Effect of different salts and organic solvents on Stability of thermo stable Lipase Enzyme from *Bacillus cereus*

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Abstract:
Microbial lipases are currently receiving much attention with the rapid development of enzyme technology. Stability of Lipase enzymes with good activity has been an interesting field for researchers. Lipases are a class of enzymes which catalyze the hydrolysis of long-chain triglycerides. Production of extracellular lipase in submerged culture of Bacillus cereus MTCC 1272 strain has been investigated. The lipase production was optimized in shake flask experiments. Lipase enzyme was purified and molecular mass determined by SDS-PAGE. The purification steps included ammonium salt precipitation, dialysis and ion exchange chromatography on DEAE-cellulose. Enzyme exhibited high activity in the presence of calcium ion. Enzyme activity reduced in the presence of EDTA confirming enzyme to be a metalloprotein. The lipase enzyme found to be stable with high activity in boric acid and sodium taurocholate. Enzyme activity found to be increased more than 200% in salts and 150% in organic solvents like acetone and butanol. Enzyme was stable for more than 4 hours at 60°C to 75°C in salts and organic solvents. And the enzyme was also stable for more than 16 hours at room temperature. Combination of salts like boric acid and bile salts had shown high activity and stability at 80°C.

Key Words:
Lipase, lipase activity, Salts and Organic Solvents, SDS PAGE, DEAE-cellulose.

Introduction:
Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), are a group of enzymes, which have the ability to hydrolyze triacylglycerols at an oil-water interface. These are widely distributed in animals, plants and microorganism, exhibit great potential in commercial application for they catalyze the production of free fatty acids, interesterification of oils and fats as well synthesis of esters and peptides [1]. Lipases are present in wide range of properties depending upon their sources [4]. Based on the fact that certain reactions are performed at higher temperature, organic solvent and salts, attempts have been made to find the lipase thermostable. Thermophilic microorganisms are found to be potential and good alternative source of thermostable enzymes. 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 [6]. 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 [7]. Microbial lipases are high in demand due to their specificity of reaction, stereo specificity and less energy consumption than conventional methods. 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 industries and promising application in the biodegradation of bioplastics is illustrated.

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.

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 [13, 14, 15].

The stability of these enzymes in organic solvents has 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. 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 [16].

Microbial lipases have wide application in the processing of food, leather, domestic, industrial wastes, cosmetic, detergents and pharmaceutical industries [10]. The most commercially important field of application for lipases is their addition to detergents, which are used mainly in household and industrial laundry [11]. Washing and degreasing by using lipases allows for smaller amounts of surfactants and operation at low temperatures. The lipase component causes an increase in detergency and prevents scaling, since enzymes can reduce the environmental load of detergent products [12].

In the present study, lipase from Bacillus cereus MTCC 1272 strain has been isolated, characterized and purified. The stability of the enzyme on different salts and organic solvents at different temperatures was focused.

Materials and methods:

Bacterial Strain

Bacterial strain used for the study was *Bacillus cereus* MTCC 1272 strain for the lipase enzyme production.

Enzyme Production and purification

The lyophilized culture was reconstituted in 200 ml nutrient medium under aseptic conditions. The medium of the enzyme production was composed of 0.5% tryptone, 0.5% yeast extract, 0.2% sodium chloride, 0.75% tributryn and pH 7.5. After 24 hours

of incubation the culture was centrifuged at 10000g and the cell free culture supernatant fluid was used as the enzyme source. All purification procedures were carried out at 4°C. The crude enzyme was subjected to 70% saturated ammonium sulphate precipitation. The precipitate was dissolved in minimal amount of 0.01M Tris HCl buffer of pH 7.0, the enzyme activities were assayed and then dialysed extensively against the same buffer. The enzyme activities in the dialysate were assayed. Ten milliliters of the dialysate was loaded on to a DEAE cellulose column which had been pre-equilibrated with 0.01M Tris HCl buffer of pH 7.0 containing 0.01M NaCl and eluted with the same buffer at a flow rate of 30ml/hour. All eluted fractions (2ml) were assayed for enzyme activities and the fractions with high activities were pooled. The pooled fractions were subjected to dialysis against Tris HCl buffer and the enzyme activities in the dialysates were determined. Purified product was collected and stored.

Enzyme Characterization

The optimum temperature of the enzyme was studied by pre-incubating the enzyme mixture for 30 min at temperatures ranging from 30 ºC to 70 ºC.

The optimum time for highest enzyme activity was studied by incubating enzyme mixture with substrate olive oil at different intervals of time up to 30 minutes.

The optimum pH of lipase present in the partially purified enzyme mixture was studied over a range of 5.0 to 12.0 by incubating the enzyme mixture for 30 min with olive oil.

The effect of activators and inhibitors was studied by incubating the enzyme mixture with activator like calcium (100mg/10ml) and inhibitor like EDTA (100mg/10ml) for 30 minutes.

Effect of substrate studied by incubating enzyme with olive oil, concentration ranging 50 to 250 micro liters.

The purified enzyme mixture was subjected to electrophoresis on 10% acrylamide gel and molecular weight was determined by comparing the relative mobility of the protein band with that of molecular weight markers.SDS PAGE and Native PAGE used to determine the molecular weight.

Enzyme assay and Protein estimation

Lipase activity was estimated by titrimetric method, using olive oil as a substrate. The reaction mixture containing 1 ml of 200mM tris buffer and 2.50 ml water and 3 ml substrate olive oil with 1 ml of enzyme solution. In blank lipase enzyme sample was replaced by equal amount of water. After 30 minutes incubation at 37 ºC, enzyme activity blocked by 3 ml of alcohol and liberated free fatty acids from triglyceraldehydes were titrated with 50mM NaOH using phenolphthalein as indicator. Amount of NaOH required to achieve the end point (colorless to pale pink) was recorded.

Lowry's method used to estimate the concentration of protein present in the enzyme. Reactivity of the peptide nitrogen with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids [17]

Stability of Lipase enzyme

Stability of the lipase enzyme was determined at various temperatures for different time period. Salts and organic solvents were used to stabilize the enzyme activity. Stabilizers added with the reaction mixture.

A number of organic solvents (2-Propanol, Ethanol, Methanol, Butenol, n-Hexane, Acetone etc) were used (0.5 ml to 1ml) and incubated for 30 minutes to 2 hours.

Various salts (Boric acid, Sodium Taurocholate, Potassium chloride, Potassium sulphate, Sodium sulphate and Bile salt) used for the enzyme stability assay 100mg/10ml. These stabilizers used for 30 minutes to 4 hrs at 40°C, 60°C and 80°C of temperature.

All results were interpreted with statistical analysis.

Results:

In the present work *Bacillus cereus* MTCC 1272 strain was used for producing lipase. Lipase production was estimated by lipid hydrolysis assay on agar plates. Production of lipase in LB medium was optimized and 24 hrs culture used for the production. Cell free culture supernatant as a crude enzyme used and it was partially purified by ammonium salt precipitation method and dialysis and maximum lipase activity was recorded in the fraction precipitated by 30% saturation. Further purification followed by ion exchange chromatography which results in 10 fold purification of enzyme.

Generally lipase of *Bacillus* cereus strains found relative thermo stable. In present study of *Bacillus cereus* MTCC 1272 strain it found thermo stable and has exhibited high activity at 30°C to 40°C. The protein concentration in the enzyme was calculated and with that value specific activity was measured.

The activity of crude enzyme was 400 U/ml and IEC enzyme activity was 4000 U/ml. The specific activity of crude enzyme was 1923.07 U/mg and IEC purified specific activity was 57971.01 U/mg.
The enzyme activity was found maximum in the range of 45°C to 65°C temperatures when incubated for 1 hr.

When the enzyme was incubated for 5 minutes to 30 minutes, highest activity found at 10 minutes and the parabolic curve formed of time and activity.

EDTA and copper was found the inhibitor for the lipase enzyme activity as activity decreased on increasing concentration and calcium found activator for the activity as activity increased on increasing concentration. Calcium ions found to be stimulatory effect on cell growth and proliferation. Calcium also reported an increase in thermostability of lipase enzyme.
Lipases catalyze reactions involving triglyceride hydrolysis, ester synthesis, transesterification and interesterification of fats, etc. Since substrates of such reactions are often insoluble in aqueous solution, organic solvents or organic aqueous two-phase media are required. However, enzymes generally denature in the presence

of organic solvents and require methods for stabilizing [18]. Optimum pH range for the lipase enzyme was 7 to 8.5.

The enzyme activity increased as substrate concentration increased. The activity was constant after 0.15 ml. Band found on Native PAGE electrophoresis showing the presence of protein monomer and SDS PAGE results in a dark band which corresponds to albumin marker.

Stability of enzyme

The enzymes that remain stable in the presence of organic solvents could be industrially very useful. Lipase produced by *Bacillus cereus* showed good stability in organic solvents. The enzyme activity increased at 27°C and 37°C temperature for 2 to 4 hr. It was not stable for more time on high temperature. Incubation of the enzyme in a strong dehydrating solvent, like acetone, methanol or ethanol, did not cause much loss of its activity; 90% activity remaining after 1h incubation. However, 2 propanol and hexane resulted in considerable loss in activity. Other lipases from *Pseudomonas* and *Bacillus* could also be activated in the presence of several water miscible organic solvents [19]. It has been proposed that organic solvents disaggregate the enzyme and keep it in open conformation. This may lead to enhanced enzyme activity or at least stabilization of the enzymes. However, certain organic solvents completely change the enzyme structure, resulting in the inactivation of the enzyme [20].

Enzyme stability remained increased on adding salts. It has shown high activity in the presence of salts.

Organic Solvents

The enzyme activity was increased in the acetone (200%) and butenol (40%) solvents for 2 to 3 hrs at high temperature 70°C to 85°C and other solvents like propanol and hexane also given preservation of enzyme activity at 37°C also. The activity was stable for initial 30 minutes then it increased and further decreased with time.

Salts

Boric acid found to be a very favorable salt for the lipase enzyme activity. Boric acid increased the activity (50% to 250%) till 2 to 3 hrs on different temperatures. After 1 hour, sodium taurocholate gave increased activity at 40°C to 80°C. Other salts like sodium sulphate, potassium sulphate, potassium chloride, bile salt etc given optimum activity for 2-3 hrs. A combination of salts also gave better activity. Boric acid and bile salt together gave more than four times enzyme activity at 80°C for 30 minutes and double activity till 1 hr and stability of activity more than 4 hrs. Potassium chloride and Potassium sulphate were also effective on activity for 2 hrs. Salts with propanol also effective at 70°C

Conclusion:

The lipase enzyme produced by *Bacillus cereus* MTCC 1272 strain was characterized and purified, was stable at various temperatures for different time. Stability was enhanced by using salts like boric acid and sodium taurocholate. Also organic solvents like acetone and butenol used for stabilization. Boric acid and other combination of salts can stabilize enzyme

without loosing the activity. Stability was for more than 3 hours at high temperature and room temperature also. Thus, the lipase produced by *Bacillus cereus* MTCC 1272 strain can be summarized as having high working temperature, good thermostability and high stability towards organic solvents as well as salts. By virtue of these features, this enzyme can be widely used in industries, especially in fats and oil industry.

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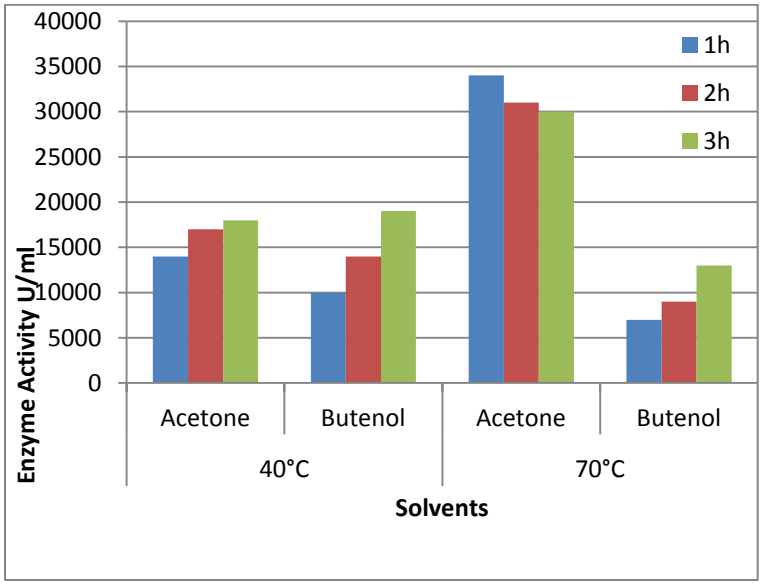

Fig: 1

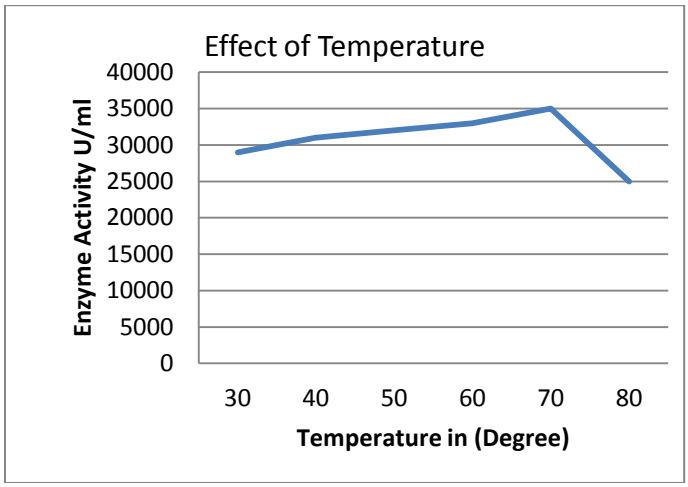
Effect of Temperature
Enzyme Activity U/ml
40000
35000
30000
25000
20000
15000
10000
5000
0
30
40
50
60
70
80
Temperature in (Degree)

Fig: 2

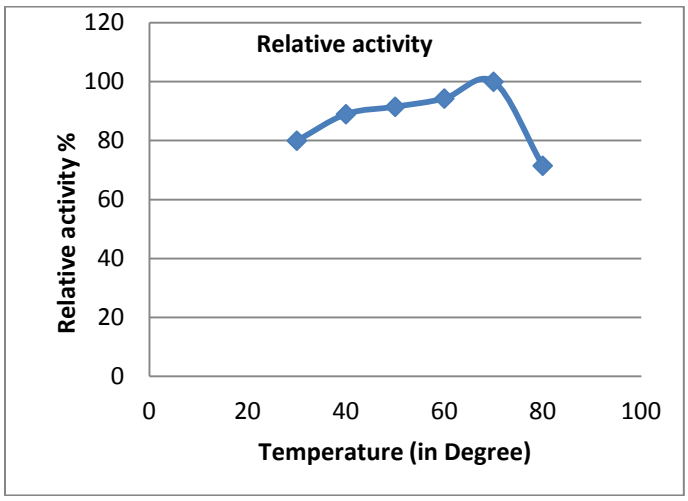
Relative activity
Relative activity %
120
100
80
60
40
20
0
0
20
40
60
80
100
Temperature (in Degree)

Fig: 3

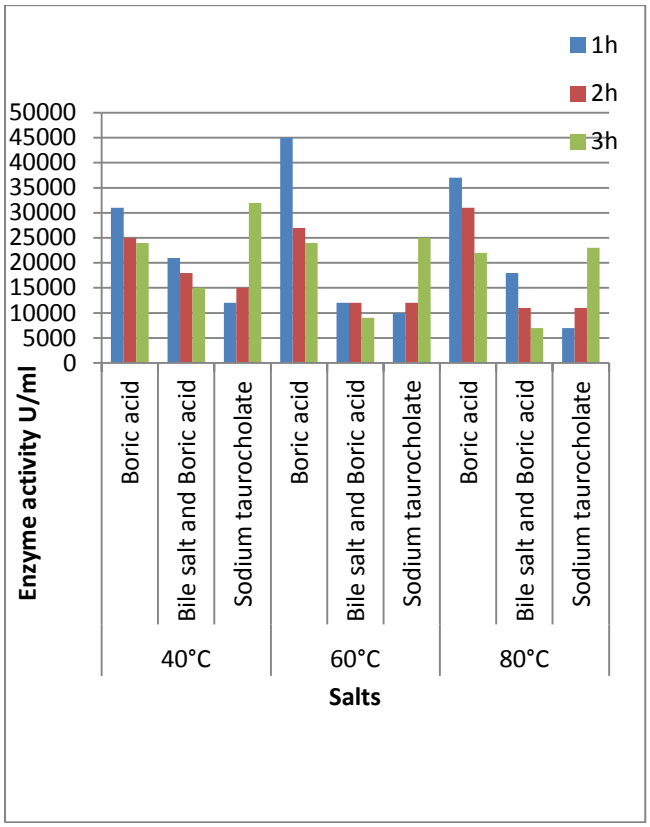
1h
2h
3h
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Enzyme activity U/ml
Boric acid
Bile salt and Boric acid
Sodium taurocholate
Boric acid
Bile salt and Boric acid
Sodium taurocholate
Boric acid
Bile salt and Boric acid
Sodium taurocholate
40°C
60°C
80°C
Salts

Fig: 4