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Production and Characterization of a Thermostable and Ca^{++} Independent Amylase Enzyme from Soil Bacteria

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Authors' contributions

This work was carried out in collaboration between all authors. Author DCS has designed the experiment and author RS has done the experimental work. Author VKS has worked on the manuscript preparation. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Present study was done with the aim of isolation of efficient amylase producing bacterial isolates from soil. Amylase enzyme production, purification, SDS-PAGE and characterization of different important parameters as pH, temperature, substrate concentration etc. was done for obtaining maximum enzyme activity.
Study Design: An experimental study.
Methodology: Isolation was done by serial dilution and spread plate method on nutrient agar at 50ºC and 11 pH. Then screening of bacterial isolates for amylase production was done using starch agar media and on addition of iodine solution bacterial colonies confirmed the amylase enzyme production by decolourization of media from purple to white due to starch hydrolysis. Bacterial isolates with highest zone of starch hydrolysis were selected for enzyme production. Amylase production was done using amylase screening broth at 50ºC, at 250 rpm for 72 h in

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incubator shaker. Enzyme activity was determined after centrifugation of the culture broth at 10,000 rpm for 10 min. Identification of bacterial isolate was done by performing different morphological and biochemical tests. Then enzyme purification was done using acetone and ammonium sulphate precipitation methods. Molecular weight determination was done by SDS-PAGE. For obtaining maximum enzyme activity evaluation of optimum values for important parameters such as pH, substrate concentration, temperature, reaction time, thermostability, effect of cations, effect of chelating agents and different raw starches was done.

Results: Bacterial isolate 16A showed the highest zone of starch hydrolysis and maximum amylase production (48.86 UmL^{-1}) was obtained on 48 h of incubation at 50ºC. On the basis of morphological and biochemical characterization 16A was identified as *Bacillus sp.* Enzyme showed 1.6 fold purification with acetone precipitation and 1.54 fold with ammonium sulphate precipitation. Molecular weight of amylase enzyme determined was 97.4 kD approximately, by SDS-PAGE. Enzyme characterization showed that maximum enzyme activity was obtained at 15 minutes reaction time, 75% substrate concentration, 7 pH and 50ºC temperature. A good thermostability was showed by amylase enzyme for 24 h at 50ºC and 15 h at 100ºC. The enzyme activity was enhanced by Fe^{++} (2 X), Mn^{++} (3 X) and Triton X-100 (2 X) while was completely inhibited Zn^{++}, Hg^{++}, Cu^{++}, Fe^{++}, Co^{++}, SDS, tween-20 and EDTA. On enzyme characterization maximum enzyme activity of 138 UmL^{-1} has been observed that is a very good activity.

Conclusion: On the basis of results during present study this is visualized that the enzyme is very suitable for many industrial applications because of its desirable qualities for its industrial applicability *as* Ca^{++} *independent nature and thermostability at 80-100ºC.* The broad range of pH and moderate thermostability makes this amylase enzyme a useful additive to liquid detergents that can function in hard and warm water. Calcium independent amylase is suitable for manufacturing of fructose syrup, where Ca^{++} is an inhibitor of glucose isomerase. So this enzyme can be very useful in different industrial applications.

Keywords: α- Amylase; thermostability; production; enzyme characterization, SDS-PAGE.

1. INTRODUCTION

Amylases are among the most important industrial enzymes that degrade starch and related polymers to yield dextrin and other smaller products. Intensive research has been performed with focus on the isolation of thermostable and thermoactive amylases from thermophiles and hyperthermophiles. The α-amylase (1, 4-glucan-4-glucanohydro-lase; EC 3.2.1.1) family consists of a large group of starch hydrolases and related enzymes, currently known as glycosyl hydrolase family 13 [1]. Starch is the most abundant form of stored polysaccharides in plants. Starch constitutes an inexpensive source for production of syrups containing glucose, fructose and maltose that are widely used in food industries [2].

Thermostable α-amylases have had extensive commercial applications in brewing, baking, sugar production, paper industry, desizing in textile industries and detergent manufacturing processes etc. [3]. Their commercial production from microorganisms represents 25–33% of the world enzyme market [4]. Many microorganisms are able to produce amylases including *Bacillus subtilis, Lactobacillus, Escherichia, Proteus, B. licheniformis, Bacillus steriothermophilus, Bacillus megaterium, Strepotmyces* sp., *Pseudomonas* sp. *etc*. α- amylases from genus *Bacillus* have been extensively studied in terms of structure, function, secretion, and industrial applications [5-6]. Production of thermostable amylases is of special interest as they could be used for saccharification processes occurring at high temperatures [7] and for instance, a reduction in cooling costs, a better solubility of substrates, reduced risk of microbial contamination, resistant to denaturing agents, solvents, and proteolytic enzymes [8].

The present study was outlined with the aim of isolation of a thermostable amylase producing bacterial isolates at 50ºC and 11 pH from soil sample. Production, purification, molecular weight determination and characterization of important parameters for maximum amylase enzyme activity were done. It resulted in the isolation of a*n efficient amylase enzyme producing bacterial isolate* and showed enhanced enzyme activity with optimized values of parameters.

2. MATERIALS AND METHODS

2.1 Isolation

Amylase producing bacteria were isolated from the garden and park soil samples of C.C.S. University, Meerut, UP. Isolation was done by serial dilution and spread plate method on nutrient agar at 50ºC and 11pH. The pure cultures were obtained and preserved on agar slants and in glycerol stocks at 4ºC and -20ºC, respectively for further study.

2.2 Screening for Amylase Production

The plates containing amylase screening medium (yeast extract 4 g, K_2HPO_4 1 g, $MgSO_4$ 0.5 g, soluble starch 5 g, agar 20 g, distilled water 1L, 11pH) were point inoculated with bacterial isolates and incubated at 50ºC for 24-48 h. The plates were then flooded with Lugol's iodine to detect the zone of starch hydrolysis around the colony. The bacterial culture 16A, showing maximum zone of hydrolysis was selected for further study.

2.3 Identification of 16 A

For identification of bacterial isolate 16A different morphological and biochemical tests were performed according to Bergey's manual.

Morphological tests- By performing Gram's staining cell shape and arrangement were observed. Endospore staining was done to determine the presence of endospore. It is very important for classifying bacteria.

Biochemical tests- For identification of 16A different biochemical tests were performed as-indole, methyl red, voges proskauer, simmon's citrate agar, oxidase, catalase, nitrate and mannitol fermentation.

2.4 Enzyme Production

The inoculum was prepared by inoculating a loop full of amylase producing bacterial culture in amylase screening broth (50 ml) and incubated at 50ºC, at 250 rpm for 24 h in shaker incubator. Amylase screening broth (50 ml) was inoculated with 1% inoculum (CFU≈10^8/ml) and incubated at 50ºC in incubator shaker at 250 rpm for 72 h. Periodically 6 ml of sample was withdrawn from flask after 24 h, 48 h and 72 h of incubation. After centrifugation of culture broth, cell-free culture filtrate was obtained of at 10,000 rpm for 10 min. This cell-free culture filtrate was used as the source of extracellular amylase and enzyme activity was determined.

2.5 Enzyme Assay

Enzyme in the cell-free culture filtrate was assayed by determining the amount of glucose liberated from starch using DNSA (Di-nitro Salicylic Acid) reagent [9]. The reaction mixture containing 0.5 mL appropriately diluted enzyme and 0.5 mL of starch (0.5%) as substrate (in phosphate buffer, 11pH) was incubated for 10 min at 50ºC. Then 1mL DNSA was added and incubated at 100ºC for 8 minutes in a boiling water bath followed by the addition of 0.4 mL freshly prepared sodium potassium tartarate, mixed thoroughly and optical density was measured at 540 nm with spectrophotometer (Jenway, 6305). A standard curve was drawn with glucose levels according to Miller [10].

2.6 Enzyme Purification

Purification of the amylase was performed using conventional methods of purification by acetone and ammonium sulphate precipitation. In acetone method chilled acetone (-20ºC) was used at different saturation levels (0-10%, 10-30%, 30-60% and 60-90%) at 4ºC, followed by overnight (12 h) storage at –20ºC. After centrifugation at 10,000 rpm for 20 min, the precipitate was recovered and dissolved in minimum amount of sodium phosphate (0.01 M) buffer (7pH) and dialyzed against the same buffer. In second method, ammonium sulphate also was used at different saturation levels (0-10%, 10-30%, 30-60% and 60-90%). The precipitates obtained after each trial were dissolved in minimum amounts of phosphate buffer (0.1 M, 7pH) and the enzyme activity and specific activity of each sample were determined after dialyzing again.

2.7 Protein Estimation

Extracellular protein content was determined by Lowery method [11]. In Lowry method 0.1 mL of enzyme was taken in sterilized test tubes, 0.1 mL of 2N NaOH was added. Test tubes were incubated at 50ºC for 5 min in a boiling water bath and cooled to room temperature. Then 1 mL of freshly prepared mixed complex reagent was added and left at room temperature for 10 minutes. Then 0.1 mL of freshly prepared Folin reagent was added and mixed thoroughly and

left at room temperature for 30-60 min. The optical density of blue coloured complex was directly measured at 550 nm.

2.8 Molecular Weight Determination of Amylase

The purity of the enzyme was ascertained by SDS-PAGE while its activity was demonstrated on native PAGE using 10% gels. The gel was stained with Coomassie Brilliant Blue R-250 [12] for overnight and then stained with silver nitrate to visualize the number of protein bands [13].

2.9 Effect of Different Parameters on Enzyme Activity

2.9.1 Reaction time vs. enzyme activity

In order to find out the optimum reaction time, enzyme activity was determined by incubating the reaction mixture at (50ºC) for different time intervals (1, 3, 5, 10, 15, 20, 30, 40, 50 and 60 min).

2.9.2 Effect of substrate concentrations

For determining the optimum substrate concentration soluble starch solutions of varied concentrations (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0%) were prepared in buffer and used in reaction mixture. Then enzyme activity was determined by incubating the reaction mixtures for 15 min at 50ºC.

2.9.3 Effect of pH on enzyme activity

To find out the effect of pH on enzyme activity was assessed by maintaining the pH of reaction mixture from 3-10 (3, 4, 5, 6, 7, 8, 9 and 10) by using various buffers (0.1M). Specific enzyme activity was determined by incubating the reaction mixtures for 15 min at 50ºC.

2.9.4 Effect of temperature on enzyme activity

To determine the effect of temperature on the enzyme activity reaction mixtures were incubated the at different temperature range from 30 to 100ºC. Then enzyme activity was determined by incubating for the reaction mixtures for 15 min at 50ºC.

2.9.5 Thermo-stability

Thermo-stability of enzyme was determined by incubating 25 mL of suitably diluted enzyme sample at 50 and 100ºC (optimum and maximum) over a period of 24 h and then enzyme activity was assayed as above mentioned.

2.9.6 Effect of divalent cations on enzyme activity

Effect of cations was studied by incubating the reaction mixtures after adding different cations (Ca^{++}, Co^{++}, Mn^{++}, Fe^{++}, Cu^{++}, Hg^{++}, Zn^{++} and Mg^{++}) at concentration of 1mM and 5mM. Enzyme activity was measured after incubating for 15 min at 50ºC.

2.9.7 Effect of detergents on enzyme activity

To determine the effect of detergents on the enzyme activity SDS, Tween-20, Tween-80, triton X-100 and EDTA were used. Reaction mixtures were incubated after adding different detergents for 15 min at 50^{o}C and followed by enzyme activity determination.

2.9.8 Effect of stabilizers on thermo-stability

Effect of stabilizers on the enzyme activity was studied by incubating the reaction mixtures with different stabilizers (starch, glucose, raffinose, and glycerol) for 15 minutes at 50ºC and followed by enzyme assay.

2.9.9 Evaluating the efficiency of amylase on different raw starches

Enzyme activity was checked on different flours (gram, wheat, millet, rice, water chest, oat, corn, and starch as control). For this reaction mixtures were incubated with above flours and starch at concentration of 0 .75% for 15 minutes at 50ºC. Flours were collected from local market.

3. RESULTS AND DISCUSSION

From thirty soil samples 52 thermophillic bacterial isolates were obtained at 50ºC and 11pH. Out of them 12 isolates were amylase enzyme producers. On the basis of measurement of zone of hydrolysis of 12 isolates, four isolates 16A, 8A, 21A and 11B with highest zone of hydrolysis (Fig. 1) were selected for determination of enzyme activity.

Among four bacterial isolate (16A) was selected on the basis of enzyme titre, the enzymes titre was quantified with the help of standard curve of glucose (Table 1). Bacterial isolate 16A was

used for amylase production under submerged fermentation in starch yeast extract broth. On the basis of morphological study (Table 2) bacterial isolate16A was found to be gram positive rod and endospore forming bacteria (Fig. 2). On the basis of biochemical characterization (indole, methyl red, simmon's citrate agar, oxidase, catalase, nitrate and mannitol fermentation) as Bergey's manual 16A was identified (Fig. 3) as *Bacillus* sp.

According to literature *Bacillus* produces a large variety of extracellular enzymes, among them particularly amylases are of considerable industrial importance [14]. In present study highest enzyme production from *Bacillus* sp. was observed at 48 h of incubation. Similarly, several

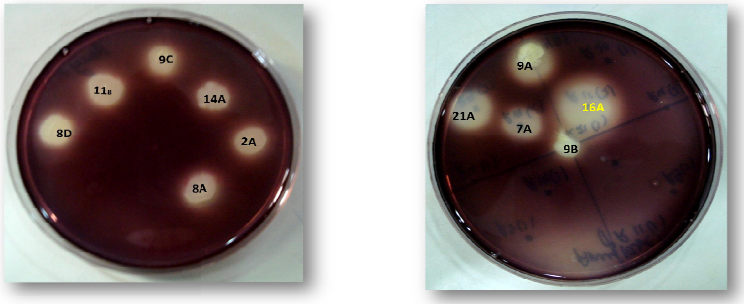

Fig. 1. Showing starch hydrolysis zones on starch agar by different soil isolates

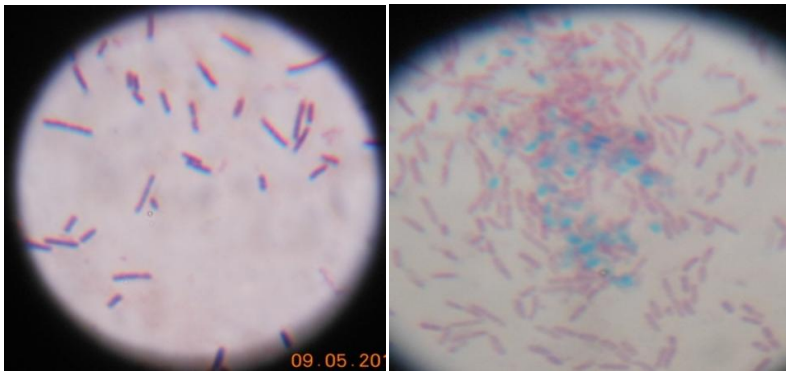

Fig. 2. Showing gram's positive, rod shape bacterial cells, endospore is present with green color and bacterial cell with pink color

Fig. 3. Showing results of IMVIC- First set showing Indole –ve, second set showing MR –Ve and VP ±Ve and third set showing citrate +Ve test

Bacillus sp. (*B. coagulans* and *B. licheniformis*) are reported to require a time of 48-72 h for optimum amylase production [9]. Asgher et al. [15] also reported the α-amylase production from *B. subtilis* JS-2004 was highest at 48 h declining gradually up to 96 h.

Further partial purification of enzyme was done using acetone and ammonium sulphate. In acetone, best activity was observed in 0-10% fraction (1.61 U/mL) with 8.06 yield and 1.6 fold purification. In ammonium sulphate best activity was also observed in 0-10% fraction (1.43 U/mL) with 6.08 yield and 1.54 fold purification (Table 3).

Similarly, Ashwini et al. [16] reported that partially purified amylase from *Bacillus marini* on ammonium precipitation exhibited specific activity of 0.035 U/mg which corresponds to 5.80 fold purification and 60.0% Yield. Krishanan and Chandra also reported that amylase enzyme from *Bacillus liquiformis* showed the best activity in 30-65% fraction with overall yield of 42% and 212 fold purification [17].

Although the molecular weights of microbial α-amylases are usually reported between 50 to 60 kD range [18], while in our experimental study electrophoretic analysis of third fraction of acetone precipitate, the molecular weight of amylase was estimated 97.4 kD approximately. Here a clear zone of starch hydrolyses is present in the zymogram study that is corresponding to the single band obtained in SDS-PAGE analysis near the top of the gel. The band is within the molecular weight range of 97.4 kD according to marker lane (Fig. 4).

Table 1. Showing enzyme titre of 3 isolates under submerged fermentation at different incubation periods

Strain no.	Activity (UmL^{-1}) (24h)	Activity (UmL^{-1}) (48h)	Activity (UmL^{-1}) (72h)
16A	40.96	48.86	39.02
8A	31.53	36.68	30.25
21A	23.71	28.97	20.71

Table 2. Showing result of morphological and biochemical tests for the identification

S. no.	Tests	Result
1	Gram staining	+
2	Endospore staining	+
3	Indole	-
4	Methyl Red	+
5	Voges proskauer	+
6	Simmon's Citrate Agar	+
7	Oxidase	-
8	Catalase	+
9	Nitrate	-
10	Mannitol fermentation	+

Table 3. Stepwise purification details of amylase enzyme produced by 16A

Steps	Volume (mL)	Activity (U/mL)	Total activity (U)	Protein (mg/L)	Total protein (mg)	Sp. enzyme activity (U /mg)	Yield (%)	Fold purification
Culture filtrate	200	48.86	9772	6.14	1228	7.95	100	1
Acetone (0-10%)	12	65.66	787.9	5.18	62.16	12.67	8.06	1.6
Amm. sulphate (0-10%)	10	59.47	594.7	4.83	48.30	12.26	6.08	1.54

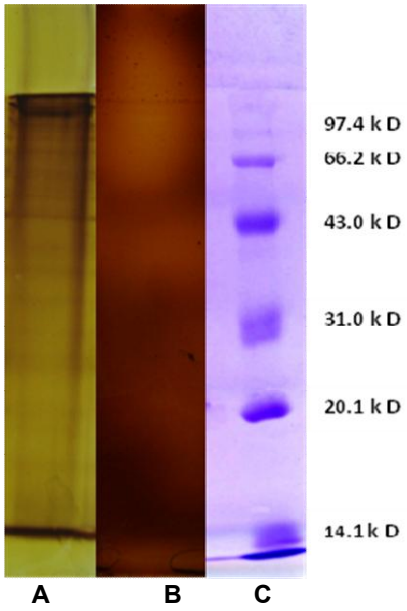

Fig. 4. PAGE analysis of the culture filtrate of (16A)

A. Partially purified protein (Acetone precipitate), B. Zymogram, C. Marker lane.

In support, Dobara Abou et al. [19] reported two α-amylase isoenzymes with a high molecular weights of (135-145 kD) were obtained from *Thermoactinomyces vulgaris*. Aguloglu and Enez also reported the molecular weight of purified α-amylase from *G. Stearothermophilus* was 63 kD as estimated by SDS-PAGE [20]. However, molecular weight of some α-amylases was found to rise owing to carbohydrate moieties [21].

3.1 Effect of Different Parameters on Enzyme Activity

For gaining the maximum of enzyme activity optimization of different cultural parameters is very important. These parameters significantly affect the enzyme activity, among many incubation time, substrate concentration, temperature and pH are very important. So in our experimental optimum values of different parameters was determined. On incubation of reaction mixtures for varying time intervals (1, 3, 5, 10, 15, 20, 30, 40, 50 and 60 min), maximum enzyme activity around 67 U/mL was observed at incubation period of 15 minutes (Fig. 5). Enzyme activity was reduced at 10 minutes of incubation time and decreasing after 15 minutes gradually.

The optimum substrate concentration was determined 0.75% with maximum amylase activity of 66 U/mL. There was a clear reduction of amylase activity below 0.75% substrate concentration (Fig. 6) while a good activity was obtained on increasing concentration.

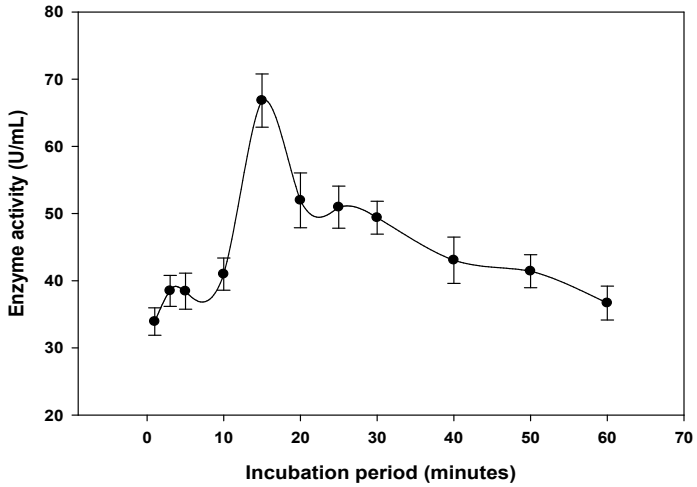

Fig. 5. Effect of incubation time on amylase activity and maximum activity is obtained at 15 minutes

Similar result was reported by Fang and Demain with a maximum activity of 66.13 U/mL at optimum substrate (starch) concentration of 0.75% [22]. Others have reported different optimum concentrations as 1.67% [23], between 2–3% [24] and moreover 0.1% (w/v) [25].

Most raw starch degrading enzymes had optimum pH in the acidic to neutral range [26,27]. In the present study maximum enzyme activity (54.9 U/mL) was observed at 7pH. There was a great variation in enzyme activity with change in pH and enzyme was active in 6 – 9 pH range. Amylase activity remained 72.70% between pH 3-5 (Fig. 7).

Our results are coinciding with Uchino and Katano, they reported that α-amylases produced by thermophilic *Bacillus* sp. was active at pH range from 5.5 to 8.5 [28] and at pH 7.0 [29]. While amylase from *T. vulgaris* showed a pH activity profile with a flat top which retaining more than 75% of the enzyme activity in the pH range 5.0-9.0, despite it was completely inhibited at 4pH [19]. In addition, the optimal activities of the purified enzymes were found to have pH optimum of 4.2 and 4.5 for GA1 and GA2 [30].

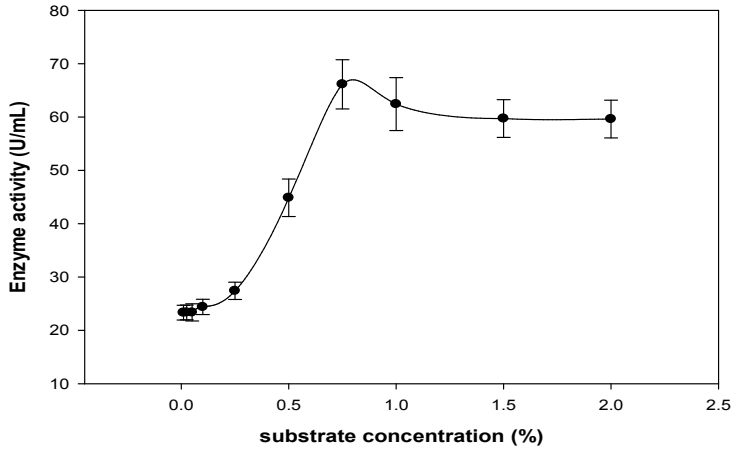

Fig. 6. Effect of substrate concentration on amylase activity and maximum activity is at 0.75% substrate concentration

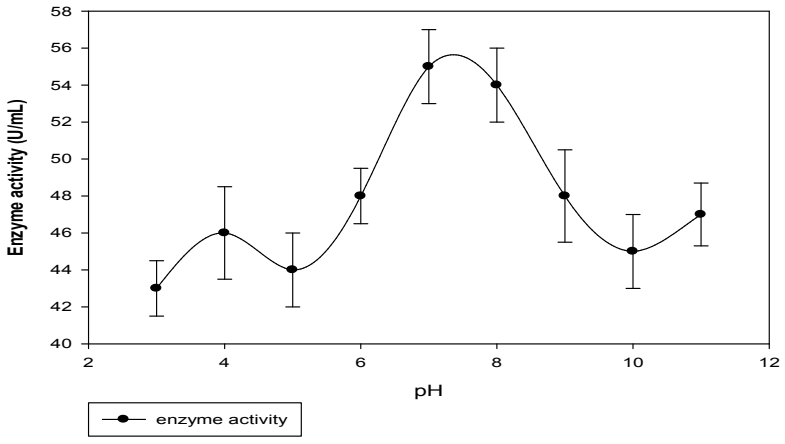

Fig. 7. Effect of pH on amylase activity and maximum enzyme activity is at 7pH

Temperature is a vital environmental factor which controls the growth and production of metabolites by microorganisms and this is usually varied from one organism to another [31]. Bacterial isolate '16A' gave the highest enzyme titre (48.86 U/mL) in submerged fermentation at 50ºC and is active up to 60ºC. The activity decreased around 23.8% in a range of 60-80ºC (Fig. 8).

In support of this study *Bacillus amyloliquefaciens, B. subtilis, B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus sp*. reported to produce α-amylase at temperatures 37–60ºC [32,33,34, 35]. A wide range of temperature (35-80ºC) has been reported for optimum growth and α-amylase production in bacteria [36,37,38,39].

The amylase enzyme showed a great promise for the saccharification process, so its stability was studied at various temperatures. The effect of temperature on amylase activity and stability was measured by incubating the reaction mixture at 50 and 100ºC. The data obtained suggested that the enzyme was stable up to 24 h at 50ºC and the enzyme was stable for 15 h at 100ºC (Fig. 9).

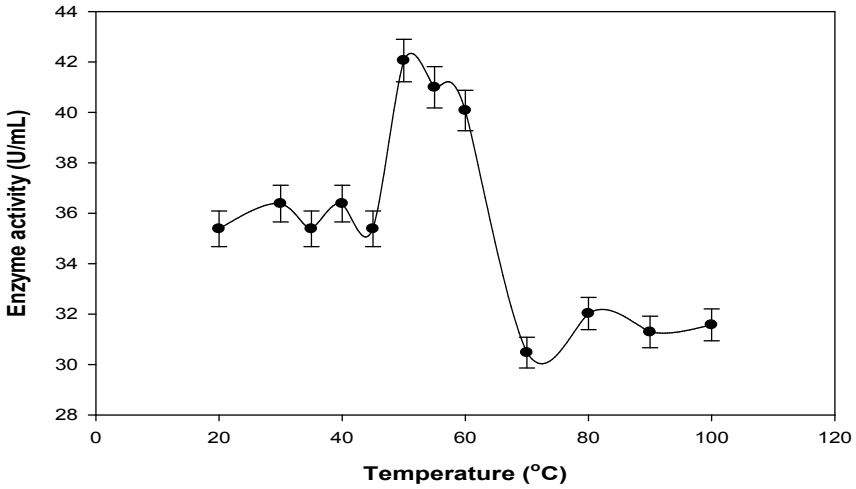

Fig. 8. Effect of temperature on amylase activity with maximum at 50ºC

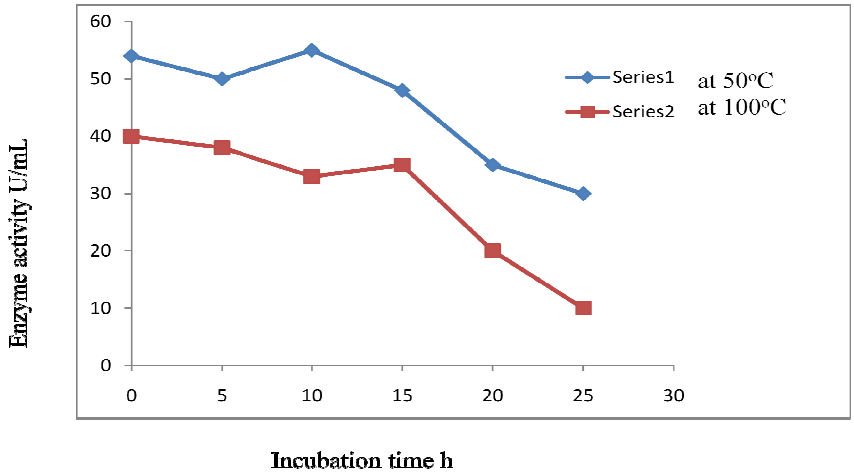

Fig. 9. Temperature stabilities at different time of incubations (blue and red line showing stability at 50ºC and 100ºC respectively)

α- amylases from *Bacillus* genus are heat stable and this is a desirable property for industrial starch liquefaction.

Dobara Abou et al. [19] reported that amylase enzyme from *T. vulgaris* was fairly stable at 50ºC over 6 h and with a half-life of about one hour at both 60ºC and 70ºC. The enzyme retained 100% of its activity at 80°C when incubated for 1 h. Aguloglu and Enez also reported that the enzyme was stable between 50 and 60ºC at the end of 2 h and it lost 50% of its activity at 70ºC [40]. Similar result was also reported that optimum temperature of α-amylase from *B. subtilis* JS-2004 was 70ºC, which is comparable to that described for other *Bacillus* α-amylases [36,19,41,39,42].

It is proposed that α-amylases belong to a new class of metalloenzymes characterized by a prosthetic group i.e., an alkaline-earth metal rather than a transition element and which plays primarily a structural role [38]. Ca^{++} had significant effects on the metabolism and physiology of bacteria. Ca^{++} had also showed an effect on enzyme activity and stabilization in defence against amylase [43,44,45]. Production of calcium independent and acid stable α-amylases has been attempted by protein engineering resulted in to Termamyl LCe. Termamyl LCe was produced via site-directed mutagenesis that has shown high calcium independence [46]. In the present study during experiment the activity of enzyme was increased by addition of Mn^{++} (3X) and Fe^{++} (2X). Enzyme activity was decreased by Zn^{++} (65.23%) while completely inhibited by Hg^{++}, Cu^{++}, Fe^{++} and Co^{++} (Fig. 10).

However, in current study Ca^{++} did not show any effect on enzyme activity. Similar results are obtained by *B. thermooleovarans*, *B. coagulans*, *B. licheniformis*, *B. sp. WN1*, etc. [19,47,48,49]. Mn^{++} and Fe^{++} showed a good enhancing effect on amylase activity. So this enzyme is *Ca $^{++}$ independent*, *active in acidic pH range and stable at 80-100°C* that is desirable qualities for its industrial applicability (starch saccharification).

Enzyme activity was enhanced in presence of Triton X-100 (76.19%), while completely inhibited in presence of SDS, tween-20 and EDTA. No significant effect of Tween- 80 was observed on amylase activity (Fig. 11).

In support Asoodeh et al. [50] determined that α-amylase activity increased with Triton X-100. Shafiei et al. [51] determined that α -amylase enzyme was quite stable against 0.5% SDS, 0.2% Triton X-100, Tween 80 and Tween 20 detergents. Similar result was observed that enzyme activity was increasing with TritonX-100 [40].

However, carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch are very expensive for commercial production of these enzymes. So enzyme activity on different flours was observed in order to find its applicability in industrial use (Fig. 12).

No enzyme activity was shown in presence of gram, wheat, millet flours while enzyme showed a good activity on kuttu, rice, water chest, oat

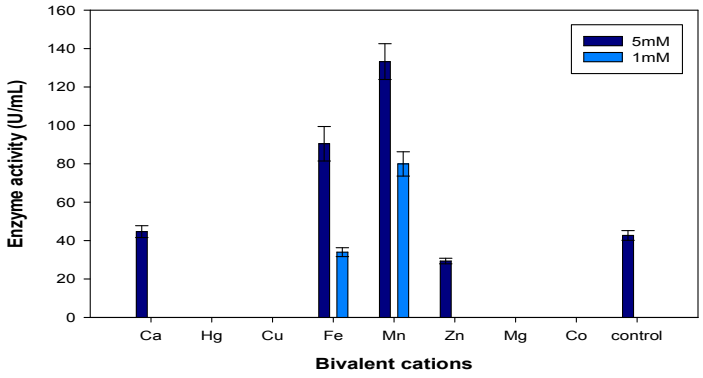

Fig. 10. Effect of different metal ions on amylase activity and enzyme activity is enhanced with Mn^{++} and Fe^{++}. Ca^{++} showed a neutral effect neither enhanced nor reduced the enzyme activity

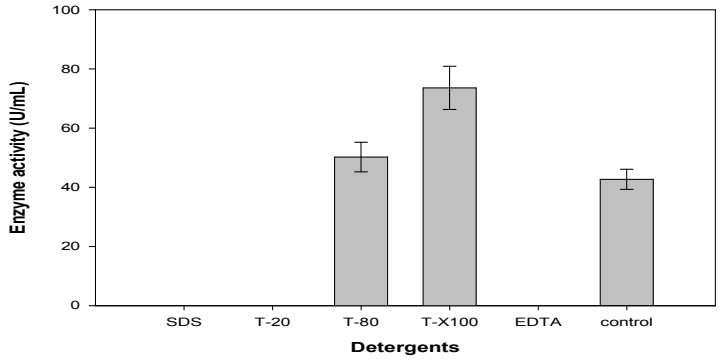

Fig. 11. Effect of detergents on amylase activity and Triton X-100 is enhancing the activity while SDS, T-20, EDTA are completely inhibiting the activity

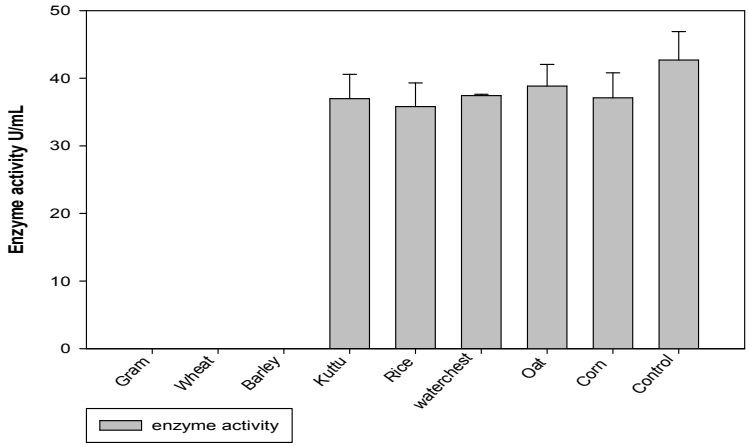

Fig. 12. Effect of different raw starches on amylase production and enzyme showed good activity on kuttu, rice, water chest, oat and corn as with purified starch (control)

and corn approximately in similar range (35-38 U/mL) as with purified starch (control). Best activity was seen in oat. While Oleveria *et al.* reported the best activity (1.59 U/mL) on corn starch [52].

4. CONCLUSION

Thermostable α-amylases are of immense importance at commercial applications in brewing, baking, sugar production, paper industry, desizing, textile industries and detergent manufacturing etc. [48]. For example, a thermophillic substitute for the mesophillic α-amylase used in cake baking industry can be advantageous because of stability at high temperature. The broad range of pH stability and moderate thermostability makes amylase enzyme useful additive to liquid detergents which must function in hard and warm water. Ca^{++} had significant effects on the metabolism and physiology of bacteria and on enzyme activity and stabilization in the defence against amylase [43,44,45]. Calcium independent amylase is suitable for the manufacture of fructose syrup, where Ca^{++} is an inhibitor of glucose isomerase [49]. So, on the basis of above discussion it is visualized that the enzyme with its characteristic features of *thermostability and Ca^{++} independent nature* will be suitable for many industrial applications.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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