1207	Wheat bran and Olive oil	Nacl, Triton X-100	500 ml EF, 45°C, pH-2.5, 24 h, MC 71.4%	630 IU/gds	[35]
Aspergillus.sps	Wheat rawa	-	250 ml EF, 30°C, pH-7.0, 96 h	1934 U/g	[38]
Penicillium restrictum	Babassu oil cake and Olive oil	-	Tray reactor, 37°C, 24 h, pH-7.0	5.8 U/ml	[107]
Rhizopus oryzae PTCC 5176	Bagasse and Urea	Olive oil and Canola oil	Tray-bioreactor, pH 8.0, 35°C, MC 80%	229.355 U/gds	[108]
Candida rugosa	Coconut oil cake	Urea, Peptone and Maltose	96 h	87.76 U/g ds	[109]
Candida rugosa DSM-2031 Yeast Species	Coconut oil cake and Wheat bran	-	28°C	48.61 U/ml 58 LU/gm dry bran	[82]
	Deoiled rice bran, Rice bran oil and Mineral salts.	-	96 h, 30°C		[76]
Aspergillus Terreus	Mustard oil cake	-	30°C, 96 h, pH- 6.0	1566.67 ± 133.33 U/ml	[106]
Aspergillus niger IIT 53A14	Wheat bran and Olive oil	-	32°C, 48 h, pH 6.3-6.6	48.6 U/gds	[110]
		Soap stock	32°C, 48 h, pH 6.3-6.6	62.5 U/gds	
Aspergillus niger	Shea butter cake	Tween 20	30°C, 7 days, pH 7.0	3.35 U/g	[111]
Aspergillus. sp	Wheat bran	-	30°C, 96 h, pH 7.0	13.1 U/ml	[112]
Aspergillus niger	Soya bean and Rice husk	-	37°C, pH 7.7, 96 h	4.23 U/ml	[113]

EF: Erlenmeyer flask; MC: Moisture content; gds: gram dry substrate; LU; lipase units; ds: dry substrate; dss: dry solid substrate; DM: dry matter; U or IU or u: The amount of enzyme that catalyzes the conversion of 1 µmoles of substrates per minute.

Table 1: Comparison of lipase production in various fungal microorganisms

as an enhancer showed a maximum lipase activity of about 30.30 U/g initial dry weight after 24 h of cultivation, whereas with peptone showed a activity of 27.80 U/g of initial dry weight [40]. Earlier investigations showed that an optimum growth of the fungal *Rhizopus oligosporous* GCBR-3 had maximum activity of 30 ± 2.1 U/g and use of *Rhizopus strains* also enhanced activity [44,45]. These fungal strains also showed greater activity with other cultures for lipase production [31,46].

Substrates with supplements/inducers: *Aspergillums niger* NCIM1207 illustrated higher levels of extracellular enzyme at 45°C. The maximum lipase activity of 630 IU/gdss was observed with wheat bran as substrate and the enzyme extraction was done with NaCl by supplementing Triton X-100 [35]. However, in the case of *Rhizopus*, *Rhodotorula* and *Aspergillus,* the lipase production seems to be independent of substrate type [47-49]. Extracellular lipase production from different microorganisms using lipids, carbohydrates and also a mixture of both has been reported on enzyme production [48-51]. The use of surfactants like Triton X-100 during SSF helps to increase enzyme production, with up to three fold increase in enzyme production both in SmF and SSF. Wheat bran has sufficient nutrients and remains loose even in moist conditions, thereby providing a large surface area for the inoculums to give maximum activity [52].

In another report, lipase production by mutant *Aspergillus niger* 11T53A14 using wheat bran with the fermentation medium containing 100 g of wheat bran grain (size ≤ 5 mm), humidified with a solution containing nitrogen source (ammonium sulfate) and inducer (sunflower soap stock) in a concentration of 3% (w/w) is demonstrated. The activity was tested for different nutrient compositions at pH 7.0, temperature 32°C, and 78 h and the maximum enzyme activity was observed to be 153.2 U/gdm at 0.6 % (w/w) nitrogen concentration [53].

Olive oil cake and Sugar cane bagasse: Cordova et al. [31] reported lipase production with an equal combination of olive oil cake and sugarcane bagasse as substrates. The lipase production was carried out using thermo stable fungal cultures of *Rhizomucor pusillus* and *Rhizopus rhizopodiform* is subjected to solid state fermentation. A mixture of 50% each substrate has shown an enzyme activity of about 43.01 U/ml. It was observed that under standard conditions, bagasse has low protein, less fat content and high level of cell wall component whereas olive oil cake possess high protein, fat and higher amount of lignin content [31].

Oily substrates: Out of 34 fungal species isolated from different oil substrates, nine exhibited lipolytic activities [54-56]. Maximum lipase production of 1152 µg/g was obtained after 96 h of incubation by

Aspergillus species [38], whereas 8 days of incubation using *A.niger* at 80% moisture content attained a high enzyme titer of 1216.0 U/g [57]. Increase in the moisture level is believed to reduce the porosity of the wheat rava, thus limiting oxygen transfer [52]. At the same time low moisture content causes reduction in the solubility of nutrients of the substrates and low degree of swelling [58]. Different optimized factors, including wheat rawa with olive oil 1%, corn steep liquor 1% and 80% moisture content increase the solubility of the nutrient with the productivity of lipase in SSF 1.94 times higher than that in SmF [38].

Regiane et al. [59] used mutant strain of *Aspergillus niger* 11T53A14 for fractional factorial design to optimize lipase production. The soap stocks which are produced during the processing of canola, sun flower and corn has rich amount of lipid by products as inducers. The maximum lipase activity of 201.5 U/gds was observed for sun flower soap stock of 0.5% nitrogen and 3% inducers, when compared with canola and corn respectively [59].

Comparison of lipase activity with substrates

Comparison studies using fungal stains of *Penicillium chrysogenum*, *Trichoderma harzianum* and *Aspergillus flavus* was carried out through solid state fermentation using agro-industrial residues as substrates for optimum extracellular lipase production. For all three strains, the growth temperature was 29 ± 1°C, and 65% w (g/gds) moisture content. The effect of fermentation conditions on production are; initial pH (6.0 and 7.0), time of fermentation (72 h, 96 h and 120 h), and type of mixed substrate (wheat bran-olive oil, and wheat bran-castor oil cake). The *Aspergillus flavus* showed maximum lipase production of 121.35 U/gds which is five and nine times more than *Trichoderma harzianum* and *Penicillium chrysogenum* at pH-7.0 for 30.3°C having 96 h fermentation time [60].

Aspergillus niger J-1 has a maximum enzyme activity of 9.14 IU/g of dry solid substrate equivalent to 4.8 IU/ml of lipase activity using the solid state fermentation techniques with wheat bran as substrate having 0.75% ammonium sulphate, 0.34% urea and nitrogen source as the medium composition at pH-6.0 for 40°C within 24 h [61]. In another report, rice bran as substrate for *Aspergillus niger* has a maximum lipase production of 121.53 U/gdss at 30.3°C. These activities can further be improved by optimizing process variables such as oil concentration, glucose concentration and humidity ratio [62]. It has also been reported that endophytic fungal strain *Colletotrichum gloeosporioides* possess more potential for producing extracellular lipolytic enzymes in SSF with sunflower oil as substrate with a production of 2560 U/g DM at 25.0°C [63]. Extracellular lipase production using *Yarrowia lipolytica* NCIM 3589 with palm kernal cake as substrate alone has a maximum lipase activity of 18.58 U/gds at 96 h [64].

Scale up studies for the production of lipase using *Aspergillus niger* MTCC 2594 was reported with a maximum activity of 745.7 U/gds with tri-fermented substrates and suggested that these techniques have a wide potential in cost-effective biofuel production [65]. It has been reported that fungal stain *Fusarium oxysporum* produced serine peptidase and lipase in alkaline condition with higher enzyme stability. Wherein, wheat bran alone showed a high activity of 228.88 U/ml for proteolytic activity and 111.48 U/ml for lipolytic activity. Study of enzyme production by varying temperature and pH suggested that these enzymes are used for application in the detergent industry [66].

Stability of lipase for different substrates

In Cordova et al. [31] study, finely ground sugar cane bagasse sample was screened to 1mm particle size and assayed for components like minerals, fat, total nitrogen, cell wall components, total fibers, lingo cellulose and lignin, fat and ash whose presence improves the enzyme production [31,52,67,68]. They observed that *Rhizomucor pusillus* and *Rhizopus rhizopodiformis* showed maximum lipase production of 1.73 U/ml and 0.97 U/ml at 16 h and 13 h respectively, whereas bagasse and olive oil cake showed a maximum production of 10.83 U/ml and 43.04 U/ml respectively. They showed that olive oil cake has more protein content than bagasse. Kamini et al. (1998) [32] showed that *Aspergillus niger* MTCC 2594 with gingelly oil cake had a good stability of enzyme at pH of 4.0-10.0 and temperature of 4-50°C. The results were compared with submerged fermentation, where lipase showed low thermal stability and specific activity of 57% at 60°C and 69% activity at 60°C which makes the enzyme stable in all detergents [32]. Similar results have been reported for lipase production from *C.cylindracea*, *A.species* and *Rhizopus species* [69,70].

Gombert et al. [33] investigated three enzymes i.e. lipase, glucoamylase and protease and observed that of three enzymes, maximum activity was obtained with media of C/N ratio [33]. A cake with 1% peptone lipase showed an activity of 4.3 times in the basal medium, whereas protease and gluco amylase showed 1.2 and 1.5 times the activity, respectively. High nitrogen concentration in culture media is effective in enhancing the production of lipase by micro-organisms [37,50,71]. Different nitrogen sources for lipase production by *Penicillium* strain in laboratory-scale fermentor have also been explored [71,72]. The basal medium rich in protein content as co-factors, amino acids which match *P.restrictum* physiological characteristics is beneficial for lipase biosynthesis. Previous investigations involving SmF showed that *P. restrictum* lipase is not stable at alkaline pH levels with a decrease in the lipase activity but a serine protease inhibitor (PMSF) when added at later fermentation stages showed improved activity [72,73]. The accumulation of proteases in SSF affecting the stability of lipases was also observed with fungus and with different species of *Penicillium* [46].

Ikram ul-Haq et al. [34] studied parameters like temperature, inoculum size etc., and showed that either lower or higher temperature result in increased energy requirements and hence, decrease the product yield [34]. Several investigations have been carried out at different cultivation temperatures and higher values as units per liter are reported [74-78]. Increase in mycelia mass, production of enzyme was shown to decline due to exhaustion of nutrients in the fermentation mash. Enzyme extraction was found to be maximum using phosphate buffer, because of permeability of membrane. Rate of enzyme synthesis using *R. oligosporous* showed maximum results at 48h with maximum enzyme synthesis at 2.16 u/g substrate per hour, whereas by using optimum concentration of substrate was found to be 0.0245-0.65 U/g per hour, 1.95 U/g per hour, 0.91 U/g per hour and 5.05 U/g per hour [46,79].

It is reported that the enzyme requires an energy of 114.25 and 116 KJ at lower and higher temperatures respectively for conversion of 1 mole substrate with the maximum activity at 45°C and pH 7.0- 8.5 [34]. Moisture content is a crucial factor in determining the process and physical properties of the solid substrate [52,80]. Whereas higher moisture content lead to decreased porosity and thus lowering oxygen transfer, lower moisture decrease the solid solubility, degree of swelling and produces a higher water tension. Maximum lipase production was obtained with wheat bran and synthetic oil based medium (SOB) medium in the ratio of 1:2.5 [35]. *A. niger* strains are known to be active at pH 4-7 and at temperatures of 40°C and 55°C. Lipase from *A. niger* NICM 1207 was found to be active at extreme acidic pH of 1.5-2.0 and over a broad pH range of 2.5-9.0 for up to 24 h at room temperature.

The optimum temperature of lipase production from *A. niger* NCIM 1207 is at 50°C and is stable at 60°C for 5 h. Addition of water to *A. niger* NCIM 1207 reduces the synthesis of esters shifting the equilibrium of the reaction towards hydrolysis, producing fatty acids. Thus, transesterification becomes dominant only when the availability of water is restricted. This enzyme also showed similar optimum pH in organic solvent. This phenomenon was well explained as pH memory and also reported in several literatures [81]. Characterization and partial purification of iso-lipases (Lip A, Lip B, Lip C) for biomedical application in pharmaceutical industry through SSF is well explained in detail by other investigators [82].

Assays for Determining Lipase Activity

Various techniques have been used to determine the lipase enzyme activity. The commonly used techniques are turbidimetry, interfacial tensiometry, atomic force microscopy, infrared spectroscopy, titrimetry, colorimetry, flourimetry, chromatography, electron microscopy and immunodetection. Colorimetry is one of the known methods used for determining the enzyme activity. In this technique the substrates used are lipids, olive oil emulsions and para-nitrophenyl esters. The principle involved in lipid water interface oil emulsion is absorbance. Safranin absorbance change is due to the net negative charge at the lipid water interface and the product analyzed with this principle is safranin. The olive oil emulsion with copper reagent as substrate involves the estimation of copper complex spectrophotometrically at 440 nm. The complex develops a pink colour absorbance at 513 nm and the product analysed is rhodamine G-FFA complex. Another method of identifying the lipase activity is using the yellow coloured product which can be measured at 410 nm with para-nitrophenyl esters as substrate to analyze the product [83-86].

Titrimetry is the easiest way of identifying the activity of the enzyme. To identify the lipase enzyme activity involves use of stirred emulsion of TAG, tributyrin, olive oil emulsified with gum Arabic as substrates and the principle involved is pH stat-method-Neutralization of released FFAs using titrated NaOH. This is the most common procedure and is sensitive to within 1 µmol fatty acid released per min [87-88].

Turbidimetry assay is a method used for determining the concentration of a substance in a solution by the degree of cloudiness or by the degree of clarification [89]. The principle used in this technique is- increase in optical density of the sample at 500 nm due to precipitation in the form of calcium salts and the substrate used is tween20 in the presence of $CaCl_2$, product analysed being is free fatty acid. Electron microscopy applies the principle of detecting fatty acids by using lipids as substrates [90]. This is simple but, initial cost of the equipment is high for this assay technique. Atomic force microscopy uses lipid bilayer supported on mica as a substrate and involves the principle of lipid dissolution which forms holes on the bilayer with time and it monitored by real time images [91]. Its main advantage is that it is applicable even for the micro as well as nano-size particles. Similarly, other assay techniques like interfacial tensiometry, infrared spectroscopy, flourimetry, chromatography, immuno detection are practiced based on the substrate availability the product is analyzed. Interfacial tensiometry, infrared spectroscopy, atomic force microscopy techniques are highly expensive and sophisticated in practice. The different methods for estimating the lipase activities by quantitatively and qualitatively are given in Table 2.

Applications of Lipases

Lipases are one of the most versatile enzymes that are extensively used in hydrolysis of fats and oils, dairy industry, detergents and degreasing formulations, anti-asthma drug, biodiesel production, oleo chemical industry, pharmaceutical industry, cosmetic industry, region selective vacillations, pulp and paper industry and flavor enhancement [92,93]. It also has medical applications like modification of castarospermine (a promising drug for the treatment of AIDS), antihypertensive agents such as angiotonsin-converting enzyme (ACE) inhibitors and synthesis of calcium channel blocking drugs such as diltiazem. Detail industrial applications of lipases are dealt in Table 3.

Engineering Aspects of Solid State Fermentation

Several bioreactors have been traditionally used in SSF processes. These can be primarily classified as: packed-beds, rotating drums, perforated drums, horizontal paddle mixers, gas-solid fluidized beds, stirred aerated beds, rocking drums and tray bioreactors. A rotating drum bioreactors, as an horizontal cylinder, with mixing provided by the tumbling motion of the solid medium aided by the baffles on the inner wall of the rotating drum (perforated or unperforated) [4]. However, in all of these reactors, the mixing is less efficient than with a paddle mixer [94]. Practically, SSF processes could be operated in batch, fed-batch or continuous modes, although batch processes are the most common [95]. This is due to the fact that uninoculated substrate particles require interparticle colonization which is slow in fed batch or continuous operation. In many reactors, *in-situ* sterilization is facilitated. A wide variety of solid substrates are employed in SSF, which vary in composition, size, mechanical resistance, porosity and water holding capacity [96].

The SSF bioreactors should be anticorrosive, non-toxic to the process organisms as well as facilitate processing aspects like substrate preparation, sterilization, loading and product recovery. Loading and unloading of substrates is accomplished using pneumatic conveying. A novel and efficient design of integrated solid matrix bioreactor called the PLAFRACTOR™ has been recently reported which has computer-controlled compact device wherein all the operations described above are made possible [97]. The entry of contaminants into the process as well as the uncontrolled release of the process organisms into the environment must be avoided by using filters on both the inlet and outlet air streams. Other factors that affect the bioreactor design are: the morphology of the fungus, its resistance to mechanical agitation and the necessity to have a sterile process.

The four major types of bioreactors used by SSF [96] are:

- Reactors without forced aeration (Tray reactor)

- Unmixed reactors with forced aeration (Packed bed)

- Continuously mixed reactors with circulation (Rotating drums)

- Intermittently mixed bed bioreactors with forced aeration

The transfer of heat into or out of the SSF system is closely related with the aeration of fermentation system. The temperature of the substrate is also very critical in SSF as it ultimately affects the growth of the micro-organism, spore formation, germination, and product formation. High moistures results in decreased substrate porosity, which in turn prevents oxygen penetration. This may help to avoid bacterial contamination. On the other hand, low moisture content may lead to poor accessibility of nutrients resulting in poor microbial

Assay	Substrate used	Product analyzed	Principle	Remarks	Reference
Turbidimetry assay	Tween20 in the presence of CaCl$_2$	Released fatty acids	Optical density increase at 500 nm due to precipitation in the form of calcium salts	Simple and quantitative but Tweens are not specific substrates for lipases	[89]
Interfacial tensiometry	Lipid monolayer spread on surface of aqueous phase	Fatty acids	Monitoring of surface pressure change due to dissolution of lipids using electro microbalance and Teflon barrier	Highly sensitive reliable measurements,	[114] [115]
	Oil water interface	Fatty acids	Tensiometers for oil drop method	Low amounts of lipids used, but requires very sophisticated equipments	[116]
Atomic force microscopy	Lipid bilayer supported on Mica	Fatty acids	Lipid dissolution forms holes in the bilayer an the increase in area of holes with time is monitored using real time images	Nano scale assay and hence requires very sophisticated instruments	[91]
Infrared spectroscopy	Triacyl glycerols (TAG)	Free fatty acids and Fatty acid esters	In the Fourier transform IR spectrum Fatty acid esters peak at 1751 cm^{-1} and FFAs at 1715 cm^{-1} and hence can be quantitated on the basis of molar extinction coefficients	Expensive and sophisticated equipments required	[117]
Titrimetry	Stirred emulsion of TAG, tributyrin, Olive oil emulsified with gum Arabic	Fatty acids	pH stat method –Neutralization of released FFAs using titrated NaOH	Most common procedure, sensitive to within 1 µmol fatty acid released per min, disadvantage if FFAs are not fully ionized	[87] [88]
Spectrophotometry (Colorimetry)	Lipid at lipid water interface Olive oil emulsion	Safranin	Absorbance change of Safranin due to change in net negative charge at the lipid water interface	Lipase activities as low as 50 mU can be detected	[83]
	Olive oil emulsion in presence of copper reagent	Free fatty acids converted to copper soaps.	Formation of a copper soap. The copper complex is estimated spectrophotometrically at 440 nm	Sensitivity and efficacy improved for specific purposes by many researchers Reproducibility is difficult	[84]
		Rhodamine G-FFA complex	The complex develops a pink color. Absorbance read at 513 nm	Convenient and quick method, used commonly. These esters are liable to spontaneous hydrolysis and also by non-specific estetrases	[85]
	Para-nitrophenly esters	Para nitro phenol	Yellow coloured product which is measured at 410 nm		[86]
Flourimetry	TAG with alkyl groups substituted with fluorescent group (Pyrenic acylglycerol derivatives)	Free fatty acids	Shift in fluorescence wavelength after hydrolysis	Rapid assay but expensive substrate, Chemically modified Tag is poorly hydrolyzed	[118] [88]
	Triaclyglycerol in the presence of fatty acid binding protein conjugated to an acrylodan fluorophore	Free fatty acids	Fluorescence emission wavelength changes from 432 nm to 505 nm upon binding	Detection of concentration as low as 1 nM. Kit commercially available	[119]
	Phosphatidylch online containing naturally fluorescent parinaric acid	Parinaric acid	Detection of parinaric acid. Excitation and emission wavelengths of parinaric acid -324 nm and 420 nm	Low quantities can be detected	[120]
Chromatography	Lipids, TAGs	Fatty acids	Thin layer chromatography and quantititative analysis of FFAs by densitometric or auto radiographic methods when TAGs are labeled	Detection of as small as a few pmoles of fatty acids. Time consuming and not continuous	[121]
Electron microscopy	Lipids	Fatty acids	Electron microscopy detection of fatty acids	----------------	[90]
Immunodetection	----------------	Lipase	ELISA using monoclonal antibodies specific to antigens on Lipase	Detection of both active and inactive form	[122]

Table 2: Different assay methods used for Lipase activity

growth [98]. Aeration is achieved in the reactors by blowing air through the substrate or flowing air around a static bed. Aeration rate depends on the rate of heat removal. Water transfer, air supply and heat removal are achieved simultaneously through proper aeration. In the same way,

maintenance of uniformity within the substrate bed could be as effective as possible. The effect of the shear forces generated by mixing of both the substrate and the microorganism should be also considered. In this way, it has been observed that these forces can damage the penetrative

Industries	Action	Application
Detergents industry	Hydrolysis of fats	Remove oil strain from fabrics
Food industry	Flavour improvement, Aroma, quality improvement, Transesterification, Hydrolysis of fats, modification of butter fat	Shelf-life prolongation, Fat removal, Whippings, health foods
Chemical industry	Transesterification, Hydrolysis, Enantioselectivity, Synthesis.	Chiral building blocks, Triacylglycerides to mono- and diglycerides, Cosmetics, digestive aids
Leather industry	Softening, Quality improvement	Tanning
Pulp and paper industry	Hydrolysis	Improve Quality
Cleaning	Hydrolysis	Removal of fats
Bioconversion	Hydrolysis	Aqueous media
		Organic media
		nonaqueous
		biotransformation
Biomedical	Treatment	Pharmaceutical

Table 3: Industrial application of Lipase [27]

hyphae and affect the gel substrate [95].

A SSF bioreactor is being manufactured and marketed by M/s Fujiwara, Japan [99] which consists of a rotating bed in the form of a bucket with the provision of substrate mixing by ribbon-shaped baffles. Operations like substrate sterilization and inoculation are automated in this equipment. Slow rotation of the helical screws ensures minimal damage to growing hyphae along with an effective mixing of the substrate bed. This coupled with a slow rotation of the basket allows the elimination of temperature gradients within the substrate bed. The forced aeration of humid air from the bottom allows the alleviation of oxygen gradients without lowering the moisture content of the bed [26].

Enzyme Extraction and Purification

The usual method used to extract this enzyme is homogenization of the substrate or mixing it in a rotary shaker with one of the various solvents including water, NaCl, $(NH_4)_2 SO_4$ and NaCl with tween 80, triton X-100. The temperature at which the mixing is done is kept high enough for maximum extraction of the enzyme. However, there is no marked difference in the recovery of enzyme using various solvents just by themselves [35]. The mixture is then filtered using a double layer muslin cloth and the filtrate is centrifuged at around 5000g for 20 min at low temperature of around 4°C to prevent enzyme denaturation [35]. The clear supernatant is used as the extracellular enzyme. Supplementation of the solvent with surfactants like Triton X-100 increases recovery by increasing the membrane permeability. Phosphate buffer is also used for extraction of these enzymes with suitable pH values [34].

Biomass estimation

Biomass estimation in SSF is done indirectly using certain indicators including (a) biomass constituents such as glucosamine and ergosterol when the media constituents are same (b) carbohydrate or any substrate consumption during growth. Glucosamine content is determined using Ride and Drysdale method [100]. The fungal wall chitin is completely hydrolyzed to N-acetyl glucosamine in concentrated KOH by autoclaving [101]. The resulting chitosan suspension is precipitated and washed with progressive dilutions of ethanol in water which is then deaminated and solubilized with nitrous acid. Glucosamine is calorimetrically assayed using 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) and $FeCl_3$. Ergosterol is the predominant sterol of most fungi which can be measured using gas liquid chromatography on the basis of its characteristic UV absorbance [102]. This is a sensitive measurement for the initial stages of colonization.

Sucrose consumption is also used as an estimator of biomass [102]. Dry sample is agitated with distilled water and centrifuged to remove all cell material. The sucrose remaining is hydrolyzed to glucose using β-fructosidase and glucose is estimated by enzymatic method using hexokinase and glucose-6-phosphate dehydrogenase where the absorbance of NADPH is measured at 340 nm. Glucose can also be assayed using DNS reagent (3, 5-dinitrosalicylic acid in NaOH with sodium potassium tartrate) or GOD-POD method [103]. Similarly nitrogen can be estimated using Kjeldahl method and used to estimate biomass growth [104]. Indirect measurements of growth like CO_2 production can be done using Infrared analyzer. Infra analyzer works on the principle of reflected light by the matrix surface at specific wavelengths. The amount of the measured component can be expressed as follows [105].

$$C = F_o + F_1 \log \frac{1}{R_1} + \cdots F_i \log \frac{1}{Ri} + \cdots F_n \log \frac{1}{Rn}$$

Where C is the amount of component; Fo to F are the calculation constants;

R_1 to Rn are the reflection values; 1 to n is the filter numbers. Infrared (IR) estimation of cell components (glucosamine and ergosterol) and medium residues (sucrose and nitrogen) can be done directly on solid medium.

Challenging Aspect of SSF

Solid state fermentation has achieved more importance as the substrates used are agricultural wastes for enzyme production. The detailed applications to the recent technology with a little improvement in the design made the process more desirable and acceptable compared to submerged fermentation.

The major challenges in SSF are the scale up problems, recovery and purification of the end products, separation of biomass which is followed by disposal of biomass. Among several critical challenges, water activity and nature of the solid substrate plays a major role in fermentation process. Heat removal is typically a major concern and it is difficult to remove the waste metabolic heat from the solid bed in which the inter-particle phase is occupied by air in a continuous phase. The reasons could be thermal conductivity and heat capacity of liquid water is superior to those of moist bed with inter-particle air. Mixing greatly promotes heat removal by bringing the medium into contact with the cooling surfaces within the bioreactor. The process of mixing and also the sensitivity of the fungal to withstand the mixing is also a problem during SSF. In addition, it showed major problem in transport phenomena, heat and mass transfer are greater challenges in SSF.

These challenges can be addressed by designing the bioreactor to control the conditions within the bed, such as the temperature and water activity, at the optimum condition for growth and product formation. The growth of the organism on substrate causes deviations as the fermentation process starts where it release of waste metabolic heat and the consumption of O_2. The other challenging aspects to run SSF processes in a continuous mode is to maintain a uniform temperature, optimal agitation rate and homogeneity of the carbon, energy source in the SSF substrate [13].

Conclusions

This review mainly highlights the lipase production by SSF using fungal species. It is reported that the fungal species have an excellence in obtaining extracellular enzymes with higher activities [13]. The microorganisms like *Aspergillus species, Candida species and Penicillium species* are known to produce lipases in SSF when oily substrates are used. Lipase is extracted from the fermented substrate using solvents like distilled water, NaCl, phosphate buffer etc. Lipase can be assayed by using titrimetry, fluorimetry, turbidimetry, electron microscopy and surface tension techniques. It has been observed that wheat bran and mustard oil cake has potential to give high enzyme activity when subjected to SSF with small amount of soap stocks as inducers [35,60,66,106]. Biomass estimation involves methods which estimate substrate consumption or glucosamine of chitin cell wall. Further, it is a complete utilizing process where it produces enzyme with high titer value and the biomass can be utilized for animal feed, fuel generation and fertilization. Different kinds of bioreactors used for SSF include rotary drum, packed bed, tray fermentor and gas-solid fluidized bed with mixing or without mixing. The SSF reactors are usually operated in batch mode for better enzyme yields. Microbial lipases have an upper hand due to their specificity, availability of raw materials, economic feasibility and biocompatibility to the environmental. These microorganisms are generally recognized as safe (GRAS) for food, brewing and pharmaceuticals in which lipases have good potential to replace chemical processes. Use of fungal genera for production of lipases may be beneficial for the removal of hazardous materials from the environment since SSF is being used to detoxify chemical from industries. Thus, improvements in SSF in future for fungal lipase production will aid in speeding up the challenges which can bring about higher titer values at lower cost.

Acknowledgements

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