COMPARATIVE STUDY ON LIPASE ACTIVITY OF TWO FUNGAL ISOLATES AND ITS APPLICATION

A dissertation submitted

in partial fulfillment for the award for the

degree of

Master of Science
in
Chemistry

Submitted by

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Candidate's Declaration

I hereby declare that the work which is being presented in the dissertation entitled **"Comparative study on lipase activity of two fungal isolates and its application"** in partial fulfillment of the requirements for the award of the degree of **Master of Science in Chemistry**, School of Chemistry and Biochemistry, Thapar University, Patiala is an authentic record to my own work carried out during a period of six months from January 2010 to June 2010, under the supervision of Dr. Ranjana Prakash, School of Chemistry and Biochemistry, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

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This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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This is to certify that the project entitled **"Comparative study on lipase activity of two fungal isolates and its application"** being submitted by Mr. **Amit Goyal** in partial fulfillment of the requirement for the award of degree for the Master of Science in Chemistry at the School of Chemistry and Biochemistry, Thapar University, Patiala, is a bonafied work carried out under my guidance and supervision and that no part of this project has been submitted for the award of any other degree.

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Summary

The present work entitled "Comparative study on lipase activity of two fungal isolates and its application" was focused to examine the potential of two fungus isolates viz., *Aspergillus sp* (RBD01 and RBD02) to tolerate oil as carbon source and generate lipase. The test organisms were observed to tolerate significantly high supplementation (upto 90%) of oil as carbon source in the growth medium. The bound lipase activity was observed to be optimum in oil supplementation of upto 40-60% depending on the strain, beyond which the activity decreased. Observations on the duration of incubation to obtain optimum enzyme activity indicated that duration of 5-6 day incubation was appropriate. Incubation temperature of 35^0C at pH of 7.5 resulted in optimal activity of the bound enzyme. Observations on the recycling potential of the biomass indicated that the enzyme activity reduced with repeated use of the biomass by 5^{th} cycle. Study on the influence of chemical nature of solvent on lipase activity indicated that polar solvents facilitate better activity over the non-polar solvents with water being the best solvent. The study is of significance in terms of demonstrating the recyclability of the biomass for continued lipase activity and the role of solvent in modulating the lipase activity of the strain.

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Introduction

Multi-faceted microbial lipases (glycerol ester hydrolases; EC 3.1.1.3) have an unsurpassed role in swiftly growing modern biotechnology (Pandey *et al.* 1999). Lipases are indispensable for the bioconversion of lipids (triacylglycerols) from one organism to another and within the organisms. In addition to their biological significance, lipases have tremendous potential in areas such as food technology, biomedical sciences and chemical industries.

Lipases possess the unique feature of acting at an interface between the aqueous and non-aqueous (i.e. organic) phase; this feature distinguishes them from esterases. The concept of lipase interfacial activation arises from the fact that their catalytic activity generally depends on the aggregation state of the substrates. It is believed that activation involves the unmasking and structuring of the enzyme's active site through conformational changes requiring the presence of oil-in-water droplets. Lipase activity generally depends on the available surface area. Recent structural studies on several lipases have provided some clues towards the understanding of hydrolytic activity, interfacial activation and stereoselectivity (Kim *et al.* 1997). They catalyse a wide range of reactions, which include hydrolysis and interesterification specifically; in addition, also catalyse alcoholysis, acidolysis, esterification and aminolysis. Lipases act under extremely mild conditions. They can be used in a variety of organic solvents and show selectivity for a specific reaction type. Recently, Patel *et al.* (1996) reviewed lipase-catalysed biochemical reactions in novel media. Gill and Valivety (1997) reviewed biotransformation and the biotechnological applications of polyunsaturated fatty acids, which involved the unique nature of lipase-catalysed.

Lipase-producing microorganisms have been found in different habitats such as industrial wastes, vegetable oil processing factories, dairy plants, and soil contaminated with oil and oilseeds among others (Sharma *et al.* 2001). Recently, some works reporting the use of immobilized whole biomass of filamentous fungi have also been published. Ellaiah *et al.* (2004) used the whole immobilized biomass of *Aspergillus niger* to produce lipase and obtained similar activities for both free and immobilized biomass cultivations

(4 U mL^{-1}). Elitol and Ozer (2000) immobilized the whole cell of *R. arrhizus* and the rate of lipase production was constant through several repeated batch experiments.

Naturally immobilized catalysts, cell-bound or whole-cell lipases have advantages over normal extra- or intracellular counterparts, and have attracted more attentions recently (Romano *et al.* 2005, Hama *et al.* 2006, Gandolfi *et al.* 2001, Molinari *et al.* 2000). These naturally bound lipases, mostly from filamentous fungi, can be used directly in industrial processes with cost effectivity and improved stability, eliminating the isolation, purification and immobilization procedures, and minimizing the loss of lipase activity. Moreover, the naturally immobilized cell-bound lipases are more stable to organic solvents, higher temperature and extreme pH due to the protection of the cells (Torres *et al.* 2003, Razak *et al.* 1999, Long *et al.* 1996). Some studies on cell-bound lipases and their successful applications in organic phase have been reported. Mycelium-bound lipases from *Rhizopus chinensis*, *Aspergillus oryzae* and *R. oryzae* had been successfully used as catalysts for the synthesis of flavour esters (Molinari *et al.* 2000, Xu *et al.* 2002) enantioselective esterification of the racemic mixture (Gandolfi *et al.* 2001, Molinari *et al.* 1998) and the production of biodiesel fuel (Ban *et al.* 2001). Mycelium-bound lipase from *Aspergillus oryzae* has been used to synthesize phenylacetate (Converti *et al.* 2005). Liew *et al.* (2001) reported transesterification activity of a mycelium-bound lipase from *Rhizomucor miehei* on palm kernel olein:anhydrous milk fat blends. In general, most of cell-bound lipases used in non-aqueous phase reactions were prepared by lyophilization to remove the water.

However, keeping in view the limited information available about the preparations of cell-bound lipases, the present study was focused on inducing the maximum cell bound lipase activity by using the cottonseed oil as carbon source in different fungal strains. Furthermore the fungal strain which show maximum lipase production in optimized conditions was used for esterification of short chain acids.

Literature Review

Lipases have emerged as one of the leading biocatalysts with proven potential for contributing to the multibillion dollar underexploited lipid technology and are used in *in situ* lipid metabolism as well *ex situ* multifaceted industrial applications (Joseph *et al.* 2008). Lipases find use in a variety of biotechnological fields such as food and dairy, detergents, cosmetics, pharmaceuticals, agrochemicals (insecticides, pesticides) and oleochemicals (Jaeger and Eggert 2002). They have a special contribution in waste management and improvement of tanning techniques (Jaeger and Eggert 2002, Benjamin and Pandey 1998). Many bacteria, filamentous fungi and yeasts have the ability to produce lipases (Benjamin and Pandey 1998, Sharma *et al.* 2001). Filamentous fungi have a greater potential for production of extracellular lipases, which are generally used as purified enzymes. However, due to the instability of extracellular lipases and cost-intensive purification procedures, their application is limited. Direct use of compact cells for intracellular production of lipases or fungal cells immobilized within porous biomass support particles as a whole biocatalyst represents an attractive process for bulk production of variety of industrially important products including biodiesel and polyesters (Ban *et al.* 2001, Nakashima *et al.* 1989, Adamczak *et al.* 2004, Hama *et al.* 1988). They are economical because they do not necessarily require purification or further immobilization (Ban *et al.* 2001). Moulds produce lipases of high catalytic activity and thermal stability (Sharma *et al.* 2001, Nahas 1988), which are desired characteristics of most of the industrial enzymes. Biosynthesis of lipase is markedly dependent on the type of inducer used. Lipases have mostly been produced using induction by low-cost substrates, namely, wheat bran, oil cake or wastes from plant oil industries (Nahas 1988). In industrial scale fermentation, their use may cause inconsistencies in medium homogeneity, oil-water surface problems and may involve expensive purification process (Nahas 1988).

The number of available lipases has increased since the 1980s. This is mainly a result of the huge achievements made in the cloning and expression of enzymes from microorganisms, as well as of an increasing demand for these biocatalysts with novel and

specific properties such as specificity, stability, pH, and temperature (Bornscheuer *et al.* 2002, Menoncin *et al.* 2008). The industrial demand for new sources of lipases with different catalytic characteristics stimulates the isolation and selection of new strains. Lipase-producing microorganisms have been found in different habitats such as industrial wastes, vegetable oil processing factories, dairy plants, and soil contaminated with oil and oilseeds among others (Sharma *et al.* 2001).

Some of the most commercially important lipase-producing fungi are recognized as belonging to the genera *Rhizopus sp*., *Aspergillus sp*., *Penicillium sp*., *Geotrichum sp*., *Mucor sp*., and *Rhizomucor sp*. Lipase production by filamentous fungi varies according to the strain, the composition of the growth medium, cultivation conditions, pH, temperature, and the kind of carbon and nitrogen sources (Cihangir and Sarikaya 2004). Colen *et al.* (2006) isolated 59 lipase-producing fungal strains from Brazilian savanna soil using enrichment culture techniques. An agar plate medium containing bile salts and olive oil emulsion was employed for isolating and growing fungi in primary screening assay. Twenty one strains were selected by the ratio of the lipolytic halo radius and the colony radius. Eleven strains were considered and, among them, the strain identified as *Colletotrichum gloesporioides* was the most productive. In another report, Cihangir and Sarikaya (2004) isolated a strain of *Aspergillus sp*. from soil samples from the different regions of Turkey and obtained an expressive activity of 17 U mL^{-1}. In submerged fermentation Teng and Xu (2008) investigated the lipase production by *Rhizopus chinensis* and obtained, at the optimized experimental conditions, a maximum lipase activity of 14 U mL^{-1}. Bapiraju *et al.* (2005) optimized the lipase production by the mutant strain of *Rhizopus sp*. and the optimum activity was 29 U mL^{-1}. Kaushik *et al.* (2006) studied the production of an extracellular lipase from *Aspergillus carneus* and obtained a maximum activity of 13 U mL^{-1}. In solid state fermentation Kempka *et al.* (2008) investigated the lipase production by *Penicillium verrucosum* and the optimum activity was about 40 U per gram of dry substrate (gds). Vargas *et al.* (2008) studied the lipase production by *Penicillium simplicissimum* and obtained an activity of 30 U gds^{-1}. Both *P. verrucosum* and *P. simplicissimum* were isolated from the babassu oil industry. Wolski *et al.* (2008) reported the use of response surface methodology to optimize the lipase production by submerged fermentation using immobilized biomass of a newly

isolated *Penicillium sp*. At the optimized experimental conditions, the authors reached a lipase activity around 21 U mL^{-1}, higher than the activity obtained by the same microorganism before immobilization. Yang *et al.* (2005) studied the repeated-batch lipase production by immobilized mycelium of *Rhizopus arrhizus* in submerged fermentation. The lipase productivity increased from 3 to 18 U mL^{-1} h^{-1}, changing the process from batch to repeated-batch mode. Ellaiah *et al.* (2004) used the whole immobilized biomass of *Aspergillus niger* to produce lipase and obtained similar activities for both free and immobilized biomass cultivations (4 U mL^{-1}). Elitol and Ozer (2000) immobilized the whole cell of *R. arrhizus* and the rate of lipase production was constant through several repeated batch experiments.

The study of enzyme activity and stability is an important aspect to consider in biotechnological process, as this information helps to optimize the economic profitability of the biocatalyst reaction. The use of naturally bound lipases is potentially cost effective because the biomass can be directly utilized. This allows the elimination of complex procedures of enzyme isolation, purification, and immobilization, which often results in loss of its activity. Moreover, the naturally immobilized cell-bound lipases are more stable to organic solvents, higher temperature and extreme pH due to the protection of the cells (Torres *et al.* 2003, Razak *et al.* 1999, Long *et al.*1996). Furthermore, the cell structure may act as natural matrix able to protect the enzymes from the possible negative action of external agents, providing an effect analogous to that exerted by common matrix used for enzyme immobilization. Extensive efforts have been focused on various immobilized forms of lipase, involving a variety of immobilization methods and support materials (Bagi *et al.* 1997, Mateo *et al.* 2000, Abrol *et al.* 2007).

However, survey of the literature showed that little work has been focused on cell (mycelium)-bound lipase (CBL) compared extensive work carried out with chemically immobilized lipases. CBLs provide a unique naturally immobilized form of lipase, which present efficient biocatalysts for future research and arose tremendous interest. Such naturally immobilized lipases have many advantages as they avoid expensive and laborious operations of isolation, purification, and immobi-lization (Loo *et al.* 2007). Furthermore, natural immobilization has higher recovery and yield compared with chemical or physical immobilization (Mustranta *et al.* 1993). In previous reports, various

carbon sources or inducers (olive oil, Tween 80, glucose, citric acid, triolein, and rioctanoin) and nitrogen sources (peptone, soybean meal, corn steep liquor, yeast extract, urea and NH_4NO_3) were frequently used for production of both extracellular and CBLs from *Geotrichum sp*. (Jacobsen *et al.* 1989, Zarevúcka *et al.* 2005). Loo *et al.* (2007) reported that CBL from *Geotrichum candidum* was produced by the medium with nitrogen sources (peptone, yeast extract, and $(NH_4)_2SO_4$ and carbon sources (glucose and palmolein). Jacobsen *et al.* (1989) also reported that CBL was produced using peptone as the nitrogen source and using olive oil supplemented with Tween 80 as the carbon source. Conventional methods for optimization of medium and fermentation conditions are most time consuming and expensive (Kaushik *et al.* 2006).

Regarded as naturally immobilized catalysts, cell-bound or whole-cell lipases have advantages over normal extra- or intracellular counterparts, and have attracted more attentions recently (Romano *et al.* 2005, Hama *et al.* 2005, Gandolfi *et al.* 2001, Molinari *et al.* 2000). These naturally bound lipases, mostly from filamentous fungi, can be used directly in industrial processes with cost effectivity. Some studies on cell-bound lipases and their successful applications in organic phase have been reported. Mycelium-bound lipases from *Rhizopus chinensis*, *Aspergillus oryzae* and *R. oryzae* had been successfully used as catalysts for the synthesis of flavour esters (Molinari *et al.* 2000, Xu *et al.* 2002), enantioselective esterification of the racemic mixture (Gandolfi *et al.* 2001, Molinari *et al.* 1998) and the production of biodiesel fuel (Ban *et al.* 2001). In general, most of cell-bound lipases used in non-aqueous phase reactions were prepared by lyophilization to remove the water.

Compared with the lyophilization, the organic solvent pretreatment is relatively simple and timesaving, and could be an effective method to prepare the ‘‘dry’’ enzymes for non-aqueous phase reactions. Acetone has been reported to wash cells and to prepare catalysts for interesterification (Nakashima *et al.* 1998) and whole-cell lipases (Essamri *et al.* 1998). However, little information is available about the preparations of cell-bound lipases with other organic solvents. The pretreatment of lipases with organic solvents can enhance their hydrolytic and synthetic activities (Chamorro *et al.* 1998, Zaman *et al.* 2006, Matsumoto *et al.* 2001), improve their stability (Zaman *et al.* 2005) and enantioselectivity (Zaman *et al.* 2005). However, most reported lipases pretreated with

organic solvents were free or immobilized enzymes, little research about cell-bound lipase was presented. In view of the cell structure and the localization of lipase in cells, the effects of pretreatments with organic solvents on the activity of mycelium- or cell-bound lipase might be complex and diverse. The locations of different cell-bound lipases (cell wall-bound, membrane-bound or periplasmic enzymes) on the cell surface also could have influences on their stability, catalyzing efficiency and further applications, while the localizations of cell-bound lipases have never been extensively studied. It was suggested that the cellbound lipases from *A. flavus* and *Yarrowia lipolytica* cells might be located in the periplasm (Long *et al.* 1996, Pereira-Meirelles *et al.* 2000). There is also a report about the presence of a membrane-bound enantioselective ester hydrolase from *Trichosporon* species (Vakhlu *et al.* 2005).

Romero *et al.* (2007) obtained a constitutive level of a mycelium-bound lipolytic activity from *Aspergillus niger* MYA 135 was strongly increased by 97% in medium supplemented with 2% olive oil. The constitutive lipase showed an optimal activity in the pH range of 3.0–6.5, while the mycelium-bound lipase activity produced in the presence of olive oil had two pH optima at pH 4 and 7. These mycelium-bound lipase activities were also very stable in reaction mixtures containing methanol and ethanol. In fact, the constitutive lipase maintained almost 100% of its activity after exposure by 1 h at 37°C in ethanol.

Mycelium-bound lipase from *Aspergillus oryzae* has been used to synthesize phenylacetate (Converti *et al.* 2005). Liew *et al.* (2001) reported transesterification activity of a mycelium-bound lipase from *Rhizomucor miehei* on palm kernel olein:anhydrous milk fat blends. Wang *et al.* (2007) obtained cell-bound lipase from *Rhizopus chinensis* CCTCC M201021 with high catalysis ability for ester synthesis was located as a membrane-bound lipase by the treatments of YatalaseTM. In order to improve its synthetic activity in non-aqueous phase, the pretreatments of this enzyme with various organic solvents were investigated. The pretreatment with isooctane improved evidently the lipase synthetic activity, resulting in about 139% in relative synthetic activity and 115% in activity recovery. The morphological changes of mycelia caused by organic solvent pretreatments could influence the exposure of the membrane-bound enzyme from mycelia and the exhibition of the lipase activity. When the pretreated

lipases were employed as catalysts for the esterification production of ethyl hexanoate in heptane, higher initial reaction rate and higher final molar conversion were obtained using the lipase pretreated with isooctane, compared with the untreated lyophilized one. This result suggested that the pretreatment of the membrane-bound lipase with isooctane could be an effective method to substitute the lyophilization for preparing biocatalysts used in non-aqueous phase reactions.

Yan and Yan (2008) used an integrated optimization strategy involving a combination of different designs was employed to optimize producing conditions of cell-bound lipase (CBL) from *Geotrichum sp.* Firstly, it was obtained by a single factorial design that the most suitable carbon source was a mixture of olive oil and citric acid and the most suitable nitrogen source was a mixture of corn steep liquor and NH_4NO_3. The dried CBL was used to synthesize methyl oleate in microaqueous hexane, resulting in 94% conversion after 24 h, and showed reusability with 70% residual activity and 69% conversion after eight cycles of batch operation, which indicating that CBL, as a novel and natural form of immobilized enzyme, can be effectively applied in repeated synthesis of methyl oleate in a microaqueous solvent.

Applications of Lipases

Lipases have become an integral part of the modern food industry. The use of enzymes to improve the traditional chemical processes of food manufacture has been developed in the past few years. Nowadays industrial enzymes, especially lipases, are commonly used in the production of a variety of products, ranging from fruit juices, baked foods and vegetable fermentation to dairy enrichment (McGee 1986, Zalacain *et al.* 1995). Microbial lipases have been used for the production of desirable flavours in cheese and other foods, and for the interesterification of fats and oils to produce modified acylglycerols, which cannot be obtained by conventional chemical interesterification (Novo 1985). Fats, oils and allied compounds are the main targets of lipases in food technology. During storage, one of the most important changes that occur in the lipid fraction is the hydrolysis of triacylglycerols, catalysed by lipase retaining non-esterified fatty acids, which are very important for the characteristic flavour of these products (Heidt *et al.* 1996). This aspect can be well exploited in the laboratory by using

commercial lipases. Similarly, the desired moiety of the triacylglycerol can be deleted or even added under controlled esterification and transesterification reactions by specific immobilized or free lipases (Novo 1985). Yoneda *et al.* (1996) have patented a process on *Pseudomonas* lipase, which was claimed to be useful in, for example, food processing and oil manufacture.

Esters of short chain fatty acids and alcohols are extremely important aroma compounds. Currently, most of the flavour and fragrance components are provided by traditional methods which included chemical synthesis or extraction from natural sources (Langrand *et al.* 1990). With the great recent interest for natural products, the flavour industry is more and more interested in the use of Biotechnology to produce natural flavours. Direct biosynthesis by fermentation has been described as potentially useful as a source of esters (Langrand *et al.* 1990). However, the ester concentrations and productivities obtained using fermentation methods are rather low. A different strategy for ester synthesis involves the use of isolated enzymes (Langrand *et al.* 1990). Ester synthesis from alcohols and acids has been described (Iwai *et al.* 1980), in appropriate conditions, using lipases (triacylglycerolhydrolases, EC 3.1.1.3.). The influence of the nature of acid and alcohol substrates on ester yield has been studied in biphasic aqueous systems (Iwai *et al.* 1980, Langrand *et al.* 1990) or in organic solvents (Langrand *et al.* 1990) for some lipase preparations, but no detailed study on the influence of acid and alcohol moiety is available.

The majority of enzymes used in industry are for food processing, mainly for the modification and breakdown of biomaterials (Australia Newzealand Food Authority 2002). A large number of fat clearing enzymatic lipases are produced on an industrial scale. Most of the commercial lipases produced are utilized for flavour development in dairy products and processing of other foods, such as meat, vegetables, fruit, baked foods, milk product and beer (Nagodawithana and Reed 1993). Lipases have been successfully used as a catalyst for the synthesis of esters. The esters produced from short-chain fatty acids are used as flavouring agents in the food industry. Lipase immobilized on silica and microemulsion based organels were widely applied for ester synthesis (Sharma *et al.* 2001, Ghosh *et al.* 1996). From the above literature studied very less reports are available

on the standardization of whole cell lipase production and its application in esterification process.

Keeping in view the lacunae outlined on various aspects identified in literature, present study was carried out to optimize cell bound lipase production of two different strains of *Aspergillus* sp.; and characterize esterification products of short chain acids.

Materials and Methods

Materials

Ethanol, propanol, butanol, hexane, ethyl acetate, acetone, chloroform, sodium carbonate, potassium di-hydrogen phosphate, di-sodium hydrogen phosphate,butyric acid, propionic acid, di-methyl sulphoxide, di-methylformamide p-nitrophenol laurate were sourced from SD Fine Chem., India. Cottonseed oil sourced from market was used as lipase inducer. Nitrogen sources included in the study were bi-ammonium hydrogen ortho-phosphate [$(NH_4)_2HPO_4$] and mycological peptone (HiMedia, India). Culture media used were Bushnell-Hass broth (BHB), Potato dextrose agar (PDA), Potato dextrose broth (PDB) (HiMedia).

Methods

Growth of fungus

The fungal strain (*Aspergillus* sp RBD 01, *Aspergillus* sp RBD 02) isolated from contaminated fat material was examined in the present study. Freshly, sporulating culture was inoculated under sterile conditions into 500 mL Erlenmeyer flask containing 200 mL of potato dextrose broth at 28 ºC, 120rpm for 72 h. This inoculum was further used for experimentation.

The mineral medium comprising magnesium sulphate (0.20 g/l), calcium chloride (0.02 g/l), mono-potassium phosphate (1.0 g/l), di-potassium phosphate (1.0 g/l), ferric chloride (0.05 g/l), was used for the growth of fungal strain. The medium was supplemented with mycological peptone or/and bi-ammonium hydrogen ortho-phosphate as nitrogen source. Cottonseed oil used as supplements for carbon source.

Biomass production and lipolytic activity determination

For cell bound lipase determination, the freshly grown biomass was introduced in oil: minimal media ratio of (10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30,80:20, 90:10) and incubated for 120 h at 28°C, 120 rpm. Biomass produced was separated and dried by

using simple filter paper. Dried biomass then crushed to powder with the help of liquid nitrogen.

The lipolytic activity of the dried biomass was determined by modifying the method of Sigurgisladottir *et al.* (1993). 0.1gm of biomass was added to a reaction mixture containing 0.8 ml of 0.05 M phosphate buffer (pH 7.5) and 0.1 ml of 0.02 M pNP (p-nitrophenol laurate) in ethanol. The mixture was incubated at 35°C for 30 min, followed by addition of 0.25 ml of 0.1 M Na_2CO_3 on cooling to room temperature. The activity was determined at 420 nm. One unit of lipase activity is defined as the amount of enzyme that liberates 1 µg p-nitrophenol (molar extinction coefficient 1.336×10^7 cm^2/mol at 420 nm) with pNP-laurate as substrate under standard assay conditions in 30 min.

Powdered biomass showing maximum lipolytic activity in above step was further studied. During standardization different parameters like incubation time of fungus (2^{nd} to 8^{th} day); temperature (25°C to 75°C), pH (5 to 9); and reusability potential of biomass (5 cycles) were varied to obtain optimum lipolytic activity. Initially the strain was grown from 2^{nd} to 8^{th} day to monitor the lipolytic activity. Based on the observation that the lipolytic activity was maximum on $5^{th}/6^{th}$ day depending upon the test strain, further experiments on the effect of temperature (25°C to 75°C) on lipolytic activity of the biomass were carried out at pH 7.5. The studies on effect of pH on the same activity were carried out with the biomass collected on $5^{th}/6^{th}$ day by varying pH from 5 to 9 at 35^0C. The reusability studies were carried out at pH 7.5 and 35^0C.

Esterification reaction

Esterification reaction of butyric acid was carried out with propanol and butanol where as a similar reaction was carried out for propanoic acid with butanol. The experiment was done in round bottom flask by using powdered biomass as catalyst. 2gm of biomass and 10gm of acid (butyric/pentanoic acids) were taken in 50ml round bottom flask and kept on magnetic stirrer in stirring condition at 35°C for 36 h. Alcohol was added step-wise at the ratio of 1:2 molar ratio at an interval of every 6 h. After 36 h, the product obtained was analyzed and quantified.

Characterization of product

Product was quantified by determining the residual acid in the mixture after 36 h of reaction by using the formula given by Satyarthi *et al.* (2009). The authors derived the formula for the quantification of FFA, based on the appearance of α-CH_2 peaks of fatty acids at δ values higher than those of the methyl or glyceryl esters. The difference in chemical shift (between the acid and ester) is due to the greater de-shielding effect of the carboxylic group compared to the ester group. Due to this shift, one of the peaks of the triplet of FFA (at 2.38 ppm) shifts out of the α-CH_2 region of the ester, and the other two peaks (2.34 and 2.30 ppm, respectively) are merged with those due to the ester at 2.35 and 2.31 ppm, respectively. In other words, a sample containing FFA and ester (vegetable oil or biodiesel) shows a quartet like spectral pattern in the α-CH_2 region of the 1H NMR spectrum with the intensity of the peaks depending on the content of FFA in esters. The unmerged peak of the FFA triplet can be used to determine the acid content. The area of the unmerged peak of the acid triplet (appearing around 2.38 ppm, out of the ester triplet) can be determined by integration of the spectral region 2.37-2.41 ppm. The triplet appears with an intensity ratio of 1:2:1. The total area corresponding to the α-CH_2 groups of the acid, will thus, be four times the area of the single unmerged acid peak around 2.38 ppm. The total area corresponding to α-CH_2 of both acid and ester can be determined by integrating the spectral region 2.20-2.41 ppm, an aspect that was used for the quantification of short chain acids, in the present study.

$$\% \text{ of Acid} = \frac{4 \text{ x Area of unmerged peak of } \alpha\text{-}CH_2 \text{ of acid}}{\text{Total area of both acid and ester}} \text{ x } 100$$

Result and Discussion

The present study was carried out focusing on optimization of lipase production from two strains of *Aspergillus* sp. followed by esterification of short chain acids. The parameters that were considered in the study included concentration of oil as carbon source; duration of incubation required for optimal lipase production and activity; and effect of pH and temperature on lipase activity. The reusability of the biomass as a catalyst was also examined as a part of the study.

Effect of concentration of cottonseed oil

Based on the belief that most extracellular lipases are inducible (Ota *et al.* 1968; Ibrahim *et al.* 1987), potential of lipase production by two different strains of *Aspergillus* sp (RBD01 and RBD02) was evaluated, by varying the supplementation of oil from 10%-90% in growth medium.

Observations indicated that the strains, were tolerant and could grow in medium supplemented with 90% oil as carbon source, an aspect that has not been reported in any fungus strain till date. Earlier studies carried by the research group indicated tolerance up to 50% by RBD01 (Aulakh and Prakash 2010). Table 1 represents the profile of lipolytic activity determined in culture media (MM) (without oil) and in samples with varying percentage of oil after 120 h of incubation.

Table 1. Bound lipase activity in the presence of varying concentration of oil

Percent supplementation of cottonseed oil	Lipase activity (U/g)	
	Aspergillus sp (RBD 01)	*Aspergillus sp* (RBD 02)
MM without oil	28.9	14.5
MM+10	165.0	45.0
MM+20	170.7	62.4
MM+30	205.0	65.0
MM+40	247.0	85.0
MM+50	103.0	97.0
MM+60	94.0	103.0
MM+70	93.0	11.5
MM+80	78.5	12.6
MM+90	6.0	9.0

The observations indicate that the increase in oil concentration resulted in enhanced lipase activity (247.0 U/g) till 40% of oil beyond which it decreased to 6.0 U/g at 90% supplementation in case of RBD01. RBD02 exhibited varying lipase activity with increase of enzyme activity observed till 60% oil supplementation (103.0 U/g) followed by subsequent decreasing trend. The decrease in the lipolytic activity is presumably due to stress and deactivation induced by the increasing concentration of oil, resulting in reduced expression of enzyme after mentioned concentration level.

The present observations are supported by Romero *et al.* (2007) who reported that mycelium-bound lipolytic activity from *Aspergillus niger* MYA 135 was strongly increased by 97% in medium supplemented with 2% olive oil. Omar and Ilias (1996) studied the effect of various carbon sources (0.3% w/v) on the production of bound lipase by *Aspergillus flavus* and reported that the fungus was able to utilize a wide range of carbon sources besides soluble starch with an average activity of about 35.5 U/g biomass. Furthermore, the lipase activity was not greatly influenced by the presence of various carbon sources. In the present study, the observations show that the lipase activity is significantly influenced by the concentration of oil as carbon source or inducing agent and the production of lipase varies with strain.

Effect of time of incubation

Effect of incubation time and duration at which maximum lipase activity could be obtained, was determined by varying the incubation time from 2 days to 8 days and quantifying the enzyme activity. The culture medium was set at 40:60 (oil: media) for RBD01 and 60:40 (oil: media) for RBD02 at 35ºC and 7.5 pH. Result (Fig. 2) indicates that with increase in day of incubation lipase activity increase from 2^{nd} (99 U/g), to 5^{th} day (210U/g) followed by decrease in activity to 180 U/g on 8^{th} day in case of RBD01. On the other hand, in case of RBD02, the maximum enzyme activity was observed on 6^{th} day (144 U/g) followed by decreasing trend till 8^{th} day (127.5 U/g). Earlier studies carried out by this research group showed that the activity of extracellular lipase was 21.8 U/mL (Aulakh and Prakash 2010) at 50% oil supplementation which is significantly below the bound lipase activity (103.0 U/g) observed in the same strain under similar

conditions. The observations demonstrate the influence of incubation time on enzyme activity which hitherto has not been reported with any fungus strain till date to the best of our knowledge.

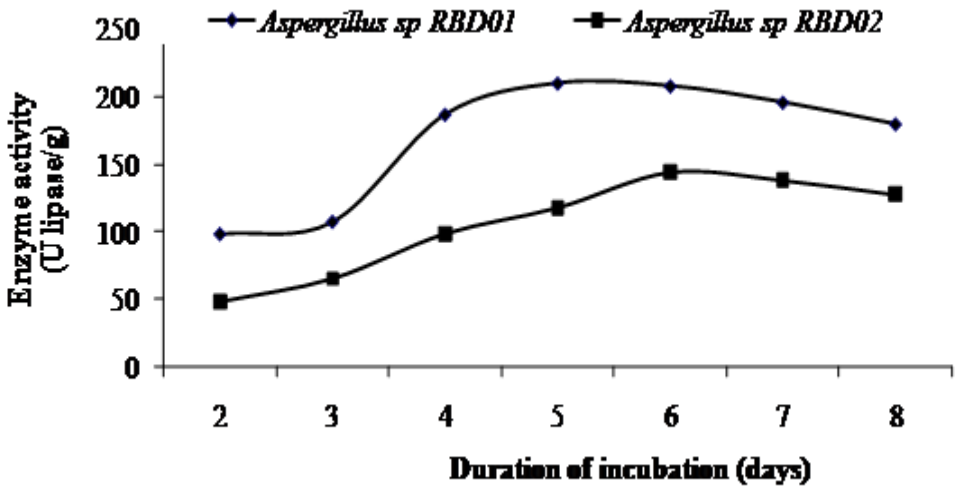

Fig 2. Effect of incubation time (days) on enzyme activity

Effect of temperature

Effect of temperature on lipase activity of whole cell biomass of *Aspergillus sp* (RBD01 and RBD02) was studied in the range of 25ºC to 75ºC. Result depicted in Fig. 3 show that both the fungal strains exhibit optimum lipase activity (RBD01: 208 U/g ; RBD02: 142 U/g) at 35ºC followed by decreasing trend till 75ºC (1.5 U/g, 1.2 U/g).

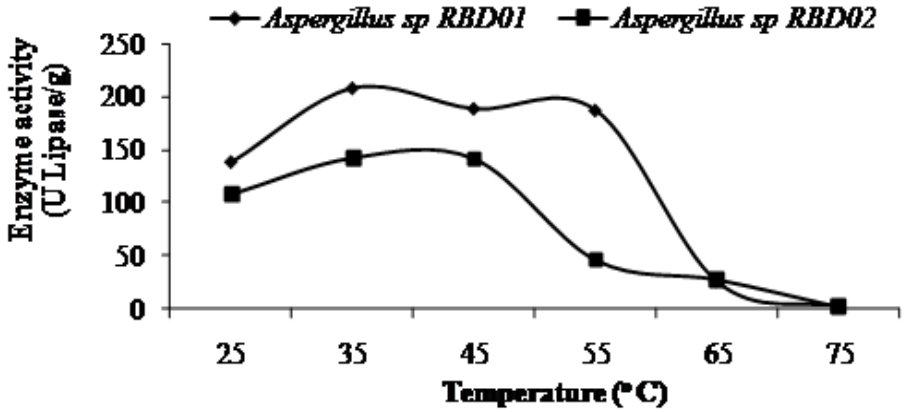

Fig 3. Effect of temperature on lipase activity

The extracellular lipase activity was also found optimal at 35^0C in the strain RBD01 as noted in the earlier studies (Aulakh and Prakash 2010). In this study, reduced activity at 65^0C and beyond is presumed to be due to significant deactivation of bound enzyme. Similar observations were also reported by Omar and Ilias (1996) wherein *Aspergillus flavus* showed maximum bound lipase activity (33.3 U/g) from dried biomass at 45ºC. Razak *et al.* (1999) obtained maximum of lipase activity from *Rhizopus oryzae* at 37ºC which is very near to our finding. Most microbial lipases exhibit optimal temperatures in the range of 25 to 40°C, as shown by lipase from *Aspergillus niger* (Fukumoto *et al.* 1963) which had an optimal temperature at 25°C, while the optima of *Synephalastrum racemosum* (Chopra *et al.* 1983) and *Mucor javanicus* were at 37°C (Ishihara *et al.*1975). Lipases from *G. candidum* (Tsujisaka *et al.* 1973), *R. japonicus* (Aisaka and Terada 1981), and *R. japonicus* NR 400 (Suzuki *et al.* 1986) exhibited optimal activities at 40°C. Aspergillus sp RBD01 exhibited optimal extracellular lipase activity at 35°C (Aulakh and Prakash 2010). While lipases of *A. niger (*Fukumoto *et al.* 1963), *R.* japonicus (Aisaka and Terada 1980), and *C.viscosum* (Yamaguchi *et al.* 1973) are stable at 50°C, lipases of thermotolerant *H. lanuginosa* and *P.* sp. *nitroreducens* are stable at 60°C and 70°C (Liu *et al.* 1973), respectively. Maximum activities of *Candida gigantea* and other lipases from mesophiles were at 30–35°C (Christakopoulos et al 1992).

Effect of pH on lipase activity

Lipase activity of *Aspergillus sp* (RBD01 and RBD02) was studied within the range of pH 5 to 9. Both the strain showed maximum lipase activity (RBD01: 227 U/g, RBD02: 143 U/g) at pH 7.5. In the case of RBD01 lipase activity was increased with increase pH from 5 (2U/g) to 7.5 (227 U/g) followed by continuous decrease from pH 8 (201.5U/g) to 9 (108 U/g). Similarly, RBD02 showed increased lipase activity from pH 5 (4U/g) to pH 7.5 (143U/g) followed by decrease in lipase activity to 104 U/g at pH 9. Thus, both the strains showed optimal lipase activity slightly towards alkaline region. However, the activity of lipase was notable even at pH 9 indicating significant stability of the bound lipase towards alkaline range.

Similar observations reported by Romero *et al.* (2007) indicated significant lipase activity in the pH range of 4-7 by *Aspergillus niger* MYA 135. Optimal pH values of 2.5 and 6.0 as well as 5.5 and 10.0 have been reported for *Penicillium roqueforti* (Lamberet and Menassa 1983) and *Aspergillus terreus* (Yadav *et al.* 1998) respectively.

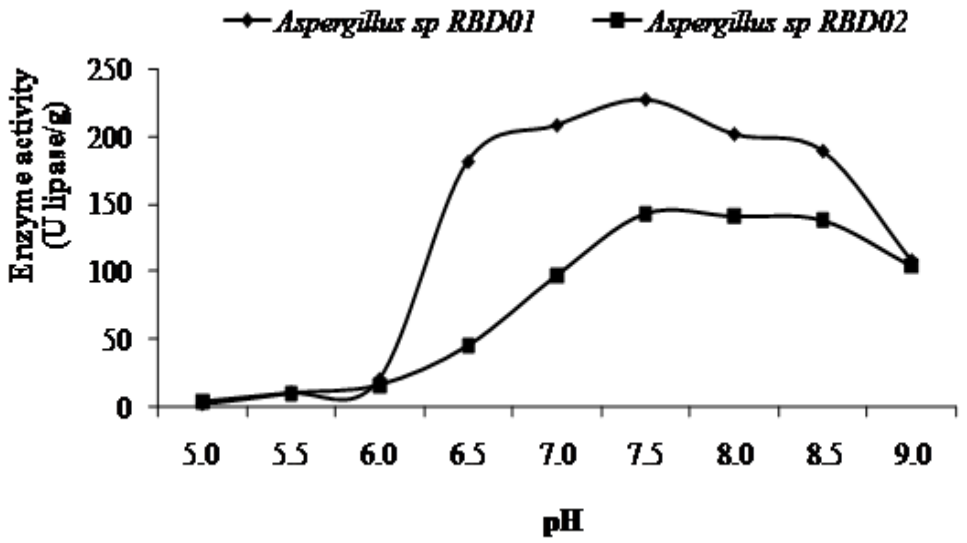

Fig 4. Effect of pH on lipolytic activity

Similar pattern of enzyme activity was reported from *Bacillus thermoleovorans* CCR11, which showed an increase in its residual activity after pre-incubation at pH 7 during 26 h (Castro-Ochoa *et al.* 2005). The lipases that have been studied, showed profound stability around pH 6.0–7.5 with considerable stability at acidic pH up to 4 and at alkaline pH up to 8 (Saxena *et al.* 1999). Extracellular lipase of *A. niger*, *Chromobacterium viscosum* and *Rhizopus* sp. are active at acidic pH (Yamaguchi *et al.*1973, Fukumoto *et al.* 1963, Laboureur and Labrousse, 1966). An alkaline lipase active at pH 11.0 has been isolated from *P. nitroreducens* (Watanabe *et al.* 1977).

A comparative profile of lipolytic activity of RBD01 and RBD02 at optimized conditions of 35^0C, pH 7.5, 40:60 (RBD01) / 60:40 (RBD02) on 5^{th} (RBD01) / 6^{th} (RBD02) day incubation, in general, showed mean activity of 223.0 ± 18.13 U/g and 143.0 ± 1.0 U/g respectively.

Reusability potential of biomass

The re-usability potential of biomass was explored by observing the lipolytic activity of RBD01 and RBD02 through 5 cycles. Biomass was used repeatedly at 35ºC and 7.5 pH after washing with water and drying. The lipase activity was measured after $5^{th}/6^{th}$ day dependent on the strain. Fig. 5 shows the lipase activity after each cycle of the use of RBD01 and RBD02. Results indicate decrease of activity from 1^{st} cycle (RBD01: 216.0 U/g, RBD02: 142.0 U/g) to 5^{th} cycle (RBD01: 181.6 U/g, RBD02: 92.0 U/g), However, rate of decrease was more significant in the case of RBD02 than RBD01. The present study indicated that the repeated use of biomass decreases the lipolytic activity to very less extent but is variable with strain. The extent recyclability of biomass and retention of the enzyme activity is important in perspective of industrial application.

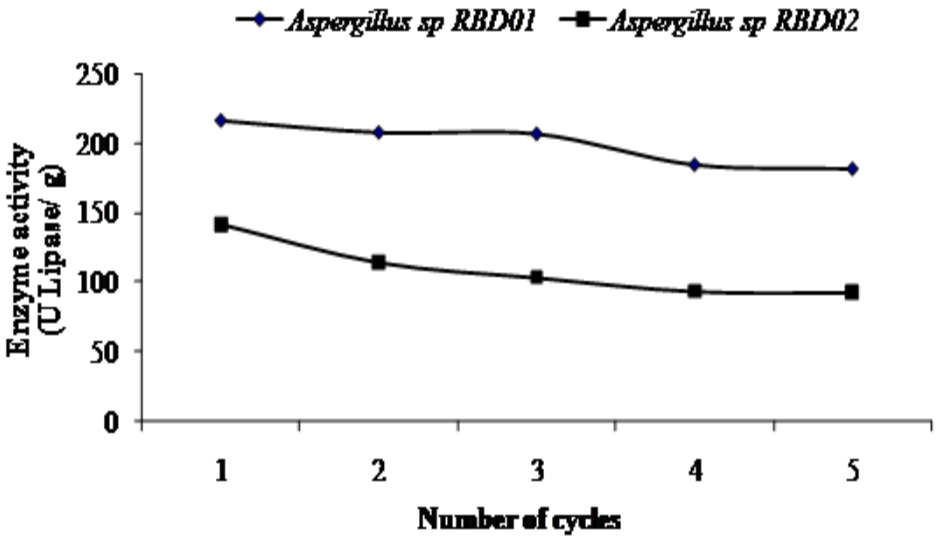

Fig 5. Lipolytic activity during different cycles of biomass

Effect of solvent on lipolytic activity

The role of solvent on the lipase activity in non-aqueous medium was examined as non-aqueous conditions are presumed to modulate lipase activity significantly (Romero *et al.* 2007). To check the effect of solvent, lipolytic activity was determined by adding 1ml of organic solvent in non-aqueous reaction mixture at 35^0C. The solvents considered in the present study included polar [dimethyl formamide (DMF); dimethyl

sulphoxide (DMSO) and ethyl acetate (EA)] as well as non-polar [diethyl ether (DE); chloroform (Chl); and hexane (Hex.)] solvents.

Results shown in Fig. 6 indicates that in case of RBD01, presence of DMF resulted in maximum lipase activity (119.0 U/g) followed by DMSO (115.0 U/g), EA (103.0 U/g), Hex., (81.0 U/g), Chl., (58.0 U/g) and DE (19.5 U/g). On the other hand, maximum lipolytic activity was shown in the presence of DMSO (112.0 U/g) followed by DMF (107 U/g), EA (106.0 U/g), Hex., (73 U/g), Chl., (43.0 U/g) and DE (30.0 U/g) in the case of RBD02. The notable aspect in the study is a significant decrease in the lipolytic activity in the presence of non-polar solvents when compared to the polar solvents by over 100 percent. This is presumed to be due to high dielectric constant of polar solvents which someway seems to influence lipolytic activity. Higher lipolytic activity observed in aqueous medium may be attributed to high dielectric constant and protic nature of water whereas other solvents are aprotic in nature. The presence of organic solvent, therefore, in general, followed a similar trend in both the strains and significantly reduced the lipolytic activity when compared to the activity in aqueous medium (RBD01: 247.0 U/g ; RBD02: 103.0 U/g). Romero *et al.* (2007) also found that organic solvents decrease the bound lipase activity.

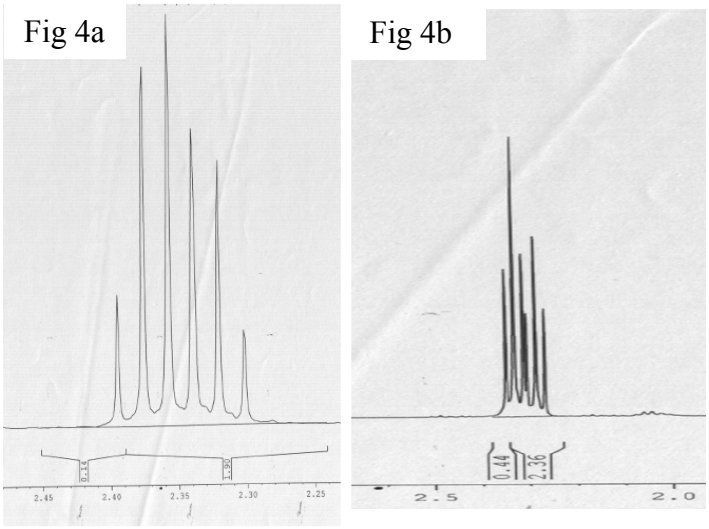

Fig 4. ^{1}H NMR of esters generated through esterification reaction between (a) propionic acid and butanol; and (b) butyric acid and butanol

With reference to the results of esterification, the reaction of butyric acid with propanol and butanol resulted in 47.8% and 37.15% of conversion respectively. On the other hand propionic acid was esterified with butanol resulting in 72.6% of conversion (Fig 4). Similar studies carried out by Li *et al*. (2008) using oleic acid and *tert*-butanol indicated an yield of 75.5% of methyl esters. As on date, there are limited studies on generation of alkyl esters with whole cell as biocatalyst, which dominantly were carried out only with pure lipase as a catalyst.

Conclusion

The present study, thus, demonstrates the potential of two fungus isolates viz., *Aspergillus sp* (RBD01 and RBD02) to tolerate high levels (90%) of oil as carbon source and also exhibit significant bound lipase activity in the presence of oil. The parameters such as pH, temperature and incubation time, associated with optimal lipase activity were also determined for both the strains. In addition, the study is of significance in terms of demonstrating the recyclability of the biomass for continued lipase activity and the role of solvent in modulating the lipase activity of the strain.

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