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

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A thermophilic amyloytic 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 *Km* 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℃ and 80℃ 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 Hg2+ 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 geothermal 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 such as amylase, protease and lipase with noble properties in thermophiles isolated from geothermal springs. 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 tengchongensis* was first isolated by zhang et al from tengchong hot springs of China (Zhang et al., 2011). In this study, a highly amyloytic *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 °C 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 °C corresponding to temperature of the sample site. The culture that showed positive growth was inoculated in nutrient broth at 37 °C. The culture that showed growth at 70 °C but do not show significant growth at 37 °C was screened for amylase activity. Alpha amylase activity was tested by streaking the pure culture in 1% starch agar plates. The amyloytic activity was detected by the formation of clear zone around the isolates after addition of Lugol's iodine.

**Characterization of the isolates**
The amyloytic 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.

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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
vials. The cultures were incubated at 40 °C, 50 °C, 60 °C, 70 °C
and 80 °C in orbital shaker at 150 rpm. The OD 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
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
different basal medium M1 (Upton & Fogarty, 1977),
M2, M3 (Pfueeller & Elliot, 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; CaCl2, 0.2; MgSO4.7H2O, 0.5;
K2HPO4, 4.0; (NH4)2SO4, 1.0 and trace metal solution 10 ml
of composition (milligram per liter) : CuSO4.5H2O, 16.0;
FeSO4.7H2O, 100.0; ZnSO4.7H2O, 80.0; MnCl2.4H2O, 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; CaCl2.2H2O, 0.250. pH
adjusted to 7.0 after autoclaving. Medium M3 had the
following composition (gram per liter) : Casein Hydrolysate,
5.0; Yeast Extract, 0.5; K2HPO4, 3.0; KH2PO4, 1.0; Soluble
starch, 1.0; Trace metal 10 ml of the composition (grams per
liter) : FeCl3, 0.3; MgCl2.6H2O, 0.5; CaCl2.6H2O, 0.85;
NH4Cl, 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; KH2PO4, 0.5; MgSO4.7H2O, 0.5;
CaCl2, 0.1; Sterile glycerol, 15% v/v. Medium M5 had the
following composition (grams per liter) : Bacterial peptone,
5.0; Corn starch, 20.0; K2HPO4, 2.0; (NH4)2SO4, 5.0;
MgSO4.7H2O, 1.0; Sodium lactate, 8.0; CaCO3, 2.0;
Inositol, 0.01; Trace metals: 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 ℃. The precipitated solution was
centrifuged at 21,000 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).

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**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 °C, 70 °C and 80 °C. 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 °C. 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 maltotriose) on a pre-coated silica plate activated at 110 °C. 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$_4$, MnSO$_4$, 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                              |
| Colon morphology              | Colony morphology (48-hour incubation on Nutrient broth, 55 °C) | Circular, pale yellow colour, Entire/lobed margin, Flat elevation, translucent with dot like appearance in the centre 2.2-2.5 mm diameter |
| Physiological test            | Growth on Nutrient broth     | Optimum temperature 50 °C, Optimum pH 8.0, Optimum salt tolerance 1 %  |
| Motility in SIM agar          | -ve: voses-proskauer test, gas from fermentation (TSI), phenylalanine deaminase, +ve: nitrate reduction, catalase, oxidase, anaerobic growth |
| Other physiological tests     | +ve: starch, tributyrin, cellulose, gelatin, -ve urea, casein, protease, esculin |
| Biochemical Test              | Substrate hydrolysis         | +ve: glycerol, salicin, dulcitol, mannitol, esculin, sorbitol, pyruvate, erythritol |
| Acids from Sugar              | -ve: maltose, fructose, dextrose, raffinose, galactose, trehalose, melibiose, sucrose, mannose, inulin |
| Utilization of carbohydrates and derivatives | +ve: inulin, sodium gluconate, adonitol, melezitose, xylitol, ONPG, D-arabinose, citrate, malonate, acetate, inositol, sorbitol, arabinol, alpha-Methyl-D-glycoside, rhamnose, cellobiose, alphamethyl-D mannoside, sorbose |
| Antimicrobial 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 °C 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 characteristics 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 °Cd 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 ($\mu_{\text{max}}$) at different temperature. B: Maximum specific growth rate ($\mu_{\text{max}}$) at 50°C and different pH. C: Maximum specific growth rate ($\mu_{\text{max}}$) 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 thermophilic nature of the enzyme.

**Thermostability study and determination of kinetic parameter**

The thermal stability of the enzyme was calculated at 60°C, 70°C and 80°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 $V_{\text{max}}$ 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 $V_{\text{max}}$ 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 (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 |
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 °C while closely associated *A. flavithermus* DSM2641T (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* DSM2641T 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. 3. Amylase activity of medium supernatants after 48-hour incubation

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
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 ℃.
This showed that the enzyme is active over a wide temperature. The enzyme was thermostable with half-life of 51.96 min at 80 °C. Although the optimum growth of A. tengchongensis was 50 °C, the enzyme optimum was at higher temperature of 70 °C. 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 °C for Anoxybacillus flavithermus (Veyes Okumus, 2015), pH 8.0 and 80 °C, Anoxybacillus flavithermus (Agüloǧlu Fincan et al., 2014) and pH 7.0 and 55 °C for Anoxybacillus rupienis (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.

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

The A. tengchongensis RA1-2-1 alpha-amylase showed significant increase in enzyme in the range of 1 mM -10 mM for Ba²⁺, Fe²⁺ and Zn²⁺ ions. The enzyme showed significant inhibition for Ca²⁺, Cu²⁺, Mg²⁺ and K⁺ within the concentration range studied. The presence of metal ions Co²⁺, Mn²⁺, Ni²⁺, Sn²⁺ and Pb²⁺ did not show significant change in activity. Hg²⁺ was completely inhibited at both concentrations. Such complete inhibition of alpha-amylase at 1 mM Hg²⁺ concentration has been reported in amylase from Aspergillus oryzae (Blanja Dec & 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 °C. The enzyme was tolerant to significant metal inhibition except Hg²⁺. 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.

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