

## Effect of culture conditions on growth and extracellular lipase production by *Sporosarcina* sp. isolated from oil extraction mill soil

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*Sporosarcina* sp. was isolated from oil extraction mill soil by enrichment culture technique that had the ability to produce lipase enzyme. Culture conditions of *Sporosarcina* sp. were optimized at shake flask level. Maximum enzyme production was achieved in between 96h and 120h of cultivation. The rice bran oil was found to be the most suitable lipid for enzyme production (8.52U/ml) among coconut oil, olive oil, refined oil and mustard oils. The enzyme had temperature optima at 37°C and its peak activity was at pH 4.0. Among different nitrogen sources, ammonium sulphate was found to be the most suitable nitrogen source at which it showed the highest activity (10.41U/ml). Potassium dihydrogen phosphate enhances the enzyme production as an inorganic salt into the medium. Tween 20 was used as biosurfactant which increases the enzyme titer to 1.5 times.

**Keywords:** Lipase, *Sporosarcina* sp., enzyme assay, nutritional parameters

### INTRODUCTION

Lipases (EC 3.1.1.3) comprise a group of enzymes which catalyze the hydrolysis of triglycerides to diglycerides, monoglycerides, fatty acids and glycerol at oil water interface<sup>1,2</sup>. In addition to hydrolytic activity on triglycerides they catalyze esterification, interesterification, acidolysis, alcoholysis and aminolysis reactions. One attractive feature of lipases is their specificity with respect to the glyceride position and fatty acid type, which could seldom be constructed by chemical catalysis<sup>3</sup>. Lipases are ubiquitous enzymes found in animals, plants and microorganisms including fungi and bacteria<sup>4,5</sup>. Microbial lipases are widely diversified in their enzymatic properties and substrate

specificity, which make them very attractive for industrial applications. Lipases are used in two distinct fashions. They are used as biological catalyst to manufacture other products such as food ingredients and by their applications such as in making fine chemicals. The lipases catalyzed transesterification in organic solvents is an emerging industrial application such as cocoa butter equivalent, human milk fat substitute "Betapol", pharmaceutically important polyunsaturated fatty acid (PUFA) rich/low calorie lipids", designers fats or saturated lipid" and production of biodiesel from vegetable oils<sup>4,6</sup>.

Lipases can be used to resolve the racemic mixtures and to synthesize the chiral building blocks

for pharmaceuticals, agrochemicals and pesticides. Some lipases retain their activity in non-polar organic solvents. Thus these can be used in the hydrolysis of water-insoluble esters such as in resolution of racemic mixtures through stereo specific hydrolysis<sup>7</sup>. Lipases are also drug targets or marker enzymes in the medical sector. Lipases are used in the enzymatic determination of serum triglycerides to generate glycerol, which is subsequently determined by enzyme linked colorimetric reactions<sup>8</sup>. Lipase producing microorganisms have been found in diverse habitats such as industrial waste, vegetable oil processing factories, dairies, soil contaminated with oil, oil seeds and decaying food<sup>9</sup> compost heaps, coal tips and hot springs<sup>10</sup>.

## MATERIAL AND METHODS

### Isolation

Two samples were collected from oil extraction unit soil of District Kangra of Himachal Pradesh, India and one from cotton seed oil extraction mill soil, Dhillon Oil Mill, Distt Bhatinda of Punjab, India. The samples were enriched in LB medium supplemented with olive oil. Further the Screening medium (Olive oil, 10 g/l; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g/l; MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 g/l; KH<sub>2</sub>PO<sub>4</sub>, 1.0 g/l; pH, 7.0) was used to screen lipolytic isolates. Lipase production was determined from the cell free culture supernatant fluid. Characterization and identification of the isolate was made following Bergeys Manual of Systematic Bacteriology<sup>11</sup>.

### Enzyme production

The medium of the enzyme production was composed of Olive Oil, 10 g/l; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 g/l; MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 g/l; KH<sub>2</sub>PO<sub>4</sub>, 1.0 g/l; Bile salts, 1.0 g/l; pH 7.0. The medium was inoculated with 2 ml of overnight culture and incubated at 37°C. After 24 h of incubation the culture was centrifuged and the cell free culture supernatant fluid was used as the crude enzyme.

### Enzyme assay

The titrimetric method was used for calculating lipase activity as described elsewhere [12]. Culture broth was centrifuged in a cooling centrifuge at 8000 rpm for 10 min. The pellet was discarded and 1ml of supernatant was taken. To

this, 2ml of 0.1M phosphate buffer of pH 7.0 and 1ml of olive oil was added. The above contents were incubated for 30 min at 40°C in rotary shaker water bath. 5ml of 96% ethanol was added to it exactly after 30 min to stop the reaction. 2-3 drops of phenolphthalein indicator were added to the above contents. The resulted content was titrated against 0.05 N KOH. Volume of KOH used was noted for each titration.

“1 unit of lipase activity was defined as imole of fatty acids liberated per minute at 40°C”.

### Effect of culture variables on lipase production Physical parameters

pH, temperature and incubation period was optimized for the production of optimum titre values of lipase enzyme in shake flask conditions. With the view to study the effect of pH on lipase production medium was inoculated with active culture of microorganism with pH range of 3-10 at an interval of one. For temperature optimization lipase production was studied at 25°C to 65°C at an interval of 5°C. Incubation period was optimized for enzyme production for six days at an interval of 24 h. Enzyme activity was determined by titrimetric method.

### Chemical Parameters

Effect of different biosurfactants was studied on lipase production. Different biosurfactants, *viz.*, tween 80, tween 20, SDS (Sodium dodecyl sulphate), and SLS (Sodium lauryl sulphate) were supplemented in production medium. Flasks were incubated for 24 h at 30°C in orbital incubator shaker. Different inorganic salts were used for optimum lipase production. The salts used were: CaCl<sub>2</sub>, MnCl<sub>2</sub>, MgCl<sub>2</sub>, KH<sub>2</sub>PO<sub>4</sub>, NaCl and MgSO<sub>4</sub>. Flasks were incubated for 96 h at 37°C in orbital incubator shaker. After incubation enzyme activity was calculated by titrimetric method.

### Nutritional Parameters

The effects of different lipids were studied by using various lipid sources *i.e.*, olive oil, coconut oil, rice bran oil, mustard oil and refined oil. Oils were added to the production medium at 1% concentration. Flasks were incubated for 96 h at 37°C in orbital incubator shaker. The effect of nitrogenous additives on lipase production was

studied using, inorganic (ammonium sulphate and potassium nitrate) and organic (peptone, trypton and yeast extract) nutrients.

## RESULTS AND DISCUSSION

### Isolation and Identification

On the basis of level of the productivity of the lipase production, one potent lipase producing strain was selected for subsequent studies. The isolate was rod shaped, aerobic, endospore forming, catalase positive and Gram positive. Based on biochemical, morphological, and physiological properties, the strain has been identified as *Sporosarcina* sp.

### Growth and lipase production

#### Effect of Temperature

Lipase production was studied in temperature range of 25°C to 65°C. The results (Fig.1) showed that maximum lipase was produced at the temperature 37°C (9.70U/ml). The production declines at above and below 37°C. Hence, 37°C was selected as the optimal temperature for further studies. Similar findings were reported by other researchers<sup>13,14</sup>. Our results are in parallel with that the optimum lipase production was achieved from *Pseudomonas* sp at 37°C<sup>15</sup>.

#### Effect of pH

Lipase production was studied in the pH range of 3-9 varied at an interval of 1.0 pH. The results showed that maximum lipase production was achieved at pH 4.0, suggesting that the strain is acidophilic. The graph (Figure 2) showed that at pH 4.0, enzyme production was 11.59U/ml. Besides this, at other pH almost constant values were achieved. Thus pH 4.0 was selected as optimum pH for further studies being higher lipase production. Other findings, that optimum lipase was produced from *Aspergillus niger* at pH 5.0 [16], maximum lipase production at acidic pH from *Rhizopus oryzae* KG-5 [12] and the optimum lipase production from *Pseudomonas* sp. at pH 5.5 [15] are in agreements with our results.

#### Effect of Incubation Period

Lipase production was studied for 144 h at an interval of 24 h. The result showed that

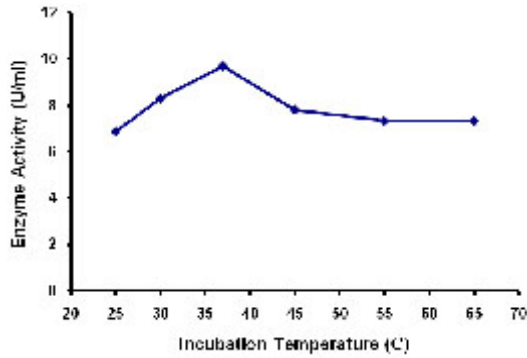
maximum lipase (10.41U/ml) was produced during 96-120 h. Above 120 h and below 96 h lipase production declined (Figure 3). Hence, lipase production was studied further for 96 h of incubations.. The study is in lines with the finding<sup>15</sup> in which it has been reported that the lipase from *Pseudomonas* sp. is active at 96 h of incubation. Other study<sup>17</sup> also reported the similar results.

### Effect of Biosurfactants

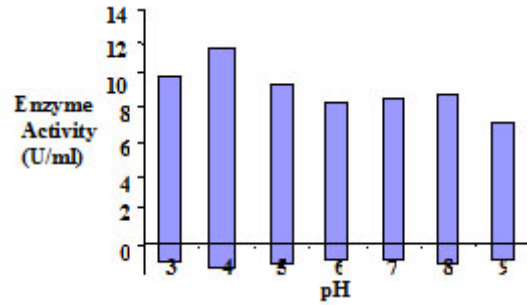
Nonionic (tween 20, tween 80) and ionic (Sodium Dodecyl Sulphate, Sodium Lauryl Sulphate) surfactant were studied. The observed lipase production was almost constant to an extent with slight variation (Table 1). Although tween 20 have synergistic effect on lipase production (13.96 U/ml). In the presence of Tween 20 the level of lipase increases<sup>18</sup>. Another finding<sup>19</sup> also supports the increased level of lipase production in the presence of Tween 20. The results are also presented in terms of relative activity, considering 100% activity in control (having no biosurfactants in production medium).

### Effect of metal salts

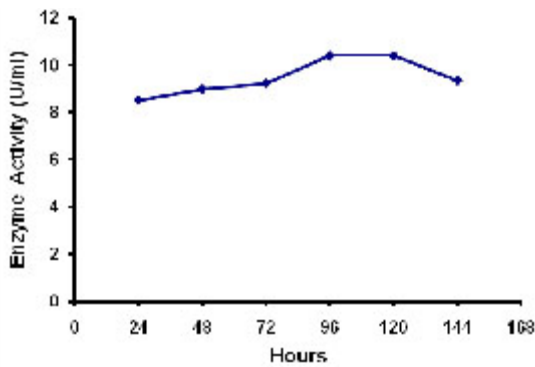
The lipase production was also studied using various inorganic salts. Among all, KH<sub>2</sub>PO<sub>4</sub> further enhances lipase production to 10.41U/ml. With divalent ions, MnCl<sub>2</sub> MgCl<sub>2</sub>, NaCl and MgSO<sub>4</sub> the enzyme activity declined (Table 2). In this study effect of inorganic salts did not affect the enzyme production drastically, except KH<sub>2</sub>PO<sub>4</sub>. It suggests that the divalent ions are not needed for the activity of lipases. Addition of metal ions (Mn, Ca, Na and Mg) did not affect markedly the lipase production and they play structural role rather than in catalytic process<sup>18</sup>. It was suggested that metal ions function as electrophiles, which seek the opportunity to share electron pairs with other atoms such that a bond or charge-charge interaction might be formed. Meanwhile, transition metal ions, such as Fe<sup>3+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup>, and Zn<sup>2+</sup>, change the conformation of the protein, rendering it less stable as the result of ion toxicity<sup>20</sup>. This effect was attributable to the interaction between salt ions and the enzyme surface charge, which might markedly affect the ionization of some amino acid residues thus changing the enzyme conformation and altering enzyme activity<sup>21</sup>.



**Fig. 1: Effect of temperature on lipase production**



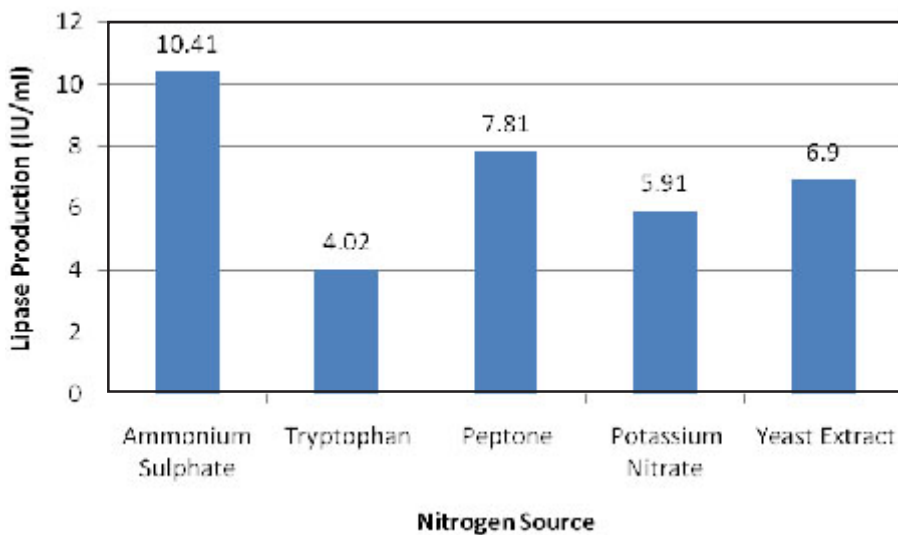
**Fig. 2: Effect of pH on lipase production**



**Fig. 3: Effect of incubation period on lipase production**

**Effect of Different Lipids**

Five different oils namely coconut oil, rice bran oil, mustard oil and refined oil were tested in addition to olive oil (conventional oil for lipase production). Table 3 indicates the comparative lipase production with all these carbon sources. The maximum lipase activity was found in case of rice bran oil (8.52 U/ml) and it was higher than conventionally used olive oil. The results are in agreement with the finding in which the maximum lipase production (14.0 IU/g wet weight) with rice bran oil was reported<sup>22</sup>. Other oils show 8.28U/ml (mustard oil), 7.81U/ml (coconut and refined oil). Lipids may act as inducer or inhibitor for enzyme production. In this study the lipase production in natural lipid supplemented medium gave slightly



**Fig. 4: Effect of different nitrogen sources on lipase production**

**Table 1: Effect of biosurfactant on lipase production**

S. No.	Biosurfactant	Enzyme Activity(U/ml)	Relative Activity(%)
1.	Tween 80	13.3	125.8
2.	Tween 20	13.9	132.0
3.	SDS (Sodium Dodecyl Sulphate)	13.7	129.6
4.	SLS (Sodium Lauryl Sulphate)	13.0	122.9

**Table 2: Effects of metal salts and chemical reagents on lipase production**

S.No.	Metal ion	Enzyme activity(U/ml)
1.	CaCl <sub>2</sub>	7.80
2.	MnCl <sub>2</sub>	6.38
3.	MgCl <sub>2</sub>	7.57
4.	NaCl	7.33
5.	MgSO <sub>4</sub>	7.57
6.	KH <sub>2</sub> PO <sub>4</sub>	10.41

**Table 3: Effect of different carbon sources on lipase production**

S.No.	Carbon source	Enzyme activity(U/ml)
1.	Olive Oil	7.33
2.	Coconut Oil	7.81
3.	Rice Bran Oil	8.52
4.	Mustard Oil	8.28
5.	Refined Oil	7.81

increased production. Better result was obtained when medium was supplemented with rice bran medium. This study is in agreement with the study that olive oil and sunflower oil increases the lipase production<sup>23</sup> and suggest that lipase has a wide range to utilize the studied natural oils.

### Effect of nitrogen sources

The effect of nitrogenous additives (ammonium sulphate, trypton, peptone, potassium nitrate and yeast extract) on lipase production was studied. Among all, ammonium sulphate showed maximum lipase production (10.41U/ml). Peptone also enhanced markedly the production of lipase (Fig. 4). Inorganic nitrogen source induces the lipase production<sup>24</sup> when basal medium is supplemented with inorganic salts the production increases while in case of organic salts the production was constant suggesting that the organic salts are complex molecule and the initial degradation is difficult. *Penicillium citrinum* produces lipase free of extraneous proteins and citrinin when cultured using, nitrogen source ammonium sulphate (lipase activity of 7.88 U/mg or ml) and yeast extract (lipase activity of 4.95 U/mg) with olive oil as carbon source<sup>25</sup>.

### CONCLUSION

Isolated strain, *Sporosarcina* sp. have the ability to produce lipase enzyme at low pH. Many gastric enzymes are active at low pH, in this view the lipase from *Sporosarcina* sp. could be a potential source for those enzymes. It can be concluded from the above study that the *Sporosarcina* sp. could be a useful source of Lipase enzyme and has the potential of medical and industrial applications.

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