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Production of Organic Solvent Tolerant lipase from *Bacillus* SRR-11

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Abstract

Lipases are vital industrial biocatalysts used in number of diverse fields like detergent, textile, oleo-chemical and food processing industries. They have the potential to hydrolyse triacyl glycerol to glycerol and fatty acids in aqueous condition. In non aqueous medium product recovery and substrate solubility can be increased by using different organic solvents. As we know in the presence of organic solvents almost enzymes deactivated, but if we isolate a strain which can produce an enzyme which can retain its activity in any harsh condition (in the presence of organic solvents), that enzyme could be an exceptional biocatalyst for the same. Consequently, bacterial strain was isolated and screened which could grow, multiply and produce an enzyme in solvent containing nutrient amended medium. This organism was also checked for its potential to produce lipase. The isolate Bacillus SRR-11 is a Gram positive rod, producing lipase in the medium comprised of 40% (v/v) solvents viz. benzene, methanol, butanol, acetone, isooctane, toluene and xylene. The log P value of all these solvents is less than 2.5. In spite of this lower log P value, the potential of lipase persists for 72 h in the presence of these organic solvents. As per our knowledge scanty reports in the literature so far are that of the solvents with log P values lower than 2.5. The isolate showed lipolytic activity at pH range of 6.0 to 9.0. Optimal lipase production was obtained at 35±2°C under shaking condition. Effect of different oils (ground nut oil, cotton seed oil, castor oil, sun flower oil, olive oil) on lipase production was also checked. Maximum lipase production was 32.26 U/ml with groundnut oil. Effect of different metal ions was checked with Mg^{2+}, Mn^{2+}, Fe^{3+}, Ba^{2+}, EDTA, Ca^{2+} enhancing the lipase activity. While Na^{+} and Cu^{2+} has negative effect.

Keywords: Lipolytic activity, organic solvent, tolerance, biocatalyst.

Introduction

Triacyl glycerol hydrolases (EC 3.1.1.3) are universal hydrolytic and industrially significant enzymes having demand in different fields like, fat-oil transformation, pharmaceuticals, lipophilization of primary compounds and phenolic compounds, detergent, surfactant production, cosmetics, agrochemicals, biodiesel production and bioremediation of oil contaminated sites[1-5]. They have the potential to hydrolyse triacylglycerol to glycerol and fatty acids in aqueous condition, in addition to esterification, transesterification, stereospecific hydrolysis of racemic esters and synthetic reactions under non aqueous environments[1,3,6,7]. Currently, as many enzymatic reaction media used, contain organic solvents, it requires the enzyme that have the ability to maintain its functionality in non aqueous environment. As we know many solvents affect the structure and efficiency of the cells[8], it is known to be toxic to most bacteria[9,10]. Despite this, solvent resistant bacteria reveal definite mechanisms to conquer the lethal effect of the solvents; they have solvent exclusion force, repairing mechanism, lower hydrophobic outer membrane, lower cell porosity and higher membrane inflexibility.

Initially, *Pseudomonas putida* IH-2000 was discovered by Inoue and Horikoshi that was able to grow in 50% toluene containing medium[11]. This strain produced the enzymes that can tolerate the solvent containing environment[12]. Ogino et al. studied that lipase activity of *P. aeroginosa* LST-03 was increased in solvent (acetone, ethanol, toluene and cyclohexane) containing medium[13]. After that research was started to isolate solvent resistant bacteria that can produce lipase; mostly from *Pseudomonas* and *Bacillus*. Bacillus was isolated and check for lipolytic activity. In this study, lipolytic activity, optimization and its stability at different temperature, pH, in presence of chemical inhibitors and organic solvents was studied.

Material and Methods

Oil contaminated soil samples were collected from auto mobile garage from Ahmedabad, India[14]. 1 gram oil contaminated soil sample was added in 10 ml sterile distilled water, followed by vortex. 100 µl of supernatant was transferred on tributyrene agar (Himedia India) plates consisting of (g/L): 0.5 tri-n-butyrin, 1.0 Ammonium sulphate, 0.35 Dipotassium phosphate, 0.1 Monopotassium phosphate Sodium chloride, 2.5 NaCl , 0.05 Magnesium sulphate $MgSO_4$-$7H_20$, and 15 agar adjusted to pH 7.5[15,16]. The plates were kept at 37 °C for 2 days. Colonies with zone of hydrolysis around them were isolated. The purified isolate with largest zone was identified using the 16S rRNA sequencing. All other chemicals used in this study were of AR grade. Before use all the solvents passed through 0.45 µm pore size membrane filter[17]. For estimating lipase production and to perform enzyme assay centrifugation was carried out at 10,000 g for 10 min.

One single colony of pure culture was transferred to 50 ml sterile YPDH broth to prepare inoculum. YPDH medium having (g % w/v): yeast extract (1.0), peptone (1.0), dextrose (1.0) and olive oil (1 ml (v/v)) at pH 7.0 and incubated for 24 h in orbital shaking condition rotating at 150 rotation per minute at 35±2°C[18,19]. The liquid culture thus obtained was used as inoculum. This medium was also used as production medium. Solvent tolerance was determined by inoculating the culture in YPDH medium with 40% solvents eg; benzene, toluene, butanol, acetone, methanol, xylene and isooctane in screw capped tubes for 24 h. After that 100 µl culture was spread over nutrient agar medium. Allow to incubate at 35±2°C for 24 h. Isolate SRR-11 was grown in YPDH medium, at the same conditions previously shown. At every interval of 24 h, 5000 µl of fermentation broth was recovered centrifugation was performed at 10,000 g, at 4°C for 20 min and that supernatant was used to determine lipase activity[20]. During the hydrolysis liberated free fatty acids were measured[21]. To determine lipase activity olive oil was added for hydrolysis. 500 µl enzymes was mixed with 1250 µl olive oil, and 20 mM of 10 µl calcium chloride. Than the enzyme was allowed to react for 5 min, 150 rpm at 35±2 °C. As we know in highly acidic environment enzymes lose their activity, we have added 500 µl 6 N HCl. Generally fatty acid dissolves in solvents, 2500 µl isooctane was added in the reaction mixture. Due to less density isooctane get separated in upper layer, 2000 µl was collected in another test tube. In the same tube 500 µl cupric acetate pyridine (5% w/v) was added. The absorbance was recorded at 715 nm against isooctane as blank, FFA was determined. From the oleic acid standard curve lipase activity was measured. One unit of lipase activity (U) can be defined as the enzyme, require liberating 1 µM of FFA in 1 min at 37ºC.

The production of lipase and its optimization by the isolate was achieved by performing a variety of parameters viz. agitation, temperature, pH, different oils as substrates, carbon sources and nitrogen sources. All the parameters were run in triplicate in Erlenmeyer flasks having capacity of 250 ml contain 50 ml medium. If otherwise mentioned YPDH medium was used of 7.0 pH, 120×10^8 cells/ml were inoculated as inoculum and incubated on shaker at 35±2ºC temperature[22]. To study the influence of agitation one set of flasks were incubate under static condition. Effect of incubation temperature was studied at 15±2, 25±2, 35±2, 40±2 and 50±2ºC. Initially pH of the production medium was kept to 4, 6, 7, 8, 9 with 0.5 N NaOH or 0.1 N HCl[23]. To determine the influence of inducers in production medium olive oil was substituted with different oils eg; ground nut oil, cotton seed oil, castor oil, sun flower oil, tributyrene, mustard oil. To know the effect of carbon source (glucose, fructose, maltose, lactose and sucrose) and nitrogen source (ammonium nitrate, ammonium chloride, peptone, tryptone, urea and yeast extract) were checked. After every 24 h of incubation sample was withdrawn and lipase activity was assayed. Na^{+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ba^{2+}, and EDTA ions were mixed with the crude enzyme at a concentration of 1000 µM, and were allow to stand at 37ºC at neutral pH for 60 minutes under static conditions. After that residual activity was determined, weather metal ions affect lipase activity or not[24]. Various concentrations of Ca^{2+} were added in reaction mixture and its effect on lipase activity was checked. Benzene, toluene, isooctane, butanol, xylene, methanol and acetone were added (40% v/v) with crude enzyme, incubated for 60 min after that residual activity was checked.

Results and Discussion

Isolation was performed using Tributyrene agar plates having 1% tributyrene oil. Among the isolates, isolate SRR-11 showed the highest zone of hydrolysis was selected and was identified as *Bacillus licheniformis* SRR-11 through 16S rRNA sequencing.

Bacillus licheniformis SRR-11 shows tolerance in presence of benzene, toluene, butanol, acetone and methanol having log P value of less than 2.5. Upto 40% solvent concentration this bacterium shows visible turbidity as well as growth when streaked over tributyrene agar plates.

Temperature affects the growth as well as production of lipase as shown in figure-1. Generally it is found that optimum temperature for lipase production was between 20-45˚C. It can be seen in fig.1 highest amount of lipase was produced at 35±2°C which was 28.42 U/ml[5].

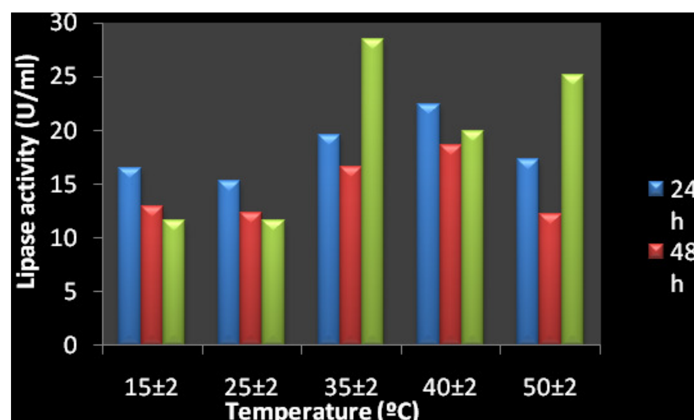

Figure-1
Effect of temperature on lipase production

After temperature aeration is also one of the significant factor which affect the availability of oxygen and maintain the level of oxygen which is directly related to respiration of living cells, as it get decreases it will affect the growth and simultaneously the enzyme production[25]. It was studied by providing static and shaking (150 rpm) conditions during incubation and the obtained data is shown in figure-2. It was found that 1.33 fold higher lipase activities was attained at 37 °C when medium was agitated with the speed of 150 rpm giving 22.45 U/ml as compared to the static condition where only 16.42 U/ml of production was achieved at 37°C. This confirmed that the

metabolic pathway for lipase biosynthesis require oxygen[25]. But it is not necessary that all the bacterial strains require same amount of oxygen, it varies with different bacterial strain. As for *Bacillus* sp. lipase production was lower at 100 rpm as compared to 300 rpm[26], whereas *Rhizopus clinensis* produced highest amount of lipase at 200 rpm[27]. pH of the medium play a vital role in functioning of different molecules, their charges and consequently their interactions. At each and every step starting from the nutrient uptake, development of bacteria, switch on the proper pathway for enzyme production and finally to liberate the extracellular enzymes initial pH is very significant[28]. During the growth if the pH get change it may affect the enzyme stability[18].

The change in pH during growth of the organism may have an effect on the stability of the enzyme in the medium[18]. *Bacillus licheniformis* SRR-11 was allow growing at pH 4.0-9.0 in YPDH medium. Highest lipase activity of 28.59 U/ml was obtained when the pH of the medium was 6.0 at 37°C as shown in figure-3[18]. Lipolytic activity was found to have a maximum value at pH 6.0 and 37ºC[18]. Mainly, most of the bacteria grow best and produces maximum lipase at pH 7.0, which is reported in the case of *Bacillus* sp., *Acinetobacter* sp. and *Burkholderia* sp.[29]. On the other hand, in many cases lipase production was reported higher at alkaline pH. As pH of the medium goes down to acidic side lipase activity was decreased comparatively.

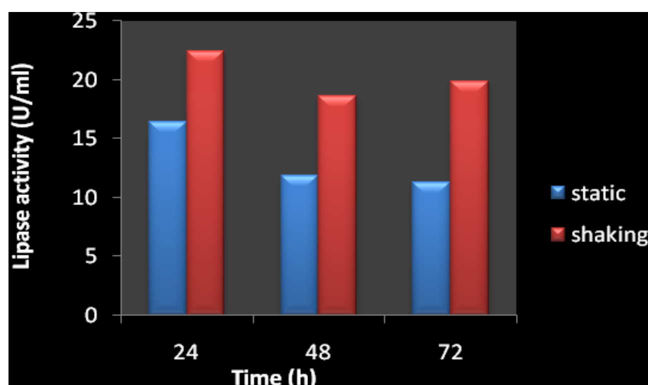

Figure-2
Effect of agitation on lipase production

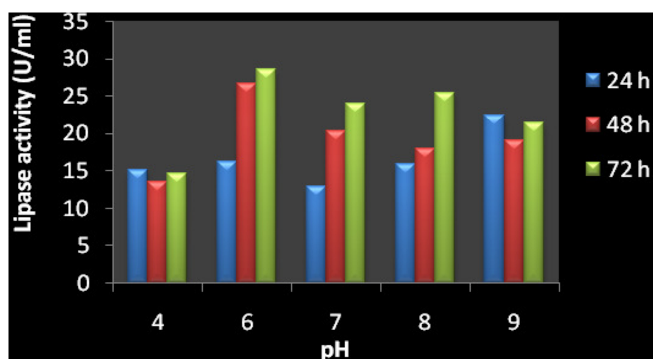

Figure-3
Effect of pH on lipase production

Raising the pH of the medium from 5.5 to neutral there was a considerable reduction in production of enzyme while declining the pH to more acidic have not a drastic change in activity[5].

Fairly rapid decline in the reaction velocity on either side of pH 6.0 and 8.0. Apparently, the fall on the alkaline side was may be due to the destruction of the enzyme, whereas the fall on the acidic side was may be partly due to a diminishing attraction of the lipase for its substrate and partly due to an irreversible destruction of the enzyme protein[30,31]. Maximum 18.40 U/ml and 24.78 U/ml lipase was produced in the occurrence of 2% fructose (figure-5) and 3% urea (figure-4) respectively in the production medium[32].

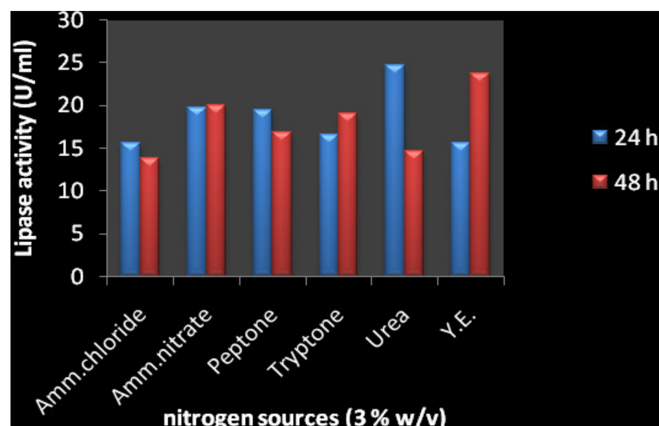

Figure-4
Effect of nitrogen source on lipase production

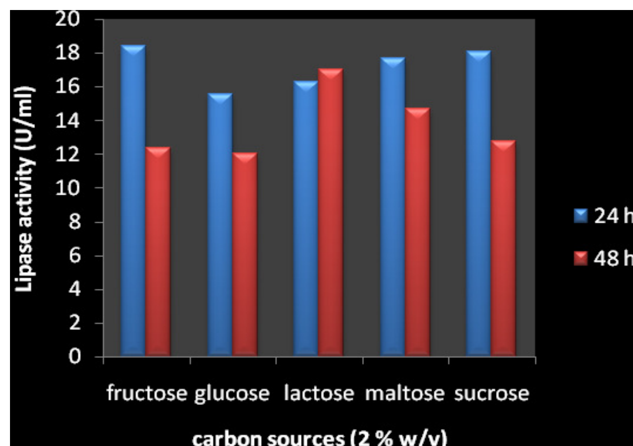

Figure-5
Effect of carbon source on lipase production

Among the various oils checked, groundnut oil gave highest lipase production of 32.26 U/ml where as olive oil, sunflower oil, caster oil, cotton seed oil, soybean oil, mustard oil resulted in 29.92, 21.02, 26.85, 27.52, 29.66 and 22.66 U/ml of lipase production[33] (figure-6). It was seen that Na^+ and Cu^{2+} decreased residual activity (96.43%) and (81.09 %) respectively, where as Ca^{2+} (129.07%), Mg^{2+}(129.10%), Mn^{2+} (133%), Ba^{2+}(126.27%), Fe^{3+}(121.71%) and EDTA (119.54%) increased the residual activity significantly as compare to control where no metal ion

was incorporated in the system (figure-7). Ca^{2+} (>130%) activated enzyme, while Na^{+} (102.9%) stimulated *H. pylori* EstV enzyme[34]. Calcium was also found to be enzyme activator by some other workers[35-38]. Some workers also found it as inhibitory[30,34-40]. These ions may have negative effect on lipase, it may cause blockage on the catalytic site[40]. These positively charged radicals have tendency to react with free fatty acids which have been attached to the droplets and thus enlarge their surface area[42,43]. The concentration of Ca^{2+} showed influence on lipase activity upto 2mM after that it shows reducing affect on lipase activity. In presence of 40% acetone, benzene, butanol, methanol, xylene, toluene and isooctane the lipase showed 47.5, 82.5, 57.5, 62.5,100, 112.5 and 70 % activity (figure-9).

Conclusion

The isolated lipolytic bacterium was identified as *Bacillus licheniformis* SRR-11 and screened for organic solvent tolerant lipase production. This bacterium shows tolerance in presence of benzene, toluene, butanol, acetone, methanol having log P value of less than 2.5. Upto 40% solvent concentration this bacterium shows visible turbidity as well as growth when streaked over tributyrene agar plates. In this study, the organic solvent tolerant SRR-11 produced mximum amount of lipase under shaking conditions for about 72 hours of incubation at 35±2 ºC and pH 6.0. Maximum 18.40 U/ml and 24.78 U/ml lipase was produced in 2% fructose and 3% urea containing production medium. When different oils were checked for their inducing effect highest amount of lipase was produced in ground nut containing medium. Metal ions like Ca^{2+} (129.07%), Mg^{2+}(129.10%), Mn^{2+}(133%), Ba^{2+} (126.27%), Fe^{3+} (121.71%) and EDTA (119.54%) showing positive response, while Na^{+} and Cu^{2+}(96.43%) and (81.09 %) showing decrease in lipolytic activity. The concentration of Ca^{2+} showed influence on lipase activity upto 2mM after that it shows reducing affect on lipase activity. In presence of 40% acetone, benzene, butanol, methanol, xylene, toluene and isooctane the lipase showed 47.5, 82.5, 57.5, 62.5,100, 112.5 and 70 % activity. Due to the tolerance to solvent this bacterium could be useful in industry.

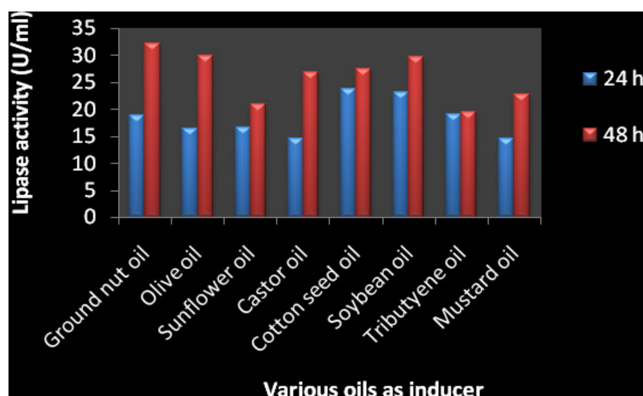

Figure-6
Effect of various oils as inducer on lipase production

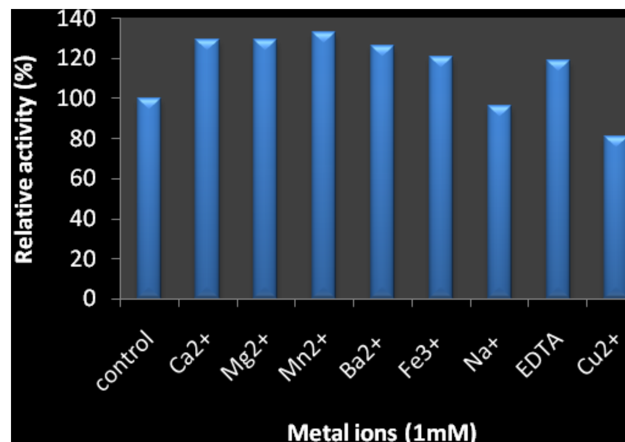

Figure-7
Effect of Metal ions on lipase activity

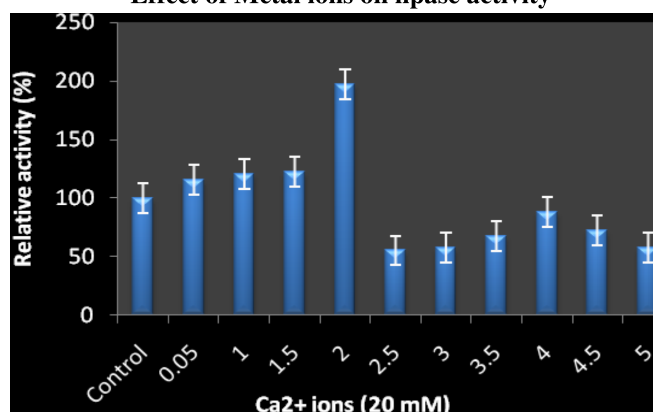

Figure-8
Effect of Ca^{2+} ions on lipase activity

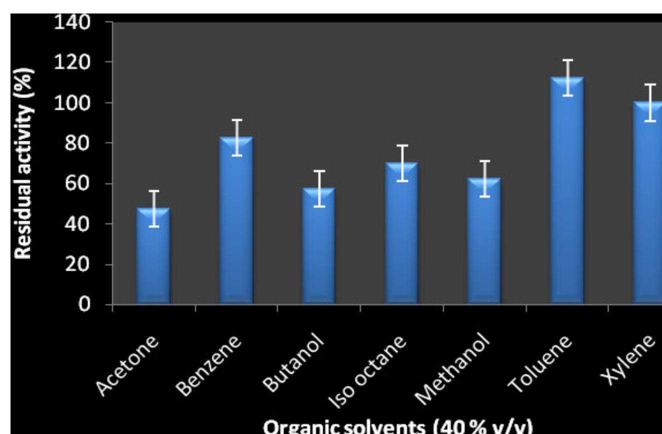

Figure-9
Effect of organic solvents on lipase activity

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