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Production and Characterization of an Alkaline Thermostable Crude Lipase from an Isolated Strain of *Bacillus cereus* C_7

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Abstract A bacterial strain isolated from spoiled coconut and identified as *Bacillus cereus* was found capable of producing alkaline thermostable extracellular lipase. Optimum temperature, time, and pH for enzyme substrate reaction were found to be 60 °C, 10 min, and 8.0 respectively. Common surfactants except Triton X 100 and cetyltrimethylammonium bromide have no or very little inhibitory effects on enzyme activity. The enzyme was found to be stable in presence of oxidizing agents and protease enzyme. The maximum lipase production was achieved at 30–33 °C, pH 8.0 on 24 h of fermentation using 50 ml medium in a 250-ml Erlenmeyer flask. The superior carbon and nitrogen sources for lipase production were starch (2%) and ammonium sulfate (nitrogen level 21.2 mg/100 ml), peptone (nitrogen level 297 mg/100 ml), and urea (nitrogen level 46.62 mg/100 ml) in combination, respectively. The maximum enzyme activity obtained was 33±0.567 IU/ml.

Keywords Lipase · *Bacillus cereus* · Alkaline · Thermostable · Production · Characterization

Introduction

Lipases (EC 3.1.1.3) are glycerol ester hydrolases that catalyze the hydrolysis of triacylglycerols into fatty acid, partial acylglycerols, glycerol, and under low water condition catalyze the reverse reaction [1, 2]. Although lipases are found in animals, plants, bacteria, yeast, and fungi, however, microbial lipases are commercially significant for their potential use in various industries such as food, dairy, pharmaceutical, detergents, textile, biodiesel, cosmetic industries, and in synthesis of fine chemicals, agrochemicals, and new polymeric materials [3]. As the applications increase, the availability of lipase possessing satisfactory operating characteristics is a limiting factor. For example, lipase added in prewash soaking agents and detergent powders needs to be stable under alkaline pH and to function in the presence of surfactants [4]. Thermal stability is another major requirement

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for commercial lipases because of their high activities at the elevated temperatures and stabilities in organic solvents. Therefore, a thermostable alkaline lipase is needed for industrial applications.

In the present paper, we describe the process optimization for maximum production of a bleach stable, protease stable, and thermostable alkaline lipase from an isolated strain of *Bacillus cereus* (GenBank accession number AB 244464) and characterization of the crude enzyme.

Materials and Methods

Microorganism

An alkaline thermostable lipase-producing bacterial strain was isolated from spoiled coconut and identified as *B. cereus* was used for the present study. It was maintained by monthly subculturing at 30 °C and stored at 4 °C.

Medium Composition

(a) The medium for plate and slant culture was composed (g/l) of olive oil, 20; $(NH_4)_2SO_4$, 5; $(NH_2)_2CO$, 2; $MgSO_4.7H_20$, 1; yeast extract, 0.5; and agar, 20 [5]. The mixture was heated and emulsified and the pH was adjusted to 8 with 1 N Na_2CO_3. (b) The inoculum medium and fermentation medium used for lipase production contained (g/l) soluble starch, 20; peptone, 20; KH_2PO_4, 5; $(NH_4)_2SO_4$ 1; $MgSO_4.7H_2O$ 1; $(NH_2)_2CO$, 1; and pH 8 (5).

Isolation of Lipase Producing Microorganism

Thirty organisms were isolated from soil and spoiled coconut using olive oil medium following plate and dilution technique [6]. Each isolate was tested for its lipase activity.

Preparation of Crude Enzyme

Inoculum was prepared by transferring one loop of culture from slant to the inoculum medium (50/250 ml Erlenmeyer flask) and incubating the flask at 30 °C in a rotary shaker at 120 rpm for 24 h. Fermentation medium (50/250 ml Erlenmeyer flask) was inoculated with 2% (*v*/*v*) inoculum and incubated for 24 h under the same conditions. The cell-free supernatant obtained by centrifugation at 4,000 rpm for 15 min was used for determining extracellular lipase activity.

Assay Method

Unless otherwise stated, all experiments were run in triplicate and repeated twice. Olive oil emulsion was prepared as follows: 25 ml of olive oil and 75 ml of 2% polyvinyl alcohol solution was emulsified using a homogenizer. The reaction mixture containing 5 ml olive oil emulsion, 4 ml 0.2 M Tris buffer (pH 8.0), 110 mM $CaCl_2$ (final concentration 10 mM), and 1 ml enzyme solution was incubated at 60 °C for 10 min. Control containing inactivated enzyme (boiled) was treated similarly. Immediately after incubation, the emulsion was destroyed by the addition of 20 ml acetone–ethanol (1:1) mixture and the liberated free fatty acid was titrated with 0.02 N sodium hydroxide. One unit of lipase was

Table 1 Taxonomical characteristics of strain C_7.

Parameters	Characteristics	
Cellular characteristics		
Morphology	Straight rods, 3.24×1.85 μm, occurring single, pair or in short chains, nonmotile	
Staining characteristics	Gram positive, spore former, spores are terminally located	
Cultural characteristics		
Nutrient broth (stationary condition) 48 h	Moderate growth, flocculent sedimentation, ring formation, no pellicle formation	
Nutrient broth (shaking condition) 48 h	Abundant growth, turbidity, ring formation, no pellicle formation, off white color	
Nutrient agar colonies	Irregular shaped (1 mm diameter), opaque, smooth, flat, off white with entire edge, dry	
Physicochemical characteristics		
Growth at different temperatures		
45 °C	Moderate	
30 °C	Abundant	
10 °C	Scanty	
Growth at different NaCl concentration		
2%	Abundant	
5%	Scanty	
7%	None	
10%	None	
Growth at different pH		
5.4	Scanty	
6.8	Abundant	
9.6 (with 6.5% NaCl)	Moderate	
Biochemical characteristics		
Ammonia from arginine	Positive	
Arginine used as a sole source	Positive	
Nitrate reduced to nitrite	Positive	
Hydrolysis of starch	Positive	
Hydrolysis of urea	Positive	
Catalase test	Positive	
Growth under anaerobic condition with/with out beef extract	Positive	
Voges–Proshaker reaction	Positive	
pH<6		
pH>7		
Litmus milk test	Negative	
Indole formation test	Negative	
Carbohydrate fermentation test	Acid formation	Gas production
Glucose	Positive	Negative
Fructose	Negative	Negative
Sucrose	Negative	Negative
Lactose	Negative	Negative
Galactose	Negative	Negative
Xylose	Negative	Negative
Mannose	Negative	Negative
Maltose	Positive	Negative
Starch	Positive	Negative
Dextrin	Negative	Negative

Table 1 (continued)

Parameters	Characteristics	
Inositol	Negative	Negative
Mannitol	Negative	Negative
Sorbitol	Negative	Negative
Salicin	Negative	Negative

defined as the amount of enzyme, which liberated 1 μmol of fatty acid per minute. One milliliter of 0.02 N NaOH is equivalent to 100 μmol of free fatty acid [5].

Taxonomical Studies

The selected strain was identified following Bergey's Manual of Determinative Bacteriology [7]. DNA base composition was determined by Bangalore Genei.

Results and Discussion

Screening of Lipase Producing Organism

Of the 30 isolates, only ten samples were found to be capable of producing lipase. The isolate C_7 was selected as the potent strain and used in further studies (results not shown).

Taxonomical Studies of the Isolate C_7

Taxonomical characteristics of the strain C_7 are shown in Table 1. On the basis of 16s ribosomal RNA sequence analysis (GenBank accession number AB 24464), the isolate has been identified as *B. cereus*. From the construction of a phylogenic tree, its nearest homolog species is found to be *Bacillus thuringiensis* (GenBank accession number EF 210288; Fig. 1).

Characterization of Crude Enzyme

Effect of Reaction Time

Enzyme substrate reaction was performed at 60 °C and pH 8 for different time period, viz., 5, 10, 15, and 20 min, other conditions remaining the same. Reaction reached its optimum value after 10 min and maximum activity was found to be 33±0.567 IU/ml (Fig. 2). Then, the activity decreased with increase in reaction time.

Effect of Temperature

Enzyme substrate reaction was carried out at different temperature, viz., 30 °C, 40 °C, 50 °C, 55 °C, 60 °C, 65 °C, and 70 °C, other conditions remaining the same. Lipase activity increased progressively with increase in temperature and maximum activity was found to be 32 IU/ml at 60 °C (Fig. 3). Then, the activity decreased with increase in

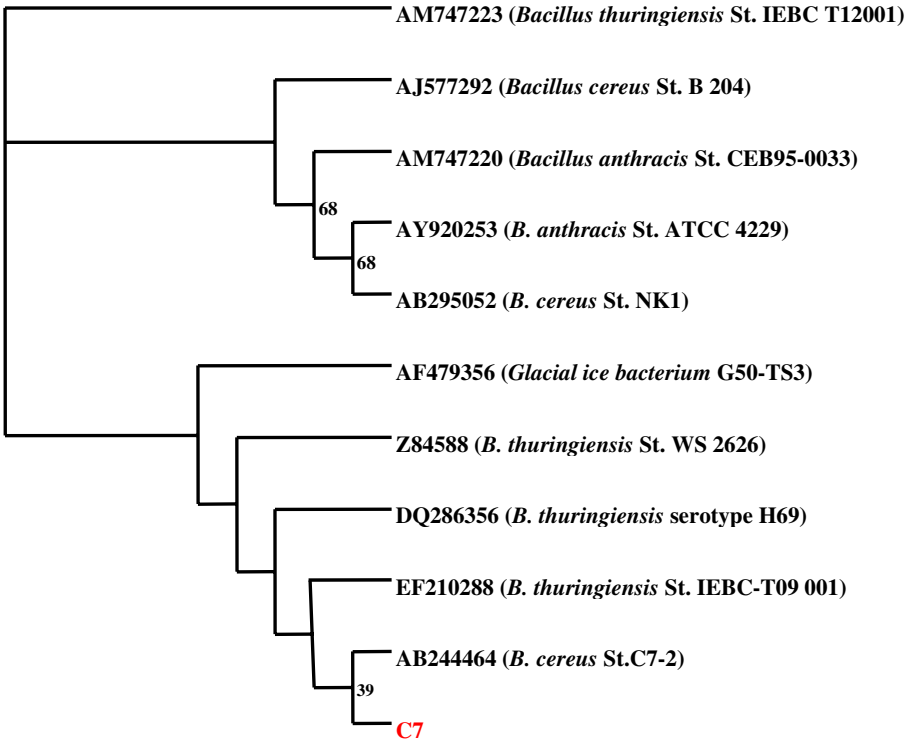

Fig. 1 Phylogenic tree (using neighbor joining method)

temperature. Ghanem et al. in 2000 reported the temperature maxima of 60–65 °C for an alkalophilic thermostable lipase activity from *Bacillus alkalophilus* [8]. Lipase from *Bacillus stearothermophilus* P_1 and *Bacillus* sp. RSJ-I also have high temperature optima [9, 10].

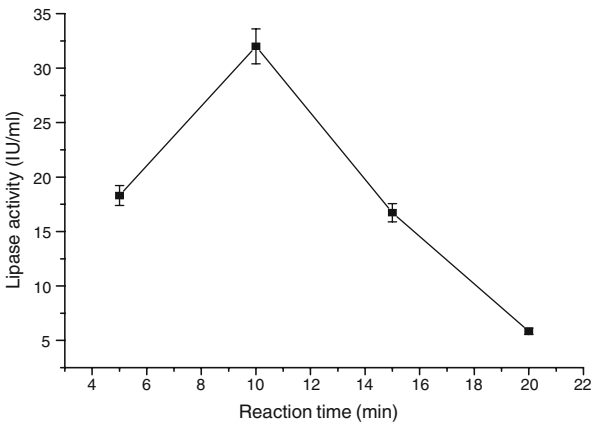

Fig. 2 Effect of reaction time on activity of the crude lipase from *B. cereus* C_7

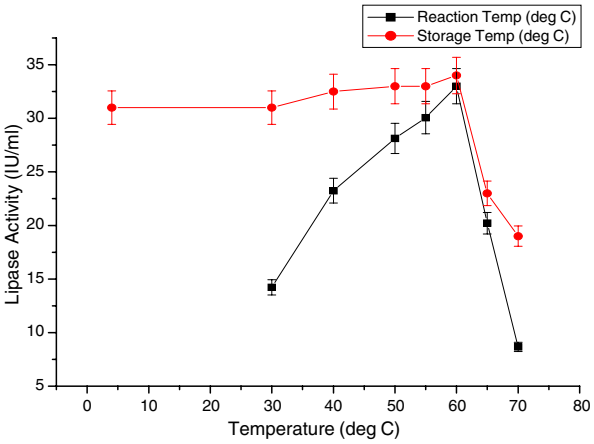

Fig. 3 Optimum temperature and thermostability of crude lipase from *B. cereus* C_7

Effect of pH

pH-activity profile was studied over the pH range of 7.2–9 at 60 °C, using Tris-HCl buffer and over pH range 9–10 using glycine-NaOH buffer, other conditions remaining the same (Fig. 4). The enzyme was active in the pH range 7.6–9 and maximum activity was shown at pH 8.0. There are a few reports of microbial lipases working optimally in the alkaline range of pH [11–13].

pH Stability and Thermal Stability

The alkaline pH stability of lipase was determined by incubating them at pH values 7.2 to 9 for 1 h and then the preservation stability of lipase was determined by standard assay

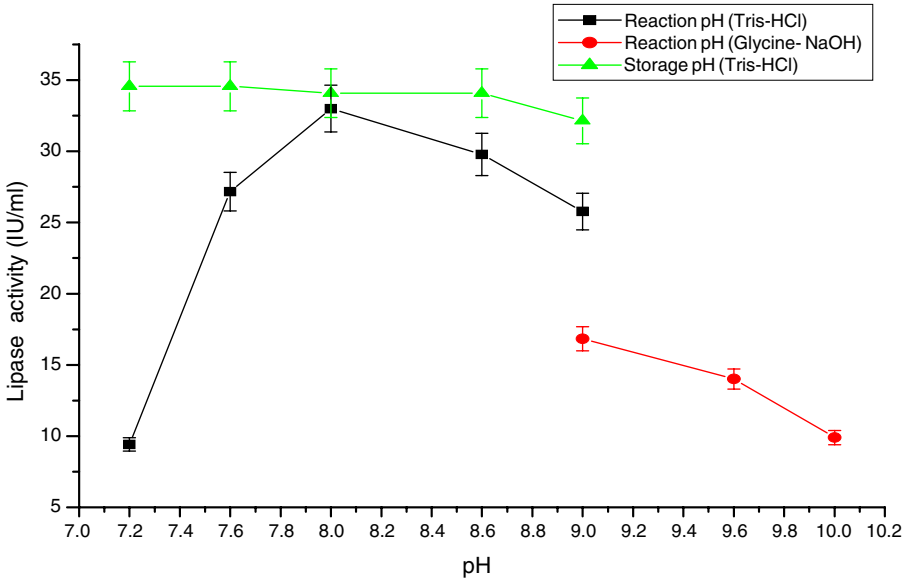

Fig. 4 Optimum pH and pH stability of the crude lipase

Table 2 Effect of various metal ions on the activity of crude lipase.

Salt used	Relative activity (%)		
	Concentration of metal ions (mM)		
	0.1	1	5
Control	100	100	100
$ZnCl_2$	131.03	141.5	72.41
$CaCl_2$	138.66	204	132
$MgCl_2$	146.66	100	100
$MnCl_2$	30.05	12	0
$CoCl_2$	212.56	100	38.83
$CuCl_2$	183.03	141.77	105.94
$FeCl_3$	75.03	34.89	0
$SnCl_2$	53.55	26.83	0

procedure. Figure 4 shows that the enzyme is stable in this pH range, so it can be said that crude lipase is active at alkaline pH.

To determine the thermal stability of the crude lipase, the enzyme was incubated at different temperature (4–70 °C) for 1 h at pH 8.0 using 0.2 M Tris-HCl buffer. The residual activities were evaluated according to the standard assay procedure. As shown in Fig. 3, the enzyme is thermostable up to 60 °C.

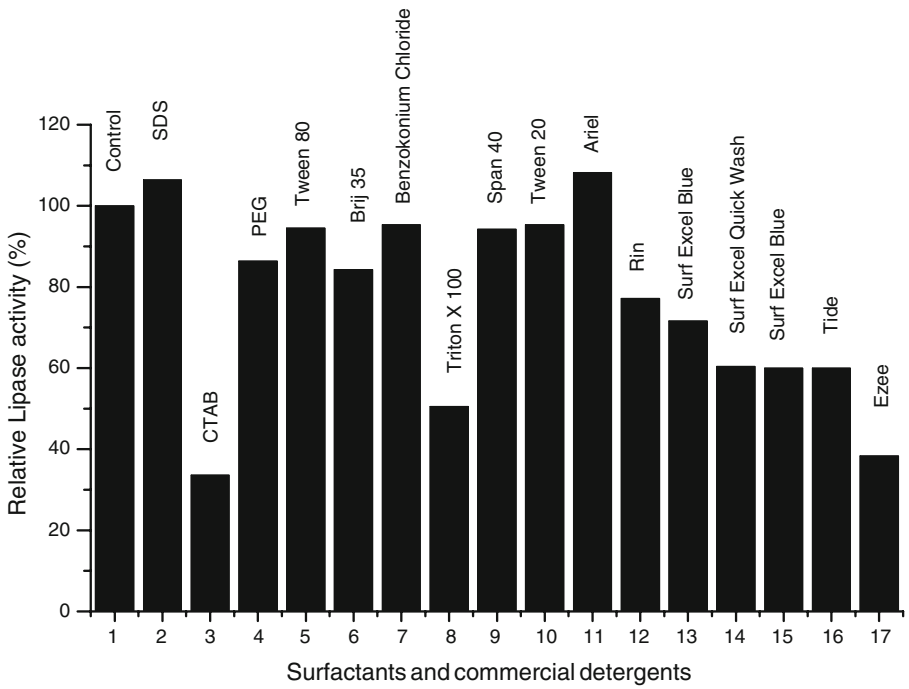

Fig. 5 Effect of surfactants and commercial detergents on lipase activity by *B. cereus* C_7

Table 3 Effect of bile salts on crude lipase activity.

Bile salts (0.5% *w*/*v*)	Relative activity (%)
Control	100
Cholic acid	90
Sodium taurocholate	120
Sodium deoxycholate	123

Effect of Metal Ions

The effect of various metal ions, viz., Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Fe^{3+}, and Sn^{2+} on enzyme activity, was studied by adding the metal ions at different concentrations (viz., 0.1, 1, and 5 mM) in enzyme–substrate reaction mixture during enzyme assay. Other conditions were as usual. The results were shown in Table 2. Among the tested metal ions, Ca^{2+} (1 mM), Co^{2+} (0.1 mM), and Cu^{2+} (0.1 mM) significantly increased lipase activity. Mg^{2+} (0.1 mM) and Zn^{2+} (1 mM) also have stimulatory effect on lipase activity. Mn^{2+}, Fe^{3+}, and Sn^{2+} ions were found to reduce lipase activity even at 0.1 mM concentration. The negative effect of ions on the lipase is generally the result from direct inhibition of the catalytic site like many other enzymes [14]. A number of lipases produced from other microorganisms were found to be Ca dependent [15–18].

Effect of Surfactants and Commercial Detergents

Different surfactants, viz., Triton X 100, Tween 20, Tween 80, Brij 35, Span 40, benzokonium chloride, sodium dodecyl sulfate (SDS), poly(ethylene glycol) (PEG), and cetyltrimethylammonium bromide (CTAB) at the concentration of 0.2% *w*/*v*, were added to the enzyme–substrate reaction mixture, other conditions remaining the same. Among the surfactants tested, SDS has little stimulatory effect; Tween 20, Tween 80, Brij 35, Span 40, benzokonium chloride, and PEG have a little inhibitory effect; and Triton X 100 and CTAB have inhibitory effect on lipase activity (Fig. 5).

Commercial detergents, viz., Ariel, Tide, Rin, Surf excel blue, Surf excel quick wash, Sunlight, and Ezee from local market, were also used at 0.2% concentration in enzyme–substrate reaction mixture and enzyme assay was carried out as usual. It has been observed that Ariel enhanced the enzyme activity, whereas the enzyme retained 60% or more activity in presence of commercial detergents like Rin, Surf excel blue, Surf excel quick wash, Sunlight, and Tide. Only Ezee showed significant inhibitory effect on lipase activity.

Effect of Bile Salts

Bile salts, viz., cholic acid, sodium deoxycholate, and sodium taurocholate at 0.25% and 0.5% (*w*/*v*) concentrations, were added separately during assay of lipase enzyme [5].

Table 4 Stability of the crude lipase in presence of oxidizing agents.

Oxidizing agents (1% *v*/*v* or *w*/*v*)	Relative activity (%)
Control	100
Hydrogen peroxide	100
Sodium hypochlorite	100

Table 5 Stability of the crude lipase in presence of protease.

Protease (0.5 mg of 1240 U/mg)	Relative activity (%)
Control	100
Trypsin	100

Sodium deoxycholate and sodium taurocholate increased the lipase activity while in presence of cholic acid, enzyme retained 90% of its activity (Table 3).

There are several reports which show that bile salts are stimulatory to the activity of some microbial lipases [19]. Watanabe et al. [20] reported inhibition of lipase activity in presence of bile salts.

Effect of Oxidizing Agents

The present lipase is highly stable (relative activity 100) toward oxidizing agent, viz., hydrogen peroxide (1% *v*/*v*) and sodium hypochlorite (1% *w*/*v*) after 1 h incubation (Table 4). Bleach stability is an important property and bleach stable enzymes are not very common. Bleach stability may be attained by site directed mutagenesis [21–23] or protein engineering [24, 25]. But the present lipase is inherently stable toward oxidizing agents. Rathi et al. in 2001 [4] studied lipase stability in presence of oxidizing agents like hydrogen peroxide, sodium perborate, and sodium hypochlorite at 1% *w*/*v* or *v*/*v*. Gulati et al. [26] reported bleach stability of a novel alkaline lipase by *Fusarium globulosum* to 0.1 M hydrogen peroxide and sodium perborate.

Effect of Protease

The *B. cereus* lipase was found to be stable in presence of protease (trypsin 0.5 mg of 1240 IU/mg; Table 5). So, it has an added advantage when used in combination with proteases in detergent formulations. Protease and lipase, both enzymes, are used in detergent formulation, so the lipase used as additive in the detergent must be resistant to protease. Rathi et al. [4] in 2001 reported that *Burkholderia cepacia* lipase was stable to different alkaline proteases (0.5 mg) from different microbial sources. Gulati et al. [26]

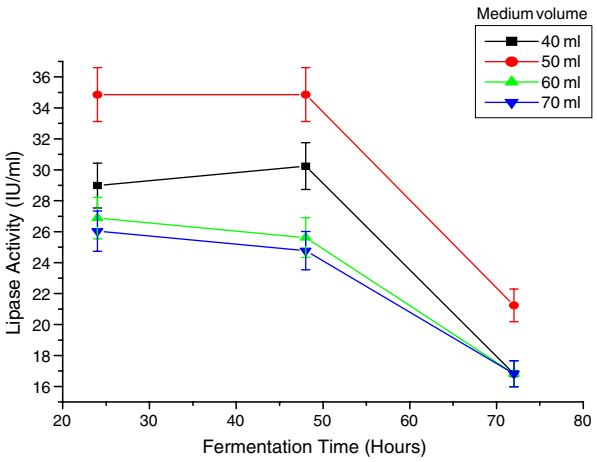

Fig. 6 Effect of fermentation time and medium volume on production of lipase by *B. cereus* C_7

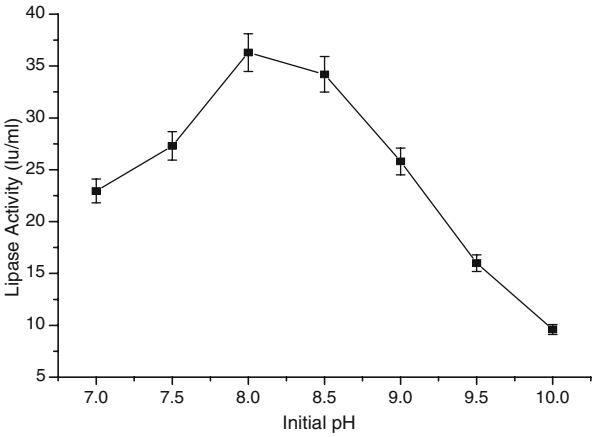

Fig. 7 Effect of initial pH on lipase production by *B. cereus* C_7

reported that *F. globulosum* lipase exhibited excellent stability in presence of *Aspergillus saitoi* protease (88% residual activity) and *Bacillus licheniformis* protease (100% residual activity) even after 1 h incubation.

Effect of Environmental Conditions on Production of Lipase

Lipase production was carried out using different volumes of medium, viz., 40, 50, 60, and 70 ml in 250 ml Erlenmeyer flask, other conditions remaining the same. Samples were collected at 24-h intervals up to 72 h and tested for enzyme activity. Volume of medium and fermentation time had a significant effect on maximum production of lipase (Fig. 6). Maximum lipase production was observed at 24 h when 50 ml medium was used. Effect of initial pH of the culture medium on lipase production was studied using a wide range of pH 7–10 and maximum production was found at pH 8.0 (Fig. 7).

Lipase production was carried out at different temperatures, viz., 25 °C, 30 °C, 33 °C, 35 °C, and 38 °C. Maximum lipase activity 35±0.826 IU/ml (Fig. 8) was achieved by

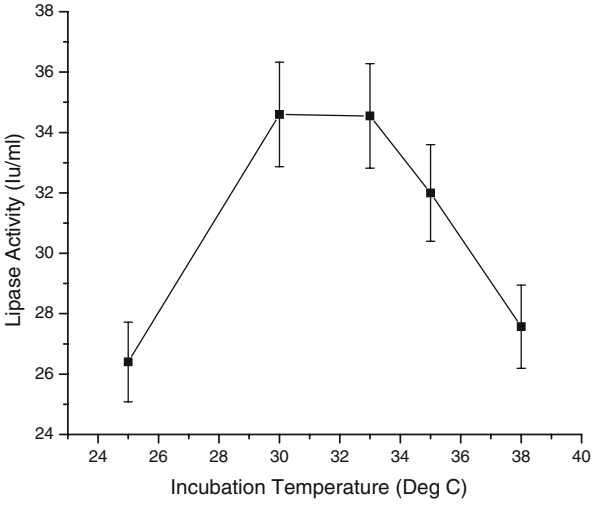

Fig. 8 Effect of temperature on lipase production by *B. cereus* C_7

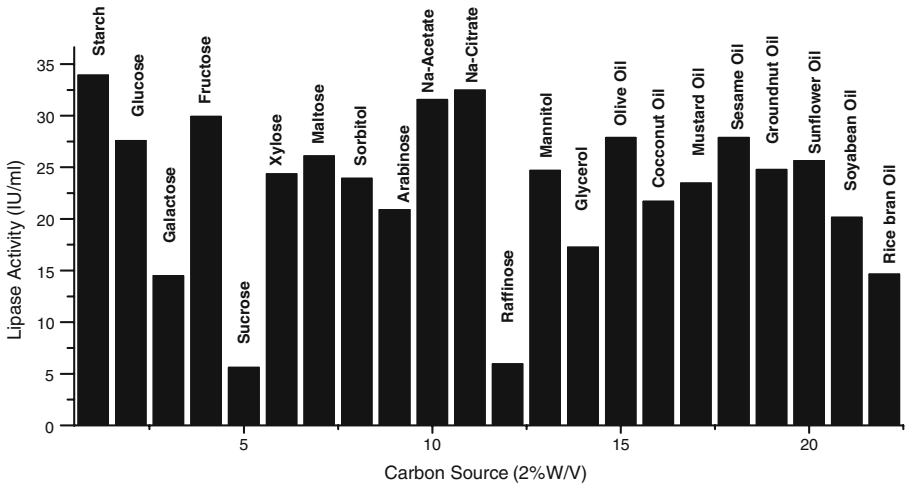

Fig. 9 Lipase production by *B. cereus* C_7 using various carbon sources (2% *w*/*v*)

growing the selected strain at 30–33 °C and 24 h of fermentation. Effects of age of inoculum (range used 20–48 h) and volume of inoculum (range used 0.5–3%) on lipase production were also studied; most suitable conditions were found to be 24 h and 2%, respectively (results not shown).

Nutritional Factors Affecting Lipase Production

Carbohydrates, a variety of oil and some salts, were used as carbon source for lipase production, other ingredients of the medium and production parameters remaining the same. The results were shown in the Fig. 9. From the experiment, starch was found to be the superior carbon source (enzyme activity 33.5±0.987 IU/ml). Moderate to good amount of lipase activity was obtained with sodium acetate, sodium citrate, fructose, glucose, maltose, xylose, mannitol, sorbitol, and arabinose. Among the different types of oil tested, except rice bran oil and soybean oil, moderate to good amount of lipase activity was observed with other oils. Then, different levels of starch were used for enzyme production and maximum enzyme production was observed at 2% starch.

Simple nitrogen sources like ammonium nitrate, ammonium chloride, ammonium sulfate, diammonium hydrogen phosphate, ammonium dihydrogen phosphate, and sodium

Table 6 Effect of inorganic nitrogen sources on production of lipase.

	Lipase activity (IU/ml) in presence of	
	Peptone 2% (N_2 level 297 mg/100 ml) + urea 0.1% (N_2 level 46.62 mg/100 ml)	Peptone 2% (N_2 level 97 mg/100 ml)
Ammonium nitrate	30.6	28.98
Ammonium chloride	27.8	21
Ammonium sulfate	31.95	30.07
Diammonium hydrogen phosphate	23.24	21.25
Ammonium dihydrogen phosphate	19.5	17
Sodium nitrate	24.07	22.1

nitrate (N_2 level 21.2 mg/100 ml) were used along with peptone (N_2 level 297 mg/100 ml) (a) in presence of urea (N_2 level 46.62 mg/100 ml) and (b) in absence of urea (Table 6). Ammonium sulfate (nitrogen level 21.2 mg/100 ml) along with peptone (N_2 level 297 mg/100 ml) and urea (N_2 level 46.62 mg/100 ml) having total nitrogen level 364.82 mg/100 ml gave the maximum production of lipase. According to Ferie et al., peptone contains certain cofactors and amino acids, which match the physiological requirements for lipase biosynthesis [27]. Lima et al. found that lipase production in *Penicillium aurantiogriseum* was stimulated using ammonium sulfate [28]. Urea was found to increase lipase production from a bacterial isolate SJ-15 [29] and a fungus *Rhodotolura glutinis* [30].

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