

Full Length Research Paper

Production of extracellular lipase by a new strain *Staphylococcus aureus* NK-LB37 isolated from oil contaminated soil

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A total of 20 bacterial isolates were obtained by screening using tributyrin agar medium. Among them the isolate which exhibited greater clearance zone and higher lipase activity was subsequently screened using spirit blue agar and rhodamine B agar medium. Based on morphological, biochemical and 16S rRNA sequence analysis, the potent isolate was identified as *Staphylococcus aureus*. The lipase production of the isolate was increased by improving the conditions of production medium. Maximum lipase production (8.11 U/ml) was achieved when 2% punnakka oil was utilized as sole carbon source at pH 7.0 and 37°C after 2 days of incubation. Addition of 3% tryptone as nitrogen source and 0.01% MgCl₂ resulted in a significant increase of lipase production (10.73 U/ml). The lipase production was slightly enhanced in the presence of 20% n-propanol and highly stable in the presence of n-butanol, toluene and n-hexane. The study resulted in isolation and production of inducible, mesophilic and solvent tolerant lipase with industrial potential.

Key words: *Staphylococcus sp.*, tributyrin agar, 16S rRNA, medium optimization, solvent-tolerant lipase.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of ester linkages of triglycerides at water-oil interface (Gupta et al., 2011). Apart from hydrolysis, some lipases can catalyze reverse reactions including synthesis of esters by esterification, transesterification and interesterification (Franken et al., 2011). Existence of lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing units, dairies, soil contaminated with

oil, compost heap, hot springs etc (Sztajer et al., 1988; Wang et al., 1995).

Although lipases are widely found in animals, plants and microbes, microbial lipases have gained special industrial attention due to their selectivity, stability and substrate specificity (Treichel et al., 2010). Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical parameters such as temperature, pH, nitrogen and carbon sources (Gupta et

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Abbreviations: TBA, Tributyrin agar; SBA, spirit blue agar; RBA, rhodamine B agar; p-NPP, p-nitrophenylpalmitate.

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al., 2004). Generally, organic solvents are known to have detrimental effect on microorganisms and so they lose their activity and cease growing (Ogino et al., 1994). However, certain microbial lipases have gained importance due to their ability of being active in the presence of organic solvents (Sellek et al., 1999). Only a few species of bacteria have been exploited for the production of solvent tolerant lipases.

Recently due to huge variation in industrial applications, the availability of lipases with specific characteristics is still required to be explored. Further screening may lead to isolation of novel lipases with desired properties. Therefore, the present study has been attempted to screen and identify a new bacterial strain for the production of extracellular solvent tolerant lipase. It was further investigated in liquid medium in order to optimize the lipase production conditions.

MATERIALS AND METHODS

p-Nitrophenylpalmitate was obtained from Sigma Aldrich (USA). Microbiological media such as potato dextrose agar, tributyrin agar, spirit blue agar, rhodamine B and sodium deoxycholate were purchased from HiMedia Laboratories, Mumbai, India. All other chemicals utilized in the study were of analytical grade.

Sample collection

Soil samples were collected from oil contaminated areas of different oil mills in and around Coimbatore, Tamilnadu in sterile containers. The samples were transferred to the laboratory and stored at 4°C until its needed.

Screening and selection of lipase producing bacteria

Ten gram of soil sample was suspended in 100 ml of sterile distilled water by shaking (160 rpm) at 37°C for 4 h. Then the soil samples were filtered using sterile cheese cloth and the filtrate was used for isolation of bacteria. The isolation process was performed by serial dilution (10^{-1} to 10^{-6}) of soil sample (200 μ L) on tributyrin agar selective medium (Booth, 1971). The plates were incubated at 37°C for 2 days for the development of bacterial colonies. Twenty colonies showing clear zones around them were individually spot inoculated on tributyrin agar medium. Based on zone of clearance and lipase activity, the isolate LB5 was selected for further studies.

The efficiency of the isolate LB5 to produce lipase was confirmed by further screening using spirit blue agar and Rhodamine B agar media. The colonies showing blue color zones on spirit blue agar was subsequently streaked on Rhodamine B agar medium according to the method described by Kouker and Jaeger (1987). Lipase production was detected by irradiating the plates with UV light at 350 nm. Bacterial colonies having lipolytic activity showed orange fluorescent halo around the colony. All screening media used in the study were adjusted to pH 7.0 and plates were incubated at 37°C for 2 days. The pure culture was subcultured on nutrient agar slants and stored at 4°C for further use.

Identification of bacterial isolate

The morphological and biochemical characterization of the isolate

LB5 was performed according to the Bergey's Manual of Determinative Bacteriology (Holt et al., 1989). The identification was further confirmed by the 16S rRNA gene sequencing analysis. Briefly, genomic DNA was extracted from the culture LB5 using GeNei Ultrapure bacterial genomic DNA isolation kit (KT162). The DNA was amplified by PCR using 16S rRNA gene specific primers (Forward primer: 5' AGA GTT TGA TCM TGG CTC AG 3' and Reverse primer: 5' TAC GGY TAC CTT GTT ACG ACT T 3'). The PCR program comprised initial denaturation at 94°C for 5 mins, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1.30 min and a final extension step at 72°C for 10 min using Thermal cycler ABI 2720. The amplified PCR product was confirmed by agarose gel electrophoresis (1.0%). The amplicon (1500 bp) was purified by PCR purification kit (KP153). The PCR product was sequenced by ABI 3100 XL Genetic Analyzer using Big Dye Terminator version 3.1^o Cycle sequencing kit. The 16S rRNA gene of the sample was compared with NCBI-GenBank and RDP databases. Sequences were aligned using the Clustal W program. A distance matrix was generated based on sequence homology using Kimura-2 Parameter and phylogenetic tree was constructed using Neighbour joining method.

Lipase production

The bacterial culture was grown in 250 ml Erlenmeyer flask containing 50 ml of basal medium of following composition CaCl₂.2H₂O, 0.01%; MgSO₄.7H₂O, 0.04%; FeCl₃.6H₂O, 0.04%; pH 7.0 (Eltaweel et al., 2005). Olive oil (1% v/v) and peptone (1% w/v) were used as sole source of carbon and nitrogen respectively. The flasks with medium were inoculated with 1×10^6 spore suspension and was incubated at 37°C for 2 days under shaking (200 rpm). After incubation, the culture was centrifuged at 10000 rpm for 20 min at 4°C. The supernatant obtained was used as crude enzyme source for lipase assay

Lipase assay

Lipase activity was assayed using pNPP as substrate with some modifications (Winkler and Stuckmann, 1979). In brief, 10 ml of isopropanol containing 30 mg of pNPP was mixed with 90 ml of 0.05 M phosphate buffer (pH 7.6) containing 200 mg of sodium deoxycholate and 100 mg of gum arabic. A total of 2.4 ml reaction mixture was added with 0.1 ml of crude enzyme source and incubated at 37°C for 15 min. The release of p-nitrophenol was determined by measuring the absorbance at 410 nm against an enzyme free control as blank. One unit of lipase activity (U) was defined as the amount of enzyme releasing 1 μ mol of p-nitrophenol per minute under the assay conditions. All the experiments were performed in triplicates and the mean values were taken for analysis.

Optimization of cultural conditions for lipase production

The incubation time required for the maximum lipase production was studied by measuring the lipase activity at different time intervals of 12 to 60 h. In order to determine the optimum pH of the culture, pH of the medium was adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. The optimal temperature for lipase production was studied at different temperatures (25, 30, 37, 40, 45 and 50 °C). Variation in lipase production in response to carbon sources (jatropha oil, neem oil, punnakka oil, cotton seed oil, karanja oil, castor oil) was studied by replacing the olive oil in the basal medium at a concentration of 1% (v/v). The organic nitrogen sources were incorporated to the basal medium at 1% (w/v) by replacing peptone which include yeast extract, tryptone, corn steep liquor, casein and soyabean meal. Inorganic nitrogen sources (0.2% w/v) added to the medium includes ammonium tartrate, ammonium carbonate, ammonium nitrate and ammonium dihydrogen phosphate.

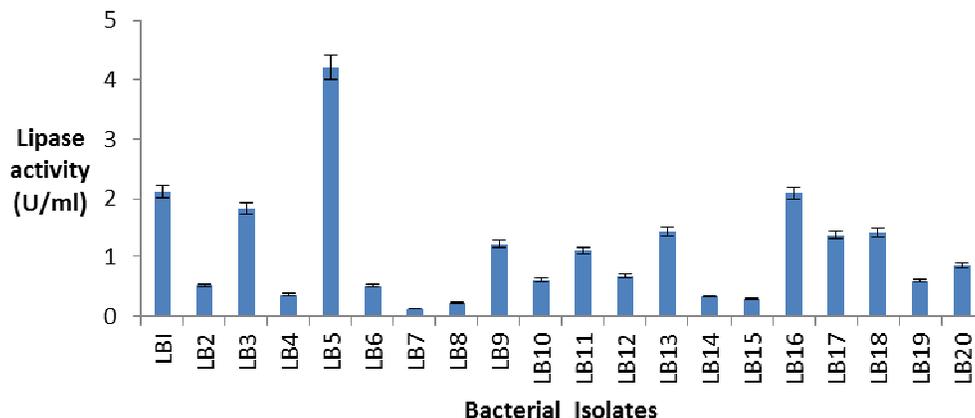


Figure 1. Lipase activity of different bacterial isolates.

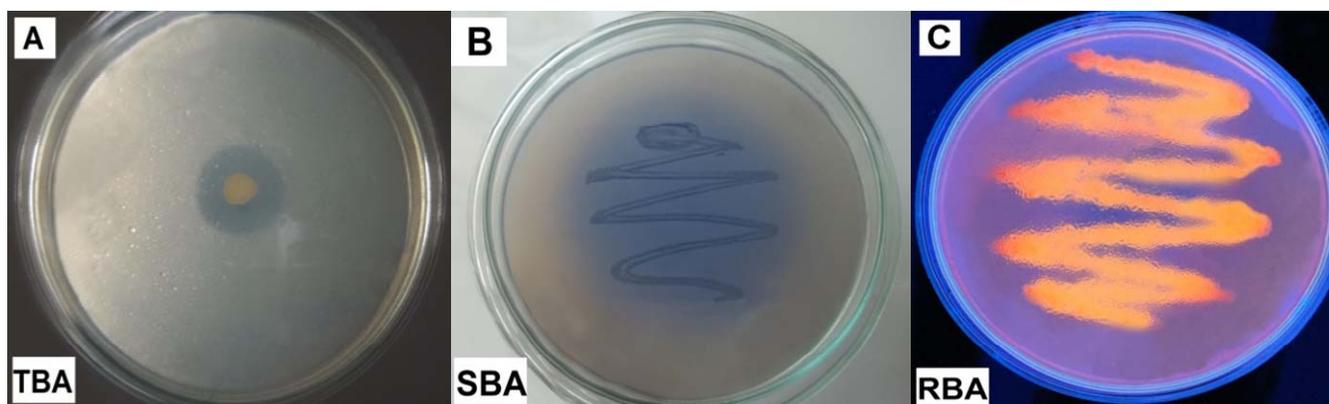


Figure 2. Growth of the potent bacterial isolate LB5 on different lipase screening medium. A. Tributyrin agar (TBA). B. Spirit blue agar (SBA); C. Rhodamine B agar (RBA).

The effect of different chloride salts of metal ions (Cu^{2+} , Ni^{2+} , Mg^{2+} , K^+ , Co^{2+} , Zn^{2+} and Mn^{2+}) at 0.01% concentration was evaluated to observe the changes in lipase production.

Effect of organic solvents on crude enzyme stability

The influence of various organic solvents (n-propanol, n-butanol, methanol, ethanol, toluene and n-hexane) at 20% concentration was studied. One ml of organic solvent was added to 4 ml of crude enzyme and incubated at 37°C for 1 h under shaking condition (160 rpm) to facilitate proper mixing of enzyme and solvent. The enzyme stability was designated as residual activity under the standard assay conditions.

RESULTS AND DISCUSSION

Screening and selection

Several methods have been proposed for screening of lipase production but there is still more scope for finding lipases with novel and specific properties through screening using tributyrin agar and plate assay (Rohit et al., 2001). Therefore the present study employs three

screening strategies to study the efficiency of the new bacterial isolate for lipase production.

Twenty bacterial isolates (LB1-LB20) were found to produce lipase as indicated by the clearance zone around the colony on the tributyrin agar plates. Among them, LB5 showed the maximum zone of clearance (11 mm) and higher lipase activity (4.21 U/ml) as shown in the Figure 1. The isolate LB5 was further screened using spirit blue agar and rhodamine B agar to determine its lipase producing efficiency which too was found to be positive (Figure 2)

Identification of potent bacterial isolate

The isolate LB5 was identified as *Staphylococcus sp.* based on its morphological and biochemical characterization (Table 1 and Figure 3). The identification was further confirmed by molecular characterization.

Molecular characterization

The molecular identification was carried out by the isola-

Table 1. Morphological and Biochemical characterization.

Test Name	LB5
Colony morphology	
Size	Small
Form	Circular
Shape(Negative staining)	Cocci
Arrangement	Clusters
Margin	Entire
Elevation	Raised
Density	Opaque
Texture	Smooth
Pigmentation	Creamy white
Gram reaction	Positive
Biochemical Tests	
Indole	-
Methyl red	-
Vogues-proskauer	-
Citrate Utilization Test	-
Catalase	+
Oxidase	-
Urease	-
Nitrate Reduction Test	+
Glucose Fermentation	+
Sucrose Fermentation	+
Lactose Fermentation	+
Starch Hydrolysis	+
Casein Hydrolysis	+
Gelatin Hydrolysis	-
Cellulose Hydrolysis	+
Tween-80 Hydrolysis	+
Motility Test	Non-motile
Coagulase Test	+

tion and sequencing of the 16S rRNA of the isolate LB5 (Figure 4). The 16S rRNA gene sequence of isolate LB5 contains 1505 bp. The sequence was compared with 10 different closely related sequences available in GenBank and RDP databases. To analyze the phylogenetic relationship of strain LB5, 16S rRNA gene sequence was determined and a dendrogram was constructed. The phylogenetic analysis revealed that the strain LB5 had highest homology with *S. aureus strain SR-05-03* (GenBank Accession Number: JQ247719.1) followed by *S. aureus strain: NBRC 12* (GeneBank Accession Number: AB680330.1). Based on these results, the isolate LB5 was designated as *S. aureus* (Figure 5). The 16S rRNA gene sequence of isolate LB5 has been deposited in the NCBI - GenBank database under the Accession Number KF152912

Optimization of parameters for lipase production

Bacterial lipases are influenced by the composition of the

growth medium, cultivation conditions and many physico-chemical (pH and temperature) and nutritional factors (carbon, nitrogen and lipid sources) (Jaeger et al., 1994). The influence of the components of the growth medium on microbial lipase production varies from one organism to another. The strategy adopted to optimize the parameters for improved lipase production was one-factor-at-a-time method

Effect of incubation time on lipase production

The lipase production was observed during a period of 12 to 60 h at 12 h interval. Substantial lipase production was detected after 24 h in late log phase. Maximum lipase production was obtained at 48 h at the early stationary phase (Figure 6A). Further incubation beyond 48 h was found to have negative effect on lipase production. This may be due to the release of by-products during decline phase. This finding is in accordance with Pogaku et al. (2010) who observed that 48 h of incubation time to be desirable for *Staphylococcus sp.* Lp12

Effect of pH

The pH of the growth medium is one of the important parameters affecting microbial cell growth and biochemical metabolism. The lipase yield was observed in pH range 7.0 to 8.5, the optimum being pH 7.0 (Figure 6B). Lipase production dropped significantly beyond pH 8.5. The finding agreed with Sirisha et al. (2010) who observed maximum lipase activity at pH 7.0 by *Staphylococcus sp.*

Effect of temperature

Lipase production was maximum at the optimum temperature of 37°C. Lipase production showed gradual increase with increase in temperature from 25 to 37°C. And with further increase of temperature beyond 40°C, the enzyme production decreased (Figure 6C). Various industrial application of microbial lipases for the hydrolysis of glycerides, inter-transesterification of fatty acid moieties etc., basically involved the use of mesophilic enzymes.

Effect of carbon source

Lipase production by the isolate LB5 cleaved all the tested non-edible oils with the highest affinity to punnakka oil (7.36 U/ml) followed by karanja oil (Figure 7A). While all the other oils also showed considerable amount of enzyme production indicating the inducible nature of the enzyme most bacterial lipases are generally induced in medium that contains the proper fatty acids and oils (Immanuel et al., 2008; Sharma et al., 2009).



Figure 3. Growth of the bacterial isolate LB5 on nutrient agar (NA).

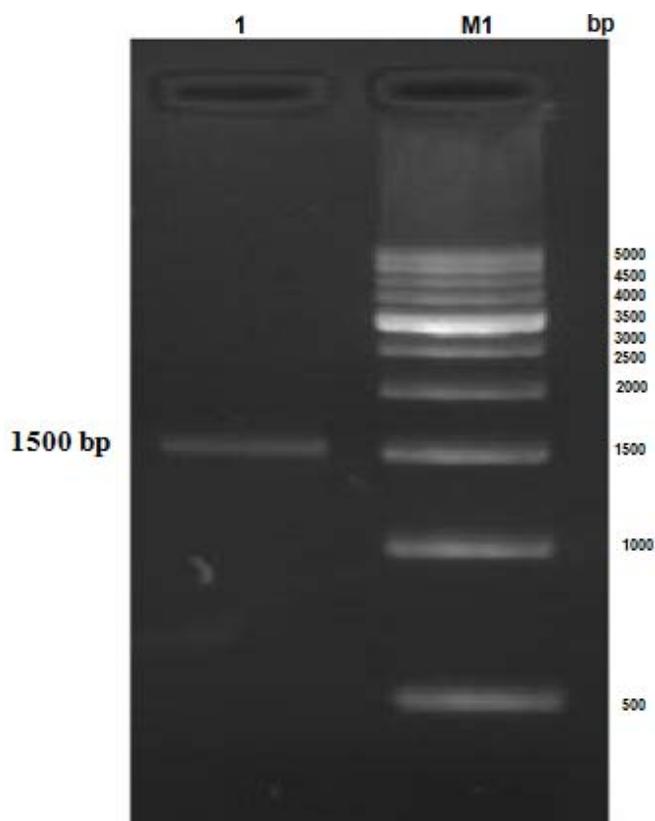


Figure 4. Agarose gel electrophoresis of the amplified PCR product. Lane 1, sample (Isolate LB5); lane M1, Stepup™ 500bp DNA ladder.

Many reports have shown that natural oil stimulate lipase production (Abdel-Fattah, 2002; Kaushik et al., 2006; He and Tan, 2006). In attempts to optimize the concentration

of punnakka oil, it was observed that upto 2% (v/v) gave maximum lipase production (8.11 U/ml) (Figure 7B). Higher levels of punnakka oil showed a deleterious effect on lipase production which might be due to the inhibition by fatty acid concentration which was liberated during the hydrolysis of triglycerides.

Effect of nitrogen source

Besides carbon source, the type and concentration of nitrogen source in the medium also play an important role in the synthesis of enzymes. Among the different organic nitrogen sources used, tryptone was found to be the most suitable nitrogen source showing maximum lipase production (9.82 U/ml) (Figure 8A). Further studies conducted by incorporating tryptone at different concentrations showed that addition of 3% (w/v) tryptone stimulated lipase production (10.33 U/ml) (Figure 8B). All the tested inorganic nitrogen sources were found to exhibit inhibitory effect on lipase yield indicating that organic nitrogen sources were preferred to inorganic nitrogen sources for lipase production (Figure 9A). So the inorganic nitrogen sources were not included in the optimized medium. Generally microorganisms exhibit high yield of lipase when organic nitrogen sources were used in the medium (Mobarak-Qamsari et al., 2011)

Effect of metal ions

Metal ions enhance the enzyme activity and confer thermostability to them (Chakraborty and Paulraj, 2008). Many enzymes in the presence of metal ions facilitate the maintenance of their active structures (Sharma et al., 2002). Different lipases show different response to metal ions. The presence of $MgCl_2$ in the culture medium was found to stimulate lipase production (10.76 U/ml) (Figure 9B). Other ions such as Mn^{2+} and K^+ slightly inhibited lipase production. This finding is in accordance with Kalpana et al. (2013) who reported that *Staphylococcus* TUL1 showed maximum lipase production in the presence of Mg^{2+} . Heavy metals such as Cu^{2+} , Ni^{2+} , Zn^{2+} and Co^{2+} strongly inhibited lipase production. Generally lipase activity in the presence of heavy metals like Co^{2+} , Ni^{2+} , Hg^{2+} and Sn^{2+} was inhibited (Patkar and Bjorkling, 1994).

Effect of organic solvents on the lipase stability

Lipases are diverse in their sensitivity to organic solvents (Raku et al., 2003). In this study, the stability and activity of various organic solvents were tested at 20% (v/v) concentration as depicted in Figure 10. Organic solvent n-propanol slightly enhanced the lipase activity. The crude lipase was stable in n-butanol, n-hexane and toluene with residual activity of 79.3, 88.6 and 90.0% respectively, whereas methanol and ethanol showed negative effect on lipase activity. Therefore, it can be inferred that the

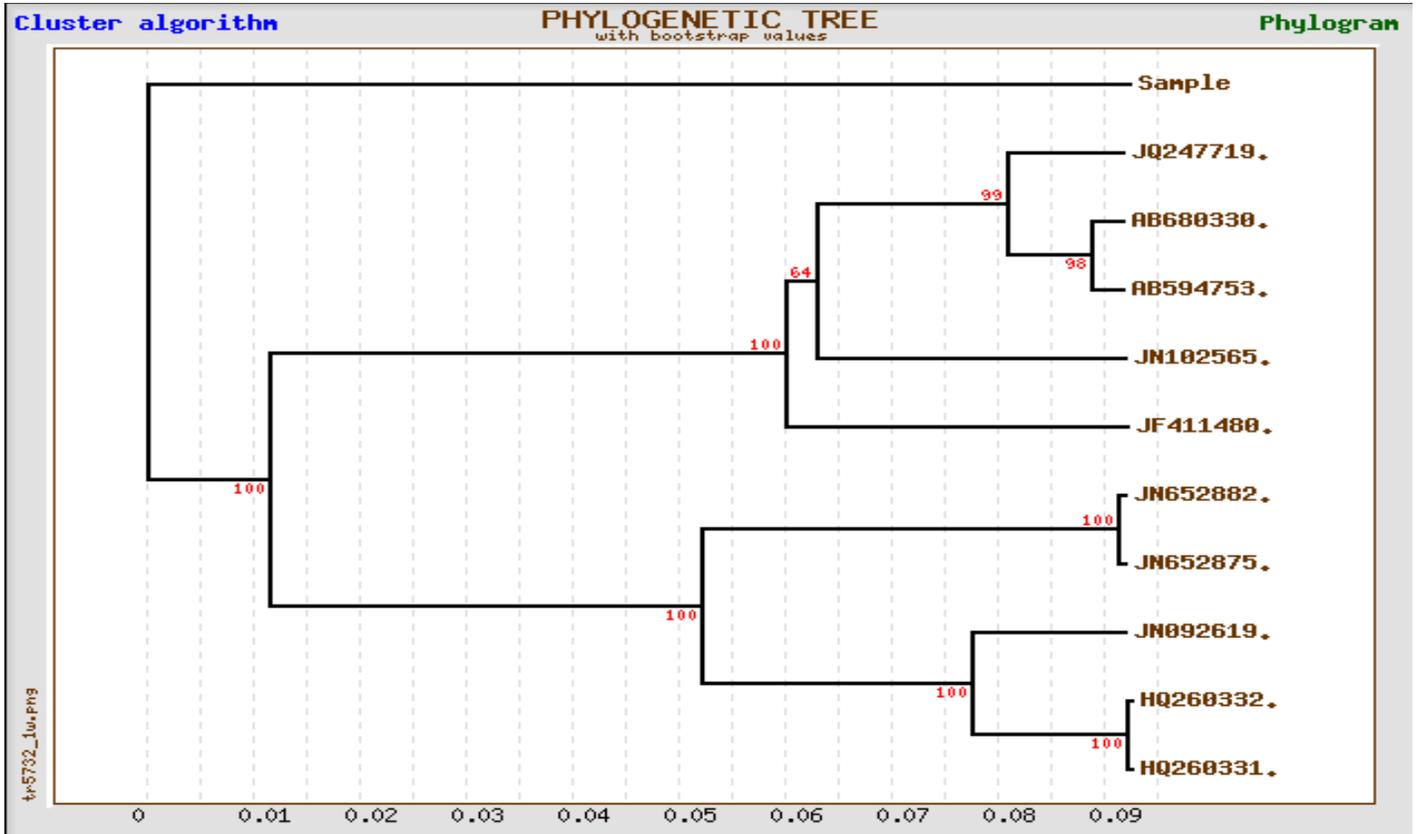


Figure 5. Phylogenetic tree based on 16S rRNA gene sequence showing the relationships between the Sample (isolate LB5) and related members of the genus *Staphylococcus*.

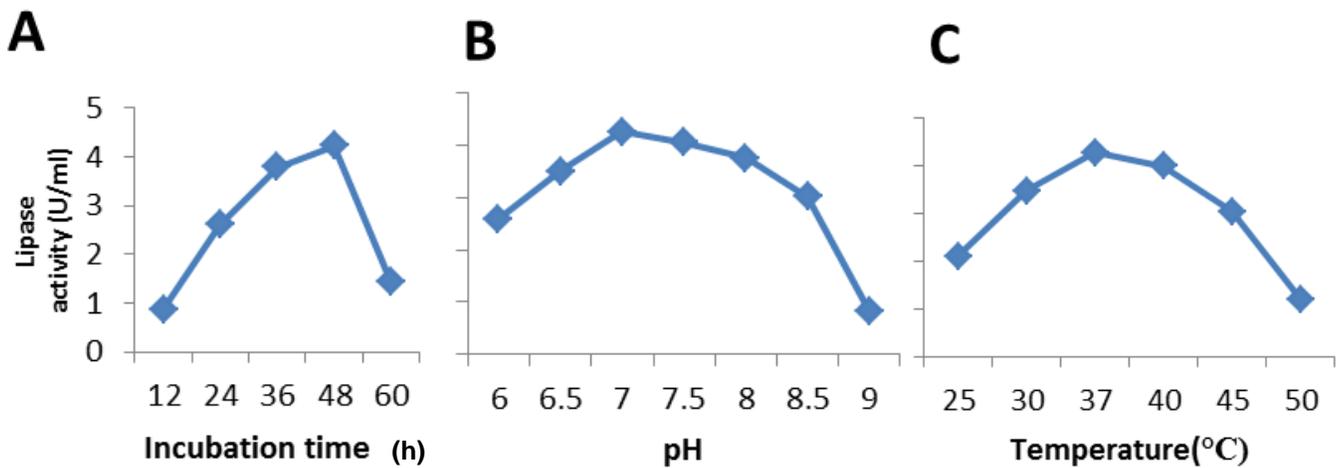


Figure 6. Effect of Incubation time (A) , pH (B) and temperature(C) on lipase production.

strain NK-LB37 exhibited different extents of tolerance to various organic solvents. Organic solvent tolerant lipases are effective catalysts in the transesterification reactions and synthesis of biopolymers (Dizge et al., 2009; Singh et al., 2010).

Conclusion

Lipases occupy a prominent place among biocatalysts with a wide spectrum of industrial applications. Considering this, in the present study, the production of lipase

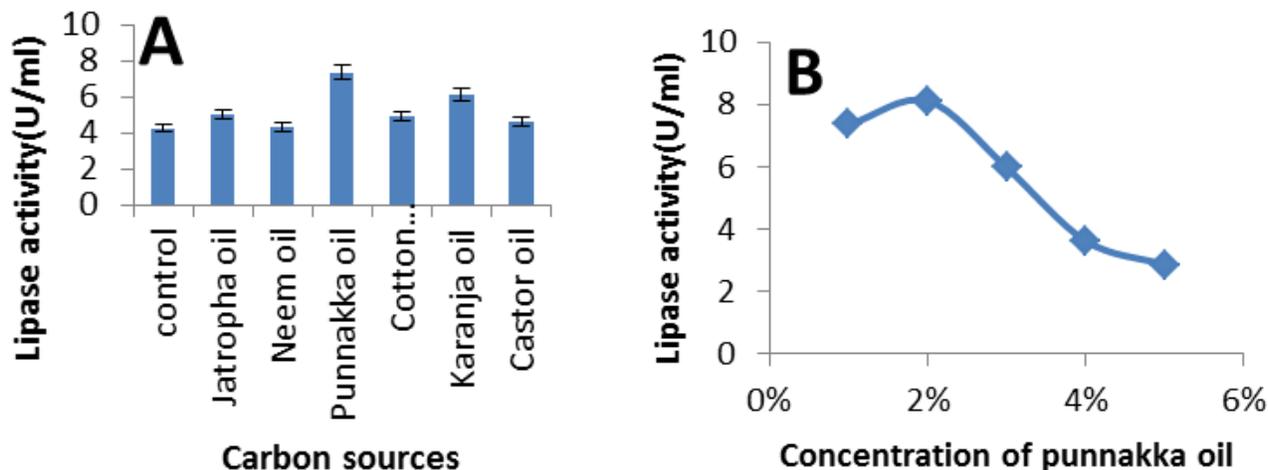


Figure 7. Effect of carbon sources (A) and concentration of punnakka oil (B) on lipase production.

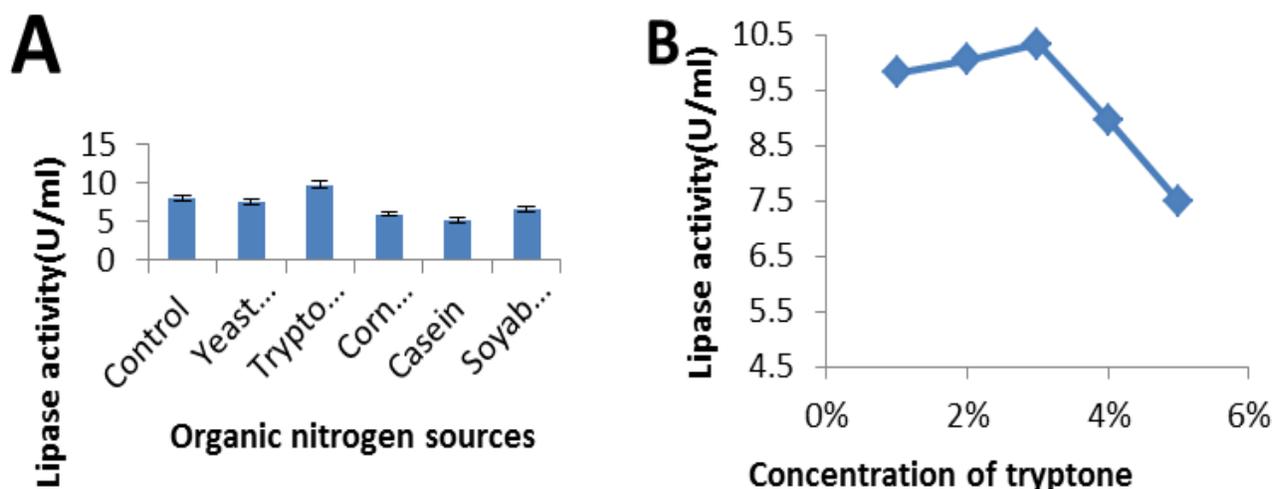


Figure 8. Effect of organic nitrogen sources (A) and concentration of tryptone (B) on lipase production.

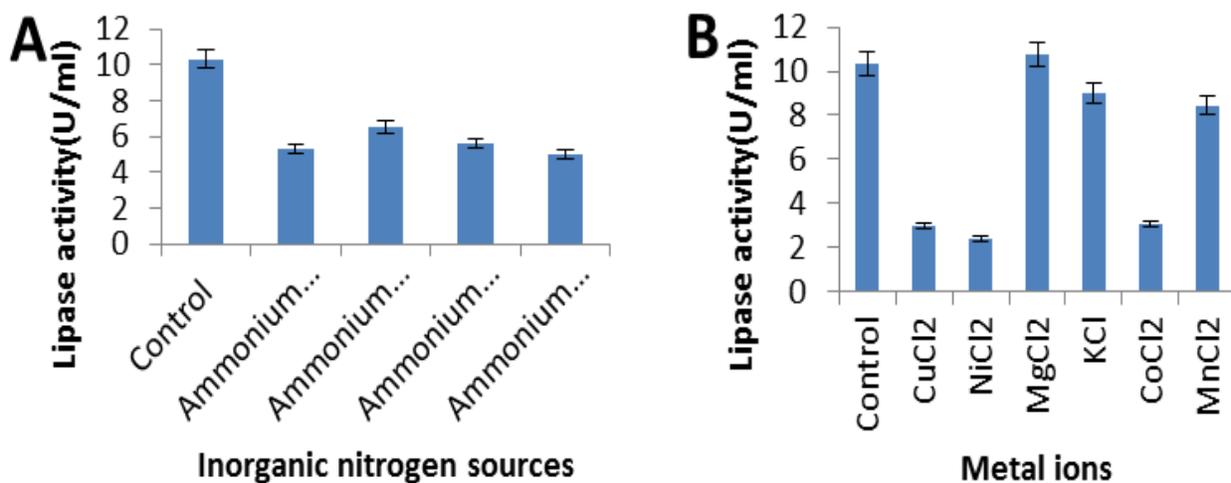


Figure 9. Effect of inorganic nitrogen sources (A) and metal ions (B) on lipase production.

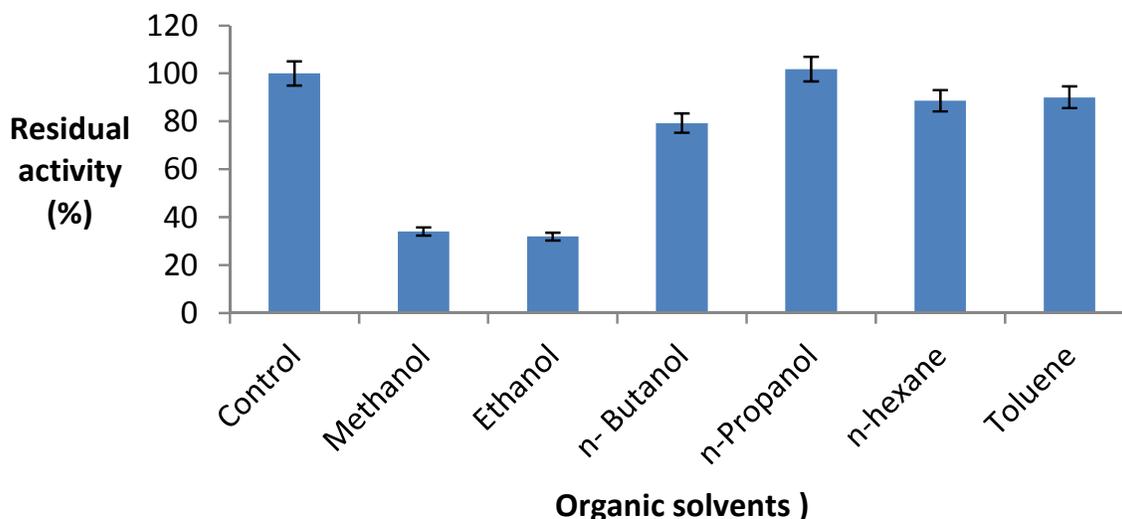


Figure 10. Effect of organic solvents on lipase stability. Residual activity was compared with the control without organic solvent. 100% was assigned to the control sample.

from a new isolate *Staphylococcus sp.* was optimized. The final optimized medium resulted in an overall 2.5 fold enhanced lipase production. This study presents significant observation on the use of 2% punnukka oil and 3% tryptone as carbon and nitrogen sources to significantly enhance the synthesis of lipase. It was also observed that the crude lipase was stable in various organic solvents with slight increase in activity when n-propanol was added to the basal medium. Therefore based on the above characteristics, this lipase may find applications in biodiesel production, oleochemical industry, polymer synthesis and triglycerides synthesis.

Conflicts of interest

The authors declare that they have no conflict of interest.

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