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Isolation and screening of potential fungi and standardization of a process for the production of extracellular lipase

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ABSTRACT

Eighty fungi species were isolated from samples obtained from different areas in and around Delhi. These fungi were screened using tributyrin agar medium. Six of the isolated fungi revealed a clear zone of hydrolysis as compared to others, indicating extracellular lipase activity. These six fungi were selected and identified at the Plant Pathology Division, Indian Agricultural Research Institute, New Delhi under the expert guidance of fungal taxonomist considering morphological and physiological characteristics. These fungi were identified as; *Alternaria alternata* (A); *Penicillium aurantiogriseum* (B); *Penicillium pinophilus* (C); *Aspergillus fumigatus* (D)*; Penicillium purpurogenum* (E); *Penicillium citrinum* (F). These fungi were point inoculated on four different production media viz, M1, M2, M3 and M4 to understand the best possible production medium for optimal lipase production. *Penicillium aurantiogriseum* (isolate B) showed highest lipase activity on M4 as compared to other production medium. The lowest lipase activity was recorded on M2 followed by M1 and M3 media. The most favorable pH and temperature for lipase production by isolate B was 6.8 at 30°C after 96h of incubation period. Besides, enzymatic production increased when the organism was cultured in medium augmented with glucose as a source of C and corn step liquor and til oil as sources of N and fat respectively. The results of this study revealed that lipase activity (1 µM of p-nitro phenol ml^{-1} min^{-1}) increased from 0.065 IU ml^{-1} min^{-1} under unstandardized conditions to 16.718 IU ml^{-1} min^{-1} under standardized conditions. *P. aurantiogriseum* could be considered as a potential source of extracellular lipase production under mild acidic environment.

Key words: fungal lipase, hydrolytic enzyme, *P. aurantiogriseum*, process standardization, screening

INTRODUCTION

Lipases (member of class serine hydrolases) catalyze the hydrolysis of triglycerides under natural conditions to diglycerides, monoglycerides, fatty acids and glycerol at the oil-water interface (Saxena et al 2003a), while under certain experimental conditions like in non-aqueous medium catalyze the reverse reaction of esterification (Saxena et al 2003b). Lipases show a broad range of substrate specificity on a variety of glycerides and non-glyceride esters. Taking into consideration their ability to bring about a diversity of hydrolytic and synthetic reactions in both aqueous and non-aqueous media, and novel and multifold applications in different industrial processes, lipases occupy a prominent place among other industrially important biocatalysts (Saxena et al 2003b; Naqvi et al 2012;Thota et al 2012).

Lipase producing microbes have been reported from a wide range of environments such as industrial wastes, compost heaps, oilseeds, deteriorated food vegetable oils processing factories and dairy products (Sharma et al 2001). Soil contaminated with oils also possesses a diversity of microorganisms producing enzymes. These microorganisms are being exploited for their potential as lipase producers. Although lipases are widely distributed among plants, animals, fungi and bacteria, fungal lipases are being used for diverse biotechnological purposes (Singh et al 2012; Sharma & Kanwar 2014). Amongst these, the filamentous fungi are considered as an ideal source of lipases as they produce an extracellular enzyme. Nonetheless, *Mucor, Penicillium, Aspergillus, Rhizopus* and *Geotrichum* have been established as the most luxuriant sources of enzyme lipase (Singh & Mukhopadhayay 2012; Gopinath et al 2013). Because of their distinctive properties and the ease of mass production as compared to the lipases from plant and animal sources fungal lipases are important for different industrial purposes (Gopinath et al 2013). Lipases have shown their tremendous applications in industries such as oleochemistry, organic synthesis, detergent formulation, nutrition, dairy, textiles, tea and paper (Ghosh et al 1996). The most traditional application of lipases has been flavor development in cheese which has now been extended to a variety of other dairy products and confectionaries like butter, milk-chocolate and sweets which is achieved by selective hydrolysis of triglycerides to release free fatty acids that act as flavor precursors (Sharma & Kanwar, 2014.). Therefore, the importance of fungal lipase research has increased tremendously (Griebeler et al 2011; Thota, et al 2012; Sharma & Kanwar 2014). Realizing the demand of lipases for different industrial uses, which are expected to further increase in the near future, a massive screening programme was initiated in the search of potential extracellular lipase releasing fungi for which samples were collected from a variety of habitats and screened on different media.

METHODOLOGY

The samples were collected from different sites in and around Delhi. These sites include barren land, garden soils and humus, near mother dairy, oil refineries, and also from dung of blue bulls (Nilgai). Soil samples which were not fine enough in texture were crushed down to the extent that it transformed into fine small particles. All the different types of soils were sieved to avoid roots, stones, dry leaves, and other unwanted materials and were air dried. For wet and humid soil the trays were exposed to the sunlight till the time the soil got adequately dried. Ten gram of dried processed soil was mixed with 100 ml of sterile distilled water in a conical flask of 250 ml and was shaken well. This homogeneous soil suspension is referred to as stock suspension. Thereafter, 9 ml of sterile distilled (SD) water was added to a test tube with the help of sterile glass pipette followed by 1 ml of aliquot from the stock suspension. This tube was rolled between the palms to develop a homogenous suspension. Subsequent dilution was processed in a

similar manner by transferring 1 ml from the previous dilution to the next tube containing 9 ml of SD water. This was continued up to 10^{-5} dilution. Half ml of the aliquot from 10^{-5} dilution was spread over the petri plates bearing potato dextrose agar (PDA). Each species of fungi produces a colony that looks different than the colonies produced by other fungal species. Since the soil had the presence of different kinds of fungi, it led to the formation of colonies of different types of fungi on a single PDA petri plate. Each different colony of fungi was point inoculated on different petri plates and slants containing PDA for getting a pure culture of a single fungal isolate. The pH of medium was adjusted by 1N NaOH/1N HCl.

Qualitative estimation of lipase production

To check whether the fungi is producing lipase or not, tributyrin agar plates (TAP) were prepared and subsequently point inoculated with respective fungi. A zone of clearance was observed around the fungal colony due to the hydrolysis of fat, which showed extracellular lipase producing potential by the fungal colonies. Six fungi exhibited the zone of clearance-lipolytic activity hence considered as lipases producers.

Identification of lipase producing fungi

Lipase positive fungi were further point inoculated on PDA slants and incubated in BOD incubator for 96 hours at 30 ℃. Based on the morphological and physiological characteristics these fungi were identified under the expert guidance of scientists of Indian Type Culture Collection, Plant Pathology Division, IARI, New Delhi.

Optimization of culture medium

Four different media for lipase producing fungal isolates were prepared. All these media were inoculated with the loop full of fungal cultures for incubating at 30℃ and 200 rpm. The fungal growth in all media was checked by taking optimal densities at 24, 48, 72 and 96 h of incubation to observe best medium for lipase production. The composition (M) of four different media g/L was as; M1 (KH_2PO_4-0.25, KCl-0.5, $MgSO_4.7H_2O$-0.5, Peptone-8.0, Glucose-8.0, temp- 37℃, pH 8.0 and Sunflower Oil-15 ml/l); M2 ($NaH_2PO_4.2H_2O$- 0.1, $CaCl_2$-0.05, $MgSO_4.7H_2O$-0.25, $FeSO_4.7H_2O$-0.2, KH_2PO_4-20.0, $ZnSO_4.7H_2O$-1.0, $CuSO_4.5H_2O$-0.5, Maltose-30.0, Glycine-2.25, $MnSO_4.H_2O$-0.005, T-aurocholic Acid-0.06, temp 37℃, pH-5.5 and Corn Oil-1.57 ml/l); M3 ($NaNO_3$-2.00, KCl-0.5, $Cu(NO_3)_2$-traces, Glucose -2.0, Casein-1.0, temp-30℃, pH- 7.0 and Corn Oil-20.0 ml/l); M4 (peptone-6.0, Casein hydrolysate-3.0, Yeast extract-0.5, temp 30℃, pH- 6.2 and vitamin stock solution-10 ml^{-1}).

Estimation of lipase activity

For enzyme production, *P. aurantiogriseum* was grown in modified Czapek-Dox medium containing 1.5% olive oil (broth). The cultivation of fungi was carried out at 30^oC for 96 h in 250 ml shake flasks each containing 50 ml of the above mentioned medium. The fungal culture was filtered through 4 or 5 layers of muslin cloth. The filtrate obtained thereafter was centrifuged at 10,000 rpm at 4^oC for 10 min to obtain supernatant, which was considered as crude enzyme. The extracellular lipase activity was measured by Spectrophotometric procedure using p-nitrophenylpalmitate (p-npp) (Winkler & Stuckmann 1979). Freshly prepared 1.2 ml of p-npp solution was incubated while it was shaking in a water bath at 37ºC for 10 min. After 10 min, 0.5ml of crude enzyme sample was added and the reaction mixture was further keep alive at 37ºC in a water bath for 30 min. The formation of yellow color due to release of p-nitro phenol was indicative of lipase activity. To terminate the reaction, 0.1ml of 100 mM $CaCl_2.2H_2O$ was added to the solution. The absorbance of yellow color was calculated at 410 nm against a control (enzyme free). One International Unit (IU) of enzyme was defined as the amount of enzyme, which released 1 μM of p-nitro phenol ml^{-1} min^{-1} under standard assay conditions.

RESULTS AND DISCUSSION

Lipase catalyzes the breakdown of long-chain triglycerides, therefore are being used for various industrial purposes. Considering their importance in industry, it is required to exploration a wide array of microbes for searching new and potential lipase producers as there are several applications of them for a variety of purposes. In the present investigation, 80 fungi were isolated and purified from the samples collected from different types of soils and industrial wastes and animal dung (Nilgai), through serial dilution method. However, out of 80 fungi only six namely, *Alternaria alternata; Penicillium aurantiogriseum; Penicillium pinophilus*; *Aspergillus fumigatus; Penicillium purpurogenum*; and *Penicillium citrinum* were lipase positive (Figure I).

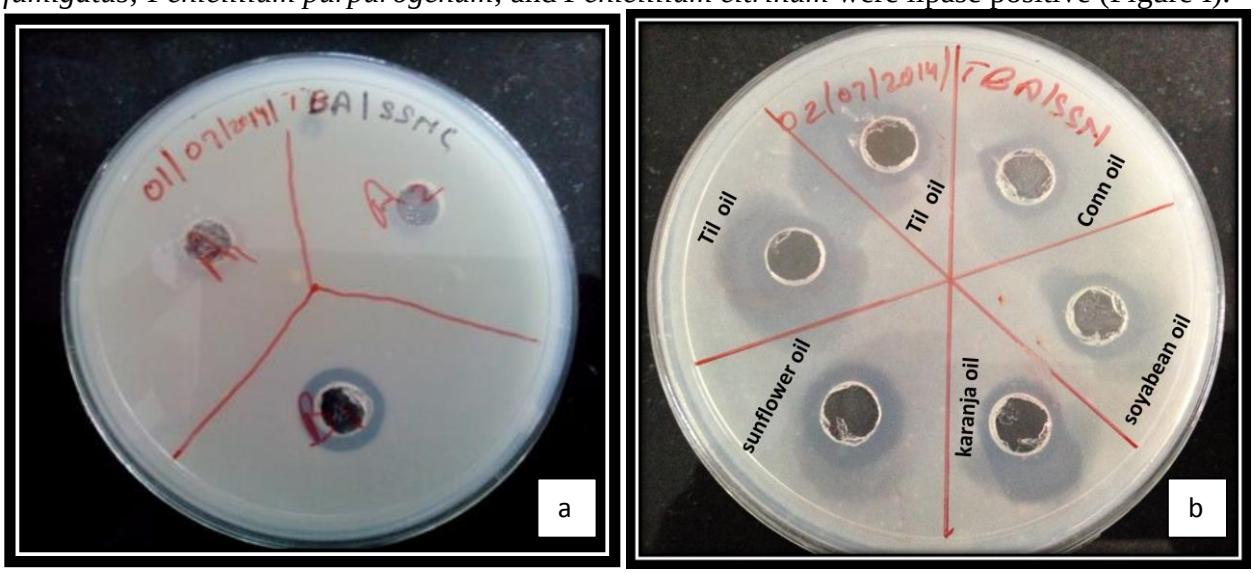

Figure I: Fungi showing zone of hydrolysis on tributyrin agar plates (a) and M4 medium (b) containing different oil sources.

Results of this investigation are consistent with the previous studies demonstrating that lipase producing microorganisms are widely distributed among different soils and agro-industrial wastes (Sharma et al 2011; Thota et al 2012). The study substantiates the fact that different types of soils and/or organic residues can be used for searching new and potent lipase producers.

Massive screening for potential lipase producers on agar containing tributyrin petriplates yielded six fungi with visible halos after 72 or 96 hours of incubation period. The zones of hydrolysis produced by the tributyrin breakdown after 72 to 96 hours of incubation period were due to the presence of lipase released by fungal isolates. The analysis of the results showed *P. aurantiogriseum* (isolate B) to be a potent lipase producer in comparison to the other fungi (isolate A, C, D, E and F) hence was selected for the further course of this study. The halo size (the diameter of the zone of clearance) of this strain increased with the increasing time of incubation and became significantly apparent after 96 hours of incubation.

The influence of nutritional and physiological factors on the production of extracellular fungal enzymes has been reported by a number of researchers. These studies demonstrate that the production of lipase is rigorously influenced by different C and N sources present in the

production medium ((Kiran et al 2008; Immanuel et al 2008; Mahler et al 2000). The requirement of specific C source and its concentration generally differs between microorganisms. Therefore, in present study, the culture medium was supplemented with various C sources and their effects on lipase secretion were examined. Four different media namely M1, M2, M3 and M4 containing different carbon, nitrogen and lipid sources with different pH and temperature were selected to increase the lipase production by *P. aurantiogriseum*. The activity *P. aurantiogriseum* lipase was observed between 24 to 120 hours (Figure II).

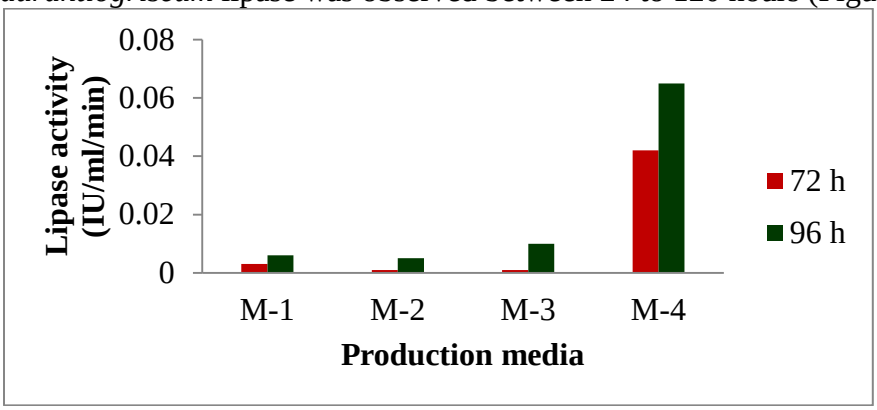

Figure II: *P. aurantiogriseum* (isolate B) showing lipase activity (IU/ml/min) in different types of production media.

The highest enzyme activity was recorded by growing *P. aurantiogriseum* on M4 production medium compared to the M1, M2 and M3 media. The lipase activity drastically decreased on M2 and M1 media followed by M3 medium. Five different oil (til oil, corn oil, sunflower oil, karanji oil and soybean oil), four carbon (glucose, lactose, maltose and sucrose) and four nitrogen (beef extract, ammonium acetate, tryptone, corn step liquor) sources were applied to find out the best source of oil, C and N to develop a process for greater production of lipase enzyme (Figure III, IV, V).

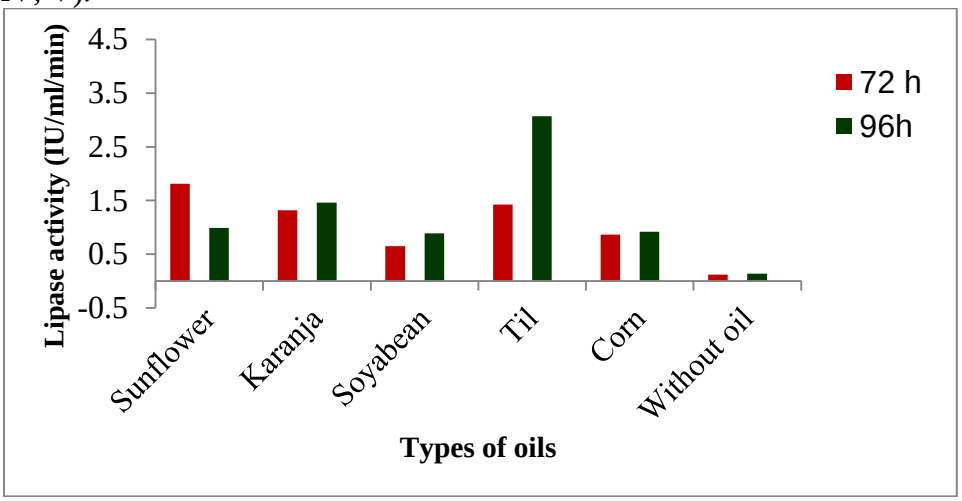

Figure III: *P. aurantiogriseum* (isolate B) showing lipase activity (IU/ml/min) in production medium M-4 supplemented with different types of oil sources.

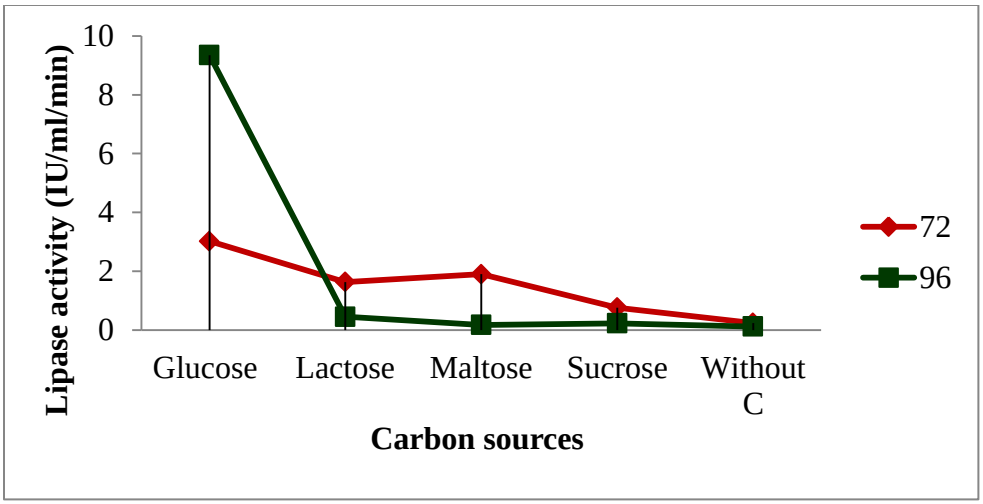

Figure IV: *P. aurantiogriseum* (isolate B) showing lipase activity (IU/ml/min) in production medium M-4 supplemented with different types carbon sources.

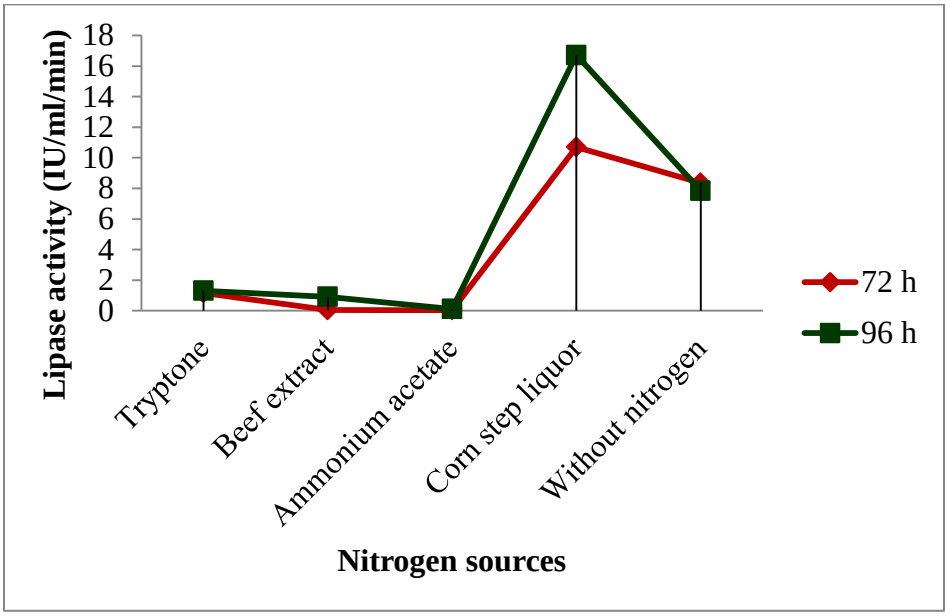

Figure V: *P. aurantiogriseum* (isolate B) showing lipase activity (IU/ml/min) in production medium M-4 supplemented with different types nitrogen sources.

The highest enzyme activity (3.072 IU ml^{-1} min^{-1}) was recorded when M4 medium was supplemented with til oil. However, the activity of lipase further increased in response to supplementation of the culture medium with glucose as a source of carbon (9.352 IU ml^{-1} min^{-1}) and corn step liquor as a source of nitrogen (16.718 IU ml^{-1} min^{-1}) after 96 h of incubation period (Figure IV). In the present investigation, greater lipase activity was found at pH 6.8 and temperature 30°C after 96 h of incubation period (Table I).

Table I: Parameters optimized for the increased production of lipase by *Penicillium aurantiogriseum* .

Parameters	Optimized condition

Temperature	30ºC
pH	6.8
Incubation time	96 h
Nitrogen source	Corn step liquor
Carbon	Glucose
Oil	Til
Initial yield of lipase	0.065 IU/ml/min
Final yield of lipase	16.718 IU/ml/min

These results substantiate the fact that the optimal production of lipase depends on the pH, temperature, incubation period as well as sources of C, N and fats (Mahler et al 2000; Naqvi et al 2012; Thota et al 2012).

CONCLUSION

Results of the present study conclude that amongst the six different lipase producing fungi; *P. aurantiogriseum* is a potent lipase producer. The fungus demonstrates its best potential at pH 6.8 and temperature 30^{o}C along with glucose as a source of carbon, corn step liquor as nitrogen and til oil as lipid. Thus, *P. aurantiogriseum* may be recommended for its exploitation for a variety of industrial purposes.

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