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Biosynthesis of Novel Alkaline Lipase Production from Penicillum Chrysogenum Suitable For Detergent Formulation

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ABSTRACT

The filamentous fungi Penicillium chrysogenum produced high levels of lipase when cultured for 5 days at 30° C and pH 9 with ground nut oil cake as substrate. Enhanced production occurred on addition of lactose, urea and $CaCl_2$ as nutritional factors. The crude extract was purified to 45.48 fold with recovery of 60 % by DEAE-column chromatography and the molecular weight was estimated to be 52KDa by SDS-PAGE. The lipase was found to be stable at higher pH 8-10 and temperature 30-50° C ranges The substrate concentration exhibiting Vmax value of 88 U/mg and Km value of 1.14 mg/ml. Regarding the stability of detergent process, the enzyme was highly stable in the presence of various surfactants and commercial detergents. For these characteristics, the lipase from Penicillium chrysogenum showed good potential as an additive in laundry detergent formulation.

Keywords: Lipase, Penicillium chrysogenum, Solid state fermentation, Optimization, Characterization & detergent.

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INTRODUCTION

Lipases (glycerol ester hydrolase, EC 3.1.1.3) are ubiquitous enzymes produced by plants, animals and microorganisms belonging to sub class 1 of hydrolytic enzymes class 3 [1]. In comparison to animal or plant lipase, extracellular microbial lipase can be produced relatively inexpensively by fermentation and in large quantities [2]. Microbial lipase can be produced by using agricultural residues like sugarcane baggase, wheat bran and rice bran by SSF which is highly economical [3]. Microbial lipases are widely used in the processing of fats and oils, decreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, production of cosmetics, paper manufacture, waste management, biosensors etc. [4, 5]. Recently, attention has been focused on the application of alkaline lipase in detergent formulations.

Microbial lipases are produced mostly by submerged culture [6], but solid-state fermentation methods can be also used [7]. In general, solid state fermentation is a well-adapted and cheaper process than submerged fermentation for the production of a wide spectrum of bioproducts and it is a high recovery method for the production of industrial enzymes [8]. This technique involves the growth and metabolism of microorganisms on moist solid without the involvement of free flowing water. Solid state fermentation (SSF) has many advantages over submerged fermentation including economy of space needed for fermentation, simplicity of fermentation media, no requirement of complex machinery, equipment and control system, compactness of fermentation vessel owing to lower water volume, superior yields, less energy demand, lower capital and recurring expenditure [9, 10].

The functional importance of lipases in the detergent industry is related to the removal of fatty residues in laundry, dishwashers as well as for cleaning of clogged drains. This enzyme showed a variable specificity/hydrolytic activity towards various fats and oils. All these properties and its resistance towards various surfactants and tolerance to commercial detergents make this lipase a potential additive for detergent formulation.

However, little attention has been paid to the process stability for detergent formulations. In the present study, an alkaline lipase was produced from solid-state fermentation of agro-industrial wastes by lipase producer strain Penicillium chrysogenum. Typically a detergent lipase needs to be active and stable in alkaline environments (pH=8–11, temperature 20–50 °C) and in the presence of salt and surfactants [11]. Here, Penicillium chrysogenum producing low-temperature alkaline lipase in a stable process is described.

MATERIALS AND METHODS

Organism

40 fungal species were collected from Karpagam culture collection center and were subjected for preliminary screening for lipase production. Among the 40 species, 28 species

shows lipase activity. Among them P.chrysogenum was found to be best lipase producer and the same was used in the study.

Substrates

Different substrates viz., Ground nut oil cake, Gingely oil-cake, Cotton seed oil-cake, Coconut oil-cake, Rice bran, Wheat bran, Paddy straw, Cotton seed, Black gram husk and Green gram husk. Agro waste substrates were purchased from local markets.

Screening method for lipase production

The screening of the fungal strains for lipase production was studied by inoculating them on Rhodamine B medium [12]. Lipase production was detected by irradiating the plates with UV light at 350 nm. Fungal colonies that have lipolytic production showed orange fluorescent halo.

Screening of alkaline lipase production

Selective agar medium (0.5 g yeast extract, 5.0 g (NH4)2SO4, 2.0 g (NH2)2CO, 1.0 g MgSO4.7H2O, 1.0g NaCl, 2.0 G bile salts, 10.0 ml olive oil, 20.0 g bacteriological agar, pH 7.0 (per litre)) for the isolation of alkaline lipase producing fungi was prepared using Colen et al [13] and observed for clear hydrolytic halos.

Solid State Fermentation

Ten grams of agro industrial waste (rice bran, wheat bran, coconut oil cake, gingelly oil cake, cotton seed cake groundnut oil cake, sugarcane baggase and) was taken in 250 ml Erlenmeyer flasks, moistened with 10 ml of sterile distilled water in the ratio of 1:1, w/v and sterilized. After cooling, the flasks were inoculated with 1 ml of spore suspension (10^6 spores/ ml) and the contents were mixed and incubated at 30°C for seven days. Enzyme extraction was performed by adding 100 ml of distilled water to the solid moulding medium and shaking the mixture in a rotary shaker (100 rpm) for 1 hour. The extracts were squeezed through a muslin cloth and clarified by centrifugation at 10,000 x g for 5 minutes [14]. The supernatants were used as crude enzyme.

Enzyme assay

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis [15]. One ml of the culture supernatant was added to the reaction mixture containing 1ml of 0.1M Tris-HCl buffer (pH 8.0), 2.5 ml of deionised water and 3 ml of olive oil. The reaction mixture was mixed well and incubated at 37 °C for 30 minutes. Both test and blank were performed. Immediately after starting the incubation 1 ml of the culture supernatant was pipetted into a 50

ml Erlenmeyer flask marked blank and stored at 4° C. After 30 minutes the test solution was transferred to a 50 ml Erlenmeyer flask. 3 ml of 95% ethanol was added to stop the reaction. Liberated fatty acid was titrated against 0.1M NaOH using thymolphthalein blue as indicator. A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per minute under specified assay conditions. Enzyme activity was expressed as units per gram of dry substrate.

Protein assay

Protein content of the supernatant was quantified by the method of Lowry et al. [16] with bovine serum albumin as standard and was expressed as mg/ml.

Optimization studies

Using different agro-industrial waste materials (wheat bran, rice bran, cottonseed oil cake, coconut oil cake groundnut oil cake, sugar cane bagasses and), lipase production was studied at different pH (7 - 11), temperature (30 – 70° C), moisture content (10-50%) and incubation period (3-9 days).

Different carbon sources (lactose, maltose, mannitol, starch and sucrose), nitrogen sources (peptone, yeast extract, casein, urea and albumin) and metal ions (magnesium sulphate, calcium chloride, sodium chloride, ferrous sulphate and zinc chloride) were supplemented separately to a final concentration of 1% (w/v) in solid media. After fermentation, the lipase activity was estimated.

Partial purification and enzyme characterization

Crude extract was precipitated by 70% saturation with ammonium sulphate and then dialyzed against 0.2M phosphate buffer (pH 6.2)) for 24 hours at 4° C. The filtrate was loaded onto a DEAE-cellulose chromatographic column (2.4 x 45 cm) pre equilibrated with 5 mM phosphate buffer (pH 6). The enzyme was eluted with the same buffer at a flow rate of 10 ml/h [17].

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12 % (w/v) acrylamide slab gel with 25mM Tris/192 mM glycine buffer (pH 8) that contained 0.1% (w/v) SDS as the running buffer, as described by Laemmli [18].

Optimum temperature for activity of the lipase was determined by carrying out at selected temperatures from 30 to 70 °C. In each case, the substrate was preincubated at the required temperature before the addition of enzyme. The optimum pH was determined by monitoring lipase activity at pH values between 4 - 8.

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The optimum substrate concentration for maximum enzyme activity was determined in terms of maximum retention velocity (V_{max}) and Michaelis constant K_m at which the retention velocity is half maximum. For this various concentration of protein in 0.05 M sodium acetate buffer were incubated with purified enzyme preparation. The accurate values of V_{max} and K_m were obtained from the Lineweaver – Burk plot and Eadie- Hofstee plot [19].

Effect of detergents on lipase stability

Different detergents were added to the enzyme solution extracted from the moldy substrate at a concentration of 1mg/ml and incubated for 1h at 30ºC. Samples were taken at 15min intervals and assayed for enzyme activity.

RESULTS AND DISCUSSION

All the 40 isolates collected from KMCCC were subjected to rapid screening by using Rhodamine B method (Table 1). Out of these isolates, 28 showed a zone of fluorescence at 350 nm. Among the active isolates, one isolate (Penicillim chrysogenum.) found to produce maximum growth zone of fluorescence (Fig. 1). Those 28 strains which showed growth in Rhodamine B agar were further screened for alkaline lipase production using the selective media. Out of 28 microbial sp., Penicillium chrysogenum showed the maximum hydrolytic halo around the colony, which indicates it, produces the highest production of alkaline lipase (Fig. 2).

Table: 1 Rapid screening of fungi for their lipase production using Rhodamine B agar medium

S.no	Fungal genera	Zone of fluorescence
1	Acremonium furcatum	-
2	Acremonium murorum	-
3	Aspergillus alutaceus	-
4	Aspergillus erythrocephalus	-
5	Aspergillus flavipes	+
6	Aspergillus flavus	+
7	Aspergillus fumigatus	+ +
8	Aspergillus glaucus	+ +
9	Aspergillus japonicus	+
10	Aspergillus melleus	-
11	Aspergillus nidulans	+ +
12	Aspergillus niger	+ + +
13	Aspergillus ornatus	+
14	Aspergillus oryzae	+ +
15	Aspergillus sclerotorum	+ +
16	Aspergillus sydowii	+ +
17	Aspergillus terrus	+
18	Aspergillus ustus	+
19	Aspergillus versicolor	+
20	Aspergillus wentii	+ +
21	Fusarium culmorum	-

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22	Fusarium dimerum	+
23	Fusarium merismoides	+
24	Humicola insolens	-
25	Mucor circinelloides	-
26	Penicillium brevicompactum	+ +
27	Penicillium chrysogenum	+ + +
28	Penicillium fellutanum	-
29	Penicillium frequentans	+
30	Penicillium livindum	-
31	Penicillium nigricans	+ +
32	Penicillium restrictum	+ +
33	Penicillium sacculum	+
34	Penicillium thomii	+
35	Penicillium oryzae	+ +
36	Penicillium canescens	-
37	Penicillium citrinum	+
38	Penicillium claviforme	+
39	Trichoderma koningii	-
40	Rhizopus oryzae	+ + +

++ + = Very good, ++ = good, + = Average, - = No zone

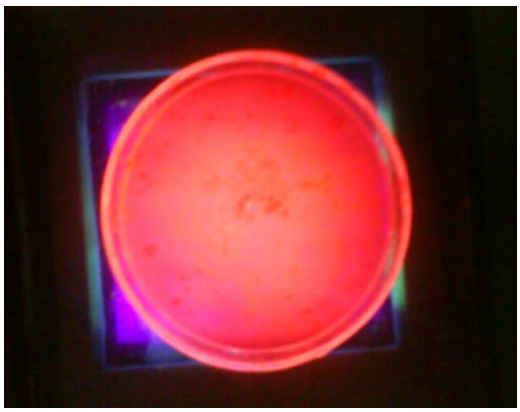

Figure 1: Growth of Penicillim chrysogenum showing fluorescent halo on Rhodamine B agar medium
Figure 2: Penicillim chrysogenum showed the maximum hydrolytic halo around the colony

Optimization of SSF system for lipase production

Effect of different solid substrates on lipase production

Among all the substrates, the maximum lipase activity of 107 U/ gds was observed with groundnut oil cake (Fig 3), these results were in accordance with observed lipase production from Singh et al., 2010. Different substrate occupied surface area according to their sizes was

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an important parameter in solid-state fermentation.10 gram of substrate yields maximum production of lipase. Due to its easy penetration, the microbial mass showed high growth rate with Groundnut oil cake as a substrate due to which more lipase production was observed. The less lipase production at higher level was due to low mass transfer rate and difficulty in penetration of the organism[20].

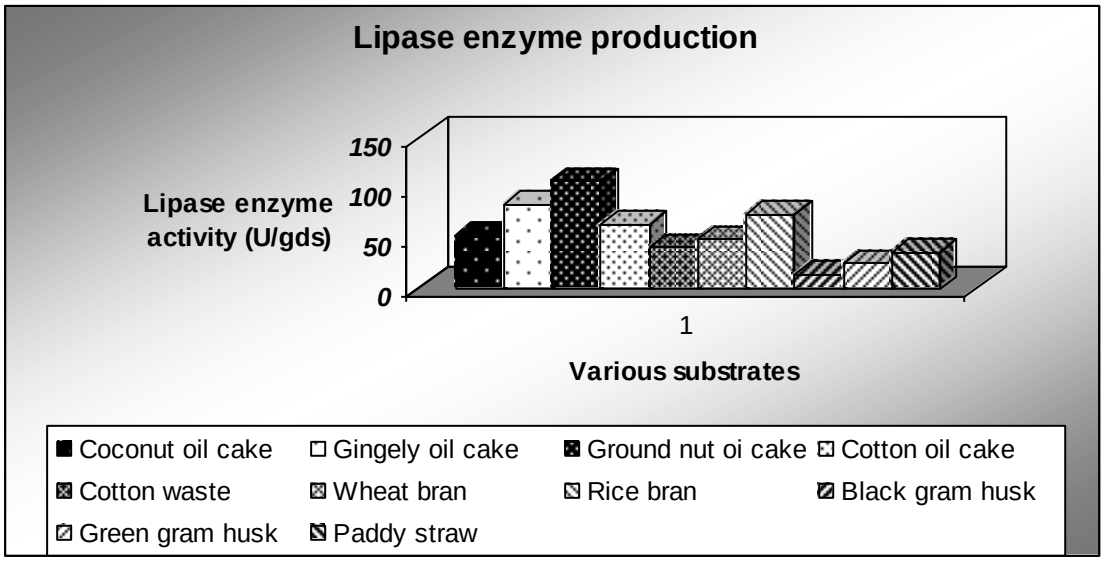

Figure 3: Effect of different solid substrates on lipase production

Effect of various physico – chemical parameters

Effect of temperature and pH on enzyme production

The effects of various temperatures ranging from 10°C to 80°C were carried out in P.chrysogenum for enzyme production. The lipase enzyme production was maximum at 30°C. P.chrysogenum showed maximum enzyme production of 110 U/gds respectively. Similar results were found in C.gigantea and other mesophiles for lipase production at 30 to 35° C [21].

The lipase enzyme production for P.chrysogenum was studied on different pH variations from 4 to 10. Penicillium chrysogenum showed the maximum enzyme production on pH 9 with 120 U/gds. Mala et al., reported the optimum pH of 8.6 for lipase production for F.solani maintained around 80% of its initial activity when incubated for 1 h at alkaline pH (7.2-8.6), with a decrease in lipase activity at pH beyond in this range [22].

Effect of various incubation periods and moisture on enzyme production

The lipase enzyme production for P.chrysogenum was examined on various incubation days ranging from day 3 to 7 days. P.chrysogenum showed the maximum of 110 U/gds enzyme

production on day 5. The results are similar to that of Cho et al., [23] observed maximum lipase activity (40U/ml) for Penicillium chrysogenum when incubated at 20ºC on the fifth day of incubation.

The effect of moisture content was examined in the range from 10% to 50% for the analysis of lipolytic activity. P.chrysogenum showed the maximum enzyme production at 20% with 92 U/gds respectively. An increase in moisture level is believed to reduce the porosity of the substrate, thus limiting oxygen transfer [24]. Low moisture content causes reduction in the solubility of nutrients of the substrate and low degree of swelling.

Effect of various carbon, nitrogen sources and metal ions on enzyme production

Several carbon sources like glucose, maltose, sucrose, lactose and mannitol were assayed for the enzyme production. Penicillium chrysogenum showed the maximum enzyme production with lactose with 140 U/gds. According to sumitra et al., [25] the supplementation of the substrate with different carbon sources showed an increased production of the enzyme. Several nitrogen sources like urea, albumin, peptone, casein and yeast extract were assayed for the enzyme production. Penicillium chrysogenum showed the maximum enzyme production with Urea 120 U/gds. The supplementation of nitrogen sources resulted in an increasing growth in a range of 6.75 U/mL for A. flavus [26].

Several metal ions like $ZnCl_2$, $FeSO_4$, $MgSO_4$, NaCl, and $CaCl_2$ were assayed for the enzyme production. Penicillium chrysogenum showed the maximum enzyme production with $CaCl_2$ 80U/gds. Pokorny et al., 1994 reported that lipase production by Aspergillus niger was enhanced in the presences of Mg2 + [27]. The result suggested that concerned metal ions apparently protected the enzymes against denaturation and played a vital role maintaining active conformation of the enzyme at high temperature [28].

Purification and Enzyme characterization

Table: 2 Purification of lipase enzyme from Penicillium chrysogenum species

Fractions	Total volume (ml)	Activity (U/g dry substrate)	Protein (mg/ ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/g dry substrate)	Purification (Fold)	Recovery (%)
Culture filtrate	100	150	0.140	14	15000	107.14	1	100
Ammonium Sulphate	50	260	0.132	6.6	13000	1969.69	18.38	86.66
Dialysis	25	320	0.102	2.55	8000	3137.25	29.28	61.54
DEAE cellulose Chromatography	10	480	0.0985	0.985	4800	4873.09	45.48	60

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The purification of lipase from Penicillium chrysogenum is showed in Table 2. The specific activity of the final purified preparation was 3428.57 U/g dry substrate protein, representing a total purification factor of 22.51. Our result was in consonance with the work of Fuh lin et al., 1996, who purified the enzyme with a recovery yield of 15% with the specific activity 5,920 U/ mg in Pseudomonas pseudoalcaligenes F-111 [29]. The molecular weight of lipase on SDS-PAGE was found to be approximately 52 kD (Fig. 4).

The maximum stability of the enzyme was observed in the tempertature range of 40 to 60°C, when the enzyme was incubated for 1 h (Fig. 5). The effect of pH on the activity of lipase was studied with various pH from7-11. The optimum pH for lipase enzyme from Penicillium chrysogenum was determined as 9. The pH stability curve showed that the lipase was stable at pH 8 – 10 (Fig. 6). Most microbial lipases are stable in the pH range 2 to 10.5 as reported by many researchers. Similar results have been reported for other fungal lipases [30, 31].

Figure 4: SDS-Polyacrylamide gel electrophoresis of purified Lipase from Penicillim chrysogenum at various stages of purification: lane 1: crude enzyme, lane 2: Ammonium sulphate precipitation, lane 3: DEAE-column chromatography**.**

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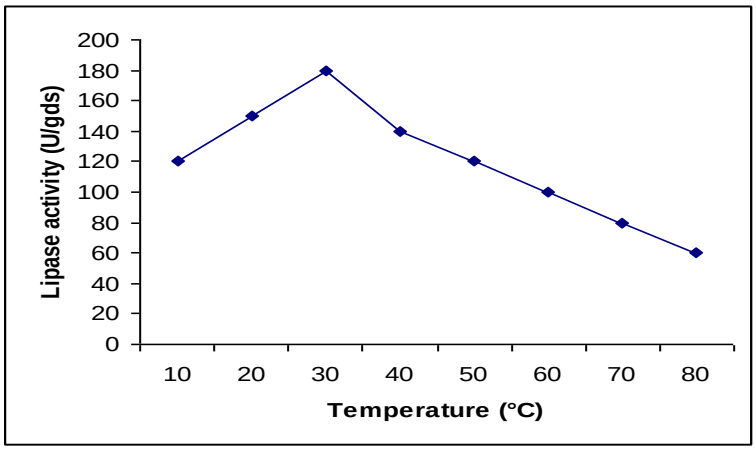

Figure 5: Effect of various temperature on lipase activity

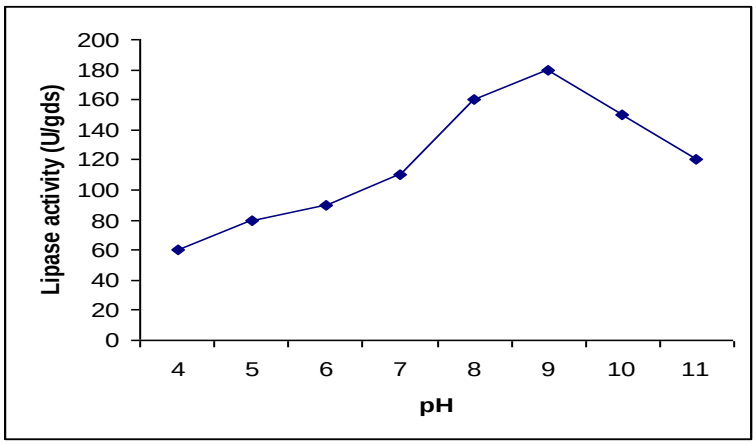

Figure 6: Effect of various pH on lipase activity

Substrate concentration

The kinetic parameters for purified extracellular lipase activity were determined using ground nut oil cake, in the concentration range of 0.2 – 1.0 mM. The values of K_m and V_{max} calculated from the Lineweaver-Burk plot were 1.14 mg/ml and 88 U/mg, respectively (Fig.7 & 8).

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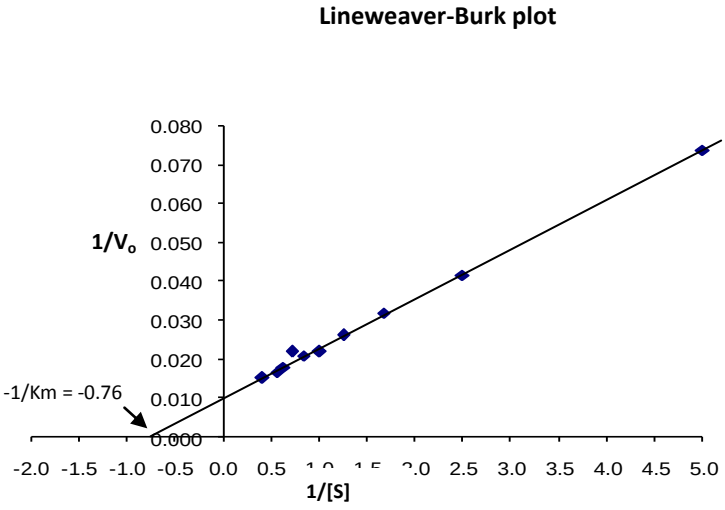

Figure - 7: Lineweaver-Burk plot for lipase from Penicillim chrysogenum. Lipase assay was conducted at various substrate concentrations at pH 9.0 and temperature 30°C. The values are means of three experiments done in duplicate

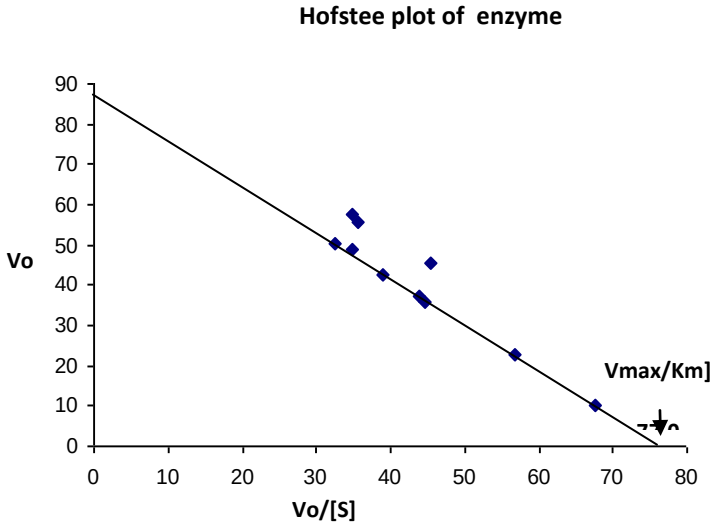

Figure -8: Eadie-Hofstee plot for lipase from Penicillim chrysogenum. Lipase assay was conducted at various substrate concentrations at pH 9.0 and temperature 30°C. Each value is average of three independent experiments.

Effect of detergents on lipase stability

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The fungal lipase exhibited considerable stability in the presence of all detergents at a concentration of 1mg/ml. The enzyme was stable in the presence of the non- ionic detergent, Tween 80, anionic detergent, sodiumdodecyl sulphate (SDS) and all commercial detergents for 45 min and the stability was maximum at 30 min with the non anionic detergent Tween (110 U/ml) and with commercial detergents such as Tide (150 U/ml) and Ariel (140 U/ml) (Table 3).

Table: 3 Lipase enzyme compatability with various commercial detergent

Lipase activity (U/ g dry substrate)				
Detergents (1mg/ml)	15 min	30 min	45 min	60 min
Control	150	147	142	142
Tween	142	137	130	80
SDS	138	132	120	100
Surf excel	143	138	133	100
Rin	134	127	120	100
Ariel	146	140	132	110
Tide	152	150	140	120
Wheel	138	130	124	120

Enzyme activity and stability in presence of some available commercial detergent were studied with a view of exploit the enzyme in detergent industry. Besides pH, a good detergent lipase is expected to be stable in the presence of detergents. The lipase showed excellent compatability in the presence of locally available detergents (Tide, Surf excel, Ariel, Rin, Wheel) Lipase from Aspergillus niger showed compatability with wide range of commercial detergents at 40˚C for 1 hour. The enzyme was stable in the presence of all detergents and similar in results have been reported for lipase from Candida cylindraceae [32], Aspergillus species [33]. Hence it could be used in detergent industry.

CONCLUSION

In this study, Penicillium chrysogenum, a strain that produces alkaline lipase at low-temperature. The lipase was purified and the relative molecular mass of the enzyme was determined to be 52 kDa by SDS-PAGE. The results obtained in this study show that Penicillium chrysogenum lipase exhibited maximum activity at 30° C and pH 9 tolerance to commercial detergents, make this enzyme a potential additive for detergent application. All these results indicate that Penicillium chrysogenum lipase has a good potential for the application in the detergent industry.

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