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EXPLORING α AMYLASE FROM RICE-FIELD *BACILLUS SUBTILIS*: PRODUCTION AND BIOCHEMICAL CHARACTERIZATION

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Abstract: α-Amylase is one of the utmost noteworthy industrialized biocatalyst, catalyzing the hydrolysis of α-1, 4-glycosidic bonds in starch to yield maltose, dextrins, and glucose. In this study, an α-amylase- yielding bacterial strain was isolated from rice field soil and characterized as Bacillus subtilis. Fermentation conditions were optimized to enhance enzyme production, followed by partial purification.T he most favorable production conditions were 30°C and pH 7.0 with starch (10.0 g/L) as the carbon source, peptone (5.0 g/L) as the nitrogen source, and yeast extract (2.0 g/L) supplementation. Calcium chloride (0.15 mg/dl) served as a stabilizing agent, substantially increasing enzyme yield. Partial purification using ammonium sulfate (40%) enhanced enzyme retrieval. The findings emphasize Bacillus subtilis as a dependable source of α-amylase with possible use in constant biocatalytic starch breakdown for industrial use.

Key words: α-Amylase, *Bacillus subtilis*, Fermentation, Enzyme production, Partial purification, Starch hydrolysis, Industrial application, Enzyme optimization

INTRODUCTION

The Alpha-amylase is one of the key hydrolytic enzymes associated to the glycoside hydrolase family. It performs a central role in the breakdown of starch and further polysaccharides by cleaving internal α-1, 4-glycosidic bonds, yielding maltose, maltotriose, and glucose(Gupta *et al*., 2003). Amongst the several forms of amylases α and β amylase (E.C.3.2.1.1) are the most broadly studied and industrially pertinent enzyme owing to its broad substrate specificity and catalytic efficacy (Sivaramakrishnan *et al*., 2006). It is yielded by plants, animals, and microorganisms; though, microbial α-amylases are ideal for industrial use as of their high yield, stability, low cost, and ease of production (Pandey *et al*., 2000; Tanyildizi *et al*., 2005). Microbial α-amylases are principally effective because of their constancy through wide ranges of temperature and pH, making them appropriate for numerous industrial practices for instance food, textile, paper, fermentation, and detergent production (Simair *et al*., 2017; Mageswari *et al*., 2012). Amongst microbes, Bacillus species are the most proficient producers of α-amylase, particularly *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*, owing to their high enzyme secretion capability and thermal stability (Gupta *et al*.,2003; Sivaramakrishnan *et al*., 2006). Enzymes from these thermophilic Bacillus species can endure elevated working temperatures (90-110°C), which augments reaction rates and reduces contamination throughout starch hydrolysis (Bharathiraja *et al*., 2017). Fermentation persists the prime method for α-amylase production, with together solid-state and submerged fermentation systems being used. Production proficiency is affected by factors for instance nutrient intensity, carbon and nitrogen sources, pH, and temperature (Haq *et al*., 2010). The use of substrates comprising α-1, 4-linked polysaccharides, for instance starch or maltose, works as an inducer for enzyme production. Solid-state fermentation, in actual, poses benefits like higher product concentration, modest technology, and lower energy necessities (Pandey *et al*., 2000). Moreover, recent progresses in molecular biology have allowed the genetic alteration of Bacillus strains to enhance enzyme yield and constancy via mutagenesis and recombinant DNA methods (Haq *et al*., 2010).

Thermostable and alkaline α-amylases have vast industrial significance, principally in starch liquefaction, fermenting, fabric resizing, and cleansing agent preparations (Gupta *et al*., 2003; Hmidet *et al*., 2009). In detergents, α-amylases aid eliminate starch-based marks and are designed to retain activity in oxidizing surroundings and at low washing temperatures. In the food sector, they are used for making maltose syrups, enhancing bread consistency, and slowing staling (Van Der Maarel *et al*., 2002). In addition, α-amylases perform a crucial role in pharmacological and fermentation

sectors, assisting in the production of bioethanol and several biochemical compounds.

The continuous worldwide demand for α-amylases has made them one of the major enzymes of commercial importance, contributing almost 30-65% of the entire enzyme market (Abdullah *et al*., 2014).Continuous enhancements in fermentation technology, and strain growth are expected to promote enhance production efficacy and industrial suitability. Due to their catalytic flexibility and broad industrial practice, α-amylases predominantly from Bacillus species remain essential biocatalysts in biotechnology and applied microbiology.

MATERIALS AND METHODS

Sample collection

Soil samples were gathered from rice-growing fields as possible sources of Bacillus subtilis for α-amylase production. Samples were taken in sterile containers, labeled properly, and transported to the laboratory under aseptic conditions for bacterial isolation and enzyme production studies.

Serial dilution

To obtain suitable bacterial concentrations for isolation, 1.0 g of soil was re suspended in 100 ml of sterile distilled water and shaken thoroughly. From this mixture, 1 ml was added to 9 ml of sterile deionized water in a series of tubes to prepare dilutions ranging from 10^{-1} to 10^{-9}. Each tube was gently mixed before transferring to the next dilution to ensure uniform distribution of cells.

Strain isolation

Isolation of the bacterial strain was performed using the spread plate method. From the final dilution (10^{-9}), 0.1 ml was streaked onto starch agar plates. Incubation of plates were carried out at 37°C for 24 hours. Colonies showing clear hydrolytic zones after iodine staining were considered α-amylase-producing Bacillus subtilis isolates

Media preparation

Starch-agar medium was formulated by dispersing 1.3 g agar and 10 g starch in 100 ml of deionized water. The pH was adjusted to 7.0, and the medium was sterilized at 121°C for 15 minutes. Following sterilization, the medium was introduced into petri plates and left to solidify naturally.

For production studies, different nutrient compositions were evaluated to identify the optimal medium for α-amylase synthesis. The medium formulation yielding maximum enzyme production contained: starch (10.0 g/dl), NaCl (0.5 g/dl), $MgSO_4$ (0.5 g/dl), $CaCl_2$ (0.15 g/dl), peptone (5.0 g/dl), and yeast extract (2.0 g/dl). The pH was adjusted to 7.0 a head of sterilization.

Procedure

The inoculation was performed by transferring 0.1 ml of the bacterial suspension onto the sterile starch-agar plates near a flame to maintain aseptic conditions. The glass spreader was rinsed with ethanol, flamed, and used to evenly distribute the inoculum. Plates were maintained at 37°C for 24 hours. Post-incubation, the clear zone surrounding colonies, visualized by iodine staining, confirmed starch hydrolysis and enzyme activity.

Streak plate method

To obtain a pure culture, the streak plate technique was used. The inoculum was streaked across successive quadrants of the agar plate to dilute the bacterial density. Plates were incubated at 37°C for 24 hours, and well-separated colonies were picked and sub-cultured for additional experimentation.

Optimization of Fermentation Conditions for α-Amylase Production

Effect of Substrate Concentration

To optimize the concentration of starch for α-amylase synthesis, media were prepared with varying starch levels from 0.25% to 2.0%, while other constituents were kept constant. The cultures were incubated at 37°C for 24 hours, and enzyme activity was measured from the supernatant.

Effect of temperature

To assess the impact of temperature on enzyme production, bacterial cultures were incubated at different temperatures (30°C, 40°C, 50°C, and 60°C).The enzyme activity was analyzed after 24 hours to identify the optimum incubation temperature.

Effect of pH

Media were formulated at diverse pH values within the range of 6.0 to 8.0 using phosphate buffers to determine the optimal pH for maximum enzyme activity. The cultures were incubated at 37°C for 24 hours, and the activity of the enzyme was assessed.

Effect of incubation time

To determine the impact of incubation time on α-amylase biosynthesis, cultures were incubated for varying durations (12-60 hours). Samples were collected at regular intervals, and enzyme activity was measured to determine the peak production period.

Effect of Nitrogen Sources

Various nitrogen sources were examined to enhance enzyme yield. Peptone and yeast extract were tested in varying concentrations while maintaining constant starch and salt levels. The cultures were incubated at 37°C for 24 hours, and α-amylase activity was quantified.

Effect of Calcium Chloride

To study the effect of calcium ions, varying concentrations of $CaCl_2$ (0.01-0.04 g/dl) were incorporated into the medium, while other components were unchanged. The bacterial cultures were incubated at 37°C for 24 hours, and enzyme activity was analyzed.

Partial Purification of α-Amylase

The crude α-amylase enzyme underwent partial purification using ammonium sulfate precipitation. The cell-free supernatant obtained after centrifugation was treated with ammonium sulfate at 4°C under constant stirring to achieve fractional saturation. The mixture was left overnight at 4°C to allow complete precipitation. The precipitate was gathered by centrifugation at 15,000 rpm for 15 minutes at 4°C, solubilized in phosphate buffer (pH 7.0), and subjected to overnight dialysis against the same buffer. The dialyzed enzyme was used for subsequent assays and kinetic studies.

Kinetic Studies of Partially Purified α-Amylase

Effect of Substrate Concentration

The influence of substrate concentration on enzyme activity was determined by varying starch concentrations from 0.25% to 2.0% under standard assay conditions.

Effect of pH

The effect of pH on enzyme activity was examined using phosphate buffer solutions adjusted between pH 6.0 and 8.0. Reactions were performed at 37°C under standard assay conditions.

Effect of Temperature

The temperature dependence of enzyme activity was assessed by conducting assays at 30°C, 37°C, 40°C, and 50°C.

Effect of Reaction Time

The enzyme-substrate reaction was incubated for varying durations (10-60 minutes) to determine the optimal reaction time for maximal catalytic activity.

Enzyme Assay

The α-amylase activity was quantified by measuring the reducing sugars released through starch hydrolysis using the Nelson method. For each assay, 0.5 ml of enzyme was mixed with 1.0 ml of substrate (1.0 g/dl starch in phosphate buffer, pH 7.0) and incubated at 37°C for 15 minutes. In control tube, NaOH (0.5 ml) was added before incubation to inactivate the enzyme. The reaction was stopped up by adding 0.5 ml NaOH to the test tube. One ml of reaction mixture was transferred to a fresh tube, and 1.0 ml of Nelson reagent was introduced. The tubes were placed in a boiling water bath for 20 minutes, cooled, and treated with 1.0 ml arsinomolybdate reagent. The absorbance was recorded at 680 nm against a blank.

Total Protein Estimation (Lowry Method)

Total protein content was assessed by the Lowry method using bovine serum albumin (BSA) as the standard. Absorbance was evaluated at 650 nm, and protein concentration was determined from the standard curve.

Reducing Sugar Estimation (Nelson Method)

Reducing sugars were measured using the Nelson method with maltose as the standard. Absorbance was read at 680 nm, and results were expressed as µmol of reducing sugar released per minute.

Enzyme Units

One α-amylase unit was defined as the amount of enzyme required to release 1.0 µmol of reducing sugar (maltose equivalent) per minute under the assay conditions (pH 7.0, 37°C, 15 minutes).

RESULTS AND DISCUSSION

Isolation and Identification of α-Amylase Producing Bacteria

Soil samples were screened for α-amylase-producing bacteria on starch agar plates. Colonies showing clear hydrolysis zones after iodine staining were selected. The isolate was recognized as Bacillus subtilis based on its morphological and biochemical characteristics (Fig. 1). These findings agree with earlier reports describing Bacillus species as efficient α-amylase producers (Unakal *et al*., 2012).

Fig 1: Colonial morphology of Bacillus subtilis grown on nutrient agar plates.

Screening of α-Amylase Producing Bacteria

The ability of the isolated strain to produce α-amylase was confirmed by the starch agar plate method. Afterwards incubation, the colonies were immersed in iodine solution; the appearance of clear zones against a dark-blue background confirmed starch hydrolysis and α-amylase production (Fig. 2).The intensity and diameter of the clear zones were used to identify the most active strain (Shah and Naqvi. 2025). The selected *Bacillus subtilis* isolate demonstrated significant extracellular enzyme activity, qualifying it for further optimization studies.

Fig 2: Screening of α-amylase-producing Bacillus subtilis using starch agar medium

Improvement of Fermentation Parameters for α -Amylase Biosynthesis

Effect of Incubation Time on α-Amylase Production

To Fermentation time significantly affected enzyme yield, with maximum α-amylase production observed after 24 hours (Fig. 3). Prolonged incubation reduced enzyme synthesis due to nutrient depletion and metabolite accumulation, consistent with previous findings (Sundarram *et al*., 2014).

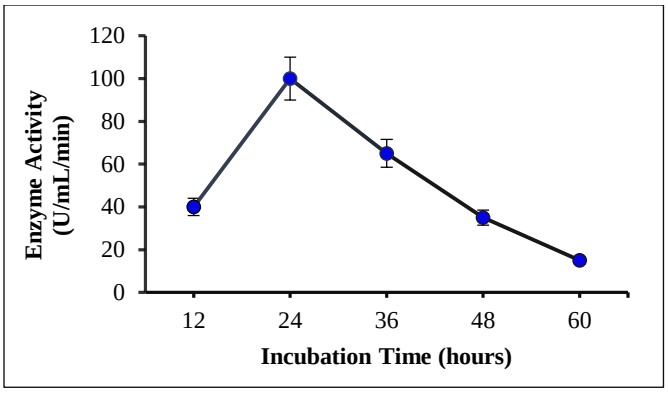

Fig 3: Effect of incubation time on α-Amylase production

Effect of Substrate and Nitrogen Sources

Among tested starch concentrations, 0.1 g/mL yielded the highest α-amylase production (Fig.4).Increased concentrations caused repression, likely due to glucose accumulation. Optimal nitrogen supplementation was obtained at 0.25% (w/v) peptone (Fig. 5a) and 0.25% yeast extract (Fig. 5b). Higher levels inhibited enzyme release, possibly by altering cell wall properties (Babu and Satyanarayana, 1993).

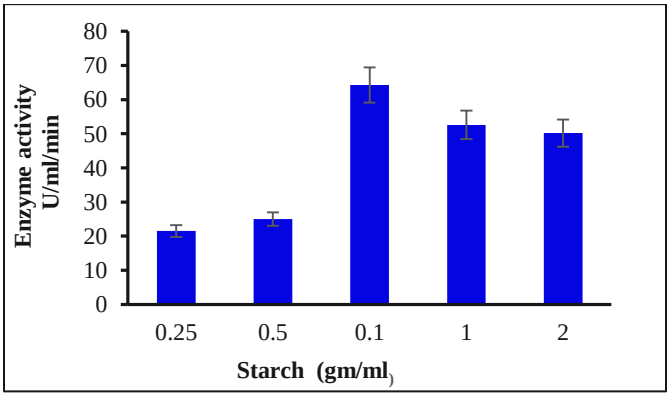

Fig4: Effect of starch concentration on α-amylase production

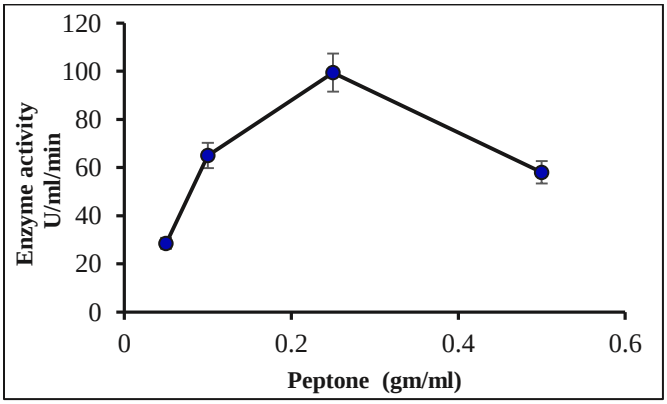

Fig 5a: Effect of peptone concentration on α-amylase production

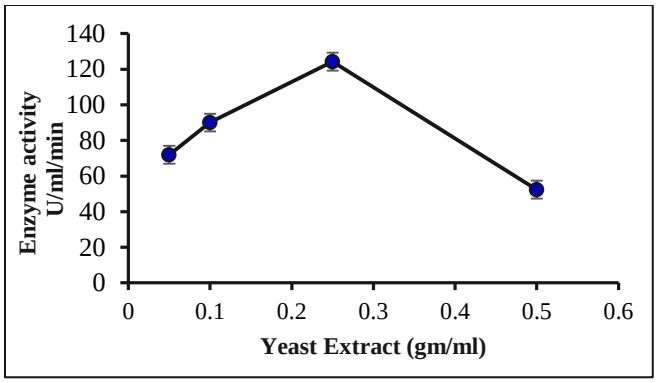

Fig 5b: Effect of yeast extract concn. on α-amylase production

Effect of Mineral Ions

Calcium (0.03 mg/mL) and magnesium (7.5 mg/mL) ions enhanced α-amylase production, confirming their stabilizing and cofactor roles (Fig. 6a&6b). Moderate NaCl (0.05 mg/mL) improved yield, while higher salinity decreased enzyme synthesis (Fig. 6c). This indicates that Bacillus subtilis exhibits facultative halophilic behavior.

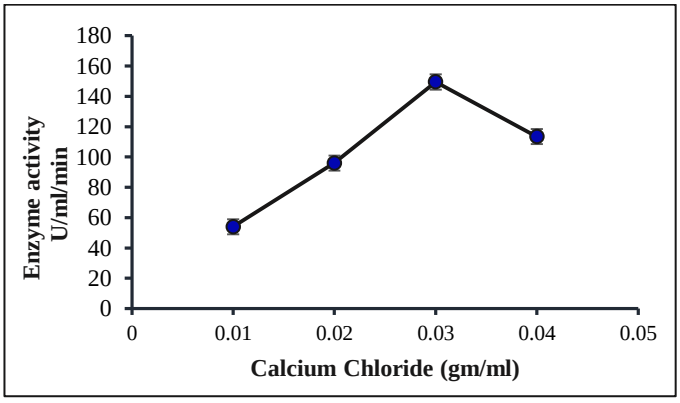

Fig 6a: Effect of $CaCl_2$ on α-amylase production

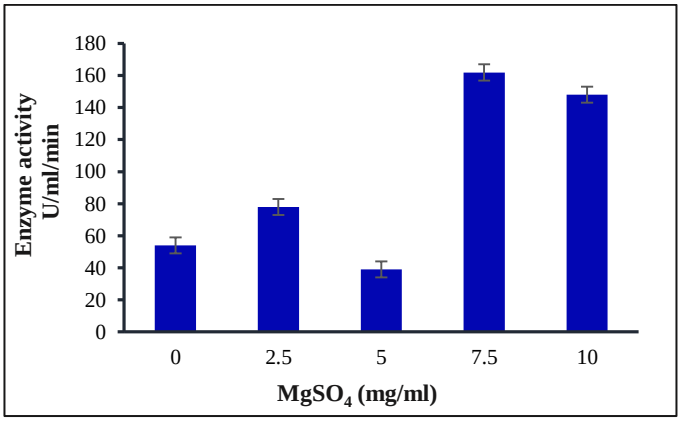

Fig 6b: Effect of $MgSO_4$ on α-amylase production

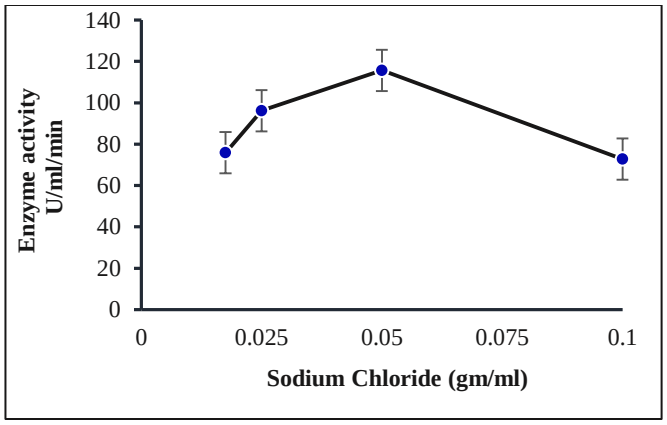

Fig 6c: Effect of NaCl on α-amylase production

Partial Purification of α-Amylase

Precipitation with 40% ammonium sulfate gave the maximum enzyme activity (specific activity 830.36 U/mg), while higher concentrations of ammonium sulphate resulted in enzyme inactivation, as also observed by Bano *et al*. (2009).

$(NH_4)_2SO_4$	Enzyme activity (U/ml/min)	Total protein (mg/ml)	Specific activity (U/mg)
Cell free Filtrate (CFF)			
30%	14.00	0.150	93.33
40%	279.00	0.336	830.36
60%	-	0.080	Nil

Kineic Studies of Partially Purified α-Amylase

Time maxima of Enzyme-substrate reaction

The enzyme exhibited maximum activity after 10 minutes of incubation with soluble starch at 37°C, after which a gradual decline was observed (Fig. 7a). This indicates that prolonged substrate exposure leads to product inhibition and enzyme denaturation**.**

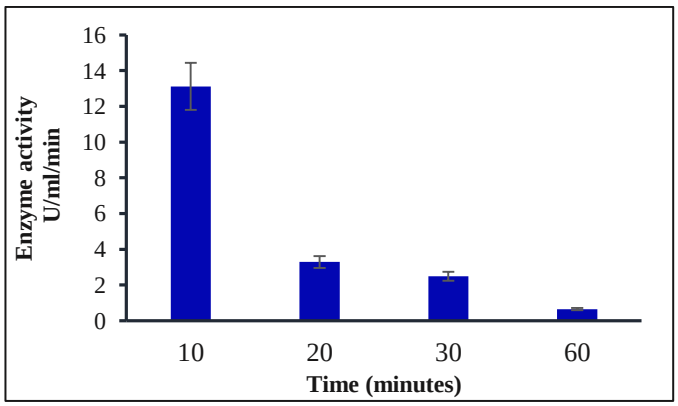

Fig 7a: Reaction-time dependence of α-amylase activity

Effect of Substrate Concentration on Enzyme-substrate activity

Enzyme activity increased with starch concentration, reaching an utmost at 0.05 g/mL (Fig. 7b). Similar behavior has been reported for *Bacillus* species using soluble starch as the preferred substrate (Al-Qodah *et al*., 2007).

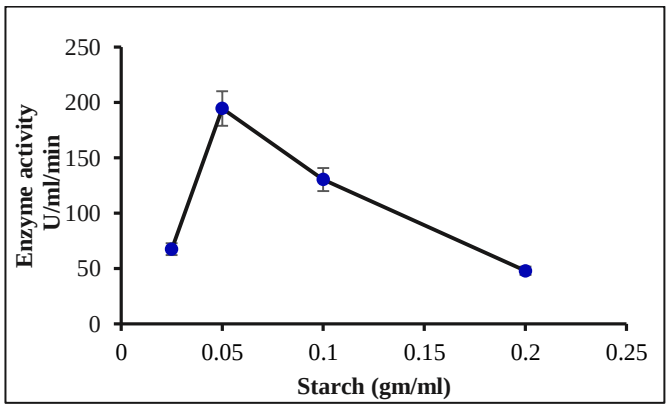

Fig 7b: Effect of substrate concentration on α-amylase activity.

Effect of Temperature on Enzyme-substrate activity

The α-amylase showed optimum activity at 30°C, with activity decreasing at higher temperatures due to denaturation (Fig. 7c). These results align with Chakraborty *et al*. 2000; Najafi *et al*. 2005), who observed similar patterns in *Bacillus* strains.

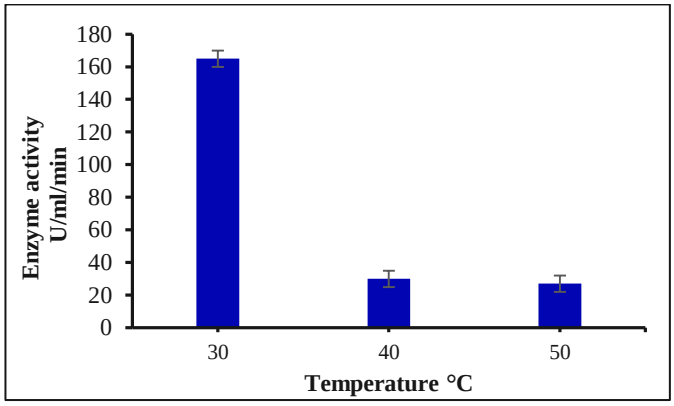

Fig 7c: Effect of temperature on α-amylase activity.

Effect of pH on Enzyme-substrate activity

The enzyme displayed maximum catalytic activity at pH 7.00 (Fig. 7d), with activity decreasing under acidic and alkaline conditions. The findings are consistent with previous studies showing neutral pH as optimal for bacterial α-amylases (Pandey *et al*., 2000; Ivanova *et al*., 1993).

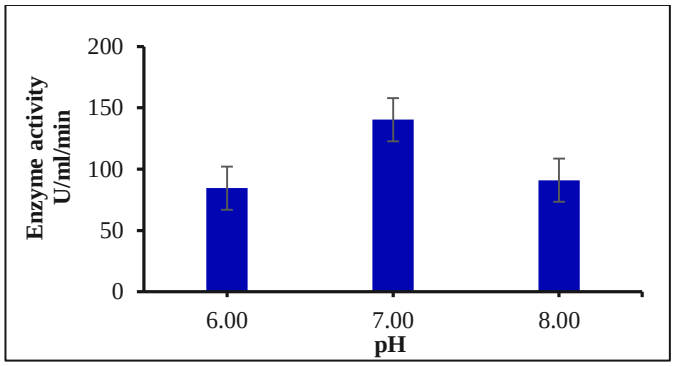

Fig 7d: Effect of pH on α-amylase activity

CONCLUSIONS

This research demonstrates the productive isolation and optimization of an innovative thermolabile α-amylase-producing strain of *Bacillus subtilis* from soil samples. The isolate was recognized in accordance with its morphological and biochemical properties and showed high possibility for extracellular α-amylase production. Optimization of the fermentation factors determined that highest enzyme yield was accomplished after 24 hours of incubation at 30°C and pH 7.0. Starch (0.1 g %) worked as the most efficient carbon source, while peptone (0.5 g %) and yeast extract (0.25 g %) were the most promising nitrogen complements.

The enzyme needed trace amounts of calcium ions (0.03 mg/ml) for activation, demonstrating its strong affinity for Ca^{2+}.Partial purification through 40% ammonium sulfate saturation augmented α-amylase activity by up to sevenfold. The enzyme showed maximum activity after 10 minutes of incubation with substrate and displayed a neutral pH most favorable. Even though highly thermolabile, the α-amylase retained substantial activity within an intermediate temperature range, highlighting its suitability for controlled industrial practices.

Summing up, this study underscores a promising Bacillus subtilis strain capable of producing a highly active, thermolabile α-amylase under low-nutrient and moderate conditions. The enzyme's stability and upgraded performance underline its potential for industrial biocatalysis and additional uses in starch processing, food, and pharmaceutical industries.

Conflict of interest

Authors declare no conflict of interest.

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