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Production and characterisation of extracellular lipase from *Bacillus stearothermophilus* MTCC 37 under different fermentation conditions

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ABSTRACT

Lipases have potential application in different fields of natural science. It is used as an enantioselective catalyst in aqueous as well as in low-water media. The present study aim for the standardization and optimization of lipase production by *Bacillus stearothermophilus* MTCC 37 under various physico-chemical conditions in the submerged medium**.** The production and lipase activity were found maximum 18U/mL at pH 7.5, 17.181U/mL at 55^0 temperature. The incubation period for maximum production was found to be 18 hours with 9.495U/mL. Under optimum conditions with 1.5% molasses as carbon source and soy extract as nitrogen source productivity under submerged fermentations conditions were found to be 12.91U/mL and 24.66U/mL respectively. The addition of 1% $CaCl_2$ as an activator proved effective with an increase in the activity to 20.128U/mL and with $HgCl_2$ taken as an inhibitor showed continuous decrease in the activity and drastically reduced to 1.66U/mL. The agro-waste products are found to be nutritionally enriched and thus can be used for production of many industrial products. One such attempt has been made through this study through solid state fermentation. Groundnut oil cake was used for the production of lipase under SSF conditions. At 36hrs the productivity was 22.91U/g. Cereal mixture in combination with groundnut oil cake showed higher 27.83U/g productivity. Whereas only the cereal mixture used for SSF was found to decrease the productivity to 19.08U/mL.

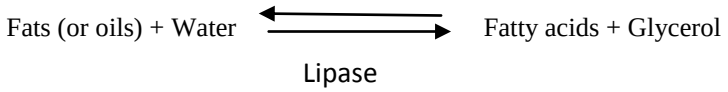
Keywords: ***Bacillus stearothermophilus*, Cereal mixture, Groundnut oil cake, Lipase, Molasses, Soy extract.**

INTRODUCTION

In this study, a comprehensive and illustrious survey is made of the applied aspects of microbial lipases in modern biotechnological practices. Lipases are the most versatile biocatalyst and bring about a range of bioconversion reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. The pivotal role of lipases in the processes and products of the food and flavouring industry, a promising application in the biodegradation of bioplastics is illustrated [26]. The panorama of lipases in the manufacture of fine chemicals is depicted with special emphasis on pharmaceuticals, pesticides, cosmetics, biosensors and detergents. Widening applications such as those in waste management and improved tanning techniques are other novel aspects of lipase utilization. A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or the other. Current developments in biotechnology are yielding new applications for enzymes [27]. The lipase enzyme find immense application in food, dairy, detergent and pharmaceutical industries etc. *Bacillus stearothermoplilus* MTCC 37, is a gram positive, thermostable, lipolytic organism. The advent of enzymology represents an important breakthrough in the biotechnology industry, with the world wide usage of enzymes being nearly U.S. $ 1.5 billion in 2000 [1].

Lipases (triacylglycerol acylhydrolases (E.C.3.1.1.3) are a class of hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over an oil-water interface and can reverse the reaction in aqueous and non-aqueous media. The reaction is reversible and catalyses the formation of acylglycerol from glycerol and fatty acids. The catalytic potential of lipases can be further enhanced and made selective by the novel phenomena of molecular imprinting and solvent engineering and by molecular approaches like protein engineering and directed evolution [2,3]. The important characteristics properties of lipases are substrate specificity, sterospecificity and the ability to catalyse heterogeneous reactions at the interface of water soluble and water insoluble systems [4]. Many enzymes denature or become inactivated by organic solvents but due to biotechnological potentials lipase results to high stability. The lipase enzyme even shows stability to extremes of pH, temperature, region and enantio-selectivity. This property of the enzyme provides chemical and pharmaceutical importance [5,6,7]. The stability of these enzymes in organic solvents have pushed them into the frontier areas of organic synthesis leading to the designing of novel drugs, surfactants, bioactive compounds and

oleo chemicals. In addition, lipase-catalysed trans-esterification and inter-esterification reactions have been exploited in the fat industry. Mode of action of lipase is illustrated as:



Lipases are serine hydrolases which act at the lipid–water interface. The catalytic triad is composed of Ser-Asp/Glu-His and usually also a consensus sequence (Glyx-Ser-x-Gly) is found around the active site serine [2].

Extensive studies on many microbes have been made for their potential production of extracellular lipases. Of these important ones are: *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium and Pseudomonas* including yeast and few endophytic fungi [8,9,10]. Fungi species are preferably cultivated in solid state fermentation (SSF) while, bacteria and yeast are cultivated in submerged fermentation (SmF). Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as an enzyme source [1, 29]. The study and development of lipase production in SSF system [9, 26 , 27] has been investigated on peanut press-cake using *Neurospora sitophila* and *Rhizopus oligosporus*. Rivera-Munoz *et al.* [28] compared SmF systems and SSF systems for lipase production using several filamentous fungi. Enzyme titres by SSF processes were higher and stable. Among the tested microbial strains, *P. candidum*, *P. camembertii*, and *M. miehei* proved the best for lipase production. The bioconversion studies attribute to the environmental safety management and health by effective utilization of agro residues [30].
The present study refers to the optimization conditions for the production of lipase using various parameters such as, pH, temperature, incubation time etc. The important energy sources that express for the higher production of lipase activity are carbon and nitrogen sources under submerged and solid state fermentation conditions have been compared.

MATERIALS AND METHOD

Selection of the strain A strain that produces lipase, *Bacillus stearothermophilus* MTCC 37 procured from MTCC was used for this study.

Media and culture condition

The lyophilized culture was reconstituted in 200mL of nutrient medium under aseptic conditions and incubated at 55ºC for 24 hours, maintained at pH 7.0. This pure culture was used as working culture and master culture preserved (in 10% glycerol) for the further use. Stock culture were also maintained by monthly transferring into the culture medium. The composition of the medium was peptone 2%, starch 2%, KH_2PO_4 0.5%, $CaSO_4$ 0.1%, $MgSO_4.7H_2O$ 0.1%, Distilled water 100mL, pH of the medium adjusted to 7.5 [18]. Tributyrin agar was prepared and sterilized by autoclaving at 121ºC. The culture was grown on the agar plate at 55^0 C for 24 hours and on completion of incubation period the clear zone was observed [11,12].

Effect of incubation time

The effect of incubation time on lipase production was studied by inoculating 2% of seed culture into the production medium [18] and incubated at 55^0 C for 24 hours under shaking conditions at 120 rpm. Lipase assay was performed after 12 hours for every 4hours of time interval.

Effect of pH

To study the effect of pH on the lipase production different pH ranges of media viz., 5, 7.5, 9 and 10; were taken and inoculated with 2% seed culture, the inoculated flasks were incubated under shaking condition at 120 rpm at 55ºC for 18 hours. Lipase activity was determined after 18 hours of incubation.

Effect of carbon source

Different carbon sources were taken for the study as a major carbon source for the enzyme production in our experiment. Molasses of different concentration (0.5% -- 2.0%) were incorporated into the production medium (KH_2PO_4, 0.5; $(NH_4)_2SO_4$, 0.1% ; $CaCO_3$ 0.1% ; $MgSO_4.7H_2O$ 0.1% ; pH 7.5); followed by incubation at 55ºC for 18hours. Lipase activity were determined after 18hours of incubation at 55 ºC under shaking conditions.

Effect of nitrogen source

For the optimization of nitrogen source soy extract were substituted in production medium. From the stock of 10% soy extract solution, various concentrations ranging from 0.5 until 2.0 ml were taken and flasks were incubated at 55ºC for 18hours.

Effect of substrate concentration

The enzyme production was estimated by using substrates viz., castor oil, coconut oil and olive oil. different concentrations from 0.5mL – 5mL of olive oil ,castor oil and coconut oil was taken as substrate, at 55ºC temperature and pH of 7.5 for 30 mins. Enzyme activity was estimated with 0.1mL of the substrate by performing assay on both the substrates and compared for the highest activity.

Effect of activator concentration

To obtain high productivity $CaCl_2$ was added in the production medium as a activator for the production of lipase enzyme. The concentration of activator was optimized from a stock solution of 10% of $CaCl_2$. Enzyme sample of 0.1ml were incubated with different concentrations of $CaCl_2$ (0.2ml – 1.0ml), at 55ºC temperature and pH 7.5 for 30 min. Lipase assay was performed to study the high productivity by the microorganism.

Effect of inhibitor concentration

Further studies were performed on the effect of inhibitors on the lipase production and $HgCl_2$ selected for the study. The concentration of inhibitor was optimized from a stock solution 10% of $HgCl_2$. 0.1 ml of enzyme was incubated with different concentrations of $HgCl_2$ (0.2ml – 1.0ml), at 55ºC temperature and pH 7.5 for 30 min. Activity of lipase enzyme was determined by olive oil assay.

Solid State Fermentation

The study also includes production of lipase enzyme by solid state fermentation to determine the effect of carbon and nitrogen sources on the growth as well as on the production. 70% moisture was maintained to operate the solid state conditions for the microorganism. 1.5% w/w of the dry mass substrate was taken and minimal medium was added to attain moisture level. The flasks were then incubated at 55^0 C. The assay was performed for the enzyme produced and its activity were measured after every 18 hours of incubation. The optimum conditions measured for Smf were only maintained for SSF with pH 7.5, temperature 55^0C and 1% activator. Various agro-waste products rich in nutritional properties were selected for the SSF . For this study groundnut oil cake, cereal mixture, and cane molasses were taken as carbon and nitrogen sources.

Enzyme assay

Lipase activity was estimated using olive oil substrate. The assay mixture (consisted of 3ml olive oil; 1ml Tris HCl buffer, pH 7.7 ; 1ml distilled water; pinch of bile salt (for emulsification) then incubated with 0.1ml of the enzyme for 30min at 55ºC. The reaction was terminated by adding 3ml ethanol (100%) followed by titration against 50mM NaOH to determine the amount of fatty acid liberated. One unit of enzyme activity was defined as one micro equivalent of fatty acid released from a triglyceride in one hour at pH 7.5 and 55°C.

Purification of the enzyme

Ammonium sulphate precipitation method was used for the purification of the extracted enzyme. Concentration of 70% cold saturated ammonium sulphate solution to a cell free supernatant was added (cells was removed by centrifugation at 6000rpm for 10min). The precipitates were suspended in 0.1M Tris HCl buffer. The enzyme was dialysed using cellulose acetate membrane against Tris HCl buffer. This partially purified enzyme was then subjected to anion exchange chromatography on DEAE-Cellulose column. 5ml of the enzyme eluted with 1M NaCl in the column.

RESULTS AND DISCUSSION

Effect of incubation time

The effect of incubation time under shake flask conditions has resulted to 18 hours as optimum incubation time for the growth of the organism and lipase production of 9.495U/ml/min. Lipase production by *Bacillus* sp. for maximum production was observed between 15-24 hours [13]. This may be an interesting property because it could allow harvesting of the enzyme for the shorter period of time (Fig. 1).

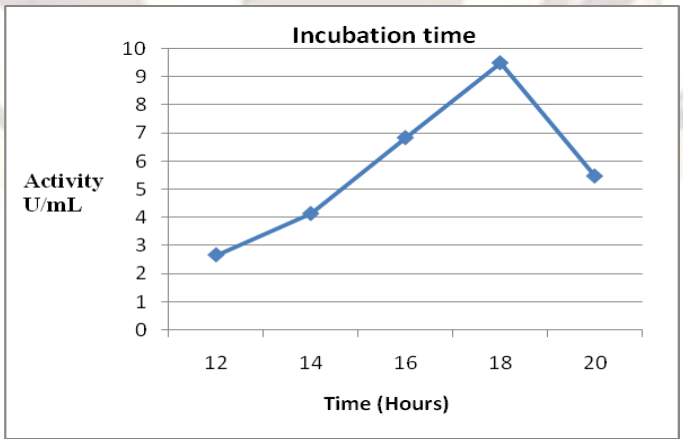


Fig 1: Lipase activity at different incubation time.

Effect of pH

The pH of the lipase enzyme was investigated using Tris HCl buffer with pH range of 5-10. The lipase yield was obtained at pH of 7.5 with an activity of 15U/mL found to be highest (Fig. 2). The optimal production of lipase by *Bacillus megaterium* AKG-1 was also reported at pH 7.5 [14]. A similar trend was observed

in *Bacillus thermocatenulatus* at pH 7.4 [15]. The use of lipase that is active at relatively alkaline pH is of great industrial application especially in detergent industries and could able to remove the dirts [18].

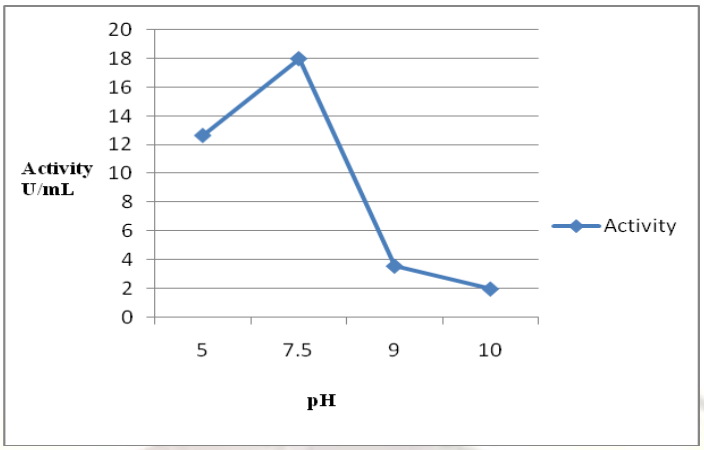


Fig 2: Effect of pH on lipase production using Tris HCl buffer at different pH

Effect of carbon source

Molasses as a raw material at different concentration was taken for the study to estimate the yield for lipase. The lipase yield was maximum at 1.5% concentration with an activity of 12.91 U/mL. The carbon concentration at 1.0% and 2% results to the sharp decline in the lipase production to 5.18 U /mL & 3.99 U/mL (Fig. 3). Thus the requirement of energy source by the microbe for high productivity is retained at lower concentration and since it is a type of raw material it is capable of supplementing the nutrient for the growth and production through complex form of the essential nutrients. *Rhodotorula mucilaginosa*-MTCC 8737 [16] also resulted good production with molasses. This substrate can be considered for industrial production as it doesn't release toxicity to the culture medium even it is considered as one the complex substrate. The decrease in the productivity at higher concentration of molasses could be due to the viscosity of the medium component that can be maintained using diluents [16]. According to Imandi et al [29] the presence of palm kernel cake with 70% moisture content yielded the maximum lipase activity (18.58U/gds) in four days.

Effect of nitrogen source

According to reports peptone was preferred for the production as a nitrogen source [14]. However tryptone also yields high for *Yarrowia lipolytica* [18] . In our study soy extract was used as nitrogen source and found it shows significant activity for lipase to 24.66 U/mL (Fig. 3). Different concentrations of nitrogen source showed constant increment in the activity and highest was 1% then sudden decline at 2% concentration. It shows that1.5% of the concentration can be considered as ideal state for good production and yield.

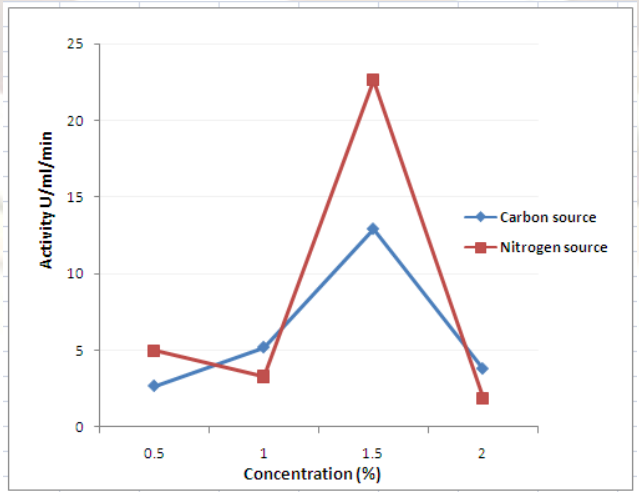


Fig 3: Effect of different concentrations of carbon and nitrogen source on lipase activity. carbon source used is molasses and nitrogen source used is soy extract.

Substrate concentration

To study the enzyme production different substrates were taken viz., olive oil, coconut oil and castor oil and it was observed for various concentration levels. It was found that the highest activity was found with olive oil and reduced activity for coconut oil. The lipase activity was found to be maximum at concentration of 3.5% for

all the substrates, with activity 20.471U/mL, 17.518 U/ mL and 15.39 U/mL (Fig. 4). Stimulatory effects of olive oil as a substrate on lipase production has been reported in *Y. lipolytica* [19] .

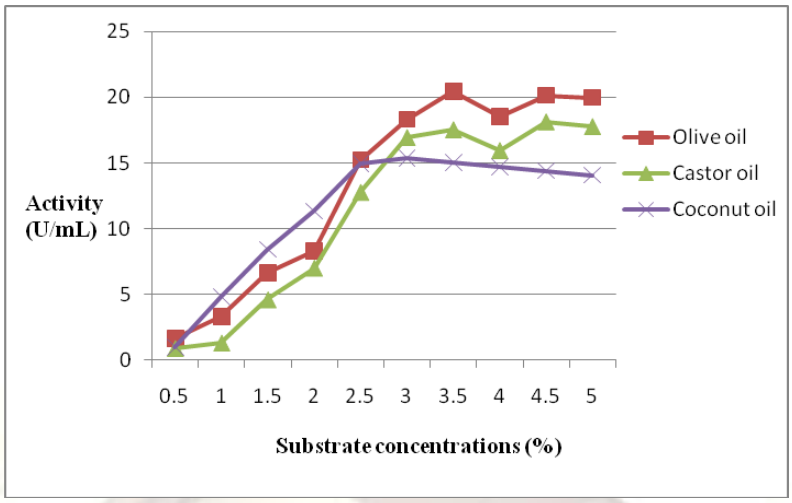


Fig 4: Effect of coconut oil, olive oil and castor oil as substrate to study the lipase enzyme activity under shaking conditions.

Activator concentration

$CaCl_2$ has found to stimulate enzyme activity. The optimum concentration of $CaCl_2$ was found to be 1% with a lipase activity of 20.128 U/mL (Fig. 5). Further increase in the concentration gave constant activity values. This may be due to the formation of calcium salts of long chain fatty acids[18]. Stimulatory effect of calcium activity on lipase been reported in the case of *B. subtilis* 168 [20], *B. thermoleovorans* ID-1 [21], *P. aeroginosa* EF2 [22]. Each divalent cation influenced the enzyme activity dependent on the temperature at which protein-ion metal interaction occurred [18].

Inhibitor concentration

The inhibitory effect on the lipase production by $HgCl_2$ was studied and it was found to be very effective as there is a decline in the activity oto 1.66 U/mL at a concentration of 1.2% (Fig. 5). According to reports lipase activity was almost completely inhibited by $HgCl_2$ in dormant seeds of the African oil bean [23]. Similar results have been reported with thermostable *Bacillus* H1 [24]. The inhibition is due to the binding of Hg^{2+} to the reactive –SH groups forming mercaptide compounds [25].

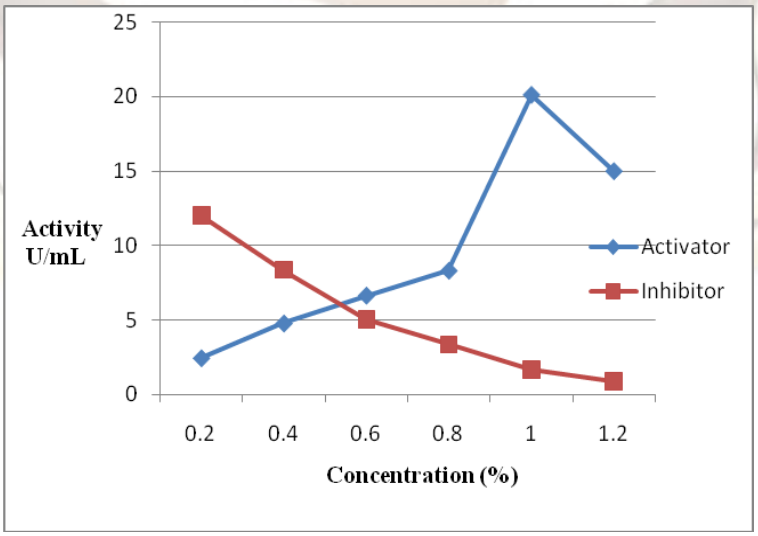


Fig 5: Effect of activator and inhibitor on lipase activity. Activator used was $CaCl_2$ and $HgCl_2$ used as inhibitor with different concentrations at 55°C for 18hours and pH 7.5.

SSF using agro-waste products

To determine the effect of different carbon and nitrogen supplements added into the medium for the production of lipase were estimated. Under same optimized conditions of pH, temperature and activator concentration with 1.5% w/w of substrate it was observed that at 36 hours for all the substrates was highest. Cereal mixture

and groundnut oil cake combination produced high 28.83U/g lipase and for molasses 19.51 U/g as shown in the Table 1. The moisture content was maintained with minimal medium to 70%. The assay for the enzyme activity with olive oil as a substrate was performed. Bhusan et al [31] reported lipase production in SSF system from an alkalophilic yeast strain belonging to *Candida* sp. Rice bran and wheat bran, oiled with different concentrations of rice bran oil were used as the substrate. Rice bran supplemented with olive gave higher lipase yields.

Comparative study of SmF and SSF for the lipase productivity

As per the result shown in Table 1, the maximum lipase activity under solid state system is found to be 22.91U/mL groundnut oil cake media, 28.83U/mL for cereal mixture and groundnut media (50% each), 19.08U/mL cereal mixture media and 19.51U/mL cane molasses. The maximum activity was observed for SmF at 18 hours where for SSF it was 36 hours. The reason could be the complexity of the medium components and its rate of utilization by the microorganism ; its bioconversion pathway to lipase [30]. The SmF conditions were comparatively lesser to SSF and the operating cost were also very high. The study indicates that the SSF with agro residues is more suitable for enzyme production like lipase since its extracellular nature can minimise the recovery process. This indicates that the harvesting time of the enzyme is less time consuming in submerged conditions and also provides better result than solid state conditions for production of lipase using *Bacillus stearothermophilus.* However, SSF condition can be improvised as the substrates used are industrial by products which are much cheaper than the SmF substrates.

Table 1: Different samples of 1.5% concentration was used for the study of lipase activity under SSF conditions.

Samples	Enzyme activity (U/g)		
	18 (hrs)	36 (hrs)	48 (hrs)
Groundnut oil cake media	8.33	22.91	12.72
Cereal mixture+ groundnut media	15.0	28.83	14.64
Cereal mixture media	9.33	19.08	11.39
Cane Molasses	10.67	19.51	9.85

Purification of the enzyme

The enzyme purification was carried out with DEAE- Cellulose column using ion exchange chromatography to obtain pure and homogenous enzyme. The lipase activity was characterised for the eluted enzyme sample and the molecular weight was found under both fermentation conditions to be 45 kDa by SDS-PAGE using silver staining.

CONCLUSION

The broad applications of biotechnology and the necessity for continued research and development on fats and oils in the food industry suggest that microbial lipase have increased importance and their role could be exploited. Application of SSF for the improvement of productivity and use of renewable resources can play an vital role in the industrial production of various enzymes. The use of these agro-wastes will help in the removal and management of waste and also could control in the generation of non-pollution wastes.

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