JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2012. Vol. **6**(4), p. 1759-1773

Molecular Characterization of Industrially Viable Extreme Thermostable Novel α-amylase of *Geobacillus* sp. Iso5 Isolated from Geothermal Spring

D.M. Gurumurthy and S.E. Neelagund*

Department of PG studies and Research in Biochemistry, Jnana Sahyadri, Kuvempu University, Shankaraghatta, Karnataka, India.

(Received: 06 April 2012; accepted: 12 May 2012)

A thermotolerant alkali-resistant gram positive, rod shaped *Geobacillus* sp.Iso5 was isolated from the thermal water. Identification was performed by biochemical tests and 16S rRNA gene sequencing. The isolate comprises of 55%±0.2 mol % G+C content and iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$ type of cellular fatty acids. Phylogenetic analysis revealed 98-99% sequence similarities was observed with thermophilic members of group *Geobacillus*; *G.kaustophilus* (99.5%), *G. thermoleoverance* (99%). A hyperthermostable α-amylase was purified using Sephadex G-150 gel filtration chromatography and a DEAE-cellulose column. The SDS-PAGE pattern of purified enzyme was showed an apparent molecular weight of 43 kDa. The MALDI-TOF/TOF mass finger printing analysis of purified enzyme has a 31% close homology with the surface layer protein of *Geobacillus* sp. The CD and FTIR spectra for secondary structure of enzyme revealed about 55% α –helix, 5% β-strand and 40% of unordered structure. The optimum activity of enzyme was determined to be 90°C and pH 8.0 and can stable up to 90°C for 10 min. The DSC-TGA thermogram of purified enzyme was showed only 10% weight loss at 200°C. The effect of EDTA and Zn^{2+} on enzyme activity was shown maximum inhibitory activity. Whereas, Ca^{2+}, Mg^{2+}, Fe^{2+} and Cu^{2+} were did not affect on the activity of the purified enzyme.

Key words: Thermal springs, *Geobacillus* sp. Iso5; 16S rRNA, α- amylase; MALDI-TOF/TOF, DSC-TGA, Circular Dichroism, FTIR.

The universal ancestor thermophiles, among the most studies of the extremophiles are diversified in wider geographical areas. These include hydrothermal vents, deep marine trench, thermal springs and permafrost [1, 2]. Thermophile, from these ecological niches gains potential interest in adaptability and applicability in industrial process condition. The stabilization at higher range of temperature with its biological system and molecules was developed and evolved during early primordial days of the earth [3]. Isolation of these groups of bacteria has its own importance in thermostable proteins, enzymes, herbicide metabolism, quorum quenching and some other industrial applications [4]. The thermophilic bacterial group, which is driven by the demand for biocatalysts, found to be convenient in commercial uses. The unique properties of these biocatalysts resulted in several novel applicability in industrial process [5]. In particular, the synthesis of polymers, agro based products and pharmaceuticals [6, 7]. The stability of these enzymes was developed by the internal and environmental factors [8]. Despite of

* To whom all correspondence should be addressed.
Mob.: +91-9448234456;
E-mail: neelgund@gmail.com

other synthetic chemical catalyst, these biocatalysts offer with fewer reaction steps, improved solubility of substrate, reduced risk of microbial contamination, lower viscosity rates and thrive in high pH or low water concentration [9, 10]. The thermophilic member *Geobacillus* sp. have greatly convinced over other mesophilic members. The bacteria of this genus with growth temperatures ranging from 35 to 78 °C are widespread in various geographical areas on earth[11-13]. More than 96.5% of 16S rRNA gene sequence similirities of *Geobacillus* were embraced in the 5th genetic group [11]. To date, more than 10 species of genus is available [12]. Members of the genus contain iso-branched saturated fatty acids like iso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$ as the major fatty acids. The polymer degrading enzymes were the most extensive studied thermozymes from *Geobacillus*; these include amylases, pullulanases, xylanases and proteases.

α- amylases (endo-1, 4 α- D –glucan glucohydrolase; E.C. 3.2.1.1) families covers more than 20 different ECs. They belongs to the glycoside hydrolases in the system they form their own families 13, 14 and 15, respectively. The endo-fashion hydrolyses of α- 1, 4 linkages in starch resulting in the liberation of α-anomeric products with linear and branched oligosaccharides like maltose, glucose and limit dextrin [14, 15]. There are two types of α- amylases; starch hydrolyzing and starch modifying. They have wider applications in conversion of starch to fermentable sugars in brewing industries; launder the cloth in laundry industries, in paper industries for sizing and in the preparation of sweet syrup in food industries. Other various applicability of this enzymes accounts for about 30% of the world enzyme production [16].

In this paper, we report a novel thermophilic *Geobacillus* sp. Iso5 was isolated from the thermal water from the geothermal spring. Further, a hyperthermostable α- amylases was purified and characterized by conventional biochemical methods.

MATERIALS AND METHODS

Sampling site

A moderate alkaline type of natural Irde (12°48´N; 75°13´E) thermal spring is located on the bank of river Siri near Puttur taluk, Dakshina Kannada District, Karnataka state, India. Like other thermal springs on the West coast, this spring was grouped under west coast geothermal provenance. Based on the seasonal variation in temperature of the fluids, the average temperature falls between 40 to 60°C [17]. The thermal water sample was collected in December 2008. The temperature and the pH of the thermal water was recorded immediately after sampling.

Strain isolation

The PBTA media (Peptone, 0.2%, Beef extract, 0.5% Tryptone, 0.1% NaCl, 2% agar) containing diluted thermal water sample was used for enumeration of thermophilic bacterial colonies. The plates were incubated at 60°C for 24 hours. The amylase production of the isolates was determined by sub cultured in SYPB media (2% starch, 1% yeast extract, 0.1% peptone, 0.1% beef extract, 0.05% $MgSO_4$, 0.04% $CaCl_2$ and 2% agar). The pH of the medium was adjusted to 7.0 and incubated at 60°C for 24 hours. A clear zone around the colonies after flooding with 1% iodine solution indicates the amylase activity of the isolates. Among the bacteria showing high amylase activity, the starin Iso 5 was selected for the amylase production.

Morphological and physiological characterization

The morphology of strain was determined under an Olympus binocular stereo microscope (Olympus, Japan). Growth and biochemical studies were performed according to Bergey's Manual of Systematic Bacteriology [18]. The optimum growth range of the isolate was determined on the PBTA medium containing 1% starch and then incubating at temperature range 40 to 90°C. The ability to grow at 1%–10% NaCl concentration was observed in T_1N_1 liquid media (1 % Bacto tryptone containing the appropriate amount of NaCl). The physiological characteristic of isolate was determined by conducting Voges-Proskauer test, lysozyme broth, and fermentation of D-Cellobiose, D- xylose, D-Galactose, Glycerol, Inositol, and D-Lactose. The hydrolysis of starch, gelatin, casein and utilization of citrate, acetate were also determined.

G+C content

The DNA was isolated and G+C content of the isolate was determined using RP-HPLC as described in the previous methods [19, 20, 21].

Fatty Acid Methyl Ester (FAME) analysis

The fatty acids were extracted and analyzed by Sherlock microbial identification system (MIDI Inc, USA) using *Bacillus thermocatenulatus* as standard [10].

16S rRNA gene sequencing and phylogeny

Genomic DNA was isolated according to the manufacturer protocol of DNA extraction kit (Aristogene, Bangalore, India). Two universal primer selected for PCR amplification were; Reverse primer 5'-ACGGCTACCTTGTTACGACTT and forword primer 5'-AGAGTTTGATCCTGGCTCGA were used to amplify 10 to 12 ng of DNA template. The amplified 1.5 kb product was purified by QIAquick gel extraction kit (Qiagen, Germany) and directly sequenced on ABI (Applied BioSystem, Sigma, Mumbai) automated sequencer as recommended by manufacturer. The partial 1493 bp 16S rRNA gene sequence was aligned with submitted sequences available in the NCBI database using clustalX software. The phylogenetic tree was constructed using neighbor-joining tree making algorithm of the MEGA 3.1.

Enzyme assay

The enzyme activity was determined after each successive step in the purification of α-amylase using glucose as standard[22-24]. One unit of α-amylase activity was defined as the amount of enzyme that release 1 µmol of reducing sugars per minute at 80°C at pH 7.0.

Estimation of protein

Protein estimation was performed using the standard procedure [25]. Bovine Serum Albumin (BSA) was used as standard protein.

Purification and characterization of α-amylase

The purification of α-amylase from isolate was carried out in the PBTA medium using starch as a sole carbon source. The optimal culture condition was 65°C at pH 8.0. The cultures were incubated for 2-3 days to yield high α- amylase production. Then the culture was centrifuged at 10000 X g for 10 min in 4°C. Solid ammonium sulfate was added to the culture supernatant to attain the 75% precipitation level and kept overnight at 4°C in refrigerator. The precipitated supernatant was again subjected to centrifugation at 15,000 X g for 20 min at 4°C and pellet was retained. The precipitate was dissolved in minimum volume of 10 mM potassium phosphate buffer pH 7.0. The dissolved protein was directly applied on Sephadex G-150 (Sigma, USA) in a 1X 40 cm column for desalting by gel filtration. The desalting column was previously equilibrated with 10 mM potassium phosphate buffer of pH 7.0 and the enzyme was eluted with the same buffer. The enzyme active fractions were collected and absorbance of the peak fraction was observed at 280 nm in UV-Visible spectrophotometer (Shimadzu, Japan). The active fractions containing enzyme were pooled and freeze-dried. The freeze-dried enzyme sample was further purified chromatographically on DEAE-Cellulose anion resin in 1 X 5 cm column (Genei, Bangalore, India) with the liner gradient of 0.1 to 1 M NaCl containing 10 mM potassium phosphate buffer pH 7.0. The active enzyme fraction was dialyzed in 10-kDa cutoff membrane dialysis bag and dialysate was concentrated by lyophilization.

Starch hydrolysis assay by TLC

Hydrolysis of 1% (w/v) soluble starch with purified amylase was performed according to the previously described method [26]. The starch, glucose and maltose were used as a standards for TLC. The hydrolysed product and the standards were analyzed on silica gel 60 (Merck, India) thin layer chromatography using butanol-acetic acid-water (3:2:1) as a solvent system. After spraying with 25% sulphuric acid in ethanol, the plates were kept in an oven at 100°C for one hour to visualize the spots.

Molecular weight Determination

The molecular weight of purified enzyme was determined using 12.5% Sodium Decodyl sulfite polyacrylamide gel electrophoresis (SDS-PAGE) [27].Protein bands were visualized by staining the gel with colloidal protein strainer. The apparent molecular weight of the purified enzyme was determined by comparing with Rf values of standard proteins; Bovine serum albumin (66 kDa), Ova albumin (43 kDa), Soybean Trypsin Inhibitor (20 kDa) and Lysozyme (14 kDa)

MALDI-TOF/TOF Peptide mass finger printing

In gel tryptic digestion of thermostable α- amylase in SDS-PAGE was performed to obtain peptide fragments using MALDI-TOF/TOF [Ultraflex TOF-TOF Bruker Daltonics, Bremen, Germany] equipped with nitrogen laser (337 nm). The enzyme digested samples were mixed with equal volume of saturated matrix solution of 2, 5-dihydroxybenzoic acid in 50% acetonitrile/H_2O with 0.1% trifluroacetic acid. This mixture (1 µl) was

spotted on a MALDI target plate, the spectra were recorded in the reflectron positive ion mode, and the spectral data were processed by Bruker Daltonics FLEX analysis software (version 2.0). The Peptide Mass Fingerprint (PMF) was determined by MALDI-TOF/TOF data and interpreted by reference to the Mascot database.

Effect of temperature and pH on purified α – amylase

The optimum temperature and pH of purified α –amylase enzyme was determined by its residual activity. The enzyme reaction mixture in the absence of $CaCl_2$ was incubated in the range of 40-100 °C in 0.1 M citric acid buffer for 30 min. The three different buffers [0.1 M sodium phosphate buffer (pH 7), 0.1 M citrate buffer (pH 8), and 0.1 M glycine-NaOH buffer (pH 9 - 12)] were used for the pH valve of 7 to 12 determine the optimum pH required for its activity.

Effect of EDTA and some metal ions on enzyme activity

The effect of metal ions on α-amylase activity was determined by pre incubating at a final concentration of 5 mM Zn^{2+},Fe^{2+},Ca^{2+},Mg^{2+},Cu^{2+} (Chloride form) and 10 mM EDTA at 65°C at pH 8.0 for 15 min. The enzyme activity was determined after incubation with the substrate for 30 min.

Thermo stability of α- amylase by DSC-TGA

Thermal stability and weight loss of α- amylase was performed and determined using SDT Q600 DSC-TGA (TA instruments, USA). Approximately 3 mg of sample was placed in standard 70 µl aluminium pans. The analysis was carried out over the temperature range from 40ºC to 200ºC at a rise in temperature of 10ºC/min. The flow rate flow rate of the gas was 20 ml/min. The weight loss is recorded as a function of temperature.

Circular Dichroism (CD)

Circular dichroism spectral analysis of the secondary structure of the enzyme was performed by the method previously described [28]. In brief, the protein concentration of 1gm/dl was scanned over a wavelength range of 190 to 250 nm with a data pitch of 0.2 nm, bandwidth of 1 nm, scanning speed of 100 nm.min^{-1} and a response time of 1 second using Jasco-710 spectropolarimeter (Jasco UK, Great Dunmow, UK) at 60°C. Ultrapure water was used as a reference. At a given wavelength, the resultant spectrum was expressed in molar ellipticity (θ) are (deg·cm^2/dmol) and the percentage of conformation evidence was analyzed by using web service tool http://www.ogic.ca/projects/k2d2/

Fourier Transform Infrared Spectroscopy (FTIR)

A Nicolet 380 FTIR spectrometer (Thermo Electron, USA) was used. The instrument used a diamond crystal to measure absorbencies from 30000cm^{-1}-200cm^{-1}, although this study only examined the mid-infrared region of 4000cm^{-1}-1000cm^{-1} where the structural bonds appear. Each result was an average of 32 scans. A subtraction from a pure water standard was made for all scans.

RESULTS

Isolation and characterization of α-amylase producing isolates

Morphologically, the isolate was gram positive, rod shaped *Bacillus*, approximately 1.3-1.5 µm in width and 3-6 µm in length and having an irregular margin with central swelling. The characterization of amylase producing isolate was observed maximum growth at temperature 85-90°C and pH 9.0. The isolate was found to be stable at 10% NaCl. The physicochemical characteristics of the isolate was observed The strain utilized acetate, lactate, citrate, starch and casein, but not gelatin. The strain was also able to produce acids from glycerol, cellobiose, galactose, xylose, but not from inositol and lactose. The methyl red and the urea decomposition tests were negative [Table 1]. The DNA G+C base composition of the strain was 55%±0.2 mol %. The fatty acid profile of isolate was largely consists of iso-C15: 0, iso-C16: 0 and iso-C17: 0, which is relevant to the genus *Geobacillus*. The phylogenetic tree of the partial 16S rRNA gene sequence of length 1493 bp was constructed using the neighbor- joining method. The tree pattern attributed the genetic similarities among *G. thermoleovorans* (99%), *G. kaustophiles* (99.5%) and *G.* sp N60 (99%) [Fig.1]. The partial 16 s rRNA gene sequence was deposited in NCBI as *Geobacillus* sp. Iso5 with the accession number GQ140232. Based on the results of the phenotypic and genotypic analyses, we conclude that strain belongs to the member of *Geobacillus* and the starin isolate number was 5; for which it is designated as *Geobacillus* sp. Iso5.

Purification of α- amylase

α- amylase was purified by using

Sephadex G-150 Desalting column [Fig. 2] and DEAE-Cellulose anion- exchange chromatography [Fig. 3]. After 80 % ammonium sulfate precipitation of the extract, the specific activity of α- amylase during desalting steps was observed to be 372 U/mg, 33.85-fold purity with the total yield of 33.3%. Further, purification was achieved using Ion exchange chromatography yielded only one major protein peak. The purity of the enzyme was enhanced upto 49.54 fold with a 24.88 % yield. The specific activity after ion exchange chromatography was determined to be 545 U/mg. The detailed chromatographic steps and purification factors is summarized in the table 2. The catalytic type of mechanism (α or β type amylase) in TLC was based on the Rf values of standards and product relased. It was concluded that, the purified enzyme was α-amylase [Fig 4].

Table 1. Comparative physiological characteristic of thermophilic *Geobacillus* sp. Iso5 isolated from Irde thermal spring with reference strains. Detailed description of characteristics features of Taxa, 1. *Geobacillus* sp. Iso5; 2. G. *Thermoleovorans* (DSM 5366^T); 3, G. *Kaustophilus* (DSM 7263^T)

Characteristic	1	2	3
Cell width (μm)	1.3–1.5	1.5	0.9
Cell length (μm)	3.0–6.0	3.5	3.0
Motility	No	ND	ND
Production of acids from			
Cellobiose	+	+	+
Galactose	+	+	V
Glycerol	+	ND	ND
Inositol	-	-	+
Lactose	-	-	-
D-xylose	+	V	+
Hydrolytic property			
Casein	+	+	+
Gelatin	-	-	+
Starch	+	+	ND
Utilization of Acetate	+	+	+
Lactate	-	+	-
Citrate	+	+	+
Voges-Proskauer reaction	-	-	-
NaCl Stability	0–10%	ND	5%
pH	8.0	6.2-7.5	6.0-8.0
Temperature(°C)	45-90	45-70	37-68
G+C (mol %)	55	52-58	51-55

*Characteristics are describes as, '+' as positive, '-' as negative, 'V' as variable and 'ND' as not determined in the available literature

Table 2. Purification steps involved in the *Geobacillus* sp. Iso5 α -amylase

Purification	Volume (ml) (U/ml)	Activity (mg/ml)	Protein (U/mg)	Specific activity	Fold purity	Yield (%)
Crude	100	482	43.2	11	1.0	100
Desalting Column (Sephadex G-150)	5	160	0.42	372	33.85	33.3
DEAE-Cellulose	2	120	0.22	545	49.54	24.80

Characterization of gb5 α- amylase

Molecular weight and MALDI-TOF/TOF peptide mass fingerprinting

The molecular weight of purified α-amylase was determined by comparison with Rf values of standards in 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A single protein band was observed which corresponds to the molecular weight of 43 kDa [Fig. 5]. The aminoacid sequence from the fragments of gel tryptic digest for MALDI-TOF/TOF peptide mass fingerprinting with sequence intensity coverage of 62.2% and sequence coverage of 12.6 % was shown 31% identity with the S- Layer glycoprotein of *Geobacillus kaustophilus* HTA426 [Fig. 6]. The possible amino acid sequences to individual peptide mass fragments and matched amino acid sequence with S- Layer glycoprotein of *Geobacillus kaustophilus* HTA426 were described in Table 3a & 3b.

Table 3(a-b). (a); MALDI TOF MS/MS peptide mass fragments showing possible aminoacid sequence of hyperthermostable α- amylase. (b); Yellow color indicates the matched amino acid sequence of α- amylase with surface layer glycoprotein of *Geobacillus* sp. HTA426

Mass Mr	Dev.	Range	P Sequence
809.453	0.050	174-180	VYGQSTR
1092.552	0.008	337-346	FVVNDAPFK
1289.565-	0.006	491-502	AMDASTFAGYTR
1334.695	0.012	225-236	AALDQVNQYVSK
1361.682-	0.012	781-974	DCQYALNINNIK
1466.726-	0.015	62-74	LPNISDYYAAYNK
1604.798-	0.018	781-794	DGQYALNINNIK
1614.769-	0.027	663-677	VGNDVVQDLSTQANR
1621.746-	0.027	447-460	DYANNFLQPNPTTK
1649.777-	0.028	248-263	AAQDANAAYEAALPFK
2646.310-	0.055	248-273	AAQDANAAYEAALPFKVESVAVVNAK

Table 3(b).

MDKKKAVKLATASAVAASAFVAANPHTSQAATDVATVVSQAKAQMKQAYYTYSHTVTETGKLPN
ISDVYAAYNKAKQAYANAVAVVNKAGGAKKDAYLADLQATYETYVFKANPKSGEARVATYIDAYNYATKLD
KTRQELKAAVDAKDLKKAEELYHKISYELKTRTVILDRVYGQSTRELLRSQFKAEAQKLRDSLIYDIT
VAMKAREAQDAVKAGNLDKAKAALDQVNQYVSKVTDAFKAELQKAAQDANAAYEAALPFKVESVAVVNA
KQIEIKFNKAVDRSTASNKNNYKIQKSSDSSASTLATLDGSADISWSDDGKTVTITTTSGIINKFGV
VNDAPFKFIVENIKDKYGKTVDSYSTNLVVKDTVAPTLKEVKATAKSTTTKVTLVFSEPVQASGAIAYVGG
QAASVQDGSNPNEIILTTAQALEAGKTYDLTLLNFKDYANNFLQPNPTTKSFTVSSDAVAPTVQDVKVVRD
NLIEVTFDKAMDASTFAGYTRVLDLNGNPQGGTITATIKSGTAGKTVRLALGSAVPFNDSGVFSGTLVLGNGIK
DVNGNAKSATTHSITLTKDTARPTVAGASYVAAGGQYAGNTYANGAIVLKFNEDVAKIGSD
FRLITAGGEDVTSRLNAGGVAVNNDNNTEVIIPLNSSLSAGTYTLRVGNDVVQDLSTQANRNAAAVTTVTVG
ASSDSSKPVVNEGSVSAVAATSQTSGTTISLTMTDNVGLDLATVQDVNNYLLNGKPLPSGSYVTIAHGS
GSSSSAATNITVTLNIPAKSITKDGQYALNINNIKDKAGNIADPKVANVRLNDDVSPELKTATISSNGLLVL
GFSESVNSVGATTPSDFQFVVNGVEVATTSNGNIVQFNDGTGTDAGKYVVTFKAKVDQGADNNVST
TADNRLYLDVNGNNAFDSGTDILVQTGTTKAVGDVTLDVNLLSALKVKVVNNAAVKDQSTLQNPIQVGTTITVK

Table 4. The effects of EDTA and metal ions on the activity α- amylase

Effectors	Concentration	Residual Activity (%)
Control	None	100
EDTA	5mM	54
$CaCl_2$	5mM	90
$ZnCl_2$	5mM	55
$MgCl_2$	5mM	82
$FeCl_2$	5mM	86
$CuCl_2$	5mM	77

Effect of Temperature and thermal stability

The optimum temperature required for α-amylase active was 90°C [Fig. 7] and activity was

Fig. 1. Phylogenetic position of *Geobacillus* sp. Iso 5 among the same genetic group 5 *Geobacillus* with validly described species of the *Bacillus*. The phylogenetic tree was inferred by using neighbour-joining methods. MEGA 3.1 was used for analysis. Number nodes represent the percentage of boot strap valve obtained from 1 000 sampling. Bar 0.01 nucleotide substitutions per site with *E.coli* were used as an out-group. The well known members of both genus include, *Geobacillus* sp. N60; G. *kaustophilus* HTA 426; G. *thermoleoverance* DMS3; *Thermus aquaticus; Bacillus caldotex* BCRC 1196

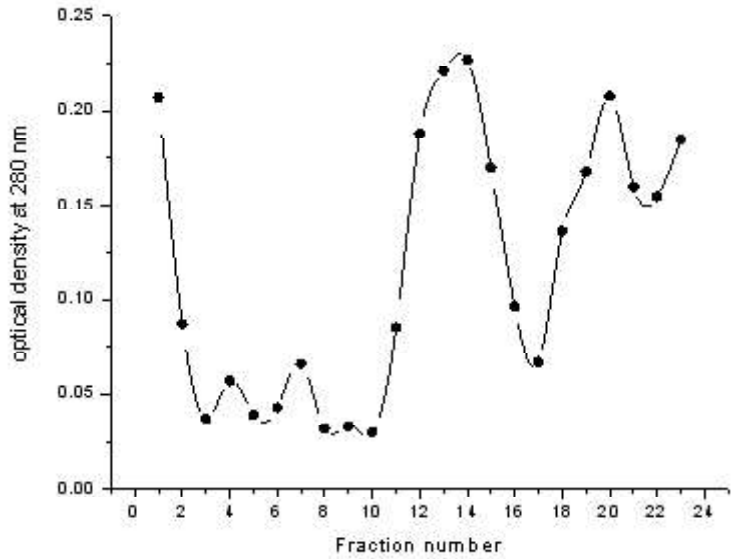

Fig. 2. Sephadex G-150 gel filtration and desalting elution of ammonium sulphate precipitated culture supernatant of *Geobacillus* sp. Iso5 containing α -amylase

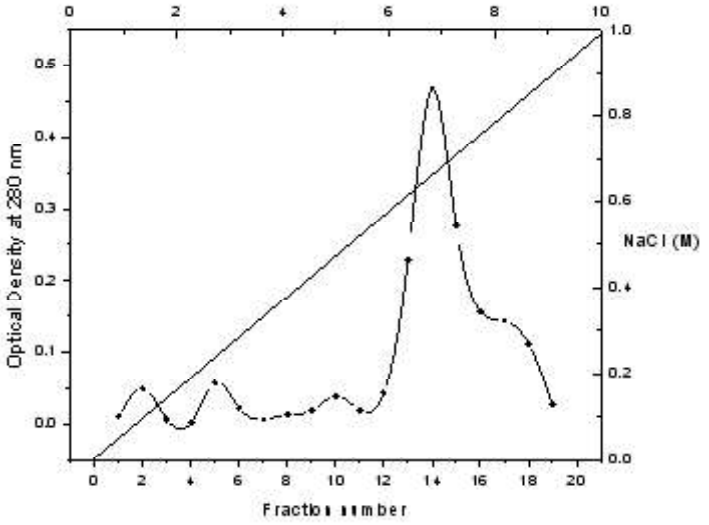

Fig. 3. DEAE-Cellulose anion exchange chromatographic purification of *Geobacillus* sp Iso5 α-amylase using 0.1 to 1.0 M NaCl in phosphate buffer

Fig. 4. Thin layer chromatography of enzyme hydrolysis products from *Geobacillus* sp. Iso5. Lane 1: soluble starch 1% (w/v);Lane 2: 10 µL of Glucose 1% (w/v); Lane 3: 10 µL of enzyme substrate reaction product; Lane 4: 10 µL of Maltose 1 % (w/v).

stable up to 90°C for 10 min at pH 8.0. To determine the accurate thermal stability, the α- amylase was further analyzed by DSC-TGA. It was revealed that, higher thermal stability even at the temperature of 200°C with only 10% weight loss at constant heat flow. In typical DSC-TGA curve, the initial decaling in the 3.5 mg weight of enzyme was occurred at the temperature at 35°C. A sharp endothermic peak was observed at the temperature of 70°C due to the initial evaporation of water from the residue. During this only about 4% of weight loss was observed. At the temperature of 80°C to140°C, the Tm of enzyme appeared to be stable with the loss of only 1% weight. However, increasing the temperature, the weight residues started to decline sharply at 200°C due to the exothermal degradation of enzyme [Fig. 8]. This indicates the thermal degradation (Tm) of enzyme was extremely high and thermal stability of the enzyme was between 80°C to 140°C.

Effect of pH and metal ions

Keeping the temperature optimum at 90°C, the enzyme activity was measured at different pH values and the data indicates that the pH optimum was 8.0 [Fig. 9]. At the temperature of 90°C and pH 8.0, the effect of EDTA on residual activity of α- amylase was shown to be 54% inhibiton. However, the residual activity of the enzyme was also inhibited by metal ions like, Cu^{2+} and Zn^{2+} showed 77% and 55% respectively. Whereas, other metal ions such as Ca^{2+}, Mg^{2+}, Fe^{2+}

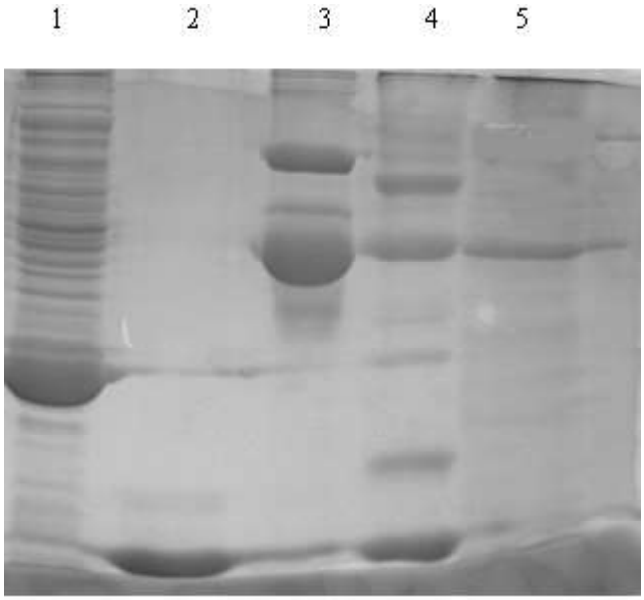

Fig. 5. Polyacrylamide gel electrophoresis analysis (12%) of the *Geobacillus* sp. Iso5 α- amylase. Lane 1: crude enzyme extract; 2: crude enzyme treated with 75 % ammonium sulfate precipitatation; 3: amylase active fraction from Sephadex G-150 column; 4: standards molecular weight markers from 94 to 14 kDa. Markers as indicated consisted of Bovine serum albumin (66 kDa), Ova albumin (43 kDa), Soya bean Trypsin inhibitor (20 kDa) and Lysozyme (14 kDa); 5: Final purified amylase fractions eluted from DEAE-Cellulose anion exchange chromatography

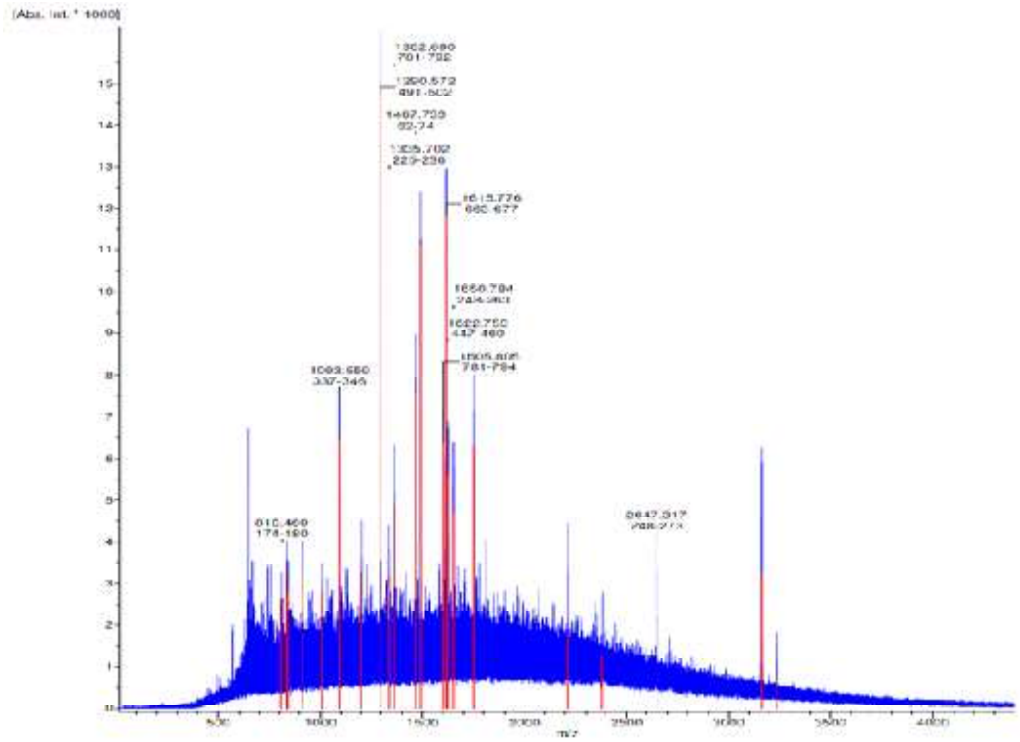

Fig. 6. InGel tryptic digest MALDI-TOF/TOF peptide mass fingerprinting spectra of purified α- amylase

did not affect much on the activity. The effect of EDTA and other metal ion on the residual activity on α-amylase was shown in Table 4.

Secondary structure analysis

The secondary structure of α- amylase was deduced by Circular Dichromism spectroscopy and Fourier Transform Infrared spectroscopy respectively. The structural deconvolution of Far-UV CD spectrum of the enzyme, revealed the presence higher order secondary structure with half of the conserved domain. The α- helical content of the native enzyme was calculated to be 55%. The β -strand and unordered and random coil was estimated to be 5% and 40% in the enzyme native form [Fig. 10].

Because of the uncertainty in the structural variations, the enzyme was further investigated by Fourier Transform Infrared spectroscopy at optimum pH 8.0. The determination of the secondary structure was based on the vibrational bands of the protein. In particular, the amide I band (1600–1700 cm^{-1}), which

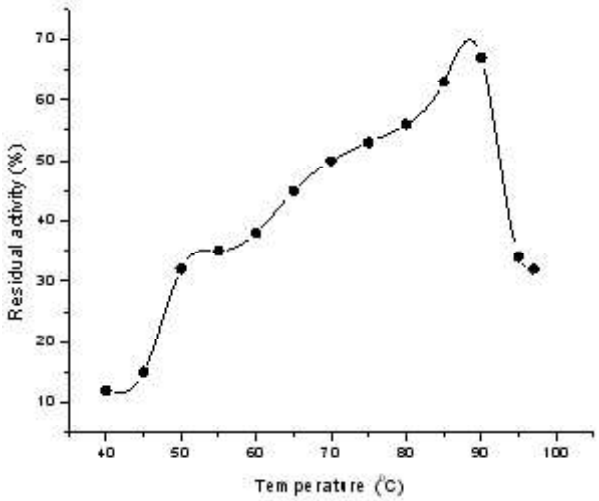

Fig. 7. The effect of temperature on the activity *Geobacillus* sp. Iso5 α- amylase after the incubation of the reaction mixture without $CaCl_2$ in 0.1 M citric acid buffer for 30 min at pH 8.0.

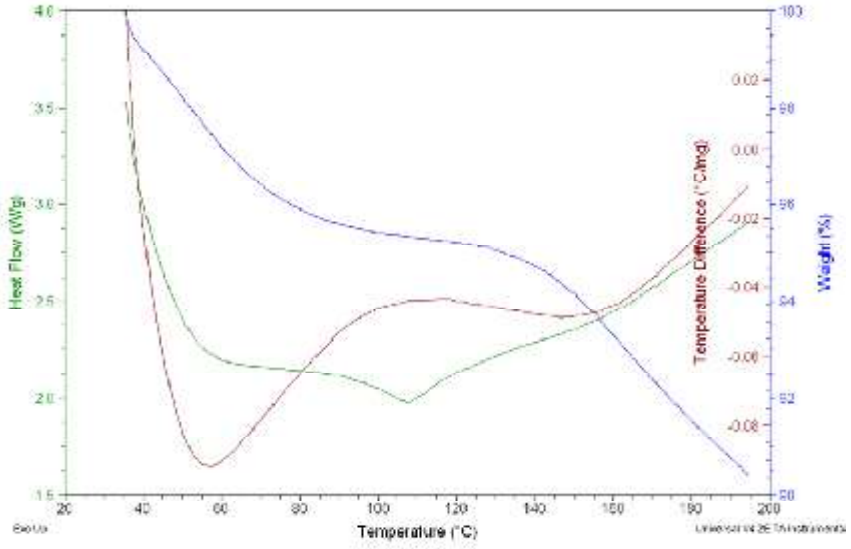

Fig. 8. DSC-TGA thermogram of purified α- amylase

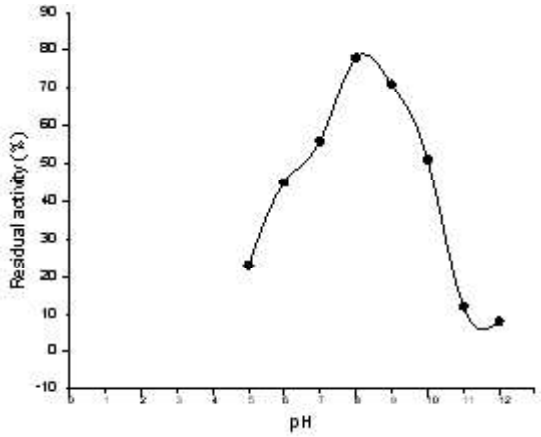

Fig. 9. The effect of pH on α- amylase activity was determined by the three different of buffers system [0.1 M sodium phosphate buffer (pH 7), 0.1 M citrate buffer (pH 8), and 0.1 M glycine-NaOH buffer (pH 9 - 12)] were used for the pH valve 7 to 12

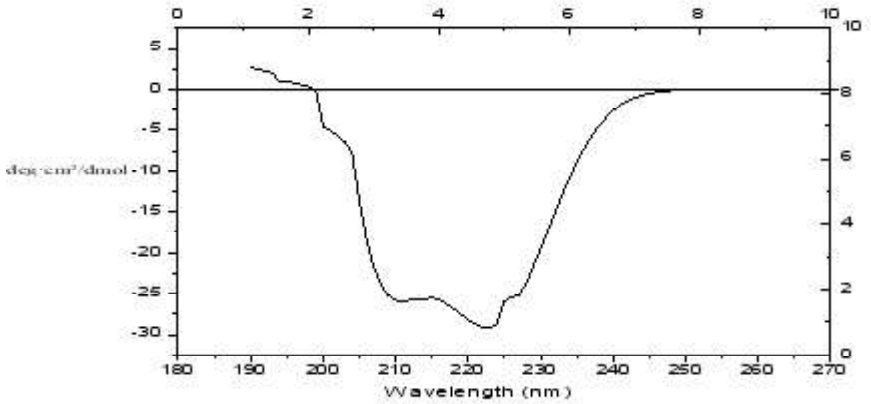

Fig. 10. Circular dichromism (CD) spectrum of the *Gb-5 α-* amylase. The enzyme was analyzed at a concentration of 0.68 mg/ml, in KH,PO, at pH 8.0

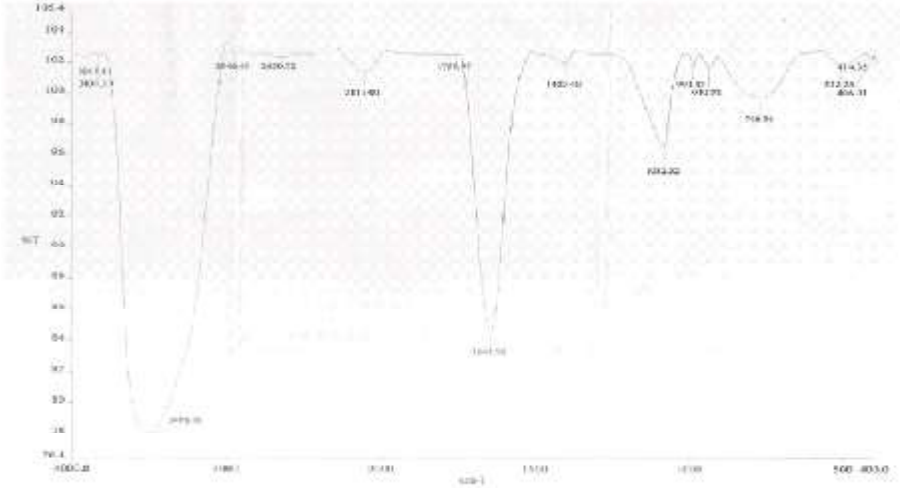

Fig. 11. Fourier transformer Infrared Spectroscopy of purified α- amylase at pH 8.0

is sensitive to the secondary structure. In FT-IR analysis, the characteristic amide I band arises principally from the C=O stretching vibration of the peptide group at 1,641.97 cm^{-1}, which is attributed to the lower shift in the normal α- helical frequency range 1645–1660 cm^{-1} for water soluble protein [Fig. 11]. However, N–H deformation at 1,403 cm^{-1} for amide II has been contributed by shift in the form of a loss of absorbance at 1550 cm^{-1} to monitor ^{1}H to ^{2}H exchange in proteins for β-strands. There was weak amide III frequency for random structure was observed in the spectrum, due to the amide III absorption is normally very weak in the infrared spectroscopy, arising primarily from N–H bending and C–N stretching vibrations and conformational shift in the protein bonds at 1230-1300 cm^{-1} for amide III frequency. Form these spectral datas, it was revelaed that, α- amylase from *Geobacillus* sp. Iso5 in its native form contain largest portion of helical content and random structure.

DISCUSSION

In this study, the hyper thermostable α-amylase producing bacterium was isolated from hot water samples. The phenotypic and genotypic characterization of the isolate was identified as the member genus *Geobacillus*. Further, a 43 kDa α-amylase was purified from the culture supernatant using sephadex G-150 gel filtration chromatography and DEAE-cellulose anion exchange chromatography. The optimum activity of purified α- amylase was showed at 90°C in pH 8.0. The enzyme was stable up to 90°C for 10 min at pH 8.0. However, the DSC-TGA thermogram of enzyme was revealed only 10% weight loss at 200°C which was higher than previosuly reported α- amylases.

The isolation and characterization of thermophilic *Geobacillus* spp. from thermal springs was previously reported [11, 12]. The morphology and physiology of the isolate was shown close resemblance with the genus *Geobacillus* group [18]. The growth temperature of *Geobacillus* sp. Iso5 was 90°C, which slightly varies with the other type strains. [12] The pH of 8.0, was close to moderate alkaline resistance like strains *G. kautophilus* [29]. The similarities in the iso-branched saturated fatty acids iso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{17:0}$ of *G.* sp. iso5 with *G. thermoleoverance* making up to 61% of total cellular fatty acids. [11] Although, G+C content of the *G*. Iso5 was 55 mol%. Whereas, *G. zalihae* TI [T] was 52 mol %, *G. Kautophilus* 51-55 mol% and *G. Thermoleoverance is* 52-58 mol% [7, 30]. The 16S rRNA gene sequence of the isolate was shown close homology with *G. kaustophilus* (99.5%) and *G. thermoleoverance* (99%). The phylogenetically coherent groups of thermophilic *Geobacillus* with sequence homology valve of 96.0% -99.4% are embrased in fifth genetic group of *Geobacillus*[11,12,31].

The purification and characterization of thermostable enzymes from *Geobacillus* sp. was previously described[10,32]. Two steps chromatographic purification of *Geobacillus* sp. Iso-5 α-amylases was shown the molecular weight of 43 kDa. Although, Ca^{2+} independent, high maltose forming and 28 kDa α- amylase from *G. thermoleoverance* was purified and shown relatively enhanced in optimum temperature of 100°C and enzymes stability at pH 8.0 [32]. *Geobacillus* sp. IIPTN produces 97 kDa high molecular weight α- amylase was isolated and characterized[33]. Many other *Bacillus* species like *B. brevis* produces high molecular weight α-amylases (205 kDa) [34]. However, internal sequencing by the peptide mass finger printing of 43 kDa amylase was shown to be unique and has a 31% close homology with previously described surface layer proteins of *Geobacillus* sp.

Industrial processing of starch into glucose, maltose and oligosaccharide syrup involves two steps, liquefaction and saccharification. These two steps are involved by the utilization of high temperature up to 100^{0}C in improved starch processing Thus, the involvement of high temperature resistance hyperactive amylase from the newer species can cope the industrial processing[35]. The α- amylases from *B. licheniformis* (100°C), *B. stearothermophilus* (70–80°C), *B. amyloliquefaciens* (70°C), and *B. stearothermophilus* (70°C) are typically used in industries were inactivated at temperature beyond 105°C [36]. Hence, the hyper thermostability of α-amylase will greatly influence the industrial processing condition. The temeprature stability which is useful in protein engineering was characterized by the certain amino acid residues which originally adapted by the other members of the genus [7, 34, 35]. Few hyperthermostable amylases

are avaliable to catalyze at 100°C in acedic pH. Like *Thermococcus profundus* with optimal activity at 80°C, pH of 4.0–5.0, *Pyrodictium abyssi* with optimal activity at 100°C, pH 5.0 and *Staphylothermus marinus* optimal activity at 100°C, pH 5.0 [32]. The characteristic behavior of enzymes in DSC-TGA was described previously[37-42]. The major goal of the DSC-TGA experiments was to measure the thermodynamic parameters associated with the temperature-induced denaturation of α-amylase in the absence of Ca^{2+}. The analysis was restricted up to 200°C because; the thermal stability of biopolymer above 100^0C is quite unstable and irreversible[35, 38, 39].

Most of the amylases used in industry are reported to be metal ions dependent like, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}. The hyperthermostability of metaloenzymes like amylase requires Ca^{2+} for its compact structural stability. It has been reported that α-amylases specifically bind to Ca^{2+}. The α-amylase catalytic site is located in a cleft between two domains (an $[\alpha/\beta]_8$ barrel and a large loop). Coordinated by ligands belonging to these two domains, Ca^{2+} is essential for the enzyme's catalytic activity and thermostability. However, in recent investigation, Ca^{2+} independent α- amylase was reported [32, 43]. In case Gb 5 α- amylase, the activity was influenced by the absence of Ca^{2+}. The influence of other metal ions like Mg^{2+}, Mn^{2+} and Fe^{3+} was shown profound enhancing the amylase activity. The effects of EDTA and Zn^{2+} largely influenced in the decreased activity of the enzymes and inhibiting its thermostability [14].

From the structural point of view, the secondary structure of enzymes was determined by far-UV circular dichromism and FTIR spectroscopy[28, 32, 44, 45]. The purified gb5 α-amylase was shown 55% α–helix, 5% β-strand and 40% of unordered structure. However, the α-amylase from *G. thermoleoverance* NT60 was reported to contain 25% α-helices, 21% β-sheets, and 54% random coils and showed similarity with that reported for *Bacillus* spp.[32, 46]. In the presence of urea both the α-helix as well as β- sheet conformational changes in the protein could be responsible for a decline in the α-amylase activity. The helical content of α-amylase in the absence of CTAB is 50%, while in the presence of low concentration of CTAB [L/P¼2) it increases about 7%. Then, by more increase in the concentration of CTAB (molar ratio of L/P, 5to15), the α-helix decreases strongly. In the molar ratio of 15, the a-helix content decreases to 16% and the random coil increases to 45%. This behavior shows a significant deformation of a-amylase in the high concentration of CTAB[47]. In addition, the FTIR spectrum of the purified α-amylases represents the covering of largest proportion of α -helical content of amide I region. However, the β-strand for N–H deformation at 1,403 cm^{-1} for amide II has been contributed by shift in the form of a loss of absorbance at 1550 cm^{-1} [45].

CONCLUSION

There is need of α-amylases, which can operate above 100°C at alkaline pH values for improved bioprocessing. The isolated novel Iso5 α-amylase can efficiently work up to 140°C at pH 8.0 to 9.0. The high-temperature stability, alkaline pH, and structural integrity of α-amylase revealed that this enzyme has the potential to fit into industrial requirements.

REFERENCES

1. Dejan, B.S., Oliver, O.F., Aleksandra, V.D.C., Kristina, O.C., Tamara, I.D., Zorica, B.S. Extremophiles-Link between Earth and Astrobiology. *Proc. Nat. Sci. Matica. Srpska. Novi. Sad.*, 2008; **114**: 5-16
2. Jonathan, D.T. Extremophiles in Astrobiology: Per Ardua ad Astra. *Gravit. Space. Biol. Bull.,* 2000; **13**: 5-11.
3. Karl, O.S. Hyperthermophiles in the history of life. *Phil. Trans. Roy. Soc. Biol. Sci.*, 2006; 361**:** 1837-1843.
4. Marieke, E.B., Anja, E.M.J., Remko, M.B. Thermozymes and their applications. *Appl. Biochem. Biotechnol.*, 2001; **90**: 155-186
5. Bertus, V.D.B. Extremophiles as a source for novel enzymes. *Curr. Opin. Microbiol.*, 2003; **6:** 213-218
6. David, C.D., Francisco, M.V., Constance, S.C. Enzymes from Extremophiles. *Curr. Opin. Chem. Biol.*, 2001; **5:** 144-151
7. Joseph, G., Walter, S. The Biocatalytic potential of extremophiles and extremozymes. *Food. Technol. Biotechnol.*, 2004; **42**: 223-235
8. David, W.H., Michael, J.D. Extremozymes. *Curr. Opin. Chem. Biol.*, 1999; **3**: 39-46
9. Pernilla, T., Gashaw, M., Eva, N.K. Potential and utilization of thermophiles and thermostable

enzymes in biorefining. *Microb. Cell. Fact.*, 2007; **6**:1-23

10. Raja, N.Z.R.A.R., Thean, C.L., Abu, B.S, Mahiran, B. *Geobacillus zalihae* sp. nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. *BMC Microbiol*., 2007; **7**:77
11. Nazina, T.N., Tourova, T.P., Poltaraus, A.B., et al. Taxonomic study of aerobic thermophilic *Bacilli*: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius* and *G. thermodenitrificans*. *Int. J. Syst. Evol. Microbiol.*, 2001; **51**: 433–446.
12. Nazina, T.N., Tourova, T.P., Poltaraus, A.B., Novikova, E.V., Ivanova, A.E., Grigoryan, A.A., Lysenko, A.M., and Belyaev, S.S. Physiological and phylogenetic diversity of the thermophilic spore-forming hydrocarbon-oxidizing bacteria from oil fields. *Microbiology.*, 2000; **69:** 96–102
13. Tamara, N., Nazina., Elena, V.L., Andrei, B.P, Tatyana, P.T., Alexandre, A.G., Diyana, S.S., Anatoliy, M.L., George, A.O. *Geobacillus gargensis* sp. nov., a novel thermophile from a hot spring, and the reclassification of *Bacillus vulcani* as *Geobacillus vulcani* comb. nov. *Int. J. Syst .Bacteriol.*, 2004; **54**: 2019–2024
14. Swetha, S., Dhanya, G., Kesavan, M.N., Carlos, R.S., Ashok, P. α-amylases from Microbial Sources – An Overview on Recent Developments. *Food. Technol. Biotechnol.*, 2006; **44**: 173–184
15. Viera, H., Stefan, J., Ernest, S. Amylolytic enzymes: their specicities, origins and properties. *Biologia.*, 2000; **55:** 605-615
16. Ferus, G.P. Extracellular enzyme synthesis in the Genus *Bacillus*. *Bacteriol. Rev.*, 1977; **41**: 711-753
17. Sarolkar, P.B: Geochemical characters of hot Springs of West Coast, Maharashtra State, India. In: *Proceedings World Geothermal Congress*; Turkey, 2005; pp 24-29.
18. Sneath, P.H.A. Endospore forming gram positive rods and cocci. In Bergey's Manual of Systematic Bacteriology. (Hensyl, WM, ed) Baltimore : Williams & Wilkins Co, 1994; pp 1104–1139
19. Cashion, P., Holder, F.M.A., McCully, J., Franklin, M. A rapid method for the base ratio determination of bacterial DNA. *Anal. Biochem.*, 1977; **81**: 461-466
20. Yoon, J.H., Kim, H., Kim, S.B., Kim, H.J., Kim, W.Y., Lee, S.T., Goodfellow, M., Park, Y.H. Identification of *Saccharomonospora* strains by the use of genomic DNA fragments and rRNA gene probes. *Int. J. Syst. Bacteriol.*, 1996; **46**: 502-505
21. Tamaoka, J., Komagata, K. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS. Microbiol. Lett.*, 1984; **25**:125-128
22. Bernfeld, P. Amylases α and β. *Methods. Enzymol*., 1955; 1:149-158
23. *Miller, G.L.* Use of Dinitrosalicylic acid Reagent for Determination of Reducing Sugar. *Anal. Chem*., *1959;* **31:** 426-428
24. Shaw., Lin, J.F., Chen, S., Chen, H. Purification and properties of extracellular alpha amylase from *Thermos* sp. *Bot. Bull. Aca. Sin.*, 1995; **36**:195-200
25. Lowry, O.H., Rosen, R.N.J., Farr, A.L., Randall, R.J. Protein estimation with the Folin phenol reagent. *J. Biol. Chem.*, 1951; **193**: 265–75
26. Ashabil, A., Burhan, A., Hatice, K., Sadik, D., Omer, C. Highly Thermostable and Alkaline α-amylase from a Halotolerent Alkaliphilic *Bacillus* sp. AB68. *Brazl. J. Microbiol*., 2008; 39: 547-553
27. Laemmli. Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature.*, 1970; **227:** 680-685
28. Jianguo, L., Zhiqiang, Z., Hu, Z., Hongyue, D., Jianren, L., Zhanfeng, C. Isolation and characterization of á-amylase from marine *Pseudomonas* sp. K6-28-040. *African. J .Biotechnol.*, 2011; **10:** 2733-2740
29. Sunna, A., Tokajian, S., Burghardt, J., Rainey, F., Antranikian, G., Hashwa, F. Identification of *Bacillus kaustophilus, Bacillus thermocatenulatus* and *Bacillus* strain HSP as members of *Bacillus thermoleovorans*. *Syst. Appl. Microbiol.*, 1997; **20**: 232–237
30. Zarilla, K.A., Perry, J.J. *Bacillus thermoleovorans*, sp. nov., a species of obligately thermophilic hydrocarbon utilizing endosporeforming bacteria. *Syst. Appl. Microbiol.*, 1987; **9:** 258–264
31. Avanish, S., Anita, P., Yogesh, S.S., Bhavesh, K., Girish, K. Characterization and identification of *Geobacillus* spp. isolated from Soldhar hot spring site of Garhwal Himalaya, India. *J. Basic. Microbiol.*, 2009; **49**: 187–194

32. Uma, M.R.J.L., Satyanarayana, T. Biophysical and Biochemical Characterization of a Hyperthermostable and Ca^{2+}-independent α-amylase of an Extreme Thermophile *Geobacillus thermoleovorans*. *Appl. Biochem. Biotechnol*., 2008; **150:** 205–219
33. Pratibha, D., Sachin, K., Yogesh, K.J, Dilip, K.A. Characterization of hyperthermostable á-amylase from *Geobacillus* sp. IIPTN. *Appl. Microbiol. Biotechnol.*, 2010; **86**: 1857–1866
34. Roy, M.D., Michel, J.D., Robert, E., Charlkes, K.L., Michele, E.P. The effects of temperature on enzyme activity: New insights and their implications. *Extremophiles.,* 2008; **12:** 51-56
35. Claire, V., Gregory, J.Z. Hyperthermophilic enzymes: sources, uses and molecular Mechanisms for thermostability. *Microbiol. Mol. Biol. Rev.*, 2001; **65**: 1-43
36. Haki, G.D., Rakshit, S.K.. Developments in industrially important thermostable enzymes: a review. *Biores. Technol.*, 2003; **89:** 17–34
37. Conejero, L.F., Mateo, P.L., Aviles, F.X., Sanchez, R.J.M. Effect of Zn^{2+} on the thermal denaturation of carboxypeptidase B. *Biochemistry.*, 1991; **30**:2067- 2072
38. Freire, E. Thermal denaturation methods in the study of protein unfolding. *Methd. Enzymol.*, 1995; **259**:144-168
39. Hernandez, A.A., Rojo, D.A., Altamirano, M.M., Calcagno, M.L. Differential scanning calorimetry of the irreversible denaturation of *E. coli* glucosamine-6-phosphate deaminase denaturation of *E. coli* glucosamine-6-phosphate deaminase. *Biochemistry.*, 1993; **32**: 3644-3648
40. Sanchez, R.J.M. Theoretical analysis of Lumry-Eyring models in differential scanning calorimetry. *Biophy. J.*, 1992; **61**: 921-935
41. Sturtevant, J.M. Calorimetric studies of biopolymers. *Prot . Sci.,* 1996; **5**: 391-394
42. Thórólfsoon, M., Ibarra, M.B., Fojan, P., Petersen, S.B., Sanchez, R.J.M., Martínez, A. L-Phenylalanine binding and domain organization in human phenylalanine hydroxylase: A Differential Scanning Calorimetry study. *Biochemistry.*, 2002; **41**: 7573-7585
43. Malhotra, R., Noorvez, S.M., Sathyanarayana, T. Production and partial characterization of thermostable and calcium independent alpha amylase of an extreme thermophile *Bacillus thermoleoverance* NP54. *Lett. Appl. Microbiol.*, 2000; **31**: 378-384
44. Kelly, S.M., Price, N.C. Protein structure and molecular enzymology. *Biochim. et Biophy. Acta.*, 1997; **1338,** 161
45. Paule, C., Catherine, V., Jean, M.R., Charles, G., Georges, F. The precursor of a psychrophilic α-amylase: structural characterization and insights into cold adaptation. *Biochim et. Biophy. Acta* ., 2003; **1649**: 119–122
46. Brzozowski, A.M., Lawson, D.M., Turkenburg, J.P., Bisgaard, F. H., Svendsen, A., Borchert, T.V., et al. Structural analysis of a chimeric bacterial α-amylase. High resolution analysis of native and ligand complexes. *Biochemistry.*, 2000; **39**: 9099–9107
47. Omidyan, R., Kazemi, S.H., Bordbar, A.K., Zaynalpour, S. Spectroscopic study on the interaction of Bacillus subtilis α-amylase with cetyltrimethyl ammonium bromide. *J. Lumin.***,** 2011; **131**: 1229–1233