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Research Article

STUDY OF ENHANCED LIPASE PRODUCTION USING AGRO-WASTE PRODUCT BY *BACILLUS STEAROTHERMOPHILUS* MTCC 37

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ABSTRACT

The agro-waste products have been proved to be nutritionally enriched and thus can be used for production of many industrial products. This study reveals the utilization of renewable resources for their cost effective production and influence on the process under mild conditions.The physico-chemical conditions were optimised for the productivity. In the second experimental stage the best substrate in terms of carbon and nitrogen were estimated for the lipase production and activity. According to the optimum conditions the production and activity were measured as; at pH 7.518U/mL, temperature 55^{0}C, 17.181U/mL 18 hrs 9.495U/mL of incubation time. Maintaining these optimum conditions of physical parameters different carbon and nitrogen sources of 1.5% concentration were measured for activity. Wheat bran and cereal mixture produced maximum lipase activity of 22.04U/mL and 27.54U/mL respectively. To enhance the productivity 1% $CaCl_2$ as an activator is added into the medium and it proved effective with an increase in the activity to 20.128U/mL.

Key words: *Bacillus stearothermophilus,* Cereal mixture, Groundnut oil cake, Molasses.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases (E.C.3.1.1.3) belongs to the class of hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over an oil-water interface and reverse the reaction in aqueous and non-aqueous media. 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 [1, 2]. The important property of lipases are substrate specificity, sterospecificity and the ability to catalyse heterogeneous reactions at the interface of water soluble and water insoluble systems [3]. The lipase enzyme finds immense application in food, dairy, detergent and pharmaceutical industries etc. Enzymes are among the most important products obtained for human needs through microbial sources**.** Lipases are the most versatile biocatalyst and bring about a range of bioconversion reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. The important role of lipases in the processes of food and flavourings industry, also a promising application in the biodegradation of bioplastics is illustrated [25]. Applications 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.

The lipase enzyme even shows stability to extremes of pH, temperature, region and enantio-selectivity. This property of the enzyme provides biotechnological potential , chemical and pharmaceutical importance [4, 5, 6]. The esterification reactions have been exploited in the fat industry. Many enzymes denature or become inactivated by organic solvents but lipase results to high stability. *Bacillus steareothermophilus* is a thermostable organism that could withstand higher trremperature and can be used for higher productivity under industrial scale.

The bioconversion studies attribute to the environmental safety management and health by effective utilization of agro residues [26]. The substrate that could provide all the necessary nutrients to the microorganisms during the growing period and also bring stability during the production stage can be used as an ideal substrate. To simplify the process of nutritional uptake pre-treat (chemically or mechanically) of some of the substrates before using in fermentation processes (e.g. ligno-cellulose), thereby making them accessible for microbial growth. The agro-based renewable resources are considered as most cost-effective , nutritionally having high calorific value and support the microbial growth under various optimal conditions thus increasing its utilization for many industrial productions.

The study and development of lipase production in SSF system [24, 27] investigated SSF on peanut press-cake using *Neurospora sitophila* and *Rhizopus oligosporus*. Few important microorganisms used for the studies on extracellular lipase production are: *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium and Pseudomonas* [7,9,27], including yeast and few endophytic fungi. Among the tested microbial strains, *P. candidum*, *P. camembertii,* and *M. miehei* proved the best for lipase production. Fungi species are preferably cultivated in solid state fermentation (SSF) while, bacteria and yeast are cultivated in submerged fermentation (SmF).

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 fermentation conditions has been reported in this paper.

MATERIALS AND METHOD

Media and culture condition

Bacillus stearothermophilus MTCC 37 was used for the study of lipase production. 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 glycerol) for the completion of the experiment. 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 [8, 16]. The culture was grown on the tributyrin agar plate at 55^0 C for 24 hours and on completion of incubation period the clear zone was observed [10,11].

Effect of physical conditions: incubation time and pH

The study on the effect of incubation time and pH were performed by inoculating 2% of seed culture into the production medium [8, 16] and incubated at 55^0 C for 24 hours under shaking conditions at 120 rpm. Lipase assay was performed for every 4 hours of time interval after an initial incubation of 12 hours. The effect of pH on the lipase production at different pH ranges of media viz., 5, 7.5, 9 and 10.

Effect of carbon source

To study the efficiency of renewable substrates from different agricultural – waste products and selection of an high producer carbon sources cane molasses, groundnut oil cake, tamarind seed, mango

seed and wheat bran 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 18 hours. Lipase activity was determined after 18 hours of incubation at 55 ºC under shaking conditions.

Effect of nitrogen source

Similarly, different nitrogen sources were used for the optimization. Soy extract, cereal mixture, corn steep liquor were substituted in the production medium as nitrogen components. From the stock of 10% substrate solution, various concentrations ranging from 0.5 until 2.0 ml were taken and flasks were incubated at 55ºC for 18 hours.

Effect of substrate concentration

The enzyme production was estimated by using substrates viz., castor oil, coconut oil and olive oil for the experiment. Different concentrations of olive oil, castor oil, and coconut oil (0.5ml – 5ml) as substrate were taken, at 55ºC temperature and pH of 7.5 for 30 min. Enzyme activity was estimated by performing assay with the substrates and compared for the high activity.

Effect of activator concentration

In order to increase the 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), and lipase assay were performed.

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 $HgCl_2$. 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.

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

The enzymes were purified by ammonium sulphate precipitation method. Cold saturated ammonium sulphate solution to a cell free supernatant was added (cells were removed by centrifugation at 6000rpm for 10min), to a final concentration of 70%. The precipitates were suspended in 0.1M Tris HCl buffer. The enzyme was dialysed using cellulose acetate membrane against Tris HCl buffer. The partially purified enzyme was subjected to anion exchange chromatography on DEAE-Cellulose column. 5ml of the enzyme eluted with 1M NaCl in the column. The purified sample was further used for the estimation of the molecular weight of the protein by SDS-PAGE using silver staining.

RESULTS AND DISCUSSION

Effect of incubation time and pH

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 [12]. This may be an interesting property because it could allow harvesting of the enzyme for the shorter period of time (Table 1 & 2) [8]. 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 [14]. 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 [17].

Effect of carbon source

The raw materials at different concentration were taken as major carbon sources 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 for molasses. Comparatively higher productivity was observed with wheat bran and groundnut oil cake 22.04U/mL and 19.48U mL respectively. As it was seen that even tamarind seed and mango seed also contribute for productivity and it was found to be 10.11U/mL and 7.96U/mL respectively. The lipase production was very less for these substrates. In case of mango seeds it was observed that oil droplets were synthesized more with the increase in the substrate concentration thus could be a reason for decrease in the production of lipase produced.

The carbon concentrations at 1.0% and 2% results to the sharp decline in the lipase production by all the substrates supplemented into the medium. (Fig. 1). Thus the requirement of energy source by the microbe for high productivity is retained at the concentration of 1.5% and since it belongs to the category of raw materials, it is capable of supplementing with many essential nutrients for the microbial growth and production through complex form. *Rhodotorula mucilaginosa*-MTCC 8737 [15] 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 [15]. Several carbon sources – individually and in combinations – were tested for their efficiency to produce lipases. Raw cake supported the growth and lipase synthesis by the yeast culture. According to Imandi et al [25] 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

In our study soy extract, corn steep liquor and cereal mixture were used as nitrogen source and observed significant activity for lipase to 24.66 U/mL,19.41U/mL and 27.54 U/mL respectively (Fig. 2). According to reports peptone was preferred for the production as a nitrogen source [13]. However tryptone also yields high for *Yarrowia lipolytica* [17, 26] . Different concentrations of nitrogen source showed constant increment in the activity and highest was 1.5% then sudden decline at 2% concentration. It could be interpreted that 1.5% of the concentration can be considered as ideal state for good production and yield.

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. Stimulatory effects of olive oil as a substrate on lipase production has been also reported in *Y. lipolytica* [17] .

It was found that the highest activity was found with olive oil and lowest activity with castor and very much reduced activity for coconut oil [Fig. 3]. The lipase activity was found to be maximum at concentration of 3.5% for olive and castor oil but for coconut it was maximum at 2% of the substrates concentration, with activity 20.471U/mL, 17.518 U/ mL and 15.39 U/mL respectively (Table 3) [8].

Activator concentration

The optimum concentration of $CaCl_2$ was found to be 1% with a lipase activity of 20.128 U/mL (Fig. 4). Further increase in the concentration gave constant activity values. $CaCl_2$ has found to stimulate enzyme activity. Stimulatory effect of calcium activity is measured in terms of reduction in the surface tension and increase in the permeability of the cell membrane. The increasing concentration were found not supporting may be because of the high

viscosity in the medium. This may be due to the formation of calcium salts of long chain fatty acids [16]. The effect of calcium ions on lipase been reported in the case of *B. subtilis* 168 [18], *B. thermoleovorans* ID-1 [19], *P. aeroginosa* EF2 [20]. Each divalent cation influenced the enzyme activity dependent on the temperature at which protein-ion metal interaction occurred (Table 3).

Inhibitor concentration

The study of inhibitor was performed to observe any bifurcation the synthetic pathway which could enhance the production. 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 (Fig. 4) in the activity to 1.66 U/mL at a concentration of 1.2% (Table 3). Reports on dormant seeds of the African oil bean has shown that lipase activity was completely inhibited by $HgCl_2$ [21] thus stops the biosynthetic pathway by disrupting the metabolite formation that could lead for the final product formation. Similar results have been reported with thermostable *Bacillus* H1 [22]. The inhibition is due to the binding of Hg^{2+} to the reactive –SH groups forming mercaptide compounds [23].

Purification of the enzyme

The lipase activity was characterised for the eluted enzyme sample and the molecular weight was found to be 45 kDa by SDS-PAGE .The enzyme purification was carried out with DEAE cellulose using ion exchange chromatography to obtain pure and homogenous enzyme.

CONCLUSION

The use of agro-wastes will help in the removal and management of waste and also could control in the generation of non-pollution waste. Another breakthrough is by genetically modifying the strains, suitable for high yield processes, would play an important role in this field of biotechnology. The conventional physico-chemical means of lipolysis could now been under shadowed by the biocatalysis using microbial lipases. The applications of biotechnology in various fields 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.

Table 1: Activity of lipase at different incubation time

Incubation time (Hrs)	12	14	16	18	20	24
Activity (U/mL)	2.66	4.13	6.83	9.495	5.465	1.37

Table 2: Study of effect of different pH on lipase activity

pH	5	7.5	9	10
Activity (U/mL)	12.66	18	3.57	1.98

Table 3: Study on the influence of various substrates (coconut, castor and olive oil) for the estimation of lipase activity under shaking conditions

S. No.	Concentration	Activator (%)	Inhibitor (%)
1.	0.2	2.46	12
2.	0.4	4.79	8.33
3.	0.6	6.66	5
4.	0.8	8.33	3.33
5.	1.0	20.128	1.66
6.	1.2	15.01	0.853

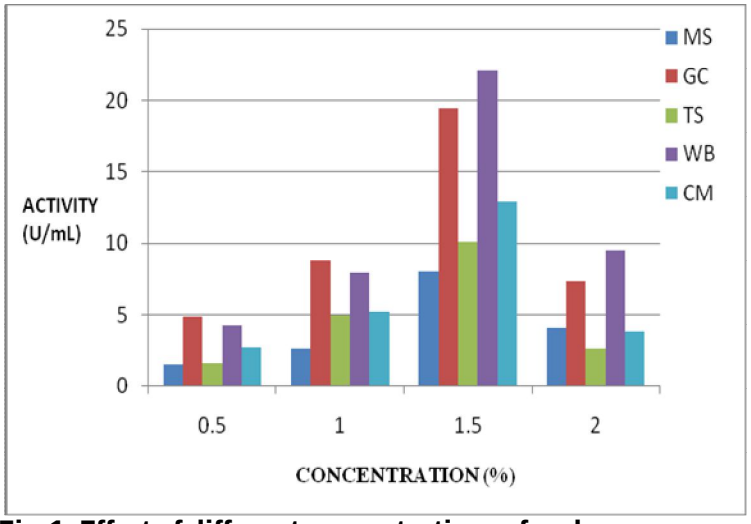

Fig. 1: Effect of different concentrations of carbon source on lipase activity. 1% w/v of the carbon sources: mango seed, groundnut oil cake, tamarind seed, wheat bran, and cane molasses

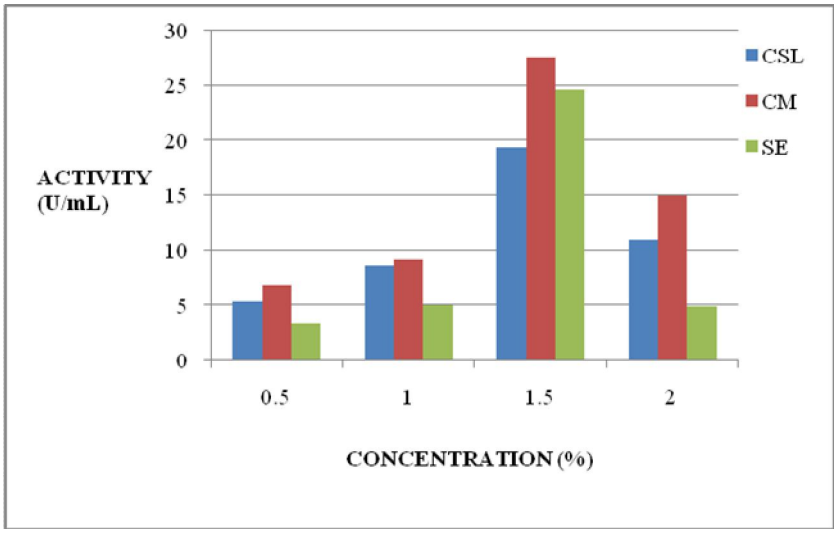

Fig. 2: Effect of different concentrations of nitrogen source on lipase activity. 1%w/v of nitrogen source used : Corn steep liquor, Cereal mixture and Soy extract.

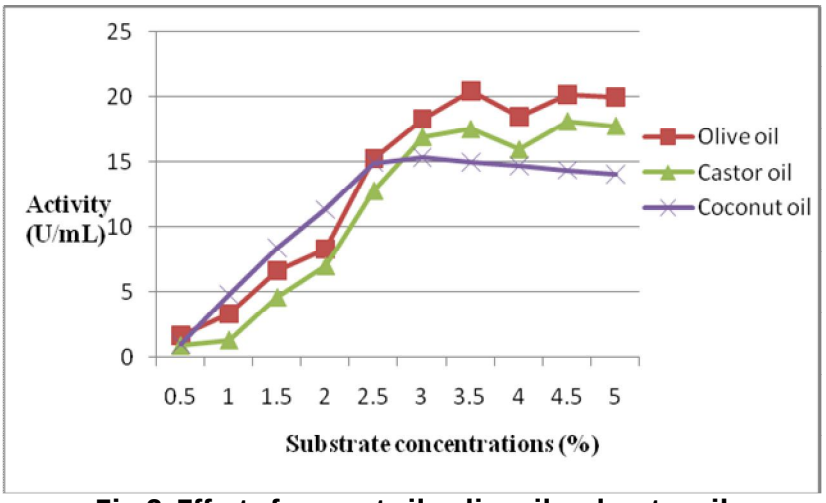

Fig. 3: Effect of coconut oil, olive oil and castor oil as substrate to study the lipase enzyme activity under shaking conditions

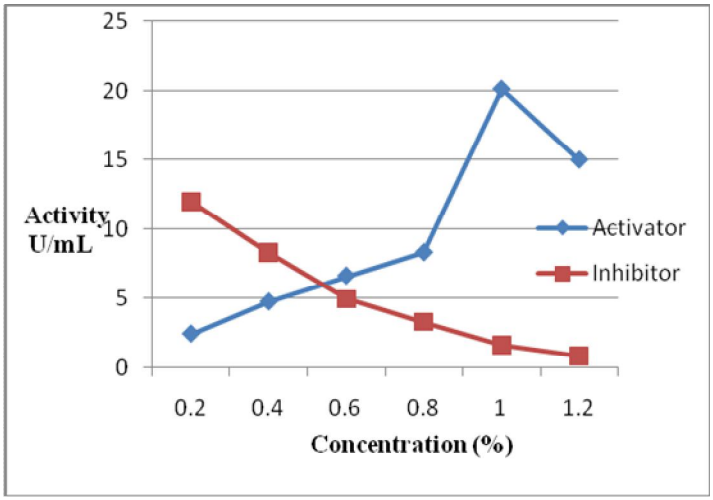

Fig. 4: Effect of activator and inhibitor on lipase activity. Activator used was $CaCl_2$ and $HgCl_2$ used as inhibitor with different concentrations

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