Development of modified medium for enhanced production of lipase by *Streptomyces* halstedii strain ST 70 obtained from Bhitarkanika mangroves

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Abstract
The lipase production by *Streptomyces halstedii* strain ST 70, a mangrove isolate was enhanced through the modifications of cultural conditions and nutritional amendments. The strain produced 26.1 U/mL enzyme in presence of starch, glycine and ammonium chloride that was enhanced upto 56.1 U/ml with 50% ammonium sulphate precipitation. Results showed that tween 80 is required as inducer to enhance the lipase production. The enzyme was found to be stable at various temperature and pH where as saline condition did not favour higher enzyme activity. The present study reveals importance towards the industrial level exploitation of this strain due to its origin and only few *Streptomyces* strains are reported for lipase production.

Key words: Lipase, *Streptomyces*, mangrove, nutrient, carbon source, nitrogensource

INTRODUCTION

The lipases are enzymes and important for physiological and biotechnological point of view. They catalyze both to the hydrolysis and synthesis of esters. Most of these enzymes are commercially available, being mostly from microbial source produced in extracellular condition (Sztajer et al, 1988, Rapp and Backhaus, 1992; Sharma et al., 2004). Microbial lipases have biotechnological impact due their practical properties, like stability in organic solvents, independence of cofactors, widespectrum substrate specificity (Lescic et al, 2004). Lipases producers have been isolated mainly from soil, or spoiled food material that contain vegetable oils. Lipases with novel properties have been discovered from microorganisms isolated from hot springs (Lee et al., 1999), compost heaps (Rathi et al., 2000) and highly salty and sugary environments (Ghanem et al. 2000). Mangrove ecosystem is also one of the important habitats for microbial activity and not studied well as far as lipase producers are concerned. In search of novel lipases, we have targeted *Streptomyces* from Bhitarkanika mangroves of Orissa.
Streptomyces are soil bacteria and produces numerous metabolites and enzymes including lipases (Xiang et al., 2006; Sommer et al., 1997; Siva Kumar et al, 2005). According to previously described results it seems that members of this genus are not typically lipase producers compared to other bacteria (Vujaklija et al., 2003) . Despite their potential , only a few studies have been reported on their esterase and lipolytic activities (Molinari, 2000; Lescic et al., 2001 ; Gandolfi et al., 2000 ; Udeya et al., 2005). So far, only few Streptomycetes lipases have been described mainly *S.albus, S. coelicolor, S. exfoliates, S. cinnamomeus , Streptomyces rimosus, S. lividans S. clavuligerus, S. diastatochromogens* (Large et al. 1999; Tesch et al., 1996 ; Abamic et al., 1999). A gene encoding an extracellualr lipase from *Streptomyces* sp M11 was cloned in the high copy number vector using *S. lividans* 66 as host (Perez etal.,1993).
Natural enzymes including lipases, don't always exhibit the desired. Therefore, further steps to optimize biocatalyst should be employed (Bancerz et al., 2005). Improvement of lipase production still depends on the optimization of culture conditions, including the composition of culture medium. There are several reports on optimizing carbon and nitrogen sources (Wei et al., 2004; Dalmau et al., 2000). The present paper deals with the screening of lipase producing Streptomycetes strains isolated from mangrove ecosystem of Bhitarkanika and the improvement of culture conditions for enzyme production by the selected strains. We also characterize the activity and stability of crude extracellular filtrate containing lipase from *S. helstedii* , a *Streptomyces* isolate in presence of differentpH and temperature etc.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade and were purchased from Hi media, SRL, Merck. Various oil substrates were from local market.

Sourceof Organism:
Streptomyces were isolated from mud flat and phyllosphere of mangrove tree species found naturally in mangroves of Bhitarkanika, Orissa state ofIndia.
Isolation : *Streptomyces* strain were isolated by serial dilution method on ISP 3, 4,5,6,7 and 9 media (Hi – media) which were afterwards incubated at 30º C and 37 ºC. *Streptomyces* were isolated from plates that

contained well separated colonies.
Preliminary screening of strains for lipase activity
The preliminary selection of lipase producers was done on lipase test medium in which positive strain had shown clear zone. Second screening was done by titrametric method and the organisms given higher activity was selected for the nutritional amendments experiments.

Basal growth medium and culture conditions:
Selected strain of *Streptomyces* was grown in two different media starch casein medium and lipase test medium for two incubation period (7 and 15 days) along with the different combination of inducer substrate in the medium and substrate for enzyme activity. The enzyme produced by this organism was tested for 1 hr and 4 hr. duration. Finally, Starch casein medium of 7.2 pH (Starch 10g, Casein 0.3g, K2HPO4-2g, KNO3-2g, NaCl-2g, FeSO4-0.01g, CaCO3-0.02g,MgSO4-0.05g per litre) , 7 days incubation, Tween 80 as inducer substrate (1ml/ 100ml medium) , tween 20 for enzyme substrate and 4 hr. as enzyme incubation was selected for the further improvement of nutritional and cultural conditions.
Enzymeassay:
At the end of incubation period, the mycelium was removed from the Culture of *S. helstedii* by filtration and centrifugation at 6000 rpm for 30 min. The supernatant used as the source of lipase was termed as crude lipase. Lipase activity was determined by the titrametric method. In which, 25 ml of olive oil was homogenized with 75 ml of 2% polyvinyl alcohol and used as the substrate. The substrate emulsion (5ml) and 4 ml of 0.1 M sodium phosphate buffer, pH 7.0 were preincubated at 37° C for 10 min and 1 ml of the enzyme solution was then added and incubated at 37° C for 20 min. The reaction was terminated by the addition of 20 ml of acetone and titrated against 0.01 M sodium hydroxide. The heat-inactivated enzyme was added to the reaction mixture as control. One unit of lipase activity was defined as the amount of enzyme that released free fatty acids in 1 min. under standard assay conditions. Controls were performed with preheatedenzymesamples. All assays were carried out in triplicate and cold conditions.
Experiment1:
Standardization of basic starch casein medium .The organisms was grown with following modifications in basic starch casein medium of 7.2 pH supplemented with Tween 80 (1%) at 37ºC for 7days.
1.1 Effectof carbonsources:
The effect of carbon sources was studied by carrying out the lipase production in basic starch casein medium with varying types of carbon sources (1 %) namely , starch, dextrose, sucrose, lactose, manitol, carboxyl methyl cellulose, inulin , raffinose, fructose and inositol.

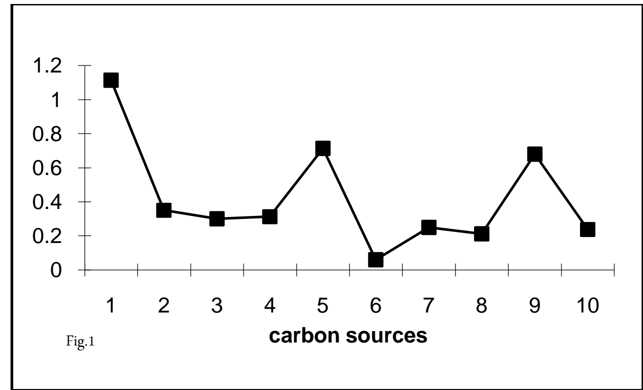

Fig. 1 Effect of carbons sources on lipase production
1=starch,2=dextrose,3=sucrose,4=lactose,5=manitoal, 6=CMC,7=inulin8=raffinose,9=fructose,10=inositol
1.2 Effect of nitrogen source
With basal starch casein medium four different nitrogen sources were taken i.e. casein, aspargin, gelatin and peptone @1% separately.
1.3 Effect of aminoacids
Twelve different types of amino acids viz., thronine, glutamine, phenylalanine, lysine, leucine, alanine, tryptophan, proline, tyrosine, arginine , glycine, methionine were taken in the basal starch casein medium in place of casein @ 1 %.

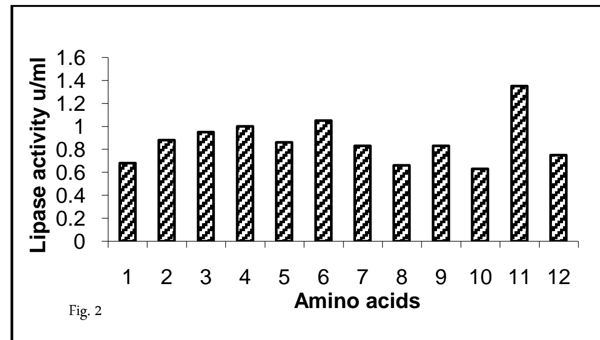

Fig.2 Effect of amino acids on lipase production
1= threonine, 2=glutamine, 3=phenylalanine, 4=lysine, 5=leucine, 6=alanine, 7=tryptophan, 8=proline, 9=tyrosine, 10=arginine, 11=glycine, 12= methionine
1.4 Effect ofconcentrationof starch
In starch casein medium finally starch was selected as carbon source and taken into the different vessel @ 1%, 3%, 5%, 7%, 11%, 13% and 15%.

1.5 Effect of glycine%
In basal starch casein medium (without casein), glycine was taken in different percentage (0.5, 1.0, 1.5, 2.0, 3.0, 3.5, 4.0, 4.5, 5.0) to see the effect on lipase activity.

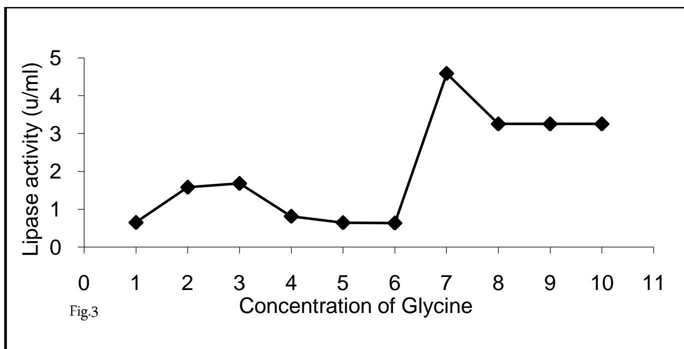

Fig. 3 Effect of concentration of glycine on lipase activity
1=0.5, 2=1.0, 3=1.5, 4=2.0, 5=2.5, 6=3.0, 7=3.5, 8=4.0, 9=4.5, 10=5.0

1.6 Effect of phosphatesource
To see the effect, two potassium and sodium phosphate source (K2HPO4, KH2PO4, Na2HPO4 and NaH2PO4) were added in the basal starch casein medium at the rate of 1 %.
1.7 Effect of NaCl
The effect of NaCl was determined by addition of NaCl in different concentration (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 %) separately in to the basal Starch casein medium.
1.8 Effect of salts in combination with starch and glycine.
With starch (5%) and glycine (1.5%), KNO3 & KH2PO4 (0.2, 0.5, 1.0, 1.5%), NaCl (0.5, 1.0 %) was added to determine the enzyme activity in culture filtrate of *Streptomyces* strain .
Experiment 2: modification of carbon and nitrogen sources
To determine the individual effect of selected carbon and nitrogen source following media were prepared (7.2 pH), supplemented with 1% Tween 80 and lipase assay was done in culture filtrate from 7days old culture developed at 37 °C.
2.1 Effect of starch and glycine in combination
Different combination of starch (3, 5, 7, 9, 11, 13, 15, 17, and 20 %) and glycine (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 %) to see the effect on enzyme activity. Medium (7.2 pH) containing only these two ingredients were prepared.

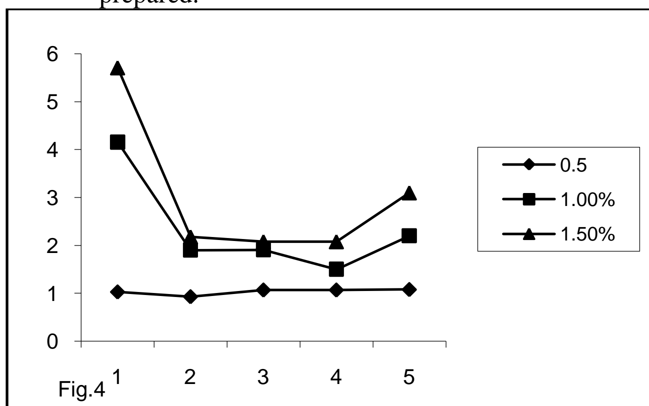

Fig.4 Effect of Higher concentration of starch and lower concentration of glycine on lipase activity
1=11%, 2=13%,3=15%, 4=17%, 5=19%

Experiment 3: Addition of other component in modified medium
3.1 Effect of urea
With starch (11%) and glycine (1.5%) , different concentration of urea (0.5,1.0, 1.5 and 2.0%) was added to prepared a medium and organisms was grown to see the lipase production.
3.2 Effects of salts
With starch (11%) and glycine (1.5%), KNO3, KH2PO4 (0.2% both), NaCl (1%) and Urea (0.5 %) was added separately to determine the enzyme activity in culture filtrate of *Streptomyces* strain . In separate experiment, these salts were added into the medium (containing starch 11 % and glycine 1.5%) in different combination with similar concentration.
3.3 Effect of ammonium salts
Nine different ammonium salts viz., chloride, ferrous sulphate, fluoride, metavandate, molybdate, sulphate, dihydrogen orthrophospahte, nitrate, oxalate at the rate of 0.5% concentration were added separately with Starch 11 % and glycine 1.5 % and medium was prepared.
3.4 Effect of concentration of ammonium salts three ammonium salts i.e. chloride, nitrate, and dihydrogen phosphate (1.0, 1.5, 2.5, 3.5%) were selected for the preparation of medium in addition to starch 11 % and glycine 5% and culture was grown to see the enhancement in lipase activity.
3.5 Effect of glycerol
Glycerol with the concentration of 1, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5 % was added with starch 11 % and glycine 5% and new mediumwas prepared.
3.6 Effect of tween 80
In new combination of starch 11 % and glycine 1.5%, tween 80 was in four concentration i.e. 0.5, 1.0, 3.0 and 5 % added and medium was prepared.
3.7 Effect of ammonium chloride
Different concentration of ammonium chloride (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5,10.5,11.5, 12.5, 13.5, 14.5, 15.5, 16.5, 17.5, 18.5, 19.5, and 20.5%) was added into the new medium along with starch 11% and glycine 1.5%.
3.8 Effect of ammonium nitrate
Different concentration of ammonium nitrate (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5 %) was added into the new medium along with starch 11% and glycine1.5%.
3.9 Effect of other amino acids
Other than starch 11% and glycine 1.5 %, lysine, alanine (1.5%) was added separately and in combination and medium was prepared.
Experiment–4
To determine the combined effect of selected carbon and nitrogen source, ammonium chloride following media were prepared (7.2 pH), supplemented with other amino acids and salts and lipase assay was done

in culture filtrate from 7day oldculture developed at 37 ℃.

4.1 Effect of lysine
Other than starch 11% , glycine 1.5 %, and 15.5 % ammonium chloride, lysine was added in concentration of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5 % and medium was prepared.
4.2 Effect of alanine
Other than starch 11% , glycine 1.5 %, and 15.5 % ammonium chloride, alanine was added in concentration of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5 % and medium was prepared.

4.3 Effect of combination of lysine and alanine
Other than starch 11%, glycine 1.5 %, and 15.5 % ammonium chloride, lysine and alanine was added in combination of equal concentration i.e. 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5 % and medium was prepared.

4.4 Effect of other ingredients in modified medium
Other than starch (11%), glycine (1.5 %), and ammonium chloride (15.5 %), lysine (0.5 %) and alanine (0.5 %), KH2PO4, KNO3, NaCl, Urea and glycerol separately and in combination was added and modified medium was prepared.

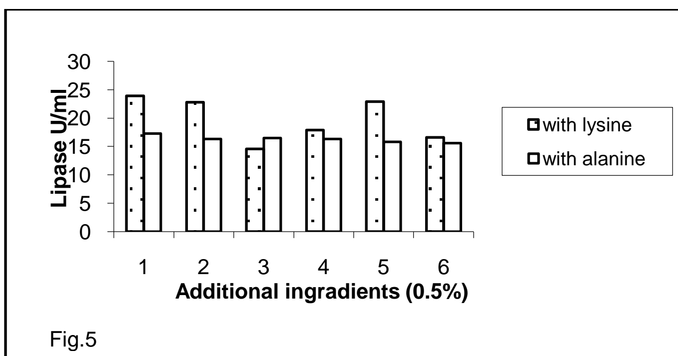

Fig.5 Effect of additon of ingredients in the modified medium Starch 11%, Glycine 1.5%, Amonium chloride 15.5% Lysine 0.5% or alanine 0.5%
1= KH2PO4, 2=KNO3, 3=NaCl, 4= KH2PO4+ KNO3+NaCl, 5= Urea, 6= Glycerol

Experiment –5 Effect of culture conditions
The modified medium containing starch 11 %, glycine 1.5%, ammonium chloride 15.5% , lysine 0.5% and 0.2 % KH2PO4 was inoculated with *Streptomyces* strain and grown under static and shake condition with different rpm (50,75 and 100) and culture vessel (100, 150, 250, 500 and 1000 ml) to determine the lipase activity.
Experiment –6 Partial purification of lipase produced in modified medium
6.1 Ammoniumsulphate purification
The enzyme produced by the *Streptomyces* strain grown in in modified medium under shake culture condition (50 rpm and/ or 100 rpm) was partially purified in separate way by different concentration of ammonium sulphate i.e. 5, 10,15,20, 40,50, 55, 60,65, 70, 75 and 80%.
6.2 Effect of pH on lipase
The pH optimum was tested using 0.1 M sodium phosphate buffer in the lipase (crude and partial purified) assay at wide range of pH (5.7, 6.5, 7.0, 7.5, 8.0).
6.3 Effect of NaCl on crude and purified enzyme
The crude and partially purified enzyme was treated with NaCl (1 and 3 %) and assayed for the activity.
6.4 Effect of Temperature
The effect of temperature on activity of lipase produced in modified medium under shake culture condition (50 rpm) was studied by carrying out the enzyme reaction at different temperature at 30, 37, 42 and 25 ℃ at pH 7.2.

RESULTSAND DISCUSSION
Microbial lipases and esterases are important industrial enzymes. Only few reports exist on production of these useful enzymes from *Streptomyces* strains (Xiang et al., 2006). Hence, present study was planned to obtain novel enzymes from microbes of difficult sources.
In the present study, the Starch casein medium was tested to maximize lipase production by the selected strain *S. helstedii*. The initial lipase activity was 2.5 units /ml in 7 days in the culture supernatant of the basal medium and lipase production was substantially enhanced by consecutive optimization of the basal medium.
Carbon is the main component of cells. It has been reported that various fats, fatty acids, plant oils, triglycerids, ester based detergents, and other substances were the best inducers of lipase synthesis by microorganisms and sources of carbon (Benzamine et al, 1995, Petrovic et al., 1990). A range of different carbon sources mainly carbohydrates were screened for their capacity to support growth of *S. helstedii* cultures and lipase production. As shown in Fig. 1 and 2 starch appeared to be the best carbon source for lipase production. This finding is in accordance with studies on other microbes (Ray et al., 1999).The activity of lipase produced under this condition was about 1.13 u/ml. Starch, manitol and fructose were mainly used for better growth and enzyme production. On the basis of enzyme activity, it was concluded that good growth but poor lipase activity, was obtained on media supplemented with dextrose, sucrose, lactose, CMC, inulin, raffinose and inositol as sole carbon sources. The organism was given preference to 5 % starch more than other carbon sources and produced 1.65u/ml (Fig.1). High nitrogen concentration are typically used for the production of lipases. To this effect different nitrogen sources were tested to obtain increased lipase production by the *Stretpomyces helstedii* strain ST 70. The highest level of extracellular lipase was detected in medium supplemented with glycine as nitrogen source (Fig. 2).

Interestingly, the natural nitrogen source did not effect much the enzyme production than the synthetic amino acids. Medium without a nitrogen source did not induce lipase production by *S. halstedii*. However, glycine (1.5 %) produced more enzyme activity i.e. 1.68 u/ml (Fig 3). Change in the phosphate source from basal starch casein medium did not effect much the lipase production and its activity. However addition of 0.5 % NaCl produced 1.43 u /ml lipase from the Streptomyces strain.Surprisingly, addition of potassium salts gave comparatively higher enzyme than the alone 5% starch and and 1.5 % glycine.
The amount of lipase was raised upto 5.71 u/ml in presence of higher concentration (11%) starch into the medium. (Fig. 4). It was 4.46u/ml and 3.18 u/ml with KNO_3 and KH_2PO_4, respectively. But higher concentration of starch with combination of glycine didn't prefer supplementation of salts of potassium. In addition to glycine , Addition of urea did not effect much the lipase production . This result is contradictory to the report of P. chrysogenum (Bencerz et al., 2005) that used urea as best nitrogen source for increasing the lipolytic activity.
It was demonstrated that a composite nitrogen source was a better substrate than a single nitrogen source. Experiments on the effects of nitrogen source composites were carried out. Composite organic and inorganic nitrogen source enhanced lipase production while composite organic and organic nitrogen source had little effect. This could be attributed to the indispensable ions as NH4, SO4 and Cl^- which promoted lipase reduction, Supplementation of nitrogen source was much effective in the case of addition of inorganic salts, with the highest lipase activity (Lima et al., 2003). The activity of lipase was reached with addition of ammonium salts in medium containing 11 % starch and 1.5% glycine especially ammonium chloride , ammonium nitrate and ammonium orthophosphate Whereas glycerol has exhibited much change in lipase production in the same modified medium . No role was played by glycerol in enhancing the lipase activity by this strain. Indeed it was inhibitory to enzyme production or activity as poor enzyme activity was observed.
Tween 80 (1%) certainly played role in inducing the enzyme system of this strain. Higher concentration upto 5% gave stability in enzyme activity. This study is corroborated with fungus Fusarium salani FS1 reproted by Maia et al., 1999. Higher oil concentration could be affecting the aeration rate of the culture and promoting a delay in growth and lipase production. Our results regarding use of Tween 80 as inducer is in support to the observation mentioned for *Calvatia, Rhizopus, Aspergillus, Mortierella vinacea and Rhodotorula* (Dalmau et al., 2000; Gaspar et al., 1999) showed that lipase production seems to be constitutive and independent of the addition of lipid substrates to the culture medium, although their presence enhanced the level of lipase activity produced. It is likely that tween 80, in addition to inducing lipase biosynthesis, increases cell permeability (Christova et al., 1996) .However, several reports are presented tween80 as sole carbon source (Li et al., 2001 and 2004).Experiment done with higher concentration of ammonium chloride besides ammonium nitrate , raised the enzyme activity upto 23.51 u/ml at 15.5 % concentration level .A comparison of the lipase activities towards different combination of amino acids demonstrate the important difference in nitrogen metabolism. The combinations of glycine with lysine and/or alanine certainly elicit the enzyme response and it reached upto 26.3 u/ml with 0.5% lysine and 26.0u/ml with 1 % alanine. However these amino acids didn't work in combination. It was observed that inclusion of KH_2PO_4 with lysine produced higher enzyme activity.
Third experiment was planned to determine the culture condition required for enhanced level production of lipase in broth culture. It is known that the aeration level plays an exclusively important role in the cultivation of aerobic producers (Yang et al., 2005). To study the effect of aeration on lipase biosysnthesis by *S. halstedii*, two aspects were studied, namely, rotating speed and the medium quantity in the flask. The speed was changed to 50-100 rpm and the medium quantity in the range of 20-175 ml Shake culture maintained at 50rpm was better than to high speed shaking and / or static condition. (Fig. 5). Aeration enhanced the lipase yield in some cases (Chander et al., 1983). In some organism lipase production depended on the oxygen concentration in the culture medium (Giuseppin et al., 1984). Genovefa et al. (1994) reported that high levels of aeration decreased lipase production in Staphylococcus carmosus. Long et al. (1996) also reported that high levels of aeration decreased lipase production by *Aspergillus flacus*. The present results also suggested that the lipase activity was enhanced with increasing levels of aeration. However, high rotation speed decreased lipase production and may contribute to the strong mechanic strength which restrained the growth of mycelia(Yang et al., 2005).

Culture medium is also important to achieve more biological product. To mean it, experiment was conducted while growing culture of *S. halstedii* in modified liquid medium of 7.2 pH in sizes of vessel ranging 50 ml to 1000 ml. Volume of medium and vessel inner area had a significant effect on production of lipase. The maximum lipase was produced by the culture prepared in 85 ml (in flask of 500 ml) i.e. 27.9 U/ml and 175 ml (in flask of 1000 ml) i.e. 26.1 U/ml. This was also supported by the maximum lipase production by *Corynebacterium* in 50 medium at 72 hr of fermentation (Ray et al. 1999).
The partially purified and crude enzyme analyzed under different temperature treatments. The highest activity of lipase obtained from ammonium sulphate partial purification was observed at 37 ºC i.e. 52.8

u/ml that was higher as compared to crude lipase. Both crude and purified enzyme was particularly stable at elevated temperature. Similar thermal stability was reported by lipase from other organisms (Bencerz et al., 2005). The partial purified enzyme was 25 times higher than that of wild strain growth in basic starch casein medium.

CONCLUSION

After optimizing the media composition and culture growth conditions for the production of an extracellular lipase by *S. halstedii*, we achieved a maximum of 56.2 U /mL. These results are promising because this strain produces lipase in an inexpensive inorganic medium and we succeded in increasing the maximum lipolytic activity approximately 62 fold over the initial values obtained with the non-optimized medium.
To conclude, the *Streptomyces halstedii* lipase has several properties of significant industrial applications in particularly an activity and stability at various temperature and pH. The lipase was quite stable at low temperature that could be exploited further for other industrial applications. However, further studies should be done with purified preparation of enzyme obtained through gel filtration or iron exchange chromatography.
The importance of the data presented here points to the possibility of obtaining an active lipase producer from Streptomyces group. *S. halstedii* as the bacteria of highest lipolytic activity will be of great interest in molecular and biotechnological research, as it is fungal like organisms easy to explore characteristics of both prokaryotic and eucaryotic system. However, the extraction, media manipulation and prediction of lipase by this organism on bench scale needs some more research on large scale. Recently it has been reported that *Streptomyces* are unique among the prokaryotes in using triglycerols as storage compounds under the speculation that it serve as carbon source during stationary stage of growth. Hence, lipase production by this strain should be tested periodical according to growth phases (Vujaklijaet al., 2003).

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