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Strain Improvement of *Pseudomonas sp.*VITSDVM1 for Optimization of Lipase Production by Chemical Mutagens.

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ABSTRACT

Lipase enzymes are currently attracting the most considerable amount of attention due to their unique characteristics. The isolate Pseudomonas sp. VITSDVM1was mutated using chemical mutagens: Ethyl Methyl Sulfonate (EMS), Ethidium bromide (EtBr), Sodium azide and N-methyl-N'-nitro-N-nitrosoguanidine (NTG), in order to enhance its production and lipolytic potential. During the treatment, mutants were qualitatively and quantitatively selected. The results revealed that EMS was found to be the best inducer which showed maximum lipase production (4.96U mL-1). This mutant showed an overall increase in activity over its parent strain for the production of extracellular lipase. Optimisation parameters for the lipase activity was found maximal at pH 8 and at 37oC, utilizing glucose and yeast extract as carbon and nitrogen sources. The study indicated that the enzyme activity of the mutant strain using chemical mutagen was 2-fold higher than the wild strain. The partially purified lipase enzyme was analysed by high-performance liquid chromatography, revealed its RT at 3.065min. Based on the findings of present study *Pseudomonas sp*. is an ideal candidate for extracellular lipase production for industrial uses and it can be exploited in various purposes.

Keywords: *Pseudomonas sp*, VITSDVM1, lipase, mutagens.

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INTRODUCTION

Lipases hydrolyze triacylglycerols to fatty acids, diacylglycerols, monoacylglycerols and glycerol and under certain conditions, catalyze reverse reactions such as esterification and transesterification[1, 2].In recent years, research on microbial lipases has increased because of their practical applications in industry, as hydrolysis of fats, production of fatty acids and food additives, synthesis of esters and peptides, resolution of racemic mixtures, or additives in detergents [3]. Lipase enzyme have a long array of industrial application in the product and process of detergents, oil, fats and dairy coupled with enormous therapeutic uses. Bacteria are the important enzyme producers since their enzymes are produced extracellularly.The exponential increase in the application of lipases in various fields in the last few decades demands extension in both qualitative improvement and quantitative enhancement. Quantitative enhancement requires strain improvement and medium optimization for the overproduction of the enzyme as the quantities produced by wild strains are usually too low [4]. Microbial production of lipases depends on the activity and stability of the producers, which can be enhanced by induced mutagenesis. Optimization of physico-chemical parameters such as the composition of production medium is an important step for its commercial usage of the enzyme. There is growing interest in effect of mutations on the biosynthesis of lipases has caused tremendous interest among scientists and industrialists. Hence the present study has been undertaken to reveal the efficiency of lipase activity from *Pseudomonas* sp. by chemical mutagenesis.

MATERIALS AND METHODS

Strain Selection for optimization Studies

Pseudomonas sp. VITSDVM1isolated from cow milk (Gen Bank Accession number: KJ775757) [5] was procured and subjected for mutagenesis and optimization process in order to enhance the production of lipase.

Mutagenesis by chemical method

EMS(Ethyl Methyl Sulfonate), EtBr (ethidium bromide), sodium azide and N-methyl-N'-nitro-N-nitrosoguanidine (NTG), were the chemicals mutagens used for mutagenesis of the parent culture, *Pseudomonas sp.* VITSDVM1.To 9 mL of bacterial suspension, 1mL of sterile solution of respective mutagens (50 µg mL^{-1} in phosphate buffer) was added individually. The reaction was allowed to proceed. Samples were withdrawn from the reaction mixture at intervals of 30, 60, 90, 120, 150 and 180 min and immediately centrifuged for 10 min at 5000 rpm. The pellet was washed three times with sterile phosphate buffer and suspended in 10 mL of the same buffer (pH 7.0). The samples were serially diluted using the same buffer and plated on nutrient agar. The plates were incubated at 37ºC for two days. The selected sodium azide treated mutants were designated as CS30, CS60, CS90 and CS120. Submerged fermentation of wild type and mutant strains of *Pseudomonas sp.* VITSDVM1was used for this study. 0.5 mL of each strain (O.D = 1.0) was inoculated into 100mLCzapek-Dox broth containing 1% olive oil as inducer. Sucrose and 10% olive oil was the carbon source. The flasks were incubated at 37°C for 96h. Un-inoculated Czapekdox broth served as control. Each experiment was done in triplicates.

Enzyme assay

Lipase activity was determined. The substrate emulsion was prepared with olive oil.[6]The amount of fatty acid liberated was determined by titration with 0.05N NaOH and the lipase activity was calculated. One unit (U) of lipase activity is defined as the amount of enzyme required to liberate one micromole equivalent fatty acid $min^{-1}mL^{-1}$ under the above assay conditions.

Estimation of total protein

The absolute extracellular protein estimated from the culture filtrate was purified by ammonium sulfate precipitation method and estimated by Lowry's method [7].

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Optimization

pH and temperature

The pH of the growth medium was adjusted to 4,5,6,7,8 whereas other parameters were unaltered. The temperature optimum for lipase producing enzymes were preferred by changing or varying the temperature of media containing growth (70°C, 4°C, 30°C) at 7.0 pH whereas other parameters was not altered. It is kept at 37°C for 72h or 48h depending on the requirement with agitation at 200rpm.The buffers included in the media were of different pH. The buffers used are sodium phosphate buffer (pH 6.0 and 7.0) sodium acetate buffer (pH 4.0 and 5.0) and Tris-HCl (pH 8.0). It is kept at 37°C for 72h or more than that with agitation at 200rpm.

Carbon and nitrogen sources

Carbon sources such as fructose, sucrose, glucose, mannose and galactose was added in medium at 1% percentage (w/v) concentration following all the another parameters similar. Nitrogen sources such as yeast extract, beef extract, peptone extract, malt extract, was included in the medium at 1% percentage (w/v) concentration and the enzyme activity f was checked at 24h.

Enzyme purification

Precipitation and dialysis; Ammonium sulfate was added to the culture supernatant in small quantities with constant stirring in order to achieve saturation. The supernatant was then concentrated by precipitation with ammonium sulfate to 60%–70% levels and the precipitates were dissolved in 50mM phosphate buffer (pH 7).The precipitates were immersed in the Tris-Cl and the solution was dialysed against Tris-Cl buffer (at neutral pH) for an overnight using dialysis bag. The dialysed solutions were concentrated using vacuum and this was adjusted to column of a Sephadex-100 which are pre-equilibrated with buffer containing citrate and eluted using the similar buffer. The flow rates were adjusted to 1.2mL/ 4min and 35 fractions was analysed and collected.

HPLC

The High Performance Liquid Chromatographic (HPLC) separation of the partially purified lipase enzyme was carried out on a LC1-10 AT vs model HPLC using 250 - 4.60 mm Rheodysne column (C 18). The protein was eluted with 75% (v/v) acetonitrile as taken the mobile phase at the flow rate of 1 mL/h at 285 nm with C18 column (3.0 mm - 300 mm).

RESULTS AND DISCUSSION

Lipase activity of *Pseudomonas sp.* VITSDVM1 treated by chemical mutagens revealed the maximum activity which is shown in table 1. EMS was found to be the better mutagen which showed more lipase production (4.96 U mL^{-1}). The present study also indicated that the strain of *Pseudomonas sp.* VITSDVM1treated with EMS produced 2-fold higher amount of lipase compared to wild strain. The lipase activity was found considerably increased depending on the nutritional conditions. The maximum lipase concentration when *Pseudomonas sp.* VITSDVM1 suspension was exposed to chemical mutagenesis using EMS and very few colonies were observed on the plate which indicates possible mutation with the strain. The growth rate of the mutant strain studied in the production broth was determined. When compared to wild type strain, the mutated strain showed maximum growth rate with increased lipase production. This result indicates that the enhancement of lipase production by the mutant strain is not due to increase in growth but due to the enhancement in production of the lipase. Similar observations were made in *Pseudomonas*[8]. Similarly reports on the lipase activity increased in isolated bacterial strains by chemical mutagens [9]. The results indicated that lipase activity was more in olive oil medium after 24 h of EMS treatments. The enzyme activity was found to be higher in mutant strains than the wild type. The lipase activity considerably increased depending on nutritional conditions. The activity of enzymes was influenced after the rise in temperature above 27°C. The following results were reported by *Pseudomonas xinjiangansis* that the highest amount of lipase production were at room temperature [10].It was evident that the growth of lipase and production of enzymes in *Pseudomonas fluorescens* were of highest amount at the temperature of 37°C [11]. Previous

studied have shown lipase production of *Pseudomonas aerugiinosa* EF2 works optimally at 55ºC. The production of lipase was greatly induced by the temperature. The optimisation parameters revealed the temperature optimum for the activity of lipases at the fermentation conditions at 37 ºC (4.92 U mL^{-1}). The other temperatures such as at 15°C, 25°C and 45°C were also seen to induce the less amount of lipase production.The maximum lipase enzyme was attained in the range of at pH 6.5 (4.39U mL^{-1}). The pH effects on the activity of lipase were analyzed with different pH ranges from pH 5.5- 8.5 and found maximum level at pH 6 with 2.21U/ml. Lipase enzyme of *Pseudomonas aeruginosa* EF2 works optimally at pH 8 [12]. The enzyme activity were found to have efficient activity by utilizing glucose as carbon source (4.19U mL^{-1}) and yeast extract as nitrogen source (4.39U mL^{-1}). In previous reports it was suggested that the lipase production were more in glucose, sucrose. Lipase enzymes also present in oils such as vegetable oils and also in sunflower oil [13]. Normally, when organic sources of nitrogen were used in production high amount of lipase enzyme were observed in various organisms such as *Pseudomonas spp.* and *Bacillus spp.* in older reports [14, 15]. In various sources of nitrogen utilized the highest amount of enzyme activity were found in yeast extract at 3.32 U/ml. Similar results were also reported for organic source of nitrogen by *Pseudomonas sp, i*n the five various sources of nitrogen used and yeast extract was found to show the most accurate source of nitrogen for the maximum activity of lipase [16, 17]. Hence the process parameters for the isolate *Pseudomonas sp.*VITSDVM1 revealed the optimum conditions for lipase production at pH 6.5, 37^{o}C and by utilising glucose and yeast extract as carbon and nitrogen sources. HPLC chromatogram for partially purified enzyme produced from *Pseudomonas sp.* VITSDVM1 indicates the presence of lipase enzyme with the retention time at 3.065 min. This study identified a lipase strain with a maximum lipase activity that could be used for industrial scale.

Table 1: Lipase activity of *Pseudomonas* sp. treated by EMS

EMS treated *Pseudomonas* sp strains	Lipase activity after 96 h(Unit/g of substrate)
CS30	3.23±0.05
CS60	4.28±0.11
CS90	4.96±0.05
CS120	4.53±0.05
Wild type	3.10±0.06

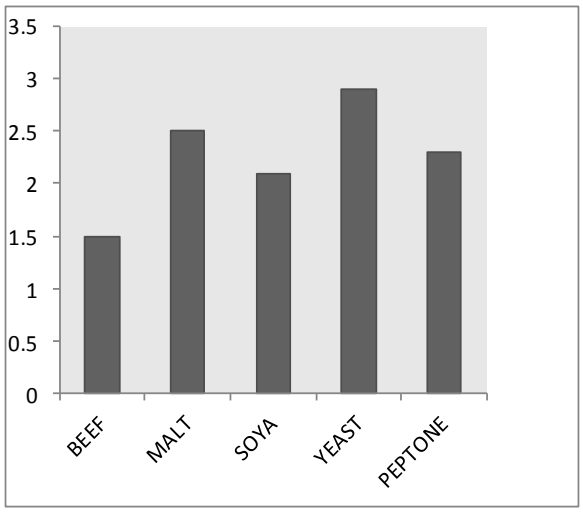

Figure 1: Effect of temperature on enzyme production by *Pseudomonas sp.*

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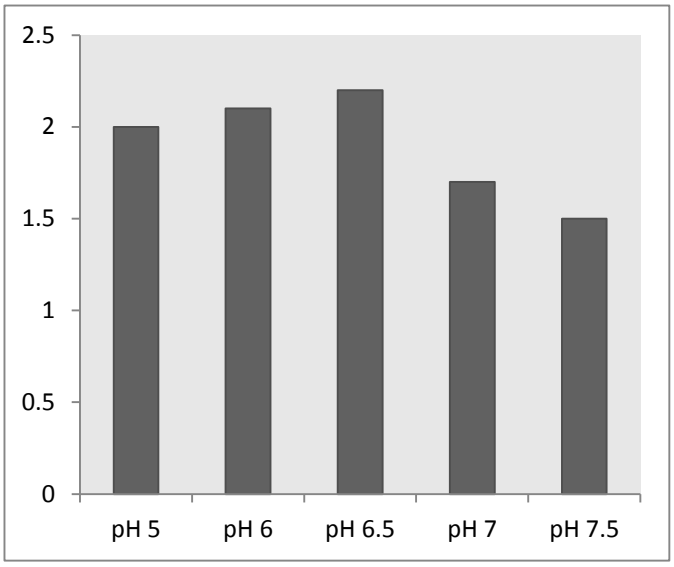

Figure 2: Effect of pH on enzyme production by *Pseudomonas sp.*

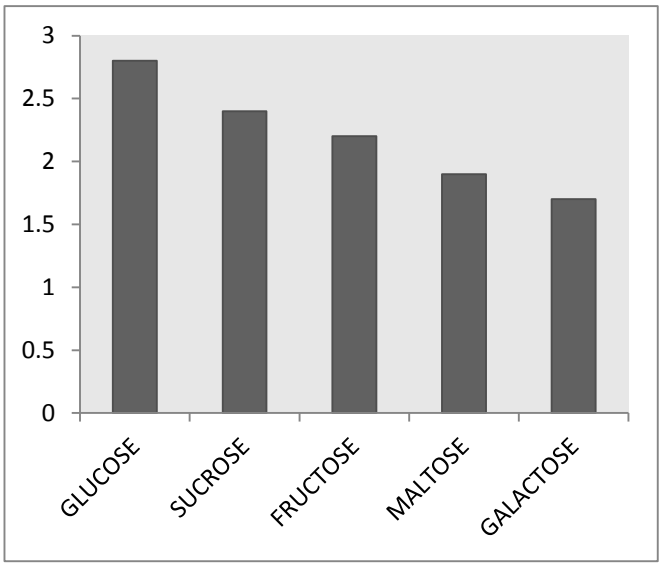

Figure 3: Effect of carbon sources on enzyme production by *Pseudomonas sp.*

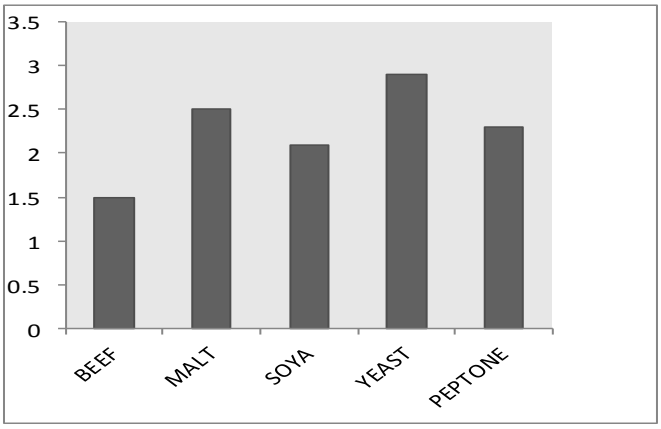

Figure 4: Effect of nitrogen sources on enzyme production by *Pseudomonas sp.*

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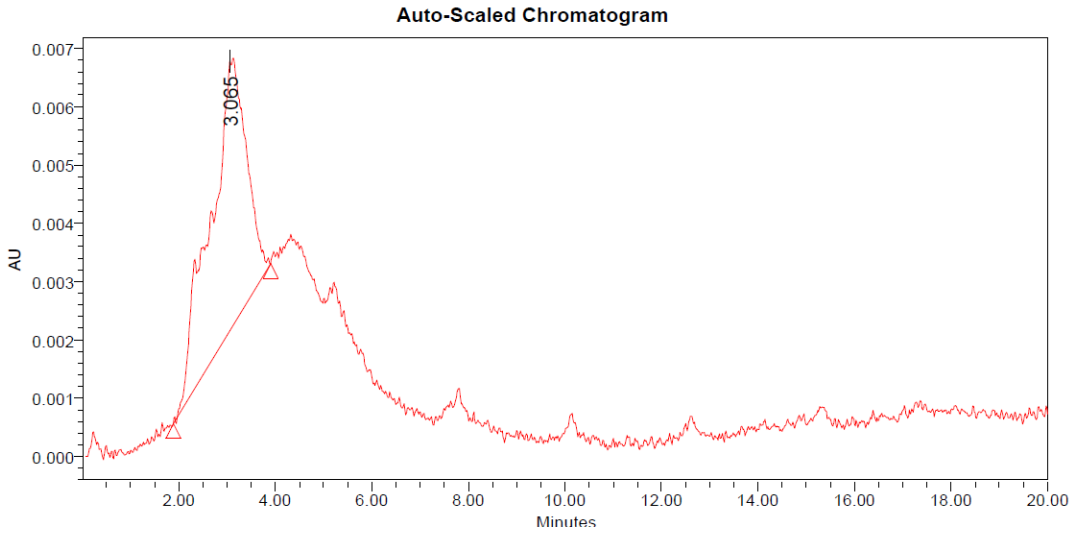

Figure 5: HPLC chromatogram of partial purified lipase

CONCLUSION

Mutation is the primary source of all genetic variation and has been used extensively in industrial improvement of metabolite production. The use of mutation and selection to improve the productivity of cultures has been strongly established for over fifty years and is still recognized as a valuable tool for strain improvement of many bioactive producing organisms. Lipases were important increasingly in high-value applications in the pharmaceutical industries and involved in the production of variety drugs and chemicals. The current study revealed that the enzyme lipase can be used in large scale industries which might hold various applications in different fields.

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