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Research Article [Araştırma Makalesi]

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Production and partial purification of α-amylase from *Pseudomonas* sp. 2 under solid-state fermentation

[Katı-durum fermentasyon kullanılarak *Pseudomonas* sp. 2 α-amilaz'ının kısmi saflaştırılması ve üretimi*]

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ABSTRACT

Purpose: The purpose of the present study was to isolate *Pseudomonas* sp. 2 from rhizosphere and screen for best amylase producer and to check for its suitability for industrial applications.

Methods: Four strains of *Pseudomonas* were isolated from rhizosphere soil, by serial dilution method, of which *Pseudomonas* sp. 2 gave best results to α-amylase production. This strain was used to determine the enzyme production by SSF on wheat bran.

Results and conclusion: The optimal enzyme production was obtained at 37 ºC and pH 7.5. Vitamins B_{12} (50 mg) followed by Pyridoxine (50 mg) were found to be enhancing the enzyme production. Amino acids cysteine (30 mg), tyrosine (40 mg) and alanine (30 mg) were stimulating the enzyme production. The enzyme was partially purified by ammonium sulphate precipitation, and was further purified by ion exchange DEAE-cellulose column Chromatography. The purity of the enzyme was 45.2 fold greater than the crude enzyme. The molecular weight of the enzyme was found to be ~62 kDa by SDS-PAGE. This is the first report of any *Pseudomonas* sp. from rhizosphere being used for amylase production. The purified enzyme was not thermostable but it was acid tolerant making it suitable for industrial application.

Key words: *Pseudomonas* sp. 2, α-amylase, SSF, Vitamins, Amino acids, Partial purification.

Conflict of interest: The authors declare no conflicts of interest of any kind.

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ÖZET

Amaç: Çalışamanın amacı Rizofer'den *Pseudomonas* sp. 2 bakterisini izole etmek, bundan en iyi amilaz üretimini bulmak ve bunun endüstriyel aplikasyonlara uygunluğunu kontrol etmektir.

Metot: Seri dilüsyon metodu ile 4 tane *Pseudomonas* suju izole eidilmiştir. Bunların arasından *Pseudomonas* sp. 2 en iyi α-amilaz üretimini vermiştir. Bu suj, buğday kepeğinde SSF ile enzim üretiminde kullanılmıştır.

Sonuçlar ve Tartışma: Optimum enzim üretimi 37 ºC and pH 7.5'da elde edilmiştir. Vitamin B_{12} (50 mg) ve takiben piridoksin (50 mg) muamelelerinin enzim üretimini arttırdığı bulunmuştur. Sistein (30 mg), ıirozin (40 mg) ve alanin (30 mg) amino asitlerinin de enzim üretimini arttırdığı gözlenmiştir. Enzim, amonyum sülfat presipitasyonu ve DEAE-selüloz kolon kromatografisi ile kısmen saflaştırılmıştır. Saflaştırma verimi, ham enzimden 45.2 kez fazladır. SDS-PAGE kullanılarak enzimin molekül ağırlığı ~62 kDa bulunmuştur. Bu çalışma, Rizosfer'den elde edilen *Pseudomonas* sp.'dan ilk defa amilaz elde edilmesi ve kullanılmasını sunmaktadır. Kısmen saflaştırılmış enzim termostabil değildir, fakat aside dayanıklıdır ki bu enzimi endüstriyel uygulamalar için uygun hale getirmektedir.

Anahtar Kelimeler: *Pseudomonas* sp. 2, α-amilaz, SSF, Vitaminler, Aminoasitler, Kısmi saflaştırma.

Çıkar çatışması: Yazarlar hiçbir çıkar çatışması bulunmadığını beyan ederler.

Itroduction

Amylases have been reported to occur in microorganisms, although they are also found in plants and animals. Two major classes of amylases have been identified in microorganisms, namely α-amylase and glucoamylase [1]. Among various extracellular enzymes, α-amylase ranks first in terms of commercial exploitation [2]. Spectrum of applications of α-amylase has widened in many sectors such as clinical, medicinal and analytical chemistry. Besides their use in starch saccharification, they also find applications in baking, brewing, detergent, textile, paper and distillery industry [3-4]. Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques. The aim of the present study was to isolate bacteria from the Rhizosphere, screen for amylase production and select the best producer for enzyme purification and characterization so as to analyze it's suitability for industrial applications.

Materials and Methods

Isolation and Screening of Bacteria from Rhizosphere Soil

Rhizosphere soil from the roots of *Leucas aspara, Coleus aromaticus and Ocimum sanctum* was collected and brought to the lab in Ziploc bags. The *Pseudomonads* were isolated by serial dilution method, plating on King's B agar plates [5] and identified by using standard biochemical tests and protocols listed in the Bergey's Manual for Systematic Bacteriology [6]. Primary screening was done by starch agar plate method.

Inoculum Preparation

Bacterial cultures were transferred from stock to 100-mL sterile distilled water, and the optical density (OD) was read at 660 nm. Accordingly, suspension with an OD_{660} of 0.5 was selected as the standard inoculum size which contains 5.5 x 10^5 cells/mL.

Medium Composition

Wheat bran was procured from Bangalore local market. The substrate was dried and ground into coarse powder with a blender. Production media contained 5 g of solid substrate and 8 mL of sterile distilled water was used to adjust the moisture content in 250 mL Erlenmeyer flasks [7]. Fermentation was carried out for five days. For production of enzymes in SSF, 1 mL of bacterial cell suspension was used as the inoculum.

Optimization of Culture Conditions for Enzyme Production

Effect of Temperature, pH, Vitamins and Amino acids

To ascertain the effect of culture conditions on enzyme production, the present study was carried out on different days, at different temperature (25, 30, 37 and 40 ºC) and pH of the medium (3.5, 4.5, 5.5, 6.5, 7.5 and 8.5). Various concentrations (30-60 mg/ml) of different vitamins (ascorbic acid, thiamine, pyridoxine, nicotinic acid, B12) and amino acids (alanine, glycine, cysteine, tyrosine and arginine) were added to the solid substrate to determine their effect on amylase production.

Enzyme Extraction and Assay

22 mL of distilled water was added to the culture flasks and mixed well in a rotary shaker (200 rpm) at room temperature (25 $\pm$ 2 ºC) for 30 min. Filtered and centrifuged at 10,000 rpm for 10 min. Supernatant was used as the crude enzyme source. Estimation of amylase activity was carried out according to the dinitrosalicylic acid (DNS) method of Miller [8]. 0.5 mL of 1 % starch was incubated with 0.5 mL of enzyme extract with 1 mL of phosphate buffer of pH6.5. The reaction mixture was incubated for 10 min. Reaction was stopped by the addition of 0.5 mL DNS reagent, incubated in water bath for 15 min. and cooled. 2.5 mL of distilled water was added and the absorbance was read at 540 nm with the help of a colorimeter against glucose as the standard. One unit of enzyme activity is defined as the amount of enzyme which releases 1 µmole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min). The experiments were carried out in triplicates. Protein content of the enzyme extracts were estimated by the method of Lowry *et al.* [9], using bovine serum albumin as the standard. Enzyme activity is expressed as specific activity which is equivalent to U/mg protein. All the experiments were carried out in triplicates and the standard error was calculated.

Enzyme Characterization

Amylase purification by ammonium sulphate precipitation

500 milliliter of enzyme extract was used. Solid Ammonium sulphate was slowly added to yield 40 % saturation and the mixture was kept overnight at 4 ºC. The precipitate was collected by centrifugation, dissolved in 0.1 M phosphate buffer (pH 6.5) and dialyzed overnight against 0.05 M phosphate buffer. All the above steps were conducted under freezing temperature and dialyzed sample was lyophilized and stored at -20° C for characterization.

DEAE-Cellulose ion exchange Chromatography

DEAE-Cellulose column was pre-equilibrated with 0.5 M phosphate buffer of pH 6.5 and the column was then washed twice with lower concentration of the same buffer (0.1 M). The enzyme was eluted with buffer containing increasing concentrations of NaCl (45 mM-180 mM), at a flow rate of 1.6 mL/min[10]. The active fractions (15 mL) were pooled and lyophilized.

Molecular Mass Determination by SDS-PAGE

Phosphorylase *b* (97 kDa), albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and lysozyme (14.3 kDa) were used as molecular mass markers. Protein bands were visualized by staining with 2.5 % Coomassie brilliant blue.

Activity Staining

Non denaturing gel electrophoresis for activity staining (PAGE) was performed in a 7.5% polyacrylamide gel containing 0.5M Tris-HCl (pH 9.1) at 4°C. To identify the location of amylase activity (clear zones in a blue background), the gels were incubated for 1 h at 30°C in 2% soluble starch in 0.2M phosphate buffer (pH 6.5) and stained with an acidic solution (0.2% I_2 and 2% KI in 0.2N HCl) [11]. Molecular weight determination by SDS PAGE was determined with a mixture of standards from Bangalore Genei Ltd., catalog no PMWM 105979.

Thermo Stability and pH stability of Amylase

The thermal stability of the enzyme was determined by incubating the partially purified enzyme without the substrate fractions at various temperatures between 30 to 60 ºC for 1 h. At 10 min. intervals, aliquots of 0.1 mL of the incubated enzyme were assayed for activity.

For checking the pH stability, equal volumes of the enzyme and the buffer of different pH (5.0-9.0) were incubated at room temperature overnight (24 hrs). The effect of pH on amylase activity was determined by DNS method using the above enzyme.

Effect of Metal Ions

Enzyme assays were performed using 10 mM concentration of various metal ions (Hg^{2+}, Fe^{2}, $Ba^{2+,}$ Zn^{2+}, Pb^{2+}, Cu^{2+}, Mg^{2+}, Mo^{+}, Li^{2+}, Co^{2+}). The chloride salts of these metal ions ($HgCl_{2,}$ $BaCl_2$, $PbCl_2$), sulphate salts of these metal ions ($MgSO_{4,}$ $FeSO_4$, $ZnSO_4$, $CuSO_{4,}$ $LiSO_4$), nitrate salt of Cobalt [$Co(NO_3)_2$] and ammonium salt of molybdenum. The relative activity of the enzyme was compared with the activity obtained in 0.1 M phosphate buffer.

Statistical Analysis

All experiments were carried out in triplicates. The results were calculated as mean ± standard error (SE) values. Statistical significance was calculated using one-way analysis of variance (ANOVA) and Duncan's test. A value of $p < 0.05$ was taken as statistically significant.

Results and Discussion

Strain Selection

Different strains of *Pseudomonas* were isolated from Rhizosphere soil and they were tested for the production of amylase by the starch hydrolysis test (Figure 1). By rapid screening, one out of five *Pseudomonas* isolates (*Pseudomonas* sp.2) was selected for further studies on α-amylase production.

Effect of pH

For the selected *Pseudomonas* sp.2, (Figure 2) as pH increased, enzyme production also increased with the highest value at pH 7.5. With further increase in pH, enzyme production was found to be decreased.

Effect of Temperature

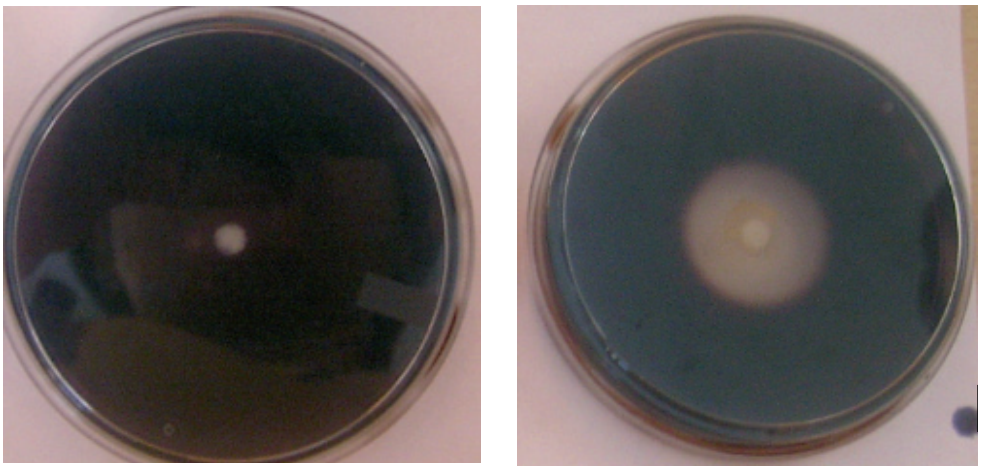
For different incubation temperatures (Figure 3), 37ºC was found to be favorable for enzyme production. At 40ºC there was a decrease in enzyme production. The enzyme production in solid state is greatly affected by the temperature. For the different temperatures tested for enzyme production, there was significant difference at 5 % level of significance.

Effect of Vitamins

Addition of vitamins viz. thiamine, ascorbic acid, pyridoxine, B_{12} and nicotinic acid at different concentrations (30 mg/mL-60 mg/mL) to the medium resulted **(**Figure 4**)** in a considerable change (increase/ decrease) in the production of amylase as compared to the control. Medium supplemented with vitamin B_{12} (50 mg) gave the maximum amylase activity. Vitamin B_{12} (50mg) supplementation enhanced enzyme production by 3 fold, which was found to be statistically highly significant ($p<0.01$). Pyridoxine was the next vitamin which enhanced enzyme production. According to our analysis, media supplemented with nicotinic acid, ascorbic acid and thiamine, at all concentrations, decreased the enzyme production when compared to that of the control. The difference was found to be significant between the control and the treatments.

Effect of Amino acids

Supplementation of amino acids viz. glycine, alanine, cysteine, arginine and tyrosine resulted in substantial difference in the amylase production**.** Cysteine, glycine and tyrosine addition resulted in enhanced enzyme production **(**Figure 5**)**. Supplementation with cysteine (30 mg) gave maximum amylase activity compared to other amino acids such as glycine (at 50 mg conc.) and tyrosine (at 40 mg conc.). We found that alanine was repressing the enzyme production compared to the control. The repression was more as the concentration of alanine increased.



NEGATIVE **POSITIVE**

Figure 1: Rapid screening showing (a) negative (*Pseudomonas* sp. 4) and (b) positive (*Pseudomonas* sp.2)

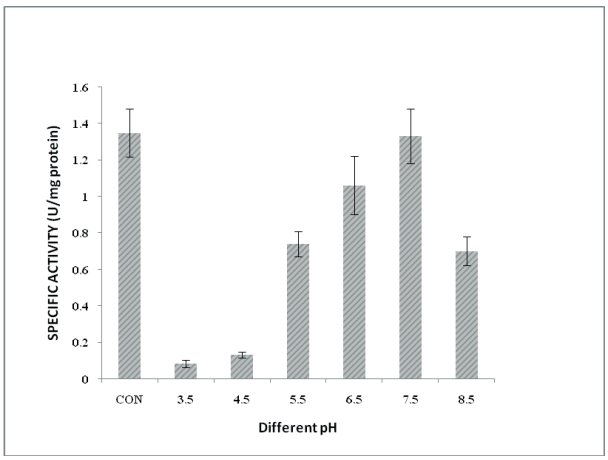


Figure 2. Effect of different pH on α-amylase production by *Pseudomonas* sp2.

(pH 3.5, 4.5 & 5.5- acetate buffer; pH 6.5 & 7.5- phosphate buffer; pH 8.5- Tris-HCl buffer)

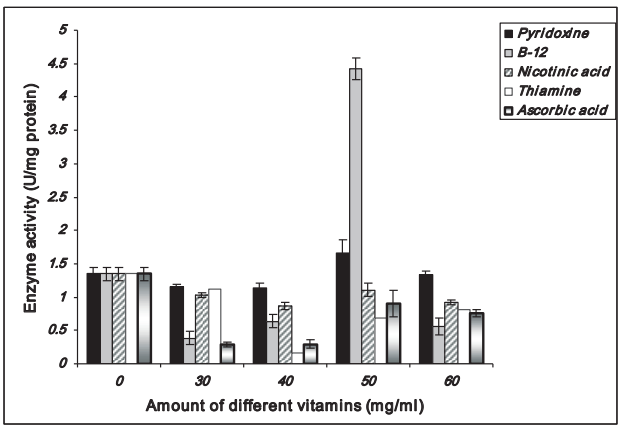


Figure 4. Effect of varying amounts of vitamins on α-amylase production by *Pseudomonas* sp2.

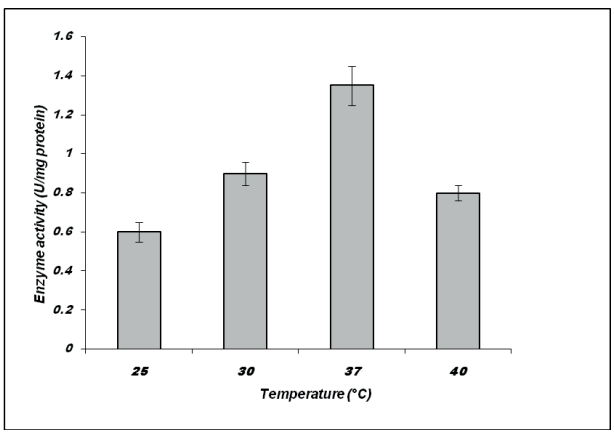


Figure 3. Effect of temperature on α-amylase production by *Pseudomonas* sp2.

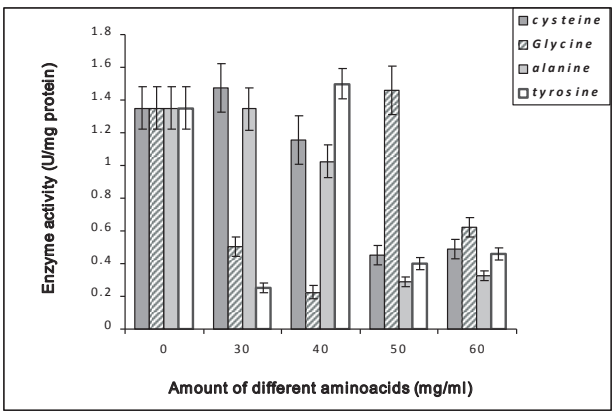


Figure 5. Effect of varying amounts of amino acids on α-Amylase production by *Pseudomonas* sp2.

Ammonium Sulphate Precipitation and Purification

α-Amylase from *Pseudomonas* sp. 2 was partially purified by ammonium sulphate precipitation. The purity of the enzyme was 3.4 fold greater than the crude enzyme (Table 1).

Purification by DEAE-Cellulose Ion Exchange Column Chromatography

α- Amylase was further purified by Chromatography. The purity of the enzyme was 45.2 fold greater than the crude enzyme (Table 1).

SDS-PAGE

The purified enzyme was finally subjected to SDS-PAGE for molecular weight determination. From the gel, it could be predicted that the molecular weight of α- amylase to be ~62 kDa **(**Figure 6**).**

Temperature stability

When the enzyme was subjected to temperature stability studies, it was found that the enzyme was not thermostable (Figure 7). At 30 ºC, 40 ºC and 50 ºC, the enzyme activity decreased with time. At 60 ºC, the enzyme showed least stability retaining negligible activity.

Effect of pH on enzyme stability

Citrate buffer of pH 5.0, 5.5, 6.0, Phosphate buffer of pH 7.0, 7.5, 8.0 and Tris HCl of pH 8.0, 8.5 and 9.0 were used to study their effect of pH on enzyme stability, of which pH 5.5 resulted in maximum enzyme activity (Figure 8)**.** There was a 3.25 fold in enzyme activity after incubating the enzyme for 24hrs with a buffer of pH 5.5. The amylase from *Pseudomonas* sp. 2 was highly stable at pH 5.5.

Effect of Metal Ions on Enzyme Activity

The enzyme activity in the presence of various metal ions was determined and it was found that the enzyme activities were enhanced by all the metal ions (magnesium, mercury, ferrous, barium, lead, copper, cobalt, ammonium and lithium) while zinc had least effect on the enzyme activity (Figure 9).

Discussion

Vitamin supplementation found to have significant difference in amylase production. Productivity was affected by incubation temperature, presence or absence of mineral, amino acids and vitamin supplements. We obtained maximum enzyme production when the solid substrate was supplemented with 50 mg of vitamin $B_{12.}$ There was actually a three fold enhancement in the enzyme activity at this level of vitamin B12 supplementation. This clearly indicates that not only the quality of the nutrient but also its concentration determines the rate of enzyme activity and production by microorganisms. Sodhi et al, [12] reported maximum enzyme production on wheat bran supplemented with Vitamin B complex 0.1 % and other essential nutrients. Sindhu [13] evaluated the effect of vitamins on enzyme synthesis by fungi during SSF by incorporating Folic acid, Ascorbic acid, Nicotinic acid and Riboflavin at 0.1 % w/w level in the medium. The induction in enzyme production by vitamins was reported by Kapoor and Kuhad, [14] in case of polygalacturonase from *Bacillus* sp. Dhake and Patil [15] reported that the addition of vitamins did not affect the enzyme xylanase production. Sapre *et al* [16] reported that vitamins had no influence on xylanase production by *S. recemosum* which is contradictory to our results.

We found the amino acids cysteine, glycine and tyrosine addition resulted in enhanced enzyme production by *Pseudomonas* sp. 2. Amino acids were found to have a significant effect on the production of secreted α-Amylases. Ensari *et al* [17] reported that secreted α-Amylase production was increased by alanine, glutamine, arginine, glycine, leucine, phenylalanine, proline whereas cysteine greatly increased the production, while aspartic acid, asparagine, glutamic acid, lysine, methionine, serine, threonine and tyrosine repressed the α-amylase production. The influence of addition of amino acids and vitamins was studied by Sapre *et al.* [16] who reported the increased production of xylanase by cystein and leucine. Zhang *et al.* [18] reported amino acids stimulate the production of enzymes like α-amylase. They found that glycine addition increased the production of α-amylase by *Bacillus amyloliquifaciens*. Gupta *et al*. [19] and Beg *et al.* [20] reported that amino acids stimulated the production of xylanase.

We found that the effect of addition of vitamin B_{12} alone at 50mg/ml concentration was significantly ($p<0.01$) enhancing the production of α-amylase from our isolate *Pseudomonas* sp. 2, when compared to the addition of other vitamins or amino acids to the solid substrate.

The molecular weight of α- amylase from our isolate *Pseudomonas* sp. 2 was found to be ~62 kDa. This value is very close to that reported by earlier workers. Other researchers found that molecular weight of amylase produced from *Bacillus* sp. KR-8104 as 66 kDa [21], Ueda *et al*. [22] reported that Amy I and Amy II produced from *Eisenia foetida* had molecular weights 60 kDa and Hamilton *et al*. [23] reported that amylase produced from *Bacillus* sp. IMD 435 had a molecular weight of 63 kDa.

At 60 ºC, the enzyme from *Pseudomonas* sp.2 showed least stability retaining negligible activity. Similar

Table 1. Purification summary of α-amylase from *Pseudomonas* sp. 2

Sl. No	Extract	Volume (ml)	Activity (μmoles/ min/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Purification fold (%)
1	Crude extract	492	8.53	6.2	1.375	1
2	Ammonium Sulphate ppt.	24	17.07	3.6	4.742	3.4
3	DEAE cellulose purified	5	112	1.8	62.22	45.2

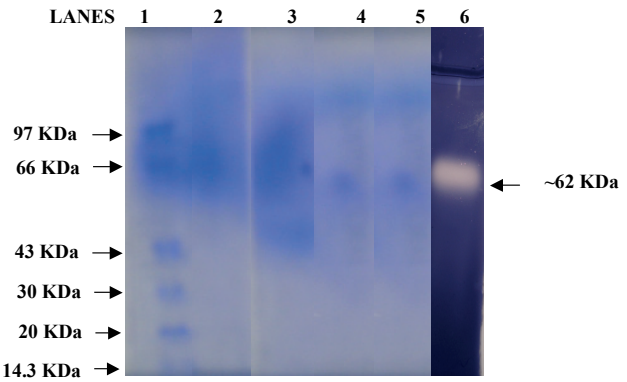


Figure 6. SDS-PAGE of the purified α-amylase. The amylase was visualized by Coomassie Brilliant Blue staining. Lane: 1-Molecular mass marker; Lane: 2 and 3-Crude extract; Lane: 4 and 5 - Purified enzyme; Lane: 6 Zymograph (activity staining)

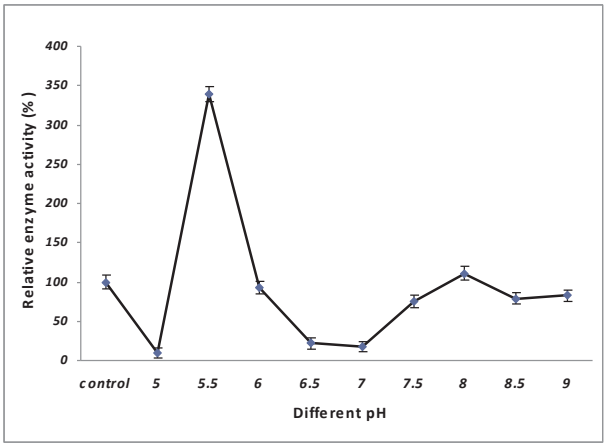


Figure 8. Effect of pH on the stability of α-amylase purified from *Pseudomonas* sp2. Enzyme activity is expressed as percentage relative activity. pH 5.0, 5.5 & 6.0- citrate buffer; pH 6.5, 7.0 & 7.5- phosphate buffer; pH 8.0, 8.5 & 9.0- Tris-HCl buffer)

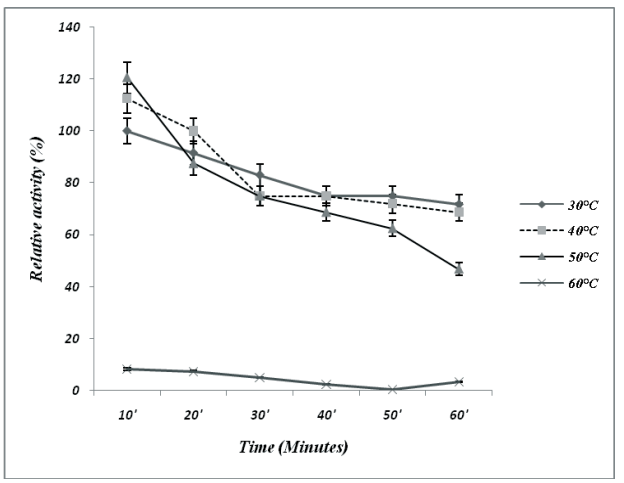


Figure 7. Effect of temperature on the stability of α-amylase purified from *Pseudomonas* sp2 (expressed as percentage relative activity)

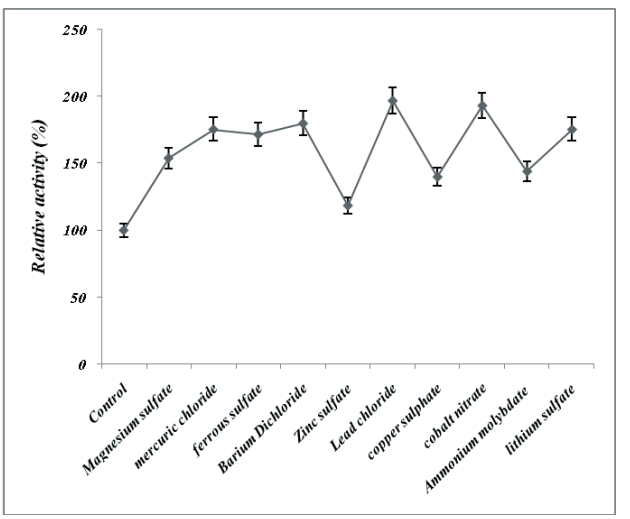


Figure 9. Effect of different metal ions on α-amylase activity (expressed as percentage relative activity).

observations that high temperature inactivation may be due to incorrect conformation due to hydrolysis of the peptide chain, destruction of amino acid or aggregation, were made by earlier workers [5, 24].

The amylase from *Pseudomonas* sp. 2 was highly stable at pH 5.5. This acid tolerance and stability of the enzyme makes it suitable for industrial applications. Goyal *et al* [25] reported that α- amylase from *Bacillus* sp. I-3 was stable at pH range 5.0-5.5 and had optimum activity at pH 7.0.

Most amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} etc. [26]. Ca^{2+} was reported to increase amylase activity of an alkaliphilic *Bacillus* sp. ANT-6 [27]. Calcium ions activated enzyme, while cadmium ions completely inactivated it. α- amylase from our isolate, *Pseudomonas* sp. 2, was activated by most metal ions like, Mg^{2+}, Hg^{2+}, Fe^{2+}, Ba^{2+}, Pb^{2+}, Cu^{2+}, Co^{2+}, $(NH4)^{2+}$ and Li^{2+}, while Zn^{2+} had no effect on it. Zn^{2+} has varied effect on different amylases. Zn^{2+} was reported to inhibit thermo stable α- amylases from a thermophilic *Bacillus* sp. The presence of Zn^{2+} decreased the activity of ANT-6 enzyme [27].

Conclusion

We isolated four strains of *Pseudomonas* from rhizosphere soil, of which *Pseudomonas* sp. 2 gave best results to amylase production. This strain was used to determine the enzyme production. Temperature 37 ºC and pH 7.5 were found to be the best for enzyme production. The enzyme was highly stable at the acidic pH of 5.5, which property could be exploited for its suitability for industrial applications. Vitamins B_{12} (50 mg/ml) was found to be significantly enhancing the enzyme production. Amino acids Cysteine (30 mg), Tyrosine (40 mg) and Alanine (30 mg) were stimulatory. We could establish that not only the quality of the nutrient but also its concentration influences the activity and production of α- amylase by *Pseudomonas* sp.2. The molecular weight of the enzyme was found to be ~62 kDs. This is the first report of *Pseudomonas* species from any rhizosphere soil being used for α-amylase production.

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Conflict of interest: The authors declare no conflicts of interest of any kind.

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