



CARBON AND NITROGEN SOURCES ENHANCE LIPASE PRODUCTION IN THE BACTERIA *Bacillus subtilis* KPL13 ISOLATED FROM SOIL SAMPLES OF KOLLI HILLS, SOUTH INDIA

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ABSTRACT

The aim of the study was to investigate the production of lipase enzyme from *Bacillus subtilis* KPL13 isolated from Kolli hills. An *in vitro* study about the *Bacillus subtilis* KPL13 in to various parameters for optimizing the lipase production gave an insight about the role of carbon and nitrogen source in lipase synthesis. The maximum lipase production was exhibited by *Bacillus subtilis* KPL13 found to 3.58 U/ml. When subjected to different carbon sources such as glucose, tributyrin and olive oil. Maximum activity was observed in Olive oil and tributyrin. The maximum production of lipase in various nitrogen sources shows the maximum activity was found in peptone. These studies have shown the role of carbon and nitrogen as a crucial factor in the lipase production. When compared with the nitrogen source, altering the carbon source yields high amount of lipase. The Lipase activity was observed in various pH and temperature. Lipase has extremely superior activity at pH 6 and found to have activity in acidic condition. *Bacillus subtilis* KPL13 has activity in temperature around 35°C. The activity of enzyme declined in higher temperature and it proved those lipases are mesophilic. It is also subjected in various metal ions and detergent. Interestingly lipase shows enhanced activity in few metal ions like Ca²⁺, Zn²⁺ and Mg²⁺ where the activity is found to be above 95% and in detergent the maximum activity *i.e.*, more than 100% was found in Triton X-100 and Tween-80. These findings give valuable information about the lipase from Kolli hills.

KEYWORDS: *Bacillus subtilis* KPL13, Lipase, Lipase assay, Temperature, pH, peptone in lipase production.

INTRODUCTION

Lipases are ubiquitous enzymes which are found in animals, plants, fungi and bacteria. Microbial lipases are high in demand due to their specificity of reaction; stereo specificity and less energy consumption than the conventional method (Saxena *et al.*, 1999). The fungal lipases are more useful than lipases derived from plant and animal sources because of their extracellular in nature, low production cost and high stability in various organic solvents (Pandey *et al.*, 1999). Such type of microorganism has been found in diverse habitat and especially oil processing industries. Lipases are widely used in numerous biotechnological process such as cosmetic, food, leather, detergent, and pharmaceutical industries (Sztajer *et al.*, 1998). Microbial lipases production has increased for the past one decade, because of its potential application in various industries (Hasan *et al.*, 2009). When compared with plant and animal lipases, bacterial lipases are well studied by several peoples. The microorganisms are usually grown in nutrient medium supplemented with carbon source, nitrogen and phosphorous sources. Glycerol, triglycerides and bile salts are usually used as inducer for the production of lipases (Kishore *et al.*, 2011). Due to its wide range of significance, lipases remain a subject of intensive study (Alberghina *et al.*, 1991; Bornscheuer, 2000). Microbial lipases are produced mostly by submerged culture (Ito *et*

al., 2001). The lipase production is influenced by the type and concentration of carbon and nitrogen sources, culture pH and the growth temperature and dissolved oxygen concentration (Elibol *et al.*, 2001). The objective of the present study is to maximize the lipase production in *Bacillus subtilis* KPL13 isolated from the soils of Kolli hills and to check the enzyme activity in various pH and temperature for industrial application.

MATERIALS & METHODS

Sample collection site

Soil samples were collected from Kolli hills. Eight diverse soil sites were selected and the entire samples were transferred to the laboratory and were used for the analysis.

Isolation of Lipolytic Bacteria

Dilution plate method was performed for isolation of lipolytic bacteria from collected soil sample. One gm of soil samples were transferred to 10 ml of 0.85% sterile saline water. Serial dilution was performed by transferring one ml of aliquot from each of the samples to 9 ml of 0.85% saline water upto 10⁻⁶ dilutions were prepared. From the diluted samples 0.1 ml of 10⁻⁴ and 10⁻⁵ was plated on nutrient agar and nutrient agar supplemented with Tween 80. Plates were incubated for 48-72 h at 37°C. Colonies with zone of clearance was picked and stored

into sterile nutrient slant for further studies (Prasanna *et al.*, 2013).

Lipase production medium

The production medium consist of (%w/v) Peptone 0.2, $\text{NH}_4\text{H}_2\text{PO}_4$ 0.1; NaCl 0.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.04; Olive oil 2.0 (v/v); pH 7.0; 1-2 drops of Tween 80 as emulsifier. Overnight cultures were inoculated into the 250ml Erlenmeyer flasks containing 100ml media and were kept in rotary shaker for 150rpm. Sample were collected after 24 hours and centrifuged at 10,000 rpm for 10mins at 4°C. The cell filtrate was used as a source of extracellular enzyme lipase (Prasanna *et al.*, 2013).

Assay for lipase activity

The activity of lipase was demonstrated by using spectrophotometrically at 30°C by using *p*-nitrophenol palmitate (pNPP) as a substrate (Winkler *et al.*, 1979). The composition of reaction mixture was 700 μl pNPP solution and 300 μl of lipase solution. The pNPP solution was prepared by adding the solution A (0.001 g pNPP in one ml isopropanol) into solution B (0.01 g gum arabic, 0.02 g Sodium deoxycholate, 50 μl Triton X-100, 9 ml of 50 mM Tris-HCl buffer pH 8) when it was dissolved. The absorbance was measured at 410 nm for the first 2 min of reaction. *Enterococcus faecium* MTCC 5695 was used as a standard for lipolytic bacteria. One unit was defined as that amount of enzyme that liberated 1 μmol of pNP per minute (:1500l/mol cm) under the test conditions (Karadzic *et al.*, 2006)

Effects of variable carbon and nitrogen source on lipase production

Lipase production was assayed by olive oil, glucose and tributyrin as a sole carbon source (1% w/v). The lipase production observed with the effect on nitrogen sources and it was analyzed by using supplementing production medium with different categories of nitrogen sources (0.2% w/v) such as yeast extract, ammonium dihydrogen phosphate, peptone and enzyme activity were assayed. To Investigate the effect of different carbon and nitrogen sources on lipase activity of *Bacillus subtilis* KPL13 was done at pH: 7.0, 30°C and 150 rpm throughout 24 h of cultivations (Karadzic *et al.*, 2006).

Lipase activity on temperature and pH

The culture broth was used as the crude enzyme after the separation of cells and various particles. Generally the enzyme was stored at 4°C until used. The activity was determined at different temperatures (30–65°C), at pH 8.0 for 10 min. The optimal pH was determined at 30°C in buffer solutions of pH values ranging from 5 to 11 (0.05 M citrate-phosphate pH 5-7; 0.05 M Tris-HCl pH 8-9; 0; 0.05 M Glycine -NaOH pH 11). By using pNPP as the substrate the enzymatic activity was measured (Karadzic *et al.*, 2006).

Effects of different ions and detergents on lipase activity

A concentration low as 1mM of some metal ions can affect the enzyme activity. Thus, the effect of several ions (Fe^{2+} , Na^+ , K^+ , Zn^{2+} , Cu^{2+} , Mn^{2+} , Ca^{2+} and Mg^{2+}) on these isolates and the control was determined. The enzyme solution was stored for 1 h at 30°C in the presence of 1 mM of various ions (as chloride salts) prior to the colorimetric assay for remaining lipase activity. In the case

of chemical detergents, activity was determined after 1h of storage of enzyme solution at 30°C in the presence of various chemical detergents (SDS, Tween 80 and Triton X-100) at 1% concentration. Activity was calculated by the spectrophotometric assay after incubation time. Remaining enzymatic activity was determined by a standard method with pNPP. Final enzyme activity was calculated by relative to control activity (a parallel enzyme reaction without additions) (Karadzic *et al.*, 2006).

RESULTS & DISCUSSION

Screening of isolate for Lipase activity

Lipase producing microbial culture were isolated from different sites of Kolli hills by serially diluting the samples and plated in Tween 80 plate. Lipolytic bacteria were isolated from the plate and enriched in Nutrient broth by periodic sub culturing. KPL13 was isolated and characterized by using 16srDNA sequencing. The obtained sequence was deposited in GEN Bank NCBI Database and the accession number was obtained KC823231. The KPL13 was identified as *Bacillus subtilis* KPL13 and used for the present study.

Assay for lipase activity

Bacillus subtilis KPL13 was subjected for lipase production along with the standard lipolytic bacteria *Enterococcus faecium* MTCC 5659. This *E. faecium* MTCC 5659 showed a maximum lipase activity of 4.280 U/ml. This *Bacillus subtilis* KPL13 has produced at a rate of around 3.58 U/ml [Fig. 1].

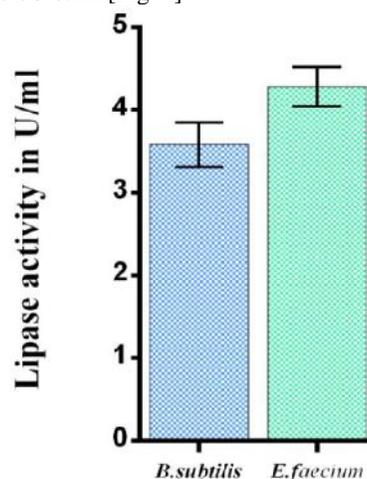


FIGURE 1: Lipase assay test organism *Bacillus subtilis* KPL13 and *E. faecium* as standard. (Temp: 30°C, pH: 7.0, Agitation: 150 rpm).

Effect of different carbon and nitrogen sources

Among the different carbon sources such as tributyrin, glucose and olive oil were used for the present study. Olive oil is considered the most appropriate source and subsequently tributyrin plays a crucial role in inducing the enzyme production [Fig. 2]. Apart from carbon source, the type of nitrogen source in the medium also has influence over the lipase production, in our work peptone was found to be the most suitable source and maximum production (3.88 U/ml) was found [Fig. 3] The most important factor for the expression of lipase has always been carbon, since the lipase enzymes are inducible enzyme [Lotti *et al.*, 1998). They are synthesized only in the presence of lipid

source such as oil or any other inducer which includes fatty acids, hydrolysable ester, tweens, triacylglycerols, bilesalts and glycerol. However their production is extensively influenced by other carbon sources such as sugars, polysaccharides and other complex sources. The results are very similar to the previous study which states that natural oils stimulate lipase production (Abdel-Fattah 2002; Kaushik *et al.*, 2006; He and Tan, 2006). Lipase production increases in thermophilic *Bacillus* sp tremendously during the presence of olive oil as a carbon source in the culture medium (Eltaweel *et al.*, 2005; Lee *et al.*, 1999).

Other research also suggests that the glucose supplementation to the basal medium inhibits the production of lipase due to the catabolic repression (Dharmstithi and Kuhasuntisuk, 1998; Lotrakul and Dharmstithi, 1997). High glucose concentration gradually reduced lipase production. This is clearly observed in the present experiment and reported. Apart from carbon source, the type of nitrogen source in the medium also has influence over the lipase production. In general microorganism gives high yields of lipase when organic nitrogen sources are provided, such as peptone and yeast extracts (Chen *et al.*, 1992; Sugihara *et al.*, 1991).

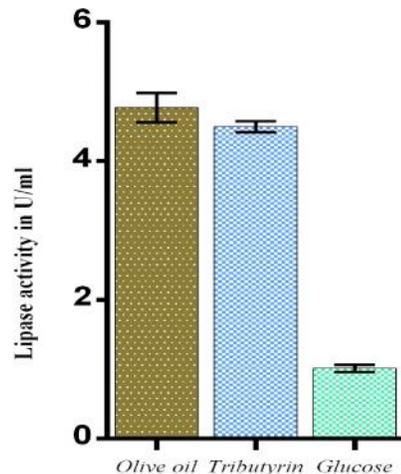


FIGURE 2: Effect of different carbon sources (1% w/v) in medium for lipase production by *Bacillus subtilis* KPL13. Temp: 30°C, pH: 7.0, Agitation: 150 rpm.

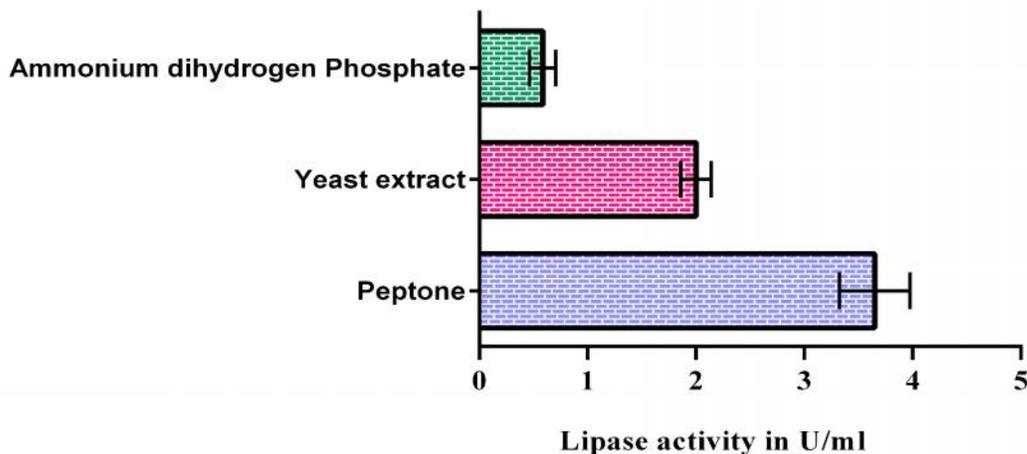


FIGURE 3: Effect of different nitrogen sources as additives (0.2% w/v)

Lipase activity and stability on various pH and temperature

The bacterial lipase may be either active in acidic or alkali condition. In the present study the lipase obtained from *Bacillus subtilis* KPL13 shows that the maximum activity in pH 6 [Fig. 4]. The lipase range has proved to be potentially acidic lipase and can be used in many reactions which are acidic.

The temperature preference of this enzyme reveals higher activity at temperature 35°C [Fig.5]. It shows that *Bacillus subtilis* KPL13 lipases is a mesophilic enzyme. A comprehensive review of all bacterial lipase stated that the maximum activity of lipases at pH values higher than 7 has been observed in many cases. Even though few studies suggest that *Bacillus* sp are capable of withstanding high temperature, the lipases produced by specific organisms are not thermostable and has activity only at 35°C.

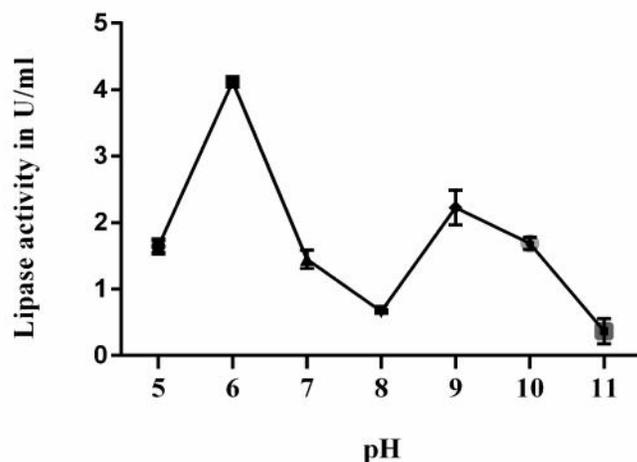


FIGURE 4: Effect of pH on activity of lipase. The activity was determined at 35°C

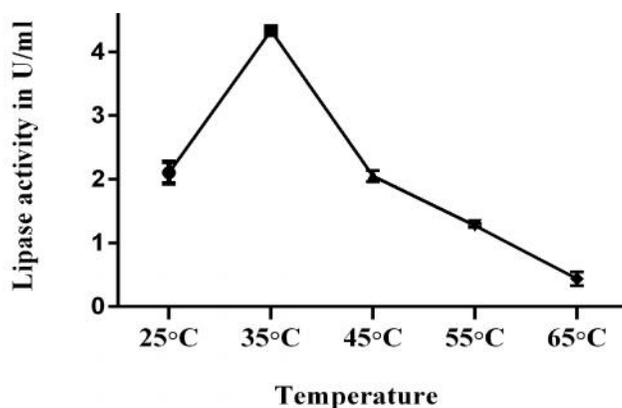


FIGURE 5: Effect of temperature on activity of lipase.

Effect of different Metal ions and detergents on activity of lipase

The effect of different metal ions on the activity of *Bacillus subtilis* KPL13 was given in [Table.1]. In present

study shows that the metal ions inhibit the activity of lipase to certain extent as a result 59% to 63.5% of lipase activity was lost in the presence of Fe²⁺ and Na⁺ ions.

TABLE 1. Effect on different Metal ions on lipase activity

Inhibitors	Lipase residual activity in (%)
Control	100
Fe ²⁺	41.1
Na ⁺	36.6
K ⁺	89.7
Zn ²⁺	97.7
Cu ²⁺	46.7
Mn ²⁺	83
Ca ²⁺	105.2
Mg ²⁺	96

The activity was increased up to 5% in Ca²⁺ ions, indicating the Ca²⁺ ions enhanced the activity of lipase. The effects of various detergents on enzyme activity of *Bacillus subtilis* KPL13 was shown in Table 2. *Bacillus subtilis* KPL13 lipase enzyme was sensitive to SDS than the other detergent. The enzyme activity was increased by 12% to 18%, when stored with Tween - 80 and Triton X -

100 respectively. The effect of metal ions could be attributed to change in the solubility and the behavior of the ionized fatty acids at interfaces and from a change in the catalytic properties of the enzyme itself. It was found that the total loss of activity in presence of SDS, as revealed the results were similar to our study. Tween- 80 and Tween- 20 can inhibit the activity of lipase, but the present

study reveals that the lipase enzyme incubated in Tween - 80 and Triton X -100 showed increased activity.

TABLE 2: Effect of various detergents on lipase activity

Inhibitors	Lipase residual activity in (%)
Control	100
EDTA	77
SDS	14.5
Tween-80	112.5
Triton X-100	118.5

CONCLUSION

In the present study extracellular lipase enzyme produced from *Bacillus subtilis* KPL13 is capable of active at optimal temperature and pH for the production of lipase was 35°C and pH 6 respectively. The lipase stability is lost in highest temperature. The lipase activity has been increased when subjected to metal ion and detergent.

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