A Surfactant, Oxidant and Inhibitor Compatible Thermo-solvent-tolerant Amylase From a Novel Extrimophilic Bacillus Subtilis Strain Clb-34 Mk443366: Study on Purification and Characterization

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Research

Keywords: Bacillus subtilis, 16S rDNA, Solvent stability, Heavy metal resistance, Thermotolerant

Posted Date: September 4th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-63514/v1

Abstract

It is the first time when thermo-tolerant, heavy metal resistance amylase producing strain *Bacillus subtilis* isolated from soil sample. Amylase was purified 3.8-fold with a specific activity of 11,305.0 U mg^{-1}. The molecular weight of purified amylase was 67 kDa as revealed by SDS-PAGE and activity gel analysis. The amylase was active in broad pH and temperature range of 4.0-11.0 and 35-110°C, respectively, with maxima at pH 7.0 and 85°C temperature. The amylase has Km and Vmax value of 2.181 mg ml^{-1} and 909.09 µg $ml^{-1}min^{-1}$, respectively when starch used as substrate. The amylase was not only stable but also its activity enhanced in the presence of n-dodecane, iso-octane, n-decane, xylene, toluene, n-butanol, acetone, and cyclohexane, after prolonged incubation (7 days). Amylase activity and stability was inhibited in the presence of Hg^{2+}, benzene, sodium perborate. The unique property of solvent tolerance and heavy metal resistance proves the potential candidature of this isolate not only for starch liquefaction for food application but also for bioremediation strategies involved in environmental cleanup.

1. Introduction

In modern times, the products of biological origin, particularly enzymes, are attracting the attention of researchers. Their role in several biological and commercial processes has been duly emphasized. Among all the enzymes, α-amylases constitute a class of industrial enzyme having approximately 30% of the world enzyme production (Van der Maarel Marc et al. 2002) and represent one of the three largest groups of industrial enzymes and account for approximately 25% of total enzymes sales in the world (Rao et al. 1998) and are an important enzyme, particularly in the process of starch or glycogen hydrolysis.

The amylases can be derived from several sources such as plants, animals and microbes. The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics (Aiyer 2005; Vidyalakshmi et al. 2009). Based on their mode of action, they are further classified into three categories α-amylases, β-amylases and glucoamylases. All amylases are glycoside hydrolyser and act on α-1,4 glycosidic bonds (Maton et al. 1993). Industrially, α-amylase is used particularly in starch liquefaction, brewing, textile, pharmaceuticals, paper, detergents, drugs, toxic wastes removal and oil drilling (Ajayi and Fagade 2003). Since α-amylases are active over a broad pH (5–9) and temperature (35–105 ^{o}C) ranges, they are world wide center of attraction for researchers. Many microorganisms like fungi, yeast, bacteria and actinomycetes produce this enzyme; however, enzyme from fungal and bacterial sources has dominated applications in industrial sectors (Kim et al. 2012). However, bacterial amylases are preferred as they grow rapidly, need less space, can be easily maintained and are accessible for genetic manipulations. The important amylase producing bacteria are species of *Bacillus, Pseudomonas, Halomonas, Arthrobacter* and *Serratia.* Among the bacterial sources Bacillus sp., *Bacillus subtilis*, *B. staerothermophilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. acidocaldarius*, *Bifidobacterium*

bifidum, *Bacillus atrophaeus* and *B. macerans* (Aiyer 2005; Konsoula and Liakopoulou-Kyrikides 2006; Enhasy 2007; Kim et al. 2012; Simair et al. 2107; Abd-Elaziz et al 2020) play an important role in production of amylase. Several species of *Bacillus* are industrially employed to produce thermostable amylase as they grow easily under extreme pH and temperature conditions.

The application of an amylase in industrial reactions depends on its unique characteristics, such as its action pattern, substrate specificity, major reaction products, optimal temperature, and optimal pH (Yun et al. 2004). They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetraose syrup. In detergents production, they are applied to improve cleaning effect and are also used for starch de-sizing in textile industry (Chengyi et al. 1999). Generally production of this enzyme has been carried out by submerged fermentation (Enhasy 2007) because of the ease of sterilization and process control easier to engineer in these systems. The purpose this studies to isolate a novel thermoloterant amylase producing bacteria in the presence of organic solvent. Purification and characterization of amylase by bacteria was also performed in this study.

2. Materials And Methods

2.1. Materials

All analytical grade reagents and media components were purchased from Hi-Media (Bombay, India) and Merk (India). Column chromatography materials, protein ladder for electrophroresis, and starch were procured from Sigma-Aldrich Pvt. Ltd., USA.

2.2. Microorganism

Bacillus subtilis strain CLB-34 MK443366 used in this study was isolated from the soil sample and identified on the basis of phenotypic (16S r DNA) and biochemical tests. Analysis of 16S rDNA sequence (799 bp) revealed its 95.0% homology with *Bacillus subtilis* strains, and was designated as *Bacillus subtilis* CLB-34. The 16S rDNA sequence of *Bacillus subtilis* was submitted to Gene bank [MK: 443366] and a link to the dataset is https://www.ncbi.nlm.nih.gov/nuccore/MK443366 (All details are submitted in the form of Supporting Data). The strain CLB-34 was in the same cluster of phylogenetic tree (Fig. 1) with different strains of *Bacillus subtilis.* It was maintained on starch nutrient agar slants at 4 °C.

2.3. Crude enzyme preparation

One full loop of 24 h grown culture take from nutrient starch slant was transferred in 50 ml basal broth (2.0%, starch; 0.5%, peptone; 0.3%, beef extract; 0.5%, NaCl) and incubated at 55 °C for 24–48. To obtain crude enzyme, culture broth was transferred to micro-centrifuge tubes and centrifuged at 10000 rpm for 10 min. Cells were discarded and resultant supernatant was used as the crude enzyme for various enzyme assay.

2.4. Enzyme assay

The activity of α-amylase was assayed by measuring the reducing sugar released by reaction on starch. Amylase assay was done (Nelson 1944; Somogyi 1952) by using a reaction mixture consisting 500 µl of substrate solution (1.0% soluble starch in 1.0 M phosphate buffer pH 7.0), 100 µl of the enzyme solution and 1 ml volume make up by adding 400 µl distilled water. The reaction mixture was incubated for 10 min at 55 °C. Reaction was stopped by adding 1 ml of alkaline copper tartrate solution and incubated in boiling water bath for 10 min and cooled, then added arsenomolybdate solution for color stabilization. Optical density of each sample with reaction mixture was taken at 620 nm in a spectrophotometer (Shimadzu, Japan). One unit of enzyme activity was defined as the amount of enzyme that liberates 1.0 µg of glucose min/ml.

2.5. Extraction and Purification of Amylase

A three step purification method was used to purify the amylase produced by the *B. subtilis* CLB-34. All the purification steps were performed at temperatures between 0 and 4 °C unless otherwise stated.

2.5.1. Enzyme extraction

The crude culture supernatant obtained from 24 h old cultures of *B. subtilis* CLB-34 grown under optimal conditions was subjected to purification. The crude culture filtrate was subjected to a cooling centrifugation at 10,000 rpm to remove the cells and the residual medium. The resulted supernatant was used as crude enzyme extract.

2.5.2. Acetone precipitation

The cell free crude enzyme was saturated by the addition of different volumes (45%, 55%, 65% and 75%) of enzyme grade chilled acetone with gentle stirring on ice-bath. The mixture was left at 4 °C for 4 h and the precipitate was recovered by centrifugation at 10,000 g for 10 min. The supernatant was drained off and precipitate was kept at room temperature for few minutes to remove traces of acetone. The precipitate was dissolved 100 mM phosphate buffer (pH 7.0). The corresponding precipitates were recovered, dissolved individually in fresh buffer and assayed for both total protein content and amylase activity.

2.5.3. Ion exchange chromatography

An anion-exchanger (Q-sepharose) column (Sigma-Aldrich Pvt. Ltd., USA; 15 × 70 mm^2), pre-equilibrated with 100 mM phosphate buffer (pH 7.0) was used for further purification of the enzyme. The active fraction of acetone precipitates was suitably diluted (final volume 3 ml) with 100 Mm phosphate buffer (pH 7.0) prior to loading on column and the flow rate was adjusted to 20 ml/h. Afterthat, the diluted enzyme fraction was allowed to bind with matrix for 2 h at 4 °C. Then the unbound fraction was collected and analyzed for enzyme activity and for protein content. After collecting the loaded sample, column was washed with the same buffer until OD620 of the effluent was zero. The bound fractions were eluted with a linear gradient of NaCl (0.1–0.5 M, 10 ml each) in the same buffer.

2.5.4. Gel filtration chromatography on Sephadex G-75 column

The partially purified enzyme was applied to gel-filtration chromatography for purification up to homogeneity. The Sephadex–75 column (Sigma Aldrich Pvt. Ltd., USA, 1.5 × 40 cm) was equilibrated with sodium phosphate buffer (100 mM, pH 7.0) and 1 ml of concentrated sample was applied to the column. The flow rate was adjusted to 5–6 ml/h and fraction of 2 mL each was collected. Amylase activity and estimation of protein content were determined for each individual fraction.

2.6. Determination of protein concentrations

Quantitative estimation of protein content was done by the method of Lowry et al. (1951) using Bovine serum albumin (BSA) as standard and expressed as mg/ml. The protein content of individual fraction obtained after different steps of chromatography was monitored by measuring the extinction at 280 nm.

2.7. Polyacrylamide gel electrophoresis

The active fraction, with maximum specific activity, obtained after gel filtration chromatography along with crude, acetone precipitate and anion-exchange chromatography was electrophorezed by Sodium Dodecyl Sulphate–Poly Acrylamide Gel Electrophoresis in a 12.5% polyacrylamide gel according to the method of Laemmli (1970). Approximate molecular weight of the amylase was estimated by SDS-PAGE against the molecular mass markers i.e. lysozyme (14.3 kDa), β-lactoglobulin (20 kDa), Carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa) and phosphorylase B (97.4 kDa) (Sigma-Aldrich Pvt Ltd., USA) run with the samples.

2.8. Characterization of Purified Enzyme

2.8.1. Effect of temperature on enzyme activity and stability:

The influence of temperature on activity of amylase was studied by incubating the reaction mixture at different temperatures (35–110 °C). The enzyme was incubated at different temperatures 35–110 °C for 1 h to study the stability of the enzyme. The residual amylase activity was measured by conducting the reaction at temperature 55 °C and pH 7.0. The activity of the enzyme was considered as 100% under standard assay conditions.

2.8.2. Effect of pH on enzyme activity and stability:

The effect of pH on amylase activity was measured in the pH range of 4 to 11, using the appropriate buffers at concentration of 100 mM (4.0–6.0, sodium acetate; 6.0–8.0, sodium phosphate; 8.0–10.0, Tris-HCl; 9.0–11.0, glycine-NaOH) under standard assay conditions. To study stability as a function of pH, 100 µl of the purified enzyme was mixed with 100 µl of the buffer solutions and incubated at 55 °C for 1 h then aliquots of the mixture were taken to measure the residual amylase activity (%) under standard assay conditions.

2.8.3. Effect of metal ions on activity and stability

The effect of various metal ions (5 mM and 10 mM) on enzyme activity was investigated using $FeSO_4$, $CaCl_2$, KCl, NaCl, $MgCl_2$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, $HgCl_2$ and $NiCl_2$. The enzyme was incubated with different metals at 55 °C for 1 h to study metal ion stability and assayed under standard assay conditions.

2.8.4. Effect of organic solvent on amylase stability

Cell free supernatant having maximum amylase activity was filtered with nitrocellulose membrane (pore size 0.22 µm) and incubated with 30% (v/v) of different organic solvent viz., n-dodecane, n-decane, iso-octane, xylene, n-hexane, n-butanol, cyclohexane, acetone, toluene, benzene, ethanol, methanol and propanol for 1 week in screw crapped tubes at 55 °C and 120 rpm. The residual amylase activity was estimated against the control, in which solvent was not present.

2.8.5. Effect of inhibitors, surfactants, commercial detergents and oxidizing agents on enzyme stability

The amylase sample was incubated with inhibitors *viz*., ethylene diamine tetra acetic acid (EDTA), *β*-mercaptoethanol, Phenyl methyl sulphonyl flouride (PMSF) and urea (5 mM and 10 mM), surfactants *viz*., Triton-X-100, Tween-40, Tween-60, Tween-80, SDS (0.1 and 1.0%, v/v), commercial detergents *viz*., surf, aerial, ghari, henko and fena (0.1 and 1.0%, w/v), and oxidizing agents *viz*., H_2O_2 (0.1 and 1.0%, v/v), sodium perborate and sodium hypochlorite (0.1, 0.5 and 1.0%, v/v) for 1 h at 55 ºC and then the residual activity (%) was tested under standard assay conditions.

2.7.3. Substrate specificity

Substrate specificity of the purified enzyme was determined by assaying with different substrates (Soluble starch, Dextrin, Pullulan, α-cyclodextrin, β- cyclodextrin, γ-cyclodextrin, wheat starch, Potato starch, Rice starch) using 1% (w/v) concentration at pH 7.0 and 55 °C. The enzyme activity on soluble starch was defined as 100%, and the enzyme activities on other substrates were calculated as relative activities.

2.8.6. Kinetic analysis

The influence of substrate concentration on the reaction velocity of the purified amylase was studied with starch. The purified amylase was incubated with various concentration of starch. The final concentration ranged from 0.25-4.0 mg/ml. In all cases, the enzymatic activity was assayed under standard conditions. The Michaelis constant (K_m) and maximum velocity (V_{max}) was determined from Lineweaver-Burk plots of Michaelis-Menten equation.

The linear velocity data was plotted as the function of concentration of the substrate by linear transformation of the Michaelis–Menten equation and usual non-linear curve fitting of the Michaelis–Menten equation for the calculation of K_m and V_{max} of the reaction.

2.9. Enzymatic reaction product analysis

The purified enzyme was used at a dose of 0.5 U/mg soluble starch in 100 mM phosphate buffer (pH 7.0) at 55 °C and at different time intervals (2 h, 8 h and 12 h). Hydrolysis products were subjected to analyzed by High-performance liquid chromatography (HPLC) using a Hypersil NH_2 column at 50 °C using a 75% acetonitrile (V/V) was used as mobile phase at a flow rate of 1.0 ml/min and the hydrolysis products were detected using a RID-10A SHIMADZU refractive detector. Authentic chromatographic grade glucose, maltose, maltotriose, maltotetrose, and maltopentose were used as standards for identification of the hydrolysis products in the reaction mixture.

2.10. Statistical analysis

Each experiment with the required controls was performed in triplicate and the data are presented as the mean ± one standard deviation (SD). Significance of the differences between means was tested for by analysis of variance (ANOVA) and Duncan's multiple means tests (DMMT) on the parametric or arc-sine square root transformed data using the SPSS software, where a value of less than 0.05 was considered as significant.

3. Results And Discussion

3.1. Culture identification

The 16S rRNA gene sequencing has been by far the most common housekeeping genetic marker to study bacterial phylogeny and taxonomy attributed to (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel 2001). In the present investigation, the 16S rDNA sequence analysis revealed its maximum closeness with *Bacillus subtilis* hence the isolate was designated as *Bacillus subtilis* CLB-34. However, the 16S rDNA sequence analysis indicates that it is a different and novel strain of *Bacillus subtilis* (Fig. 1).

3.2. Purification of extra-cellular α-amylase

The crude enzyme extract was first concentrated by acetone precipitation. Maximum activity was observed in the fraction obtained by the addition of acetone in 50% with protein content of 25.67 mg/ml. This fraction had 11,305.0 U/mg of specific activity with recovery of 79.7% and with regard to purification it showed 3.8-fold purification (Table 1).

Table 1
Summary of purification of Amylase from *Bacillus subtilis*

Purification Steps	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Crude	3,63,890	123.57	2 ,944.80	100	1.0
Acetone	2,90,199.8	25.67	11,305.0	79.7	3.8
Q-Sepharose	2,48159.1	8.97	27,665.4	68.2	9.3
Sephadex G-75	98,496.5	0.99	99,491.4	27.1	33.8

The active fraction of acetone precipitation method was used for further purification by using ion exchange chromatography. Sample (1 ml) was loaded into the Q-Sepharose column pre-equilibrated with sodium phosphate buffer (100 mM, pH 7.0) and allowed to pass through the column. The unbound fraction was collected and analyzed for amylase activity and protein content. There was no amylase activity in the fraction, while 2.1 mg/ml of protein was estimated. The absence of enzyme in unbound fraction suggested that total amylase was bound to matrix. The bound enzyme was eluted by sodium phosphate buffer (100 mM, pH 7.0) having NaCl with increasing concentration at gradient of 0.1 M. Ten per ml solution of each concentration of NaCl was used to evade the bound enzyme. The amylase activity was detected in the fraction released by the addition of 0.5 M NaCl Anion-exchange chromatography of amylase on column resulted in one prominent peak at the 24th fraction (Fig. 2).

The active fraction was applied on Sephadex G-75 column. Figure 3 shows the fractionation pattern of amylase on Sephadex G-75 column. One distinctive protein peak was appeared that overlapped with the amylase activity. The purification process resulted in 33.8-fold purification factor and a final recovery of 27.1% of the enzyme with specific activity of 99,491.4 U/mg (Table 1). However, Mesbaha and Wiegelb (2014) reported the amylase was purified by a combination of 80% ethanol precipitation, ion exchange chromatography with Q sepharose and Superdex™ 75 gel filtration chromatography. The enzyme was purified 4.5 fold with 15.4% recovery and a specific activity of 250 units/mg protein

3.3. Electrophoretic analysis

The purity of the enzyme was confirmed by the presence of a single band on SDS-PAGE and its molecular weight was approximately 67 kDa (Fig. 4), which was similar to *Bacillus subtilis* and *Bacillus sp*. DR90 amylase (67 kDa) (Yandri Suhartati and Hadi 2010; Asoodeh et al 2013) but different from licheniformis Isolate AI20 amylase (53 kDa) (Abdulaal 2018).

3.2. Characterization of purified enzyme

3.2.1. Effect of temperature on enzyme activity and stability

In this experiment the amylase of *B. subtilis* CLB-34 was absolutely stable in the wide temperature range of 35–95 ^{o}C during 1 h incubation. The enzyme retained 98% activity even after treatment at 100 ^{o}C (Fig. 5). Similarly 100% activity at 90 ºC for 1 h for amylase from *Bacillus* sp. has been reported by Teodoro and Martins (2000). However, with further increase in every 5 ^{o}C temperature, there was a gradual decrease in enzyme stability ranging between 10–15% upto 110 ^{o}C. The amylase of *B. subtilis* CLB-34 retained 98, 88 and 75% activity even after treatment at 100, 105 and 110 ^{o}C, respectively (Fig. 5). The amylase of *Bacillus subtilis* CLB-34 is more thermostable than amylase studied by several other researchers. Arikan (2007) have reported a thermostable amylase stable up to 60–100 ^{o}C but retained only ~ 96% activity at 100 ^{o}C. Most other thermophile *Bacillus* amylases reported to so far, amylases exhibited higher temperature optimum for activity and showed good thermal stability (Dong et al. 1997; Horikoshi 1999). These are the properties considered to be very important for industrial starch liquefaction. Hence it is evident that the amylase of *Bacillus subtilis* CLB-34 is more thermostable, and may be applied to several biotechnological and industrial purposes.

3.2.2. Effect of pH on enzyme activity and stability

The pH stability on the purified amylase of *B. subtilis* CLB-34 was determined by measuring the enzyme activity at varying pH values ranging from 4.0–11.0 using different suitable buffers. Figure 5b showed that maximum amylase activity was established at pH 8.0, however it was found to be most stable at pH 7.0 (Fig. 6). The relative activities at pH 4.0, 4.5, 5.0 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were determined to be 48, 55, 67, 80, 95, 100, 115, 109 and 105%, respectively. At pH above 8.0, the amylase activity decreased rapidly. The amylase from *Bacillus subtilis* CLB-34 was stable in a range of pH 5.0-10.5 and at pH 11.0 approximately 65% of its activity was retained (Fig. 6). Amylases are generally stable over a wide range of pH from 4.0 to 11.0 (Fogarty and Kelly 1979). For most *Bacillus* sp. strains, the pH optima and stability of amylase has been reported to be the range of pH 6 .0 to 7.0 (Rajagopalan and Krishnan 2008; Mukherjee et al. 2009; Singh and Kumari 2016; Gbenga et al 2017).

3.2.3. Effect of metal ions on activity and stability

Results suggest that α-amylase of *B. subtilis* CLB-34 showed maximum relative activity (194%) and stability (160%) in the presence of Calcium ion (10 mM). *B. subtilis* CLB-34 α-amylases was activated by 10 mM Ca^{2+} but inhibited by all other metal ions to a variable extent. Abd-Elaziz et al. (2020) also found that thermostability of α-amylase from a *Bacillus* atrophaeus NRC1 isolated from honey was enhanced in the presence of 10 mM calcium. α-Amylases contain at least one Ca^{2+} ion and affinity of Ca^{2+} is much stronger than that of other ions (Abdulaal 2018) slight activity inhibition was observed by Ni^{2+}, Mn^{2+}, and Zn^{2+} (Table 2). A stronger inhibitory effect was observed in case of Cu^{2+} and Hg^{2+}. The same inhibitory effect by Hg^{+2} and Cu^{2+} had been reported also by *Bacillus cereus* (Annamalai et al. 2011), *Bacillus licheniformis* Isolate Al20 (Abdel-Fattah et al. 2012), *Bacillus atrophaeus* NRC1 (Abd-Elaziz et al. 2020). The inhibition by Hg^{2+} may indicate the importance of indole amino acid residues in enzyme function as has been demonstrated for other microbial α-amylases (Gupta et al. 2003). The inhibition of α-amylase

by Co^{2+}, Cu^{2+}, and Ba^{2+} ions could be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased metalloenzyme activity (Leveque et al. 2000).

Table 2
Effect of metal ions on enzyme activity and stability

Metal ions	Concentration (mM)	Residual activity (%)	
		Activity	Stability
Control		100.0	100.0
$CaCl_2$	5	166.5	135.2
	10	194.4	160.3
$NiCl_2$	5	124.5	92.2
	10	89.3	78.5
$FeSO_4$	5	125.9	110.8
	10	112.2	88.7
$MgCl_2$	5	145.9	115.6
	10	135.1	82.3
$CuSO_4$	5	89.5	84.7
	10	56.9	48.8
$HgCl_2$	5	48	50.4
	10	28	30
$MnCl_2$	5	78.6	113.9
	10	62.1	51.7
KCl	5	124.2	106.4
	10	121.1	92.1
NaCl	5	127.8	101.4
	10	121.4	100.3
$ZnSO_4$	5	98	78
	10	67	56

Enzyme activity was determined at 55 °C in the presence of metal ions in the reaction mixture directly and for stability enzyme was pre-incubated with different metal ions at 55 °C for 1 h and assayed as standard assay method. The enzyme activity without incubation with metal ions was taken as 100%. Mean standard deviation for all the values is < ± 5.0%.

3.2.4. Effect of organic solvents on amylase stability

In another experiment, the effect of various organic solvents (30%, v/v) on amylase stability was also investigated for one week, and the results are depicted in Table 3. The amylase of *Bacillus subtilis* is extraordinarily stable in the presence of all organic solvents under study. It was observed that except benzene, propanol and ethanol, presence of other solvents (n-dodecane, iso-octane, n-decane, xylene, toluene, n-haxane, n-butanol, acetone, methanol and cyclohexane) enhanced the amylase activity (Table 3). After incubation with n-dodecane, iso-octane, n-decane, xylene, Toluene, n-haxane, n-butanol, Acetone, Methanol, and cyclohexane the amylase activity increased to 252.3, 180.1, 188.8, 123, 190.2, 164.3, 115.2, 137.2 and 130%, respectively. The presence of benzene, ethanol and propanol marginally reduced the amylase with residual activities of 77, 88.3 and 85.4%, respectively (Table 3). Mesbaha and Wiegel (2014) also reported that amylase of *Amphibacillus* sp. NM-Ra2 showed enhanced activity in the presence of organic solvents (25%, v/v) of log P value reduced the protease activity by 23–38%. It is therefore; evident from our study that amylase of *Bacillus subtilis* is remarkably stable in the presence of broad range hydrophilic as well as hydrophobic organic solvents employed in this study.

Table 3
Stability of crude Amylase in presence of various organic solvents

Organic solvents (30%)	log *P*	Residual activity (%)							
		1 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Butanol	-0.80	122.7	164.3	148.2	135.4	127.6	120.3	115	105
Methanol	-0.76	103.4	135.1	137.2	128.3	120.6	115.4	108.3	99.3
Iso-propanol	-0.28	91.2	102.3	99.3	93.4	92.5	91.4	87.3	85.4
Ethanol	-0.24	99.1	108.6	107.5	102.6	99.5	95.4	91.2	88.3
Acetone	-0.23	105.1	115.2	109.6	103.1	100	98.2	95.3	90.4
Benzene	2.13	100	120	99.2	95.1	90.3	87.2	81.3	77.1
Toluene	2.5	121.9	190.2	160.1	138.1	130.8	122.4	115.6	109.3
Iso-octane	2.9	130	180.1	145.5	140.5	133.4	130.3	125.7	110.2
Xylene	3.1	91.4	117.1	123.4	117.4	110.2	104.1	99.5	94.6
Cyclohexane	3.3	90.6	112.5	130.4	109.2	100.2	99.5	90.2	87.4
Hexane	3.6	133.8	152.7	143.5	137.5	122.3	123.6	113.7	103.8
n-decane	5.6	145.3	171.3	188.8	168.3	146.2	121.7	110.1	99.0
n-dodecane	6.0	151.8	180.2	252.3	178.1	169.9	140.2	118.0	100.0

Enzyme was pre-incubated with different organic solvents at a concentration of 30% (v/v) at 55 °C for different time period and assayed as standard assay method. The enzyme activity without incubation with organic solvent was taken as 100%. Mean standard deviation for all the values is < ± 5.0%.

3.2.5. Effect of inhibitors on enzyme stability

When the *Bacillus subtilis* amylase enzyme was incubated with EDTA, PMSF, Urea and β-mercaptoethanol, the enzyme activity was retained at 96.3%, 94%, 99.4%, and 95.6% of the original activity at 10 mM (Table 4). Similarly, Kim et al. (2012) reported that Bacillus sp. AAH-31 retained approximately 85% activity in the presence of 0.1% (w/v) EDTA, while Abd-Elaziz et al. (2020) reported 28% activity with 5 mM. EDTA generally shows non-competitive inhibition of amylase activity and a slight inhibition showed us it is a metallo-enzyme. *Bacillus subtilis* CLB-34 α-amylase has also slight inhibition by 3.3% with 10 mM EDTA. It was reported that amylases from alkaliphilic *Bacillus* strains were not inhibited by 10 mM EDTA at 40 ºC but was completely inactivated by 8 M urea (Horikoshi 1999). These results show that the *Bacillus subtilis* α-amylase is an alkaliphilic enzyme. *Bacillus subtilis* CLB-34 α-amylase has also slight inhibition by 6% with 10 mM PMSF. Similarly Lin et al. (1998) were also reported similar result (97% with 10 mM) while Abd-Elaziz et al. (2020) reported that *Bacillus* sp. showed 81.3% activity with 5 Mm PMSF. *Bacillus subtilis* CLB-34 amylase has also slight inhibition by 4.4% with 1.0% β-mercaptoethanol. Similarly Ozcan et al. (2010) reported that 1.0% β-mercaptoethanol inhibit only 5%

residual activity by alkanophilic *Bacillus* sp. DM-15. On contrary to our result, Anupama and Jayaraman (2011) reported only 48% relative enzyme activity at 2 mM with β-mercaptoethanol.

Table 4
Effect of different inhibitors, surfactants, commercial detergents and oxidizing agents on amylase stability

Surfactants	Concentration (%)	Residual activity (%)
Control		100.0
β–mercaptoethanol	0.1 (%)	109.6
	1.0 (%)	95.6
EDTA	5 mM	120
	10 mM	96.7
Urea	5 mM	119
	10 mM	99.4
PMSF	5 mM	105.4
	10 mM	94
Tween-40	0.1	110.0
	1.0	99.6
Tween-60	0.1	115.7
	1.0	99.7
Tween-80	0.1	106.4
	1.0	96.5
Triton-X-100	0.1	109
	1.0	97
SDS	0.1	120
	1.0	95
Ghari	0.1	97.1
	1.0	75.2
Ariel	0.1	94.7
	1.0	61.3

Enzyme was pre-incubated with different inhibitors, surfactants, commercial detergents and oxidizing agents at 55 °C for 1 h and assayed as standard assay method. The enzyme activity without incubation with inhibitor, surfactants, commercial detergents and oxidizing agents was taken as 100%. Mean standard deviation for all the values is < ± 5.0%.

Surfactants	Concentration (%)	Residual activity (%)
Surf Excel	0.1	91.6
	1.0	55.6
Fena	0.1	99.4
	1.0	78.4
Henko	0.1	99.2
	1.0	65.9
Sodium perborate	0.1 (%)	85.8
	0.5 (%)	66.8
	1.0 (%)	55.3
Sodium hypochlorite	0.1 (%)	121.9
	0.5 (%) 1.0 (%)	113.2
		98.4
H_2O_2	0.1 (%)	114.5
	1.0 (%)	89.2

Enzyme was pre-incubated with different inhibitors, surfactants, commercial detergents and oxidizing agents at 55 °C for 1 h and assayed as standard assay method. The enzyme activity without incubation with inhibitor, surfactants, commercial detergents and oxidizing agents was taken as 100%. Mean standard deviation for all the values is < ± 5.0%.

3.2.6. Effect of surfactant on enzyme stability

In order to have applications in detergent industries, amylase must be stable to various detergent ingredients, such as surfactants. As shown in Table 4 the enzyme was appreciably stable in the presence of non-ionic surfactants like Tween-40, Tween-60 Tween-80 and Tritone-100 and detergent SDS. However, these compounds slightly inhibited the amylase activity with 99.6, 99.7, 96.5, 97.0, 95.0% of residual activity at concentration 1.0% (v/v). Similarly, Kim et al. (2012) reported that, a highly thermostable and alkaline amylase was fully stable in 1% (w/v) of Tween 20, Tween 80, and Triton X-100, and 0.1% (w/v) of SDS. Our results was indicated that, *Bacillus subtilis* CLB-34 amylase enzyme highly stable with 1% SDS (95%). Abd-Elaziz et al. (2020) also stated that *Bacillus atrophaeus* NRC1 was resistant 87.5% to 5 mM SDS, additionally Lo et al. (2001) reported that SDS had no marked effect on enzyme activity ion as concentration 8%. This resistance, which is essential requirements, suggests that the enzyme may be used as an effective additive in detergents.

3.2.7. Effect of detergent agents on enzyme stability

The amylase was substantially stable with commercial detergents at lower concentration (0.1%, w/v). RG-09 showed 99.4, 99.2, 97.1, 94.7 and 91.6% of residual at concentration 0.1% (w/v) with fena, henko, ghari, ariel, surfexcel. However, these compounds inhibited the amylase activity at higher concentration (1.0%, w/v) (Table 4). Simair et al. (2017) also reported 69.73–79.33% residual activity with amylase from *Bacillus* sp. BCC 01–50 in the presence of commercial detergents. In another study, Bancerz and Ginalska (2007) reported that lipase from *Bjerkandera adusta* R59 showed sufficient compatibility with commercial detergents.

3.2.8. Effect of oxidizing agents on enzyme stability

Among the oxidizing agents tested the amylase activity enhanced in presence of sodium hypochlorite and H_2O_2 with residual activities 121.9 and 114.5% at concentration 0.1%, whereas, higher concentrations (0.5 and 1.0%) decreased the stability except sodium hypochlorite (113.2% residual activity at concentration 0.5%, w/v) (Table 4). Likewise, Wang et al. (2009) also reported that lipase from *B. cepacia* was highly stable in the presence of hydrogen peroxide, sodium hypochlorite and sodium perborate after 1 h. Mesbaha and Wiegelb (2014) also reported that H_2O_2 enhance the enzyme activity at the concentration of 1%. The stability profile of the amylase in the presence of detergents and oxidizing agents prove its potential application in the detergent formulations as these agents are the active components of house hold detergents (Hajji et al. 2007).

3.2.9. Substrate specificity

The ability to hydrolyze several carbohydrates is a criterion of amylase potency (Grebeshova et al. 1999), however, the affinity towards a carbohydrates (substrate) may vary with substrate to substrate and for a particular substrate with the source of enzyme. Substrate specificity is defined how different substrates compete for an enzyme and determined by value of kcat/Km (Perona and Craik 1995; Hedstrom 2002). To determine the affinity of the amylase towards different substrates, nine substrates have been used in the reaction mixture (Table 5). The purified enzyme was able to efficiently hydrolyze starch and dextrin with 100 and 82.3% relative activity. Similarly, Abd-Elaziz et al. (2020) also reported that purified amylase of *Bacillus atrophaeus* NRC1 showed 100% starch hydrolysis for substrate specificity experiment. Among cyclodextrins, purified enzyme could easily hydrolyze γ-cylodextrin but could not hydrolyze α and β-cyclodextrin. This might be due to the cyclical shape of cyclodextrin contributing to the configuration of substrate and making the substrate low accessibility to the active site of the enzyme, thus making enzyme catalysis difficult. The cyclical shape of cyclodextrin with longer chain showed weaker effect on the configuration of the molecule than other cyclodextrin with shorter chain (Xian et al. 2015). These results are consistent with the similar observation reported earlier (Hashim et al. 2005; Hassan et al. 2011; Xian et al. 2015). Purified enzyme showed very low activity toward pulullan with 15.6% relative activity (Table 4), maybe as enzyme favors α-1,4 glycosidic bond to α-1,6 glycosidic bond, and the plenty of α-1,6-D-glycosidic bonds contribute to the configuration of the substrate and makes enzyme catalysis difficult (Steyn and Pretorius 1995). Most other α-amylases showed lesser activity toward pullulan (Moreira et al. 2004), except for McAA (Han et al. 2013), which reported maximum activity for pullulan. Purified amylase

showed maximum relative activity for soluble starches from potato, wheat and rice was the substrate. Similarly, Xain et al., (2015) also reported similar finding by *Talaromyces pinophilus* 1–95.

Table 5
Substrate specificity of amylase

Substrate (1%)	Relative activity (%)
Soluble starch	100
Dextrin	82.3
Pullulan	15.6
α-cyclodextrin	ND
β- cyclodextrin	ND
γ-cyclodextrin	67.9
Potato starch	98.2
Wheat starch	96.2
Rice strach	95.1

ND = Not detectable. Substrate specificity was checked by assaying enzyme with 1% (w/v) of substrates in 100 mM Phosphate buffer (pH 7.0) at 55 °C. Mean standard deviation for all the values is < ± 5.0%.

3.2.10. Kinetic analysis

Kinetic analysis with starch revealed the K_m and V_{max} to be 2.181 mg/ml and 909.09 µg ml/min, respectively by Linweaver-Burk plot (Fig. 7). Similarly, Amid and Manap (2014) & Abd-Elaziz et al (2020) reported that purified amylase enzyme showed Km and Vmax value 2.7 mg/ml and 34.30 u/min/mg & 1.63 mg/ml and 526 µM/ml.

3.3. HPLC analysis of hydrolysis products of α-amylase

The end products of starch hydrolysis by the amylase of *Bacillus subtilis* CLB-34 were analyzed by HPLC. At an early stage (2 h), the hydrolysis products were maltose, maltotriose and maltotetraose, maltopentose and with a trace amount of glucose. As the incubation time prolonged, the amount of glucose, maltose and maltotriose increased, but the amount of maltotetraose and maltopentose decreased. After 8 hours of incubation, the amount of maltotetraose was hard to be detected, which was hydrolyzed to smaller one by the amylase of *Bacillus subtilis* CLB-34. The main products were glucose, maltose and maltotriose, the contents of the three were about 16.45%, 58.99% and 27.41%, respectively, at the time of 14 hours of incubation. However, during the whole process of cultivation, the soluble starch could not be completely hydrolyzed to the three main products by the α-amylase of *Bacillus subtilis* CLB-34, at the time of 14 hours of incubation, the degree of hydrolysis was about 85.67%.

4. Conclusion

It is the first instance when a thermo-tolerant amylase being reported from a thermo-tolerant solvent tolerant *Bacillus subtilis* isolate. The strain is unique with respect to several solvents tolerance, heavy metals, surfactant and inhibitor resistance, makes the isolate applicable under stressed conditions. Outstanding solvent stability of the amylase proves its possible application under anhydrous conditions and amylase liquefaction. The amylase activity in broad pH and temperature range of 4.0–11.0 and 35–110 °C clearly indicate the thermo-alkaline nature of this enzyme. This enzyme, which possesses unique properties, could be widely used in different types of industries, especially in food and biotechnological applications.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors consent to publish this manuscript.

Availability of data and materials

Name of the repository is NCBI (National Center for Biotechnology Information) where our data's were deposited and a link to the dataset DOI are https://www.ncbi.nlm.nih.gov/nucleotide/MK443365.1 and https://www.ncbi.nlm.nih.gov/nuccore/MN370035.1.

Competing interests

The author(s) declare that they have no competing interests.

Funding

This work was supported by the University Grant Commission Delhi, India [F-4-2/2006(BSR)/BL/17-18/0049]. The funding bodies played no role in the design of the study or in collection, analysis, or interpretation of data, or in writing the manuscript.

Authors' contributions

1. S. T. carried out the research work and drafted the manuscript.
2. R. G. has designed the experiment, contributed substantially to analysis and interpretation of data and have given final approval of the version to be published.

All authors read and approved the final manuscript.

Acknowledgement

Dr. D.S. Kothari PDF Fellow is greatly acknowledged by all Authors.

Supplementary information

The supporting data also include in this manuscript as a supporting file.

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Figures

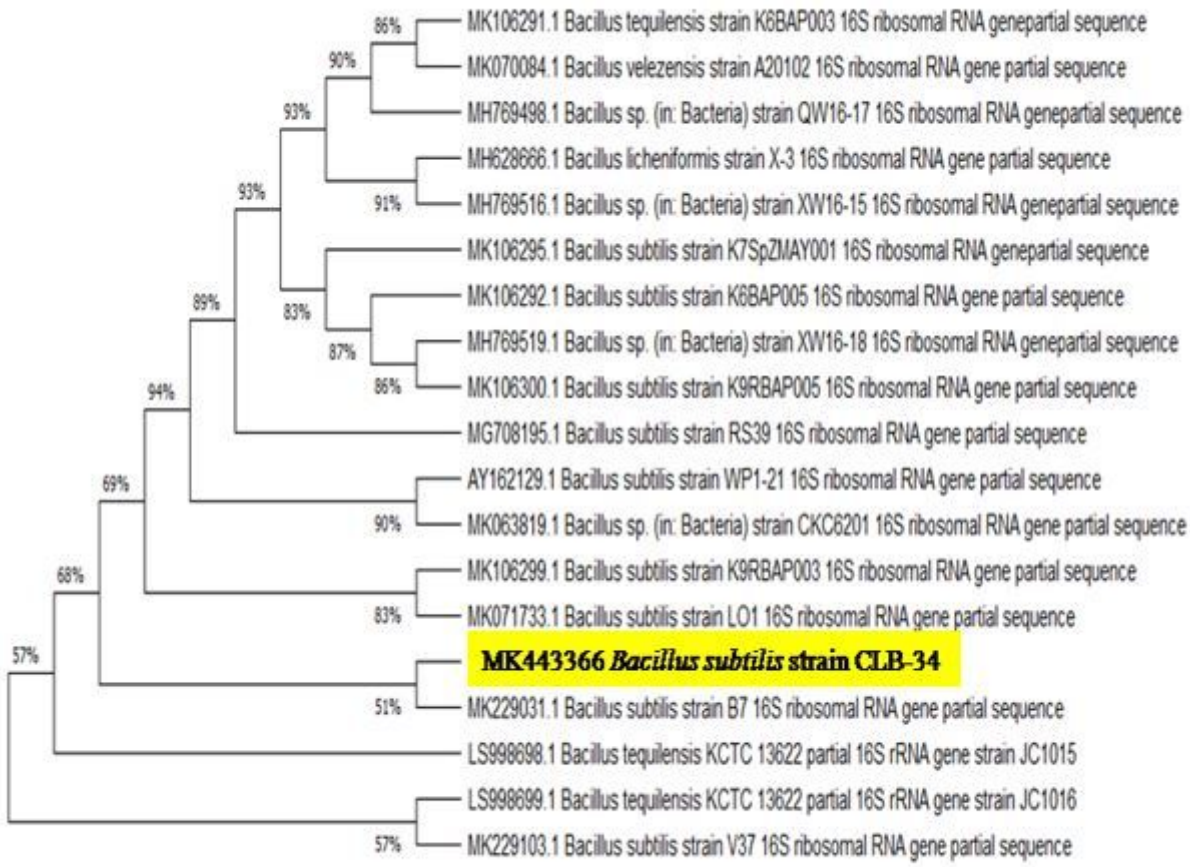

Figure 1

Phylogenetic tree showing relation between Strain CLB-34 and other Bacillus strains. In phylogenetic tree analysis, the strain was in the same cluster with different strains of Bacillus and showed only 95% homology to other Bacillus subtilis., so it could be stated that therefore it is different from reported Bacillus subtilis. The phylogenetic tree was drawn by MEGA 6 software using Neighbour-joining method and the significance of junctions was established using bootstrap method (1000 replicates).

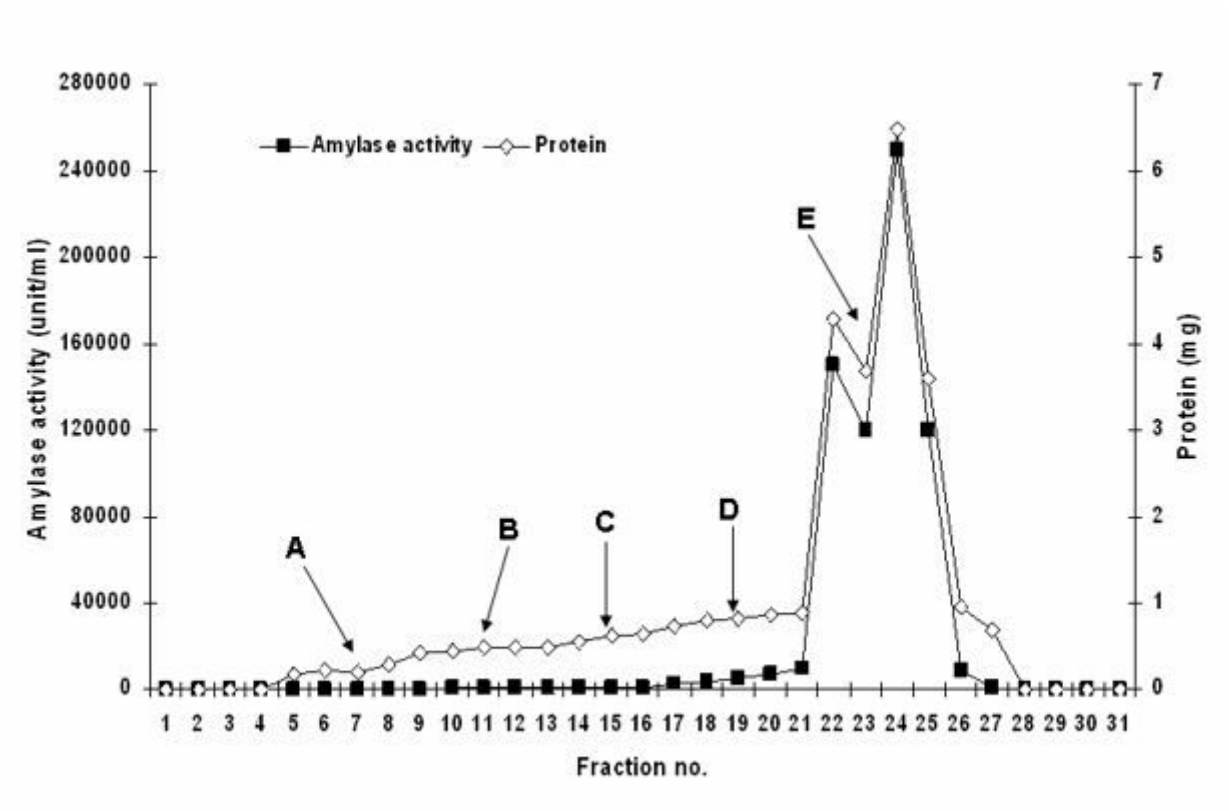

Figure 2

Chromatographic purification profile of extracellular amylase from Bacillus subtilis on anion-exchange column (Q-Sepharose) equilibrated with sodium phosphate buffer (100 mM, pH 7.0). The amylase was eluted with a gradient of sodium chloride (0.1 M-0.5 M) in sodium phosphate buffer (100 mM, pH 7.0). A-0.1 M, B-0.2 M, C-0.3 M, D-0.4 M, E-0.5 M.

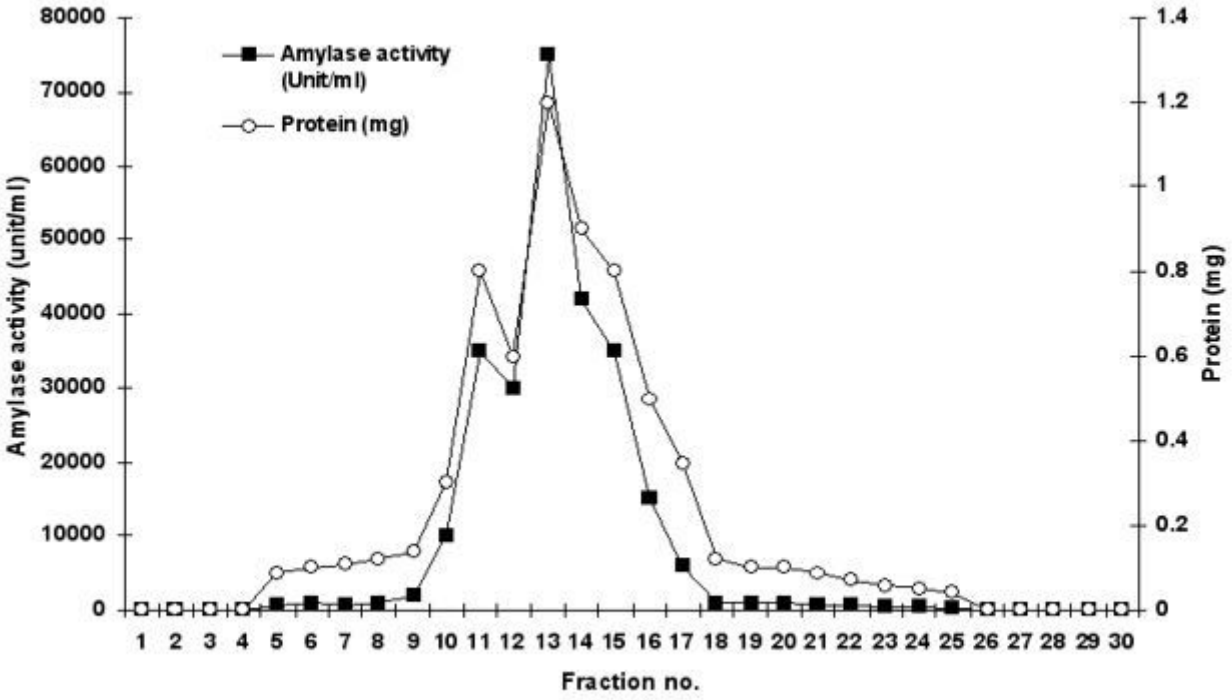

Figure 3

Chromatographic purification profile of extracellular amylase on Sephadex G-75 gel filtration chromatography. Column was equilibrated with sodium phosphate buffer (100 mM, pH 7.0). The sample was loaded and eluted with the same buffer.

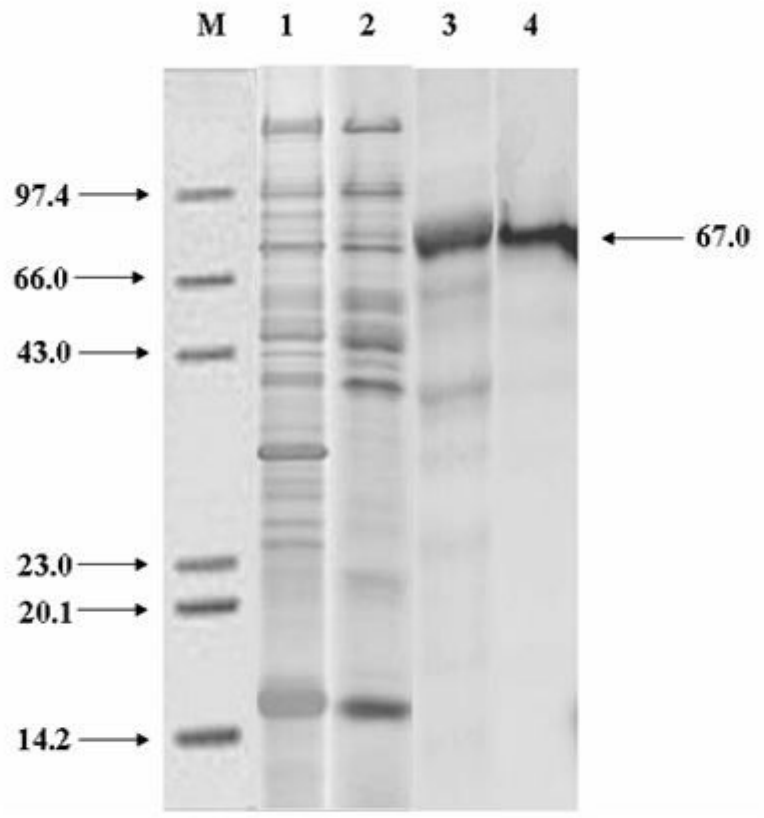

Figure 4

Photographic representation of the SDS-PAGE of different fractions of amylase of Bacillus subtilis obtained during purification steps. Lane 1: Marker proteins; lane 2: Crude supernatant; lane 3: Concentrated enzyme after acetone precipitation; lane 4: Purified enzyme obtained after anion-exchange chromatography; lane 5: Purified enzyme obtained after gel-filtration chromatography. Molecular weights were presented in the form of kDa.

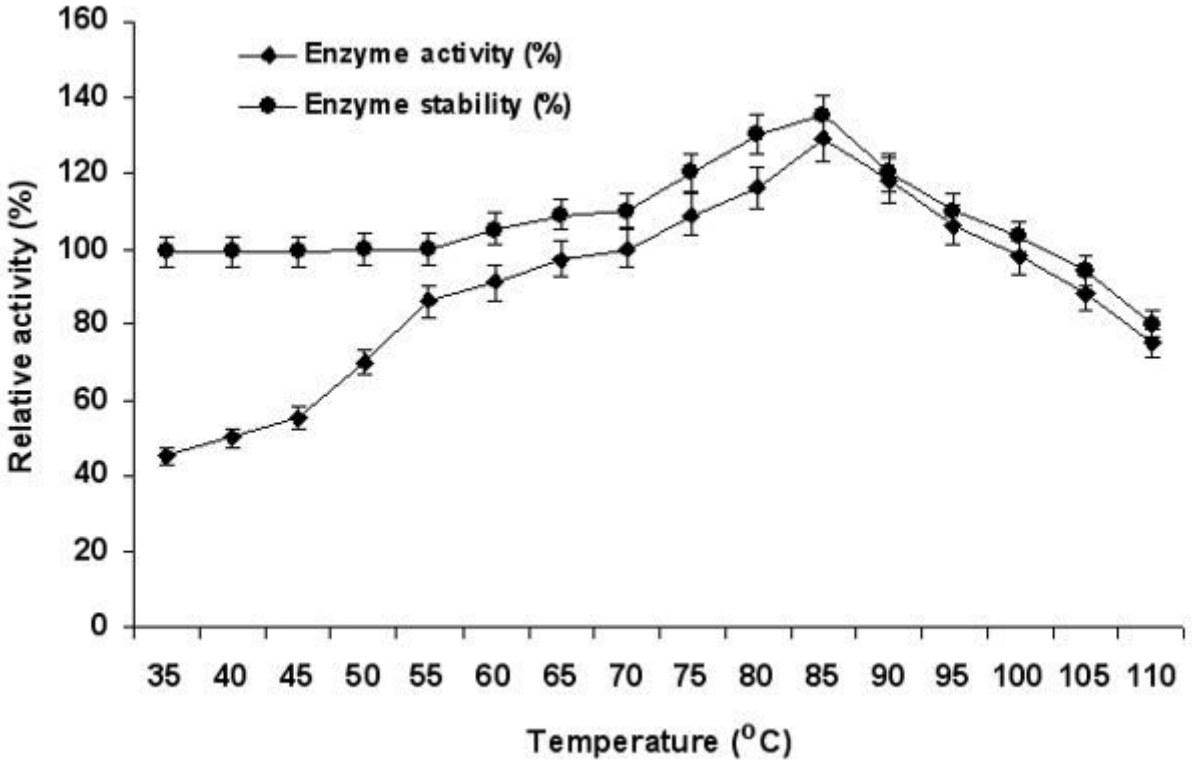

Figure 5

Effect of temperature on enzyme activity and stability. For enzyme activity reaction mixture was incubated at different temperatures (35-110°C) and for stability enzyme was pre-incubated at respective temperatures for 1 h and reaction was conducted as standard assay method.

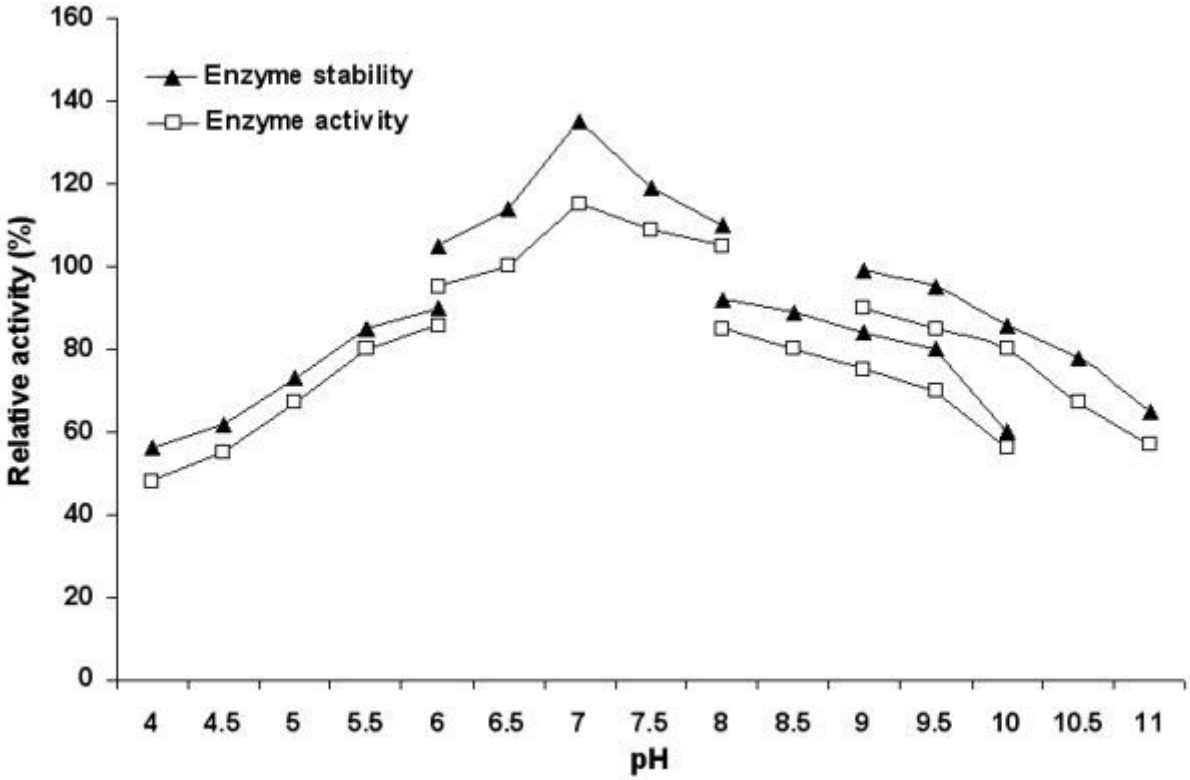

Figure 6

Effect of pH on enzyme activity and stability. For enzyme activity the reaction was assayed at respective pH and for stability enzyme was pre-incubated with buffers (100 mM, in ratio 1:1) of different pH (4-11) at 55°C for 1 h and assayed by standard assay method.

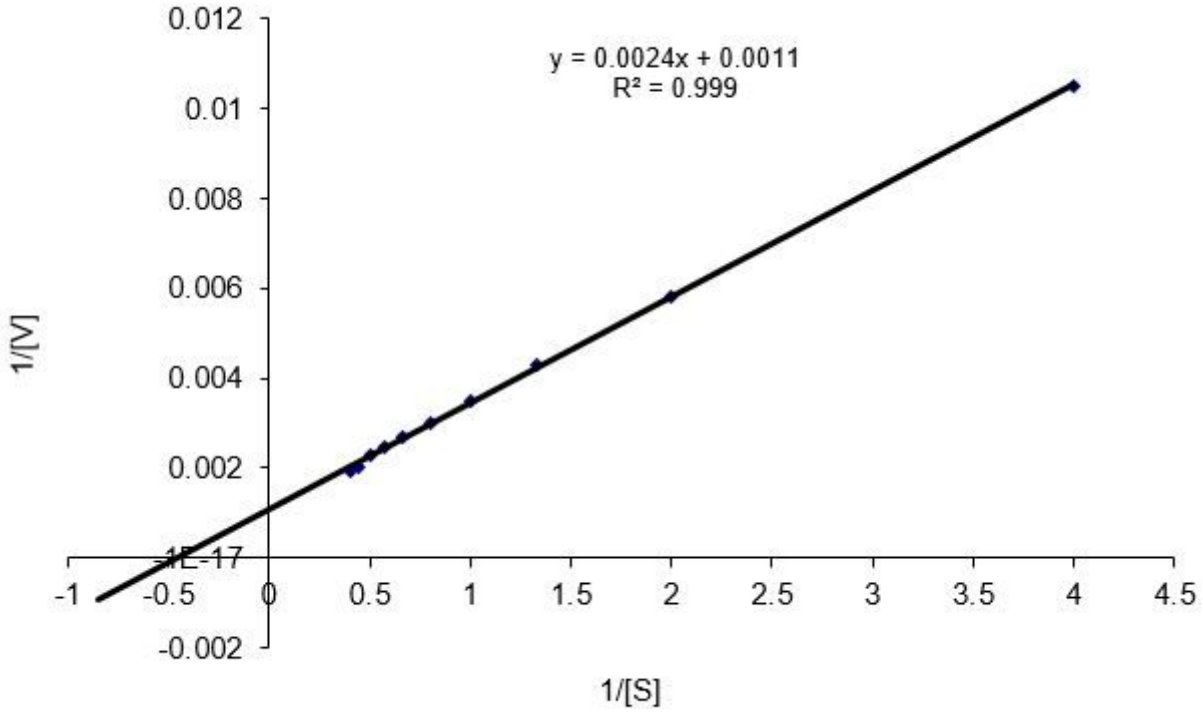

Figure 7

Lineweaver-burk plot of amylase enzyme of Bacillus subtilis.

Supplementary Files

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