ISOLATION, EXTRACTION, PURIFICATION, AND CHARACTERIZATION OF THERMOSTABLE AMYLASE FROM BACTERIA ISOLATED FROM SOIL

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Abstract:

Only a small portion of the microbes on Earth have currently been used for human benefit. It appears that microbial life is not restricted to any particular environment. It has become evident over the past few decades that microbial communities can exist in a wide range of environments, including those with extremes of temperature, pressure, salinity, and pH. These bacteria, also known as extremophiles and they synthesize biocatalysts that can operate in harsh environments. Numerous microorganisms, particularly a variety of *Bacillus* species, are known to produce a wide range of extracellular enzymes, which have a wide range of industrial applications. Amylases are particularly important among these enzymes for industry. *Bacillus* group of organisms have diverse potential to produce a variety of enzymes at different physiological conditions like high temperature, high salt concentrations, etc.

The present study aimed to track the isolation of thermostable amylase-producing *Bacillus* species from soil samples that have the ability to synthesize extracellular thermostable amylase after that was purified by ammonium sulfate precipitation followed by column chromatography and confirmed by using SDS-PAGE, it was found that partially purified amylase has a molecular weight around of 40kDa when compared to standards.

Introduction:

Polysaccharides constitute the majority of carbohydrates in nature, assuming various roles, such as energy storage and as constituents of plant cell walls. Among these, starch emerges as the principal source of carbohydrates in the human diet, constituting more than 50% of our overall carbohydrate consumption. This complex carbohydrate polymer is predominantly synthesized by plants, particularly crops, and is comprised of amylose and amylopectin, both consisting of glucose units linked by 1,4 and 1,6 glycosidic bonds. Despite its seemingly straightforward chemistry, starch metabolism is intricate, involving a multitude of (iso)enzymes and proteins, the comprehensive understanding of which still needs to be completed. Starch serves as a vital source of nutrition and energy for both humans and animals. Additionally, starch finds extensive utility in various non-food industrial applications in contemporary times [1]. The bacterium Clostridium botulinum, the source of botulinum toxin, is inherently present in soil environments. How this toxin engages with the immune system is multifaceted. When exposed to the toxin, the immune system can potentially generate antibodies, providing a certain degree of protection. Nevertheless, the botulinum toxin can influence the immune system indirectly by inducing muscular paralysis, subsequently undermining the bodily infection barriers [2].

Amylase, a digestive enzyme predominantly secreted by the pancreas and salivary glands and, to a lesser extent, by other organs [27], exhibits wide distribution across microbial, plant, and animal kingdoms. The primary role of amylases entails the hydrolysis of glycosidic linkages within starch molecules, thereby facilitating their conversion into simpler sugars. Alpha-, beta-, and gamma-amylase, representing the three main categories of amylase enzymes, exert specific effects on carbohydrate molecules [3].

Alpha-amylase is ubiquitously present across humans, animals, plants, and microbes. Conversely, beta-amylase is primarily detected in microbes and plants. Gamma-amylase, on the other hand, is observed in animals and plants; nevertheless, enzymes derived from fungal and bacterial origins have predominantly emerged as the favored choice for industrial applications [4,22].

Amylase can be derived from various sources, including plants, animals, and microorganisms. Barley and rice plants have been recognized as notable sources of plant-derived amylase. In both industrial and scientific domains, the utilization of microbial amylases derived from bacteria, fungi, and yeast has emerged as the predominant preference [21,25]. It is important

to note that the level of amylase production varies not only among different microbes but also within the same genus, species, and strain. Moreover, the synthesis of amylase is subject to the influence of microbial origin, whereby strains obtained from starch- or amylose-rich environments demonstrate heightened levels of enzyme production [6]. Notably, a multitude of factors, including pH, temperature, and the availability of carbon and nitrogen sources, exert significant influence on the rate of amylase production, particularly during fermentation processes. Implementing genetic engineering methodologies holds promise in enhancing amylase yields by facilitating improvements in microbial strains.

Furthermore, microbial strains can be optimized to enhance the production of efficient amylases that exhibit desirable characteristics such as thermostability and stability even in harsh conditions. These advancements not only mitigate the risk of contamination from extraneous proteins but also lead to reduced reaction times and lower energy consumption during the amylase reaction. Furthermore, the selection of halophilic strains proves advantageous for amylase production under extreme conditions [7,8].

As a result, various novel applications of enzymes in industrial processes have been made possible by the special qualities of these biocatalysts. Thermophilic extremophiles have attracted the most attention. [9]

Specifically, extremophilic enzymes, including amylases, proteases, lipases, and polymer-degrading enzymes such as cellulases and chitinases, have gained significant traction in industrial settings. The utilization of these enzymes is anticipated to experience a notable increase in diverse sectors, such as chemical, food, and pharmaceutical industries, owing to recent advancements in the generation and cultivation of extremophiles. Furthermore, progress in the cloning and expression of extremophile genes in heterologous hosts further contributes to the expanding repertoire of enzyme-driven transformations [10].

For numerous years, amylase has played a pivotal role in starch-based industries. While there are various microbiological sources capable of effectively producing this enzyme, the selection process for suitable strains of fungi and bacteria suitable for industrial-scale production is rigorous. The search for novel microbial candidates capable of amylase production remains an ongoing endeavor. Notably, recent studies have reported encouraging progress in developing purification techniques for amylase, facilitating its application in demanding sectors such as pharmaceuticals and clinical settings, which necessitate high-purity amylases [11].

Material and methods:

Sample Collection: Five soil samples were obtained from Mustansiriyah University in Bagdad, Iraq. The collection process involved sampling the subsurface soil at a depth of 3 to 4 cm, utilizing a sterile spatula to ensure aseptic conditions. The collected soil samples were then carefully transferred into sterile plastic bags provided by Hi-media. Subsequently, these samples were transported under strict aseptic conditions to the laboratory for subsequent isolation procedures.

Enrichment of amylase producers:

1 gm of soil sample was added in 100 ml of nutrient broth containing an extra 2 % soluble starch then flasks were kept at 55 to 60 ˚c for 48 hrs. After incubations enriched broth was subjected to isolation of thermostable amylase producer on 2 % starch nutrient agar plates.[12]

Isolation of amylase-producer bacteria:

The isolation of soil bacteria was carried out using the serial dilution method [13]. To begin with, one gram of the soil sample was subjected to sequential dilution using sterilized distilled water, resulting in a concentration range spanning from 10^{-1} to 10^{-5}. Subsequently, 0.1 ml of each dilution was aseptically transferred onto nutrient agar plates supplemented with 2% extra pure water-soluble starch. The samples were evenly spread across the plates using a glass rod and incubated at 55°C for 24 hours. Bacterial isolates exhibiting growth on the plates were further sub-cultured on corresponding media to obtain pure cultures.

Following an incubation period of 24 hours, the colonies' visual characteristics and cell morphology were observed and recorded after Gram staining. To confirm the identity of the isolates, KOH staining was also conducted. Isolates demonstrating traits consistent with the Bacillus genus, such as Gram-positive, catalase-positive, rod-shaped, and spore-forming bacilli, were identified and recorded [13,23].

Screening of isolates for amylase activity:

The amylase activity of the bacterial isolates was assessed by subjecting the starch plates to saturation with Lugol's iodine solution, resulting in the formation of clear zones surrounding amylase-producing microorganisms. These clearance zones were subsequently measured to

indicate the extent of amylolytic activity [13,24]. To determine the hydrolytic activity, the plates were flooded with Lugol's iodine solution, and the resulting zones of clearances were quantitatively measured.

Microscopic Examination of Isolates:

Microscopic examination was conducted to assess the cellular morphology of the bacterial isolates. To accomplish this, smears of the pure isolates were meticulously prepared on sterile slides labeled correctly. Subsequently, heat fixation was performed to immobilize the cells. The staining procedure involved a sequence of steps, commencing with applying Crystal violet dye, which was allowed to interact with the cells for 60 seconds before being thoroughly rinsed. Following this, Gram's iodine was applied for an equal duration of 60 seconds, followed by rinsing and subsequent decolorization utilizing 95% alcohol. After rinsing with water, safranin was applied for a specific period. The slides were then carefully rinsed, dried, and examined under the oil immersion objective lens to facilitate detailed observations [14].

Biochemical tests of amylase producer:

Essential biochemical tests were performed for the pure culture of amylase producer for further study and observations are noted down in Table No. 2

Crude amylase Production and Extraction:

Crude amylase production was carried out by using a modified protocol of Razzak et.al.,[15] In this Purified *Bacillus species* culture has been subjected to several culture conditions in order to determine the best ones for producing amylase. At particular temperatures (45, and 55°C), substrate (starch) concentrations (01.0, 2.0, and 3.0%), and pH values (6.0, 7.0, and 9.0), growth, and amylase output were estimated. In a 500 ml Erlenmeyer flask with 250 ml of starch medium (containing 0.5% peptone, 0.3% yeast extract, 1% soluble starch, 0.3% NaCl, 0.1% K2HPO4, and 0.02% $MgSo_4 7H2O$), all tests were conducted for 24 and 48 hours respectively, inoculated flasks were kept rotating at 150 rpm in a water bath shaker.

Enzyme Assay: The assay of α-amylase activity was conducted through a series of experimental steps. Firstly, 0.1 ml of the enzyme was incubated with 1.0 ml of soluble starch (1.0 w/v) prepared in 0.1 M phosphate buffer at a pH of 7.5. This mixture was subjected to incubation at a temperature of 50°C for 5 minutes. To halt the enzymatic reaction, appropriate measures were taken. The determination of the released reducing sugars was achieved by adding 2.5 ml of 3,5-dinitro salicylic acid reagent, with glucose (at a concentration of 500 µg/ml) serving as the standard. It is noteworthy that all assay sets were conducted at a temperature of 55°C [16,17].

Purification of Amylase Enzyme:

While whole cells are used as the source of enzymes in many industrial processes, the efficiency can be increased by using isolated and purified enzymes. However, the ultimate application determines the criteria for choosing a certain isolation and purification process. Amylase enzyme purification was accomplished using ammonium sulphate precipitation and dialysis.[18] For the experimentation, a volume of 100 ml of cell-free extract was subjected to centrifugation at a speed of 7000 rpm for 15 minutes. ugation, the resulting supernatant was carefully and subsequently saturated up to 70% with ammonium sulphate. The provided solution underwent a second round of centrifugation at a speed of 7000 revolutions per minute (rpm) for 15 minutes. This centrifugation step resulted in the formation of a pellet, which was carefully preserved for subsequent analysis. Subsequently, the enzyme mixture was transferred into a dialysis bag with a spatial capacity of 70 cm and placed in a phosphate buffer solution with a pH value of 7. The entire system was maintained at a temperature of 4°C for 24 hours. During this period, the buffer solution was continuously agitated using a magneticrer. To ensure the adequate semi-purification of the enzyme mixture, the buffer solution was replaced three times throughout the entire experimental procedure [15].

Column chromatography:

The precipitate showing amylase activity collected after ammonium sulfate precipitation was subjected to ion exchange chromatography. In this, the column was packed by activated DEAE cellulose of 20cm with 2.0cm diameter. Then the dialyzed sample (approximately 5 ml) was loaded on the column. After that column was washed with 150 ml

phosphate buffer (pH-7.0) and further column was eluted by 0.1 M to 1.0 M NaCl gradient solutions. The fraction of 5ml was collected at the flow rate of 1ml/min. The eluted fractions were checked for the protein content by measuring absorbance 280 nm using a UV-visible-spectrophotometer. The fractions showing the highest protein content and washing buffer were checked for starch hydrolysis activity.

Molecular Weight Determination of Amylase by SDS-PAGE:

The determination of the molecular weight of the amylase enzyme was carried out through the employment of SDS-PAGE, employing a 10% polyacrylamide gel, following the well-established methodology outlined by Laemmli [20]. The partially purified enzyme sample was meticulously loaded into distinct wells of the gel alongside standard protein markers comprising myosin (205 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). Subsequently, the protein bands were made visible via Coomassie brilliant blue staining, and their molecular weights were compared to those previously reported for thermostable amylase enzymes [20,26].

Results

Isolation of amylase-producer bacteria:

A total of 06 isolates were obtained after 48 hrs incubation at 55˚ c from those 2 bacteria that had the highest hydrolytic activity as indicated by the hydrolysis zone after iodine exposure (Fig 1)

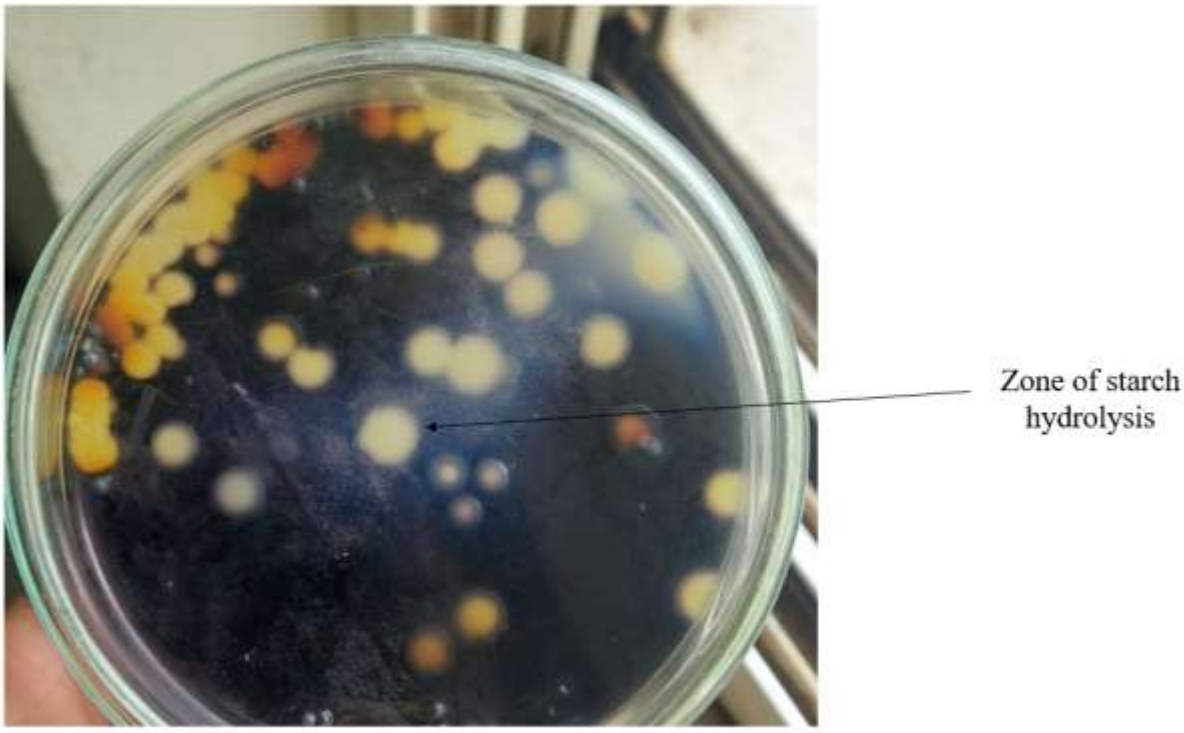

Fig 1 Starch hydrolysis showing colony after Iodine exposure.

Microscopic Examination of Isolates:

The identification of the bacterial isolates was conducted based on their Gram's nature, as depicted in Figure 2, as well as their colony morphology, as detailed in Table 1. The selected organism underwent extensive biochemical utilization testing, the outcomes of which are presented comprehensively in Table 2. Notably, the screening process revealed that the potent isolate, which demonstrated confirmed amylase production, exhibited characteristics of being Gram-positive rods without any discernible distinct microscopic morphology. A careful comparison of the microscopic observations and the results of biochemical tests, in conjunction with reference standards, led to identifying the isolate as belonging to the genus *Bacillus*.

Size	Shape	Colour	Margin	Surface	Elevation	Opacity	Consistency
5 mm	Circular	Off white	Erose	Smooth	Convex	Opaque	Sticky

Gram nature	Motility
Gram positive	Non motile

Table 1. Morphological characteristics of amylase producing isolate.

Sr.No	Test	Result
1	catalase	Negative (- ve)
2	Citrate	Positive (+ve)
3	Flagella	Non sFlagellated
4	Gas production	Negative (-ve)
5	Gelatin Hydrolysis	Positive (+ve)
6	Gram Staining	Gram Positive (+ve)
7	Indole	Negative (-ve)
8	Adonitol	Negative (-ve)
9	Arabinose	Positive (+ve)
10	Arabitol	Negative (-ve)
11	Fructose	Positive (+ve)
12	Maltose	Positive (+ve)
13	Glucose	Positive (+ve)
14	Adonitol	Negative (-ve)
15	Arabinose	Positive (+ve)
16	Arabitol	Negative (-ve)
17	Fructose	Positive (+ve)
18	Galactose	Positive(+ve)

Table No.2 Biochemical tests of isolated bacteria.

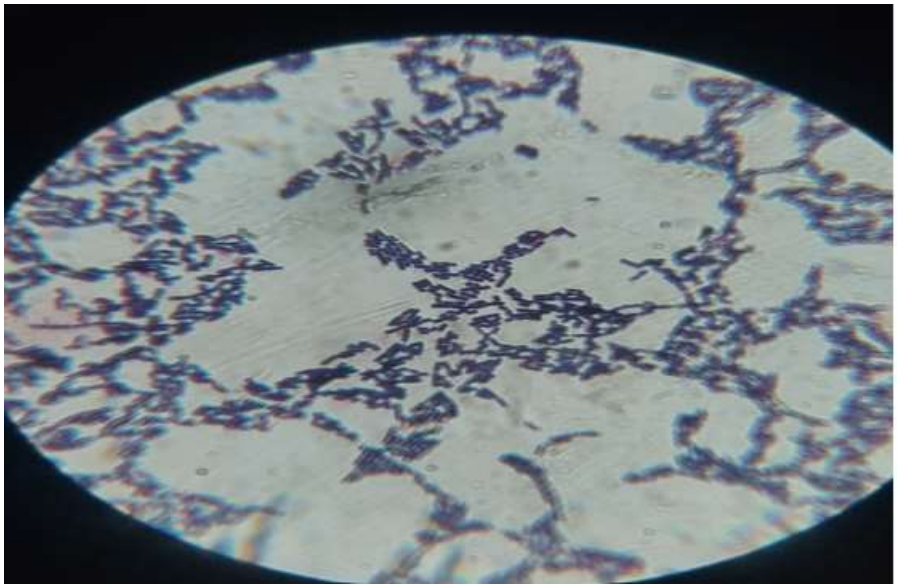

Fig 2. Gram staining of purified bacteria, showing Gram-positive short rods.

Enzyme Assay of Amylase-

1] cell-free broth:

The determination of amylase activity and the quantification of glucose concentration released by the bacterial isolate were performed at two different time points: 24 hours and 72 hours of fermentation. The results revealed that the potent isolates exhibited noteworthy amylase activity, with a recorded highest value of 56.36 U/ml after 24 hours, followed closely by 50.50 U/ml after 72 hours. These findings confirm that the isolated bacteria yield a substantial quantity of extracellular amylase after 24 hours of fermentation.

2] Partially purified amylase by Ammonium sulphate precipitation:

After 24 h of fermentation cell-free broth was used for partial purification of amylase by using 70% ammonium sulphate salt after centrifugation obtained pellet was tested for amylase activity by using starch as substrate and glucose as standard. The amylase activity results for the potent isolates after 70 % ammonium sulphate precipitation had the highest amylase activity with 60.35 U/ml this confirmed that partially purified amylase shows the highest enzyme activity as compared to the cell-free broth.

Column chromatography:

The ammonium sulfate extracted sample was loaded on DEAE-cellulose column chromatography and eluted by 0.1M to 1.0M NaCl. [19] The column was washed with 150 ml of equilibration buffer and the bound protein was eluted by 0.1M to 1.0M NaCl, Fractions (2.0 ml) were collected at a flow rate of 0.5 ml/min and assayed for enzyme activity. A total of 60 fractions were collected and subjected to spectroscopic analysis for checking protein concentration at 280 nm, it was found that 5 different proteins were separated from the column after that all proteins were tested for amylase hydrolysis.

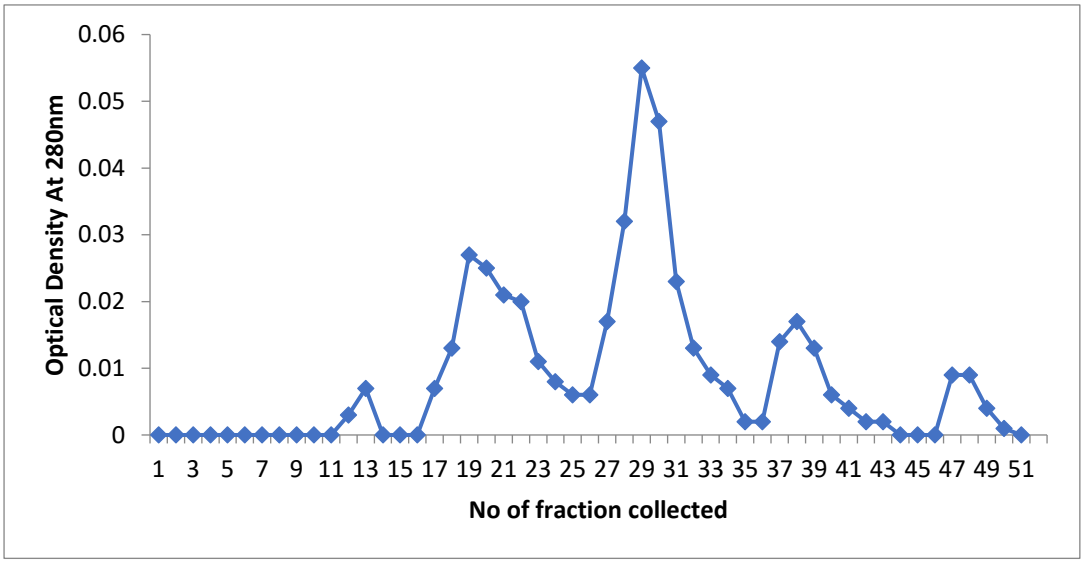

Fig 3. Purification of amylase by ion exchange chromatography

Molecular Weight Determination of Amylase by SDS-PAGE:

The purified amylase was analysed by SDS-PAGE, and it was found that the band was observed at approximately 40 kDa. (Fig No.4) [ ]

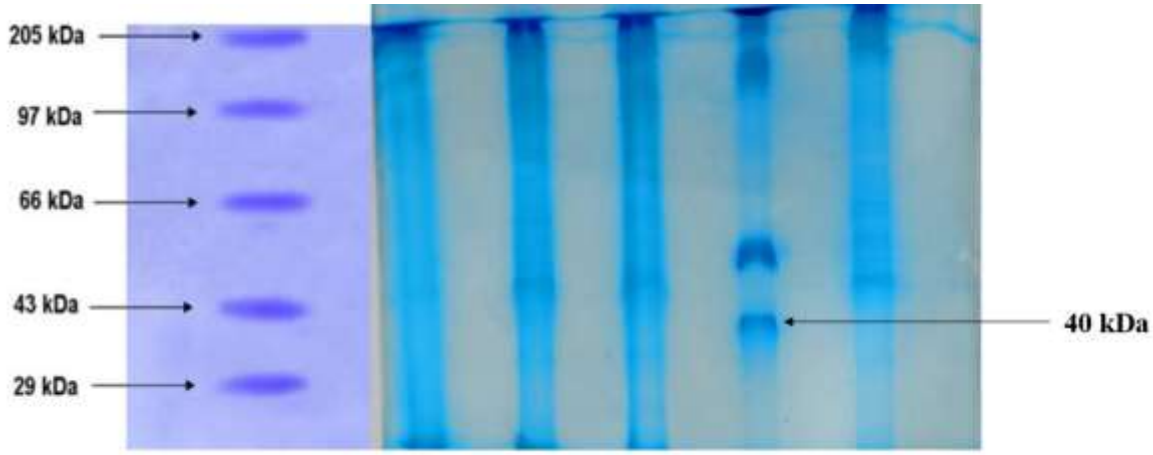

Fig No.4 Molecular weight determination of thermostable amylase by SDS-PAGE.

Conclusion:

Selecting microorganisms capable of hyper-producing amylase is a crucial aspect of the starch saccharification industry. The present investigations have focused on the isolation, extraction, purification, and characterization of thermostable amylase derived from bacteria obtained from soil samples. The findings of this study lead to the following conclusions:

1] The isolated bacteria exhibit promising potential for producing extracellular thermostable amylase.

2] Ammonium sulfate precipitation proves to be an efficient method for extracting this enzyme.

3] The specificity and thermal stability properties of the amylase hold significant commercial importance across various industries.

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