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# The Quantification of an Extracellular α-Amylase Activity from *Bacillus subtilis* in a Strach-Supplemented Medium Using Reducing Sugar Analysis

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**Abstract**

**Background:** *α-Amylase is an extracellular enzyme responsible for the degradation of starch by cleaving α-1,4-glycosidic linkages, thereby producing shorter reducing sugars. This reaction holds significance due to the abundance, cost-effectiveness, renewability, and widespread use of starch as a raw material in numerous bioprocesses. In the context of industrial starch processing, the hydrolysis mediated by amylase is integral to the liquefaction and saccharification stages, which are critical in determining the efficiency of syrup production, brewing, baking, bioethanol production, and other processes reliant on starch.* **Methodology:** *This study aims to evaluate the extracellular α-amylase activity from Bacillus subtilis cultivated in nutrient broth supplemented with 1% (w/v) soluble starch. The culture was incubated at 37°C for 24 h under shaking conditions at 130 rpm and an initial medium pH of 7.0. The crude extracellular enzyme was obtained by centrifugation at 6,000 rpm for 20 min. α-Amylase activity was determined using the 3,5-dinitrosalicylic acid (DNS) assay by measuring reducing sugars released from soluble starch hydrolysis, with maltose as the calibration standard. The enzyme activity was determined in U/mL by spectrophotometric analysis at 550 nm.* **Findings:** *The maltose standard curve produced the regression equation $y = 0.7849x + 0.0938$ and an $R^2$ value of 0.9732. The B. subtilis culture grown in starch-supplemented nutrient broth showed a mean absorbance of 2.3915 and an α-amylase activity of 0.116 U/mL. These results showed that B. subtilis secreted detectable extracellular α-amylase under the tested conditions, although the activity was low.* **Contribution:** *The addition of starch contributed to enzyme induction because starch functions as both a substrate-related carbon source and a signal for amylolytic enzyme production. Therefore, nutrient broth supplemented with 1% starch can be used as a simple medium for preliminary extracellular α-amylase production by B. subtilis*

**Keywords:** *α-amylase; Bacillus subtilis; Extracellular Enzyme; Maltose; Reducing Sugar*


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## INTRODUCTION

Enzymes are protein molecules that catalyze biochemical reactions in biological systems. They play essential roles in various reactions, including hydrolysis, oxidation, reduction, isomerization, addition, radical transfer, and carbon chain cleavage (Yao et al., 2026). In general, enzymes increase reaction rates with high substrate specificity and regulatory control. As biological catalysts, enzymes accelerate chemical reactions by lowering the activation energy required for the reaction to occur (Siddikey et al., 2025). Some enzymes can increase reaction rates by approximately $10^8$ to $10^{11}$ times compared with uncatalyzed reactions. During catalysis, enzymes convert substrates into products without being consumed or permanently altered (Farhan et al., 2025). Enzyme activity is also affected by temperature. It generally increases with rising temperature until the optimum temperature is reached. Beyond this point, temperature increases may reduce enzyme activity due to structural instability or denaturation (Egilmez & Haspolat, 2024).

In addition, amylases are among the hydrolytic enzymes abundantly produced by microorganisms. These enzymes hydrolyze glycosidic bonds in starch, producing smaller molecules such as glucose, maltose, and dextrins (Kashtoh & Baek, 2023). Starch hydrolysis by amylase is commonly associated with three main processing stages: gelatinization, liquefaction, and saccharification (Apostolidi et al., 2020). These processes are generally energy intensive (Roohi et al., 2024). Amylases can be produced by amylolytic bacteria and fungi isolated from various environments, including soil and hot springs (Alhazmi & Alshehri, 2025). Soil provides a complex habitat for bacterial growth and survival because it contains diverse nutrients that support microbial metabolism. Amylolytic bacteria are widely distributed in soil, making them potential sources of amylase for industrial applications (Thiele-Bruhn, 2021).

Moreover, the α-amylase enzyme can be produced extracellularly by several microorganisms: *Aspergillus oryzae, Aspergillus niger, Aspergillus awamori, Bacillus megaterium, Bacillus subtilis, Bacillus stearothermophilus,* and *Bacillus licheniformis. Bacillus subtilis* is a rod-shaped, Gram-positive, aerobic bacterium (Patil, 2023). In this study, *Bacillus subtilis* was used because it is relatively easy to cultivate in simple media, is generally non-pathogenic, and can grow at moderately elevated temperatures (Liu & Yu, 2025).

Furthermore, the selection of *Bacillus subtilis* as an α-amylase-producing microorganism is supported by its biological characteristics, particularly its ability to secrete proteins into the culture medium. This characteristic is important because extracellular enzymes can be more easily recovered from the culture supernatant, separated from cell biomass, and purified for enzyme activity analysis (Cong et al., 2024). Recent studies have also shown that the *Bacillus subtilis* expression system can be optimized through engineered promoters, signal peptides, and inducible expression systems to enhance extracellular enzyme production (Farouk et al., 2025).

In fact, microbial α-amylase production is influenced not only by the microbial strain but also by the medium composition, carbon and nitrogen sources, temperature, pH, and fermentation method. Solid-state fermentation is often applied because it

enables the utilization of agro-industrial residues as low-cost substrates, including rice bran, wheat bran, agricultural by-products, and other starch-containing materials (Far et al., 2020a). This strategy is important for reducing enzyme production costs, particularly when α-amylase is intended for industrial applications in food processing, textiles, detergents, and starch-based bioprocessing (Singh et al., 2022). Previous studies have also reported that agro-residues can support amylase production by *Bacillus subtilis*, while statistical optimization can further improve α-amylase yield from *Bacillus* isolates for industrial applications (Pranay et al., 2019).

Clearly, in the application, α-amylase plays an important role in the early stage of starch hydrolysis by cleaving starch chains into shorter oligosaccharides. This process can be followed by saccharification to increase the formation of simple sugars (Sigüenza-andrés et al., 2022). Several recent studies have shown that hydrolysis efficiency can be improved by optimizing pH, temperature, incubation time, and microbial strain selection, particularly strains with high enzyme activity and good thermal stability (Mahfudz et al., 2024). These findings are relevant to studies assessing extracellular amylase activity produced by *Bacillus subtilis*, as these parameters determine the enzyme's potential application in enzyme-based starch processing (Alhazmi & Alshehri, 2025). The industrial potential of α-amylase is not determined only by its ability to hydrolyze starch, but also by its stability under different processing conditions, substrate compatibility, and efficiency of extracellular production. These aspects are important because industrial enzymes must maintain catalytic activity during processing and remain suitable for scalable production systems (Farooq et al., 2021). In this context, improving extracellular α-amylase production in *Bacillus subtilis* through regulatory element modification and fermentation optimization provides a relevant strategy for developing more efficient enzyme production systems for starch-based bioprocessing (Rao et al., 2026).

The nature and mechanism of action of the α -amylase depend on its source. Extensive information has been obtained regarding amylase enzymes that have been successfully isolated, purified, and crystallized from various sources (Kathuria et al., 2023). Most α-amylases originate from microorganisms, as they can be cultivated in large quantities under well-controlled conditions. Microbial α-amylases are relatively easy to isolate and purify, and their production can be tailored according to specific functional requirements and protein structures. Some α-amylases, such as those from *Bacillus stearothermophilus,* are thermostable and possess relatively stable cellular components (Siddikey et al., 2025). Given the biological importance of α-amylase, numerous studies have been conducted. Therefore, this study aimed to quantify the extracellular α-amylase activity produced by *Bacillus subtilis* after 24 hours of cultivation in nutrient broth supplemented with 1% soluble starch. Enzyme activity was assayed using the 3,5-dinitrosalicylic acid (DNS) method described by Bernfeld (1955) under assay conditions of pH 7.0 and 27°C for 20 minutes, with extracellular α-amylase activity expressed in U/mL as the main measured variable.

## METHOD

### Materials and tools

The equipment used in this study consisted of a spectrophotometer, centrifuge, test tubes and racks, serological pipettes, vortex mixer, hot plate, and 1 L beaker glass. Then, the materials included *Bacillus subtilis* culture, nutrient broth (NB), soluble starch, maltose stock solution at 1 mg/mL, DNS reagent, 0.05 M phosphate buffer at pH 7.0, aluminum foil, and distilled water.

### Preparation of Crude Enzyme Extract

A *Bacillus subtilis* culture was inoculated at 5% (v/v) into 50 mL of nutrient broth (NB) medium supplemented with 1% (w/v) soluble starch in a 250 mL Erlenmeyer flask. Then, the culture was incubated at 37°C for 24 h. After incubation, the culture was centrifuged at 6,000 rpm for 20 min. Next, the supernatant obtained was collected as the crude extracellular enzyme extract and used for quantitative determination of α-amylase activity (Amina et al., 2026).

### Preparation of NB Medium, Bradford Reagent, DNS Reagent, Maltose Stock Solution, and Phosphate Buffer

Bradford reagent was prepared in a fume hood by mixing 25 mL of 95% ethanol, 50 mL of 85% *orthophosphoric acid*, and *Coomassie Brilliant Blue* G-250 to an initial volume of 75 mL. The solution was then diluted with 425 mL of sterile distilled water to obtain a final volume of 500 mL. Before use, the Bradford reagent was diluted at a ratio of 1:4 by mixing 1 mL of reagent with 4 mL of sterile distilled water. Meanwhile, a 1 mg/mL maltose stock solution was prepared by dissolving 100 mg of maltose in sterile distilled water and adjusting the final volume to 100 mL (Izebe et al., 2020).

### Preparation of Crude Enzyme Extract

Cultures of *Bacillus subtilis* were incubated for 24 hours in *Nutrient broth* (NB) medium supplemented with 1% soluble starch and in NB medium without starch. After incubation, the cultures were centrifuged at 6.000 rpm for 20 minutes. The resulting supernatant, referred to as the crude enzyme extract, was used for the quantitative amylase enzyme assay (Yao et al., 2023).

### Maltose Standard Curve Preparation

A maltose standard curve was prepared using a 1 mg/mL maltose stock solution. The stock solution was serially diluted with distilled water to obtain standard concentrations of 0; 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8; 0.9; and 1.0 mg/mL. The ratio of maltose stock solution to distilled water is presented in Appendix 2. A volume of 0.5 mL from each maltose standard solution was first combined with 0.5 mL of buffered substrate solution and incubated at 27 °C for 20 minutes. Following incubation, 1 mL of DNS reagent was introduced, and the mixture was thoroughly homogenized using a vortex mixer. The reaction mixture was then heated at 100 °C for 5 minutes, subsequently cooled to room temperature, and analyzed

spectrophotometrically. Absorbance readings were recorded at a wavelength of 550 nm using a spectrophotometer (Wahyuni et al., 2017).

**Measurement of $\alpha$ -Amylase Enzyme Activity**

$\alpha$-Amylase activity was determined using the 3,5-dinitrosalicylic acid (DNS) assay by measuring reducing sugars released from soluble starch hydrolysis, with maltose as the calibration standard. The absorbance of the red-brown reduced ANS complex was measured spectrophotometrically at 550 nm. Although the classic DNS assay often specifies 540 nm, measurement at 550 nm is highly valid due to the broad absorption maximum of the 3-amino-5-nitrosalicylic acid complex within the 540–550 nm range, which minimizes potential background interference from remaining media constituents. To ensure assay accuracy, a negative control (enzyme blank) containing the inactivated enzyme or replacing the enzyme volume with distilled water/buffer was utilized to subtract any background reducing sugars present in the crude extract. Additionally, a positive control using known concentrations of standard maltose or a commercial $\alpha$-amylase preparation was routinely evaluated to verify the precision of the colorimetric reaction.

## RESULTS AND DISCUSSION

The corrected absorbance values derived from maltose standard solutions were employed to construct a calibration curve. The linear regression analysis produced the equation $y = 0.7849x + 0.0938$, with an $R^2$ value of 0.9732. This result indicated a strong linear relationship within the evaluated concentration range. Table 1 presents the corrected absorbance for each concentration, whereas Figure 1 illustrates the resulting standard curve.

**Table 1.** Maltose absorbance values

| Maltose Concentration | Absorbance of Repeat 1 | Absorbance of Repeat 2 | Mean Absorbance | Corrected Abs |
|---|---|---|---|---|
| 0.0 | 0.108 | 0.134 | 0.121 | 0.121 |
| 0.1 | 0.189 | 0.194 | 0.192 | 0.192 |
| 0.2 | 0.239 | 0.239 | 0.239 | 0.239 |
| 0.3 | 0.293 | 0.232 | 0.263 | 0.263 |
| 0.4 | 0.389 | 0.414 | 0.402 | 0.402 |
| 0.5 | 0.505 | 0.519 | 0.512 | 0.512 |
| 0.6 | 0.522 | 0.585 | 0.554 | 0.554 |
| 0.7 | 0.667 | 0.668 | 0.668 | 0.668 |
| 0.8 | 0.618 | 0.646 | 0.632 | 0.632 |
| 0.9 | 0.630 | 0.642 | 0.636 | 0.636 |
| 1.0 | 0.586 | 0.623 | 0.605 | 0.605 |

The corrected absorbance values were calculated by subtracting the blank absorbance from the absorbance values of the samples and controls, as follows:

corrected Abs = sample/control Abs − blank Abs. The corrected absorbance value was then used as the y-value in the regression equation y = 0.7849x + 0.0938 to determine the corresponding x-value, representing the maltose concentration. The maltose concentrations of each treatment, including *Bacillus subtilis* cultured in starch medium and *Bacillus subtilis* cultured in non-starch medium, are presented in Table 2.

**Table 2.** Maltose Concentration

| Sample | Mean Sample Absorbance | Mean Control Absorbance | Mean Blank Absorbance | K-B | S-B | Xk | Xs | Xs-Xk | Activity Unit |
|---|---|---|---|---|---|---|---|---|---|
| *Bacillus subtilis* strach | 2.392 | 2.175 | 0.119 | 2.056 | 2.273 | 2.49 | 2.70 | 0.21 | 0.116 |

Note: K-B: control net abs.; S-B: sample net abs.; Xk: control maltose conc.; Xs: sample maltose conc.; Xs-Xk: enzymatic maltose conc. Yield

Enzymes function as biological catalysts, mainly proteins in nature, that increase the rate of biochemical reactions without being consumed during the process. During an enzymatic activity, substrates were transformed into specific products through enzyme-mediated catalysis. Amylases are enzymes responsible for starch degradation and can be obtained from microbial, plant, or animal sources. In particular, α-amylase is an extracellular enzyme widely produced by bacteria and fungi. It catalyzes the hydrolysis of α-1,4-glycosidic linkages in starch molecules such as amylose and amylopectin, resulting in smaller carbohydrate units including maltose, maltotriose, and dextrin fragments (Far et al., 2020b).

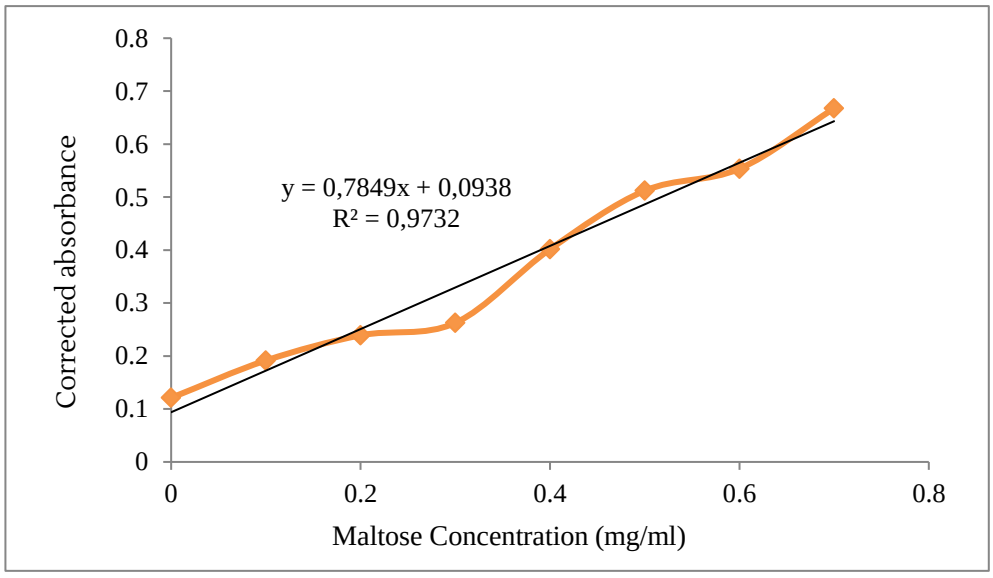


**Figure 1.** Maltose Standard Curve Graph

The mode of action of α-amylase (1,4- α-D-glucan glucanohydrolase) involves the hydrolysis of α -1,4-glycosidic bonds in starch. This enzyme is an extracellular, endo-acting enzyme that cleaves internal α-1,4-glycosidic linkages within starch molecules, producing shorter oligosaccharides and dextrins. *Bacillus subtilis* is known as a microorganism capable of producing α-amylase (Almanaa et al., 2020).

In this study, reducing sugar concentration was determined using the 3,5-dinitrosalicylic acid (DNS) method. The DNS method is based on detecting reducing sugars produced during enzymatic hydrolysis, in which DNS is reduced to form 3-amino-5-nitrosalicylic acid, yielding a reddish-brown color whose absorbance is proportional to the reducing sugar concentration in the sample (Sokač et al., 2023). This method was selected because it is commonly used to estimate α-amylase activity with starch as the substrate and has been reported as a simple, rapid, practical, precise, and accurate colorimetric assay (Zin et al., 2022).

The quantification of α-amylase activity using the 3,5-dinitrosalicylic acid (DNS) method relied on the reduction of DNS by the free reducing groups of sugars released during starch hydrolysis, primarily maltose. Under alkaline conditions and high temperatures, the dinitrosalicylic acid was reduced to 3-amino-5-nitrosalicylic acid, resulting in a distinct color shift from yellow to reddish-brown, which could be measured spectrophotometrically at 550 nm. The application of a heating step was critical in this colorimetric assay because it simultaneously drives the chemical reduction process and effectively terminates the enzymatic reaction, ensuring an accurate endpoint evaluation of the α-amylase catalytic capacity (Zin et al., 2022; Sokač et al., 2023).

Furthermore, the absorbance readings obtained using a spectrophotometer reflect the concentration of reducing sugars produced through amylase activity. The controls were used in the enzyme activity assay to ensure that the measured response resulted from the reaction between the enzyme and the substrate during the incubation period. The absorbance values were maintained within the recommended measurement range of 0.2 – 0.8 to ensure reliable spectrophotometric readings. All crude enzyme extracts obtained in this experiment were diluted twofold before analysis.

The α-amylase activity assay, the enzyme extract obtained from the *Bacillus subtilis* + NB + 1% starch treatment, showed an absorbance value of 2.3915 and an enzyme activity of 0.116 U/mL. This activity was higher than that observed in the *B. subtilis* + NB treatment without starch. The higher of α -amylase activity in the starch-containing medium may be attributed to the role of starch as an inducer of enzyme production. In addition to enzymes produced through bacterial metabolism, the presence of starch in the medium could stimulate of α -amylase production and enhance the hydrolysis of -1,4-glycosidic bonds in soluble starch. This hydrolysis results in the formation of shorter carbohydrate molecules, such as maltose, maltotriose, and glucose. In contrast, in the *B. subtilis* + NB treatment without starch, enzyme production depended mainly on bacterial cells in the absence of starch as an inducer. One unit of α-amylase activity is defined as the amount of enzyme required to produce 1 µmol of maltose per minute from the hydrolysis of soluble starch under the assay conditions.

In this study, α-amylase activity was determined by converting absorbance values to glucose concentration using a standard curve, followed by calculation with the appropriate enzyme activity formula. Notably, the primary advantage of the method presented in this study was its exceptional cost-effectiveness and operational simplicity, as it successfully induced and detected enzyme activity using a straightforward 1% soluble starch in a standard nutrient broth formulation without relying on expensive chemical additives or complex multi-stage purification steps. This showed that a practical baseline medium could effectively facilitate extracellular enzyme production, establishing a highly accessible platform for preliminary screening and industrial bioprocess planning.

To further contextualize these findings, the observed activity of 0.116 U/mL was compared with relevant *Bacillus subtilis* research from the last six years, which typically reported a baseline activity range of 0.5–5 U/mL depending on the degree of environmental and nutritional optimization. In a study by Yao et al., (2023), extracellular α-amylase yields in wild-type *B. subtilis* strains under simple shake-flask conditions naturally fall at the lower end of the generalized threshold. The authors emphasized that transitioning to higher volumetric units required intensive genetic engineering or complex modifications to expression elements, highlighting the distinct value of our standard, non-engineered isolate as a pure and reliable natural baseline.

However, the slight variation in activity observed in this experiment could be systematically explained through critical parameters, primarily media composition and incubation time. Regarding nutrition, Almanaa et al., (2020) showed that maximizing catalytic stability and output in *B. subtilis* usually requires the integration of optimized metal co-factors—such as calcium α-amylase or manganese α-amylase ions—and specialized nitrogen-to-carbon ratios. The omission of these metabolic enhancers in our basic nutrient broth explains why the reaction maintained a stable, controlled baseline output rather than a highly accelerated catalytic rate.

Furthermore, physical parameters such as pH (7.0) and temperature (37°C) were closely aligned with optimal biomass growth conditions, though they may require further fine-tuning to trigger peak enzymatic accumulation. Additionally, Mahfudz et al., (2024) reported that extracellular amylase yields fluctuate markedly with incubation duration and substrate availability, typically remaining near the 0.5–1.5 U/mL threshold in baseline, unoptimized trials. The kinetic behavior observed in our 24-hour cultivation was reasonably attributable to the moderate shaking speed of 130 rpm, which likely influenced the oxygen transfer rate required for maximum secretion. Consequently, the quantified activity of 0.116 U/mL served as a highly valid, stable, and realistic benchmark for crude extracellular α-amylase production. Rather than indicating a limitation, the systematic evaluation of these unoptimized nutritional and physical parameters established a robust and realistic foundation for future upscaling and optimization strategies.

## CONCLUSION

Based on the α-amylase activity assay, the *Bacillus subtilis* + NB + 1% starch sample yielded an activity of 0.116 U/mL. This result showed that starch successfully induced functional extracellular enzyme production to cleave α-amylase-1,4-glycosidic bonds under the tested conditions. Functionally, these findings offer vital implications primarily for eco-friendly starch biodegradation frameworks and cost-effective bioremediation strategies. The capacity of this non-engineered strain to secrete active amylase using a highly simplified, inexpensive medium established an economically viable blueprint for the sustainable bioprocessing of carbohydrate-rich agro-industrial waste and standard industrial starch liquefaction. Consequently, this study provides a realistic baseline benchmark that justifies future upscaling models for environmental biotechnological applications.

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