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Full Length Article

Effect of Physicochemical Parameters on Lipase Production by *Penicillium fellutanum* using Canola Seed Oil Cake as Substrate

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Abstract

Microbial lipases show a unique behavior among other enzymes due to their ability to catalyze various reactions in aqueous and non-aqueous media. The present study was planned to explore new potential fungal strain for lipase production using agro-industrial wastes under solid state fermentation (SSF). Among different residues used as substrate such as rice bran, wheat bran, canola seed oil cake, sunflower hulls and peanut shells, the canola seed oil cake showed the highest lipase activity. Various production parameters like initial pH of production media, moisture content, incubation time, inoculum size, incubation temperature and substrate level were optimized to enhance extracellular lipase production. Effect of different carbon and nitrogen sources was also studied. A maximum lipase activity of 521 units/gram dry substrate (U/gds) was obtained after 48 h of reaction time in a media containing 10 g canola seed oil cake as a substrate supplemented with 2% olive oil as inducer and 50% moisture content at initial pH of 4.0 using 2 mL inoculum at 30°C. Additional carbon sources also affect significantly the fermentation process. Among different carbon sources glucose exhibited maximum potential to enhance the lipase production.

Keywords: Lipase; *Penicillium fellutanum*; Fermentation; Optimization

Introduction

Chemical catalysts compel several disadvantages and generate a variety of by-products and toxic effluents. Enzymatic reactions are striking alternatives to expensive chemical methods and in addition, they are biodegradable and perform its action under eco-friendly conditions (Contesini *et al*., 2010). Lipases (EC 3.1.1.3) are enzymes having the ability to catalyze the hydrolysis of glycerol ester bond at fat and water interface (Hosseinpour *et al*., 2011). They show a unique behavior among other enzymes due to their ability to catalyze various reactions in aqueous and non-aqueous media. Due to this characteristic of these enzymes, scientists feel great interest for their intensive use in industrial bio transformations. Lipases have a large number of applications in various industries as food, cosmetic and pharmaceutical, due to their hydrolytic reactions. Prospective applications comprise production of flavor esters in the food industry, the resolution of racemic mixtures, alteration of sugars and production of biofuel (Shu *et al*., 2010). This makes the lipases one of the most prominent classes of enzymes in the field of organic synthesis.

Lipases occur naturally in plants, animals, and microorganisms (Melo *et al*., 2005; Paques *et al*., 2008; Gangadhara *et al*., 2009). Among these sources, microbial lipases prove to be best owing to their substrate specificity, greater stability and lower production costs. Microorganisms which are effective lipase producers include bacteria, fungi and yeast (Abada, 2008). Filamentous fungi are the preferred sources for lipase production since they produce extracellular lipases which are convenient for their extraction (Carvalho *et al*., 2005).

In the preceding two decades, solid state fermentation (SSF) has fascinated the scientists owing to its several advantages in the production of enzymes such as higher rate of product formation, lower catabolic repression etc. (Holker *et al*., 2004), that's why fungi are cultivated preferably in SSF (Dutra *et al*., 2008). SSF is a valuable implement for processing agro-industrial wastes. Many developing countries, like Pakistan produce agricultural wastes in large quantities but their consumption is restricted as feeds for animals or simply as landfills. Nevertheless, agricultural wastes stand for large prospective assets for use in biotechnological processes largely due to their ease of access, low cost and nutrients like carbon, nitrogen and minerals (Graminha *et al*., 2008). Oil cakes provide ideal nutrient support in SSF representing both carbon and nitrogen sources. Using fungal species, oil cakes have been reported to be good substrate for enzyme production (Ramachandran *et al*., 2007).

Due to limited foreign exchange and very few substrates for the production of lipases, the import of these enzymes in the country is very cost effective leading to the high cost of their production. Therefore, it is need of hour to investigate other nearby available sources used for the

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production of this important enzyme. In nature, the Penicillia, are versatile and opportunistic fungi. They are good producers of extracellular enzymes such as cellulases, lipases, xylanases and proteases (Li and Zong, 2010). Thus, the objectives of this investigation were to explore the potential of *Penicillium fellutanum* for the production of lipase under SSF and to study the effect of various carbon and nitrogen sources on the enzyme biosynthesis.

Materials and Methods

Microorganism and Inoculum Preparation

Fungal species *Penicillium fellutanum* used during the study was obtained from the Fungal Bank, Punjab University, Lahore, Pakistan. Spores of *P. fellutanum* were maintained on PDA slants, inoculated with spores of the fungus under aseptic conditions in a laminar air flow, were incubated at 30ºC till sporulation and stored at 4ºC for further use. Inoculum was prepared by transferring spores of *P. fellutanum* in 100 mL of Kirk Basal medium. Properly washed small glass beads (4-5) were added to obtain the homogenous suspension of spores by the breakdown of the mycelia. The medium was then autoclaved at 121ºC for 15 min. Inoculum was developed by inoculating a loop of three days old culture from the slants to the autoclaved medium under aseptic conditions, followed by incubation at 30°C and agitation at 120 rpm. The 72 h old culture with spore count of 1×10^8 spores/mL was used as inoculum.

Lipase Production in SSF

Five different agro-industrial wastes were collected from the local market of Faisalabad Pakistan and used as substrate. All the substrates before use were dried, ground in an electric mill and then sieved. Ten grams of substrate was transferred in a series of 250 mL Erlenmeyer flasks, moistened with water (50%), and sterilized at 15 lbs/in at 121°C for 15 min. Afterwards the flasks were inoculated with 2 mL of spore suspension and incubated in favor of fermentation at 30°C for 72 h.

Optimization Studies

The fundamental factors influencing the lipases production studied were moisture content (%), pH, incubation time (h), amount of substrate (g), inoculum size (mL), incubation temperature (°C) and olive oil concentration (%). Effect of supplementary carbon and nitrogen sources was also investigated. At the end of fermentation, crude enzyme was extracted by mixing the fermented substrate with 100 mL of phosphate buffer (0.1 M; pH 7) and then shaking the mixture in an orbital shaker at 100 rpm. The obtained extract was filtered and the supernatants were used for lipase assay.

Enzyme Assay

Lipase activity was determined by following the method of Yagiz *et al.* (2007), by means of para nitrophenylpalmitate (PNPP) as a substrate. Crude enzyme extract (100 µL) was mixed with 0.9 mL (900 µL) of solution which contains 3 mg PNPP substrate dissolved in 1 mL of propane-2-ol diluted in 9 mL of the 50 mM Tris-HCl, pH 8, having 40 mg triton X-100 and 10 mg of Gum Arabic. This mixture was incubated at 37 ºC for 30 minutes and liberated para nitrophenol was recorded at 410 nm.

Activity units were determined by the following formula:

$$Lipase\ Activity\ \left(\frac{U}{mL}\right) = \frac{Absorbance\ of\ sample\ \times Standard\ factor}{Time\ of\ reaction\ \times enzyme\ extract\ (mL)}$$

One unit of lipase activity is defined as the amount of enzyme liberating 1µmol of para-nitrophenol per mL/min under standard assay conditions. Total protein was estimated by using Bradford method (Bradford, 1976). Bovine serum albumin (BSA) was used as standard.

Statistical Analysis

Experimental design used throughout this study was Completely Randomized Design (CRD). Data represents the mean of three independent trials and results are presented as mean ± SD values. One-way ANOVA was used for testing the differences between parameters.

Results

Five different agro-industrial wastes including canola oilseed cake, wheat bran, rice bran, peanut shells and sunflower hulls as substrates were used. Among these residues tested for lipase production, canola oilseed cake offered better enzyme production than any other residue by *P. fellutanum* (Fig. 1a). At moisture level 50%, pH 5.5 and 72 h of incubation period, canola oilseed cake produced 323.62 U/gds lipase by *P. fellutanum*, so in subsequent studies canola oilseed cake was used as a substrate.

Effect of Physical Parameters

The physical parameters such as moisture content, pH, amount of substrate, inoculum size, incubation time and temperature usually affect the lipase production. After the selection of suitable substrate, all above parameters were studied one by one using classical statistical approach. To observe the effect of moisture content on production of lipase by *P. fellutanum*, different amounts (30-80%) of distilled water were added to canola oilseed cake. Fig. 1b depicted that moisture content had a significant effect on lipase production ($p < 0.05$) and maximum lipase activity (323.62 U/gds) was achieved at 50% moisture level. Further increase in moisture content gradually decreased the lipase yield.

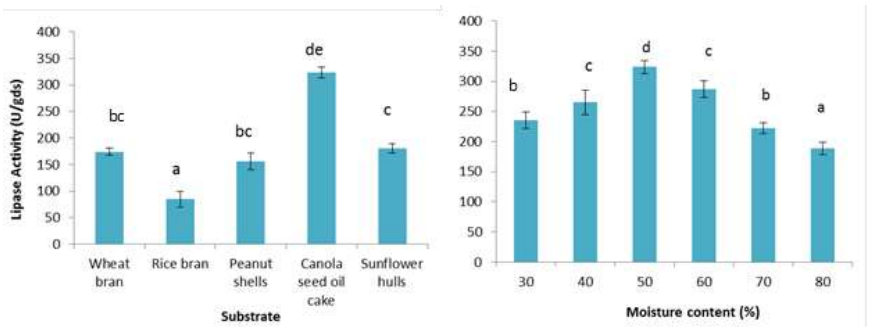

Fig. 1: Lipase production by *P. fellutanum*: Screening of substrates (left) and effect of moisture content (right). Reaction time 72 h, T = 30°C, substrate amount 10 g, inoculum size 2 mL and pH 5.5. Error bars indicate standard deviations (n=3). Different letters on bars indicate significant variation ($p < 0.05$) between the responses observed

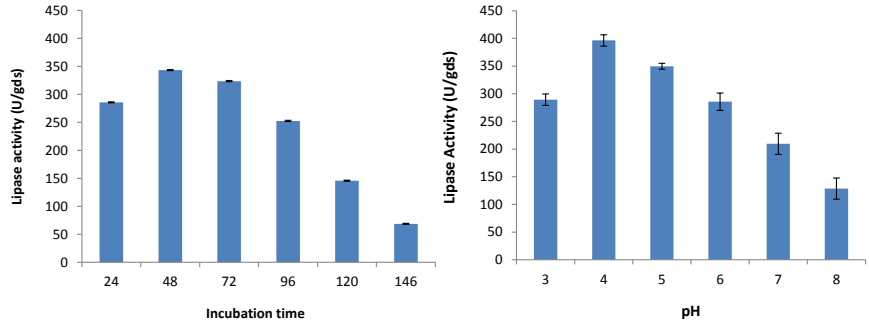

Fig. 2: Lipase production by *P. fellutanum*: Effect of incubation time (left) and effect of pH (right). Moisture content 50%, T 30°C, substrate amount 10 g and inoculum size 2 mL. Error bars indicate standard deviations (n=3). Different letters on bars indicate significant variation ($p < 0.05$) between the responses observed

The amount of lipase produced was investigated after every 24 h up to 144 h. The result indicates that incubation time affected lipase production very significantly ($p < 0.05$) and maximum lipase activity (373.56 U/gds) was observed after 48 h of incubation time (Fig. 2a). After long incubation time, lipase production was turned down as its activity was found 68.65 U/gds after 6 days (144 h) of incubation whereas mycelial biomass was rapidly stimulated throughout the fermentation period.

To optimize the initial pH for lipase production, experiments were conducted by varying the pH of production medium from 3.0 to 8.0 and data represented that pH played a significant role ($p < 0.05$) in enzyme production. Lipase production was increased from pH 3.0 to 4.0 and the maximum lipase activity (396.59 U/gds) was observed at pH 4.0 (Fig. 2b). When the pH value exceeded 4.0, the lipase activity decreased steadily so present study revealed that the lipase production by *P. fellutanum* needs acidic environment.

Optimization of substrate level was carried out by varying the amount of substrate (5-25 g) in the fermentation process. The results showed that 5 g of substrate (canola oilseed cake) yielded maximum lipase activity (415.33 U/gds) by *P. fellutanum*. Results clearly indicated that lipase production was decreased by increasing the amount of substrate but it retained lipase activity (188.78 U/gds) up to 25 g of substrate as illustrated in Fig. 3a.

Different inoculum levels (1-6 mL) were tried to investigate their effect on lipase production so as to achieve an optimum inoculum level. The results regarding the effect of inoculum size are shown in Fig. 3b, which revealed that the lipase production was decreased with the increase in fungal mass and optimal inoculum size was found to be 2 mL with 397.95 U/gds lipase activity.

Incubation temperature is a significant parameter which plays a key role in the biochemical activities of microorganism. To examine the effect of temperature on lipase activity, the temperature of incubating chamber was varied from 25 to 45°C with an increment of 5°C. Results depicted that temperature had affected the fermentation process significantly ($p < 0.05$). The crude enzyme exhibited maximum activity (401.37 U/gds) at 30°C. It was observed that increase or decrease in temperature caused the enzyme production with much low activities (Fig. 4a). At temperature below 30°C, there was a slight decrease in the lipase activity, while above 30°C the drop in enzyme production was steep.

Effect of Nutritional Parameters

Medium components significantly affect the fermentation process (Zhang *et al*., 2009) and for this reason it is essential

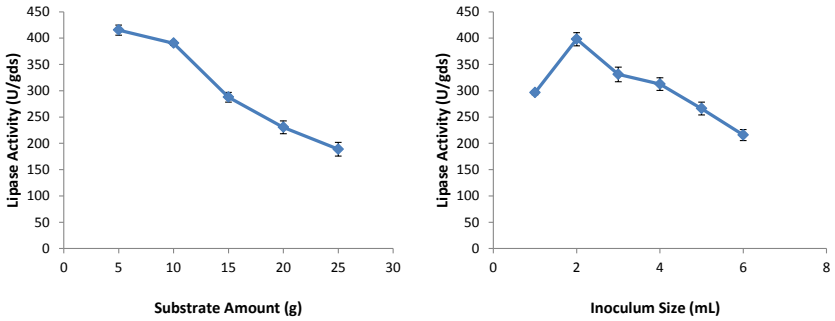

Fig. 3: Lipase production by *P. fellutanum*: Effect of amount of substrate (left) and effect of inoculum size (right). Moisture content 50%, reaction time 48 h, T 30°C and pH 4.0. Error bars indicate standard deviations (n=3). Different letters on bars indicate significant variation ($p < 0.05$) between the responses observed

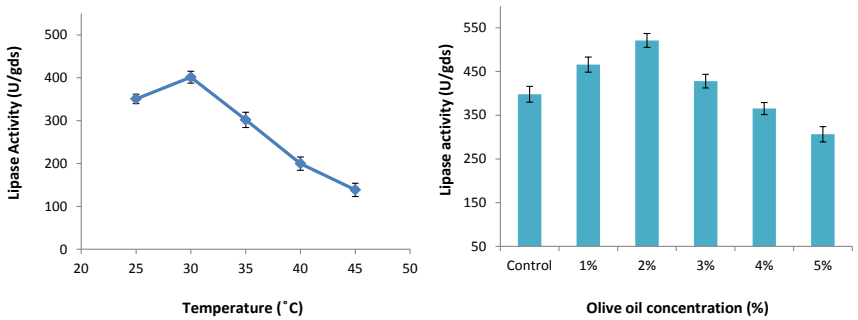

Fig. 4: Lipase production by *P. fellutanum*: Effect of temperature (left) and effect of olive oil concentration (right). Moisture content 50%, reaction time 48 h, substrate amount 10 g, inoculum size 2 mL and pH 4.0). Error bars indicate standard deviations (n=3). Different letters on bars indicate significant variation ($p < 0.05$) between the responses observed

to assess the nutritional requirements (lipidic carbon and nitrogen sources etc.) towards enhance productivity. In the recent study, all the nutritional sources were observed to affect lipase production significantly ($p < 0.05$). Effect of olive oil was investigated by supplementing the growth medium with 1-5% olive oil concentrations. Control experiments were also conducted to evaluate the influence of olive oil clearly. In the presence of olive oil with substrate as an inducer, lipase activity was reached to its maximum level and optimum concentration was found to be 2%, increasing the lipase activity up to 521 U/gds (Fig. 4b).

Different carbon sources i.e. glucose, lactose, maltose, fructose, sucrose, sodium acetate and tri sodium citrate were added to canola oil cake at concentration of 1% (w/w) to investigate their effect on lipase production. The effect of supplemented carbon sources was stimulatory with the exception of tri sodium citrate which had inhibitory effect while sodium acetate did not affect the lipase production. Results revealed that glucose was the most effective supplement for lipase production by *P. fellutanum* (Fig. 5a). It enhanced the lipase activity from 392.65 U/gds to 496.36 U/gds, which indicated a decent increment in lipase activity.

Both organic and inorganic nitrogen sources have been traditionally used for lipase production. The growth medium consisting of canola oilseed cake was supplemented with different nitrogen sources (ammonium sulfate, urea, peptone, ammonium nitrate, yeast extract, sodium nitrate and diammonium tartarate) to determine the effect of nitrogen on lipase production. Ammonium sulfate was found to be an excellent nitrogen source giving lipase activity as 479.69 U/gds (Fig. 5b).

Lipase activity with optimized growth conditions by *P. fellutanum* was observed to be 51.976 U/mL or 519.76 U/gds. Optimization of different physicochemical factors led to 1.6 folds enhancement in lipase production. Total units of lipase and total protein contents calculated in crude extract were 36383.2 U and 275.37 mg respectively. Specific activity of crude enzyme extract was found 132.12 U/mg.

Discussion

Solid state fermentation is gaining more and more attention in recent years due to the possibility of using cheap agro-industrial wastes as substrates. In this study, five different agro-industrial wastes were used as substrates and lipase production was more prominent with canola oilseed cake which indicates that canola oilseed cake had provided all the necessary nutrients for the growth of fungus. The decent lipase production by canola oilseed cake proved it the good lipase producer, therefore, it was used as substrate in subsequent study. The technique of solid state fermentation

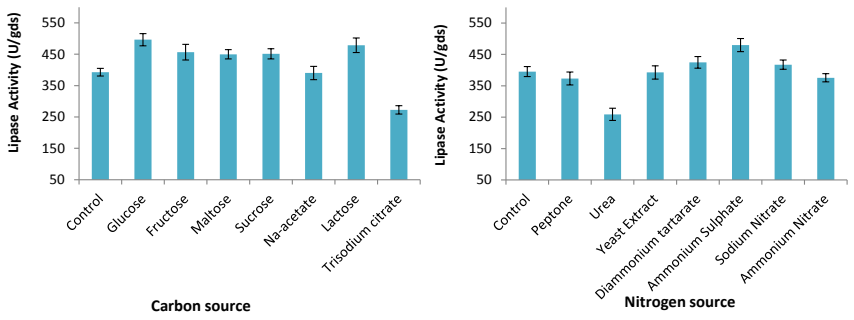

Fig. 5: Lipase production by *P. fellutanum* (a) Effect of additional carbon sources (b) Effect of nitrogen sources (moisture content 50%, reaction time 48 h, T 30°C, Substrate amount 10 g, inoculum size 2 mL and pH 4.0). Error bars indicate standard deviations (n=3). Different letters on bars indicate significant variation ($p < 0.05$) between the responses observed

involves the growth of microorganisms on moist solid substrates without any free flowing water. Optimum moisture content is an important parameter because it is responsible for the efficiency of fermentation process as it can vary from one microorganism to another due to its metabolic activity, heat evolution, environmental factors and type of micro-organisms as well as substrate. Results revealed that variation in the moisture content significantly affect the production of lipase. It is usually observed that higher moisture levels maintain the stickiness, reduce the porosity, and oxygen transfer in substrate, while lower moisture contents cut down the nutrients solubility in the substrate (Contesini *et al*., 2010). So, in the present study, an indecent lipase production was observed at very low or high moisture levels. Mahanta *et al.* (2008) reported initial moisture content at 50% of substrate as ideal for lipase production using *Jatropha curcas* seed cake as the substrate. In the present study, maximum lipase activity was observed after 48 h of incubation and thereafter, it was declined, however mycelial growth was going on increasing till 144 h of incubation. It might happen due to the loss of nutrients and moisture or poor oxygen supply due to the compaction of fungal mycelia.

The pH being a measure of acidity or alkalinity of a medium, plays an important role in determining the type of organisms that can colonize a particular substrate. Each microorganism grows and acts at a unique optimum pH, because change in pH affects its growth and metabolic activity. Filamentous fungi are thought to flourish over a wide range of pH under SSF due to enhanced buffering capacity of solid substrate (Shankar and Mulimani, 2007; Amin *et al*., 2008; Sun and Xu, 2008). In the recent study, it has been observed that *P. fellutanum* has the ability to grow over a wide range of pH but with an optimum of pH 4 which means that it needs an acidic pH to grow well and produce lipase. The concentration of substrate is vital in solid-state fermentation. The level of substrate per unit area of working volume of the flask influences the porosity and aeration of the substrate. In this study, highest lipase activity was observed with 5 g of substrate which might be due to easy penetration of microbial mass in small amount of substrate, which produced more lipase owing to high growth rate with 5 g canola oilseed cake as a substrate. Lipase production was less at higher substrate level due to the difficulty for the organism in penetration of substrate (Singh *et al*., 2010). Inoculum density is also an important factor in an SSF processes since higher inoculum levels, besides increasing spore concentration, also increase water content of the solid substrate, thereby inhibiting fungal growth and enzyme induction. On the other hand, lower inoculum levels require more time for fermenting the substrates in SSF still cultures. Therefore, inoculum size must be distributed homogenously and must be sufficient for the microorganism to grow well. It may be suggested that lower inoculum size caused a slow lag phase due to which no fungal growth and enzyme production was obtained (Ramachandran *et al*., 2004). However, high inoculum sizes are inhibitory in nature that often leads to oxygen and nutrient exhaustion in the fermentation media and thus affecting the overall productivity (Rahman *et al*., 2005).

Growth temperature is a very critical parameter which varies from organism to organism and slight changes in growth temperature may affect enzyme production. In this study optimum temperature for lipase production was 30°C. At higher temperature, due to the production of large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation (Bhatti *et al*., 2007). An increase in temperature increased the number of effective collision between the enzyme and substrate to form the activated complex and thus the rate of reaction increased. There is a limit to the increase in enzyme activity with the increase in temperature. When the rate of enzyme catalysed reactions is measured at several temperatures, there is an optimal temperature at which the reaction is most rapid, but when it is above that temperature, the reaction rate decreases sharply mainly due to the denaturation of enzyme by heat (Murray *et al*., 2003). Temperature also influences secretion of extraceIlular enzymes by changing the physical properties of the cell membrane (Pirt, 1975). It may be correlated with the increased production of protease at higher temperatures which lead to deactivation of lipase (Palma *et al*., 2000).

Lima *et al.* (2003) reported that cultures of Penicillium for lipase production are usually developed between 25 and 30°C and mostly at 28°C.

Lipases are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, bile salts and glycerol (Sharma *et al*., 2009). Lipidic carbon sources serve as inducers and olive oil with high contents of oleic acid is a well-known inducer for the lipase production by many bacterial and fungal strains (Wang *et al*., 2008). During this study, maximum lipase activity was achieved in the presence of 2% olive oil as an inducer with substrate, which indicated that additional lipid source is more effective to enhance the lipase production than any other carbohydrate source. It was observed that further increase in olive oil concentration did not favor to boost up the lipase activity. It might be due to poor oxygen transfer at higher level which could modify the microbial metabolism leading to less lipase production. Gombert *et al.* (1999) described lipase production using babassu cake as substrate from *P. restrictum* in SSF and highest lipase activity was obtained with the addition of 2% olive oil. The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes (Gupta *et al*., 2004). Due to the presence of only 7–8% of carbohydrate in agricultural residues, the substrate was supplemented with ready sugar to enhance the growth and enzyme production. Carbon sources serve as important substrates for energy production in microorganisms. The requirement of sugar as carbon source in addition to lipids varies with the organism. The present study revealed that glucose improved the lipase productivity significantly, because fungal cells get adaptation to absorb this sugar more easily than any other so as to give good mycelia growth and ultimately greater enzyme production (Contesini *et al*., 2010). Previously it has been reported that glucose is best carbon source for production of lipase by *Bacillus licheniformis* (Bayoumi *et al*., 2007) and *Bacillus pumilus* SG2 (Sangeetha *et al*., 2008). Results indicated that out of seven nitrogen sources three (ammonium sulfate, diammonium tartarate and sodium nitrate) caused the remarkable increase in lipase production. All the other nitrogen sources used in this study either inhibited the lipase production or had no any prominent effect.

In conclusion, the use of agro-industrial wastes as substrates in SSF is promising to give the solution towards cost effective processes. Optimization of such processes could allow further reduction of total product costs so as to facilitate scaling up. This study was done with a new promising lipase producing fungal species (*P. fellutanum*), which produced extracellular lipase in an inexpensive medium. Different physical and nutritional parameters such as pH, moisture content, incubation time, temperature, olive oil concentration, carbon and nitrogen sources etc. affected lipase production significantly. Lipase produced by *P. fellutanum* is very promising and could be used for industrial purposes using low cost canola oil cake as substrate in short period of time.

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