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Potential Aspects of Lipases Obtained from *Rhizopus* Fungi

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Abstract: Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are a class of hydrolases that catalyze both, hydrolysis and synthesis of esters which are formed from glycerol and long-chain fatty acids. Lipases are potential enzymes which are used extensively in a wide range of industrial applications. The production, biochemical properties and application potentials of the extra-and intra-cellular lipases obtained from *Rhizopus* fungi have been studied by various researchers. Research efforts are being directed towards obtaining high enzyme yields of both intracellular and extracellular lipases from *Rhizopus* by optimizing production or through genetic engineering and in purifying these lipases to elucidate structure, properties and potential applications. Lipases from the *Rhizopus* genus have been found to have enantioselectivities suitable for resolution of various compounds which have potential in the synthesis of pharmaceuticals, cosmetics, agrochemicals and other products. More attention is being paid to *Rhizopus* lipases for their strong 1,3-position specificity making them especially suitable for lipid modifications. *Rhizopus* lipases either as whole cell biocatalyst or as the purified form of lipase have great potential for biodiesel production from waste oils which is an ever-expanding industrial field. Some of the purified lipases of this genus exhibit thermostability and good stability in organic solvents which therefore have a great potential in the biocatalysis industry.

Key words: Extracellular lipases, fermentation, fungi, intracellular lipases, regiospecificity, *Rhizopus*

INTRODUCTION

Lipase is the first enzyme to be discovered by Claude Bernard in 1856 and since then, researchers have obtained an increased interest in discovering potential enzyme-producing microorganisms and in identifying the unique enzyme properties. This research interest is due to the great economic potential of enzymes on the market which is increasing year by year. The global market for industrial enzymes as reported by Business Communications Company, Inc., was estimated at $2 billion in 2004 and has an average annual growth rate of 4 to 5% (Rajan, 2004).

Lipases occur widely in nature, but only the microbial lipases are commercially significant (Sharma *et al*., 2001). They are placed only after proteases and carbohydrases in world enzyme market and share about 5% of the total enzyme market (Vakhlu and Kour, 2006). Only 2% of the world's

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microorganisms have been tested as enzyme sources (Hasan *et al*., 2006) and therefore researchers in this field are investigating ways of screening for and enhancing the production of different enzymes.

Rhizopus species such as *Rhizopus arrhizus*, *Rhizopus delemar*, *Rhizopus japonicus*, *Rhizopus niveus* and *Rhizopus oryzae* are filamentous fungi and are part of the most productive species on the market for lipase which also include the genera *Aspergillus*, *Mucor*, *Penicillium* and *Geotrichum* (Godtfredsen, 1990; Ghosh *et al*., 1996; Rubin and Dennis, 1997). The systematic of *Rhizopus* fungi is as follows: Phylum Zygomycota, Class Zygomycetes, Order Mucorales, Family Mucoraceae with Genus *Rhizopus* (Punt *et al*., 2002).

Most *Rhizopus* fungi are mesophilic (Nahas, 1988) but there are some which have been reported to be thermophilic such as *R. rhizopodiformis* (Samad *et al*., 1990), *R. arrhizus* (Kumar *et al*., 1993), *R. oryzae* (Salleh *et al*., 1993) and *R. homothallicus* IRD 13a (Mateos Diaz *et al*., 2006) which are capable of growing at 45 to 50°C. Thermophilic and thermotolerant fungi are potential sources of suitable enzymes with high levels of thermal stability and activity at the mesophilic temperatures used in chemical processes (Mateos Diaz *et al*., 2006).

Lipases from fungi are important in industrial applications (Essamri *et al*., 1998) and have been widely used for biotechnological applications in dairy industry, oil processing and production of surfactants and preparation of enantiomerically pure pharmaceuticals (Hiol *et al*., 2000). Thus lipases from *Rhizopus* species have also gained more and more attention by researchers. Filamentous fungi are preferred sources of lipases as they produce extracellular enzymes (Saxena *et al*., 1999) but for a variety of fungal strains the intracellular lipases have also been found to have unique application properties.

Moulds are known to produce lipases of high catalytic potential and stability (Hameed, 1997) thus making them suitable for various industrial applications. Lipases which hydrolyze the ester bonds of triglycerides at the sn-1 and sn-3 positions, but not the sn-2 position are referred to be 1,3-specific. Increasingly more attention has been paid to lipases from *Rhizopus* for their strong 1,3-position specificity (Yan *et al*., 1999; Ul-Haq *et al*., 2002). The 1,3-specific property has been shown to be particularly useful for producing structured lipids (Shimada *et al*., 1996).

This review gives an overview on the research efforts which have been conducted regarding lipases obtained from the *Rhizopus* species with particular emphasis on the enhancement methods for their production, the subsequent purification techniques applied, their biochemical characterization and application in industry.

THE PRODUCTION OF LIPASES FROM *Rhizopus* SPECIES

The growth of the biotechnology industry has led to filamentous fungi being widely employed in the fermentation industry and becoming a principal source of enzymes and metabolites (Wang *et al*., 2005). Features such as low cost and high productivity have attracted many research efforts in both molecular-genetic techniques and bioprocess improvements (Finkelstein and Ball, 1992; Banerjee *et al*., 2003).

There are three fermentation techniques applied in industry for lipase production: Solid-state fermentation, submerged fermentation and immobilized cell fermentation (Benjamin and Pandey, 1997; Elibol and Ozer, 2000a; Elibol and Ozer, 2002; Yang *et al*., 2005). All three techniques have been investigated by researchers to enhance lipase production from the genus *Rhizopus*. According to Yang *et al*. (2005) the lipase production by *Rhizopus* species is relatively low and high at cost. But there are a number of reports that have successfully used production enhancement methods to optimize the fermentation conditions and have obtained increased folds of lipase production making the *Rhizopus* fermentation more economic. Yan *et al*. (1999) optimized fermentation conditions

with *Rhizopus* Y92 and obtained 99.15 U mL^{-1} of lipase activity (olive oil emulsion method) and *R. oligosporous* (Toshiko *et al*., 1989) showed higher productivity of lipases among other species of fungi studied.

Some *Rhizopus* fungi are able to produce several different lipases possessing different molecular forms. Davranov and Kuilibaev (1994) have studied production of the intra-and extra-cellular lipases and its individual forms during cultivation of *R. microsporus*. The fungus produces five forms of intracellular lipases, three of which are secreted by intact cells into the culture medium (Davranov and Kuilibaev, 1994). Choosing the right fermentation condition for lipase production is crucial and each strains needs its own investigative study to determine its optimum condition for optimal lipase production.

Submerged Fermentation (SmF)

Submerged fermentation has been used by the majority of researchers for the production of fungal lipases. Lipase production depends on several process variables, such as culture pH, substrate concentration, inoculum level, inducer concentration and growth temperature (Elibol and Ozer, 2002).

Effects of Inducers, Carbon and Nitrogen Sources

Inducers such as oils, fatty acids or surfactants and different carbon and nitrogen sources have been studied by many researchers to assess their effects after the addition to fermentation cultures with the main aim of enhancing the lipase production by *Rhizopus* fungi. Among the genus of *Rhizopus* fungi the production mechanism of the intra-and extra-cellular lipases differs in the response to the different media components added.

The intracellular lipase activity of *R. chinensis* for the interesterification reaction was enhanced significantly by the presence of substrate-related compounds or inducers such as oleic acid, olive oil and tea oil but the production of extracellular lipase was higher in cultures containing none of these substrate-related compounds (Nakashima *et al*., 1988). Iwai *et al*. (1967) explained this phenomenon after studying the lipase production of *R. delemar*. They suggested that in the absence of these compounds a great amount of lipase existed intracellular until the stationary phase and that beyond that point it decreased sharply implying that in the last stage of cultivation all intracellular lipase would then be secreted into the culture medium and existing then as the extracellular lipase. Although this is true for some strains it does not apply to all since some fungi did indeed yield higher extracellular amounts after addition of certain inducers (vegetable oils, surfactants, fatty acids and other).

In the application of a circulating bed fermentor for intracellular lipase production by *R. chinensis*, Nakashima *et al*. (1989) used meat extract as the nitrogen source and as the fed-substrate since its use is more economic than the use of polypeptone at large scale. Researchers have found that the presence of glucose in media containing oil reduced the production of lipases from these fungi. According to Fadiloğlu and Erkman (1999) this result may be due to limited availability of these carbon sources to the fungus in media supplemented with oil. The optimum nitrogen source for lipase production by *R. chinensis* was polypeptone and the presence of glucose together with substrate-related compounds reduced lipase production (Nakashima *et al*., 1988). The addition of glucose or lactose to the medium in presence of olive oil decreased lipase production by *R. oligosporous* (Nahas, 1988). Glucose had a repressive effect on lipase production by *R. arrhizus* NRRL 2286 if it was used in a concentration of higher than 1.0 g L^{-1} (Elibol and Ozer, 2002). The best carbon and nitrogen sources for lipase production by *R. delemar* CDBB H313 were dextrin and yeast extract, respectively with sunflower oil yielding the highest lipase production (Espinosa *et al*., 1990). The surfactant, Tween 80 exerted positive effects on its lipase production by *R. delemar* CDBB H313. The best carbon source and best

nitrogen source for lipase production by *R. rhizopodiformis* (Samad *et al*., 1990) were maltose and peptone respectively and glycerol and lecithin as substrate-related compounds also enhanced lipase production. A thermophilic *R. oryzae* capable of growing at 50°C was isolated from the effluent treatment pond of a palm oil mill and parameters affecting production of extra-and intra-cellular lipases were investigated by Salleh *et al*. (1993). All the carbon sources tested with the exception of sucrose generally inhibited the production of extracellular lipase by *R. oryzae*, but enhanced the production of intracellular lipase (Salleh *et al*., 1993). Peptone was the best nitrogen source for the production of extracellular lipase and intracellular lipase activity could be enhanced by a variety of substances such as tryptone, corn steep liquor, polypeptone and tryptic soy digest. Glycerol stimulated the extracellular lipase production by *R. oryzae* whereas the intracellular lipase was enhanced by olive oil, oleic acid and triolein and the surfactants, Tween 60 and Span 40 resulted in an approximately four times higher lipase production (Salleh *et al*., 1993). The addition of the surfactant, Tween 80 to the culture medium was required for maximum production of extracellular lipase by *R. delemar* (Martinez *et al*., 1993). Kumar *et al*. (1993) studied the production of acidic lipase from a thermophilic strain of *R. arrhizus*. Highest productivity was achieved with the addition of 2% groundnut oil. The best carbon and nitrogen sources were arabinose and soybean meal, respectively. Metal ions such as $MnCl_2$, $SnCl_2$ and $CaCl_2$ increased the lipase productivity by four fold. The *R. arrhizus* lipase productivity in the fermentor was much higher (310 U mL^{-1}) than in shake-flask (180 U mL^{-1}) (Kumar *et al*., 1993). Fadiloğlu and Erkman (1999) reported that lipase production by *Rhizopus oryzae* CBS 539.80 was higher in media containing olive oil compared to media with no olive oil. Olive oil increased growth and lipase activity nearly two fold but not lipase specific activity (Fadiloğlu and Erkman, 1999). Nahas (1988) found that lipase specific activity was not increased at the same rate as biomass production and lipase activity during fermentation with *R. oligosporous*. Highest lipase activity achieved by *R. oryzae* was with protease peptone in combination with olive oil and lowest with yeast extract and lactose. Glucose or lactose added to media with olive oil suppressed lipase production. Compared to nitrogen sources they reported that these stimulate lipase production in the presence of olive oil as carbon source.

A two level fractional factorial design was used to optimize intracellular lipase production by *R. oryzae* ATCC 24563 (Essamri *et al*., 1998). The most suitable substrates for lipase production and cell growth by *R. oryzae* ATCC 24563 were rapeseed and corn oil with best growth and optimum lipase production at an oil concentration of 3 and 2%, respectively (Essamri *et al*., 1998). The addition of vegetable oils to the culture medium increased both the lipase activity and cell growth up to three folds compared to medium without oil (Essamri *et al*., 1998). Elibol and Ozer (2002) reported that initial glucose concentration and an inducer viz. corn oil concentration were the major factors affecting lipase production by *R. arrhizus*. The combined effects of initial glucose concentration and inducer (corn oil) concentration on lipase production by freely suspended *R. arrhizus* NRRL 2286 was thus investigated using the response surface methodology with a 2^2 full-factorial central composite design. Optimum glucose and inducer concentrations were found to be 1.1 and 3.3 g L^{-1}, respectively obtaining biomass concentration of 2.4 g L^{-1} with a lipolytic activity of 370 μmol L^{-1} min (Elibol and Ozer, 2002). Soybean flour and soy protein concentrate were the best nitrogen sources for lipase production by immobilized *R. arrhizus* BUCT (Yang *et al*., 2005) and the best inducer for lipase production was oleic acid.

Fukumoto *et al*. (1966) developed a process for preparing *R. delemar* lipase free from protease. They showed that increased amounts of the nitrogen source such as peptone, or decreased amounts of the carbon source in the culture medium can substantially depress the production of free protease. The coexistence of lipase and protease has been noted by many researchers (Jonsson and Snygg, 1974; Pimentel *et al*., 1994) and this was the reason given for the rapid inactivation of lipase (Iwai and Tsujisaka 1984; Long *et al*., 1996). Optimizing culture conditions which can suppress protease production can result in significant yields of lipase.

Effects of Oxygen, pH, Temperature and Stirring Speeds

Lipase production by *R. delemar* was found to be dependent on the oxygen concentration in the culture medium (Giuseppin, 1984) where increasing oxygen concentrations enhanced the lipase production. Yang *et al*. (2005) observed increasing lipase activities by *R. arrhizus* BUCT with increased levels of aeration. However high rotation speeds decreased lipase production. The effect of oxygen on lipase production by *R. arrhizus* NRRL 2886 was studied by Elibol and Ozer (2000b) under two operating modes, controlled Dissolved Oxygen concentration (DO) and controlled aeration rate. The fermentation was studied using a 1-L capacity fermentor. A significant increase in lipase yield was obtained when the DO level was increased from 20 to 30% air saturation (Elibol and Ozer, 2000b).

For lipase production by *R. oryzae* the optimum temperature was at 30°C and pH was 8.5 (Essamri *et al*., 1998) and after 48 h the production was 12-fold greater than that obtained with the non-optimized medium. For maximum production of extracellular lipase by the mesophilic *R. oryzae* (Razak *et al*., 1988), an agitation speed of 300 rpm was necessary. Static incubation was required for maximum lipase production by *R. oligosporous* and the optimum pH for the lipase production was at 6.0 (Nahas *et al*., 1988). The optimum stirring speeds and temperature for intra-and extra-cellular lipase production by *R. rhizopodiformis* were 150 rpm, 37°C and 100 rpm, 45°C, respectively and the optimum pH for both types of lipases was at 5.0 (Samad *et al*., 1990).

Solid-State Fermentation (SSF)

Solid-state fermentation holds tremendous potential for the production of enzyme as it fulfils the demand of industrial environment and food biotechnology as compared to stirred fermentation (Pandey *at al*., 1999). Lipase production by solid state fermentation of olive cake and sugar cane bagasse using thermostable fungal culture of *R. rhizopodiformis* (ORSTOM culture collection) was studied by Cordova *et al*. (1998). Solid state fermentation of by-products such as olive cake bagasse and sugar cane bagasse could be of economic importance for the production of thermostable lipases by thermophilic filamentous fungi and a way to reduce the environmental threat created by the disposal of untreated olive oil cakes (Cordova *et al*., 1998). Cultivation of *R. rhizopodiformis* on bagasse and olive cake as solid substrates produced 79.60 U of lipase per gram of dry matter (equivalent to 43.04 U mL^{-1}) (Cordova *et al*., 1998). The production of extracellular lipase by *R. oligosporous* GCBR-3 using solid-state fermentation yielded a maximum activity (48.0±2.1 U g^{-1} substrate) using almond meal therefore *R. oligosporous* GCBR-3 can be exploited for large-scale production of enzyme for commercial purposes (Ul-Haq *et al*., 2002). The mutant strain of *R. oligosporous* T^{UV}-31 was studied by Iftikhar and Hussain (2002) for production of extracellular lipase by solid-state fermentation. Tween 80 at 0.5% was found to be the best carbon source. The best organic and inorganic nitrogen sources for lipolytic activity were soybean meal and ammonium sulphate, respectively. Egg yolk achieved a maximum production of the lipase. Solid-state fermentation for lipase production by *R. oligosporous* ISU^{UV}-16 (Awan *et al*., 2003) obtained maximum lipase production when Tween 80 at a concentration of 0.5% was used as an additional carbon source. The best organic nitrogen source for optimal lipolytic activity was ammonium sulphate.

Immobilized Fungal Mycelium

In most technical applications of immobilized cells the objective is to increase the extent of reaction and to facilitate downstream processing (Elibol and Ozer, 2000a). Nakashima *et al*. (1988) reported that when *R. chinensis* cells were immobilized in polyurethane Biomass Support Particles (BSPs), the extracellular lipase production was suppressed and the intracellular lipase activity was several-fold higher than that of the freely suspended cells. A 10-L circulating bed fermentor with a working volume of six to eight liter was applied by Nakashima *et al*. (1989) to examine the industrial preparation of immobilized acetone-dried cells of *R. chinensis*. Cubic polyurethane foam HR-40 was

used as BSPs. Both the intracellular and extracellular lipase formations by *R. chinensis* were greatly enhanced using BSPs and the circulating bed fermentor was successfully employed for their production using BSPs (Nakashima *et al*., 1989). Immobilization of *R. chinensis* cells in various BSPs made from polyurethane, nylon, polyester, stainless steel, polyvinyl alcohol and cellulose enhanced intracellular lipase activity as much as several-fold than that of freely suspended cells irrespective of the BSP material (Nakashima *et al*., 1990). Effective immobilization on polyurethane and cellulose supports was also achieved with other strains of *Rhizopus* (*R. javanicus*, *R. delemar*, *R. oligosporous*, *R. niveus*, *R. japonicus* and *R. oryzae*) with several-fold enhancement in the intracellular lipase activity. Christen *et al*. (1995) studied lipase production by *R. delemar* using polymeric resin (Amberlite) as the BSP and dextrin, maltose or glucose as the substrates. The *R. delemar* lipase was effectively adsorbed on the support and 96 U g^{-1} of initial dry matter were obtained when dextrin was used as the carbon source against only 68 and 58 U g^{-1} for maltose and glucose, respectively (Christen *et al*., 1995). Elibol and Ozer (2000a) studied lipase production by immobilised *R. arrhizus* NRRL 2286 under various fermentation conditions. Immobilisation was achieved by placing a foam slab (15 ppi, 55×20×8 mm) fixed onto a stiff L-shaped stainless steel wire into 250 mL^{-1} flask. Maximum lipolytic activity was recorded at 1.0 and 2.5 g L^{-1} glucose concentrations, pH 6.0 and 150 rpm and corn oil resulted in a 2.5 times higher lipolytic activity than in the absence of the inducer. A 2 L capacity fermentor was used to produce extracellular lipase by *R. oryzae* (Hiol *et al*., 2000) where a metallic grill, used as growth support, was placed in the fermentor. Maximal enzyme concentration was reached after four days of culture at the late logarithmic growth in a medium composed of 4% corn steep liquor and 1% peptone (Hiol *et al*., 2000). Perlite and polyurethane were used as solid supports for lipase production by *R. arrhizus* cells and lipase activity was eight times higher compared to non-immobilized cells (Chunhua *et al*., 2002). Yang *et al*. (2005) have obtained higher amounts of lipase with *R. arrhizus* BUCT in repeated batch fermentation with immobilized mycelium than in batch fermentation and the time for repeated batch fermentation was reduced greatly from 72 to 6-12 h in shake flasks and from 96 to 18-24 h using a 5 L fermentor.

Effects of Fungal Morphology

The fungal morphological change, i.e., the formation of pellets or pulp-like mycelium during growth, is susceptible to shifting under varying culture conditions of the growth medium, such as pH, agitation, DO concentration or inoculum size (Metz and Kossen, 1977). It was reported by Nakashima *et al*. (1990) that immobilization of *Rhizopus* species in BSPs could promote morphological changes, from pulp-like cells to pellet-like cells and that this change caused the enhancement of intracellular lipase formation. In freely suspended cultures the overall intracellular lipase activity of free cells, consisting of pellet cells and pulp-like cells, increased with an increase in the ratio of pellet cells to pulp-like cells therefore the trigger for enhancement of intracellular lipase seemed to be cell aggregation, such as pellet formation (Nakashima *et al*., 1990).

Genetic Engineering for Enhancement of Lipase Production

The exponential increase in the application of lipases in various fields in the last few decades demands extension in both qualitative improvement and quantitative enhancement (Bapiraju *et al*., 2004). Strain improvement and medium optimization are required for quantitative enhancement. The strain is genetically engineered to overproduce the lipase in a suitable optimized culture medium. Another method of achieving overproduction of lipase is to isolate the gene responsible in the *Rhizopus* sp. and insert it into a suitable microorganism having simple growth requirements.

UV and N-methyl-N-nitro-N-nitroso Guanidine (NTG) were effective mutagenic agents for strain improvement of *Rhizopus* sp. BTS-24 for enhancement of its lipase productivity (Bapiraju *et al*., 2004). Matsumoto *et al*. (2002) optimized the cultivation procedure for maximizing the content of

active lipase in the *Saccharomyces cerevisiae* cells. Intracellular overproduction of the pro-sequence from *R. oryzae* IFO4697 (rProROL) was carried out under the control of the constitutive Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) promoter (Takahashi *et al*., 2001) and the inducible 5-upstream region of the isocitrate lyase gene of *Candida tropicalis* (UPR-ICL) (Umemura *et al*., 1995; Kanai *et al*., 1996). The highest intracellular lipase activity of 350.6 IU/l was obtained in the inducible UPR-ICL system with an initial glucose concentration of 0.5% at 30°C (Matsumoto *et al*., 2002).

Other Methods Employed for Enhancement of Lipase Production

One of the methods used for improving the microbial strains such as yeasts and filamentous fungi is cellular engineering and particularly the electrofusion of microbiological cells (Sawicka-Ukowska *et al*., 2004). The electrofusion method was employed by Sawicka-Ukowska *et al*. (2004) to obtain products of fusion of the filamentous fungus, *R. cohnii* Rh.c./1 with an increased capacity for lipase biosynthesis in comparison with the original strain. Protoplasts of auxotrophic mutants of the parent strain Rh.c./1 obtained after UV irradiation of the spores were subjected to electrofusion (Sawicka-Ukowska *et al*., 2004). The fusion product labelled XIII-21 was selected as the best strain and its lipase activity was about 3.5 times higher compared to the original strain Rh.c./1. Minning *et al*. (1998) used the methylotrophic yeast *Pichia pastoris* to functionally express and secrete the mature lipase of the fungus *R. oryzae*. In a batch cultivation using a 5 L bioreactor, where methanol feeding was linked to the dissolved oxygen content in the cultivation solution, a lipase activity of 500 000 units per liter (60 mg active lipase per liter) of culture was obtained after initial glycerol feeding of the culture (Minning *et al*., 1998).

PURIFICATION OF LIPASES FROM *Rhizopus* SPECIES

A variety of *Rhizopus* intracellular, extracellular lipases and their isoenzymes have been purified and characterized but the reports remain to be scarce. New species of *Rhizopus* fungi are being found and there is potential in the purification of their lipases. Table 1 summarizes some of the *Rhizopus* lipases which have been isolated and purified.

Methods of Purification

The intracellular lipase from *R. microsporus* UzLT-1 has been isolated and purified by Davranov and Duyarov (1978). They reported that the intracellular lipase differs from the extracellular lipase in

Table 1: List of some purified and characterized *Rhizopus* lipases

Rhizopus species	References
R. microsporus	Davranov and Duyarov (1978)
R. delemar	Iwai *et al*. (1974)
R. japonicus	Aisaka and Terada (1981)
R. delemar (isoenyzmes)	Tahoun and Ali (1986)
R. arrhizus	Gancet and Guignard (1986)
R. japonicus	Suzuki *et al*. (1986)
R. delemar	Haas *et al*. (1992)
R. japonicus	Uyttenbroeck *et al*. (1993)
R. oryzae	Ben Salah *et al*. (1994)
R. niveus	Kohno *et al*. (1994)
R. oryzae	
R. rhizopodiformis	Razak *et al*. (1997)
R. arrhizus	Chattopadhyay *et al*. (1999)
R. chinensis	Yasuda *et al*. (1999)
R. oryzae	Hiol *et al*. (2000)
R. homothallicus	Mateos Diaz *et al*. (2006)

its pH optimum and electrophoretic mobility. The extracellular lipase from *R. japonicus* KY 521 was purified by Aisaka and Terada (1981) using ethanol precipitation, chromatography on Column-lite, affinity chromatography on Heparin-Sepharose 4B and finally, separation into two lipases, I and II, by isoelectric focusing. Tahoun and Ali (1986) have purified three intracellular lipases designated A, B and C from *R. delemar*. An Ultrogel column was applied after enzyme extraction followed by gel filtration on Sephadex G150 column. The specific activities of lipases A, B and C were 720, 480 and 320 U mg^{-1}, respectively. Hydroxyapatite, Octyl sepharose and Sephacryl S-200 columns were used by Suzuki *et al*. (1986) to purify *R. japonicus* NR 400 lipase. Oleic acid affinity and CM-Sephadex C25 columns have been applied to purify lipase from *R. delemar* (Haas *et al*., 1992). Two types of lipase were purified from *R. niveus* by Kohno *et al*. (1994) using DEAE-Toyopearl (1 pass) and CM-Toyopearl columns (2 passes). Razak *et al*. (1997) have purified and characterized extracellular lipases from *R. oryzae* and *R. rhizopodiformis*. Both species have been isolated from palm oil mill effluent. Acetone precipitation up to 80% was used followed by gel filtration with a Sephadex G-100 column (2.5×54 cm). Chattopadhyay *et al*. (1999) used ammonium sulphate fractionation followed by Sephadex G-100 gel filtration to purify lipase from *R. arrhizus*. The intracellular lipase from *R. chinensis* was purified by Yasuda *et al*. (1999) using CM-cellulofine C-500, Ether Toyopearl 650 M, Super Q Toyopearl and CM-cellulofine C-500 columns. Hiol *et al*. (2000) purified an extracellular lipase produced by *R. oryzae* by ammonium sulphate precipitation, sulfopropyl sepharose chromatography, Sephadex G-75 gel filtration and a second sulfopropyl sepharose chromatography step. Extracellular lipases from thermotolerant *R. homothallicus* isolated from solid and submerged fermentation were purified to homogeneity by Mateos Diaz *et al*. (2006). The two lipases were purified by application of Butyl Sepharose followed by Superdex G-200 chromatography. Table 2 summarizes the yields, purification folds and the molecular weights of some of the purified *Rhizopus* lipases.

Characteristics of Purified *Rhizopus* Lipases

Temperature, pH Properties and Stabilities in Organic Solvents

Extracellular lipase purified from *R. oryzae* by Hiol *et al*. (2000) showed a good stability in a variety of organic solvents and especially in long chain-fatty alcohols. The lipase retained about 65%

Table 2: Summary of yields and molecular weights of purified *Rhizopus* lipases

Rhizopus sp.	Yield (%)	Purity (fold)	Molecular weight (kDa)	References
R. japonicus				Aisaka and Terada (1981)
Lipase I			42	
Lipase II			42	
Rhizopus japonicus NR 400	31	93	30	Suzuki *et al.* (1986)
R. delemar				Tahoun and Ali (1986)
A	11.1	106	76	
B	3.3	70.6	60	
C	4.0	47	45	
R. delemar	30	10.3	30.3	Haas *et al.* (1992)
R. niveus				Kohno *et al.* (1994)
Lipase I	31.1	54.4	34	
Lipase II	3.1	67.9	30	
R. oryzae	64	158	-	Razak *et al.* (1997)
R. rhizopodiformis	38	10	-	
R. arrhizus	42	720	67	Chattopadhyay *et al.* (1999)
R. chinensis	27.6		28.4	Yasuda *et al.* (1999)
R. oryzae	22	1260	32	Hiol *et al.* (2000)
R. homothallicus				Mateos Diaz *et al.* (2006)
SmF	40	42	29.5	
SSF	15	77	29.5	

SmF: Submerged fermentation, SSF: Solid state fermentation

Table 3: Temperature and pH properties of lipases purified from various species of *Rhizopus* fungi

Species	pI	Optimum pH	pH stability range	Optimum T (°C)
R. japonicus (Aisaka and Terada, 1981)	7.0-8.5 (both)			
Lipase I	7.4			
Lipase II	7.9			
R. delemar (Haas *et al*., 1992)	8.6	8.0-8.5	7.3-9.1	30
R. arrhizus (Kumar *et al*., 1993)		3.5		45
R. niveus (Kohno *et al*., 1994)				
Lipase I	6.0-6.5			35
Lipase II	6.0			40
R. oryzae (Razak *et al*., 1997)	ND	6.0	4.0-7.0	45
R. rhizopodiformis (Razak *et al*., 1997)	ND	6.0	8.0	45
R. chinensis (Yasuda *et al*., 1999)		5.5		37
R. oryzae (Hiol *et al*., 2000)	7.6	7.5	4.5-7.5	35
R. homothallicus (Mateos Diaz *et al*., 2006)				30
SmF				40
SSF				

SmF: Submerged fermentation, SSF: Solid state fermentation

of its initial activity after 30 min incubation at 45°C. Table 3 summarizes the temperature and pH properties of lipases from various species of the *Rhizopus* fungi.

Regiospecificity and Substrate Specificity

Lipases purified from mycelia of *R. delemar* have the same catalytic activity with respect to glycerides synthesis from free fatty acids and glycerol (Tahoun and Ali, 1986). The lipases have inability to react at position 2 of glycerol and had high specificity towards caprylic and capric acids. The crude lipase preparation from *R. arrhizus* prepared by Kumar *et al*. (1993) is highly active in phospholipid triglycerides and failed to hydrolyse-methyl esters of caprylate and palmitate. Extracellular lipases from *R. oryzae* and *R. rhizopodiformis* purified by Razak *et al*. (1997) displayed 1,3-specificity. The *R. oryzae* lipase showed chain length specificity and exhibited broader substrate specificity compared to the lipase from *R. rhizopodiformis*. Purified lipase from *R. chinensis* exhibited high hydrolytic activity against fatty acid methyl esters such as methyl caprylate, methyl laurate and methyl palmitate and was also able to catalyze transesterification between olive oil and methyl laurate in n-hexane (Yasuda *et al*., 1999). The *R. oryzae* lipase purified by Hiol *et al*. (2000) had a preference for the hydrolysis of saturated fatty acid chains (C8-C18) and a 1, 3-position specificity but poorly hydrolyzed triacylglycerols containing n-3 polyunsaturated fatty acids.

Inhibition Effects

Serum had an inhibition effect on the lipases I and II purified by Aisaka and Terada (1981). Triton X100, SDS and metal ions such as Fe^{2+}, Fe^{3+}, Cu^{2+} and Hg^{2+} inhibited the lipolytic activity of the extracellular lipase from *R. oryzae* isolated by Hiol *et al*. (2000) and was enhanced by sodium cholate or taurocholate. Benzamidine and PMSF had no effect on the lipase activity (Hiol *et al*., 2000).

INDUSTRIAL APPLICATIONS

Rhizopus lipases in whole cell form and purified form have been applied in many fields such as biodiesel production, pharmaceutical, cosmetic and the food industry. *Rhizopus* lipases in their whole cell form have been shown to be more stable and are yielding higher amounts of the desired product. Naoe *et al*. (2001) have studied *R. delemar* lipase for the esterification of oleic acid with octyl alcohol in reverse micellar system of sugar ester DK-F-110. The sugar esters were employed as a non-ionic amphiphile to form reverse micelles for biocatalysis.

Modification of Oils and Fats

Nutritional and physico-chemical properties of fats and oils can be improved via enzymatic transesterification. Lipases allow for the modification of properties of lipids by altering the location of fatty acid chains in the glyceride and replacing one or more of the fatty acids with alternative ones and thus producing high value lipids from cheap and less desirable ones (Sharma *et al*., 2001). The *R. japonicus* lipase has been used to produce hard butter suitable for chocolate manufacture by interesterification of palm oil with methyl stearate (Matsuo *et al*., 1981). *R. niveus* lipase has good 1-3 positional specificity and is therefore useful as a cacao butter substitute (Matsuo *et al*., 1980) cells. Safari and Kermasha (1994) have demonstrated that, among several commercial enzymes, lipase from *R. niveus* shows an interesting potential for the production of interesterified butter fat with an increased proportion of oleic acid at the sn-2 position.

There is potential in the application of naturally immobilised lipase in the oils and fats industry to catalyse processes such as interesterification, glycerolysis, acidolysis and transesterification (Long *et al*., 1996). Nakashima *et al*. (1988) have immobilized cells of *R. chinensis* (with a 1, 3-positional specificity lipase) on Biomass Support Particles (BSPs) and compared their activity to freely suspended cells. The interesterification lipase activity was 4-7 times higher than that of freely suspended cell but the intracellular hydrolysis lipase activity could not increase in proportion to the increase of intracellular interesterification activity (Nakashima *et al*., 1988). Nakashima *et al*. (1988) suggested that various types of lipases might be produced in the cells immobilized in BSPs. No clear difference in extracellular hydrolysis activity was observed between immobilized and non-immobilized.

The interesterification of olive oil with palmitic acid catalyzed by *R. delemar* lipase was investigated in phospholipids microemulsion systems by Komatsu *et al*. (2004) using soybean lecithin as the amphiphilic component. One of the main applications of the lipase-catalyzed transesterification reaction is the incorporation of Docosahexaenoic Acid (Dha)/Eicosapentaenoic Acid (EPA) into vegetable oils (Khare and Nakajima, 2000).

Immobilized *R. oryzae* lipase appears as an efficient biocatalyst for DHA enrichment of a mixtures of fatty acids from sardine oil by selective esterification (Hiol *et al*., 2000). The hydrolysis of sunflower oil by immobilized and native *Rhizopus* sp. lipase in a reactor system in presence of surfactants, proteins and metals was studied by Sroka (1994) whereby tit was found that the immobilized lipase was more resistant to inhibition than the native one. Khare and Nakajima (2000) immobilized *R. japonicus* lipase on Celite and applied it for the enrichment of DHA in soybean oil achieving an incorporation of 25% DHA into soybean oil in n-hexane media within 24 h.

Flavor and Fragrance Compounds

Biocatalytic reactions catalyzed by lipases are of increasing interest in the food industry (Komatsu *et al*., 2004). Esters of short chain fatty acids and alcohols are known as flavor and fragrance compounds. The use of lipase for direct esterification reactions in free-solvent media is a good alternative to produce flavor compounds for the food industry (Melo *et al*., 2005). Ethyl hexanoate is a typical fragrance compound of Chinese liquor and Japanese sake with an annual demand of more than 2000 kl. Whole cell lipase of *R. chinensis* CCTCC M201021 had a much higher ability in the synthesis of ethyl hexanoate with a maximum yield of 96.5% after 72 h conversion (Xu *et al*., 2002) among ten commercial lipases studied.

Biodiesel Production

Biodiesel (fatty acid methyl ester), which is derived from triglycerides by transesterification with methanol also referred to as methanolysis, has attracted considerable attention during the past decade as a renewable, biodegradable and nontoxic fuel (Hama *et al*., 2004). It is in current production using plant oil in Europe and the USA and waste oil in Japan (Oda *et al*., 2005). Research on lipases obtained

from *Rhizopus* fungi in the production of biodiesel is currently underway and these lipases have been found to be of great potential.

The extracellular lipase from *R. oryzae* (Kaieda *et al*., 1999) was found to be a potential biocatalyst for the methanolysis of waste oil which contain water since the lipase catalyzed methanolysis in the presence of 4-30% water in the starting reagents but was nearly inactive in absence of water. Utilization of waste oil reduces its disposal and its negative environmental impacts.

The enzymatic production of biodiesel fuel from plant oils using *R. oryzae* IFO 4697 cells (with a 1,3-positional specificity lipase) immobilized within biomass support particles for the methanolysis of soybean oil was investigated by Ban *et al*. (2001). When methanolysis was carried out with stepwise additions of methanol using BSP-immobilized cells, in the presence of 15% water the methyl esters content in the reaction mixture reached 90%-the same level as that using the extracellular lipase (Ban *et al*., 2001). Glutaraldehyde-treated *R. oryzae* IFO 4697 (Ban *et al*., 2001) immobilized cells had a high durability in comparison with non-treated cells and were suitable for repeated use and thus offers a promising means of biodiesel fuel production for industrial application.

Pizarro and Park (2003) applied *R. oryzae* lipase powder (F-AP15) for methanolysis of vegetable oils from waste bleaching earths obtained from a crude vegetable oil refining process. The vegetable oils were extracted and identified as soybean, palm and rapeseed oil. Optimum conditions for methanolysis of extracted oils were 75% water content (by weight of substrate), an oil/methanol molar ratio of 1:4 and 67 IU g^{-1} of substrate with shaking of 175 rpm for 96 h at 35°C and the highest conversion yield reached 55% (w/w) was with palm oil after 96 h of reaction time (Pizarro and Park, 2003).

The effect of cell membrane fatty acid composition on biodiesel-fuel production by *R. oryzae* whole cells was investigated by (Hama *et al*., 2004). Oleic or linoleic acid-enriched cells showed higher initial methanolysis activity than saturated fatty acid-enriched c ells, among which palmitic acid-enriched cells exhibited significantly greater enzymatic stability than unsaturated fatty acid-enriched cells (Hama *et al*., 2004). They assumed that fatty acids significantly affect the permeability and rigidity of the cell membrane and that higher permeability and rigidity lead to increases in methanolysis activity and enzymatic stability, respectively.

For the industrial application of a whole-cell biocatalyst to biodiesel-fuel production, a technique for preparing whole-cell biocatalyst in large quantity is and thus Oda *et al*. (2005) scaled-up the fermentation culture to immobilize *R. oryz*ae IFO 4697 (which has a 1,3-positional specificity lipase) cells within BSPs by application of an 20 L air-lift bioreactor. The resultant immobilized *R. oryzae* cells were used as whole-cell biocatalyst in repeated batch-cycle methanolysis reaction of soybean oil. Faster cell growth and higher methanolysis activity were obtained in air-lift bioreactor cultivation than in shake-flask cultivation (Oda *et al*., 2005). It has been shown that the lipase from *Rhizopus* fungi have potential in the industry of biodiesel production since they are able to carry out methanolysis in the presence of water, are able to be re-used in the immobilized form and large quantities of immobilized *Rhizopus* cells can be produced to cater for biodiesel production at the large-scale.

Pharmaceuticals, Cosmetics and Other Applications

Wax esters, long-chain esters that are derived from fatty acids and alcohols both with chain lengths of 12 carbons or more, have potential applications as lubricants, plasticizers and cosmetics (Chen and Wang, 1997). *R. niveus* cells (IFO 4759) have been immobilized on cellulose BSPs and successfully applied to the esterification between long-chain alcohol and oleic acid for the production of wax esters (Chen and Wang, 1997).

Since lipases exhibit enantioselectivity they have become attractive enzymes used for the resolution of compounds which are then applied as precursors for synthesis of a variety of pharmaceuticals. Products of the resolution of (±)-glycidyl butyrate are used in the synthesis of (+)-testdinariol A (Takikawa *et al*., 2001), Linezolid (Brickner *et al*., 1996) and other. Palomo *et al*. (2004)

have immobilized *R. oryzae* lipase on dextran-hosphol coated Sepabeads and applied them to the enzymatic resolution of (±)-glycidyl butyrate which achieved an enantiomeric excess of >99 at 55% conversion.

Miyazawa *et al*. (1999) found that lipases belonging to the *Rhizopus* genus showed higher enantioselectivities than those of other sources. Resolution of the alcohol, 2,2-Diphenyl-1,3-dioxolane-4-methanol, was conducted via *R. delemar* lipase-catalyzed transesterification with vinyl butanoate in isopropyl ether (E = 23) and resulted in the production of the I-alcohol with 95% e.e. in 33% yield based on racemate (Miyazawa *et al*., 1999).

CONCLUSIONS

Optimization of culture conditions have shown to be effective in obtaining higher yields of *Rhizopus* lipases and it has been realized that the effect of various compounds such as oils, surfactants and fatty acids on the extra-and intra-cellular lipases vary among the species of this genus. *Rhizopus* lipases have been applied at the bioreactor scale and have potential for their production and subsequent application at large-scale. Research in the field of biodiesel production by *Rhizopus* lipases is directed towards studying the properties of whole cell biocatalysts for methanolysis. The biochemical characteristics of the purified *Rhizopus* lipases allow them to be applied in a wide range of biotechnological applications. These lipases do show potential in the resolution of racemates due to their high enantioselectivities which has usefulness in pharmaceutical, cosmetic, agrochemical and other industries.

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