EXTRACTION, PURIFICATION, AND CHARACTERIZATION OF THERMOSTABLE ALPHA-AMYLASE

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ABSTRACT

Microbes are a special capability in producing a wide range of biologically essential substances possessing numerous importance. Several enzymes like proteases, cellulases, lipases, and amylases are synthesized through microbes possessing numerous importance. The Amylases degrade starch by eliminating glucose, maltose, as well as another oligosaccharide. *The industrial enzymes' particular properties may be derived from microbes by optimizing method parameters with through enzyme engineering.* The various industrial enzymes are synthesized with the use of Bacillus. *Amylases are a group of enzymes it hydrolyses starch. Thermostability is an essential property of amylases that are useful in industrial activities. Among bacteria Bacillus species like B. licheniformis as well as B. amyloliquefaciens were well producers of thermostable amylases. The thermostable enzymes are important, such as decreased cooling value and lesser contamination chances.*

Keywords: ***Starch, α-amylase, Optimization, Thermostable, Extraction, Purification, Characterization.***

INTRODUCTION

Enzymes are very essential bioproducts required for life on the planet. Enzymes are regarded as magnificently particular along with potent biological catalysts for various kinds of biochemical reactions [1]. Microbes are a special capability to produce a wide range of biologically essential substances possessing numerous importance [2]. It consists of vitamins, antibiotics, bioactive substances, enzymes, etc. Determining different industrial importance from microbes is various it is derived from various ecological niches [3]. Enzymes are biocatalysts because of their capability to support reactions rapidly including effectively. Several enzymes are synthesized through microbes for industrial applications [4]. They have characteristics such as varied functionality, including constancy over pH and temperature ranges [5]. Industrial enzymes are properties that may be derived from microbes by optimizing method parameters through enzyme engineering [6]. Several enzymes like proteases, cellulases, lipases, and amylases are synthesized through microbes possessing numerous importance. Industrial enzymes are obtained from microbes, plants in addition to animals. The various industrial enzymes are synthesized with the use of Bacillus. Amylases are a group of enzymes it hydrolyses starch [7,8]. Starch it is a main plentiful structurally various organic polymer; each organism on the planet is mainly based on an eventual source of energy polysaccharides of nutrition including expansion. Starch is contained two polymers, such as unbranched amylose (including α-1,4-D-glucosidic attachment) along with branched amylopectin (including α-1,4-D-glucosidic linkages as well as α-1,6-D-glucosidic attachment). Starch is the glucose polymer, that is produced through a broad range of species of the plant [9]. The Starch granules consist of two kinds the α-glucans, amylose as well as amylopectin, all-inclusive describing 98–99 percent of complete dry weight. Amylose is an unbranched linear molecule polymer attached through α-1, 4 glycosidic bonds and amylopectin is a water-soluble highly branched polymer consisting of α-1, 4 jointed linear chains along with α-1, 6 jointed side chains it produced a starch compound. The α-Amylases are is capable to

degrade α-1,4-D-glucosidic linkages of the starch compound. The Amylases degrade starch by eliminating glucose, maltose, as well as another oligosaccharide. The enzymes mostly α-amylase from several microbial origins it is the main important component for industries [10,11]. α-Amylase is of various importance in sugar production for liquefaction in addition to starch gelatinization, detergent industries, biofuel formation, textile, pharmaceuticals as well as analytical chemistry. The wild-kind α-amylases need certain important alterations to tolerate serious surrounding situations [12]. Acidophilus, psychrophiles, alkalophilic, thermophiles, halophiles, as well as piezophiles, are certain extremophiles that show balance in less pH, greater pH, elevated temperature, less temperature, greater salt concentration, as well as greater osmotic pressure, accordingly. Prevailing operations need environments like pH as well as temperature for microbial growth for the formation of an enzyme. Majorly microorganisms used in industrial activities are neutrophiles including mesophiles, that may not tolerate severe environmental states including need particular environments. The extremophiles are important, they can decrease the threat of pollution. That may also decrease the value of water as well as energy, which is necessary for managing sterility. Industrial formation of bio-products includes upstream along with downstream methods. The Thermophilic enzymes show thermostability above 55 °C, extreme thermophilic enzymes show stability above 75 °C, as well as hyperthermophilic enzymes, show stability above 90 °C. Thermostability is an essential property of amylases that are useful in industrial activities. Among bacteria Bacillus species like B. licheniformis as well as B. amyloliquefaciens were well producers of thermostable amylases. The thermostable enzymes are important, such as decreased cooling value and lesser contamination chances [13].

Material and methods

Isolation of thermostable bacteria from compost piles

Sample collection

For the isolation of thermotolerant bacteria for thermostable amylase, production samples were collected from compost piles from different agricultural fields of nearby locations in the Kopargaon area. Samples were collected into sterile plastic bags [14].

Enumeration of bacteria

Samples collected from different locations were examined individually. Samples were serially diluted in 0.85 percent saline solution and dilutions were further carried up to 10^{-1} to 10^{-5}. After dilution 100 µl diluted suspension was poured into the surface of the Nutrient agar plate from the last dilution i.e., 10^{-5}, and spread by an „L" shaped spreader aseptically.

The bacteria can thus be isolated and counted by C.F.U i.e., Colony Forming Unit.

C.F. U=No. of colonies/inoculum size (g) X Dilution Factor

The above-mentioned procedure was repeated for all the samples.

Incubation conditions at varying temperatures

As the goal is to isolate thermophilic bacteria the incubation temperature was kept varying from 450C to 90 0C. For all the samples and after incubation no. of colonies were counted and CFU was calculated for all the samples. The overall procedure was repeated in triplicate.

Screening of bacterial isolates for amylase production

The thermostable bacteria that were grown at high temperatures were selected for further analysis. The selected thermostable isolates were screened for their protentional to hydrolyze starch. The starch hydrolysis indicates the synthesis of amylase enzyme by that bacterium. The assay was carried out using slandered plate assay methods. Diluted culture of screened isolates was inoculated in starch agar plates and after overnight incubation zone of hydrolysis was observed after adding iodine solution to the culture plate, clear zone surrounding the colony against a blue background indicates starch hydrolysis and confirms the amylase synthesis by bacteria. The extent of amylase synthesis depends upon the diameter of the zone larger the zone greater the amylase synthesis as well a smaller zone indicates a lesser amount of amylase synthesis. The protentional isolates were further continued for amylase production.

Laboratory-scale production of amylase

Laboratory-scale production of amylase was carried out by using standard starch-containing media. The potential isolate which was found effective for the hydrolysis of starch was further carried out for laboratory-scale production of amylase. The sterile starch-containing media was inoculated with bacterial isolate and kept for incubation at 37 0C for 5 days. During the incubation reducing sugar was calculated by the DNS method at regular time intervals to check the hydrolysis of starch [15].

Extraction and purification of amylase

The enzyme was purified by ammonium sulphate precipitation followed by dialysis and a 100-ml sample of culture filtrate was centrifuged at 8000 rpm for 20 min at 4°C to remove the cells and the supernatant brought to 60% ammonium sulphate saturation at 4°C for 12 h in an ice bath. The precipitated protein was collected by centrifugation at 8000 rpm at 4°C and dissolved in a minimum volume of phosphate buffer (0.1 M, pH 7.0). The enzyme solution was dialyzed at 4°C against the same buffer for 24 h at 4°C with continuous stirring and three changes of the same buffer. the extracted and partially purified amylase was further analyzed for enzyme activity, total protein content, specific activity, folds of purification and percent yield, etc [16].

Enzyme assay (Enzyme activity and Specific activity)

Estimation of amylase activity was carried out according to the DNSA (3, 5 dinitro salicylic acid) method. One ml of 1% starch was incubated with different dilutions of the enzyme extract and 1ml of citrate-phosphate buffer (pH 6.0). The reaction mixture was incubated at 50°C for 30 min. The reaction was stopped by adding 2ml of DNS and kept in a boiling water bath for 10min. The absorbance was read at 540nm using a Spectrophotometer (Shimadzu, Thermoelectric cell holder, S-1700), against glucose as the standard. One unit of enzyme activity is defined as the amount of enzyme, which releases 1µmole of reducing sugar as glucose per minute, under the assay conditions (U/ml/min). The experiments were carried out in triplicates and standard error was calculated.

Estimation of protein content

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Thermostability of amylase

The thermal stability of the enzyme was assessed by incubating the enzyme without the substrate at various temperatures between 30 to 60ºC for 2h. The enzyme was taken at every 30 min intervals and was assayed for activity, thus assessing the stability of the enzyme at different temperatures.

pH stability of amylase

The stability of the enzyme in different pH was assessed by incubating the enzyme for 2 hrs in buffers of different pH (citrate phosphate buffer for pH 5-8 & Tris-HCl buffer for pH 9). The stability of the enzyme was investigated by checking the enzyme activity every 30 min

Effect of Metal Ions

Enzyme activity was assayed in the presence of 5mM and 10mM concentrations of various metal ions (Na^+, Mg^{++}, Ca^{++}, and Co^{++}) in chloride salts. The relative activity of the enzyme was compared with the activity obtained in 0.1M citrate phosphate buffer.

Stain removal

As amylase has been used for several purposes in different industries like food, feed, and laundry, also it is commonly used in detergents to remove food-based stains. The partially purified amylase was accessed for its protentional was accessed by using the tube method and cloth method [17].

Tube method

In the tube method two test tubes each containing 9 ml, 1% starch solution were taken, then in both tubes 1 drop of dye was placed then in one test tube partially purified enzyme was added that tube was labeled as a test then another without enzyme was considered as a control. After incubation coloration was observed. Coloration indicates a positive test whereas the absences of coloration indicate a negative test.

Stain removal on cloth

The stain removal from cloths by amylase was checked with tomato catchup spread on two pieces of muslin cloth. After that stain removal was accessed by using amylase enzyme in combination with detergent containing water, for one piece of cloth this combination was treated as a test then another piece of stained cloth was treated with detergent only without enzyme, and this combination was considered as a control. As an enzyme is thermostable its working efficiency was checked at varying temperatures cold washing, moderate and hot washing [18].

Biochemical test

The potential isolate which was found effective for amylase production was further characterized by its morphological and Biochemical characteristics. For biochemical determination of amylase-producing microbes, several tests are carried out such as Catalase Test, Triple Sugar Fermentation Test as well as Oxidase Test..

Result and Discussion

Sampling

Fig:1 Compost pile

Table:1 Screening of thermostable bacteria at various temperatures

Temp: 45°C

Sample	R-1	R-2	R-3	Average
S-1	50×10^{-5}	45×10^{-5}	51×10^{-5}	48
S-2	60×10^{-5}	40×10^{-5}	55×10^{-5}	51
S-3	55×10^{-5}	58×10^{-5}	42×10^{-5}	51
S-4	50×10^{-5}	45×10^{-5}	48×10^{-5}	47
S-5	52×10^{-5}	50×10^{-5}	49×10^{-5}	50

Temp: 55°C

Sample	R-1	R-2	R-3	Average

S-1	30×10^{-5}	25×10^{-5}	28×10^{-5}	27
S-2	29×10^{-5}	27×10^{-5}	26×10^{-5}	27
S-3	30×10^{-5}	31×10^{-5}	29×10^{-5}	30
S-4	28×10^{-5}	27×10^{-5}	27×10^{-5}	27
S-5	24×10^{-5}	26×10^{-5}	22×10^{-5}	24

Temp: 80°C

Sample	**R-1**	**R-2**	**R-3**	**Average**
S-1	10×10^{-5}	9×10^{-5}	6×10^{-5}	8
S-2	9×10^{-5}	5×10^{-5}	6×10^{-5}	6
S-3	8×10^{-5}	10×10^{-5}	11×10^{-5}	9
S-4	11×10^{-5}	12×10^{-5}	8×10^{-5}	10
S-5	13×10^{-5}	10×10^{-5}	9×10^{-5}	10

Temp: 90°C

Sample	**R-1**	**R-2**	**R-3**	**Average**
S-1	3×10^{-5}	3×10^{-5}	2×10^{-5}	2
S-2	1×10^{-5}	3×10^{-5}	1×10^{-5}	1
S-3	1×10^{-5}	2×10^{-5}	1×10^{-5}	1
S-4	2×10^{-5}	3×10^{-5}	2×10^{-5}	2
S-5	2×10^{-5}	2×10^{-5}	3×10^{-5}	2

Serially diluted compost samples were examined for a culturable fraction of microorganisms. After incubation at varying temperatures i.e., 450C, 550C, 800C, and 900C it was found that as the incubation temperature was increased microbial count get decreased. At temperature 450C the average microbial count was found to be 47 – 51 CFU, similarly, at temperature 550C the average microbial count was between 24 – 30 CFU, this reduction in the microbial count was due to a rise in temperature by 10 0C as a further increase in the temperature up to 80 0C has significantly reduced the count between the range 6 – 10 CFU. At the highest temperature i.e., at 90 0C only 2-3 isolates were able to grow at this high temperature and these isolates were considered a thermophile. Further, these thermostable isolates were screened for their starch hydrolyzing capability.

Fig: 2. Isolates at 90 0C

Table:2 Starch hydrolyzing ability

Sample	Isolates	Starch hydrolysis
S -1	l - 1	-
	l - 2	-
	l - 3	-
S -2	l - 4	-
	l - 5	+
S-3	l - 6	-
S-4	l - 7	-
S-5	l - 8	+++

The isolates which were found thermotolerant were further tested for their capability to degrade starch. As starch hydrolysis indicates amylase production. After inoculating the cultures on starch-containing media the starch hydrolyzing ability was checked by measuring the zone of hydrolysis after the addition of iodine. It was found that among all the thermostable isolates from the respective samples only two isolates were found able to degrade starch. Only a single isolate I-8 from Sample S-5 was found very effective, which produced a large zone of hydrolysis. Another isolate i.e. I-5 from sample S-2 tested positive for starch hydrolysis but was not found suitable for further study as it produced a small zone of hydrolysis as compared to isolate I-8. Isolate I-8 was further selected for amylase production as it has produced a large zone of hydrolysis, a large zone indicates more quantity of amylase produced [19].

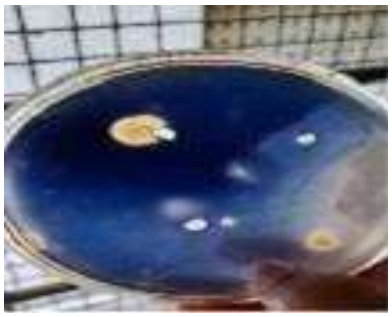

Fig:3 Starch hydrolysis by isolate I-8

Amylase production and optimization

Laboratory scale amylase production was carried out using thermostable isolate I-8, which was found suitable for amylase production. The fermentation was carried out in an Erlenmeyer flask of 100 ml capacity. Initially, the sterile starch broth was inoculated with isolate I-8. The flask was kept for incubation at room temperature for six days. During incubation, the starch hydrolysis was measured in terms of reducing the sugar by using the DNS method. after incubation, the broth was further used for extraction and purification of enzymes.

Table:3 Purification thermostable amylase

Purification steps	Total volume (ml)	Total enzyme activity (units)	Total protein (mg)	Yield (%)	Specific activity (units/mg protein)	Fold of purification
Culture filtrate (crude)	100 ml	6000	260	100	29.25	0
Ammonium sulphate precipitation	20	1742	26.9	25.9	63.14	2.0
After dialysis	25	2100	23.2	29.3	98.03	3.5

After fermentation, the broth was subjected to repeated centrifugation as per protocol to remove biomass. Before that crude filtrate was tested for enzyme activity, specific activity, and total protein content. The total enzyme activity of crude filtrate was found 6000 units, specific activity of 29.25 U/ mg of proteins, and the total protein content was 260 mg for 100 ml broth. The further crude filtrate was continued for ammonium sulphate precipitation, the total volume for precipitation was 100 ml then precipitate was tested for enzyme activity, specific activity, and total protein content it was observed that enzyme activity was 1742 Unites, get reduced as compared to the crude filtrate, the specific activity was increased up to 63.14 U/mg of protein then total protein content was also reduced to 26.9 further overall yield was 25.9 %. then further purification was carried out using dialysis after dialysis the partially purified enzyme was again tested for enzyme activity, specific activity, total protein content, and percent yield [20].

Fig:4 Partially purified amylase enzyme

Table:4 Effect of temperature on stability and activity of Amylase

Temperature (°C)	40	50	60	70	80	90	100
Enzyme activity	2200	2400	3000	3500	4000	5000	2000

A partially purified enzyme sample was further subjected to check its activity and stability at varying temperatures and pH. It was observed that enzyme activity gets increased as the temperature get increased the activity was found to be highest at 5000U at a temperature of 900C. high enzyme activity and stability at high temperatures correspond to the thermophilic nature of bacteria. The further enzyme was found less stable and active at a lower temperature. Also further increase in temperature at 1000C decreased the activity up to 2000 U (Table:4 and Fig:5).

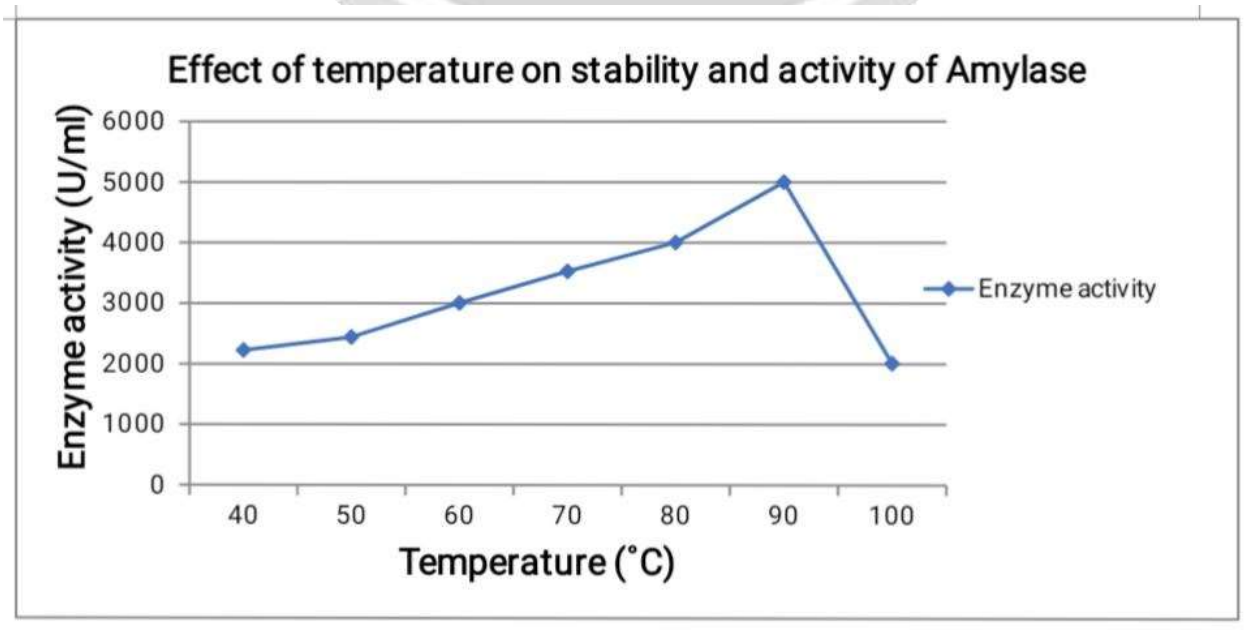

Fig:5 Effect of temperature on stability and activity of Amylase

Table:5 Effect of pH on stability and activity of Amylase

pH	5	6	7	8	9	10
Enzyme activity	4200	6000	4000	3900	2800	2000

After checking the efficiency of an enzyme at varying temperatures enzyme activity was also accessed at the varying condition of pH. It was found that at pH 6 the activity was high i.e.; 6000 U further activity was found low at pH 9 i.e 2800 at neutral pH condition activity was 4000 quite low as compared to slightly acidic pH. From the above observation, it can be concluded that enzyme activity was more at acidic pH and get reduced as the pH of the medium get increased [21].

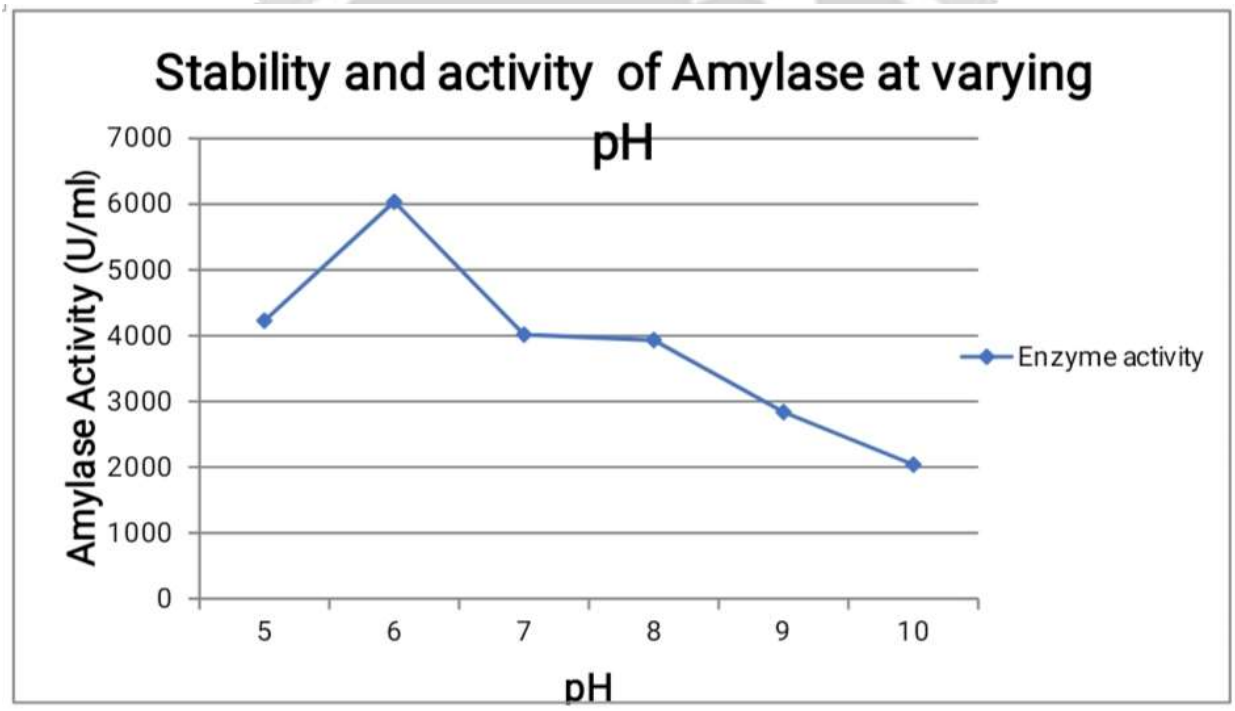

Fig:6 Stability and activity of the enzyme at varying pH

Table:6 Stain removal by tube method

Test	**Test**	**Control**
Starch 1%	9 ml	9 ml
Dye	1 drop	1 drop
A test sample of amylase	1 ml crude	-
Result	Coloration	No - color

The crude filtrate of the enzyme was checked for its efficiency to remove stains. As amylase has several applications in different areas like food, pharma, and the leather industry they are also used in detergents for washing to remove of organic stains from cloths as the enzyme was isolated from thermostable bacteria enzyme also shows stability and activity at high temperature. For this reason, it can be suitable for warm washing. The stain removal was checked by tube method and cloth method. in both methods stain was successfully removed at varying temperatures also it was almost completely removed at 90 ˚C.

Fig: 6 Stain removal by tube method

Table:7 Stain removal on cloth

Sr. No	Stain removal at different temperatures		
	Temperature	**With enzyme**	**Without enzyme**
1	90°C	+++	-
2	55°C	++	-
3	4°C	+	-

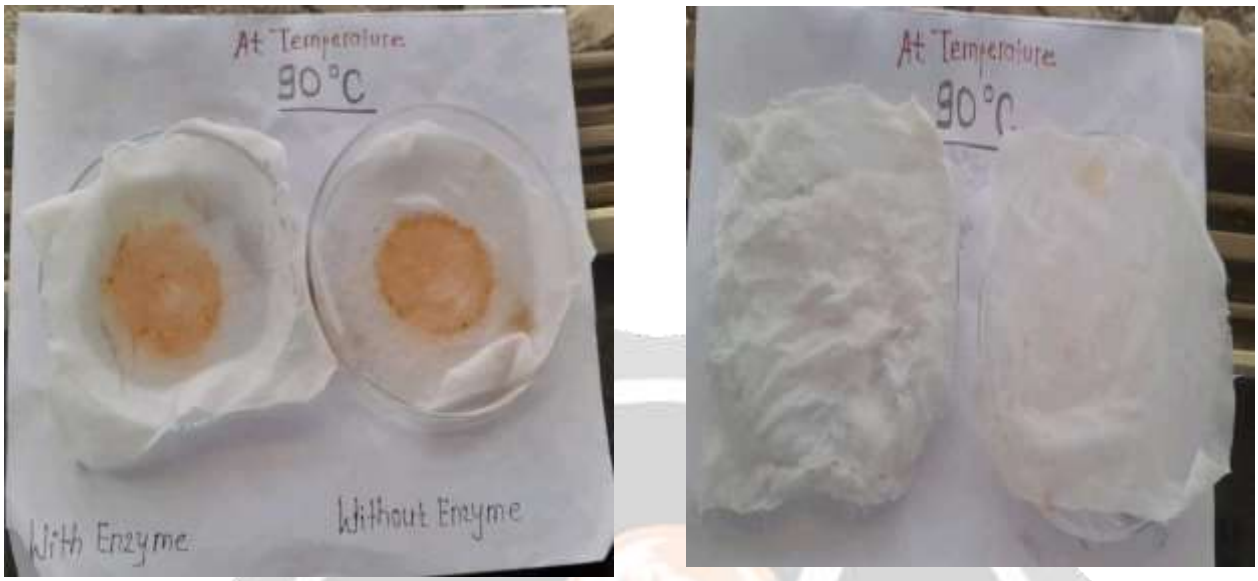

Fig: 7 Stain removal by cloth method at 90 0C (Warm Washing).

Fig: 8 Stain removal by cloth method at 4 0C (Cold Washing)

Table: 8 Effect of various metal ions on enzyme activity

Salt Concentration	Ca++	Mg++	CO++	Na+
Without salt	5000	5000	5000	5000
5 mM	7200	6500	6168	7500

10 mM	8000	3500	4800	5720

***Enzyme activity in U/mL/min**

Effect of Metal Ions

The effect of selected metal ions on enzyme activity was accessed by using metal ions at different concentrations. Initially, enzyme activity was checked without metals and it was found constant i.e., 5000 U / mL/min. After the addition of 5 mM concentration of Ca2+, Mg2+, Co2+, and Na+ enzyme activity was significantly increased in presence of all metal ions, enzyme activity was found to 7200,6500,6168, and 7500 further increase in the concentration of metal ions at 10mM the activity was again increased for single metal ion i.e., Ca ++ and it was found 8000 U for other metals like Mg2+, Co2+, and Na+ the activity gets decreased. This indicates that there is no direct relation between metal ion concentration and enzyme activity [22].

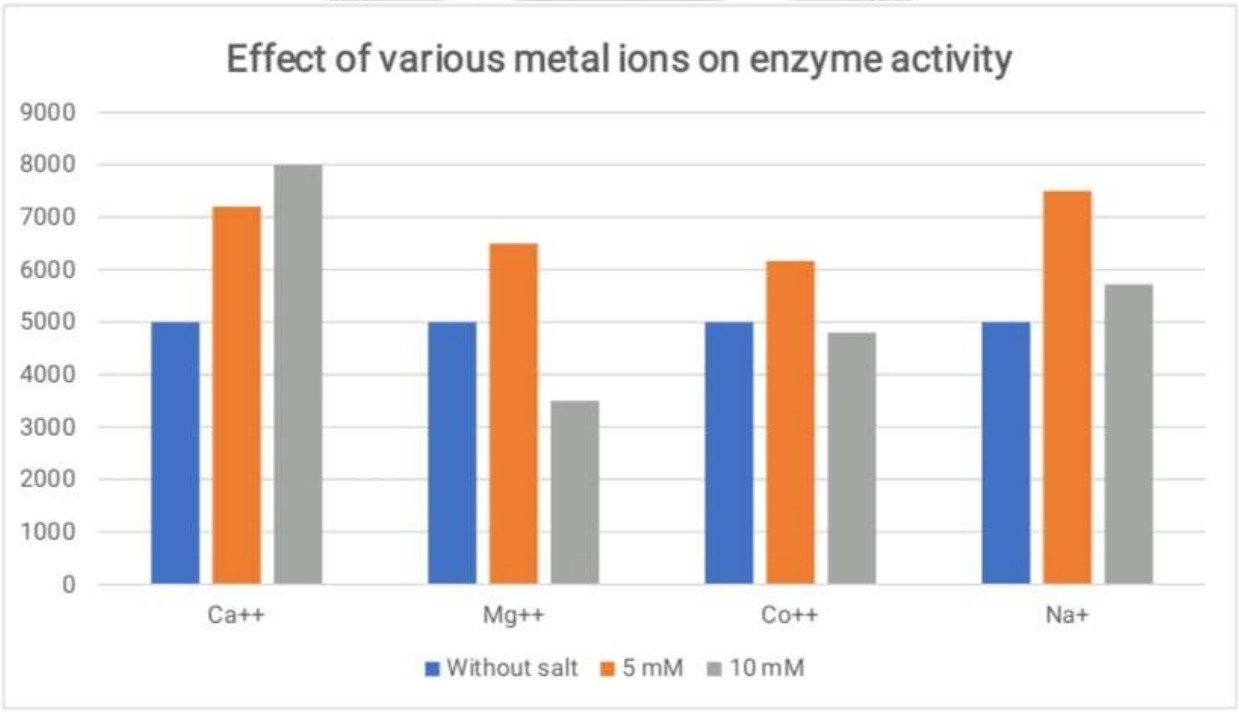

Fig:8 Effect of various metal ions on enzyme activity

Conclusion:

Thermostable α-amylases are not only of extraordinary interest at the fundamental level to investigate the thermodynamic stability of proteins but also to understand the relationship between stability, flexibility or plasticity, and their catalytic efficiency. Even though α-amylases have been used in a wide variety of technical applications for several years, there have not been many new developments. The available enzymes are good and have fulfilled, until recently, the needs of the customers. The interest in new and improved -amylase is growing, and consequently, the research is intensified as well. Research is focused on developing more thermotolerant α-amylases modifying them genetically or applying site-directed mutagenesis to acquire desired properties in the enzymes.

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