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Full Length Research Paper

Optimization of process parameters for enhanced production of lipase by *Penicillium notatum* using agricultural wastes

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In this study, cultural conditions were optimized for enhanced production of lipase by *Penicillium notatum*. Different agricultural wastes including canola oilseed cake, sesame oilseed cake, linseed oil cake, cotton oilseed cake, rice bran and wheat bran were used as substrates for their potential to be used for the production of lipase under solid state fermentation conditions. Among these, the canola oil seed cake proved to be the best substrate for production of lipase under solid state fermentation conditions. A maximum lipase activity of 5335 U/g dry substrate was observed after 96 h of incubation period using 60% moisture content, at pH 5, incubation temperature of 30°C and olive oil of 3%. Enrichment with maltose as carbon source enhanced the lipase production significantly, while nitrogen supplementation did not affect the lipase production significantly. The optimization of conditions led to two-fold enhancement of lipase activity as compared to the initial enzyme activity.

Key words: Fermentation, *Penicillium notatum*, olive oil, lipase, culture conditions.

INTRODUCTION

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are a class of serine hydrolase family, which possess the natural function of hydrolyzing triacylglycerol (TAG) to glycerol and free fatty acids at the interface of water and oil. In addition, lipases also catalyze esterification, transesterification and ammonolysis in non-aqueous media with high enantioselectivity. Due to their ability to carry out a variety of chemical transformations with regiospecificity and chiral selectivity (Sakinc et al., 2007), lipases have emerged as one of the most promising biocatalysts with immense industrial potential. Consequently, lipases have found wide usage in food, detergent, organic synthesis and pharmaceutical industries (Park et al., 2005; Gupta et al., 2007; Grbavcic et al., 2007; Franken et al., 2010).

Lipases are ubiquitous enzymes and are widely distributed in nature. They are present in plants and animals, as well as in microorganism. However, for industrial applications, lipases of microbial origin, bacteria (Gupta et al., 2004), yeast (Benjamin and Pandey, 1996; Vakhlu and Kour, 2006) and fungi (Fernandez-Lafuente, 2010), are mainly exploited because of their easy extraction and potential for an unlimited supply. Both submerged state and solid state fermentations have been exploited for the production of industrial enzymes. However, solid state fermentation has attracted immense attention, not only due to the availability of cheap carbon sources such as agro-industrial residues, but also because of several biotechnological advantages including higher concentration and stability of product and least catabolic repression (Holker et al., 2004).

Lipase activity and production depend upon the microbial strain and composition of the fermentation medium (Cihangir and Sarikaya, 2004). Continuous demand for highly active enzymes with appropriate properties has encouraged the research for the new sources of lipase. The composition of the growth medium,

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cultivation conditions, pH, temperature, and the kind of carbon and nitrogen sources greatly affect the production of lipase, and the optimization of these physiological and nutritional parameters results in the high yield of lipase. The objective of this study was to find suitable locally available agro-industrial waste as a fermentative substrate for the production of lipase by *Penicillium notatum* using solid state fermentation and the optimization of both physical and nutritional process parameters for maximum lipase production. This study is an inventive step for the appraisal of industrial relevance and utilization of *P. notatum* for lipase production.

MATERIALS AND METHODS

All the chemicals used during this study were of analytical grade and were obtained from Sigma Chemical Co., USA. Different substrates viz. canola oilseed cake, sesame oilseed cake, linseed oil cake, cotton oilseed cake, rice bran and wheat bran were obtained from the local market and tested for lipase production by employing solid state fermentation.

Microorganism and Inoculum

P. notatum was obtained from the Department of Plant Pathology, University of Agriculture, Faisalabad and maintained on potato dextrose agar slants at 4°C. Seed culture (inoculum) was prepared by inoculating a loopful of three days old stock culture from the slants to the 250 ml Erlenmeyer flak containing 100 ml medium consisting of (g/L): KH_2PO_4, 5; NH_4NO_3, 2; $(NH_4)_2SO_4$, 4; $MgSO_4.7H_2O$, 0.2; trisodium citrate, 2.5; peptone, 2 and yeast extract 0.1, followed by incubation at 30°C and agitation at 120 rpm. This 72 h old culture was used as inoculum.

Lipase production by solid state fermentation

10 g of substrates was transferred in a series of 250 ml Erlenmeyer flasks, moistened with water (50%), and sterilized at 15 lbs/in at 120°C for 20 min. After sterilization, the flasks were cooled and inoculated with 2 ml of spore suspension. After inoculation, the flasks were cotton plugged and incubated at 30°C for 72 h.

Optimization studies

Media optimization for maximum lipase production was carried out following the classical statistical approach, by varying one factor at a time, while keeping others as constant. The fundamental factors influencing the lipases production studied were: moisture (40 to 90%), pH (3 to 9), temperature (25 to 40°C), time of incubation (24 to 96 h), supplementary carbon sources (glucose, maltose, fructose and lactose), olive oil concentration (1 to 5%) and nitrogen sources (NH_4NO_3, $(NH_4)_2SO_4$, yeast extract, urea and peptone). Effect of different concentrations of selected carbon and nitrogen sources were also determined.

Enzyme extraction

At the end of fermentation, crude enzyme was extracted by mixing the fermented substrate with 70 ml of 0.1 M phosphate buffer (pH 7) and then shaking the mixture in an orbital shaker at 150 rpm. The extract was taken and 30 ml of the buffer was mixed in the residue again and shaken at 150 rpm. The obtained extract was filtered and the supernatants were used for lipase assay.

Enzyme assay

Lipase activity was determined by the method described by Amin et al. (2011) using para nitrophenyl palmitate (PNPP) as substrate. 0.1 ml (100 µ) of crude enzyme extract was mixed with 0.9 ml (900 µl) of solution containing: 3 mg PNPP dissolved in 1 ml of propane-2-ol diluted in 9 ml of the 50 mM Tris-HCl (pH = 8) containing 40 mg triton X-100 and 10 mg of Gum Arabic. The aforementioned mixture was then incubated at 37°C for 30 min. The liberated para nitrophenol was immediately recorded at 410 nm. “One unit of lipase activity is defined as the amount of enzyme liberating 1 µmol of para-nitrophenol/min under standard assay conditions”.

Statistical analysis

All experiments were performed in triplicate and data were analyzed statistically using regression analysis and analysis of variance (ANOVA). Main points were highlighted through appropriate graphs. All the data analysis was done by using Microsoft Excel 2007.

RESULTS AND DISCUSSION

The main objective of this study was to check the suitability of the locally available agro residues for the enhanced production of lipase. The solid state fermentation of agriculture and food residues has been well used for the production of enzymes (Mahanta et al., 2008). In the light of several advantages related to the solid state fermentation, lipase production was done using locally available agro residues. Different fungal species and agro-residues were screened for the lipase production. The best lipase producer strain was observed to be *P. notatum* followed by *Ganoderma lucidum* and *Pleurotus ostreatus* (Figure 1), and was selected for further studies. Both canola oilseed cake and linseed cake were the best suitable substrates with an insignificant difference for lipase production (Figure 2). However, canola oil seed cake, being cheaper and easily available compared to linseed cake was selected for lipase production in SSF by *P. notatum.* Good growth of *P. notatum* and lipase production was observed on canola oilseed cake as substrate. All the necessary nutrients for the growth of fungus were provided by the substrate. At moisture level of 50%, at pH 7 and at 72 h of incubation, canola oilseed cake produced 2693 U/g.

There has been no previous report for lipase production by using canola oilseed cake as substrate of solid state fermentation. However, other commonly exploited agro-industrial wastes such as babassu cake, wheat bran, gingelly oil cake, jatropha seed cake and castor bean

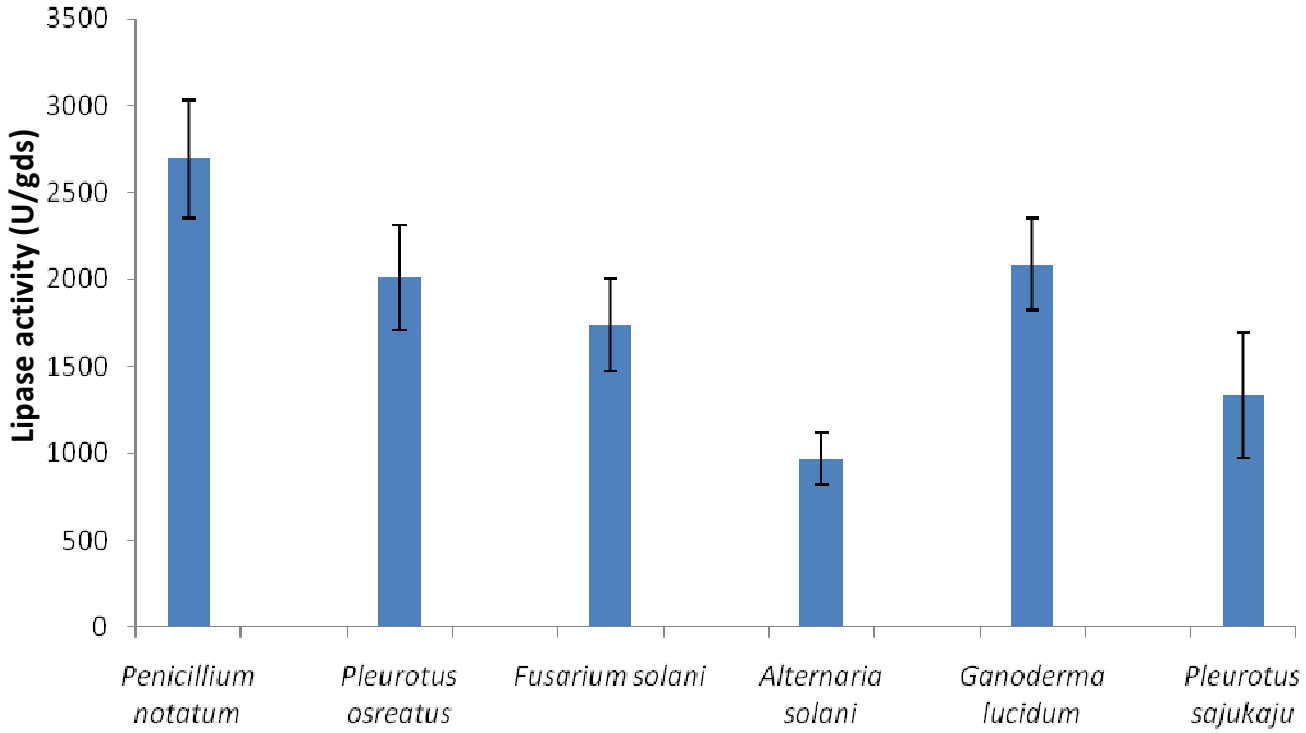

Figure 1. Screening of different fungal strains for lipase production by solid state fermentation on canola oilseed cake with 50% initial moisture contents. Experiments were done in triplicates and the error bars represent the standard deviation.

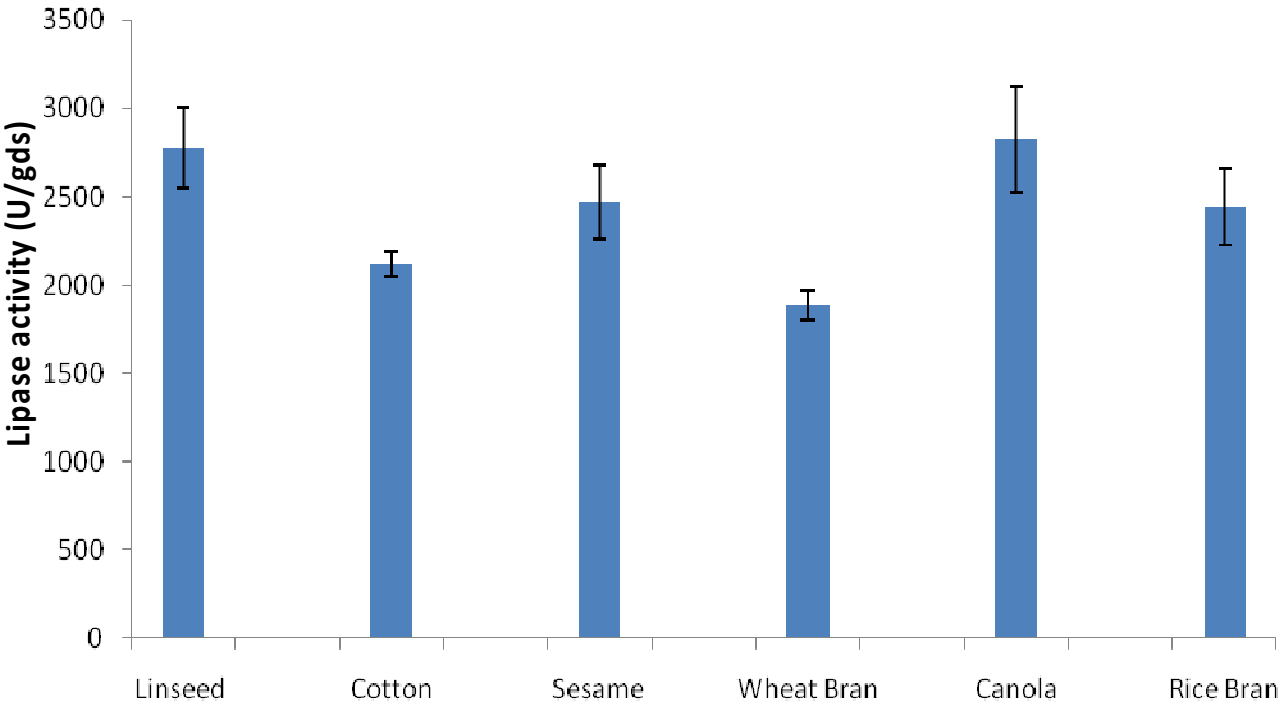

Figure 2. Effect of different substrates on lipase production by *Penicillium notatum* in SSF. Initial moisture content was 50%. Experiments were done in triplicates and the error bars represent the standard deviation.

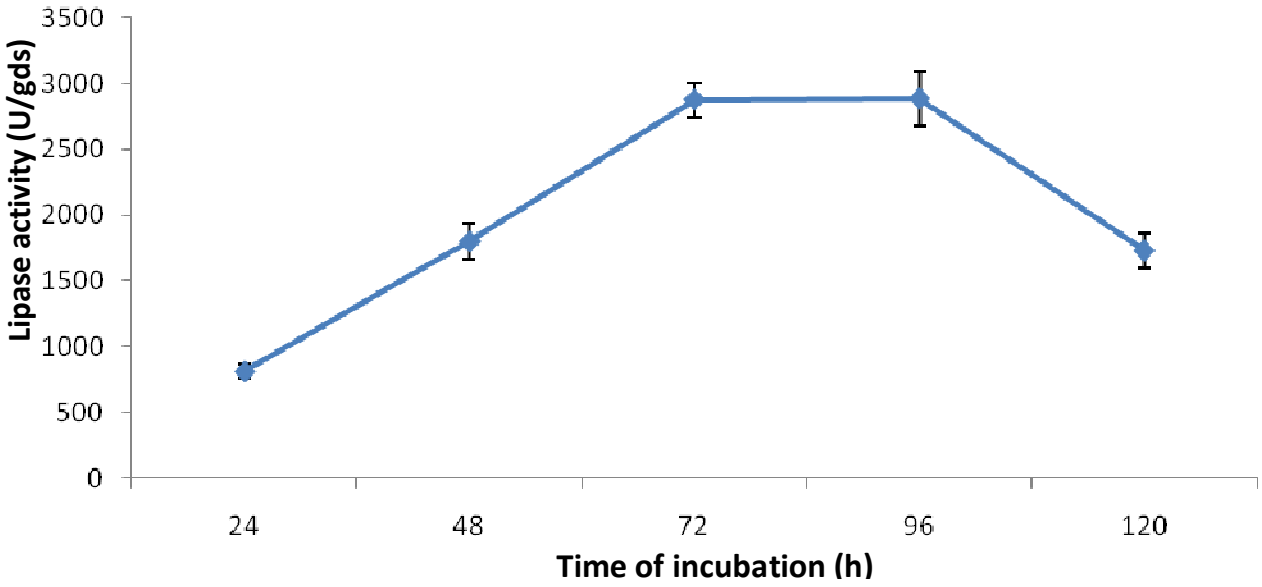

Figure 3. Effect of incubation time on lipase production by *P. notatum*. Experiments were done in triplicates and the error bars represent the standard deviation.

residues have been reported to produce lipase in SSF; 26.4 U/gds (Gutarra et al., 2005), 630 U/gds (Mahadik et al., 2002), 384 U/gds (Mala et al., 2007), 976 U/gds (Mahanta et al., 2008), 44.8 U/gds (Godoy et al., 2009), from *Penicillium simplicissimum*, *Aspergillus niger* NCIM 1207, *Aspergillus niger* MTCC 2594, *Pseudomonas aeruginosa* and *P. simplicissimum*, respectively which is much lower than our observations.

Effect of incubation period on lipase production

The time course of lipase production from *P. notatum* using canola oilseed cake as a substrate is given in Figure 3. Result indicates that incubation period affected the lipase production significantly ($P< 0.05$). Apparently, maximum lipase activity for *P. notatum* was achieved after 96 h of incubation. However the accurate optimal point as determined by regression analysis was estimated to be 81 h. Beyond these incubation periods, decrease in the enzyme activity was observed, which might be related to the depletion of the nutrients or denaturation of the enzyme caused by the interaction with other components in the medium or change in pH of the medium (Mahanta et al., 2008). Canola oilseed cake contained a residual amount of fats and fatty acids which may be acted upon by the extracellular lipase produced during fermentation. The results obtained are consistent with Kamini et al. (1998) and Gutarra et al. (2009), who obtained maximum lipase yield from *A. niger* and *P. simplicissimum* after an incubation period of 72 h. Mahadik et al. (2002) observed maximum lipase yield from *A. niger* after five days period of incubation.

Effect of initial moisture contents on the production of lipase

Appropriate moisture content is crucial for microbial growth in solid state fermentation. To determine the effect of moisture during the solid state fermentation, canola oilseed cake was moistened with different amounts of distilled water (40, 50, 60, 70, 80 and 90%) prior to fermentation. From the results, it is clear that initial moisture contents played significant role in the lipase production by *P. notatum* ($P<<0.05$). Maximum lipase activity (3426 U/gds) was observed at 60% initial moisture level (Figure 4). Statistically the optimum initial moisture level was estimated to be 60.8%. The enzyme production was significantly affected by an increase and decrease in moisture contents. It has been suggested that moisture level has great impact on the physical properties of substrate. An increase in moisture content decreases the substrate porosity, alter substrate particle structure, promote development of stickiness and reduce gas volume and exchange, leading to poor oxygen transfer and diffusion while lower moisture contents reduce the solubility of nutrients contained in solid substrate, resulting in improper swelling and a higher water tension (Bhatti et al., 2007; Sun and Xu, 2008; Mahanta et al., 2008; Amin et al., 2008).

Hence, low enzyme production at very high or low

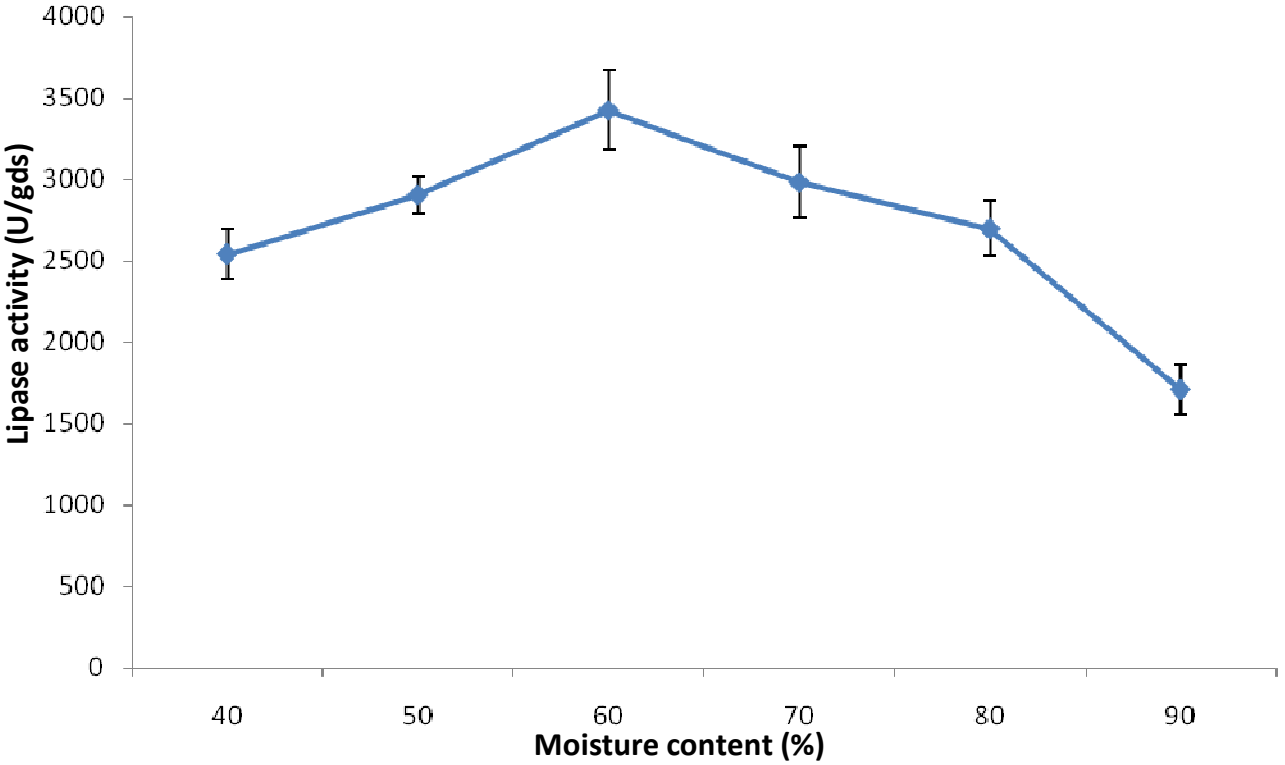

Figure 4. Effect of initial moisture content on the lipase production by *P. notatum.* Experiments were done in triplicates and the error bars represent the standard deviation.

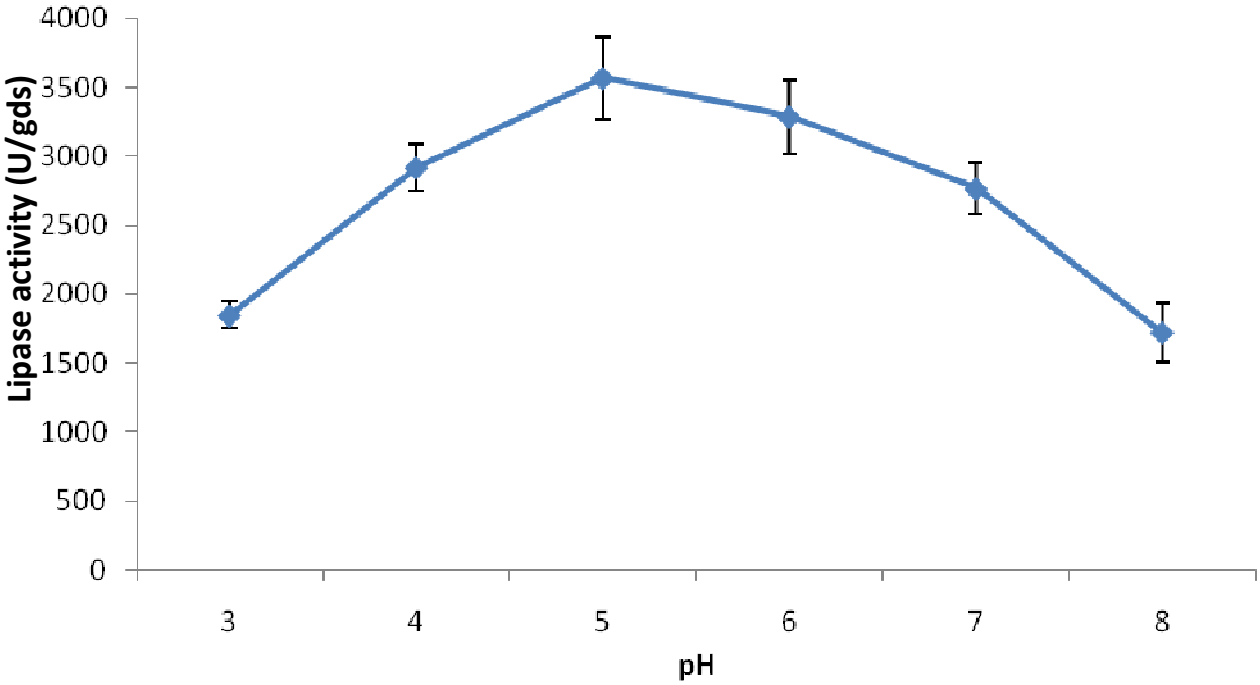

Figure 5. Effect of initial pH on the lipase production by *P. notatum.* Experiments were done in triplicates and the error bars represent the standard deviation.

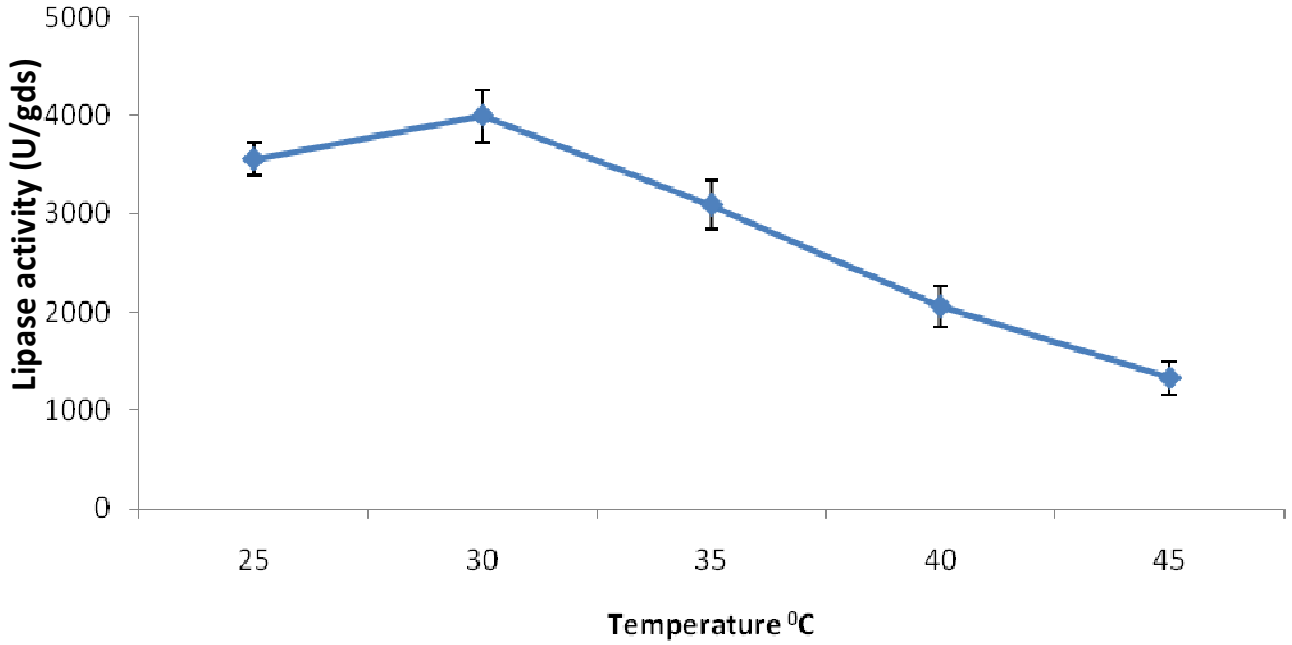

Figure 6. Effect of incubation temperature on the lipase production by *P. notatum.* Experiments were done in triplicates and the error bars represent the standard deviation.

moisture levels during this study might be related to the fact that low diffusion of nutrients and metabolites takes place in lower moisture contents whereas compaction of substrate occurs at higher moisture contents resulting in decreased enzyme production. The results obtained during this study are in agreement with the previous reports. Earlier reports indicated the requirement of 71 and 60% initial moisture content for maximal lipase production by *A. niger* with wheat bran and gingelly oilseed cake, respectively (Mahadik et al., 2002; Kamini et al., 1998). Gutarra et al. (2005) reported the requirement of 70% initial moisture content for maximum lipase production by *P. simplicissimum* on babassu cake while 70% moisture was required by *Rhizopus chinensis* for maximum lipase production (Sun and Xu, 2008). More also, a moisture content of 50% was optimal for lipase production by *Candida rugosa* (Rao et al., 1993).

Effect of initial pH of the medium on lipase production

The pH of the growth medium is an imperative factor, which greatly affects the microbial growth and enzyme production during solid state fermentation. Each microorganism possesses a unique optimum pH, as well as a pH range for its growth and activity. Filamentous fungi are supposed to thrive over a broad range of pH under solid state culture, because the solid substrate holds a better buffering capacity (Shaheen et al., 2008; Sun and Xu, 2008). Initial pH of the canola seed cake was adjusted to different levels (3.0 to 8.0) to determine the influence of pH on lipase production by *P. notatum. P. notatum* showed capability of producing lipase in the range of pH 4 to 7, with maximum enzyme activity at pH 5.43 (Figure 5) The pH values above and below the optimum pH resulted in a significant inhibition of lipase production ($P<0.05$). Similar results were reported by Lin et al. (2006), who observed pH 5.5 to be optimal for lipase production by *Antrodia cinnamomea*. However, these optimum pH values are lower than other lipase-producing organisms: *Rhizopus glutinis* (pH 8.0) (Dimitris et al., 1992), *Candida rugosa* (pH 7.0), marine bacterial lipase (pH 10.0) (Camargo de Morais et al., 1998) and *Pseudomonas aeruginosa* (pH 7.0)

Effect of incubation temperature on the lipase production

Incubation temperature is another important factor which affects the enzyme yield during solid state fermentation. Different temperatures ranging from 25 to 45°C were used to determine the optimum temperature for lipase production by *Penicillum notatum* in SSF. Results presented in Figure 6 represent that change in incubation temperature in SSF most significantly affected the enzyme yield ($P<<0.05$). Maximal lipase activity by *Penicillum notatum* was observed when the incubation

temperature was 30°C. Statistically, the optimum temperature was found to be 33.5°C. Temperature levels below the optimum caused a slight decrease in the lipase activity, whereas temperatures above the optimum levels resulted in steep drops in enzyme production. This negative effect of temperature on the lipase production by *P. notatum,* using canola oilseed cake might be due to the enhanced production of proteases at higher temperatures as Gutarra (2003) found high levels of proteases when fermentation was carried out at higher temperatures for the production of lipase by *P. simplicissimum* cultivated in babassu cake medium. Moreover, other than proteolysis, enzyme deactivation could be a factor contributing to lipase activity decrease at higher temperatures. The results are similar to that reported for *Fusarium oxysporum* (Rifaat et al., 2010) and *Rhizopus glutinis* (Dimitris et al., 1992) (28 and 30°C, respectively). However the results are significantly different from *Penicillium citrinium* (Maliszewska and Mastalerz, 1992), *Colletotrichum gloeosporioides* (Balaji and Ebenezer, 2008) and *Rhizopus arrhizus* (Yang et al., 2000). The optimal temperatures for maximum lipase production for these fungi were 22, 25 and 26.5°C, respectively.

Effect of additional carbon sources on lipase production

Considering the presence of only 7 to 8% of carbohydrate in seed cake, it was believed to be suitable to supplement the substrate with ready sugar for better growth and enzyme production. Different carbon sources (glucose, lactose, maltose and fructose) were added to canola oil cake at concentration of 2% to investigate their effect on lipase production. The affect of supplemented carbon had a stimulatory affect, however, this effect was not very highly significant ($p = 0.05$). Presence of 2% maltose resulted in the highest lipase yield (4626 U/gds) (Figure 7). The results are similar to those reported by Mahanta et al. (2008) and Rao et al. (1993), who reported that presence of maltose in the growth media enhanced the lipase production by *P. aeruginosa* and *C. rugosa*, respectively. Wheat bran supplemented with glucose as carbon source was also observed to be a good cultivation medium for lipase production by *A. niger* (Falony et al., 2006).

Effect of olive oil concentration on lipase production

Olive oil is a well known inducer for the lipase production by many bacterial and fungal strains. Effect of olive oil was investigated by supplementing the growth medium with 1 to 5% olive oil concentrations. Olive oil was observed to affect the lipase production quite significantly ($P<0.05$). The best production yield of lipase (5233 U/gds) was obtained at 3% olive oil for *P. notatum (*Figure 8). Statistically, the optimum concentration of olive oil was 2.3%. The results are consistent with those of Fadiloglu and Erkmen, (2002), Benjamin and Pandey, (1996) and Rajendran et al. (2008) who also observed olive oil to be most influential factor for lipase production. Teng and Xu (2008) observed 2.367% olive oil to be optimum for lipase production by *R. chinensis*. In addition, olive oil supplemented with babassu cake with a C/N ratio of 13:3 gave the best growth and lipase production by *P. restrictum* (Azeredo et al., 2007).

Effect of additional nitrogen sources on lipase production

The growth medium consisting of canola oilseed cake was supplemented with different organic and inorganic nitrogen sources to determine the effect of nitrogen on lipase production. Ammonium sulphate, ammonium nitrate, urea, peptone and yeast were used for this purpose**.** Supplementary nitrogen did not affect the lipase production by *P. notatum*. However maximum lipase activity was achieved in the presence of peptone (5335 U/gds). However, the results (Figure 9) show that the difference in the activity was not significant as compared to the control. Results obtained were similar to those reported by Montesinos et al. (1995), Kamini et al. (1998) and Gutarra et al. (2005), who observed no effect of nitrogen source on lipase production, but are in contradiction to those reported by Glu and Erkmen (2002), who observed significant enhancement of lipase production by *C. rugosa* in the presence of supplemented nitrogen source (yeast extract and peptone). Mahanta et al. (2008) also observed enhanced lipase production by *P. aeruginosa* in the presence of peptone.

Conclusion

In this study, among the six fungal species and agro-residues screened for lipase production in the SSF, *P. notatum* was proven to be the best lipase producing species, and canola oil seed cake as the best substrate. Furthermore, the production of lipase by *P. notatum* in solid state fermentation using canola oilseed cake was investigated, and optimization of different physicochemical factors led to two times enhancement in lipase production. Under these conditions, the enzyme activity was observed to be 5335 U/gds. It was illustrated that canola oilseed cake provided necessary nutrients for the fungus to grow and produced enzyme and it could be a good source for lipase production by SSF. In addition, the amount of lipase in initial experiments without

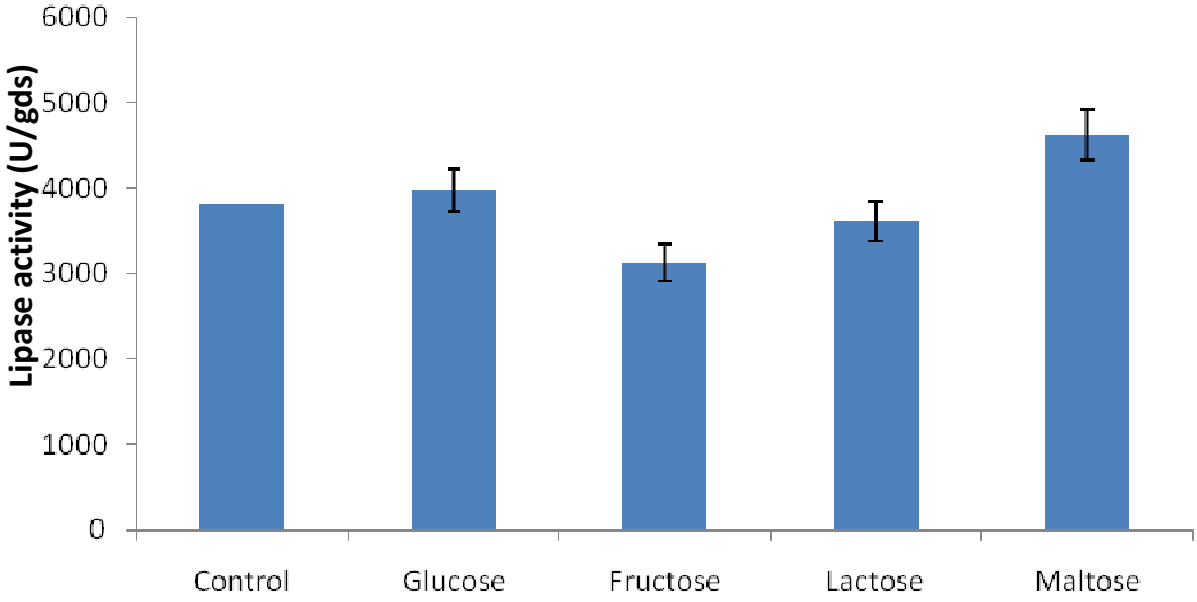

Figure 7. Effect of additional carbon source on the lipase production by *P. notatum.* Experiments were done in triplicates and the error bars represent the standard deviation.

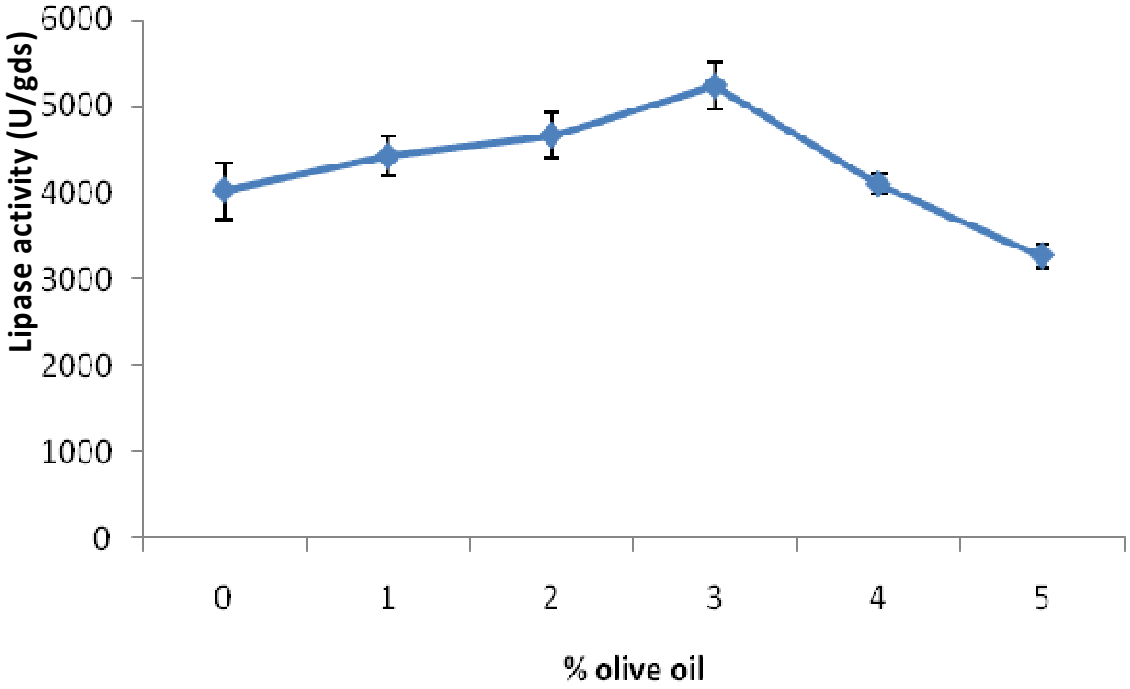

Figure 8. Effect of olive oil on the lipase production by *P. notatum.* Experiments were done in triplicates and the error bars represent the standard deviation.

optimization is promising. With these encouraging results, it will be worthwhile to use *P. notatum* and canola seed cake for the production of lipase on commercial scale.

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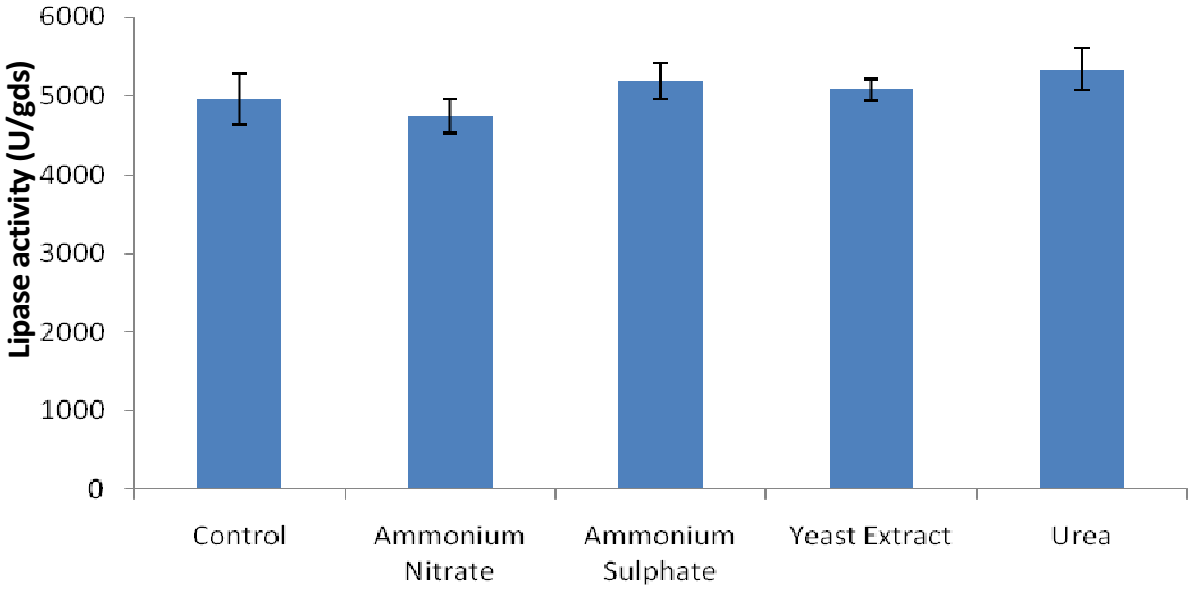

Figure 9. Effect of supplemented nitrogen on the lipase production by *P. notatum.* Experiments were done in triplicates and the error bars represent the standard deviation.

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