

Screening, Production, Partial purification & characterization of Bacterial extracellular Alpha amylase enzyme

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ABSTRACT: α -Amylase (EC 3.2.1.1) is an enzyme which breaks down alpha bonds of large, alpha-linked polysaccharides starch and glycogen, producing glucose and maltose. 4 bacterial species were primary screened on Starch hydrolysis Agar medium for its amylase activity. This enzyme was optimally active at pH 7.0 and temperature at 37°C retained 81% of its maximal relative % activity. Many enzymes require metal ions for maximal activity. If the enzyme binds the metal very tightly or requires the metal ion to maintain its stable, native state, it is referred to as a metalloenzyme. Most of the alpha-amylases are metalloenzymes, which require calcium ions (Ca^{++}) for their activity, structural integrity and stability. The enzyme activity of alpha amylase from *Bacillus subtilis* was strongly activated by Ca^{++} (5mM) however it was also strongly inhibited by ethylene diamine tetra acetic acid (EDTA) and partially inhibited by Cu^{++} .

Key words: Extracellular Amylase, *Bacillus subtilis*, Submerged fermentation, Shake flask study.

Introduction:

Enzymes are catalysts produced by a variety of living organisms ranging from bacteria, fungi, actinomycetes, yeasts, plants and humans, the microbial source generally meet the industrial demand.^{1,2} Different types of amylase are present depending on the bond they are breaking in starch molecule. They are α -amylases, β -amylases and iso-amylases etc. Alpha amylase (endo-1,4-D-glucose-D-glucosylase 3.2.1.1) belongs to the family of endo-amylases that randomly cleave the 1,4-D-glycoside linkage between adjacent glucose units in the product chain retaining the α -anomeric configuration in the product.³ Glucoamylase (exo-1,4- α -D-glucan glucohydrolase) hydrolyzes single glucose units from the nonreducing ends of amylose and amylopectin in a stepwise manner.^{4,5} These are calcium metalloenzymes, which are completely unable to function in the absence of calcium. Calcium stabilizes the interface between the central A domain (291 residues) with (β/α)₈ barrel structure and the more variable B domain (104 to 206 residues).^{6,7} The production of microbial amylases from bacteria is dependent on type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, metal ions, pH, temperature and time of incubation.

In the present day scenario, amylases have a great commercial value in biotechnological applications ranging from food, fermentation, textile to paper industries. These uses have placed greater stress on increasing amylase production and search for more

efficient processes.^{8,9,10}

This work describes the effects of culture conditions on amylase production in batch experiments in shake flasks and under controlled conditions in a laboratory incubator. In this study, we show that enzyme synthesis is affected by pH, Temperatures, activators and inhibitors. The optimum enzyme production by the bacterial isolate was found at 37°C & 42°C, whereas maximum enzyme activity was observed at 37°C. The enzyme was activated by Ca^{2+} (relative activity 27.95%). It was strongly inhibited by EDTA, but less affected by Mg^{2+} and Cu^{2+} .

Materials and methods

Iodine, Potassium iodide, Hydrochloric acid, Sodium hydroxide, Sodium acetate, Potassium dihydrogen phosphate purchased from Research - Lab Fine Chem Industries, India, 3,5-dinitrosalicylic acid (DNS) were obtained from Sigma Chemical Co. India. Other chemicals like potassium chloride, glacial acetic acid, KH_2PO_4 , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, CuSO_4 and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, sodium chloride, EDTA were used are Analytical grade.

Nutrient broth, starch, peptone, yeast extract, Agar-Agar were obtained from Hi-media.

Organism Used:

Bacillus subtilis, *Bacillus cereus*, *Bacillus coagulans*, *B. Magisterium*.

Primary Screening for Amylase Activity (Starch Iodine Test)

Pure culture of different organisms was aseptically streaked on starch agar plates with starch as the only

carbon source. After incubation at 37°C for 48 hrs., individual plates were flooded with Gram’s iodine (Gram’s iodine- 250 mg iodine crystals added to 2.5gm potassium iodide solution, and 125ml of water, stored at room temperature) to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue colour forms, which is the basis of the detection and screening of an amylolytic strain. The organisms which were showing maximum zone of clearance in starch agar plates¹¹ were maintained on to nutrient agar slants and making glycerol stocks kept it at -20°C for further study.

Preparation of seed culture

A glycerol stocks were used in the present studies. 1 ml of glycerol stock of each strain was aseptically inoculated in to sterilized 50 ml inoculums medium in 250 ml Erlenmeyer flask and cotton plugged containing nutrient broth 13 g/l, pH 7.4 ± 0.2 (It was sterilized in an

autoclave at 15 lbs/inch² pressure at 121°C for 20 min.)The flask was incubated 24hrs at 37°C and 150 rpm in a rotary shaking incubator.

Enzyme production in shake flask

Amylase production was carried out by submerged fermentation. Inoculated 10 ml of 24 hrs seed culture in 500 ml of the sterilized production medium in 2000 ml Erlenmeyer flask and cotton plugged contained (g/L) starch 10, peptone 10, yeast extract 20, KH₂PO₄ 0.05, MnCl₂.4H₂O 0.015, MgSO₄.7H₂O 0.25, CaCl₂.2H₂O 0.05 and FeSO₄.7H₂O 0.01. The flask was kept on a rotary shaker incubator at a speed of 150 rpm at different temperatures 37°C ,40°C and 45°C for 24 hrs,48hrs and 72 hrs. After incubation, fermented broth was centrifuged at 4°C ,8000 rpm for 15 min in a cooling centrifuge. Supernatant was collected and used for the estimation of amylase and protein.

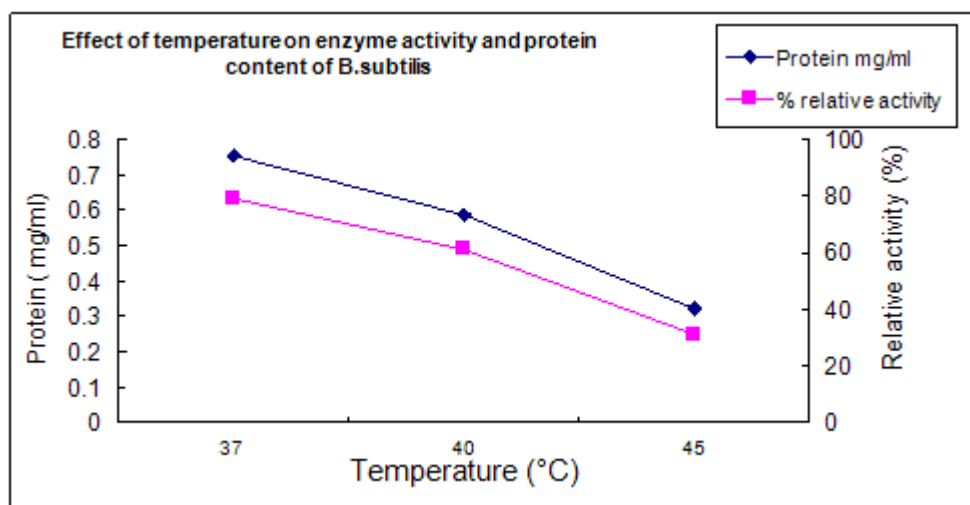


Figure 1 Effect of temperature on enzyme activity and protein content

Protein Estimation

The protein content of the extract was determined following Lowry’s method¹⁴ with bovine serum albumin as standard. 2 ml of analytical reagent was added to 0.2 ml suitably diluted test samples (enzyme solution). The mixture was mixed well and allowed to stand for 10 min at 50°C. Then 0.2 ml of the folin-ciocalteau reagent was added and shaken to mix well and incubated at room temperature for about 30 min. Optical density of the reaction mixture was measured at 600 nm, against a blank prepared with 0.2

ml buffer. A standard curve was constructed with each experiment using bovine serum albumin as a known protein. The amount of the soluble protein was calculated from the standard curve of as mg protein per ml of test samples. The protein estimated at different time intervals such as 24 hrs,48 hrs and 72 hrs and at different incubation temperatures such as 37°C ,40°C and 45°C. On the basis of protein content, it was decided that the optimum incubation time required for the production of Alpha amylase in given conditions of respective bacteria.

Table: 1 Protein content in enzyme Extract

Incubation temp	Incubtion time	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>
(°C)	(hrs)	protein mg/ml	protein mg/ml
37	24	0.462	0.531
	48	0.753	0.498
	72	0.749	0.486
40	24	0.458	0.692
	48	0.586	0.681
	72	0.573	0.613
45	24	0.386	0.723
	48	0.321	0.716
	72	0.296	0.681

*Average value of two experiments.

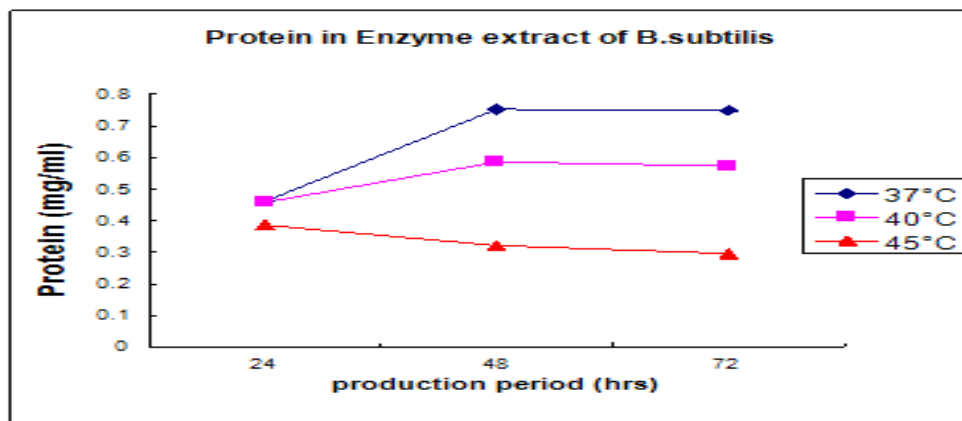


Figure 2 Protein content in enzyme Extract of *B.subtilis* at different Incubation time & temperature .

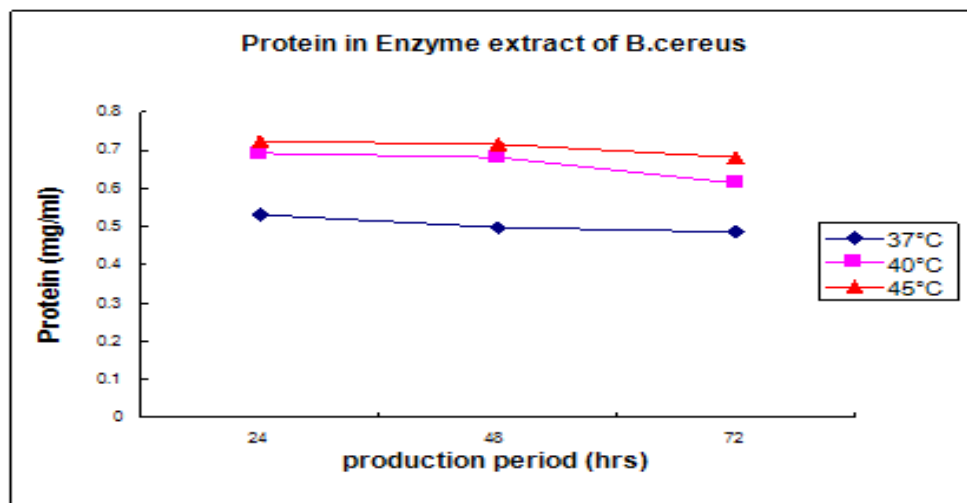


Figure 3 Protein content in enzyme Extract of *B.cereus* at different incubation time & temperature .

ASSAY OF ENZYME:-

In enzyme assay ,the rate of reaction can be known by measuring the amount of substrates (s) utilized or the amount of product (p) formed in unit time.

Enzyme activity are usually expressed in terms of specific activity which is defined as the amount of substrates utilized or product formed per mg protein per minute at definite temperature.

Amylase activity was determined according to the method of Bernfeld (1955)¹². The enzyme assay mixture contained 0.5 ml of enzyme extract, 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.0 and 1.0 ml of 1% starch. The reaction mixture was incubated for 15 min at 37° followed by arresting the reaction by the addition of 1.0 ml of dinitrosalicilic acid reagent (DNS reagent). The optical density was read at 540 nm against reagent blank after addition of 3.0 ml of distilled water.

ENZYME UNITS

One unit of enzyme activity (DUN) is defined as the quantity of enzyme that causes 1% reduction of blue colour intensity of starch-iodine solution at 37°C in 1 min¹³.

Partial Purification of Alpha Amylase from *B.subtilis*:

A)Ammonium Sulphate Precipitation:

Solid ammonium sulphate ¹⁵was added to 1L of the culture supernatant at 80 % saturation. After incubating overnight at 4 °C, and centrifugation 10,000 rpm for 30 min at 4 °C , the re suspended precipitate in 25 ml 20mM Phosphate buffer (pH 6) was dialyzed overnight against the same buffer at 4°C and re centrifuged. The supernatant was used as partially purified enzyme.

B)Acetone Precipitation¹⁶

The crude enzyme extract was taken in a glass beaker and to it chilled acetone was added slowly, with continuous stirring, up to 70% (v/v) concentration and kept at 20°C for 4 h to allow protein precipitation. The precipitates were then harvested by centrifugation at 4°C and 10,000 rpm for 30 min. The pellet thus obtained was resuspended in 34 mL of 20 mM Phosphate buffer (pH 7) to allow the Solubilization of proteins.

The unsolubilized proteins were then removed by centrifugation at 4°C,

10,000 rpm for 30 min. Supernatant was then subjected to dialyzed overnight against same buffer at 4°C. The protein content and enzyme activity were determined.

TLC analysis of the products

Starch hydrolysates was analyzed by TLC. The reaction mixture contain 1 ml of enzyme solution and 1 ml of 1% soluble starch in 4 ml of 0.1M sodium acetate buffer were incubated at 60 °C for 30 min and reaction was stopped by heating reaction mixture in boiling water bath for 10 min. 80 µl of reaction mixture was applied along with glucose and maltose as reference standards

was applied onto pre coated silica gel plates . The plates were developed with Acetonitrile : water (85:15) . After developing hydrolysates, sugar spots were made visible by applying 2% H2SO4 used and TLC plate were heated 10 minutes at 100°C for detection of spots .¹⁸ Sugar spots of hydrolysates were identified by comparing their Rf values with those similarly obtained for standard sugar spots.

Determination of optimum substrate concentration of Alpha amylase from *B.subtilis*:

For the determination of optimum substrate concentration for maximum enzymatic activity, It was measured at different concentrations of starch (0.1% to 4.0 %). Then assay was carried out as mentioned earlier, the enzyme assay mixture contained 0.5 ml of enzyme extract, 0.5 ml of 0.05 M sodium phosphate buffer, pH 7.0 and 1.0 ml of 1% starch. The reaction mixture was incubated for 15 min at 37° followed by arresting the reaction by the addition of 1.0 ml of dinitrosalicilic acid reagent(DNS reagent). The optical density was read at 540 nm against reagent blank after addition of 3.0 ml of distilled water.¹⁹

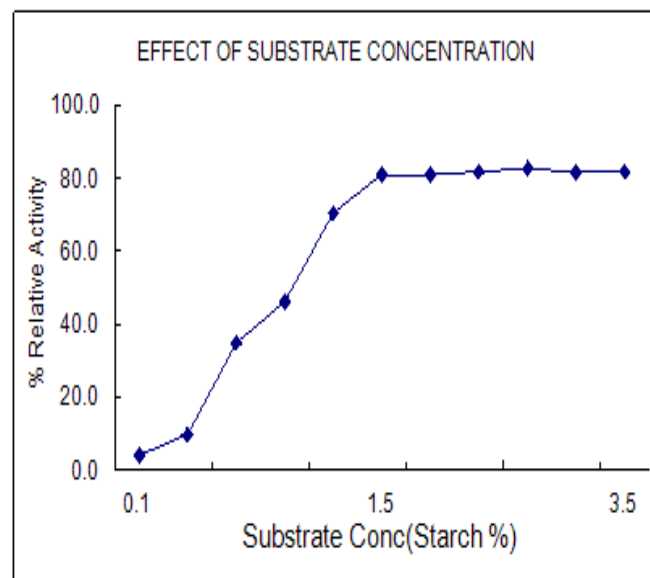


Figure 4 Effect of substrate concentration on enzyme activity

Table: 2 Effect of substrate concentration on enzyme activity.

Sr. No.	Substrate concentration Starch (%)	Activity (U/ml)*	%Relative Activity
1	0.1	2.6	4.1

2	0.2	6.4	9.7
3	0.5	22.8	34.8
4	0.7	30.2	46.2
5	1	46.1	70.5
6	1.5	53.0	81.0
7	1.8	53.0	81.0
8	2	53.6	81.8
9	2.5	54.1	82.7
10	3	53.5	81.8
11	3.5	53.5	81.8

*Average value of two experiments.

Effect of temperature on activity of purified Alpha amylase from *B.subtilis*

For determination of optimum temperature for activity of the crude and purified α -amylase, The assay was carried out as per the procedure from the incubation temperature of 25°C to 60°C at pH 7.0 with an incubation period of 10 min¹⁷.

Table: 3 Effect of temperature on activity of purified Alpha amylase from *B.subtilis*

Sr.No	Temp. 0C	Activity (U/ml)*	% Relative Activity
1	25	35.73	55.92
2	30	41.96	65.67
3	35	51.78	81.04
4	40	49.19	78.99
5	45	39.86	62.39
6	50	34.01	53.22
7	55	32.30	50.56
8	60	27.56	43.13

*Average value of two experiments.

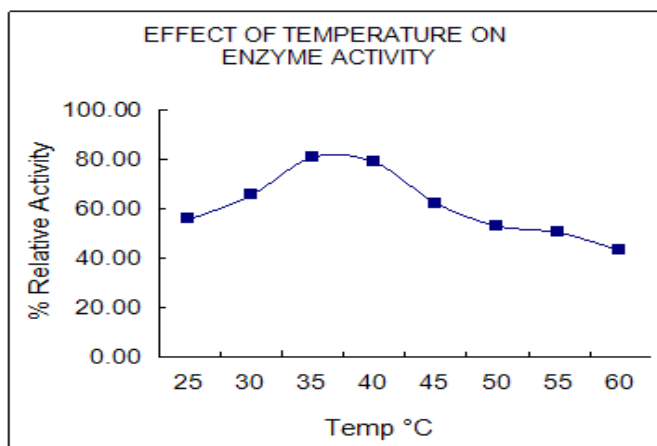


Figure 5 Effect of temperature on enzyme activity .

Effect of pH on activity of purified Alpha amylase from *B.subtilis*

The effect of pH on activity of purified Alpha amylase, buffer solution from pH range of 3.5 to 10 were prepared by using Acetate buffer of pH 5.0; Phosphate buffer of pH 6.0; and alkaline borate buffer in the pH range of 8 to 10. For pH 4 and pH 7, buffer tablets were used; then, the catalytic activity of the enzymes in the above buffers was determined with starch as substrate then the assay was carried out for each buffer solution¹¹ with an incubation period of 10 min.¹⁷

Table: 4 Effect of pH on activity of Alpha amylase from *B.subtilis*

Sr.No	pH	Activity (U/ml)*	% Relative Activity
1	3.5	17.6	26.7
2	4	32.0	52.7
3	5	42.7	64.8
4	6	48.6	73.7
5	7	53.4	81.0
6	8	50.7	77.0
7	9	39.0	59.2
8	10	21.9	33.2

*Average value of two experiments.

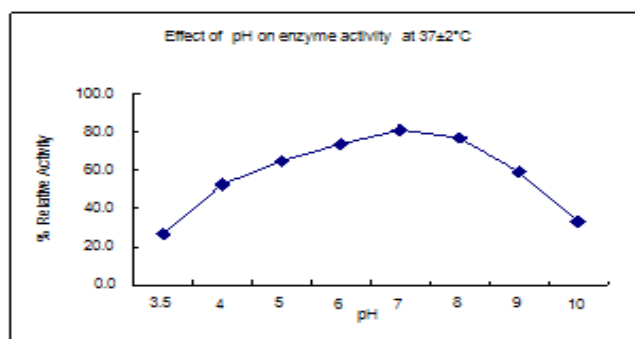


Figure 6 Effect of pH on Alpha amylase activity .

Effect of metal ions and inhibitors on activity of purified Alpha amylase

The effect of various metal ions like Mg²⁺, Ca²⁺, Cu²⁺ and EDTA on enzymatic activity of purified α -amylase carried out by adding various concentration from 1 to 10 mM. The volume taken was 1ml in reaction mixture described in the assay and the assay was carried out at 37°C and optimum pH was taken 7.0

Table: 5 Effect of metal ions and inhibitors on activity of Alpha amylase

Sr.No	Concentration (mM)	% Relative activity				
		control	Mg ⁺⁺	Ca ⁺⁺	Cu ⁺⁺	EDTA
1	1	81.04	84.3	94.8	76.83	63.04
2	5	81.04	85.1	99.7	80.77	59.76
3	10	81.04	85.1	96.6	78.30	57.13

*Average value of two experiments.

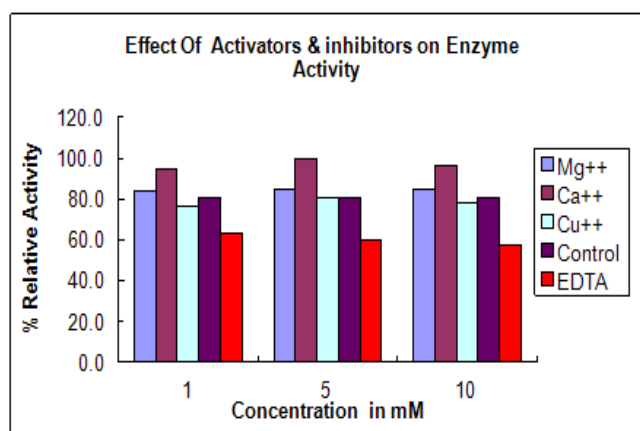


Figure: 7 Effect of Activators & inhibitors on enzyme activity.

RESULT AND DISCUSSION:

Primary screening:

Bacteria isolates were able to hydrolyze starch showing maximum zone of hydrolysis around the growth on agar medium supplemented with soluble starch indicate that the organism has ability to produce alpha amylase enzyme.

Following are the two bacterial isolates was gave zone of clearance around the colonies.

1. *Bacillus subtilis*
2. *Bacillus cereus*

After primary screening these two organisms taken for further study.

Development of seed culture :

Seed cultures were developed from glycerol stock in Nutrient broth on 150 rpm at 37°C for 24 hrs.

Enzyme production in shake flask

For the enzyme production inoculate 5% of 24 hrs grown seed culture into production medium . After inoculation incubate the flasks at different temperatures such as 37°C ,40°C & 45°C for 24 hrs ,48hrs and 72 hrs .

After estimation of amylase and protein it was observed that *B.subtilis* gave high protein content 0.753mg/ml at 37°C within 48 hrs while *B.cereus* gave high protein content 0.723 mg/ml at 45°C within 24hrs. On the basis of protein content of both the organisms at different incubation time and temperature it was observed that the protein content of *B.subtilis* was higher than *B.cereus*.

Effect of temperature on purified enzyme activity and protein content

Production of amylase by the *B.subtilis* was optimum at 37°C temperature and as the temperature increased, there was gradual decrease in the protein content and enzyme activity (Figure 1). At 45°C, the production of amylase and protein content was extremely low.

The crude enzyme extract was subjected to ammonium sulphate precipitation and by chilled acetone. Considerable loss of amylase activity was observed with acetone. On the other hand, ammonium sulphate precipitation resulted in good yield with an increase in ~4 folds purity of alpha amylase. Hence, ammonium sulphate precipitation was selected for fractionation of amylase, from the crude enzyme extract.

TLC analysis of the products

TLC analysis of reaction products of *B.subtilis* amylase on starch showed that glucose and maltose as main products. The appearance of maltose and glucose as major hydrolysis product indicates that the amylase produced by this organism is of α-type.¹⁷

Optimization of substrate concentration

For the determination of optimum substrate concentration for purified Alpha amylase substrate, solution of soluble starch in the concentration range from 0.1% to 3.5% was used. Figure 4 and Table 2 Justifying that as the substrate concentration was increased above 0.1%, rate of reaction increased progressively up to 1.5%. When concentration increased above 1.5%, the rate of reaction remained constant suggesting that Alpha amylase obtained from *B.subtilis* followed the Michaelis Menten equation. Hence, optimum substrate concentration was found to be 1.5%, as in Graph justifying that, at this concentration enzyme remained saturated with substrate for 10 min and rate of reaction was maximum at this concentration.

Effect of temperature on the activity of purified α -amylase isolated from *B.subtilis*

The initial relative activity of purified α -amylase increased to 81.04% as the temperature increased up to 37°C (Fig. 5). Maximum activity was obtained at 37°C and pH 7.0 for the substrate starch (15g/L-1). Above 37°C, α -amylase activity was decreased sharply. Hence 37°C was chosen as the optimum temperature for the assay of purified α -amylases.

Effect of pH on the activity of purified α -amylase

Among physical parameters, pH of the growth medium plays an important role in enzyme secretion. The pH range observed during the growth of microbes also affects product stability in the medium²⁰. Most of the earlier studies revealed an optimum pH range between 6.0 and 7.0 for the growth of bacterial strains and enzyme production^{2,22,23}. Previous studies have revealed that bacteria required neutral pH for optimum growth²⁴. So, the effect of initial pH on the production of amylase by *Bacillus subtilis* was investigated at different pH (3.5-10). The maximum activity of the enzyme was obtained at pH 7 as shown in figure 6. at acidic & alkaline pH the enzyme activity was extremely low (Table 4). It might be due to the fact that the enzyme was inactive in the acidic & alkaline medium^{25,26} reported that, α -amylase production at pH 7.0 by the *Bacillus subtilis* was maximum. Another study conducted by El-Tayeb O et al showed that alpha amylase production by *Bacillus subtilis* in fermentor was highest at pH 7.0²¹

Effect of metal ions and EDTA on purified alpha amylase activity:

Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca⁺⁺, Mg⁺⁺ etc. was reported to increase alpha amylase activity of an *Bacillus sp.*^{1,6}. Concerning the effect of some ions on the relative activity of purified alpha amylase from *Bacillus subtilis*, the result in figure 7 demonstrated that Ca⁺⁺ (5

mM) and Mg⁺⁺ (5mM) enhanced the enzyme activity by 18.63% and 4.0% respectively.

As shown in Table 5, the addition of EDTA and Cu⁺⁺ decreased enzyme activity by approximately by 27% & 2.7% respectively.

It was found that metal ions may stimulate the enzyme activity by acting as a binding link between enzyme and substrate combining with both and so holding the substrate and the active site of the enzyme.

CONCLUSION

Enzyme synthesis was affected by pH, temperatures & incubation period. The optimum temperature enzyme activity was found at 37°C, whereas maximum enzyme activity was observed at pH 7. The *Bacillus subtilis* α -amylase observed more activated by using Ca²⁺, which could make the enzyme from *B.subtilis* more suitable for future use in various industries. It can be concluded that, *B. Subtilis* can be a potential producer of extracellular amylase which could find applications in industry. Due to the importance of these findings, further studies need be carried on in order to commercialize the production process.

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