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PRODUCTION AND CHARACTERIZATION OF AN ALKALOTHERMOSTABLE, ORGANIC SOLVENT TOLERANT AND SURFACTANT TOLERANT ESTERASE PRODUCED BY A THERMOPHILIC BACTERIUM *GEOBACILLUS SP.* AGP-04, ISOLATED FROM BAKRESHWAR HOT SPRING, INDIA

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ABSTRACT

A thermophilic bacteria, *Geobacillus sp.* AGP-04, isolated from Surya Kund hot spring, Bakreshwar, West Bengal, India was studied in terms of capability of tributyrin hydrolysis and characterization of its thermostable esterase activity using p-nitrophenyl butyrate (PNPB) as substrate. The extracellular crude preparation was characterized in terms of pH and temperature optima and stability, organic solvent tolerance capacity and stability, substrate specificity, surfactant tolerance capacity, kinetic parameters and activation/inhibition behavior towards some metal ions and chemicals. Tributyrin agar assay exhibited that *Geobacillus sp.* AGP-04 secretes an extracellular esterase. The *Vmax* and *Km* values of the esterase were found to be 5099 U/Land 103.5µM, respectively in the presence of PNPB as substrate. The optimum temperature and pH, for *Geobacillus sp.* AGP-04 esterase was 60°C and 8.0, respectively. Although the enzyme activity was not significantly altered by incubating crude extract solution at 20-70°C for 1 hour, the enzyme activity was fully lost at 90°C for same incubation period. The pH stability profile showed that original crude esterase activity is stable at a broad range (pH 5.0-10.0). Moreover, the enzyme was highly organic solvent and surfactant tolerant. The effect of some chemical on crude esterase activity indicated that *Geobacillus sp.* AGP-04 produce an esterase which contains a serine residue in active site and for its activity -SH groups are essential. Besides, enzyme production was highly induced if fermentation medium contain polysaccharides and oil as carbon source.

Keywords: *Geobacillus,* hot spring, esterase / lipase, thermophile, thermostabilty

INTRODUCTION

Esterases (E.C. 3.1.1.1, carboxyl ester hydrolases) catalyze the hydrolysis of esters composed of short-chain fatty acids, ester synthesis, and trans-esterification reactions. They are broadly distributed in animals, plants and microorganisms (**Bornscheuer 2002**). Among the esterases, true esterases (EC 3.1.1.1, carboxyl ester hydrolases) hydrolyze esters of short chain carboxylic acids (≤12) and lipases (EC 3.1.1.3, triacylglycerol hydrolases) show maximum activity towards insoluble long chain (C≥12) acylglycerides (**Eggert *et al.* 2002**). Microbial esterases and lipases are of substantial interest because of their prospective biotechnological application. The major reasons of limiting industrial usage of known esterases are their limited thermostability, mainly at high temperatures; pH stability; and instability in the organic solvent in operating industrial conditions. Therefore, the exploration for new microbial enzyme sources is vital for the advancement of new thermostable and organic solvent resistant enzymes and their applications. The correlation between thermostability of an enzyme in water and its resistance to denaturation in organic solvent has been reported earlier (**Owusu *et al.* 1989**). For this reason thermostable enzymes are attractive not only to be used in aqueous media but also in organic media (**Kademi *et al.* 1999**). Thermostable enzymes are usually screened from thermophilic or hyperthermophilic organisms (**Gowland *et al.* 1987**). In organic media, lipases and esterases catalyze esterification, inter-esterification, alcoholysis or acidolysis reactions (**Kawamoto *et al.* 1987**). The modification of triglycerides for fat and oil industry, synthesis of flavor esters for food industry, resolution of racemic mixtures used for the synthesis of fine chemicalsfor the pharmaceutical industry can be performed (**Molinari *et al.* 1996**). The carboxylesterase produced by *Bacillus subtilis* has been used in the synthesis of naproxen as a nonsteroidal anti-inflammatory drug (**Quax *et al.* 1994**) and 2-arylpropionic acids with high enantioselectivity (**Azzolina *et al.* 1995**). Effectual kinetic resolutions were accomplished in the synthesis of primary alcohols by esterase from *Bacillus coagulans* (**Baumann *et al.* 2000**) and secondary alcohols from *Bacillus stearothermophilus* (**Molinari *et al.* 1996**). The cloning, expression, purification and biochemical characterization of esterolytic enzymes from *Bacillus* species were reported (**Kademi *et al.* 2000a; Markossian *et al.* 2000; Eggert *et al;* 2002**). Despite most organic solvents being toxic to microorganisms because of their effects on cellular membranes (**Sardessai *et al.* 2002**), when this strain was cultivated in the presence of some hydroxylic organic solvents, esterase production was higher than in the absence of an organic solvent (**Torres *et al.* 2005**). In this study, we describes the production of the thermostable, organic solvent-tolerant and alkali tolerant esterase produced by a thermophilic bacteria *Geobacillus sp.* AGP-04 isolated from Surya Kund Hot Spring of Bakreshwar, West Bengal, India and we report here the results of the production and characterization of crude esterase. It is the first report on esterase producing bacteria isolated from this hot spring.

MATERIAL AND METHODS

Materials

p-nitrophenyl acetate (pNPC2), *p*-nitrophenyl butyrate (pNPC4), *p*-nitrophenyl laurate (pNPC12) and *p*-nitrophenyl palmitate (pNPC16) were purchased from Sigma-Aldrich, USA. And the other chemicals such as DTT, PMSF, EDTA were purchased from Merck, India. The primers used in the study were purchased from Chromas Biotech Pvt. Ltd. India. Other microbiological medium and medium composition were purchased from Himedia, India.

Culture media and fermentation media composition

The Luria-Bertani (LB) liquid medium contained (g/L in distilled water) yeast extract 5.0, bactotryptone 10.0 and NaCl 5.0, pH 8.0. LB medium was used to culture the strain. The fermentation medium used for esterase production was LB medium supplemented with (g/l) soluble starch, 20; peptone, 20; KH_2PO_4, 5; $(NH_4)_2SO4$ 1; $MgSO_4.7H_2O$ 1; $(NH_2)_2CO$, 1; and tributyrin at a ratio of 15ml/l. The pH of the media was adjusted to 8.0. Fermentation was done at pH 8 and 60ºC temperature on a shaking incubator operating at 200 rpm.

Sampling and screening of thermophilic esterolytic bacteria

To isolate the thermophilic bacteria capable of producing esterase enzyme, the water and sediment sample was collected in sterile glass container from Surya

kunda hot spring (Bakreshwar) located at 23.88°N 87.37°E, Birbhum, West Bengal, India. Then the sample were serially diluted and plated on tributyrin agar plate containing 1.5% trybutyrin in Luria-Britani (LB) solid medium at 60ºC for three days incubation. After incubation presence of clear zone surrounding the colony confirmed the ability to hydrolyze ester present in the media. The bacteria were then pure cultured. The strain was identified as *Geobacillus sp.,* the strain was used for the present study. It was maintained in nutrient agar slant by monthly subculturing and stored at 4ºC.

Taxonomical Studies

To confirm the identity of the isolate AGP-04, genomic DNA extraction and purification were done as described preciously (**Redburn *et al.* 1993**). 16S rRNA gene was amplified using the universal primer 8F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-TACCTTGTTACGACTT-3´). The amplification was done by initial denaturizing at 95ºC for 6 min followed by 35 cycles of 95ºC for 30 second, 52ºC for 30second, 72ºC for 30 second, and final extension at 72ºC for 10 min. The polymerase chain reaction (PCR) product was purified and sequenced by Chromas Biotech Pvt. Ltd (India). The phylogenetic relationship of the isolate was determined by comparing the sequencing data with its closely related neighbor sequences retrieved from the GenBank database of the National Center for Biotechnology Information, via BLAST search. Phylogenetic analysis was performed using the software package MEGA version 5.0 (**Kumar et al. 2008**) after obtaining multiple alignments of data by CLUSTAL W (**Thompson *et al.* 1994**). Pairwise evolutionary distances were computed using the correction method and clustering was performed using the neighbor-joining method (**Saitou *et al.* 1987**). Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1,000 replicates (**Nei *et al.* 2000).**

Preparation of crude enzyme

Inoculum was prepared by transferring one loop of culture from slant to the inoculum medium (LB medium) (50/250 ml Erlenmeyer flask) and incubating the flask at 60°C in a rotary shaker at 200 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 esterase activity and characterization of enzyme. (**Nawani *et al*, 2000**).

Esterase assay

Esterase activity was determined by using the spectrophotometric assay described by Lee et. al (**Lee *et al.* 1999**) with slight modification. For activity assay, stock substrate solution was prepared by dissolving p-nitrophenyl butyrate (PNPB) in acetonitrile at a concentration of 10 mM. For the enzyme assay the substrate solution included stock substrate solution, ethanol and 50 mM phosphate buffer (pH 8) in ratio of 1:4:95 (v/v/v), respectively. To determine the enzymatic activity 0.3 ml of the cell free supernatant was added to 0.9 ml of the substrate solution. After the incubation of the reaction mixture at 60°C for 15 min, the change in absorbance at 405 nm was monitored spectrophotometrically (UV-VIS SPECTROPHOTOMETER 118, Systronics). The amount of released p-nitrophenol (p-NP) was determined for the esterase activity. The non-enzymatic hydrolysis was subtracted by using a blank without enzyme. One unit of enzyme activity was defined as 1µmol of p-NP formed per min under assay conditions.

Substrate specificity

The substrate specificities of the crude esterase were determined using *p*-nitrophenyl acetate (pNPC2), *p*-nitrophenyl butyrate (pNPC4), *p*-nitrophenyl laurate (pNPC12) and *p*-nitrophenyl palmitate (pNPC16) as substrates at the standard assay conditions. The substrate stock solutions were prepared in acetonitrile at a concentration of 10 mM. Other assay conditions remained same.

Optimum pH and pH stability of the crude enzyme

The effect of pH on the crude esterase activity was measured at 60°C by using 50 mM of following buffers; sodium acetate (pH 4.0-5.0), potassium phosphate (pH 6.0-8.0) NaOH-Glycine buffer (pH 9.0-11). The same buffers with 1.0 pH value increments were used to determine the pH stability of the enzyme extract. The mixture of enzyme extract and the buffers given above (1 : 1) was incubated for 24 h at 4°C and then the residual activity was assayed by using 0.3 ml of this mixture at optimum conditions with PNPB as substrate (**Yildirim *et al.* 2005; Zhang *et al.* 2005**]. The residual esterase activity was calculated by comparison with unincubated enzyme.

Optimum temperature and thermostability of the crude esterase

For the determination of optimum temperature and thermal stability of the crude esterase, enzyme activity assay was performed for different temperatures in the range of 20-90°C at the optimum pH value using a circulation water bath. The reaction mixtures were incubated for 15 min at various temperatures indicated above. Then, the relative activity was determined spectrophotometrically at 405 nm as rapidly as possible. In order to determine the thermal stability of the enzyme, the crude enzyme were incubated at temperatures of 20-90°C with 10°C increments for 1 h, rapidly cooled and brought to 25°C. After the reaction mixture reached to room temperature, the enzyme activity was assayed under the standard reaction conditions at optimum pH value (**Yildirim *et al.* 2005; Ozen *et al.* 2004**).

Effect of metal ions on esterase activity

The effect of various metal ions on the activity of crude esterase enzyme was determined by addition of 1mM of chloride salts of each metal, for monovalent ions Na^+ and K^+, for divalent Cu^{2+}, Ni^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, Hg^{2+}, Co^{2+}, and Cr^{3+} for trivalent ions. After addition of each metal ion solution on crude enzyme extract (1:1) and incubation at room temperature for 30 minutes, the enzyme activity was assayed using 0.3ml of aliquot of metal ion: crude enzyme solution and PNPB as substrate. The residual activities were measured by comparison with standard assay mixtures contain no metal ions and diluted enzyme in the ratio of 1:1 at optimum pH (**Lee *et al.* 1999**).

Effect of additives on the esterase activity

The esterase activity was observed in the presence of ethylene diamine tetraacetic acid (EDTA) and 2-mercaptoethanol (2-ME) at the concentration of 2, 2.5 and 5mM whereas dithiothreiotol (DTT) and phenyl methyl sulfonyl fluoride (PMSF) at the concentration of 50, 100, 250 µM. The activity of the crude enzyme extract in the presence of these chemicals was assayed as indicated in the section of metal ion effect.

Kinetic study of the enzyme

To determine the effect of substrate concentration on the reaction rates of the crude esterase a substrate saturation curve was obtained by plotting the final substrate concentration in the range of 50-1000µM versus esterase activity in the presence of PNPB as substrate. The Michaelis-Menten constant (Km) and the maximum velocity of the reaction (Vmax) were calculated from Lineweaver-Burk plot (**Lineweaver *et al.* 1934)**.

Effect of environmental conditions on production of esterase

Esterase production was carried out using 50 ml fermentation medium in 250 ml Erlynmeyer flask. Effect of initial pH of the fermentation medium on esterase production was studied using a wide range of pH 4-11. After 24 h incubation at the temperature of 60ºC at 200 rpm rotation the cell free supernatant was used for enzyme activity as described earlier. On the other hand to determine the effect of initial temperature on esterase production was carried out at different temperatures with 10°C increment, viz., 20ºC, 30ºC, 40ºC, 50ºC, 60ºC 70ºC 80ºC and 90º C. The other condition remaining the same, the enzyme activity was measured by the method as described earlier.

Effect of surfactants and commercial detergent on the esterase

To determine the effect of surfactant on esterase production, various surfactant viz., Triton X- 100, Tween 20, Tween 80, Benzokonium chloride, Sodium dodecyl sulfate (SDS), and Cetyltrimethyl ammonium bromide (CTAB) at the concentration of 0.2% (w/v), were added to the enzyme substrate reaction and some commercial detergent from local market viz., Ariel, Tide, Rin, Surf excel blue, Surf excel quick wash, Sunlight, and Ezee were also used at 0.2%(w/v) concentration in the enzyme substrate reaction mixture. Whereas control didn't contain any surfactant or commercial detergent in the fermentation medium. After 24 h fermentation at 60ºC temperature in a rotary shaker at 200 rpm continuous shaking, the cell free supernatant was tested for the enzyme activity. Percent residual activity was calculated by comparison with control.

Effect of carbon source on the production of esterase

To study the effect of carbon source on the esterase production in the fermentation medium various carbohydrates (Glucose, Maltose, Sorbitol, Galactose, Sucrose, Fructose, Mannitol, Starch, Cellulose) and a variety of oil (Glycerol, Olive oil, Coconut oil, Soyabean oil and Mustard oil) were used at a concentration of 2% for esterase production, other ingredients of the medium and production parameters remained same. After fermentation the cell free supernatant was used for the enzyme activity under standard assay condition.

Effect of water-miscible organic solvents on crude esterase activity

The effect of water-miscible organic solvents in enzyme stability and activity was investigated in 50mM Tris–HCl (pH 8.0) containing 50% (v/v) organic solvent.

The activity of esterase in organic solvents was measured using PNPB (10mM) as substrate at 60°C. The enzyme activity in each organic solvent was compared with the activity in water which was set at 100% (**Baigori et al. l996**). The esterase stability in organic solvent was measured by incubating the enzyme in the reaction mixtures at room temperature for 1 h. After that, the enzyme activity in each solvent was measured and compared with the initial activity (before the incubation) in the same solvent, which was set at 100%.

Statistical analysis

Determinations were made in triplicate, and results shown are the average of three or more independent experiments. Data are represented by the mean±standard deviation. Analysis of variance was performed on data sets using a significance of p values lower than 0.05. The analysis was done by GraphPad Prism software version 5.0.

RESULTS

Screening of esterase producing organism

By using tributyrin degrading assay, 8 thermophilic and moderate thermophilic bacteria showed their capability to degrade tributyrin. Among them AGP-04 showed maximum activity. Thus the isolate AGP-04 was selected as potent strain and used for the further study. It can be easily extracted from the result that, the isolate AGP-04 secretes an extracellular esterase enzyme which was responsible for tributyrin degradation.

Identification and taxonomical studies of the isolated strain AGP-04

On the basis of morphological characteristics, the isolate AGP-04 is a Gram-positive, rod-shaped, and aerobic spore forming bacterium. The isolate grew well in a wide range of pH (6-10). Optimal bacterial growth was observed at pH 8.0 and 55-60^{o}C temperature (data not shown). Phylogenetic analysis based on 16S rDNA gene sequence assessment revealed that isolate AGP-04 fell within the genus of *Geobacillus*. From the construction of phylogenetic tree (Fig.1), its closest homolog was found to be *Geobacillus stearothermophilus* strain G1017_C12 (GenBank Accession no HQ324905) (97% maximum identity). Our organisms sequence data have been submitted to the NCBI GenBank databases under the accession number JX513957 (*Geobacillus* sp. AGP-04).

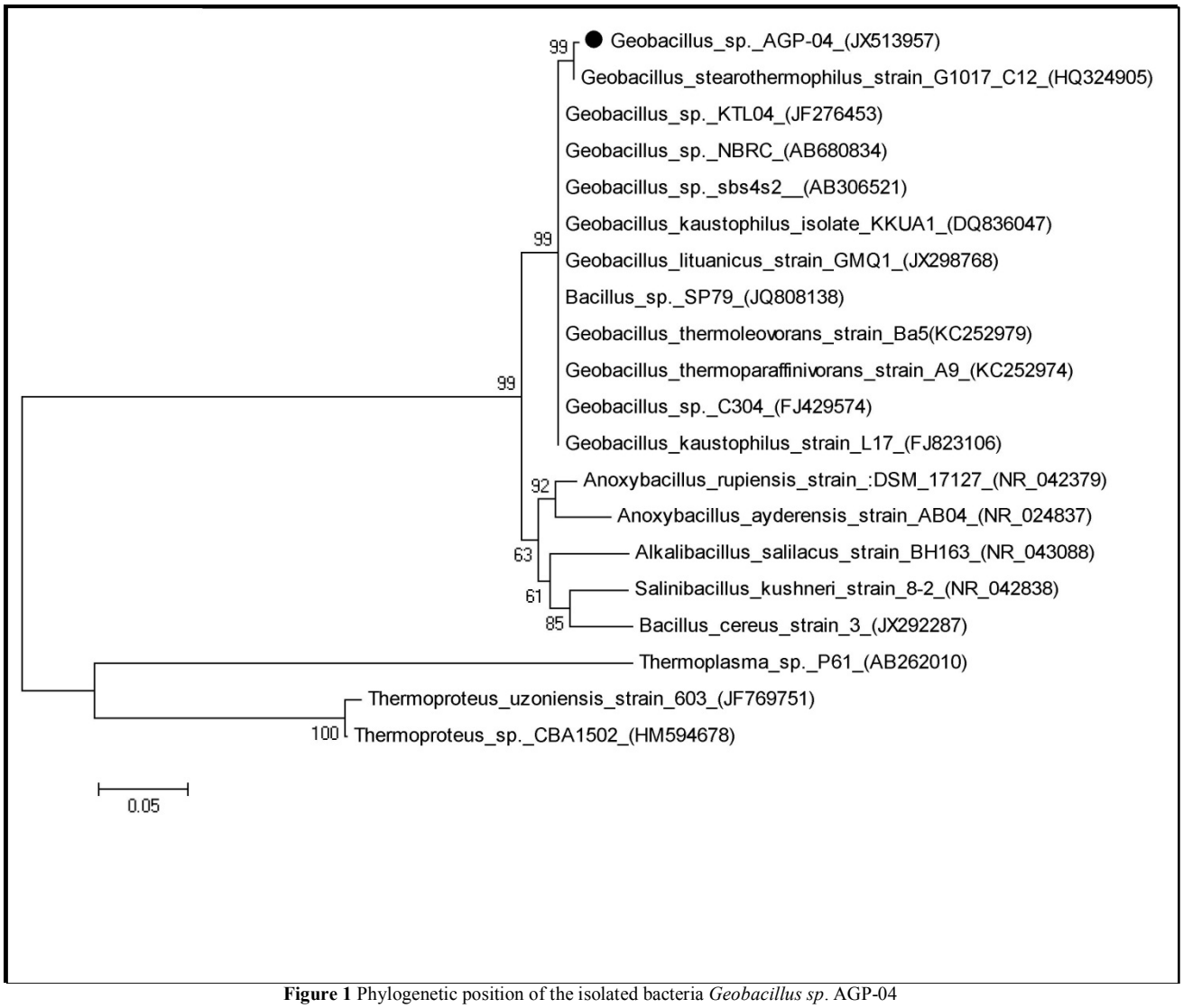

Figure 1 Phylogenetic position of the isolated bacteria *Geobacillus sp*. AGP-04

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.99375584 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 769 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. GenBank accession number in bracket

Substrate specificity

The substrate specificity of the crude enzyme produced by *Geobacillus* sp. AGP-04 was studied with p-nitrophenyl acetate (C_2), p-nitrophenyl butyrate (C_4), *p*-nitrophenyl laurate (C_{12}) and *p*-nitrophenyl palmitate (C_{16}) (Fig. 2). Maximum activity was observed on pNPB. Concerning the activity on pNPB as maximum, the activity on pNPA and pNPL was about 66% and 14% respectively, and no activity was noticed on pNPP.

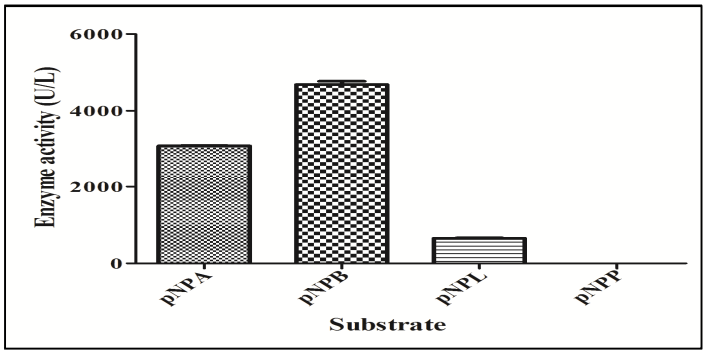

Figure 2 Substrate specificity of the isolated crude esterase enzyme. Substrate specificity of the crude enzyme was determined using 10 mM p-nitrophenyl acetate, p-nitrophenyl butyrate, p-nitrophenyl laurate and p-nitrophenyl palmitate.

pH optima and stability profiling of the crude enzyme

The effect of pH on the crude esterase produced by *Geobacillus* sp. AGP-04 was determined by using PNPB as substrate. The pH buffer range was studied in this experiment ranging from 5.0 to 11.0. As depicted in Fig. 3, the enzyme activity gradually increased from pH 5.0 to pH 8.0 and the enzyme showed its highest activity at pH 8.0. At this pH the enzyme activity reached at 4127.67 U/L. Increasing in pH value after pH 8.0 the enzyme activity gradually decreased and at the pH value of 11.0 the enzyme lost it's all the activity. Further investigations were carried out at pH 8.0. Besides, the pH stability study of the isolated esterase enzyme was performed by incubating the crude enzyme in pH buffer in a ratio of 1:1 for 24 hours at 4°C. It was observed that the isolated enzyme retained its activity in a wide range of pH (5-10). After pH 10 the enzyme activity drastically decreased and at pH 11 the enzyme lost its whole activity.

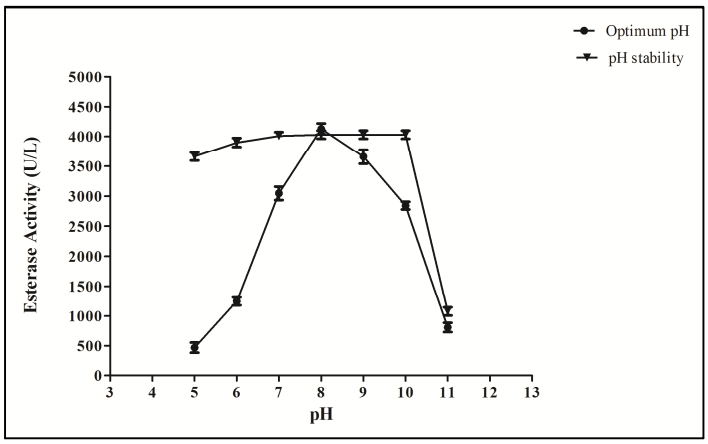

Figure 3 Optimum pH and pH stabilty of the crude esterase produced by *Geobacillus sp*. AGP-04. Effect of pH on activity of the crude enzyme in 50 mM sodium acetate buffer (pH 4.0-5.0), in potassium phosphate buffer (pH 6.0-8.0) and in NaOH-Glycine buffer (pH 9.0-11) and pNPB used as substrate. The pH stability of extracellular crude esterase residual activity after 24 h incubation at desired pH at 4°C. The activity was assayed under standard conditions, pH 8.0 at 60°C.

Study of the optimum temperature and thermostability of the crude esterase

To study the optimum temperature for the highest activity of the crude esterase, the enzyme activity was assayed at different temperature started from 20°C to 90°C. As the assay temperature was raised from lower to higher temperature the enzyme activity gradually increased and it was observed that the enzyme reached its highest activity at the temperature of 60°C. At this temperature the enzyme activity was found 4228.74 U/L (Fig. 4). Increased temperatures after 60°C gradually lowered the activity of the crude esterase enzyme and at 90°C the enzyme fully lost its activity. Moreover, the enzyme almost fully retained its original activity in a broad range of temperature from 20°C to 70°C.

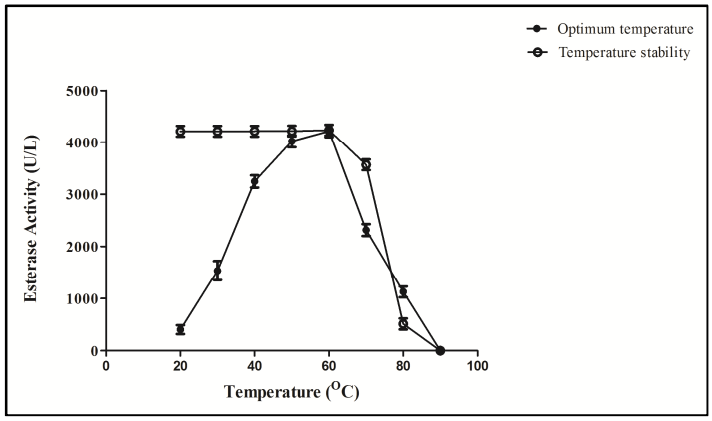

Figure 4 Optimum temperature and thermostabilty of the crude esterase produced by *Geobacillus sp.* AGP-04. Temperature optimum and stability profile of Geobacillus sp. AGP-04 esterase that hydrolyzes p-nitrophenyl butyrate. The reaction mixture in pH 8.0 phosphate buffer was incubated at different temperatures and activity was monitored at 405 nm. For thermal stability, the crude enzyme extract was incubated at temperatures of 20-90°C. The residual activity was compared with unincubated enzyme.

Metal ions effect on esterase activity

Different metal ion's effect on crude esterase activity was determined by incubating the enzyme with 1mM concentration of chloride salt of various metal ions at room temperature for 30 minutes. The residual enzyme activity was measured by using PNPB as substrate and compared with the activity of the enzyme solution which did not contain any metal ion. As shown in Table. 1, among the monovalent ions both K^+ and Na^+ ions significantly augmented the enzyme activity whereas among the divalent ions Ca^{2+} and Mn^{2+} ions had little inhibitory or no effect on the enzyme activity. For the other divalent ions Hg^{2+} and Zn^{2+} considerably inhibited the enzyme activity. In the presence of Cu^{2+} and Co^{2+} ions the enzyme retained its activity of 60% and/or above, while in case of Ni^{2+} it was 70% or higher, for Cd^{2+} it was 80% or/and higher. Moreover trivalent ion used in this study was Cr^{3+} and it was found that this ion significantly inhibited the crude esterase activity.

Table 1 Effect of some metal ions on *Geobacillus sp.* AGP-04 esterase activity. The esterase activity in the presence of a metal ion was compared with the control containing no metal ion. The control activity was taken as 100%.

Metal ion (1mM)	Residual Activity (%)
Cu^{2+}	67.32 ± 3.06
K^+	121.41 ± 5.13
Na^+	114.09 ± 5.72
Ni^{2+}	78.22 ± 3.31
Ca^{2+}	87.24 ± 4.05
Zn^{2+}	56.13 ± 3.54
Cd^{2+}	84.10 ± 3.28
Mn^{2+}	97.65 ± 5.17
Hg^{2+}	48. 26 ± 3.04
Co^{2+}	62.18 ± 4.27
Cr^{3+}	54.02 ± 4.16

Effect of some additives on the crude esterase activity

The effect of some chemicals and metal chelating agents on crude esterase activity was performed in this study. From the study it was found that dithiothreiotol (DTT) and 2 mercaptoethanol (2-ME) significantly enhanced the enzyme activity to a extent of 121.82 ± 4.41 and 152.43 ± 5.31 respectively, while Phenyl Methyl Sulfonyl fluoride (PMSF) and Ethylene Di-amine Tetra Acetic Acid (EDTA) considerably inhibited the esterolytic activity of the enzyme to a extent of 82.57 ± 4.08 and 28.62 ± 3.24 respectively of the crude enzyme (Table 2).

Table 2 Effect of some reagents on *Geobacillus sp.* AGP-04 esterase activity. The esterase activity in the presence of a reagent was compared with the control which contained no reagent other than the compounds required for esterase assay. The control activity was taken as 100%

Concentration (µM)	Relative Activity (%)		Concentration (mM)	Relative Activity (%)	
	PMSF	DTT		2-ME[a]	Na-EDTA
50	92.02 ± 5.13	106.22 ± 5.17	2	123.27 ± 5.04	82.14 ± 5.07
100	87.48 ± 4.33	113.04 ± 5.16	2.5	131.11 ± 5.21	58.26 ± 3.13
250	82.57 ± 4.08	121.82 ± 4.41	5	152.43 ± 5.31	28.62 ± 3.24

Legend: [a] 2-Mercaptoethanol

Influence of surfactants and commercial detergent on the enzyme activity

Different surfactants, viz; SDS, CTAB, Tween 20, Tween 80, Triton X-100 and Benzokonium chloride at the concentration of 0.2% (w/v), were added to the enzyme-substrate reaction mixture, other conditions were remained the same for assaying activity. Among the tested surfactant Tween 20 and Tween 80 significantly enhanced the enzyme activity whereas CTAB and Triton X-100 drastically inhibited the enzyme activity. All the other surfactants except SDS moderately inhibited the activity. SDS had little inducing ability. Commercial detergents, viz; Ariel, Rin, Sunlight, Surf Excel Quick Wash, Surf Excel Blue, Tide, and Ezee from local market, were also used at a concentration of 0.2% in enzyme-substrate reaction mixture and enzyme assay was carried out as usual. Among the commercial detergents, Ariel significantly enhanced the enzyme activity and the enzyme retained its 70% or more activity in presence of commercial detergents like Rin, Sunlight, Surf Excel Quick Wash, Surf Excel Blue and Tide. Only Ezee showed significant inhibitory effect on esterase. The effect of different surfactant on enzyme activity was depicted in Fig. 5.

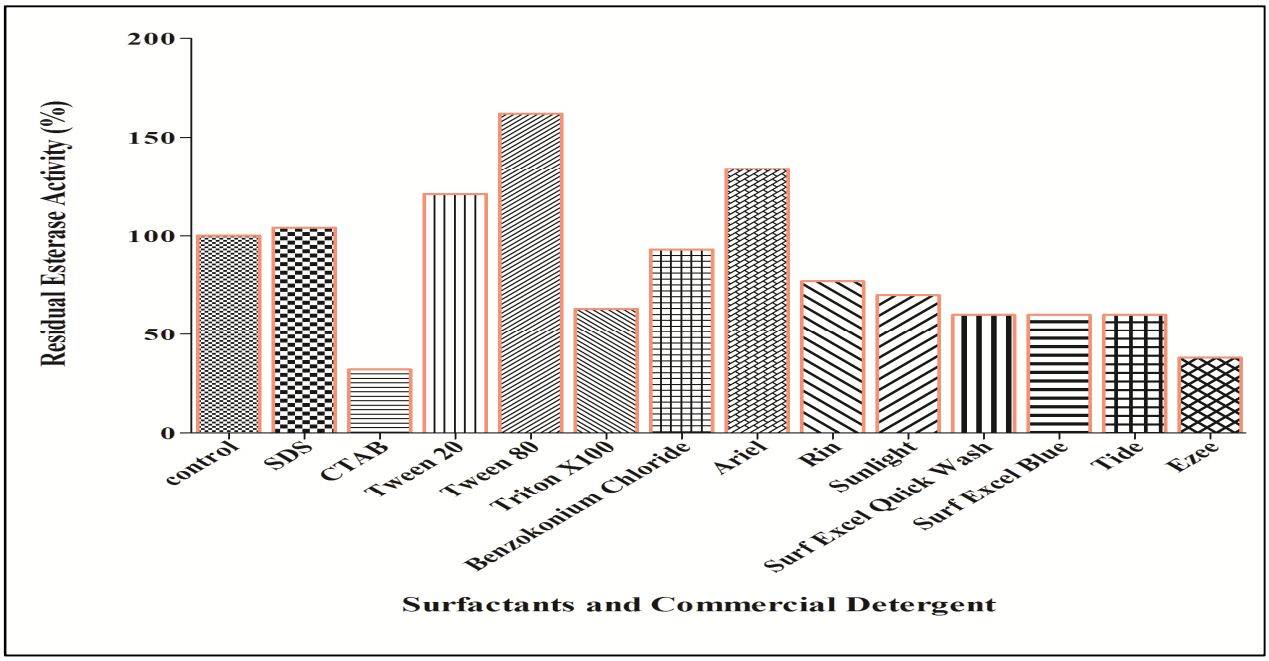

Figure 5 Effect of Surfactants and Commercial detergents on Esterase activity Produced by *Geobacillus sp*. AGP-04

Effect of water-miscible organic solvents on crude esterase activity

The enzyme activity of the crude esterase was measured in reaction mixtures containing 50% water- miscible organic solvent (Table 3). These reaction mixtures were incubated at room temperature for 1 hour, and then residual activity was measured and compared with the initial activity in the same solvent. Activity was determined in all cases, and definitely a high level of activity was found 50% glycerol and propylenglycol. Also, in these solvent the stability of the crude esterase was considerably high, similar to the water. It was found that, the isolated crude esterase retained more than 70% activity after 1 hour incubation in the presence of all the solvent except 1,3-propenodiol. Moreover, the isolated crude esterase enzyme was much more active in 50% methanol and1- propanol.

Table 3 Effect of water-miscible organic solvents on the activity and stability of the crude esterase produced by *Geobacillus sp.* AGP-04.

Organic solvent (50%)	Relative esterase activity (%)[x]	
	Activity[y]	Stability[z]
Water	100	96.03±2.00
Ethanol	15.03±1.26	75.23±1.63
Methanol	29.32±2.62	102.63±8.32
1-propanol	10.27±3.21	121.02±12.02
2-propanol	16.32±0.91	88.63±5.39
DMSO	42.36±3.98	93.21±1.48
Glycerol	86.92±2.61	98.54±8.36
Propylenglicol	58.27± 4.31	83.32±2.21
1,3-Propenodiol	6.32±0.86	42.63±2.61

Legend: [x]Data represent the mean of three independent assays ±SD. Esterase activity was assayed with PNPB at pH8.0 and 60°C.

[y]Esterase activity in water set as 100%.

[z]Residual activity after 1 h incubation in 50% organic solvent at room temperature. Remaining esterase activity in each solvent was compared with the initial activity (before the incubation) in the same solvent, which was set at 100%.

Kinetic study of the crude esterase enzyme

To determine the Km and Vmax of the isolated crude esterase, a substrate saturation curve was obtained by interpolating the substrate concentration against enzyme activity values. Here PNPB was taken as substrate. Michaelis-Menten Constant (Km) and maximum velocity (Vmax) were determined as 103.5µM and 5099 U/L respectively, from Lineweaver-Burk plot (Fig. 6).

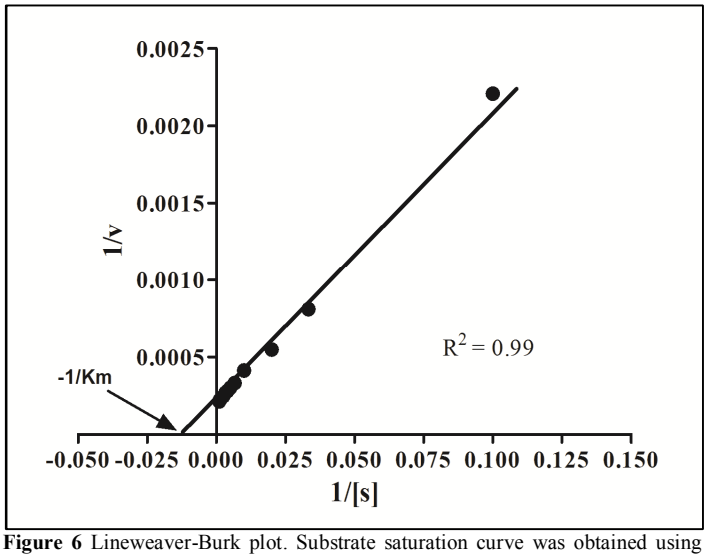

Figure 6 Lineweaver-Burk plot. Substrate saturation curve was obtained using 50-1000 µM PNPB as substrate versus enzyme activity

Study of the environmental conditions on production of esterase

To determine the optimum pH for the production of said esterase enzyme the pH of the production medium was adjusted to different pH value viz; 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0. It was found that the enzyme shows highest activity at the pH of 8.0. In this pH the enzyme activity reached up to 4155.33 U/L (Fig. 7). It was observed that the production gradually increases as pH increases from 4.0 and then reached at optimum value at pH 8.0. After that the increasing in pH of the production medium didn't show the increase in enzyme activity but the enzyme activity was lowered. On the other hand temperature optimum for the production of esterase enzyme by the isolated bacterial strain was found at the temperature of 60°C. To study the temperature optima for the highest enzyme activity, the production medium were incubated at different temperature viz; 20°C, 30°C. 40°C, 50°C, 60°C, 70°C and 80°C. It was found that at the 60°C temperature the enzyme showed its highest activity. Temperature above and

below of the 60°C, enzyme activity were found lesser than the activity at 60°C. At this temperature the enzyme showed the highest activity of 4213.47U/L (Fig. 8).

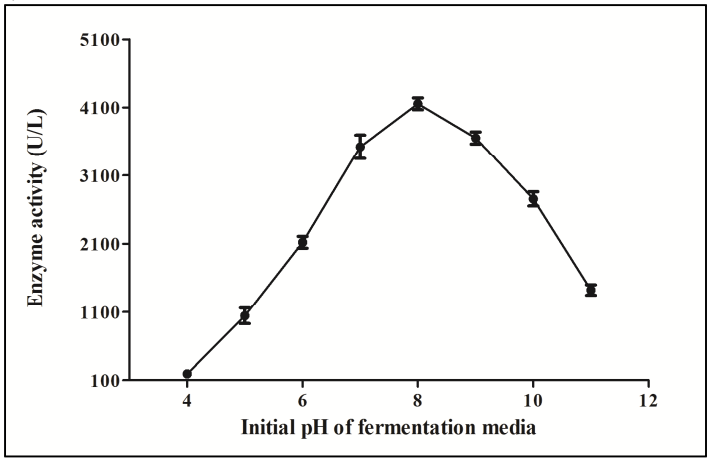

Figure 7 Effect of initial pH on estersae production by *Geobacillus sp*. AGP-04. The production medium pH was adjusted to different values and incubated at 60°C. After fermentation the extracellular crude enzyme activity was measured under standard assay conditions.

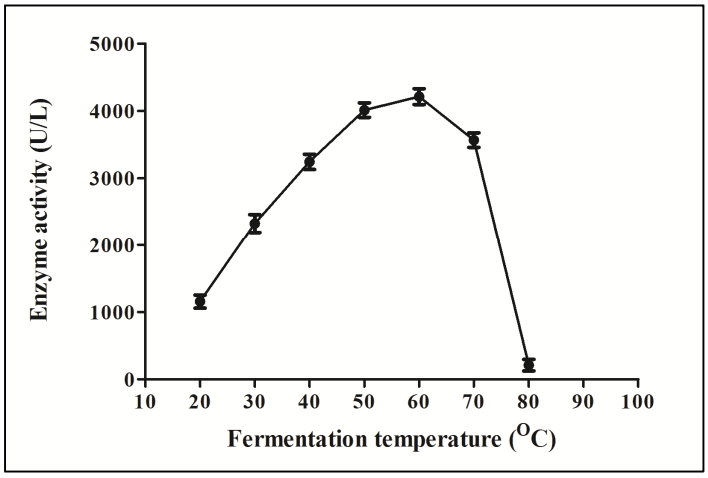

Figure 8 Effect of initial temperature on esterase production by *Geobacillus sp*.

AGP-04. The production medium pH was adjusted to 8.0 and incubated at different temperatures. After fermentation the extracellular crude enzyme activity was measured under standard assay conditions

Effect of carbon source on the production of esterase

Carbohydrate and a variety of oils were used as carbon source for esterase production, other ingredients of the fermentation medium and production parameters remaining the same. The results were shown in the Fig. 9. From the experiment, it was found that cellulose and starch are the superior carbohydrate as source of carbon. Enzyme activity using cellulose and starch as carbon source were 4636.67±34.07 U/L and 4525±117.2 U/L respectively. Whereas among the oils tested olive oil and coconut oil were found as good carbon source for the production of esterase. By using olive oil and coconut oil enzyme activity determined as 3967±113.7 U/L and 3865±46.19 U/L respectively. Moreover, moderate to good amount of esterase activity was obtained with Glucose, maltose, galactose, fructose and mannitol.

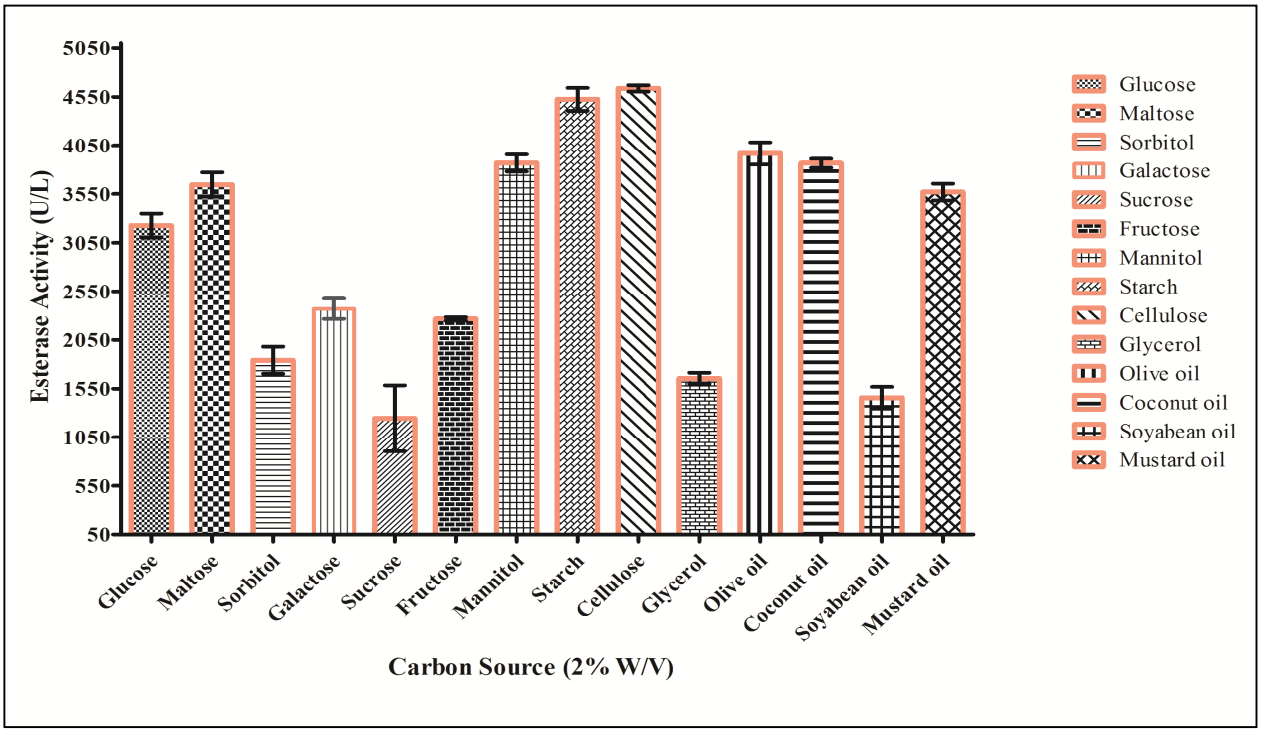

Figure 9 Esterase production by *Geobacillus sp*. AGP-04 using different carbon

DISCUSSION

In this study we are describing the esterolytic activity of a newly isolated hot spring bacteria and characterization of the crude enzyme produced by the strain. The isolated bacterial strain AGP-04 was identified as *Geobacillus sp*. by 16S rDNA profiling. As the isolated bacteria is close to its closest homolog *Geobacillus stearothermophilus* strain G1017_C12 (GenBank Accession no HQ324905) to an extent of 97% maximum identity, thus this bacteria is little different from the cited bacterium and named as *Geobacillus sp*. AGP-04 (GenBank Accession no JX513957). The esterolytic activity of the *Geobacillus sp*. AGP-04 was monitored by the tributyrin degradation in tributyrin agar plate and confirmed by the production of clear halo. Maximum substrate activity of the crude enzyme was observed in presence of pNPB as substrate. Besides crude enzyme also exhibited its activity towards pNPA and pNPL which is a unique characteristic of this enzyme. Since lipases hydrolyze esters in emulsion and usually water-insoluble substrates, typically triglycerides composed of long chain fatty acids, whereas esterase preferentially hydrolyze simple esters and usually only triglycerides bearing fatty acids shorter than C_6 (**Bornscheuer 2002**), these results strongly suggest that the enzyme used in this study showed an esterase activity. It must be pointed out that the finding about the significant activity demonstrated by the enzyme on pNPL (~ 14%) is quite interesting. This value is higher from that of esterases from *A. viscosus* NRRL B-1973 (**Lee *et al.* 1999**), *B. licheniformis* (**Alvarez Macarie *et el* 1999**), *A. nicotianae* 9458 (**Smacchi *et al.* 2000**) and *B. circulans* (**Kademi *et al.* 2000b**). Moreover, when pNPB is used as substrate, the esterase of *P.freudenreichii ssp. freundenreichii* ITG 14 (**Kakariari *et al.* 2000**) showed only 10% of maximum activity obtained by pNPA. Therefore, the new esterase is quite different from the known esterase which usually showed no activity on the substrate higher than C_{10}. Optimum activity at 60^0C and pH 8.0 was described for Geobacillus sp. AGP-04 esterase. The result was well consistent with the opinion which thermostable enzymes could be extracted for thermophilic microorganisms (**Jung *et al.* 2003; Kademi *et al.* 1999**). A thermostable esterase with an optimum temperature of 60 and 70-75^{o}C was also reported for *Bacillus circulans* (**Kademi *et al.* 2000b**) and *Bacillus thermoleovorans* ID-1 (**Lee *et al.* 1999**), respectively. The enzyme also highly thermostable, the enzyme almost fully retained its original activity in a broad range of temperature from 20^{o}C to 70^{o}C. The enzyme activity significantly decreased at 80^{o}C and it was clearly observed that the enzyme lost its full activity at the temperature of 90^{o}C. From both thermal activity and thermal stability profiles, it can be speculated that crude enzyme extract prepared from *Geobacillus sp.* AGP-04 can be used with any temperature from 20 to 70^{o}C in a biotechnological applications. These characteristics are superior to those of other notable esterases or lipases from *Bacillus thermoleovorans* ID-1 lipase (**Lee *et al.* 1999**) and *Bacillus sp.* esterase (**Burcu *et al.* 2006**). The thermostable esterases from soil thermophilic bacterial strains were also reported to be stable in the ratio of 98% at the end of 1 hour incubation period at 70^{o}C when compared with its original activity (**Kademi *et al.* 1999**). The extracted crude enzyme also showed pronounced pH stability at a very wide range of pH (4.0-10.0). Fojan et al reported that the esterase has a pH optimum at approximately 6.0 and lipases show maximum activity around 8.0 (**Fojan *et al.*** 2000). But in this study the isolated esterase have the pH optimum at 8.0. The optimum pH values for extracellular esterases were found different among bacteria (**Jung *et al.* 2003**). The stability of the enzyme in alkaline and neutral pH suggests its usefulness in industrial applications. The pH stability profile of thermophilic *Bacillus sp*. esterase showed that enzyme retained almost its original activity when it was incubated at pH 4.0-8.0 up to 10 hours at room temperature, but a decline of enzyme activity at the end of 24 hours incubation period (**Burcu *et al.* 2006**). Therefore, this esterase is highly stable in a wide pH range with comparison to the previous one. It is known that the metal ions have important role to maintain the enzyme in active and stable structure by binding to amino acid residue with negative charge in specific sites (**Colak *et al.* 2005**). The enzyme activity was significantly induced by the presence of K^+ and Na^+ ions and repressed by the Hg^{2+} and Zn^{2+}. The inhibition of enzyme activity with Hg^{2+} was also supported this result (**Kademi *et al.* 2000a**). From the results it can be suggested that the enzyme is K^+ and Na^+ dependent and this metal ions may be served as prosthetic group for the enzyme activity. The inhibition of esterase activity in the presence of EDTA can be attributed to its metal chelating effect. However, dithiothreitol (DTT) and 2- mercaptoethanol as reducing agent of disulfide bridges were also stimulated enzyme activity. It can be concluded from this result that -SH groups were essential for PNPB hydrolysis. The little inhibition of esterase activity by PMSF may be attributed to the presence of serine residues responsible for the hydrolytic reaction in the active site since this inhibitor is known to interact selectively and irreversibly with the serine hydroxyl groups. Among the surfactants tested SDS, Tween 20 and Tween 80 have stimulatory effect; whereas CTAB, Triton X-100 and Benzokonium chloride have inhibitory effect on esterase activity. It may be that as Tween 20 and Tween 80 act as mild surfactants and they break protein-lipid, lipid-lipid but not protein-protein interactions and they do not denature enzymes. Therefore, enzymes became more solubilized and remained in their native and active form. So the said surfactants increase freely available enzyme and therefore enzyme activity increases. On the other hand CTAB and Benzokonium chloride itself have the toxic effect to the enzyme as a result the enzyme inhibition occurred. Among the commercial detergents, Ariel considerably enhanced the enzyme activity, whereas the enzyme retained its 70% or more activity in presence of commercial detergents like Rin, Sunlight, Surf Excel Quick Wash, Surf Excel Blue and Tide. Only Ezee showed significant inhibitory effect on esterase. This is probably due to the composition of the commercial detergents. The extracted crude enzyme is considerably active and stable in organic solvent. The activity of *Geobacillus* AGP-04 esterase in glycerol was higher than the activity measured for *Bacillus atrophaeus* SB-2 and *B. licheniformis* SB-3 lipases in the same concentration of the solvent (10% and 30% residual activity, respectively) (**Bradoo *et al.* 1999**). And its stability was similar to that of the extremely stable esterase Est of *P. calidifontis*. However, *Geobacillus* AGP-04 esterase was much more active in 50% methanol, ethanol, and 2-propanol than the last one (**Hotta *et al.* 2002**) activity. The crude enzyme showed low Km and high Vmax value when pNPB was used as substrate. Substrate saturation curves for pNPB indicated that the *Geobacillus sp.* AGP-04 esterase follows simple Michaelis-Menten kinetics. This lower value of Km indicates a great affinity of the enzyme for pNP-butyrate. This Km value was lower than the esterase from the thermophilic *Bacillus* strain G18A7 which had a 0.1 mM Km value when pNPB was used as substrate (**Owusu *et al.* 1991**). It was reported that the Km values of most industrial enzymes are varied in the range of 10^{-1} to 10^{-5} M when acting on biotechnologically important substrates (**Fullbrook. 1996**). The environmental variables such as pH and temperature for the optimum enzyme production in the production medium were determined as pH 8.0 and 60 ^{0}C. From the result it is suggested that the isolated organism is an alkalophile and thus produce maximum esterase enzyme at the alkaline pH range, and the organism produced maximum esterase at the temperature of 60^{o}C. At lower and higher incubation temperature than 60^{o}C yielded a lower enzyme production. This phenomenon is considerably important for industrial purposes. As this organism produced esterase enzyme at alkaline pH and higher temperature, thus made this organism as an industrially important source of said enzyme. By the use of carbon source like starch and cellulose in the production medium augmented the enzyme production. This is a novel characteristic of the isolated bacteria. If organic waste is used as carbon source in the production medium this bacteria may be able to utilize the waste material as carbon source for the production of enzyme. Besides, natural oil also increased the enzyme production. In particular the organism preferred dual types of carbon source; one is starch and cellulose like polysaccharides whereas on the other hand natural oil substrates like Olive oil, Coconut oil, and Mustard oil. From the study it may be suggested that when the organism grown in simple sugar they did not require to express their esterase synthesis gene to conduct their metabolism but when the organism were grown in complex medium they need to switch on their esterase producing gene to fulfill their metabolism. As a result the fermentation medium contains high esterase enzyme. As carbon source as concern this dual character may make the organism as vital biotechnological tool for extracellular esterase production where the organism is able to use two types of carbon source for the said enzyme production efficiently.

CONCLUSION

In conclusion, a thermophilic bacterium *Geobacillus sp*. AGP-04 isolated from hot spring in Bakreshwar, West Bengal, India, secrets an extracellular hydrolase responsible for the degradation of some para-nitrophenyl esters. The extracellular crude preparation was investigated in terms of pH and temperature optima, substrate specificity, organic solvent and surfactant tolerance capacity, pH and thermal stability and kinetic parameters. It was found that that *Geobacillus sp.* AGP-04 produces a novel thermostable, organic solvent tolerant extracellular esterase, and the enzyme was highly stable at a broad range of pH including alkaline scale. The stimulation of enzyme activity in the presence of DTT and mercaptoethanol, and inhibitory effect of Hg^{2+} shows that –SH groups in specific sites are required. The enzyme production was significantly induced if fermentation medium was supplemented with polysaccharides and oily substrate as carbon source. Further study is needed to purify the enzyme and molecular characterization of the purified enzyme.

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