SCREENING OF LIPASE PRODUCING FUNGI AND ITS APPLICATION

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ABSTRACT: : *The present study is to highlight on screening of lipase producing fungi isolated from oil spilled soil by serial dilution technique. 30 fungi were isolated in the primary screening using potato dextrose agar at 28±2˚C after 3 d of incubation. Lipase production was carried out using minimal salt agar medium containing olive oil and waste engine oil as the sole source of carbon at 28±2°C for 5 d on rotary shaker. Out of 30, 25 fungal isolates showed zone of oil degradation on phenol red agar medium containing 1% olive oil. Most efficient lipid degrading fungi were further proceed for quantitative estimation by spectrophotometric method [420 nm] using oleic acid as a standard. Proficient lipase producing fungi was identified as Penicillium sp. on the basis of morphological characteristics and showed lipase activity 91.80 ±1.2U/ml. The present considered lipase has the application in Biofuel production.*

Key Words: *Lipase, oleic acid, minimal salt medium, phenol red medium*

Introduction:

The use of enzyme mediated processes can be seen from primordial civilisations. Today about 200 enzymes are in commercial use out of nearly 4000 known enzymes. The widely held industrial enzymes are of microbial origin. Lipases are well thought-out to be the third largest group based on total sales volume after protease and carbohydrates. Lipases have the capability to carry out hydrolytic and synthetic activity in both aqueous and non-aqueous media (Lanka,2017). Lipases (triacylglycerol acylhydrolases; EC3.1.1.3) are class of hydrolytic enzymes which act on the carboxylic ester bonds to liberate fatty acids and glycerol. They are serine hydrolases (Sharma,2001). Lipase producing microbes have been found in diverse sources such as agro industrial waste, vegetable oil processing factories, dairy plants and oil spilled soil (Bharathi,2018). Lipase producing fungi are present on an extensive range of substrates in the ambient surroundings and these results could also provide crucial data for supplementary investigations on fungal extracellular enzymes (Kumar,2012).

Diverse types of lipases are produced by taxonomically close strains. Some lipase producing microorganisms are *S.aureus, B.megaterium, P.aeruginosa, Rhizopus, Aspirgillus, C.rugosa, and Streptomyces.* While screening fungi for lipase production both culture pH, and assay pH are chief parameters. The permanence depends upon the charisma of substrate (Anderson,1979). Novel biotechnological applications have been productively recognized using lipases for the synthesis of biopolymers and biodiesel, the manufacture of pharmaceuticals, agro-chemicals, and flavour compounds (Jaeger and Eggert,2002).The purpose of present study is to isolate most proficient lipase producing fungi and its role in biodiesel production in greater perceptive of previously discovered enzymes.

Materials and Methods:

3.1 Chemicals: - Potato dextrose broth, Phenol red, Oleic acid was purchased from Himedia laboratories, Mumbai and all other reagents used was of analytical grade.

3.2 Isolation of fungi: -

The lipase producing fungi was isolated from oil spilled soil by serial dilution technique on potato dextrose agar medium for 3-5 d at 28± 2ºC. The lipase production was done by using a minimal salt medium containing olive oil and waste engine oil as sole source of carbon. The medium contain (g/l), KNO_3 (2.5), KH_2PO_4 (1), $MgSO_4$ (0.5), NaCl (5) and carbon source (15) with pH of 7. The oil is emulsified in the Tween 20, sterilizes separately then added to medium and incubates at 28± 2ºC for 3-5 d on shaking condition (Lanka,2017).

3.3 Screening of lipase producing fungi on Phenol red agar medium and its identification: -

Lipase production by selected fungal isolates was analysed on phenol red agar medium (Phenol red 0.01%, $CaCl_2$ 10mM, olive oil 1% and agar 2%) of pH 7.3±2. After solidification of sterilized medium, the 6mm well was filled with 100µl of culture filtrate (crude lipase) and the plates were incubated at 37ºC for 24 h. The

circular zone (yellow) appeared around the well indicates lipase production by isolates (Lanka,2017). The most efficient lipase producing fungi were further preceded for identification by morphology, pigments produced and wet mount (Wiley).

3.4 Quantitative estimation of lipase: -

Quantitative estimation of crude and standard lipase was determined by modified method of Degrassi (1999), spectrophotometrically at 420nm by using oleic acid (1%) as a substrate in 1M phosphate buffer of pH 7.8 and phenol red as an indicator, incubated in boiling water bath for 30 min at shaking condition. One unit of lipase activity was defined as the amount of enzyme which produces 1µmol of fatty acids per minute under assay conditions. Experimentation was carried out in triplicates.

3.5 Optimization parameters for lipase production:

3.5.1 Effect of inoculums and substrate: -

The effect of inoculums was evaluated by adding different inoculums 6mm disc like 1disc, 2disc, 4disc, 6disc to minimal salt medium, and the effect of substrate was studied by adding the substrate (olive oil) in different concentrations like 0.5%, 1%, 1.5%, 2% in minimal salt medium, incubate at 28± 2ºC for 3-5 d in shaking condition (Ghori,2011),media was further centrifuged and the supernatant was assayed. Testing was carried out in triplicates.

3.5.2 Effect of temperature and pH: -

For the study of effect of temperature the minimal salt medium containing culture incubated at different temperatures and pH like 10 ºC, 30 ºC, 37 ºC, 40 ºC; 4,5,6,7,8 and 9 respectively, incubate at 28± 2ºC for 3-5 d in shaking condition (Ghori,2011), media was further centrifuged and the supernatant was further proceed for assay. Experimentation was performed in triplicates.

3.5.3 Effect of metal ions and inhibitors: -

The different metal ions(0.1mM) Mn, Mg, Ca, Zn, Co, Cu, Na, Pb, Hg, DMSO, EDTA, PMSF, and Mercaptoethanol(1%) were added in the minimal salt medium and incubate at 28± 2ºC for 3-5 d in shaking condition and calculate relative activity as compared to control (Ghori,2011).

3.6 Partial purification of lipase: -

The crude lipase was partially purified by 50% and 80% ammonium sulphate precipitation, dialyzed overnight against the same buffer and then concentrated on sucrose. Total and specific enzyme activity of each fraction (50% and 80%) was analysed using standard method as described by Jayaraman.

3.7 Molecular weight determination by SDS-PAGE:-

Molecular weight determination was carried out by Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE). In order to determine molecular weight, the partially purified enzyme and known molecular weight markers (MolBio Himedia, broad range 10-245 kDa) were subjected to electrophoresis. SDS-PAGE was performed with 12% polyacrylamide gels and the protein bands were stained with a solution of Coomassie blue R-250. The molecular weight of purified lipase was estimated as described by Sambrook.

3.8 Biodiesel production from lipase: -

As per the modified method of Bueso (2015) the pre-warmed (50ºC for 20 m) raw material (waste engine oil, cotton seed oil, waste cooking oil) were mixed with 45% methanol in the ratio of 3:1 and crude lipase was added as catalyst. Then the mixture was kept for transesterification reaction at 50ºC for 2 h. After the reaction, the mixture was poured in separating funnel and allowed to separate for 6 h, two layers were observed, upper layer contains biodiesel and lower layer contain glycerol.

Result and Discussion: -

4.1 Isolation and identification of fungi: -

Out of 30 fungal isolates 20 were showing lipase activity on phenol red agar medium as mentioned in table no.1, Fig.1. The 5 proficient lipase producing fungi were identified as *Penicillium sp, Aspergillus sp, Alterneria sp, Rhizopus sp, Peciliomyces sp* by morphology, pigment produced and wet mount. The fungal species reported previously to produce lipase was *Trichoderma sp.* (Kumar,2012) . Lanka (2017) in their research used 5 dominant fungi out of 15 isolates from marine water for enzyme study. In present work most efficient fungi, *Penicillium sp.* (Fig.2) was further proceed for quantitative and optimisation study.

Table. 1 zones of lipase on phenol red agar medium

Colony	Circular zone in cm	Colony	Circular zone in cm
F2	5	C1	5
A2	5	B2	4.5
F1	4.5	A4	4.5
C5	3.9	B3	4.5

E2	3.5	D2	4
F3	3	B1	3
D4	2.2	C2	3
D5	2.2	E2	2.5
E3	2.2	A1	2.5
E1	1	D1	1.5

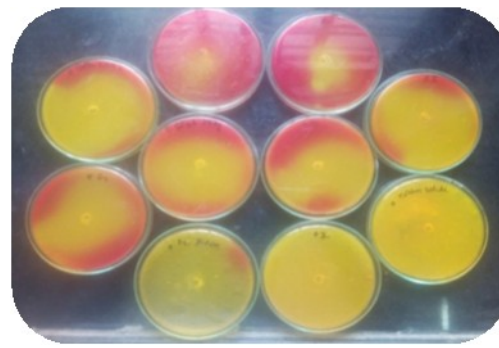

Fig.1 zone of lipase on phenol red agar medium

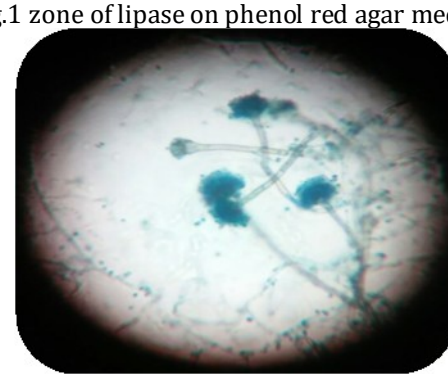

Fig.2 *Penicillium species*

4.2 Quantitative estimation of lipase: -

Quantitative lipase activity was determined by using 1% oleic acid by spectrophotometrically at 420 nm. The lipase activity found to be 91.80± 1.2 U/ml (Fig.3). Degrassi (1999) in their work the esterase activity found to be 12 U/ml by using p-nitrophenyl acetate as substrate at 420 nm on spectrophotometer.

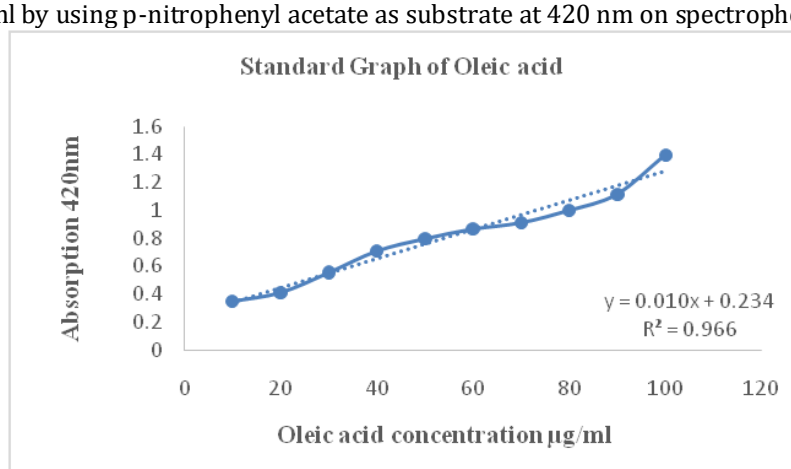

Fig.3 standard graph of oleic acid

4.3 Optimization parameters for lipase production:

4.3.1 Effect of inoculums and substrate: -

In the investigation, different inoculum disc of 6mm size and substrate concentration were tested for the production of lipase. The production was found to increase with increasing No. of inoculums and substrate

concentration at 4 discs and 2% (Fig.4, 5). Further increase in the inoculums no. and substrate concentration, decreased the production which might be due to rapid growth of fungi and depletion of essential nutrients in the early stages.

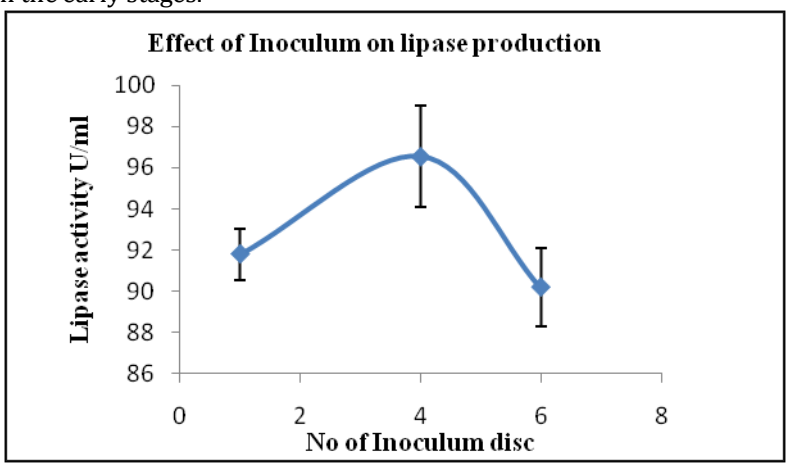

Fig.4 effect of inoculum on lipase production

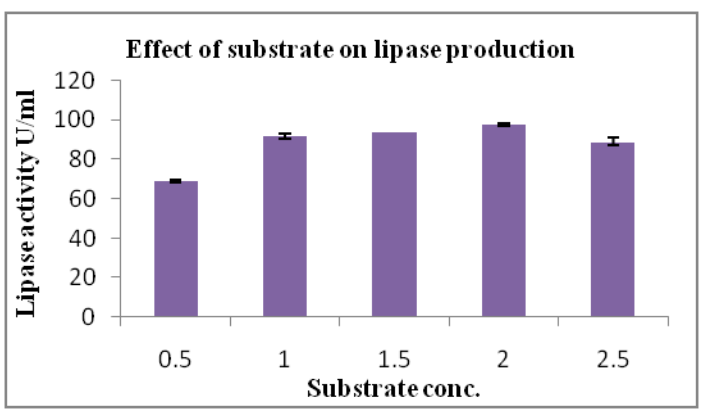

Fig.5 effect of substrate concentration on lipase production

4.3.2 Effect of temperature and pH: -

The optimum activity of lipase enzyme was found at pH 6.0 alongside temperature of 30 °C (Fig.6, 7). The lipase from *Bacillus sp.* previously reported by Ghori (2011) has optimum temperature of 60°C and pH 9. Degrassi (1999) for yeast found optimum temperature as 50°C with neutral pH (7.0). Maximum production takes place when a suitable pH in culture media is maintained. Further increase in temperature and pH beyond the optimum caused a speedy decrease in enzyme action.

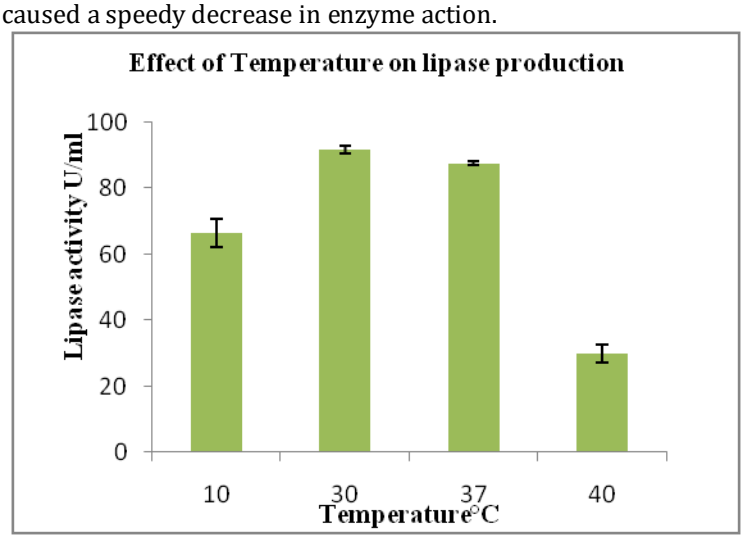

Fig.6 effect to temperature on lipase production

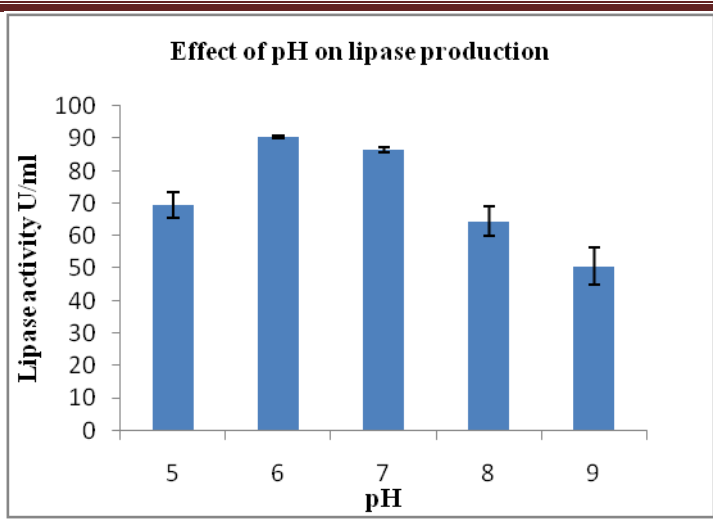

Fig.7 effect of pH on lipase production

4.3.3 Effect of metal ions: -

Relative activity of lipase by *Penicillium sp.* was found to be reduced in presence of EDTA, Cu, Hg, Co, Pb, DMSO, Mn, Mg and Zn ions while strongly inhibited by PMSF indicating it belongs to serine proteases. However, its activity was strongly enhanced by Ca^{2+} ions (Fig.8). Similar result was obtained in case of thermophilic *Bacillus sp.* RS-12 by Sidhu (1998). Previous studies by Sharon (1998) prove Mg^{2+} ions to enhance the lipase activity at 0.8 M concentration in *P. pseudoalcaligenes*.

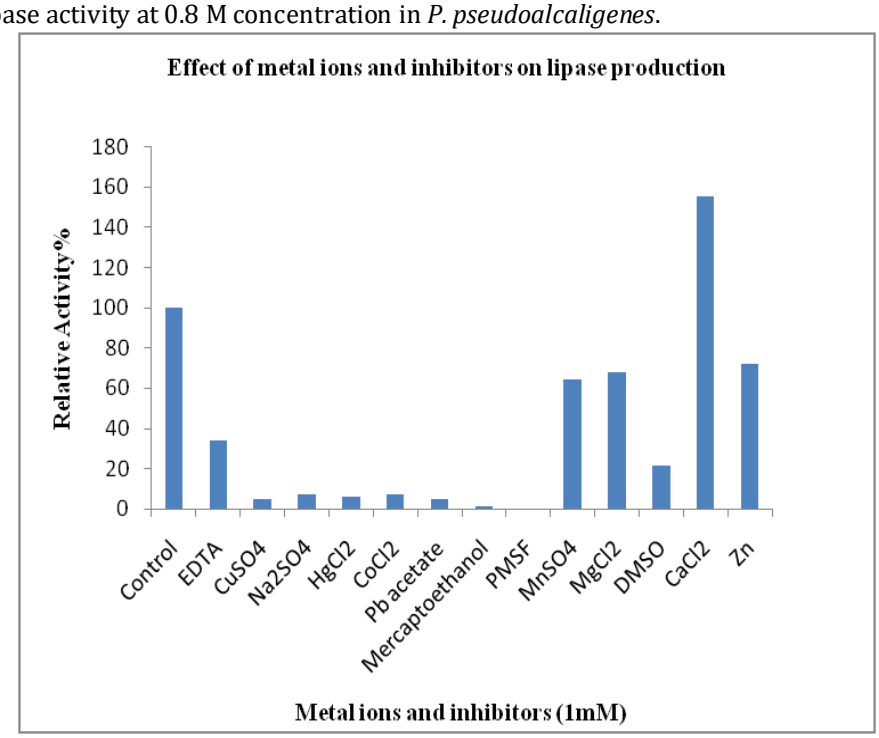

Fig.8 effect of metal ions and inhibitors on lipase production

4.4 Statistical Analysis:-

All the experiments were carried out in triplicates and the data were evaluated by analysis of variance (ANOVA).

4.5 Molecular weight determination by SDS-PAGE:-

The molecular weight of partially purified lipase was found to be 28 kDa and 45 kDa (Fig.9). Bharathi (2018) in their research stated the molecular weight of lipase to be 32-47 kDa.

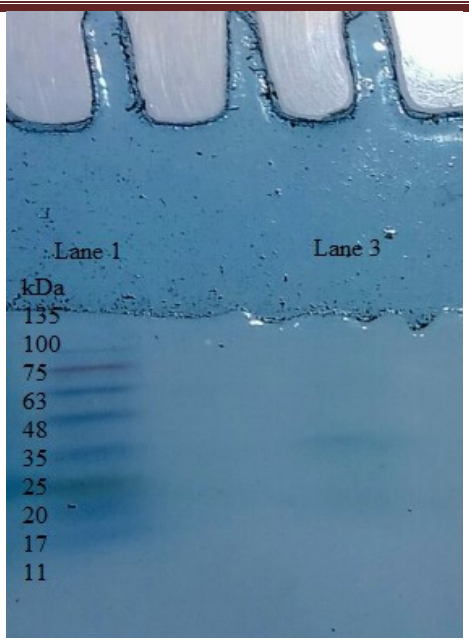

Fig.9 SDS-PAGE analysis

4.6 Biodiesel production from lipase: -

Biodiesel was successfully produced from waste engine oil, cotton seeds and waste cooking oil through transesterification process by using *Penicillium* fungal lipase as a catalyst. Crude biodiesel was purified by column chromatography using activated charcoal. The effluent was collected and used as biodiesel (Fig.10). From results of study by Bueso (2015), biodiesel was produced from jatropha by using lipase as a catalyst.

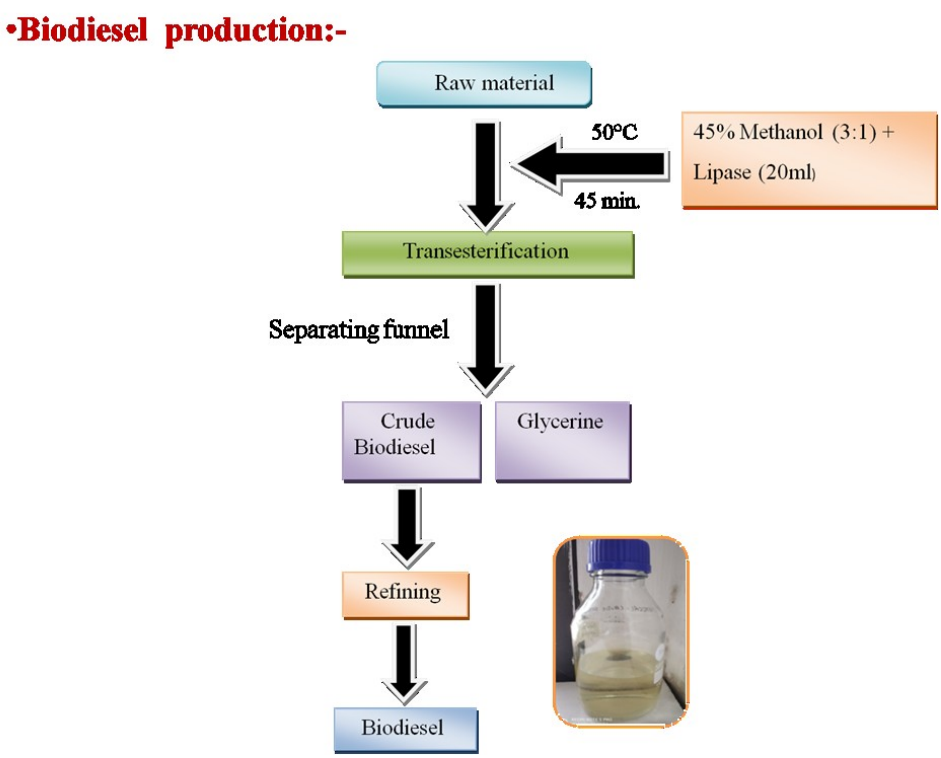

Fig.10 biodiesel production

Conclusion:

20 fungi showed lipase production. Lipase activity was confirmed by phenol red agar medium. The most efficient lipase producing fungi was found to be *Penicillium sp.* The isolates showed 91.80 ± 1.2 U/ml lipase activities using 1% oleic acid as substrate. By optimization evaluation, optimum lipase activity was found at

30ºC temperature, 6 pH, 2% substrate concentration with 4 discs inoculum. The *Penicillium sp.* fungal lipase is a class of serine protease which is inhibited by PMSF. The biodiesel was successfully produced by using a fungal lipase. The cost price of biodiesel is 50 Rs.

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