Production and Optimization of Thermostable lipase from a Thermophilic Bacillus sp LBN 4

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Abstract

The Bacillus sp isolated from the hot spring of Tawang district of Arunachal Pradesh, India was studied. Maximum enzyme production was achieved after 20 Hrs of cultivation. The olive oil and yeast extract was found to be the most suitable substrate for enzyme production. The enzyme had temperature optima of 65°C. Over 80% of its peak activity was at the pH range of 7-8, with an optimum of 8.0. The enzyme was found to be most stable at 80°C for 10 minutes. Almost 80% of the residual activity was retained at 80°C. $CaCl_2$ was found to stimulate the lipase activity by 40%.

INTRODUCTION

To satisfy the global requirement of the industrial process we should have enzymes showing optimal activities at maximum degree of extremity of pH, temperature and salt concentration. Thermophilic microorganisms are found to be potential and good alternative source of thermostable enzymes (Brock 1985). The extremophilic, especially thermophilic bacteria can be isolated from the natural high temperature environments distributed throughout the world and found in association of tectonically active sites (Brock 1985). Lipases (EC 3.1.1.3) are comprised a group of enzymes which catalyse the hydrolysis of triacylglycerols. In the recent years, the interest on lipase has grown significantly. The development of technologies using lipases for the synthesis of novel compounds will result in their expansion into new areas and increase in number of industrial applications (Bjorkling et al. 1991). Lipases are extremely versatile enzymes, showing many interesting properties of industrial applications. Microbial lipases are high in demand due to their specificity of reaction, stereo specificity and less energy consumption than conventional methods. Microbial lipases have wide application in the processing of food, leather, domestic, industrial wastes, cosmetic, detergents and pharmaceutical industries (Ghosh et al. 1996, Saxena et al. 1999). In this paper isolation of lipolytic Bacillus sp from a hot spring of Arunachal Pradesh its production and characterization of the enzyme are reported.

MATERIAL AND METHODS

ISOLATION

The samples were collected from the thermal spring of kitpi area of Tawang district of Arunachal Pradesh, India. This thermal spring is situated adjacent to the river Jung. Water and sediments showed a characteristics hydrogen sulfide odour. The samples were enriched at 50°Cin the nutrient broth supplemented with olive oil. Lipolytic isolates were selected on tributyrate agar medium (Collins and Lyne 1989) using glycerol tributyrate as substrate. Isolates having a higher ratio of clearing zone to the colony size were grown in the liquid culture and level of lipase production was determined from the cell free culture supernatant fluid. Characterization and identification of the isolate was made following Bergeys Manual of Systematic Bacteriology (Sneath 1986).

ENZYME PRODUCTION

The medium of the enzyme production was composed of peptone 2%, starch 2%, KH_2PO_4 0.5%, $(NH_4)_2SO_4$ 0.1%, $(NH_4)_2CO_4$ 0.1%, $MgSO_4$. $7H_2O$ 0.1%, pH 7.5. The medium was inoculated with 2 ml of overnight culture and incubated at 50°C. After 24 hours of incubation the culture was centrifuged and the cell free culture supernatant fluid was used as the enzyme source.

EFFECT OF CULTURE VARIABLE ON LIPASE PRODUCTION

For the study of lipase production from the crude enzyme, starch was replaced by other carbon sources such as glucose,

fructose, sucrose, and lactose and olive oil. Each of the substrates (1%w/v) was used as sole carbon source while tested for their ability to induce lipase production. The crude substrates were autoclaved for 30 minutes at 121°Cin the autoclave before use. The effect of nitrogen sources on the lipase production was analyzed by supplementing production medium with different nitrogen sources (1%w/v) like yeast extract, gelatin, tryptone, potassium nitrate and ammonium sulphate and enzyme activity was assayed.

ENZYME ASSAY

All the experiments were run in triplicate. The reaction mixture containing 5ml of olive oil emulsion composed of 25 ml olive oil and 75 ml 2% polyvinyl alcohol solution, 4ml of 0.2M tris buffer ,1ml of 110mM $CaCl_2$ and 1ml enzyme solution (Watnabe et al. 1977). The control containing boiled inactivated enzyme (at 100°Cfor 5 minutes) was treated similarly. After the incubation, the enzyme activity was blocked by 20 ml of acetone ethanol (1: 1) mixture and liberated free fatty acid was titrated against 0.02 M NaoH using phenolphthalein as indicator. One unit of lipase was defined as the amount of enzyme, which liberates 1 mol of fatty acid/ min. (1ml of 0.02N NaOH is equivalent to 100 mol fatty acid liberated per minute.)

TEMPERATURE AND PH PROFILE

The crude enzyme preparation obtained after cultivation of the organism in the production medium was assayed for lipase activity at different temperature range (45-85°C). For determining the effect of pH on enzyme activity, different buffers (Acetate, Phosphate and Tris-HCl) at a concentration of 50mM and pH 5-9 were used in the enzyme assay reaction mixture.

EFFECT OF METAL SALTS ON ENZYME ACTIVITY

The effect of different salts on lipase activity was examined by incubating various metal salts with enzyme extract in 50mM phosphate buffer (pH 7.0) at 30°Cfor 1hour.

THERMAL STABILITY

Thermostability of the lipase was studied by subjecting the enzyme sample at different temperatures (45-90°C) for different intervals of time upto 30 minutes.

RESULTS

ISOLATION

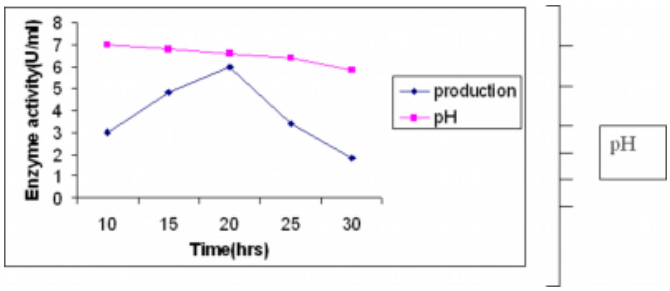
On the basis of level of the productivity and thermostability of the lipase produced, one strain designated as LBN 4, was selected for subsequent studies. The isolate was rod shaped, aerobic, endospore forming, catalase positive and Gram positive. It grew optimally at 50°C. Based on these biochemical, morphological, and physiological properties, the strain has been identified as a thermophilic strain of the genus Bacillus.

GROWTH AND LIPASE PRODUCTION

Isolate LBN 4 entered stationary phase after 20 hours and the culture pH was dropped from 7.0 to 5.0 within 16 hours. Lipase production was detected from 4 hrs to 30 hrs and reached a maximum (6.9 U/ml) after 20 hrs (Fig 1).

Figure 1

Figure 1: Time course on enzyme production by LBN 4 and culture pH.



EFFECT OF CULTURE VARIABLE ON LIPASE PRODUCTION

Among the different carbon sources used, olive oil was found to be the most suitable source to induce the lipase production. In the olive oil 1% (v/v) the maximum activity observed (6.0 U/ml) (Fig 2) . The yeast extract was found to be the most suitable nitrogen source and the enzyme activity was observed (3.4 U/ml)(Fig 3).

Figure 2

Figure 2: Effect of different carbon sources on enzyme production

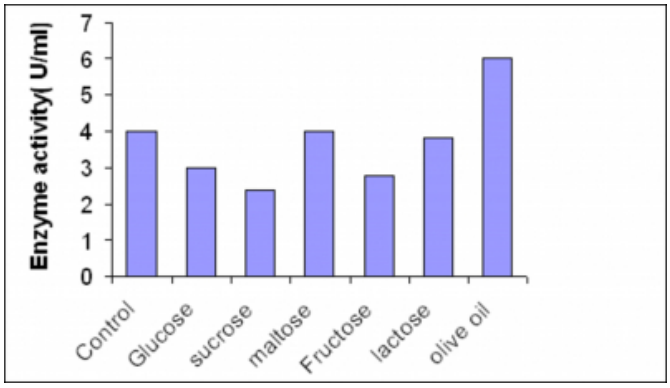
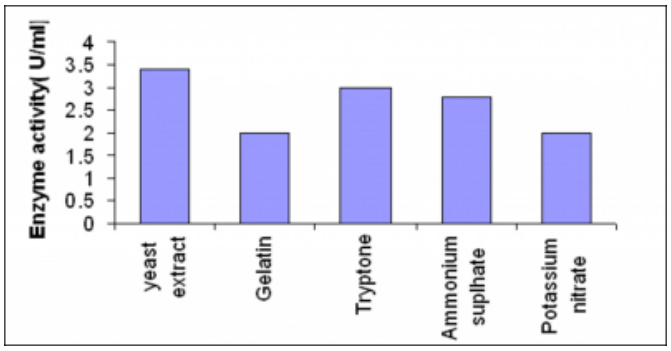


Figure 3

Figure 3: Effect of different nitrogen sources on production



TEMPERATURE AND PH PROFILE

The temperature profile of the enzyme was determined by assaying the enzyme at different temperatures. The enzyme had an optimum temperature of 65°C, with 80% of the maximum activity retained at 70°C(Fig 4). However, at 75°C a slight increase in the activity was observed. The pH profile of the LBN4 lipase was investigated using phosphate buffer with various pH values at 50°C. The enzyme has an optimum pH of 8.0(Fig 5). Moreover, the enzyme was stable from pH 7.0 - 8.5 respectively.

Figure 4

Figure 4: Effect of temperature on enzyme activity

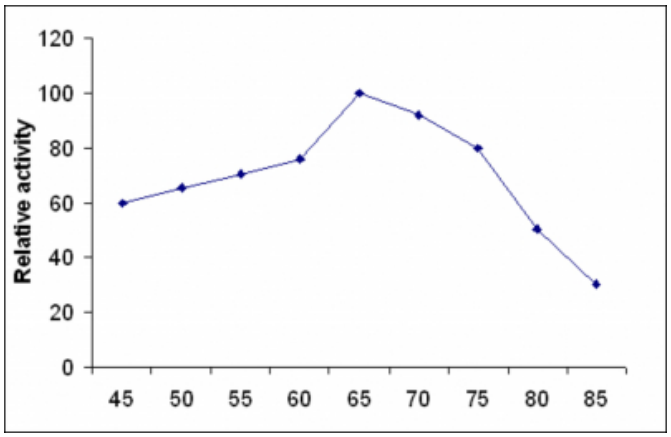
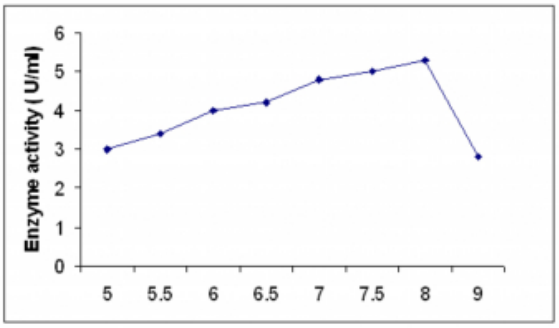


Figure 5

Figure 5: Effect of pH on activity of lipase

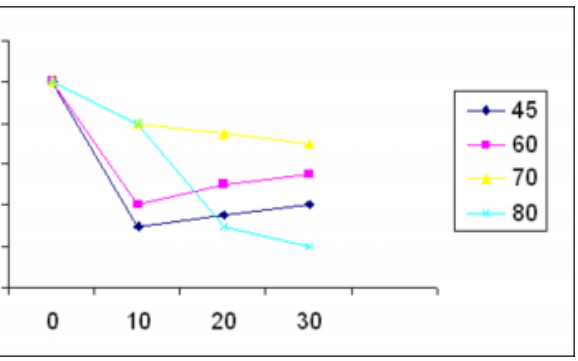


THERMAL STABILITY

Thermostability of the lipase was studied by subjecting the enzyme sample at different temperatures (45-90°C) for different intervals of time upto 30 minutes. Present study on this lipase indicated that the enzyme was thermostable up to 80°Cfor 10 minutes; and considerable activity (80%) (Fig 6) was retained. Enzyme activity sequentially decreased as the incubation time is increased. The incubation of the enzyme at 80°Cfor 20 minutes caused drastic reduction in the enzyme activity (50%). Almost all the activity was lost after 30 minutes of incubation.

Figure 6

Figure 6: Thermo stability of lipase



EFFECT OF METAL SALTS ON ENZYME ACTIVITY

The effect of different metal salts on lipase activity was examined by incubating various metal salts with enzyme extract in 50Mm phosphate buffer (pH 7.0 at 30°Cfor 1 hour. The $CaCl_2$ has found to stimulate enzyme activity whereas others have antagonistic effect.(Table 1)

Figure 7

Table 1: Effect of metal salts

Sl. No.	Compound 10mM	Relative activity %
1.	Control	100± 2
2.	$MgCl_2$	Nil
3.	$CaCl_2$	140±1
4.	$MnCl_2$	80±2
5.	$HgCl_2$	Nil
6.	$CoCl_2$	28±3
7.	NaCl	55±2
8.	$NaNo_3$	Nil

DISCUSSION

In the recent years, the emphasis has been laid on extremophiles for their potential use in the production of extremozymes, because of different mechanisms and strategies that help them to survive and function in brutal circumstances. The ability to access enzyme activities from the extreme of environmental spectrum rather than conventional domains is very much needed to develop industrial applications. The extremozymes decrease the need of the added steps in the process, reduce the cost and increase in efficiency. The present study was carried out to exploit the microbial diversity and enzymatic potential of the thermophilic bacteria from hotspring of Arunachal Pradesh, India as it has been a part of Indo- Burma biodiversity hot spot (Myers et al. 2000).

Bacillus sps are good lipase producers and are preferred in the food industry for the enzyme production due to their non-pathogenecity. The lipase production by Bacillus sp LBN4 was growth associated reaching a maximum around 20 hrs. Similar studies have been carried out in Bacillus sp where a maximum production was observed at 20 hrs (Ghanem et al. 2000) and in between 15-24 hrs (Wang et al. 1995) this may be an interesting property because it could allow harvesting of the enzyme for the shorter period of time. The mesophilic and thermophilic Bacillus sp, are the main sources of commercial lipase at present are reported to require a batch time of 24-48 hrs for optimum production.

The production of the lipase is significantly influenced by carbon sources such as sugars, oils and nitrogen sources. The lipase production from the Bacillus sp in the presence of 1% olive oil was found to be optimum which corroborated with the similar results reported earlier (Sugihara et al. 1991; Sharma et al. 2002(a)). Among the nitrogen sources yeast extract was found to be the most suitable substrate, which are more or less similar to that reported earlier (Sugihara et al. 1991 Kim et al. 1994; Sharma et al. 2002a).

The enzyme was optically active and stable at pH 8.0. A similar trend was observed in thermophilic bacteria Bacillus thermocatenulatus pH 8-9.0 (Schmidt- Dannert et al. 1996) and Bacillus strain 398, pH 8.2(Kim et al. 1994) respectively. The use of lipase that is active at relatively alkaline pH are of great industrial application especially in detergent industries could able to remove the dirt. Generally microbial lipases have temperature optima in and around 30-80°C. Dharmashiti and Luchai (1999) reported the optimum temperature of 70°C, 65°C(Kim et al. 1994) and 60-80°C(Schmidt- Dannert et al. 1996) in Bacillus sp which are more or lees similar to the reported result. For industrial application it is highly desirable that the enzymes must have higher temperature optimum.

The other important feature of the isolate LBN4 lipase is high thermal stability. Very few strains of thermophilic Bacillus sp have been reported so that to produce highly thermostable lipase. (Schmidt-Dannert et al. 1996). 80% of the residual activity was retained at 80°Cfor 10 min. Ghanem et al reported 70% of residual activity at 75°C. How ever in others the relative activity and thermo stability was found to be less in short period of time.

Cofactors are generally not required for lipase activity, but divalent cations such as calcium often stimulate enzyme activity they are needed as a cofactor for catalytic activity. Ca^{2+} has found to stimulate the lipase activity in the results given in the (Table No 1). This has been suggested to be due to the formation of calcium salts of long chain fatty acids (Macrae and Hammond 1985; Godtfredson 1990). Calcium stimulated lipases has been reported in the case of B. subtilis 168(Lesuisse et al 1993), B.thermoleovorans ID-1 (Lee et al 1999), P.aeroginosa EF2 (Gilbert et al 1991a). In the B.licheniformis H1 the enzyme activity has enhanced in the presence of 10mM Ca^{2+} that is more or less similar to the result obtained.. Each divalent cation influenced the enzyme activity dependent on the temperature at which protein-ion metal interaction occurred

CONCLUSIONS

In can be concluded from the above study, that the Bacillus sp based on its characterization could be a useful source of enzyme and has the potential of industrial application.

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