Enhanced Production of Extracellular Alkaline Lipase by an Improved Strain of *Pseudomonas aeruginosa* MTCC 10,055

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Abstract: Problem statement: Lipases are industrially important enzymes having applications in numerous industries. For easy commercialization it is necessary to produce lipases at industrial level which could be achieved by strain improvement and medium formulation. **Approach:** In the present study strain improvement of *Pseudomonas aeruginosa* MTCC 10,055 was done by chemical mutagenesis using mutagen 4-nitroquinoline1-oxide for alkaline lipase production. Different fermentation parameters affecting lipase production were optimized using one-variable-at-a-time approach. **Results:** The selected mutant (M-05) exhibited 3.6-fold higher productivity over wild type. Maximum alkaline lipase was produced when culture was incubated at 35°C with initial medium pH 9.0 in 28 h with inoculum density 0.5% (v/v) (Abs_{610}-1.0). Supplementation of production medium with combination of castor oil and starch as carbon source and Triton-X-100 as surfactant significantly influenced the alkaline lipase production. The composition of fully optimized medium was determined to be (g L^{-1}): $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.6; $MgSO_4$, 0.4; yeast extract, 0.2; castor oil, 2.0; starch 20.0; gum arabic, 5.0; Triton-X-100, 1.0. An overall 14-fold enhanced production was achieved after complete medium optimization. **Conclusion/Recommendations:** The improved strain was capable to produce high titer of alkaline lipase at flask level, which can be examined at fermentor level to obtain sufficient enzyme yield to meet the world wide industrial demand.

Key words: Alkaline lipase, chemical mutagenesis, 4-nitroquinoline1-oxide, medium optimization, *Pseudomonas aeruginosa*, gum arabic, enzyme yield

INTRODUCTION

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) belong to the family of hydrolases that break carboxylic ester bonds. The triglycerides of long-chain fatty acids are natural substrates of lipases, however, they also act on hydrophobic esters of primary and secondary alcohols (Houde *et al*., 2004). Because of versatile functions they can be widely applied in the food, detergent, pharmaceutical, leather, textile, cosmetic, organic chemistry and paper industries (Jaeger and Reetz, 1998; Jaeger and Eggert, 2002). Regio-specific and fatty acid specific microbial lipases could immensely be exploited for retailoring of vegetable oils. Tailored vegetable oils with nutritionally important structured triacylglycerols and modified physico-chemical properties have great prospective in the future market (Gupta *et al*., 2003).

Contrary to many other enzymes, remarkable activity and stability of lipases in non-aqueous environments, facilitates the catalysis of several unnatural reactions such as esterification and transesterification (Ghaly *et al*., 2010). Because of these unique properties, lipases are most attractive enzymes for use in organic chemical processes. In addition they do not usually require cofactors and they can be produced in high yields from microorganisms, especially bacteria and fungi (Jaeger and Reetz, 1998; Jaeger and Eggert, 2002).

The escalating demand of lipases in various industries, in the last few decades, necessitated the extension in both qualitative improvement and quantitative enhancement. Quantitative enhancement could be done by strain improvement and medium optimization, as the quantities produced by wild strains are low (Bapiraju *et al*., 2004). Improvement of the commercial applicability of microbial strains has been practiced for centuries (Parekh *et al*., 2000). Industrial strain improvement plays a central role in the commercial development of microbial fermentation processes (Zhao *et al*., 2010). Meanwhile, strain

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improvement of the enzyme producer offers the greatest opportunity for cost effective production without significant capital outlay (Stanbury *et al*., 1995). This is achieved when a selected strain can synthesize a higher proportion of the product using the minimal quantity of raw materials (Zhao *et al*., 2010).

The aim of the present study is to improve the alkaline lipase production by strain improvement through chemical mutagenesis and medium improvement.

MATERIALS AND METHODS

Microorganism: The wild-type strain of *P. aeruginosa*, isolated in our laboratory from oil contaminated soil was used as a parent strain for mutagenesis. It was identified and deposited at Indian Institute of Microbial Technology (IMTECH), Chandigarh, India and given an accession number MTCC 10,055.

Inoculum preparation: The culture was grown in medium containing (g L^{-1}): $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.6; $MgSO_4$, 0.2; yeast extract, 0.1; olive oil, 20; pH 9.0. The flasks were incubated at 37°C with shaking at 120 rpm for overnight.

Strain improvement by chemical mutagenesis: The chemical mutagenesis of *P. aeruginosa* MTCC 10,055 cells were carried by exposing with 4-nitroquinoline1-oxide (4-NQO, Sigma-Aldrich Chemical Pvt. Ltd, USA) by a modified method of Punt *et al*. (1987). The overnight grown culture was harvested by centrifugation at 10,000×g for 10 min, washed twice with phosphate buffer (100 mM, pH 7.2) and suspended in the same buffer (cell density-58.0×10^{10} cells mL^{-1}). This cell suspension (200 µL) was mixed with 50 µL (200 µg mL^{-1}) of 4-NQO and 750 µL of sterile double distilled water. The mixture was incubated at 37°C for different time interval (15-60 min). After incubation, mutagen was inactivated by addition of 100 µL of 10% (w/v) sodium thiocynate followed by further incubation for 5 min. The mutagenized cells were harvested by centrifugation at 10,000×g for 5 min, washed with phosphate buffer (100 mM, pH 7.2) and re-suspended in sterile distilled water.

Preliminary screening of lipase-overproducing mutants was done on tributyrin agar medium (pH 9.0) prepared by supplementing the nutrient agar with 1% (v/v) tributyrin. The treated cell suspension was diluted and spread on a series of pre-sterilized plates of above mentioned medium and incubated at 37°C for 24-48 h. The bacterial colonies forming the largest clear zone were isolated at randomly from different plates.

Screening of a potent over-producing mutant strain: The selected mutants were grown in the basal fermentation medium comprising (g L^{-1}): $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 0.6; $MgSO_4$, 0.2; yeast extract, 0.1; olive oil, 20; gum arabic, 5.0 and pH 9.0 to determine their potencies for alkaline lipase production in shake flask. Fermentation experiments were performed at 37°C and 120 rpm in 250 mL flasks containing 50 mL medium, inoculated with 1% (v/v) of the seed culture. At the end of fermentation process, the flasks were taken and harvested to measure the lipase activity in the fermented broth.

Analytical methods: Extracellular lipase activity was measured spectrophotometrically by following the method of Winkler and Stuckman (1979) with slight modifications. The substrate solution containing 10 mL of isopropanol with 30 mg of p-nitrophenyl palmitate was mixed with 90 mL of Tris-HCl buffer (50 mM, pH 9.0), containing 0.4% Triton-X-100 and 100 mg of gum arabic. Freshly prepared substrate solution (2.4 mL) was incubated at 37°C with 25 µL of s uitably diluted cell-free supernatant for 15 min. After incubation absorbance was measured at 410 nm by using a spectrophotometer (UV-1601, Shimadzu) against a control with heat inactivated enzyme. One unit of enzyme is defined as the amount of enzyme liberating 1 µg of p-nitrophenol $mL^{-1} min^{-1}$ under the assay conditions.

Development of bioprocess for lipase production by mutant strain M-05: The protocol adopted for optimization of process parameters aimed to evaluate the effect of an individual parameter at a time and to incorporate it at the standard level before optimizing the next parameter.

Selection of physical parameters: The effect of incubation period on growth and alkaline lipase production was monitored in the basal medium used previously for screening. Samples (3 mL) were harvested at every 4 h intervals for both bacterial growth and lipase production. Absorbance at 610 nm was measured for the determination of biomass. The influence of initial pH and incubation temperature on alkaline lipase production was studied by adjusting the pH of the basal medium at different levels (8.0-10.0) then incubated at temperatures 30-45°C (at difference of 5°C). The effect of inoculum sizes (0.25-2.5%, v/v) were also investigated on lipase production.

Selection of nutritional parameters: The effect of various medium supplements such as oils (2.0% v/v;

castor, neem, mustard, linseed and groundnut oil), carbon source additives (0.5% w/v; fructose, glucose, lactose, mannitol, soluble starch, sorbitol and sucrose), organic nitrogen sources (0.1% w/v; corn steep liquor, gelatin, malt extract, peptone, tryptone and yeast extract), inorganic nitrogen sources (0.1% w/v; sodium nitrate, ammonium nitrate, ammonium chloride, potassium nitrate, di-ammonium hydrogen orthophosphate and ammonium di-hydrogen orthophosphate), metal salts (0.02%, w/v $FeSO_4$, $CaCl_2$, $MnCl_2$, $ZnCl_2$, $CuSO_4$, $HgCl_2$ and $NiCl_2$) and surfactants (0.1%, Tween-80, Triton-X-100, SDS, Tween-40 and Tween-20) on enzyme production was studied by incorporating the constituent individually in the production medium.

Statistical analysis: All experiments were carried out in triplicates and the results are presented as the mean of three independent observations. Standard deviation for each experimental result was calculated using Microsoft Excel.

RESULTS

Mutagenesis and screening of mutants: The observations of strain improvement through chemical mutagenesis suggest that lethality of viable cells increased with the increase in exposure time with mutagen and only 0.12% viable cells were recorded after 60 min of incubation (data not shown). Several mutant strains (twenty four) showing zone of lipolysis on the tributyrin plates were selected at randomly from the plates of different exposure time interval and tested for lipase production in shake flask. Mutant strains such as M-01, M-05, M-09, M-12 and M-21, showed remarkable over production of alkaline lipase (data not shown). Among the various mutants, the maximum lipase production was achieved by the mutant M-05 (160.9 U mL^{-1}) which was approximately 3.6-fold higher as compared to the wild-type strain. Hence, mutant strain M-05 was selected for further study.

Optimization of cultural parameters for lipase production:
Growth kinetics and enzyme production: A time-course study was conducted in order to determine the growth and lipase production level by the mutant strain with regard to incubation period (Fig. 1). The logarithmic phase commenced at 8 h and continued for another 20 h and maximum growth was achieved after 28 h of incubation at 37°C. With regard to lipase production, it commenced in early logarithmic phase (after 12 h) and increased until maximum production was achieved (283.4 U mL^{-1}) after 28 h of incubation.

Further increase in incubation period did not lead to an increase in lipase production, rather a slow decrease in lipase yield was observed.

Effect of inoculum size on alkaline lipase production: The inoculum concentration was studied to obtain optimum size of inoculum and results indicated that lipase production increased with an increase in inoculum size (Fig. 2). Maximum lipase production (348.6 U mL^{-1}) was obtained at 0.5% (v/v) inoculum size, however, further increase in inoculum size resulted in sharp decrease in lipase production, especially when 2.5% (v/v) inoculum size was used.
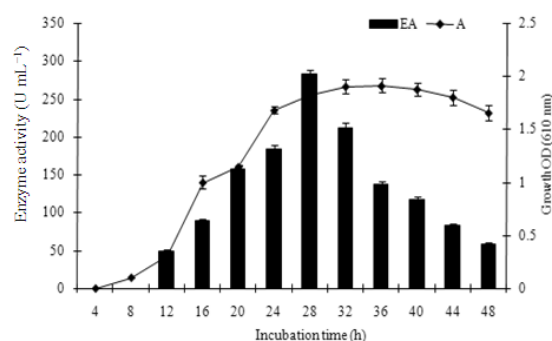

Fig. 1: Growth kinetics along with the alkaline lipase production. EA- Enzyme Activity, A- Absorbance. Inoculated flasks were incubated at 37°C at 120 rpm for 48 h. Samples were harvested at every 4 h and assayed for lipase activity and biomass determination (Absorbance at 610 nm). Bars presented are mean values of ± standard deviation of triplicates of three independent experiments
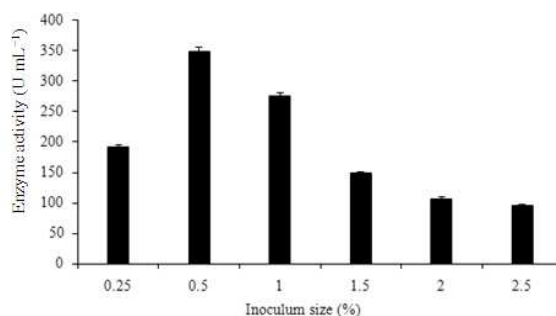

Fig. 2: Effect of inoculum size on alkaline lipase production. The inoculated flasks were incubated at 37°C for 28 h at 120 rpm. The flasks having 1% (v/v) inoculum was taken as control. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments
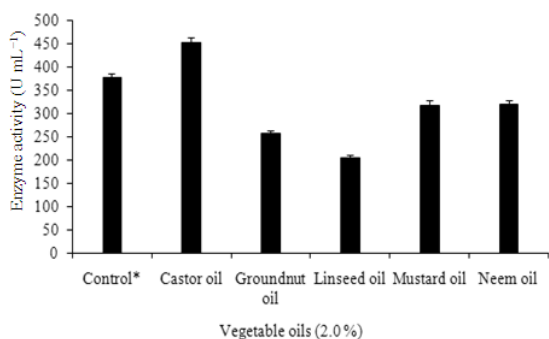
Fig. 3: Influence of various vegetable oils on alkaline lipase production. The control flasks contained olive oil (2.0% v/v). Test flasks contained different oils in the medium replacing olive oil at a level of 2.0% (v/v). Inoculated flasks were incubated at 35°C for 28 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments
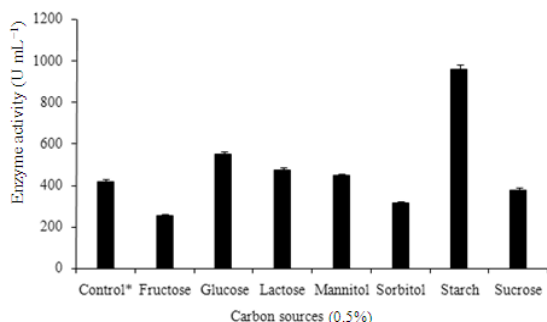

Fig. 4: Influence of various additional carbon sources on alkaline lipase production. The control contained castor oil (2.0% v/v) without any additional carbon source. Test flasks contained different sugar in the medium with castor oil. Inoculated flasks were incubated at 35°C for 28 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments

Table 1: Effect of temperature and pH on alkaline lipase production. The inoculated flasks were incubated at different temperatures for 28 h under shaking condition (120 rpm). The ± values in the columns showed the standard deviation of three independent trials

	Enzyme activity (U mL^{-1}) Temperature (°C)			
pH	30	35	40	45
8.0	150.5±2.9	170.4±5.3	165.4±5.8	108.6±0.8
8.5	161.2±3.8	254.4±8.1	184.9±4.8	134±3.9
9.0	277.4±8.0	381.8±10.7	219.4±6.1	154.4±4.5
9.5	171.9±4.6	211.3±8.7	165.3±5.6	125.8±4.4
10.0	99.3±2.4	154.9±3.9	124.1±3.8	88.6±2.9

Effect of pH and temperature on alkaline lipase production: It is evident from Table 1 that substantial quantity of lipase was produced by mutant strain at all the pH and temperature levels tested except pH 10 and temperature 45°C. The maximum lipase was produced at pH 9.0 and temperature 35°C with the enzyme activity of 381.8 U mL^{-1}.

Effect of vegetable oils on lipase production: Different lipid sources as substrate had different effects on alkaline lipase production (Fig. 3). Of all the lipidic sources tested, the highest lipolytic activity of 453.0 U mL^{-1} was obtained with castor oil followed by olive oil (377.8 U mL^{-1}) and mustard oil (318.5 U mL^{-1}). However, groundnut oil and neem oil also supported lipase production. Contrastingly, linseed oil reported very low lipase production (204.9 U mL^{-1}) which could be due to hampered growth of the strains as evident by the absence of any visible turbidity in the medium.

Effect of additional carbon sources on alkaline lipase production: Addition of different additional carbon sources had both positive and negative effect on alkaline lipase production (Fig. 4). Maximum lipase production was obtained with starch (960.8 U mL^{-1}) followed by glucose (550.9 U mL^{-1}) and lactose (476.1 U mL^{-1}), whereas, mannitol (448.1 U mL^{-1}), sucrose (380.4 U mL^{-1}) and fructose (253.9 U mL^{-1}) had negative effect on lipase production.

Owing to the effect of starch we optimized several combinations of castor oil and starch in the production medium (Table 2). Marked increase in lipase production (1098.8 U mL^{-1}) was observed with combination 1 (castor oil 0.2% v/v + starch 2.0% w/v), whereas, other combinations were ineffectual as compared to control.

Effect of different organic nitrogen sources on lipase production: A range of organic nitrogen sources were tested to evaluate their capacity to support lipase production.

Table 2: Effect of different combinations of castor oil and starch on lipase production. The inoculated flasks were incubated at 35°C for 28 h under shaking condition (120 rpm). The ± values in the columns showed the standard deviation of three independent trials

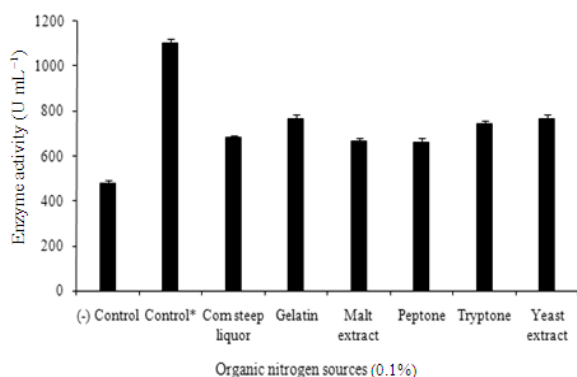| Combinations	Castor oil + starch (%)	Enzyme activity (U mL^{-1})
Control	2.0+0.0	964.1±21
1	0.2+2.0	1098.8±20.6
2	0.5+1.5	917.8±18.3
3	1.0+1.0	449.0±8.9
4	1.5+0.5	531.9±15.4
5	2.0+0.5	657.6±18.0
6	0.0+2.0	154.2±3.0

Fig. 5: Influence of various organic nitrogen sources on alkaline lipase production. Negative control was without yeast extract. Positive control contained yeast extract (0.01% w/v). Test flasks contained different nitrogen source (0.1% w/v) in the medium replacing yeast extract. Inoculated flasks were incubated at 35°C for 28 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments
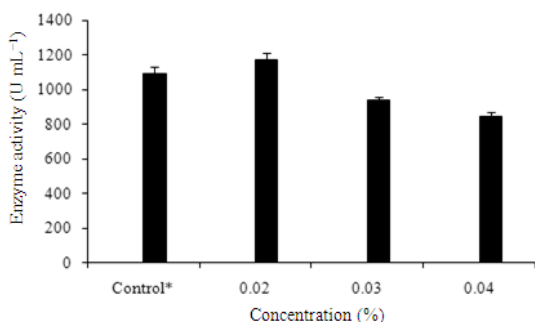

Fig. 6: Effect of different concentration of yeast extract on alkaline lipase production. The control flasks contained yeast extract (0.01% w/v). Test flasks contained different concentration of yeast extract (0.02-0.04% w/v) in the medium. The inoculated flasks were incubated at 35°C for 28 h under shaking condition (120 rpm). Bars presented are mean values of ± standard deviation of triplicates of three independent experiments

Figure 5 showed that mutant strain produced significant lipase with all the organic nitrogen sources, however, maximum lipase production (1100.6 U mL^{-1}) was attained in control (yeast extract-0.01% w/v), whereas inhibition in lipase production was observed in the medium devoid of yeast extract.
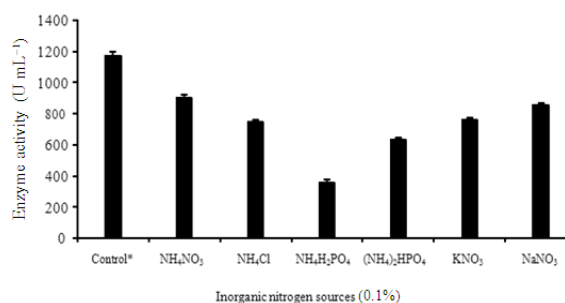

Fig. 7: Influence of various inorganic nitrogen sources on alkaline lipase production. The control flasks contained ammonium sulphate (0.1% w/v). Test flasks contained different inorganic nitrogen sources (0.1% w/v) in the medium substituting ammonium sulphate. Inoculated flasks were incubated at 35°C for 28 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments

Owing to effect of yeast extract different concentration (0.01-0.04%, w/v) were studied on lipase production. It was observed that 0.02% (w/v) exhibited marginal increase in lipase yield i.e., 1175.9 U mL^{-1} (Fig. 6). Further increase in concentration of yeast extract, resulted in gradual decrease in lipase production.

Effect of different inorganic nitrogen sources on lipase production: It is observed from Fig. 7 that all the inorganic nitrogen sources supplemented in the production medium tend to decrease the lipase production except the medium containing ammonium sulphate i.e., control (1170.6 U mL^{-1}). In case of ammonium di-hydrogen orthophosphate minimum lipase yield was observed (356.9 U mL^{-1}).

When different concentrations of ammonium sulphate were added to the medium it didn't exhibit significant increase in lipase production and maximum lipase yield (1167.4 U mL^{-1}) was attained in control (0.1%, w/v) (Fig. 8). Whereas, lipase production was completely repressed at concentration 0.4%.

Effect of metal salts on lipase production: Figure 9 depicts the influence of various metal salts on lipase synthesis. Maximum enzyme production (1187.4 U mL^{-1}) was observed in control containing $MgSO_4$ followed by $FeSO_4$ (999.9 U mL^{-1}), however, rest of the metals ($CuSO_4$, $HgCl_2$ and $NiCl_2$) did not stimulate the lipase production. Owing to effect of $MgSO_4$, we varied its concentration in the range of 0.02-0.08% into the production medium. The maximum yield

(1254.9 U mL^{-1}), with trivial increase, was obtained with 0.04% (w/v) concentration (Fig. 10). Further increase in MgSO$_4$ concentration resulted in gradual decrease in lipase production with minimum at concentration 0.08% (w/v).

Effect of surfactants on alkaline lipase production: Influence of surfactants on alkaline lipase production is presented in Fig. 11. Results revealed that the lipase production was profoundly affected by types of surfactants used.
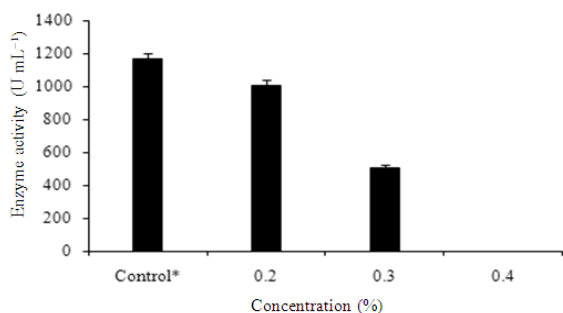

Fig. 8: Effect of different concentration of ammonium sulphate on alkaline lipase production. The control flasks contained ammonium sulphate (0.1% w/v). Test flasks contained different concentration of ammonium sulphate (0.2-0.4% w/v) in the medium. Inoculated flasks were incubated at 35°C for 28 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments
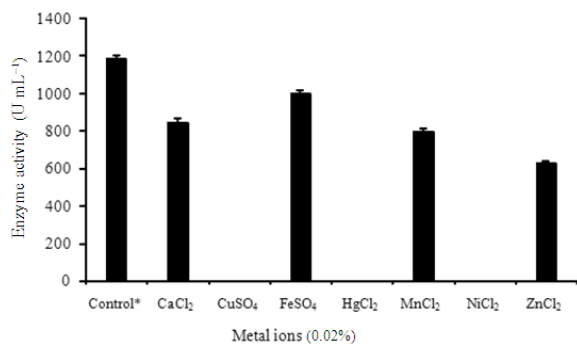

Fig. 9: Effect of various metal salts on alkaline lipase production. The control flasks contained MgSO$_4$ (0.02% w/v). Test flasks contained different metal ions in the medium replacing MgSO$_4$. Inoculated flasks were incubated at 35°C for 28 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments

Mutant strain of *P. aeruginosa* MTCC 10,055 showed maximum lipase production (2368.7 U mL^{-1}) with Triton-X-100, whereas, Tween-80, SDS and Tween-40 did not have any significant effect on lipase production as compared to control. Supplementation of Tween-40 in the production medium tend to decrease the lipase yield (454.4 U mL^{-1}).
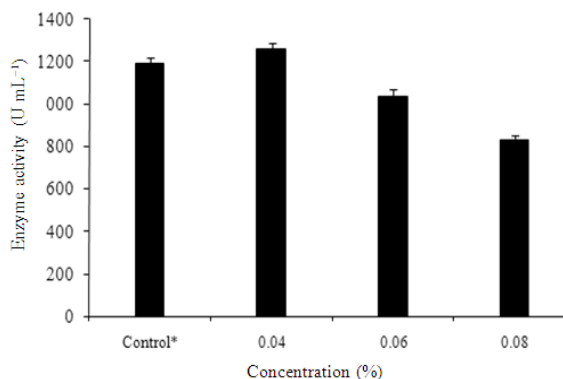

Fig. 10: Effect of different concentration of MgSO$_4$ on alkaline lipase production. The control flasks contained MgSO$_4$ (0.02%). Test flasks contained different concentration of MgSO$_4$ (0.04-0.08%) in the medium. Inoculated flasks were incubated at 35°C for 28 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments
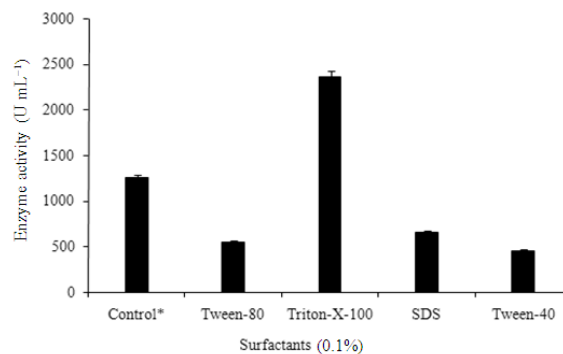

Fig. 11: Influence of various surfactants on alkaline lipase production. The control flasks were without any surfactant. Test flasks contained different surfactant (0.1%) in the medium. Inoculated flasks were incubated at 35°C for 28 h. Bars presented are mean values of ± standard deviation of triplicates of three independent experiments

DISCUSSION

The mutation and screening of industrially useful microorganisms are important for the successful development of the various strains required in the fermentation industry. Zhao *et al*. (2010) reported 2.3-fold increase in the lipase from a mutant strain of *Serratia marcescens* as compared to wild type strain after exposure to Ultra Violet (UV). Gaoa *et al*. (2000), also reported 3.25-fold increase in lipase yield by mutant of *Pseudomonas* upon exposure to UV and N-methyl-N'-nitro-N-nitrosogunidine (NTG).

Kinetic study of the growth and alkaline lipase production by the mutant strain demonstrated that optimum lipase production occurred after 28 h, thereafter decreased on further incubation (Fig. 1). Rosenau and Jaeger (2000) also reported synthesis of extracellular lipase in the late logarithmic phase of growth and suggested that the two-component system lipQ/R, which directly controls lipA gene expression is subjected to a superior quorum sensing system. Similarly, Sekhon *et al*. (2006) reported a high level of lipase activity after 27 h incubation.

The nature of inoculum as well as its size may affect the microbial process and could play crucial role in enzyme production (Elibol *et al*., 1995). In a study of lipase production by *Bacillus thermoleovorans* ID-1, Lee *et al*. (1999) obtained optimum results when 1% (v/v) inoculum was used to inoculate the fermentation medium. At a suitable inoculum size, the nutrient and oxygen levels support sufficient growth of bacteria and therefore, enhance the lipase production. Higher inoculum size may cause insufficiency of total dissolved oxygen and nutrient supply in the culture media resulting in poor product yield (Baharum *et al*., 2003). If the inoculum size is too small, insufficient biomass lead to reduced level of secreted lipase.

The present study revealed that the lipase production by the mutant strains requires alkaline pH 9.0, which is relatively high when compared to lipase production by *Bacillus megaterium* AKG-1 DSM 730 (Sekhon *et al*., 2006) and low when compared to *Arthobacter* sp. BGCC# 490 (Sharma *et al*., 2009). Invariably, the lipase production by *Serratia marcences* prefers slightly acidic pH (6.5-7.0) (Gao *et al*., 2004).

Our results indicated that the optimum temperature for lipase productivity by the mutant strains was 35 °C. Many workers investigated the relation of temperature and lipase production. Gunasekaran *et al*. (2006) observed similar results where the optimum temperature for lipase produced by *Citrobactor freundii* was 35°C. In addition, Baharum *et al*. (2003) investigated the production of lipase by *Pseudomonas*

sp. strain S5 and recorded that the optimum temperature for lipase production was 37°C.

The major factor for the expression of lipase activity has always been reported as the carbon source, since lipases are mostly inducible enzymes and sometimes constitutive, although their role in lipase synthesis and stimulation is poorly understood (Large *et al*., 1999). Among the various oils used in the present study, maximum lipase production was achieved with castor oil followed by olive oil and mustard oil. These findings are supported by the study of Kulkarni and Gadre (2002) and Sharon *et al*. (1998) who also reported the castor oil to be the most effective oil for lipase production by *P. fluorescens* NS2W and *P. aeruginosa* KKA-5, respectively. On the contrary to our results, neem oil and olive oil were found to good for lipase production by *Fusarium globulosum* (Gulati *et al*., 2005) and *Bacillus* sp. (Shah *et al*., 2007). Macris *et al*. (1996) suggested that the carbon chain moiety of the fatty acid present in the triglycerol can control the lipase synthesis.

For lipase production different lipid based carbon sources have been reported as inducer as well as carbon source (alone or in combination with carbohydrates) by several researchers. Therefore, the suitability of different carbon source additive for lipase production by mutant strain was investigated. Supplementation of production medium with starch tremendously boosted the lipase activity (about 129.3%) as compared to control. Similar results were reported by Pogaku *et al*. (2010), while investigating the lipase production by *Staphylococcus* sp. Lp12. Rathi *et al*. (2001) also reported increase in lipase production from *Burkholderia cepacia* in the presence of oil and glucose as sugar additive.

Among the various nitrogen sources tested, a significant increase in growth and lipase production was observed with yeast extract. Likewise, maximum lipase production with 0.1% (w/v) and 0.5% (w/v) yeast extract have also been reported earlier by *Bacillus* strain A30-1 (Wang *et al*., 1995) and *P. citrinum* (Pimentel *et al*., 1994), respectively. According to Gupta *et al*. (2007) yeast extract apart from acting as a nitrogen source also supplies vitamins and trace metals, thereby affecting the growth of organism in turn, increase in lipase production.

Among the inorganic nitrogen sources studied, the ammonium sulphate was found to be most suitable for lipase production, whereas ammonium di-hydrogen orthophosphate inhibited the lipase production and others had insignificant effect. Lima *et al*. (2003) also reported that ammonium salt was necessary for growth of *P. aurantiogriseum*. In contrast, Pogaku *et al*. (2010)

found that potassium nitrate was better than ammonium sulphate for increased lipase production by *Staphylococcus* sp. LP12.

As metal ions are known to influence lipase synthesis, activity and secretion mechanism (Lee *et al.*, 2007), various metal salts in the medium were evaluated for their effect on alkaline lipase production. Maximum enzyme production was observed in control containing $MgSO_4$ followed by $FeSO_4$. Shah *et al.* (2007) have also reported similar results with *Bacillus* sp. (Pa2) for increased lipase production when Mg^{+2} (0.25%, w/v) was incorporated into the production medium. On the other hand, influence of Ca^{+2} in addition to Mg^{+2} was found to enhance lipase production by *P. aeruginosa* (Kulkarni and Garde, 2002).

Among the various surfactants tested, a significant increase in lipase production was observed with Triton-X-100. The lipase production rapidly decreased when Tween-80 and SDS were used, whereas, Tween-40 did not have any significant effect. According to Wu and Tsai (2004), higher levels of lipase production were observed when the substrate formed an emulsion, thereby presenting an interfacial area to the enzyme. This has resemblance with the earlier *P. pseudoalkaligens* F-111 lipase, where addition of Triton-X-100 increased the alkaline lipase production by 50-fold (Lin *et al.*, 1995). An elaborated study was done by Lin *et al.* (1995) on effect of Triton-X-100 on lipase production. Triton-X-100 may directly act as a positive activator of lipase gene or may induce the expression of lipase gene by removing the repressor molecules. They also inferred that Triton-X-100 mechanistically promotes both uptake and exit of compounds from the cell through modification of plasma membrane permeability. Contrastingly, the studies on lipase production by *Staphylococcus* sp. indicated the positive influence of Tween-80 (Pogaku *et al.*, 2010). However, surfactants do not always increase lipase production (Deive *et al.* 2003) and their effect varied with microorganism, type of surfactant and its concentration (Dominguez *et al.*, 2003).

CONCLUSION

Improvement of microbial strains for the over production of industrial products has been the hallmark of all commercial fermentation processes. Effect of supplementation of castor oil and starch, in combination and Triton-X-100 were interesting findings where about 250 and 90% increase in lipase production was

observed, respectively. In the present study, strain improvement goes hand-in-hand with medium and process improvement and the high titers are the results of both being employed synergistically. We have successfully developed a promising mutant strain, by employing a simpler technique of chemical mutagenesis, which can be exploited at bioreactor level for industrial production of alkaline lipase, a biotechnologically important hydrolytic enzyme.

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