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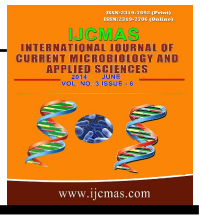

Original Research Article

Production and characterization of lipase from *Staphylococcus* sp. SDMlip

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A B S T R A C T

In this study, *Staphylococcus* sp. SDMlip was isolated from the oil contaminated Rajpardi Lignite Mine water sample. Lipase production was optimized for oils, carbon sources, nitrogen sources, emulsifier and incubation period. All the oils were studied at 2% (v/v) concentration. 3% glucose addition gave 9.09 fold higher enzyme production as compared to initial unoptimized fermentation conditions. Lipase activity of 18.75UmL^{-1} was obtained at pH 7.0, in the presence of groundnut oil and glucose as carbon sources, gum acacia as an emulsifier and a combination of NH_4Cl and yeast extract (1:1) as nitrogen sources at 30 ºC after 48 h. Process optimization resulted in 56.82 fold enhancement in lipase production. The enzyme had a molecular weight of about 43 kDa as determined by SDS-PAGE. The dialyzed lipase showed maximum activity at 30ºC and pH 6.0. The enzyme was stable between pH 5.0 and 7.0 and temperatures up to 37 ºC. Lipase activity decreased in the presence of hydrophilic organic solvents tested, at a final concentration of 10% (v/v) for 1 h. Only Tween-20 retained 100% of the residual activity as compared to other surfactants. Lipase activity was significantly enhanced by Na^+, whereas other metal ions had no significant effect.

K e y w o r d s

Lipase production, *Staphylococcus* sp., Fermentation optimization, Characterization

Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) are enzymes that hydrolyze triacylglycerol at the oil-water interface and liberate free fatty acids and glycerol (Dheeman *et al*., 2010). Lipases belong to the class of serine hydrolases (Sharma *et al*., 2009) and contains the consensus sequence G-X_1-S-X_2-G as the catalytic moiety, where G = glycine, S = serine, X_1 = histidine and X_2 = glutamic or aspartic acid (Mala and Takeuchi, 2008; Seo *et el*., 2014). Lipases are widely distributed in plants, animals, insects and microorganisms (Bhumibhamon *et al*., 2003). Lipases from microorganisms have gained much importance mainly due to their availability and stability (Ginalska *et al*., 2004). The chemo-, regio- and enantio- specific behaviour of these enzymes has gained tremendous interest among scientists and industrialists (Gulati *et al*., 1999). Though only 5% industrial enzyme market is covered, lipases are important as biotechnologically valuable

enzyme (Sangeetha *et al*., 2011). Lipases are widely used in the processing of fats, oils and food, synthesis of fine chemicals, paper manufacturing, production of cosmetics, resolution of racemic drugs, formulation of detergents and degreasing agents (Bayoumi *et al*., 2007). Moreover, a promising application field for lipases is the polyhydroxyalkanoates and polycaprolactone (Gombert *et al*., 1999).

Microbial lipases are mainly produced by submerged culture but solid state fermentation can also be used for lipase production (Mahadik *et al*., 2002; Chaturvedi *et al*., 2010). But there are some problems associated with solid state fermentation, like designing scale up, control of heat transfer and cooling, control of oxygen transfer and diffusion of products (Chaturvedi *et al*., 2010). Therefore, submerged cultivation is more favourable for microbial lipase production.

The aim of the present work was to identify the selected bacterial culture isolated from the mine water sample, to determine the cultural conditions for lipase production and to characterize the produced lipase.

Materials and Methods

Isolation of lipase producing microorganisms

Lipase producing bacteria were isolated from oil contaminated water sample of Rajpardi lignite mine, Gujarat, India, by plating 0.1 mL of 1:100 diluted samples on 1% Tributyrene Agar (HiMedia, India) plates. All the plates were incubated at 30±2 ºC temperature. After incubation, well isolated bacterial colonies, giving zone of hydrolysis were picked up and purified isolates were maintained on nutrient agar (HiMedia, India) slants.

Screening for lipolytic activity

From the isolates, the culture colony which showed a maximum ratio of lipolytic zone diameter to colony diameter on the tributyrene agar plate, was selected. The selected isolate was maintained by monthly sub-culturing on nutrient agar slants and stored at 6.0±2 ºC.

Taxonomical studies of the isolate

The selected isolate was identified using routine biochemical tests following Bergey's Manual of Systematic Bacteriology (Sneath *et al*., 1986). Genomic DNA was isolated from the pure culture pellet using Genei Pure Bacterial DNA purification kit-117290 (Genei, India). The 16S rRNA gene fragment was amplified using high frequency PCR polymerase. Universal 16S rDNA primers and instant ligation kit 105611 were used in the process. The PCR product was cloned in pGEMT vector and 1.5 kb insert were sequenced in ABI 3100 (16 capillary) sequencer.

Inoculum preparation

Throughout the study, actively growing 16 h old culture from nutrient agar slant, was harvested in sterile normal saline to get 3.4×10^{10} cells mL^{-1} and from this suspension 10% (v/v) inoculum was added in the production medium. The cell density was measured using the turbidimetic method at 550 nm against normal saline as blank.

Chemicals

All chemicals and media ingredients used were of analytical grade from HiMedia, India and SD Fine Chemicals, India. Olive oil used was from Figaro (Spain) and all

other oils were of local brand available in the market.

Enzyme production

Staphylococcus sp. SDMlip was grown in a modified basal production medium containing (g L^{-1}): glucose 2.0; K_2HPO_4 1.0; NH_4Cl 5.0; $MgSO_4.7H_2O$ 0.1; coconut oil 2.0% (v/v) and pH 7.0 for enzyme production (Rathi *et al*., 2001). The cells were allowed to grow in this medium at 30±2 ºC on a rotary shaker, shaking at 150 rpm. Samples were withdrawn at different time interval and centrifuged at 10,000 g for 10 min at 4 ºC to get cell free supernatant. Enzyme activity was determined from the cell free supernatant.

Growth estimation

From each flask, 1 mL of the sample was withdrawn and 0.2 mL of 5% Triton X-100 (v/v) was added. The samples were centrifuged at 10,000 g for 10 min. The obtained pellets were suspended in 1 mL of phosphate buffer (0.01 M, pH 7.0) and absorbance was measured at 550 nm in a spectrophotometer (Systronics model 119, India) against the buffer blank (Rathi *et al*., 2001; Gupta *et al*., 2007).

Fermentation process optimization

The physico-chemical variables namely types of oil, various carbon sources, nitrogen sources, effect of emulsifier and incubation period studied are summarized in Table 1. The obtained optimum values were used in subsequent steps. If otherwise mentioned, all the experiments were carried out in triplicates in 250 mL Erlenmeyer flask containing 50 mL production medium of pH 7.0, inoculated with 10% (v/v) actively growing (3.4×10^{10} cells mL^{-1}) inoculum and incubated at 30±2 ºC temperature.

Lipase assay

Lipase activity was determined by the method of Macêdo *et al*., (1997), with the following modifications. Lipase assay was performed with olive oil emulsion, which was prepared by mixing 25 mL of olive oil and 75 mL of 7% gum acacia solution in a homogenizer. The reaction mixture containing 5 mL of olive oil emulsion, 2 mL of 10 mM phosphate citrate buffer (pH 7.0) and 1 mL of the culture broth supernatant was incubated at 30±2 ºC for 30 min in orbital shaker shaking at 150 rpm. The reaction was immediately stopped after incubation, by adding 1 mL of acetone-ethanol mixture (1:1, v/v) and the liberated free fatty acids were titrated with 0.05 N NaOH using phenolphthalein as an indicator. The assay was also performed using a phosphate citrate buffer of pH 5.0 and 6.0. One unit of lipase activity was defined as the amount of the enzyme, which liberated 1 µmol of fatty acid per min (Sharma *et al*., 2009).

Purification of lipase

At the post-exponential phase of growth, crude supernatant was separated from bacterial cells by centrifugation (10,000 g, 10 min) at 4 ºC and filtered through a 0.45 µm membrane filter. Subsequent steps were performed at 4 ºC. Protein, containing the lipase fraction, was precipitated by the addition of ammonium sulphate to 60-70% saturation. The saturated solution was maintained at 4 ºC with slow stirring for 24 h before being centrifuged for 17,000 g for 15 min. The pellet of precipitated protein was re-dissolved in 10 mM phosphate citrate buffer (pH 7.0) and desalted for 8-10 h by membrane dialysis in the same buffer. Following dialysis, the sample was stored at 4 ºC till use.

Molecular weight determination by SDS-PAGE

Dialyzed protein sample was separated on SDS-PAGE gels according to the method of Gallagher and Wiley, (2008). Samples were prepared by boiling in the sample loading buffer and loaded onto a 7% stacking gel and separated in a 12% separating gel. After electrophoresis, the gel was stained and then destained. The molecular weight of the visualized protein bands was determined by comparing them with the molecular weight markers.

Characterization of lipase

The effect of pH on lipase activity was determined in citrate phosphate buffer (pH 5.0 and 6.0) and potassium phosphate buffer (pH 7.0). The optimum pH obtained was used for the investigation of thermostability and other parameters. These buffers were used to determine pH stability of the partially purified lipase preparation. A mixture (1:1) of enzyme and buffers was incubated for 1 h at 30 ºC, and the standard enzyme assay described previously was performed. The residual activities were calculated by comparison with the activities in the respective controls without pre-incubation.

Lipase activity was measured at various temperatures (10-50 ºC) under standard assay conditions. Thermal stability of the partially purified enzyme was investigated by pre-incubating the enzyme with equal volume of buffer of pH 6.0 at various temperatures (10-50 ºC) for 1 h followed by residual lipase activity estimation at 30 ºC and pH 6.0 under standard assay conditions.

The effect of hydrophilic and hydrophobic organic solvents (20% v/v) on crude lipase stability was investigated, for which equal volumes of organic solvent was added to cell-free supernatant and incubated at 30 ºC for 1 h at pH 6.0. The samples were then assayed for remaining activity relative to the control without solvent.

Effect of various surfactants namely, Triton X-100, Tween-20, Tween-80 and SDS, at a concentration of 0.2%, was checked by pre-incubating enzyme with equal volume of surfactant for 1 h followed by residual activity estimation.

The influence of metal ions on lipase activity was studied by incubating the enzyme in the presence of 1 mM of metal ions (Sn^{2+}, K^{+}, Ca^{2+}, Co^{2+} and Na^{+}), which were added as their chloride salts. Incubation was carried out at 30 ºC for 1 h and assayed for lipase activity. In enzyme characterization studies, all solutions were prepared in buffer of pH 6.0, except in study of pH stability.

Results and Discussion

Isolation of lipase producers

Accumulated water from the Rajpardi lignite mine showed the pH, redox potential, total dissolved solids and sulphate as 3.1, 390 mV, 1.21 ppt and 5 g L^{-1} respectively and traces of dispersed oil droplets. On 1% Tributyrene agar plate, 8 morphologically different bacterial isolates showed the zone of hydrolysis. Among the isolates studied, the Gram positive cocci, which gave the largest hydrolytic zone diameter ratio of 5.66 was selected for further investigation.

Taxonomical studies of the isolate

The isolate was found to utilize glucose, adonitol, arabinose, lactose, sorbitol, mannitol, sucrose, maltose, fructose, lysine, citrate and gave positive reactions

for ornithine decarboxylation, nitrate reduction, catalase; whereas, it gave negative results for urease, oxidase, amylase, protease production, phenylalanine deamination, citrate utilization and H_2S production. The isolate was unable to utilize xylose. Based on colony morphology, cell morphology and studied biochemical tests, the isolate was identified as *Staphylococcus* sp. Finding of 16S rRNA sequence analysis also confirmed the identity of the isolate as *Staphylococcus* sp. The sequence was deposited in GenBank with accession number HQ262547 and the strain was designated as *Staphylococcus* sp. SDMlip. The isolate showed 98% homology to *Staphylococcus hominis* strain KSI 1345 (GenBank accession number KC113159).

Process optimization for lipase production

Results on oils with long chain saturated, monounsaturated and polyunsaturated fatty acids, tested at 2% (v/v) concentration, showed that the enzyme was more efficient to act on oils with long chain monounsaturated fatty acids. As shown in Fig. 1 (a), among the various oils used in this study as an inducer, under the experimental conditions, maximum lipase production of 0.66 U mL^{-1} was achieved with olive oil followed by groundnut oil (0.5 U mL^{-1}), cottonseed oil (0.4 U mL^{-1}), coconut oil (0.33 U mL^{-1}) and sesame oil (0.16 U mL^{-1}), in the presence of 0.2% glucose. Gupta *et al*., (2004) and Acikel *et al*., (2011) have also reported variations in induction of lipase production depending on the type of lipid sources used. But keeping in mind the cost and bulk availability, groundnut oil was selected for further studies. As depicted in Fig. 1 (b), results of optimization of glucose concentration showed the highest lipase activity of 3.0 U mL^{-1} in fermentation broth having 3% (w/v) of glucose concentration and 2% (v/v) of groundnut oil. Above this glucose concentration, enzyme production decreased.

Among the various inorganic and organic nitrogen sources tested, the highest lipase activity increased from 3.0 U mL^{-1} to 12.5 U mL^{-1} in the presence of a combination of (1+1)% of NH_4Cl and yeast extract at 30±2 ºC (Fig. 2). This comes out to be 4.16 fold rise in enzyme production as compared to the production in 0.5% NH_4Cl containing medium. Yeast extract act as a nitrogen source as well as it supplies vitamins and trace metals, thereby affecting the growth of the organism and thus increasing lipase production (Gupta *et al*., 2007). Addition of urea significantly decreased lipase production. Dheeman *et al*., (2010) also reported urea as an inhibitor for lipase production by *Amycolatopsis mediterranei* DSM 43304. From the obtained results, it can be said that among the nitrogen sources studied, organic nitrogen sources are more suited for lipase production by the staphylococcal isolate as compared to inorganic nitrogen sources. Nitrogen sources were found to play more significant role as compared to carbon sources studied.

Gum acacia as an emulsifier has been reported to enhance lipase production (Gulati *et al*., 1999). It is clear from Fig. 3 that enzyme production started in exponential phase and reached a peak in stationary phase, as was also observed by many workers (Rathi *et al*., 2001; Gupta *et al*., 2004). Enzyme activity increased with time up to 48 h of fermentation and then decreased, irrespective of the presence of gum acacia. In the absence of gum acacia, cell growth was maximum at 48 h and then rapid decline was observed; whereas

in the presence of 1% gum acacia, cell growth was maximum at 29 h of incubation and then decreased slowly, although enzyme activity continuously increased up to 48 h. The enzyme activity of emulsified medium was 1.5 fold higher as compared to non-emulsified medium that gave 12.5 U mL^{-1} lipase activity. The enzyme was found to be more active at pH 6.0 as compared to pH 7.0, as the activity was 14.6 U mL^{-1} at pH 6.0 and it decreased to 13.5 and 12.5 at pH 5.0 and 7.0, respectively.

Characterization of lipase

It was observed that ammonium sulphate, between 60% and 70% of saturation, resulted in the maximum lipase recovery and it comes out to 21.85 fold increase in purification (Table 2). Dialysis was performed for this fraction and the lipase was purified to 35.50 fold with a specific activity of 486.45 U mg^{-1} protein. The lipase showed a molecular weight of approximately 43 kDa. As per the report of Mala and Takeuchi, (2008), microbial lipases are of 20-60 kDa proteins.

As can be seen from Fig. 4 (a), for the *Staphylococcus* sp. SDMlip lipase, alkaline pH was found more inhibitory as compared to acidic pH. The lipase showed activity and stability, at acidic pH values. Mahadik *et al*., (2002) found acidic lipase from *A. niger*, where optimum activity was between pH 2.5 and 3.0, with a sharp decline above pH 3.5. The lipase was found to be quite stable over a pH range of 5.0 to 7.0. At pH 6.0, the highest residual activity of 98.27% (4.83 U mL^{-1}) was achieved, which decreased to 92.46% (4.42 U mL^{-1}) and 84% (4.17 U mL^{-1}) at pH 5.0 and 7.0, respectively. Similar results were also reported in the literature (Oterholm *et al*., 1970; Ginalska *et al*., 2004); whereas Kumar *et al*., (2005) and Bhushan *et al*., (2008) have found alkaline pH more suitable for the optimum enzyme activity and stability.

The lipase hydrolyzed olive oil in the temperature range of 10-45ºC, with a maximum at 30ºC (Fig. 4b). The lipase was quite stable at temperature of 30 ºC and 37ºC, retaining 83.33% and 76% of the lipase activity after 1 h incubation. The enzyme also showed > 50% of the residual activity after 1 h incubation at 45ºC, whereas 100% activity was retained at 10ºC. Maia *et al*., (2001) also found that lipase from *F. solani* FS1 was stable below 35 ºC and above this significant losses were observed.

Solvents, namely, ethanol, acetone, isopropanol and pyridine, at a final concentration of 10% (v/v), were tested for their effect on the enzyme activity and the results are depicted in Fig. 5 (a). These solvents showed residual activity of 57.14%, 64.29%, 50.01% and 71.44% respectively; whereas an increase in residual activity by 9%, 10% and 25% was found by Sharma *et al.*, (2009) for acetone, ethanol and isopropanol, respectively. Shabtai and Mishne, (1992) reported that pyridine caused 90% loss in enzyme activity after 24 h incubation. The *Staphylococcus* sp. SDMlip lipase enzyme also showed 28.56% loss within 1 h. Lipases are diverse in their sensitivity to organic solvents, but there is a general agreement that water immiscible lipophilic solvents retain enzyme's high catalytic activity as compared to water miscible solvents because they do not strip off the crucial bound water from the enzyme's surface (Maia *et al*., 2001; Dheeman *et al*., 2010).

As evident from Fig. 5 (b), among various surfactants, Tween-20 had no effect; whereas Triton X-100 and SDS retained

85.47% and 84.55% of the enzyme activity, respectively, while Tween-80 showed 76.37% of residual activity. These surfactants were used at a final concentration of 0.2%. Similar results were also reported by Dutta and Ray. (2009). Kundu *et al*., (1987) reported that SDS (2 and 20 mM) did not affect lipase activity. The activity of lipase from *Bacillus stearothermophilus* P1 was enhanced in the presence of Triton X-100, as was reported by Bayoumi *et al*., (2007).

Table.1 Parameters studied for process optimization

No.	Parameters	Values studied
1.	Oils - coconut, olive, sesame, groundnut, cottonseed (% v/v)	2.0
2.	Glucose concentration (% w/v)	1.0, 2.0, 3.0, 4.0, 5.0
3.	Nitrogen sources (% w/v):	
	NH_4Cl	0.5, 1.0, 2.0
	Urea	2.0
	Peptone	2.0
	Yeast extract	2.0
	NH_4Cl + yeast extract (1+1)%	At a final concentration of 2.0
	Yeast extract + peptone (1+1)%	
	Yeast extract + peptone(1.5+0.5)%	
	Yeast extract + peptone (1.75+0.25)%	
	Yeast extract + peptone(0.5+1.5)%	
	Yeast extract + peptone (0.25+1.75)%	
4.	Incubation period (h) with and without 1% gum acacia	0 to 50

Table.2 Purification steps of lipase from *Staphylococcus* sp. SDMlip

Purification steps	Lipase activity ($U\ mL^{-1}$)	Total lipase activity (U)	Total protein (mg)	Specific activity ($U\ mg^{-1}$)	Purification fold	Recovery (%)
Crude enzyme	12.5	525	38.30	13.70	1.0	100
60-70% $(NH_4)_2SO_4$ fraction	33.67	134.68	0.45	299.29	21.85	25.65
Dialyzed enzyme	23.35	116.75	0.24	486.45	35.50	22.23

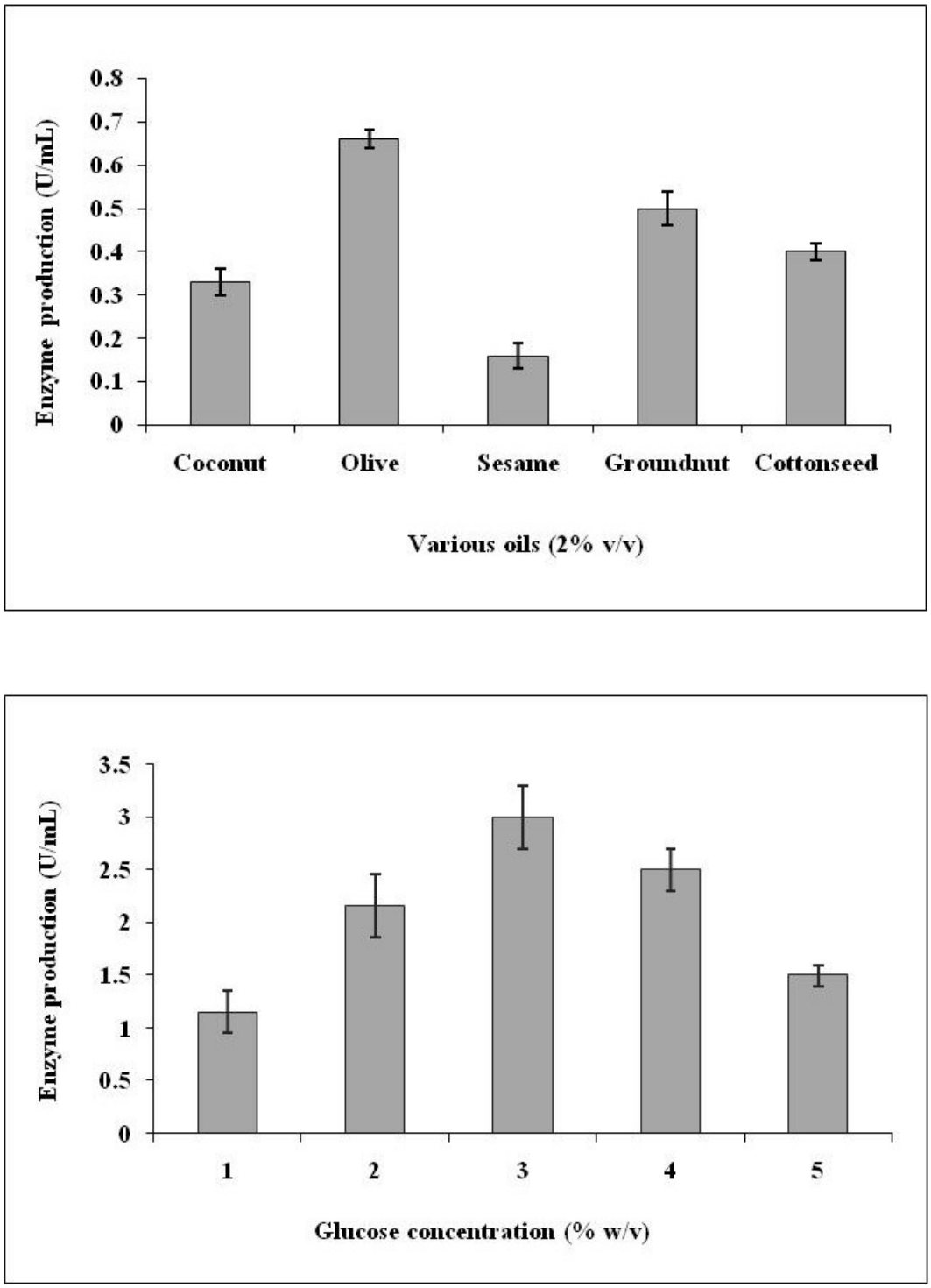


Figure.1 (a) Induction of lipase production by different oils (b) Effect of various glucose concentrations on lipase production

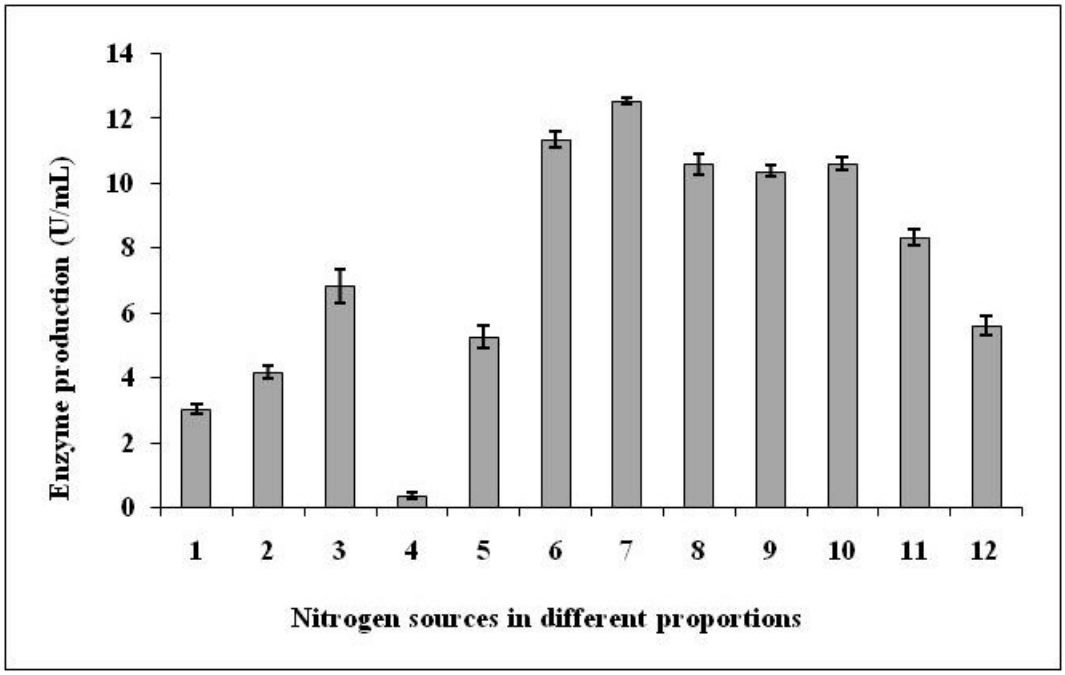


Figure. 2 Effect of nitrogen sources and their combinations in different proportions on lipase production. 1. NH_4Cl (0.5%); 2. NH_4Cl (1%); 3. NH_4Cl (2%); 4. Urea (2%); 5. Peptone (2%); 6. Yeast extract (2%); 7. NH_4Cl+yeast extract (1+1)%; 8. Yeast extract+peptone (1+1)%; 9. Yeast extract+peptone (1.5+0.5)%; 10. Yeast extract+peptone (1.75+0.25)%; 11. Yeast extract+peptone (0.5+1.5)%; 12. Yeast extract+peptone (0.25+1.75)%

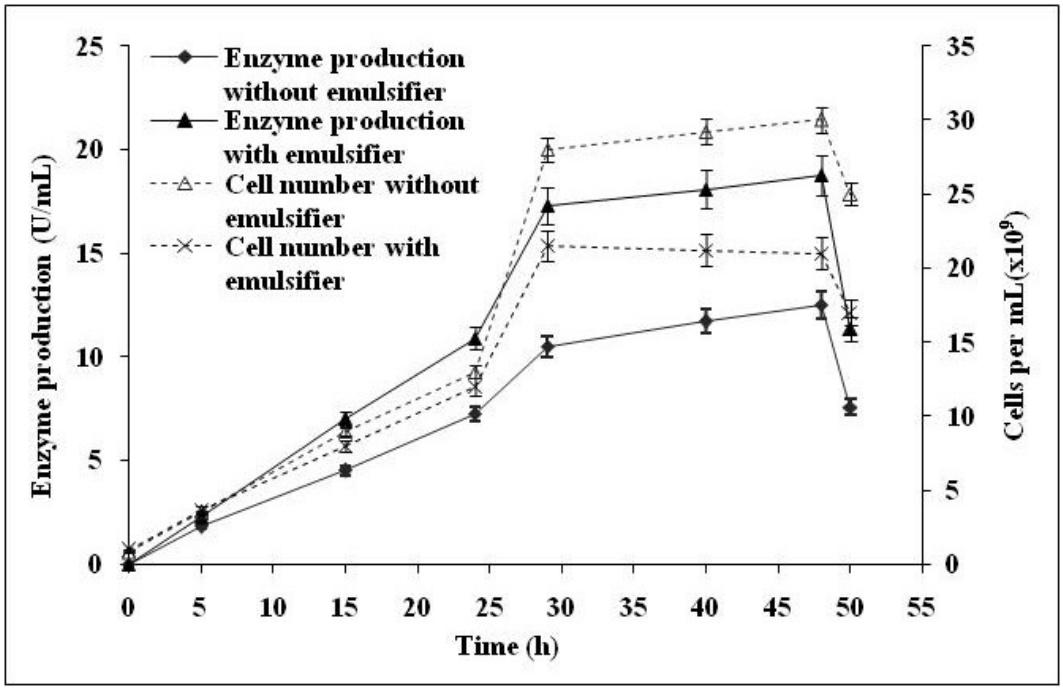


Figure.3 Effect of incubation time on lipase production and cell growth in absence and presence of 1% gum acacia

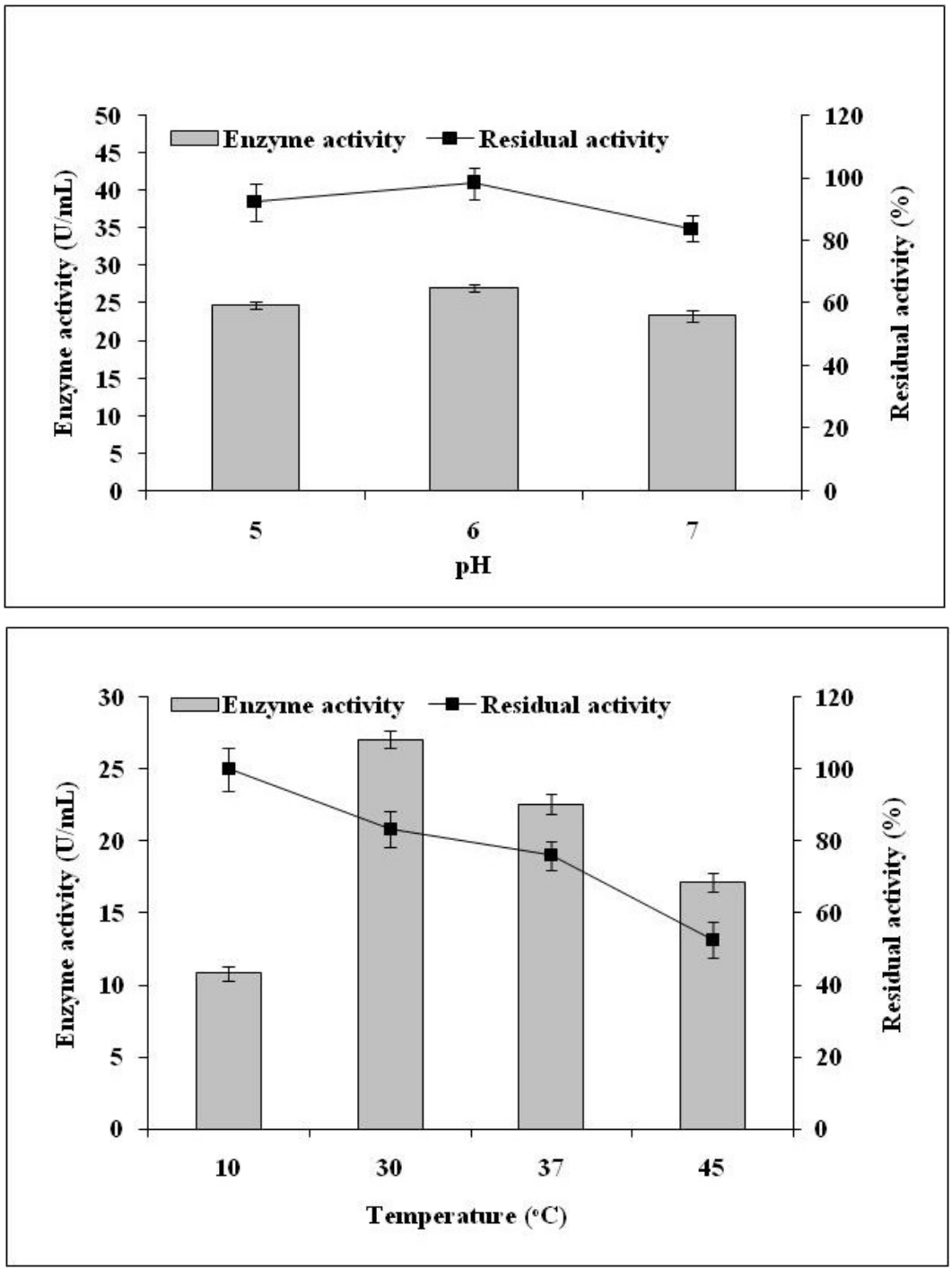
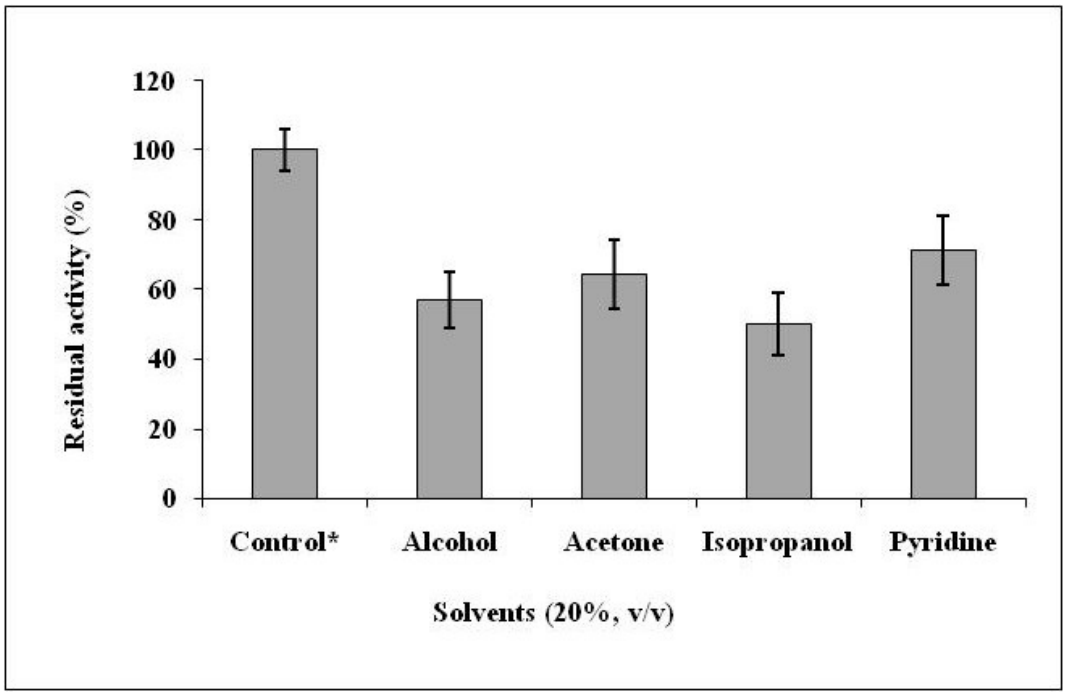
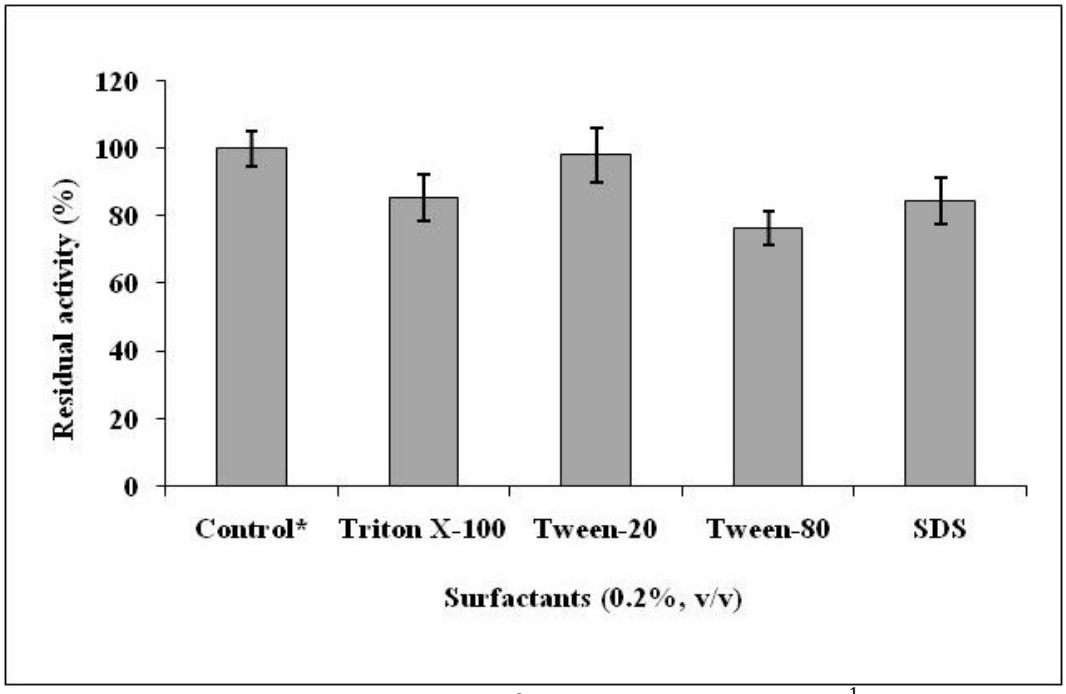


Figure. 4 Effect of (a) pH and (b) temperature on enzyme activity and stability

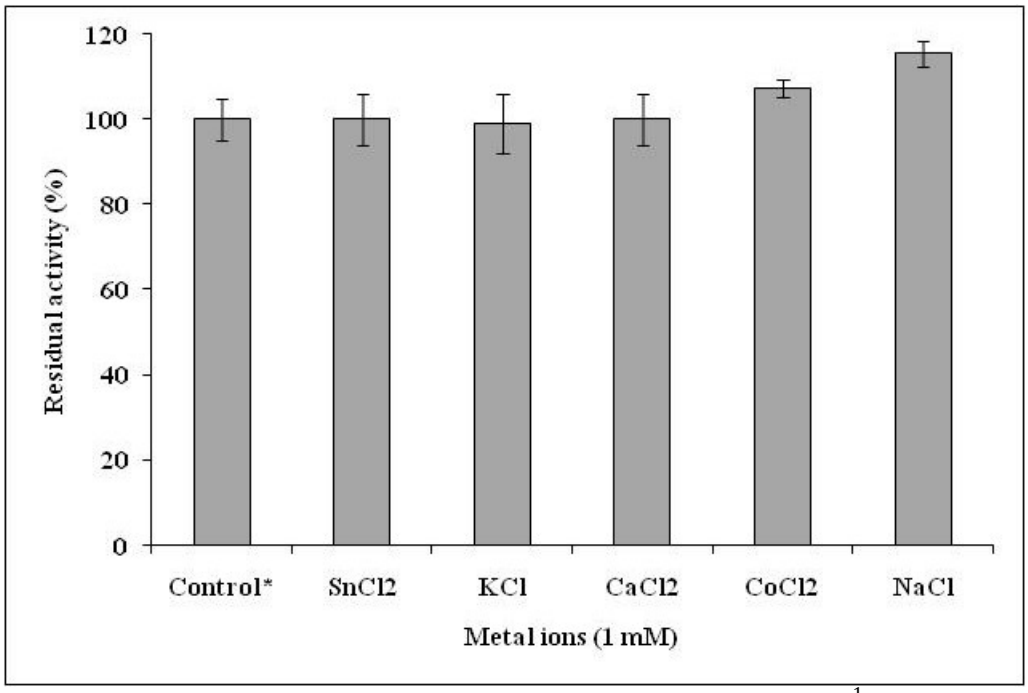


*Value of control was 14.37 U mL^{-1}.



* Value of control was 14.37 U mL^{-1}.

Figure.5 Effect of (a) solvents and (b) surfactants on lipase stability



* Value of control was 14.37 U mL^{-1}

Figure.6 Effect of metal ions on lipase stability

Staphylococcus sp. SDMlip lipase activity data in Fig. 6 showed that the crude lipase was unaffected by the presence of $SnCl_2$, $CaCl_2$ and KCl; whereas $CoCl_2$ and NaCl enhanced the residual activity by 7.29% and 15.47%, respectively. Two thoughts exist; some scientists found Ca^{2+} as enhancer (Henderson, 1971; Kim *et al*., 2009); whereas some found it inhibitory (Choo *et al*., 1998; Oterholm *et al*., 1970). The negative effect of ions on the lipase is the result from direct inhibition by the catalytic site (Dutta and Ray, 2009). The stimulatory effect of NaCl was consistent with the results of Bhumibhamon *et al*., (2003). This effect may be due to the ability of the salts to react with free fatty acids adhered to the droplets and hence increasing their surface area (Anguita *et al*., 1993).

From the obtained results, it can be concluded that the isolate *Staphylococcus* sp. SDMlip was found to be the most efficient lipase producer among the isolates studied from the mine water sample. Based on the studies, the optimized fermentation medium for lipase production by *Staphylococcus* sp. SDMlip is (g L^{-1}): glucose 30; K_2HPO_4 1.0; NH_4Cl 10; yeast extract 10; $MgSO_4.7H_2O$ 0.1; gum acacia 10; groundnut oil 2% (v/v); pH 7.0 at 30 ºC. Optimization resulted in a 56.82 fold increase in lipase production (18.75 U mL^{-1}) as compared to unoptimized conditions (0.33 U mL^{-1}) and optimum pH for the activity of the produced enzyme is 6.0. Following $(NH_4)_2SO_4$ precipitation, the enzyme was partially purified to 35.50 fold by dialysis. This 43 kDa lipase showed maximum activity at 30 ºC and pH 6.0. The enzyme was stable between pH 5.0 and 7.0 and temperatures up to 37 ºC. Lipase activity was decreased in the presence of hydrophilic organic solvents tested. Only Tween-20 retained 100% of the residual activity and the enzyme also remain active in the presence of metal ions tested, with calcium being enhancer.

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