RESEARCH ARTICLE
J. Food Sci. Technol. Nepal, Vol. 12 (49 - 58), 2020
ISSN: 1816-0727

Purification and Characterization of a Noble Thermostable Alpha-amylase from *Anoxybacillus tengchongensis* RA1-2-1 Isolated from Geothermal Spring of Nepal

PARASH MANI TIMILSINA[1], GYANU RAJ PANDEY[1, 2], ASMITA SHRESTHA, [2] MANISH OJHA[1], GARIMA BARAL[1], TIKA BAHADUR KARKI[1]

[1]Department of Biotechnology, Kathmandu University, Dhulikhel, 6250, Nepal
[2]Biotechnological Research and Developmental Centre Pvt. Ltd., Bharatpur, Chitwan, 44200, Nepal

A thermophilic amylolytic strain, Anoxybacillus tengchongensis RA1-2-1 was isolated from geothermal spring of Rasuwagadi district of Nepal. The BLAST alignment of the 16s rRNA sequence revealed 99.3% similarity with the type strain Anoxybacillus tengchongensis T-11. The morphological, physiological and biochemical properties were similar to the type strain. The enzyme from the strain was purified to 21-fold purification by DEAE-cellulose ion exchange chromatography. The K_m value of the enzyme was 0.68±0.05 mg/ml. The optimum pH and temperature were 7.0 and 70 °C. SDS-PAGE analysis showed a single band at 69 kDa. The half-life of the enzyme at 70°C and 80°C were 85.01min and 51.96 min respectively. TLC analysis of the hydrolysis product showed that the enzyme is maltogenic amylase. The calcium independent enzyme was completely inhibited by Hg^{2+} but showed inhibitory effect in the range of 100 %-30 % in the presence of other salts at 1-10mM concentrations.

Key words: Alpha-amylase, Enzyme Kinetics, Thermostability, Liquefaction, Starch

Introduction

Extremophilic microorganism that can survive harsh environmental conditions can be a potential source of industrially important enzymes. Enzymes from this extremophile have unique properties of salt tolerance, thermostability, cold adaptability and substrate specificity (Dumorné et al., 2017).

Halophilic thermophiles isolated from geo thermal springs are a potential source of noble enzymes. Hydrolytic enzymes such as amylase, protease and lipase with noble properties have been identified in thermophiles isolated from geothermal springs. Studies have been focused on the identification of these enzymes with noble properties suitable of specific application in food chemical, pharmaceuticals and biomass energy.

Alpha-amylase (EC 3.2.1.1, α-D-Glucan-glucanohydrolase) is one of the most important enzymes which has application in brewing, baking, textile, detergent and bioethanol production (Kindle, 1983; Finore et al; 2011; Fincan & Enez, 2014). Noble alpha-amylase with greater range of pH and temperature stability, tolerance to metal inhibition and higher substrate affinity has been isolated from thermophiles isolated geothermal springs.

Anoxybacillus tengchogensis was first isolated by zhang et al from tengchong hot springs of China (Zhang et al., 2011). In this study, a highly amylolytic *A. tengchongensis* RA1-2-1 strain was isolated from Rasuwagadi geothermal spring sediment of Nepal. Preliminary medium screening was done to identify optimum production medium. The enzyme was purified and characterized. Alpha-amylase from *A. tengchongensis* RA1-2-1 was found to be a potential candidate for industrial application. Owing to the high thermostability and resistance to metal inhibition, the enzymes can be used to improve the existing process for liquefaction in food processing industries.

Materials and Methods

Materials

All bacteriological grade growth medium and reagents were purchased from Himedia. All Analytical reagents for enzyme assay (Sodium phosphate, monobasic, Sodium chloride, Potato Starch,Sodium hydroxide, Potassium sodium tartrate, tetrahydrate, 3,5-Dinitrosalicylic acid, D-(+)-Maltose) were purchased from Sigma-Aldrich.

Sample collection and isolation

Samples were collected from geothermal springs located in Rasuwagadi district of Nepal. Water, bio mats and sediments were collected using three bottles of 500 ml sterile thermo flask and transported to the laboratory within 24 hours in icebox.

Enrichment of thermophiles was done in nutrient broth (pH 7.0) at 55 ℃ water-bath. Growth was monitored by measuring turbidity with a DEN-B densitometer. Isolation of obligate thermophiles was performed by incubation of 1:3 (v/v) dilution homogeneous bacterial suspension at 70 ℃ corresponding to temperature of the sample site. The culture that showed positive growth was inoculated in nutrient broth at 37 ℃. The culture that showed growth at 70 ℃ but do not show significant growth at 37 ℃ was screened for amylase activity. Alpha amylase activity was tested by streaking the pure culture in 1% starch agar plates. The amylolytic activity was detected by the formation of clear zone around the isolates after addition of Lugol's iodine.

Characterization of the isolates

The amylolytic strain was characterized by phenotypic, physiological and molecular study. The phenotypic study investigated were colony morphology, grams staining, spore staining and microscopic observations.

The physiological parameters studied were: optimum growth temperature, optimum pH, optimum salt tolerance and motility test. Optimum temperature, pH and salt concentration

*Corresponding author, email address: timipara@ku.edu.np

were obtained by OVAT (one variable at a time) method. For determination of optimum temperature, 0.5 ml overnight culture was transferred to 5 ml fresh nutrient broth in 15 ml tubes. The tubes were incubated at 40 ℃, 50 ℃, 60 ℃, 70 ℃ and 80 ℃ in orbiter shaker at 150 rpm. The optical density readings were taken every hour. For determination of optimum pH, the temperature was maintained at previously identified temperature optimum and inoculated in nutrient broth of pH 6.0-11.0 at 0.5-unit increment. For determination of optimum salt tolerance, overnight log phase culture was transferred to 5 ml nutrient broth with final NaCl concentrations of 0 %,1 %, 2 %, 3 %, 4 % and 5 %. OD reading was done in one-hour interval. Maximum specific growth rate was estimated by Gompertz growth model nonlinear regression method (Zwietering et al., 1990).
All carbohydrate utilization test was done in replicates using Himedia HiCarbo™ identification kits. Antibiotic sensitivity test for 16 different antibiotics was done with Himedia HiComb™ disc according to the manual.

Molecular characterization

Molecular characterization of the strain was done by 16s rRNA sequence analysis. Genomic DNA was isolated using DNeasy Ultraclean Microbial Kit Qiagen. PCR amplification was performed using 27F and 1492R universal primers. The PCR product was purified by Qiagen QIAquick PCR purification kit. The PCR product was sequenced in ABI Prism DNA sequencer (Macrogen, Korea) using Big Dye terminator cycle sequencing. Sequencing primers used were 785F and 907R primers. The forward and reverse sequence obtained was analysed and consensus sequence was generated in Mega-X. The FASTA sequence was analysed in NCBI blast tool. The phylogenetic tree was constructed using neighbour Joining algorithm. The sequence was published in NCBI GenBank.

Determination of amylase assay

The activity of alpha-amylase was assayed according to Bernfeld et al (Bernfeld, 1955). The unit of alpha-amylase is defined as the amount of enzyme which liberates 1 mg of reducing sugar as maltose in 3 min under the assay condition. *Bacillus amyloliquefaciens* alpha-amylase was used for test validation. Maltose released (mg) in the amylase test solution is calculated by linear regression analysis of the standard maltose curve. Protein concentration in the enzyme sample is measured by Lowry's assay (Lowry et al., 1951).
Units/ml enzyme = (mg of maltose released) * Dilution factor
Units /mg protein = (Units/ml enzyme) / (mg/ml protein)

Selection of medium for enzyme production

The bacteria were cultured in 100 ml volume in predefined five different basal medium **M1** (Upton & Fogarty, 1977), **M2**, **M3** (Pfueller & Elliott, 1969) , **M4** (Andersson et al., 1985) and **M5** (Upton & Fogarty, 1977)] and incubated at 55 ℃ . Medium **M1** had the following composition(grams per liter) : Corn starch, 15.0; $CaCl_2.H_2O$, 1.0; $MgCl_2.6H_2O$, 1.0; K_2HPO_4, 4.0; $(NH_4)_2SO_4$, 1.0; and trace metal solution 10 ml of composition(milligram per liter) : $CuSO_4.5H_2O$, 16.0; $FeSO_4.7H_2O$, 100.0; $ZnSO_4.7H_2O$, 80.0; $MnCl_2.4H_2O$, 7.0; pH adjusted to 7.0 after autoclaving. Medium **M2** had the following composition (gram per liter): Corn starch, 10.0; Yeast extract, 3.0; Peptone, 5.0; $CaCl_2.2H_2O$, 0.25.0. pH adjusted to 7.0 after autoclaving. Medium **M3** had the following composition (gram per liter): Casein Hydrolysate, 5.0; Yeast Extract, 0.5; K_2HPO_4, 3.0; KH_2PO_4, 1.0; Soluble starch, 1.0; Trace metal 10 ml of the composition (grams per liter):$FeCl_3$, 0.3; $MgCl_2.6H_2O$, 0.5; $CaCl_2.6H_2O$, 0.85; NH_4Cl, 100.0; NaCl, 100.0. pH adjusted to 7.3 after autoclaving. Medium **M4** had the following composition (grams per liter): Soluble starch, 10.0; Peptone, 5.0; Yeast extract, 2.0; NaCl, 15.0; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 0.1; Sterile glycerol, 15% v/v. Medium **M5** had the following composition (grams per liter) : Bacterial peptone, 5.0, Corn starch, 20.0; K_2HPO_4, 2.0; $(NH_4)_2SO_4$, 5.0; $MgSO_4.7H_2O$, 1.0; Sodium lactate, 8.0; $CaCO_3$, 2.0; Inositol, 0.01; Trace metals: same as in medium **M1**. pH was adjusted to 7.0. The cell free medium after centrifugation was tested for amylolytic activity by amylase assay. The medium with highest amylolytic activity was chosen as basal medium for enzyme production in 300 ml medium.

Purification of amylase

The production medium was centrifuged at 8,400 g for 15 min to separate cells and particulate matters. The supernatant was collected. Ammonium sulphate was added to the culture supernatant to get 75 % saturation level. All precipitation work was performed at 4 ^{0}C. The precipitated solution was centrifuged at 21000 g for 15 min at 4 ℃. The pellet was dissolved in 1 ml of 0.01 mM phosphate buffer, pH 7.0. Overnight dialysis was performed using 12,000 MW cut-off dialysis bags against 0.01 M phosphate buffer with three changes of the same buffer. The dialyzed solution was purified in DEAE cellulose mini column. The column was washed with 0.01 M phosphate buffer. Enzyme solution was added slowly and again washed with the same buffer. The enzyme was eluted with 1 M NaCl with a flow rate of 1 ml per min and fractions of 1 ml each was collected. All the fractions were analysed by amylase assay at 55 ℃ and pH 7.0. Three fractions with the highest amylase activity were collected and dialyzed against 0.1 M phosphate buffer overnight and freeze-dried. (Telstar Lyoquest). Denaturing SDS-PAGE of the purified enzyme was performed according to Laemmli (Laemmli, 1970) for determination of molecular weight and purity of the enzyme. The gel image was analysed by GelAnalyzer 19.1.

Determination of optimum temperature and pH

Effect of temperature

The effect of temperature on amylase activity was measured from 30 ℃ to 100 ℃ at pH 7.0. The activity of appropriately diluted stock enzyme solution was measured by amylase assay as mentioned earlier but the incubation temperature was varied accordingly. Relative activity was calculated by taking percentage ratio against the highest activity observed. The optimum temperature was determined from the plot of temperature vs relative activity.

Effect of pH

The effect of pH on the alpha amylase activity was performed in the pH range of 3.0 - 9.0. The activity of diluted stock enzymes was measured by amylase assay. The starch solution was prepared in different pH adjusted buffers. The buffers used were: 20 mM Sodium acetate (pH 3.0 - 5.0), 20 mM Sodium phosphate (pH 5.0 - 7.5) and 20 mM Tris-HCl buffer (pH 8.0 - 9.5).

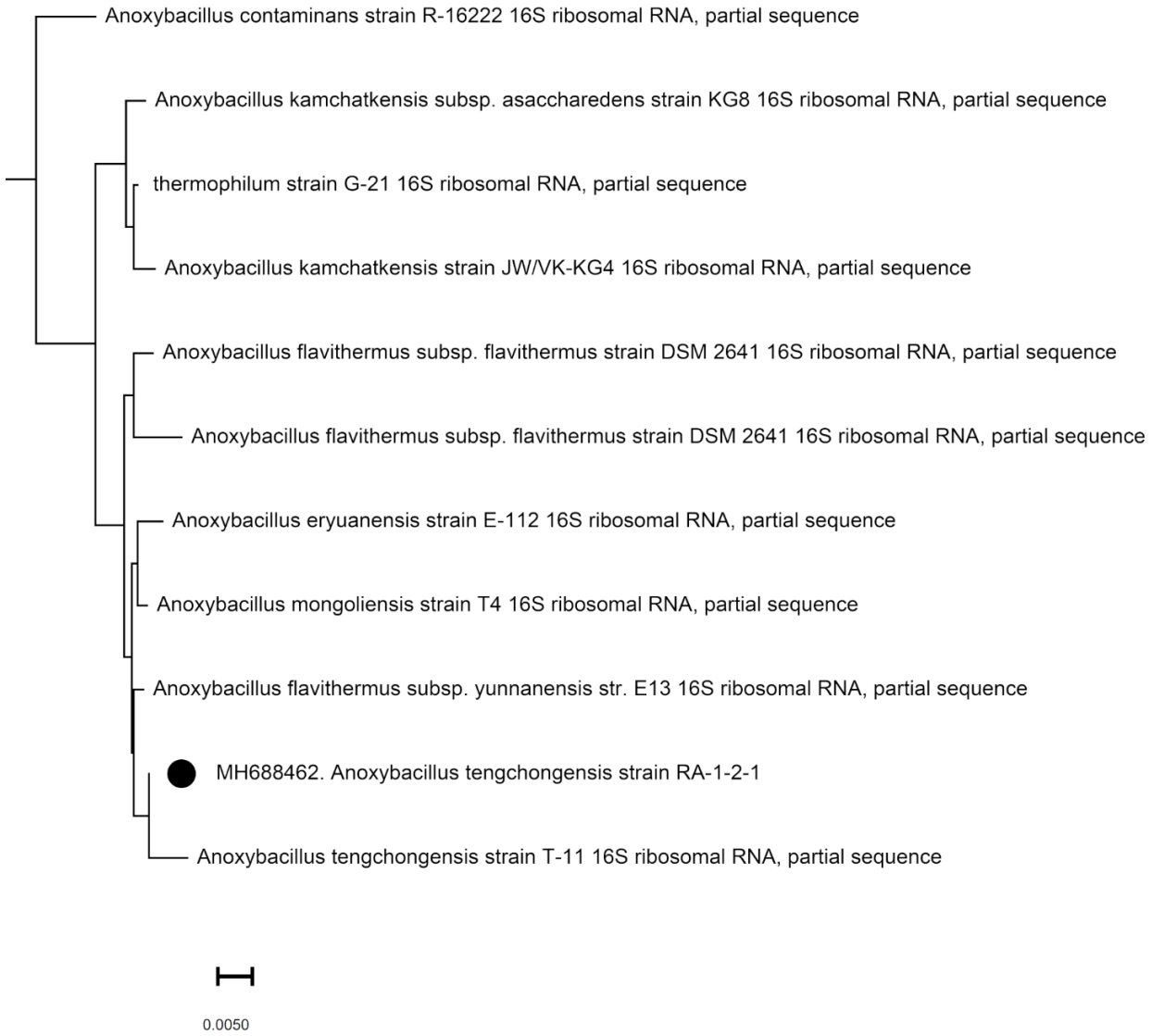

Fig. 1. Phylogenetic tree of strain RA1-2-1 isolate with 99.3% homology with Anoxybacillus tengchongensis T-11 type strain. The tree was constructed using neighbor joining algorithm in rRNA database using NCBI blast tool.

Thermo-stability Study

The thermostability of the enzyme was studied at 60 ℃, 70 ℃ and 80 ℃. 10 ml of the appropriately diluted enzyme solution in phosphate buffer pH 7.0 was transferred in test tubes and incubated in water bath. The residual activity of the enzyme was calculated after 1, 2, 3 and 4 hours of incubation by amylase assay at pH 7.0 and 55 ℃. Enzyme activity is compared with the activity at time 0 as 100 %. The data was fit into exponential function using Minitab19.0 to calculate half-life.

Thin layer chromatography of hydrolysis product

TLC of the hydrolysis product was performed according to Hansen, 1975 (Hansen, 1975). The product liberated by the action of amylase on starch was identified by spotting the starch digest and standard sugars (glucose, maltose and maltotrioses) on a pre-coated silica plate activated at 110 ℃. The plates were developed in butanol: ethanol: water solvent (5:3:2) and dried overnight at room temperature. The individual sugars were visualized with aniline-diphenylamine reagent.

Determination of kinetic parameters and data analysis

The kinetic parameter V_{max} and K_m is determined by incubating the amylase with final concentration of soluble starch ranging from 0.1 % to 0.7 % in the buffer at optimum pH and temperature. The enzyme activity is measured by amylase assay as mentioned earlier. The Michaelis-Menten enzyme kinetic model was used for parameter estimation. The kinetic parameter V_{max} and K_m is determined by nonlinear regression statistical methods using Minitab 19.

Enzyme inhibition by metals ions

The stock enzyme solution was added to equal volume of 2X metal solutions to make final concentration of 1 mM and 10 mM respectively. The salt solution used were $BaCl_2$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$, KCl, $MgSO_4$, MnSo4, $NiCl_2$, $Pb(C2H3O2)_2$, $SnCl_2$ and $ZnSO_4$. The activity of diluted enzyme was measured by amylase assay at optimum temperature and pH.

Table 1
Taxonomical Characterization of *A. tengchongensis* RA1-2-1.

Test	Study	Results
Morphology	Microscopic study	Gram positive, rods, subterminal endospores
	Colony morphology (48-hour incubation on Nutrient broth ,55 ^{0}C)	Circular, pale yellow colour, Entire/lobed margin, Flat elevation, translucent with dot like appearance in the centre ,2-2.5 mm diameter
Physiological test	Growth on Nutrient broth	Optimum temperature 50 ^{0}C, Optimum pH 8.0, Optimum salt tolerance 1 %
	Motility in SIM agar	Motile
	Other physiological tests	-ve: voges-proskauer test), gas from fermentation (TSI), phenylalanine deaminase, +ve: nitrate reduction, catalase, oxidase, anaerobic growth
Biochemical Test	Substrate hydrolysis	+ve starch, tributyrin, cellulose, gelatin, -ve urea, casein, protease, esculin
	Acids from Sugar	+ve: maltose, fructose, dextrose, raffinose, galactose, trehaloes, melibiose, sucrose, mannose, inulin -ve: lactose, xylose, L-arabinose, sodium gluconate, pyruvate
	Utilization of carbohydrates and derivatives	+ve: glycerol, salicin, dulcitol, mannitol, esculin, sorbitol, pyruvate, erythritol -ve: inulin, sodium gluconate, adonitol, melezitose, xylitol, ONPG, D-arabinose, citrate, malonate, acetate, inositol, sorbitol, arabitol, alpha-Methyl-D-glycoside, rhamnose, cellobiose, alpha-methyl-D mannoside, sorbose
	Antibiotic sensitivity assay	amoxicillin (30.25), ampicillin (15.75), azithromycin (20.75), carbenicillin (34.5), cefotaxime (29.75), cefuroxime (28.25), ciprofloxacin (30), erythromycin (23), gentamicin (18.6), levofloxacin (26.5), miconazole (13.5), moxifloxacin (28.5), penicillin (26), rifampicin (24.25), tetracycline (27.6), vancomycin (20.25)
Molecular Characterization	16s sequence analysis (PCR Primers: 27F, 1492R)	99.3% similarity with *Anoxybacillus tengchongensis* T11

Results

Sampling and isolation

Water samples, algal bio mats and sediments were collected from Rasuwagadi geothermal springs of Nepal. The maximum temperature of the water sample was 72 ℃ and pH 7.0. A total of 15 strains were isolated based on colony morphology in nutrient agar plates. Strain RA1-2-1 showed significant amylase activity in starch agar plate's assay.

Characterization

Molecular characterization

The 16s partial sequence Blast analysis of strain RA1-2-1 sequence showed 99.3% homology with the type strain *Anoxybacillus tengchongensis* T-11. The sequence was published in NCBI gene database with the gene accession no**. MH688462**. The phylogenetic tree was constructed using Neighbour Joining algorithm using NCBI Blast tool (**Fig. 1).**

Morphological, physiological and biochemical properties

A. tengchongensis RA1-2-1 was gram positive motile rods with charactertics similar to type strain *A. tengchongensis* T-11. Morphological, physiological and biochemical properties of *A. tengchongensis* RA1-2-1 are shown in (**Table 1**). The properties of *A. tengchongensis* RA1-2-1 was similar to *A. tengchongensis* T-11. The optimal pH, temperature and salt concentration were found to be 8.0, 50 ℃d 1 % respectively. The generation time in the optimal condition was 58.39 min (**Fig. 2).**

Medium selection and purification of enzyme

The maximum amylase activity was observed in Medium **M3** (1.07 ± 0.05 Units/ml). The enzyme expression in medium **M4** and **M5** (0.627 ±0.01 and 0.25 ± 0.07 U/ml) respectively while the enzyme production in medium **M1** and **M2** was insignificant (**Fig. 3)**. The enzyme from medium **M3** was purified to 21-fold purification after DEAE-Cellulose anion exchange chromatography. The elution pattern showed major peak of amylase activity for three fractions, which were, collected (**Fig. 4a)**. The purified fraction was further subjected to SDS-PAGE analysis for determination of, molecular weight. A single peak of the enzyme at 69 kDa was obtained (**Fig. 4b)**. The specific activity of the purified enzyme was 66.51 U/mg protein. The final yield was 46 % (**Table 2).**

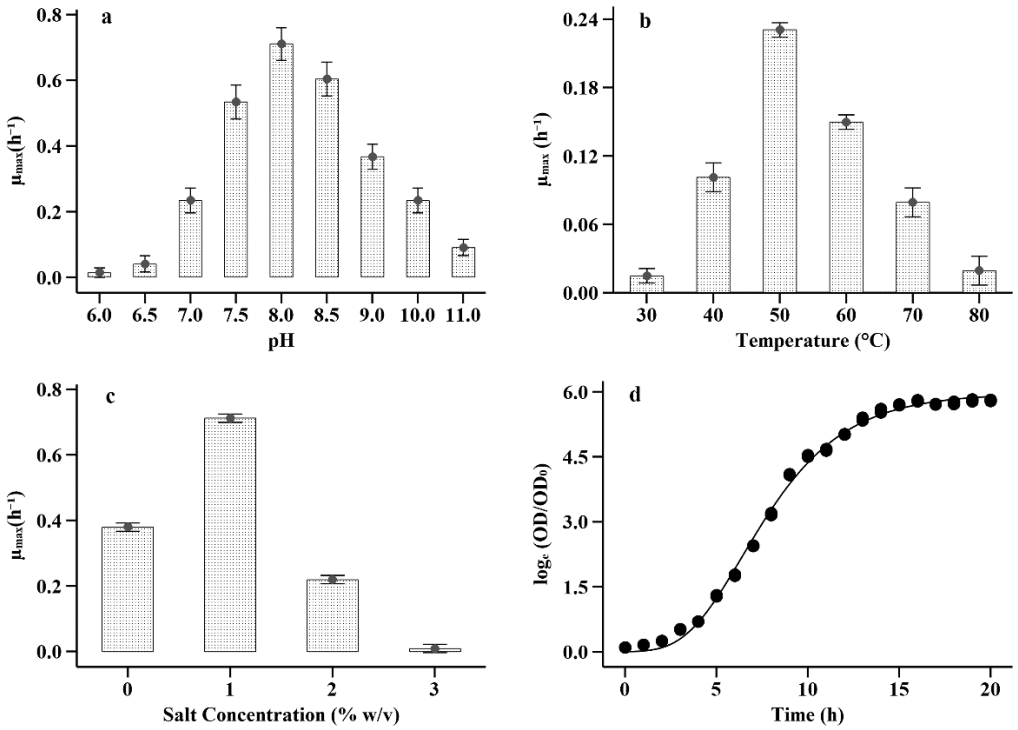

Fig. 2. Growth parameter optimization for *A. tengchongensis* RA1-2-1 in nutrient broth. **A**: Maximum specific growth rate(μ_{max}) at different temperature. **B**: Maximum specific growth rate (μ_{max}) at 50 °C and different pH. **C**: Maximum specific growth rate (μ_{max}) at pH 7.0, temperature 50 °C and different salt concentrations. **D**: Gompertz plot of optical density (OD) at different time interval.

Optimization of pH and temperature

The optimum pH was found to be at pH 7.0 (**Fig. 5a**). The enzyme had activity 38.03 ± 2.27 % at pH of 6.0 but higher retention value of 59.68 ±0.63 % in pH 8.0. The enzyme retention was 26.6 ± 0.73 % at pH 9.0. The optimum temperature of the enzyme was found to be at 70 °C. However, the enzyme retained 96.90 ±1.4 % at 60 °C, 80.72 ±0.68 % at 80 °C and and 37.00 ±3.1 % at 90 °C (**Fig. 5b)**. The data suggest the highly thermophillic nature of the enzyme.

Thermostability study and determination of kinetic parameter

The thermal stability of the enzyme was calculated at 60 ^{0}C,70 ^{0}C and 80 ^{0}C. The enzyme half-life at 60 °C ,70 °C and 80 °C were calculated to be 157.6 min, 85.01 min and 51.96 min respectively (**Fig. 6**). The K_m and V_{max} value were calculated at optimum temperature and pH using nonlinear regression method in Minitab. The Michalis Menten constant (K_m) was 0.68±0.04 mg/ml and V_{max} value was 2.12±0.02 mM(maltose)/min (**Fig. 7**).

Thin layer chromatography

To characterize the mode of action of the alpha-amylase, the hydrolysis products were analysed by thin layer chromatography. Soluble starch was converted into glucose, maltose and maltotriose (**Fig. 8)**. After 1 h of incubation, all hydrolysis product was present. The dominant product was maltose as darker spot in the maltose zone suggesting maltogenic amylase. This suggests that the alpha-amylase of *A. tengchongensis* RA1-2-1- is a maltogenic amylase.

Metal inhibition assay

The amylase did not require any specific ion for catalytic activity. A stronger inhibitory effect was observed in the presence of Hg^{2+} at both 1 mM and 10 mM (**Fig. 9**). All enzyme except Hg^{2+} showed very small inhibition at 1 mM.

Table 2
Purification of alpha-amylase from *A. tengchongensis* RA 1-2-1.

Steps	Volume (ml)	Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Broth Supernatant	281	421.5 ±0.9	253.46 ± 0.6	1.66 ±0.04	1	100
Precipitation/Dialysis	1	265.54± .8	11.23 ± 0.1	23.63 ±0.06	14	63
DEAE-Cellulose Chromatography	3	103.89±0.6	2.91 ± 0.04	35.39 ± 0.5	21	24

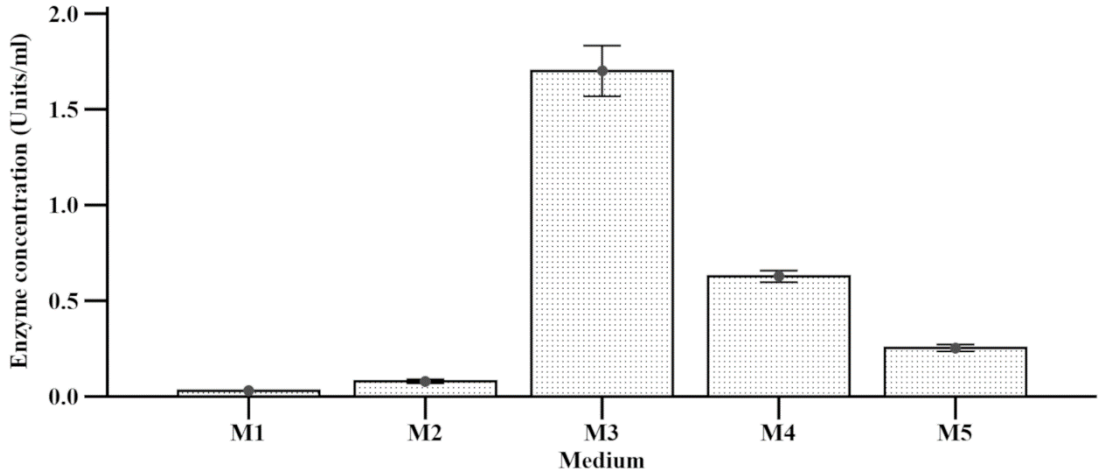

Fig. 3. Amylase activity of medium supernatants after 48-hour incubation

Discussions

An obligate thermophile *A. tengchongensis* RA1-2-1 was isolated from Rasuwagadi geothermal spring of Nepal. The strain showed positive amylolytic activity in starch agar plate. The morphological physiological and biochemical properties of *A. tengchongensis* RA1-2-1 and type strain *A. tengchongensis* T11 (zhang et al) were similar. Both were gram positive, sporulating rods. The colony characteristics of both strains were similar: circular pale-yellow, flat and opaque after 24-hour incubation in nutrient broth. Both strains have optimum temperature of 50 ^{0}C while closely associated *A. flavithermus* DSM2641^T (Heinen et al., 1982) have temperature optimum of 60-65 °C. Optimum pH for growth of *A. tengchongensis* RA1-2-1 was 8.0 while of *A. tengchongensis* T11 was 8.5 suggesting both were alkalophilic. Both were positive for catalase and oxidase, gelatine hydrolysis and nitrate reduction test. These tests were negative for *A. flavithermus* DSM2641^T as described by Heinen. BLAST analysis showed that the 16s rRNA sequence of *A. tengchongensis* RA1-2-1 was 99.3% similar with the type strain. The morphological, physiological, biochemical and molecular data showed that the isolated bacterial culture is a new strain of *Anoxybacillus tengchogensis.*

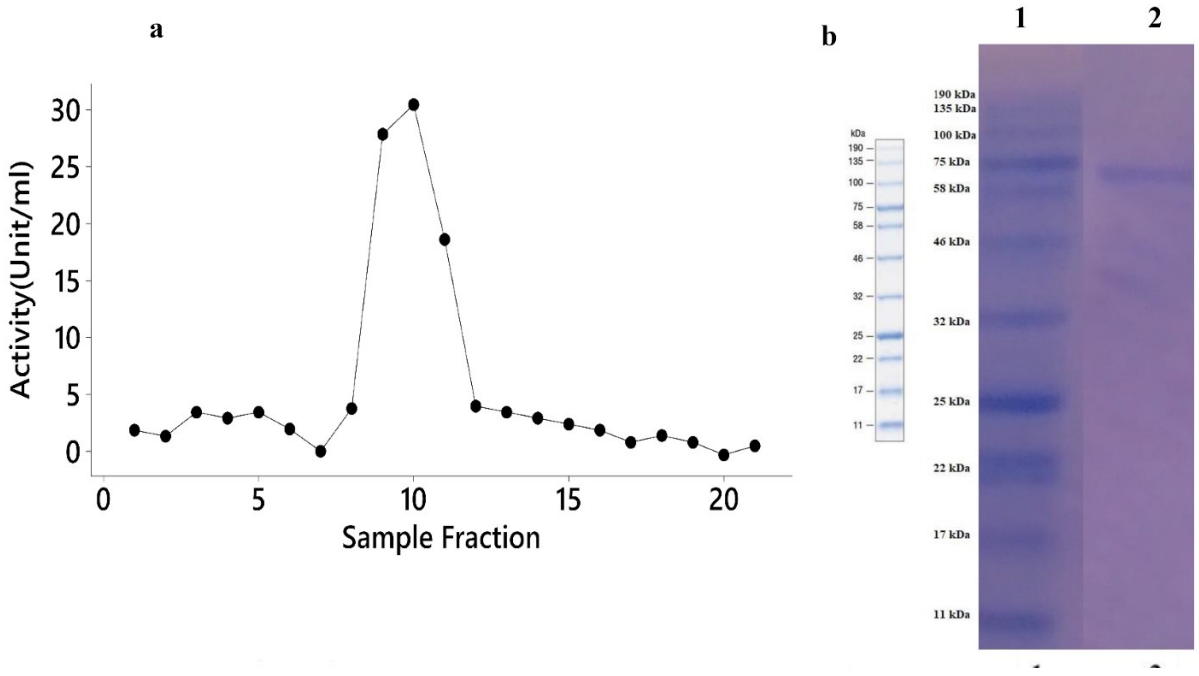

Fig. 4. Elution profile and SDS-PAGE analysis. A: Enzyme activity of different fraction after DEAE-Cellulose anion exchange chromatography, B: SDS-PAGE of purified alpha amylase from A. tengchongensis RA1-2-1; Lane 1: Molecular marker, Lane 2: Purified Enzyme

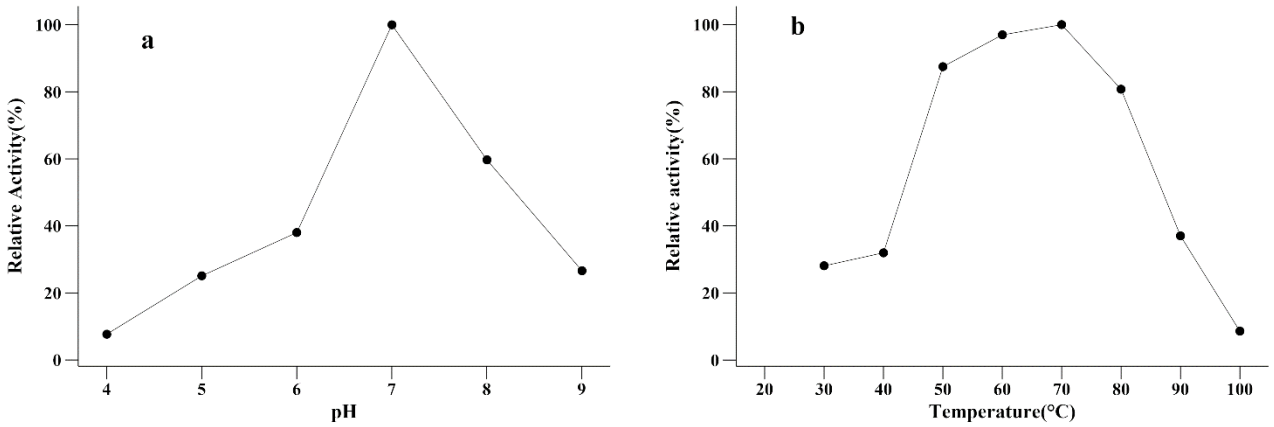

Fig. 5. Temperature and pH optimization. **a**: Effect of pH on Enzyme activity of A. tengchongensis. .100 % activity of amylase= 1.059 U/ml. **b**: Effect of temperature on the enzyme activity of alpha amylase from A. tengchongensis at pH 7.0. 100 % activity

Preliminary screening was carried out in five different medium formulation to identify optimum medium for amylase production. Higher amylase activity in fermentation broth was found in Medium **M3** while moderate expression was found in Medium **M4** and **M5**. The higher production of amylase in medium **M4** may be due to the presence of casein hydrolysates, yeast extract and mineral salts. The presence of complex source nitrogen such as yeast extract and meat extract has been found to enhance amylase production (Bano et al., 2011; Elmansy et al., 2018). These complex sources may contain growth factor necessary for growth of fastidious bacteria resulting in high amylase production.

This is the first report of purification and characterization of alpha amylase from Anoxybacillus tengchongensis strain. The optimum pH of alpha-amylase from *A. tengchongensis* was 7.0. The enzyme activity at pH 8.0 was 59.68 ± 0.63 % and at pH 9.0 was 26.6 ± 0.73 suggesting wide range of ph activity over alkaline pH. Such types of enzyme from alkalophilic bacteria has been reported and grouped under type IV class (Yamamoto et al., 1972).

The optimum temperature was 70 ℃ and the enzyme activity was 84.35 ± 0.71 % at 80 ℃ and 38.65 ± 1.34 % at 90 ℃.

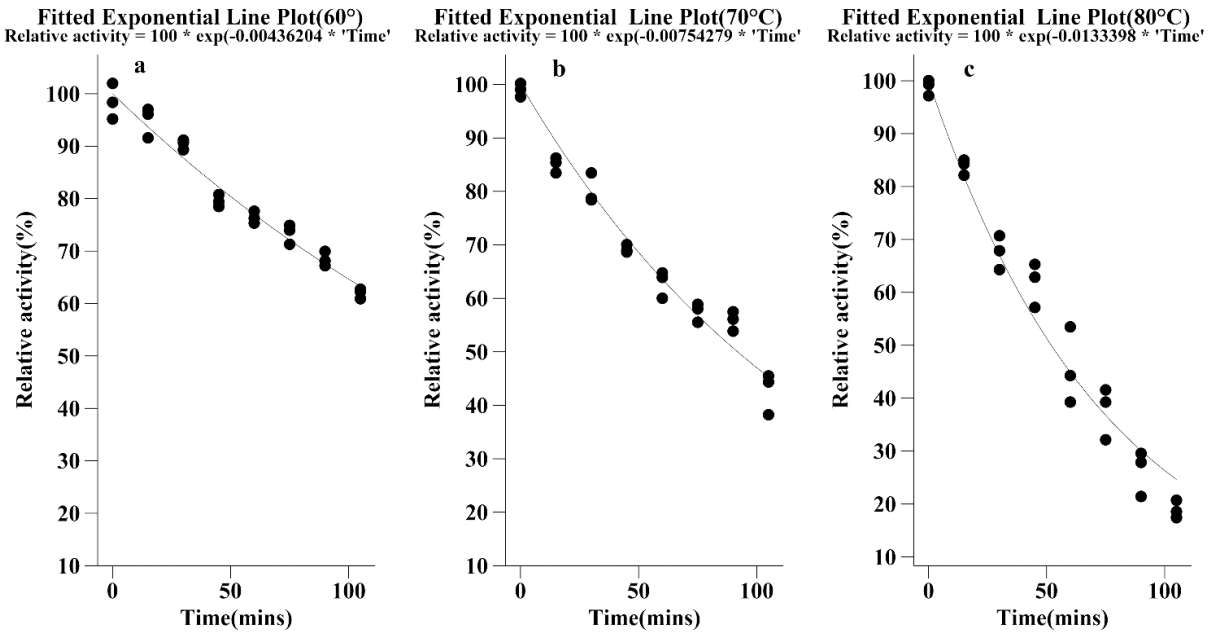

Fig. 6. Thermostability of alpha amylase from A. tengchongensis RA1-2-1 at different temperatures. Enzyme activity at 0 hour was considered as 100% activity. The half-life of enzyme was 51.96 min at 80 ºC.

This showed that the enzyme is active over a wide temperature. The enzyme was thermostable with half-life of 51.96 min at 80 ℃. Although the optimum growth of *A. tengchongensis* was 50 ℃, the enzyme optimum was at higher temperature of 70 ℃. It has been demonstrated that there is no obligatory relationship between thermophilic properties of bacteria and thermostability properties of the enzyme (Am et al., 1978). Optimum pH and temperature for the strain was similar to other reported values: pH 7.0 and temperature 80 ℃ for *Anoxybacillus flavithermus* (Veysi Okumus, 2015), pH 8.0 and 80 ℃, *Anoxybacillus flavithermus* (Agüloğlu Fincan et al., 2014) and ph 7.0 and 55 ℃ for *Anoxybacillus rupiences* (Bhavtosh A. Kikani et al., 2020). The range of pH over alkaline pH and temperature stability make the enzyme suitable for application in detergent and textile industries .

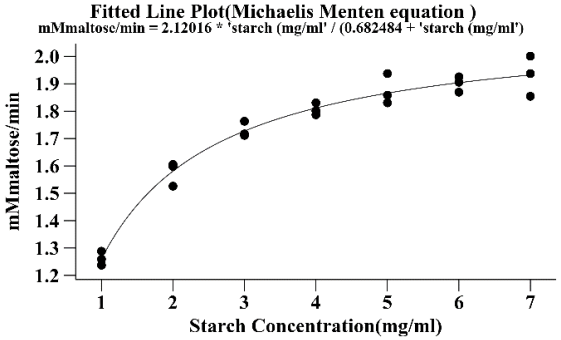

Fig. 7. Kinetics of alpha amylase from *A. tengchongensis* RA 1-2-1. The data was fit into Michaels Menten equation using nonlinear regression method. Estimated Km = 0.68 mg/ml.

SDS-PAGE result showed that the molecular mass of *A. tengchongensis* RA1-2-1 alpha amylase was 69 kDa. This is the first report of experimental calculation of molecular mass of *A. tengchongensis*. The observed molecular mass of alpha-amylase from *A. tengchongensis* RA1-2-1 is higher than other closely related Anoxybacillus species: *A. rupiensis* TS-4 :48 kDa (Kikani et al., 2020) ; *A.beppuensis* :43 kDa (B. A. Kikani & Singh, 2012) and *A.flavithrmus*: 60 kDa (Agüloğlu Fincan et al., 2014).

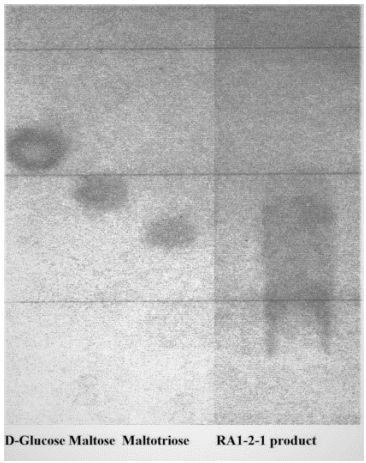

Fig. 8. TLC of the hydrolysis product of amylase from A. tengchongensis RA1-2-1 with soluble potato starch as substrate. Lane 1: D-Glucose, 2: Maltose, 3: Maltotriose, 4: Hydrolysis product after 1-hour incubation

The K_m value of the enzyme was calculated to be 0.68 mg/ml for soluble starch as substrate. K_m value *A*noxybacillus alpha-amylase have been reported earlier : 0.58 mg/ml for *Anoxybacillus rupiensis* TS -4 , 0.5mg/ml for *A. beppuenisis* (Bhavtosh A. Kikani et al., 2020), This value is also comparable to other reported value for commercially important amylase source (*Bacillus licheniformis*, K_m: 0.9mg/ml for *Bacillus amyloliquefaciens* BH072 (Ivanova et al., 1993), , K_m: 4.27 mg/ml for *Bacillus amyloliquefaciens* (Du et al., 2018) and K_m:2.68 mg /ml for *Bacillus subtilis* (Bano et al., 2011). Lower K_m value reflects the higher affinity of amylase of *A. tengchongensis* RA1-2-1 for soluble potato starch.

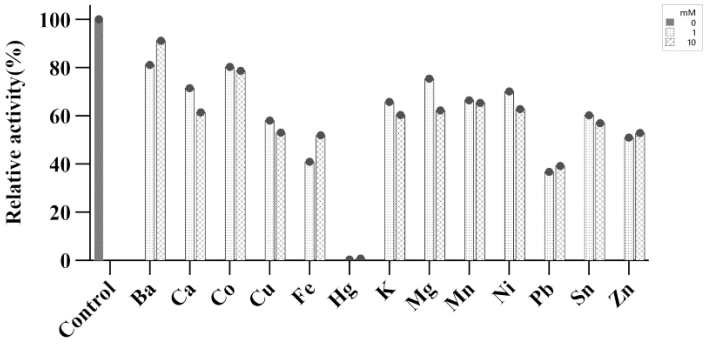

Fig. 9. Effect different metal ions on enzyme activity. The stock enzyme solution was mixed with different metal salt solution to make final concentration of 1 mM and 10 mM in buffered solution of pH 6.0.

The *A. tengchogensis* RA1-2-1 alpha-amylase showed significant increase in enzyme in the range of 1 mM -10 mM for Ba^{2+}, Fe^{2+} and Zn^{2+} ions. The enzyme showed significant inhibition for Ca^{2+}, Cu^{2+}, Mg^{2+} and K^{+} within the concentration range studied. The presence of metal ions Co^{2+}, Mn^{2+}, Ni^{2+}, Sn^{2+} and Pb^{2+} did not show significant change in activity. Hg^{2+} was completely inhibited at both concentrations. Such complete inhibition of alpha-amylase at 1 mM Hg^{2+} concentration has been reported in amylase from *Aspergillus oryzae* (Bhanja Dey & Banerjee, 2015) and *B. amyloliquefaciens* (Demirkan et al., 2005)). Due to its salt tolerance properties alpha amylase from *A. tengchongensis* RA1-2-1 could be a candidate for industrial enzymes. The genes for the enzymes can be cloned in suitable host for desirable modification, purification and expression of the enzyme.

Conclusion

The liquefying maltogenic enzyme was found to be highly thermostable with half-life of 51.96 min at 80 ℃. The enzyme was tolerant to significant metal inhibition except Hg^{+}. The enzyme was active over a wide pH range of 6.0 -9.0. The enzyme could be a potential candidate for application in food processing industries.

Acknowledgment

This research work was funded by International Foundation for Science (IFS), Sweden and Organization for the

Prohibition of Chemical Weapons (OPCW) through project ID: F/5785-1.

References

Agüloğlu Fincan, S., Enez, B., Özdemir, S., & Matpan Bekler, F. (2014). Purification and characterization of thermostable α-amylase from thermophilic Anoxybacillus flavithermus. *Carbohydrate Polymers*, *102*(1), 144–150. https://doi.org/10.1016/j.carbpol.2013.10.048

Am, T., Amylases, A., Synthesis, A. E. E., Biosynthesis, A., Induction, C., Repression, C., & Aspects, D. G. (1978). *Bacterial a-Amylases M. B. INGLE*. *24*.

Andersson, E., Johansson, A. C., & Hahn-Hägerdal, B. (1985). α-amylase production in aqueous two-phase systems with Bacillus subtilis. *Enzyme and Microbial Technology*. https://doi.org/10.1016/0141-0229(85)90112-7

Bano, S., Qader, S. A. U., Aman, A., Syed, M. N., & Azhar, A. (2011). Purification and characterization of novel α-amylase from bacillus subtilis KIBGE HAS. *AAPS PharmSciTech*. https://doi.org/10.1208/s12249-011-9586-1

Bernfeld, P. (1955). [17] Amylases, α and β. *Methods in Enzymology*. https://doi.org/10.1016/0076-6879(55)01021-5

Bhanja Dey, T., & Banerjee, R. (2015). Purification, biochemical characterization and application of α-amylase produced by Aspergillus oryzae IFO-30103. *Biocatalysis and Agricultural Biotechnology*, *4*(1), 83–90. https://doi.org/https://doi.org/10.1016/j.bcab.2014.10.002

Demirkan, E. S., Mikami, B., Adachi, M., Higasa, T., & Utsumi, S. (2005). α-Amylase from B. amyloliquefaciens: purification, characterization, raw starch degradation and expression in E. coli. *Process Biochemistry*, *40*(8), 2629–2636. https://doi.org/https://doi.org/10.1016/j.procbio.2004.08.015

Du, R., Song, Q., Zhang, Q., Zhao, F., Kim, R. C., Zhou, Z., & Han, Y. (2018). Purification and characterization of novel thermostable and Ca-independent α-amylase produced by Bacillus amyloliquefaciens BH072. *International Journal of Biological Macromolecules*. https://doi.org/10.1016/j.ijbiomac.2018.05.004

Dumorné, K., Córdova, D. C., Astorga-Eló, M., & Renganathan, P. (2017). Extremozymes: A Potential Source for Industrial Applications. *Journal of Microbiology and Biotechnology*, *27*(4), 649–659. https://doi.org/10.4014/jmb.1611.11006

Elmansy, E. A., Asker, M. S., El-Kady, E. M., Hassanein, S. M., & El-Beih, F. M. (2018). Production and optimization of α-amylase from thermo-halophilic bacteria isolated from different local marine environments.*Bulletin of the National Research Centre*, *42*(1).https://doi.org/10.1186/s42269-018-0033-2

Fincan, S. A., & Enez, B. (2014). Production, purification, and characterization of thermostable a-amylase from thermophilic Geobacillus stearothermophilus. *Starch/Staerke*. https://doi.org/10.1002/star.201200279

Finore, I., Kasavi, C., Poli, A., Romano, I., Oner, E. T., Kirdar, B., Dipasquale, L., Nicolaus, B., & Lama, L. (2011). Purification, biochemical characterization and gene sequencing of a thermostable raw starch digesting α-amylase from Geobacillus thermoleovorans subsp. stromboliensis subsp. nov. *World Journal of Microbiology and Biotechnology*. https://doi.org/10.1007/s11274-011-0715-5

Hansen, S. A. (1975). Thin-layer chromatographic method for the identification of mono-, di- and trisaccharides. *Journal of Chromatography A*. https://doi.org/10.1016/S0021-9673(00)82770-3

Heinen, W., Lauwers, A. M., & Mulders, J. W. (1982). Bacillus flavothermus, a newly isolated facultative thermophile. *Antonie van Leeuwenhoek*, *48*(3), 265–272. https://doi.org/10.1007/BF00400386

Ivanova, V. N., Dobreva, E. P., & Emanuilova, E. I. (1993). Purification and characterization of a thermostable alpha-amylase from Bacillus licheniformis. *Journal of Biotechnology*. https://doi.org/10.1016/0168-1656(93)90176-N

Kikani, B. A., & Singh, S. P. (2012). The stability and thermodynamic parameters of a very thermostable and calcium-independent α-amylase from a newly isolated bacterium, Anoxybacillus beppuensis TSSC-1. *Process Biochemistry*, *47*(12), 1791–1798. https://doi.org/10.1016/j.procbio.2012.06.005

Kikani, Bhavtosh A., Kourien, S., & Rathod, U. (2020). Stability and Thermodynamic Attributes of Starch Hydrolyzing α-Amylase of Anoxybacillus rupiensis TS-4. *Starch/Staerke*, *72*(1–2), 1–10. https://doi.org/10.1002/star.201900105

Kindle, K. L. (1983). Characteristics and production of thermostable α-amylase. *Applied Biochemistry and Biotechnology*, *8*(2), 153–170. https://doi.org/10.1007/BF02778096

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. https://doi.org/10.1038/227680a0

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*. https://doi.org/10.1016/0922-338X(96)89160-4

Pfueller, S. L., & Elliott, W. H. (1969). The Extracellular α-Amylase of Bacillus stearothermophilus. *Journal of Biological Chemistry* , *244*(1), 48–54. http://www.jbc.org/content/244/1/48.abstract

Upton, M. E., & Fogarty, W. M. (1977). Production and purification of thermostable amylase and protease of Thermomonospora viridis. *Applied and Environmental Microbiology*. https://doi.org/10.1128/aem.33.1.59-64.1977

Veysi Okumus, S. O. (2015). Isolation of a Novel Thermophilic Anoxybacillus flavithermus SO-13, Production, Characterization and Industrial Applications of its Thermostable alpha-Amylase. *Journal of Bioprocessing & Biotechniques*, *05*(07). https://doi.org/10.4172/2155-9821.1000237

Yamamoto, M., Tanaka, Y., & Horikoshi, K. (1972). Alkaline Amylases of Alkalophilic Bacteria. *Agricultural and Biological Chemistry*, *36*(10), 1819–1823. https://doi.org/10.1080/00021369.1972.10860480

Zhang, C. M., Huang, X. W., Pan, W. Z., Zhang, J., Wei, K.

B., Klenk, H. P., Tang, S. K., Li, W. J., & Zhang, K. Q. (2011). Anoxybacillus tengchongensis sp. nov. and Anoxybacillus eryuanensis sp. nov., facultatively anaerobic, alkalitolerant bacteria from hot springs. *International Journal of Systematic and Evolutionary Microbiology*, *61*(1), 118–122. https://doi.org/10.1099/ijs.0.020834-0

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., & Van't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology*, *56*(6), 1875–1881. https://doi.org/10.1128/aem.56.6.1875-1881.1990