Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 15(2), 437-447, 2025
Journal of the Institute of Science and Technology, 15(2), 437-447, 2025
ISSN: 2146-0574, eISSN: 2536-4618
Biology **DOI: 10.21597/jist.1577711**
Research Article
Received: 01.11.2024 **Accepted: 27.11.2024**

To Cite: Güler, B.E., Baygın, E. & Demirkan E. (2025). Time-Dependent Hydrolysis of Starch by Mutant *B. subtilis* EBUE 5-3 α–Amylase, Investigation of Its Effect on Starch Granules by SEM Microscopy. *Journal of the Institute of Science and Technology*, 15(2), 437-447.

Time–Dependent Hydrolysis of Starch by Mutant *B. subtilis* EBUE 5–3 α–Amylase, Investigation of Its Effect on Starch Granules by SEM Microscopy

Baran Enes GÜLER[1], Eren BAYGIN[2], Elif DEMİRKAN[1]*

Highlights:

- After 45 minutes, the mutant α–amylase hydrolyzed starch as the final main product with a small amount of G1 and a large amount of G2 and G3.
- The enzyme broke down different raw starch granules in different patterns.
- Rice ranked first in substrate specificity.

Keywords:

- Amylase
- Thin layer chromatography
- Raw starch granules
- Scanning electron microscopy

ABSTRACT:

In this study, the hydrolysis products of soluble potato starch by α–amylase from mutant *B. subtilis* EBUE 5–3 were determined in time. The enzyme was observed as hydrolysis products from starch within the first 5 minutes as G2, G3 and G5 and a small amount of G4. G4 was detected between 15-45 minutes, while G5 was observed in small amounts in 15 minutes. G4 and G5 were not observed at other times. The amounts of G1, G2 and G3 increased with the increase of time. A small amount of G1 and a large amount of G2 and G3 were obtained as the final main products. The degradation capabilities of the enzyme on raw wheat, corn, rice and potato starch granules were investigated by scanning electron microscope. While deep holes were observed in wheat, corn and rice granules, a spongy structure was formed in rice and corn granules. It was observed that the surfaces of both were completely degraded. Only superficial disintegration was determined in potato granules. It was determined that the enzyme preferred rice, corn and wheat as the best starches in terms of substrate specificity, respectively.

[1] Elif DEMİRKAN (**Orcid ID**: 0000-0002-5292-9482), Baran Enes GULER (**Orcid ID**: 0000-0001-7967-9041), Bursa Uludag University, Faculty of Arts and Science, Department of Biology, Bursa, Türkiye
[2] Eren BAYGIN (**Orcid ID**: 0000-0002-3251-0705), Karamanoğlu Mehmetbey University, Faculty of Engineering, Department of Bioengineering, Yunus Emre Campus, Karaman, Türkiye)

*** Corresponding Author:** Elif DEMİRKIRAN e-mail: edemirkan@uludag.edu.tr

INTRODUCTION

Starch is an insoluble storage carbohydrate composed of α-glucose polymers synthesized by plants and algae. Starch is soft, white, odorless, tasteless and is insoluble in alcohol, water and ether (Pokhrel, 2015). Starch is composed of two macromolecules, a linear amylose and branched amylopectin structure. While amylose is a flat structure consisting of D–glucopyranosyl polymer linked by α–1,4 glycosidic bonds, amylopectin contains 5–6% branch points linked by α-–1,6 glycosidic bonds and a flat structured α–(1–4) D-glucose unit chain (Tako et al., 2014, Hamaker et al., 2019). Native starch contains 80-90% amylopectin and 10-20% amylose (Pawar et al., 2008).

Starch has been widely used in human nutrition for many years. Apart from the food sector, it has a wide range of uses in the production of paper and cardboard, bioplastics, as packaging material, in the pharmaceutical sector and in textiles (Zhang et al., 2014). Global starch production is estimated to be between 88.1 and 97.7 million tonnes in 2020. Of this total, 75% is maize, 14% cassava, 7% wheat and 4% potato (Vilpoux et al., 2023). The global starch market is also growing as the demand for ready-made foods increases in response to the rapid growth of population and industry. The market is expected to reach a valuation of USD 75.7 billion by 2027, at a compound annual growth rate (CAGR) of 6.4% (Anonymous, 2024).

Starch as well as its degradation products are produced from starch using acids and enzymes and are used in various industrial fields. Due to the formation of undesirable by-products as a result of the hydrolysis of starch with acids, chemical hydrolysis has now been replaced by enzymatic hydrolysis at a rate of 75% due to its significant advantages (Tonkova, 2006).

Starch is hydrolyzed by the synergistic action of α–amylases (endo–amylase), β-amylase and glucoamylase (exo–amylases), pullulanase (enzymes that reduce branching) and other enzymes (Nirmala & Muralikrishna, 2003). α–Amylases and glucoamylases are the most widely used enzymes in industrial and biotechnological applications (Aquino et al., 2003).

Amylases, which constitute 25% of the world enzyme market, are important industrial enzymes used as broadcast in many industries such as food, sugar, fermentation, animal nutrition, paper and pulp, and textile. The amylase family (exoamylases, endoamylases, debranching enzymes and transferases) is the most studied group of enzymes that hydrolyze starch into polymers composed of dextrins and glucose units (Li et al., 2015). Starches are enzymatically hydrolyzed to glucose, maltose, maltooligosaccharides and dextrins. Dextrin is generally defined as a degradation intermediate from starch to maltose, but typical dextrin products are obtained by the degradation of starch to a lesser extent than oligosaccharides (Sharma et al., 2010; Nakanishi et al., 2014).

Starch fractionation products are widely used in various fields of industry. It is mainly used for food, sweeteners, flavor adjustment, osmotic pressure adjustment, moisturizing agents and powder-based materials. In the field of medicine, it is used as a carbohydrate source in enteral nutrition and as an excipient in medicines, and the starch breakdown products themselves have physiological effects such as intestinal regulation, blood sugar regulation, and triglyceride-lowering effects. It is also used as a binder in the cosmetic field to solidify cosmetics and adjust the viscosity of cream-like cosmetics. Dextrins have a low degree of sweetness and do not significantly change the sweetness of foods and beverages, so they are used as bulking agents and carbohydrate sources in many foods and beverages (Nakanishi et al., 2014)

In recent years, interest in maltooligosaccharides, which are starch hydrolysis products, has increased due to their increased use as functional foods and biopreservatives (Barreteau et al., 2006).

Maltose, glucose or fructose syrups formed from starch degradation products are widely used in the sweetener, food and ethanol industries because they are inexpensive products (Gligorijevic et al., 2014). After starch is broken down to dextrins, it is further decomposed to glucose by the addition of glucoamylase to the medium. The released glucose is obtained either in syrup form or in crystalline powder form. High fructose syrup is produced by adding glucose isomerase enzyme to glucose syrup. This product has a high sweetness and is widely used in soft drink production, dairy products, bakery products, canning, pastry and confectionery production (Parker et al., 2010)

Maltooligosaccharides produced by enzymatic hydrolysis of starch are determined qualitatively by thin layer chromatography (TLC). Sweeteners or ethanol suitable for industrial applications can be produced by looking at the products visualized by TLC as a result of hydrolysis depending on time (Lovšin Kukman et al., 1998).

In the present study, the time–dependent determination of starch degradation products of mutant *Bacillus subtilis* EBUE 5–3 α–amylase enzyme by TLC and their ability to degrade different starch granules by Scanning Electron Microscope (SEM) were investigated.

MATERIALS AND METHODS

Materials

A new isolate *B. subtilis* strain (ORBA Biochemistry, Istanbul) isolated and identified from Turkish soil was mutated using a combination of ultraviolet radiation (UV), ethidium bromide (EtBr) and ethyl methyl sulfonate (EMS), and a mutant strain called *Bacillus subtilis* EBUE 5–3 was used (Sarıkaya, 1999).

Raw rice, corn, wheat and potato starches were used as starch sources.

Methods

Enzyme Production Conditions and Purification Steps

Liquid basal medium content (%): starch 1, corn soaking liquid 0.5, peptone 0.5, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.05, $(NH_4)_2SO_4$ 0.8, K_2HPO_4 1.4, KH_2PO_4 0.6 (pH 7.0) (Sarıkaya & Gurgun, 2000). Mutant *Bacillus subtilis* EBUE 5–3 α–amylase production was performed at 37ºC on a shaker with 150 rpm for 72 h. The culture medium was centrifuged (10 min at 6000 rpm), and the supernatant was used as the crude enzyme source.

The enzyme was purified by 80% ammonium sulfate precipitation, TSK Toyopeal column, ultrafiltration, dialysis and SP sepharose column. Purity control was performed and demonstrated by Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970; Demirkan, 2011).

Profile of starch hydrolysis products

In this study, thin layer chromatography (TLC) method was used to determine the profile of the products formed as a result of the hydrolysis of soluble starch with α-amylase (Stahl, 1965).

For the chromatographic method, 5 ml of 1% soluble starch solutions were taken and incubated with 0.1 mL of purified enzyme at 37°C for 5, 15, 30, 45, 60, 120 and 180 minutes. At the end of this period, the reaction was stopped by adding 5 ml of 0.1 N HCl solution into the tubes. In order to determine the type of hydrolysis products as a result of the reaction, 20×10 cm and 0.2 mm thick silica gel plates were used (Merck, 100390). Samples were applied as 5 µl with a microsyringe (Hamilton) at room temperature, 2 cm above the bottom of the plate and at least 1.5 cm between samples, and the plates were allowed to dry at room temperature. A mixture of ethanol: distilled water: butanol (3:2:5)

was used as a solvent to separate the enzyme hydrolysis products by thin layer chromatography (Jensen et al., 1988). The plate on which the samples were applied was placed vertically in the chromatography tank containing the solvent according to the bottom-up method and immersed 1.5 cm from the bottom edge into the solvent. The plate was kept in the tank at room temperature until the solvent rose 10–15 cm above. Then the plate was removed and dried by keeping it in the oven at 110 °C for 10 minutes. In order to make the carbohydrates, which are the hydrolysis products, visible on the thin layer silica gel plate, a solution of sulfuric acid: methanol (1:3) was carefully sprayed on the plate. Then, the plate was dried in an oven for 10 minutes at 110°C, so that the carbohydrates become visible as black or brown dots on the white plate (Robyt & White 1987).

Glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltoheptaose (G5), maltohexaose (G6), maltoheptaose (G7) were used as standards to determine the type of hydrolysis products.

Scanning electron microscopy examination of raw starch granules

To determine the hydrolysis ability of the enzyme on starch grains, firstly starches from different sources were washed with distilled water several times. Then they were dried in a desiccator. Dried starches (5%) were suspended in 50 mM Na acetate buffer (pH 5.4) containing 5 mM $CaCl_2$ and 10 mM 2–ME (Mercaptoethanol). Pure enzyme solution was added to the suspended starch samples and incubated at 35 °C for 24 h. After incubation, the solution was centrijugated at 10 000 rpm for 5 minutes at +4°C.

For electron microscopy studies, pellets containing starch granules were washed twice with ethanol and then twice with t–butyl alcohol (2–methyl–2–propanol) and centrifuged (5 min at 10 000 rpm) after each wash. After centrifugation, all samples were lyophilized for 2 hours. Dry starch granules were attached to a silver-plated SEM (Scanning Electron Microscope) stand and the samples were coated with palladium/platinum using an ion coater (Hitachi, E–1030). Thus, the samples were investigated with a Scanning Electron Microscope (SEM), Hitaci, S–4500) (Demirkan Sarikaya et al., 2005).

Substrate specificity

Substrate specificity of the pure enzyme was studied using different substrates. For this purpose, rice, corn, wheat, and potato starches were used. The concentration of reducing sugars formed by amylase activity was determined by the dinitrosalicylic acid (DNS) method (Berfeld, 1955). Amylase activity was measured in a 5% raw starch solution for 3 min at 35°C. The concentrations of reducing sugars formed as a result of the activity experiment were calculated in milligrams according to a calibration curve made with maltose.

The results are the averages of three independent determinations.

RESULTS AND DISCUSSION

Starch is an important source of energy for humans. Starch and starch hydrolysis products are used in many industrial areas. It is important to determine the end products and timing of hydrolysis of different starch sources by starch degrading enzymes.

In this study, Thin Layer Chromatography (TLC) method was used to determine the substrate degradation products of α–amylase enzyme. As a result of the studies, the products formed during the hydrolysis of starch on the silica gel plate were determined qualitatively. In the study conducted with mutant *B. subtilis* EBUE 5–3 α–amylase, starch degradation products were detected between 5 minutes and 3 hours. The enzyme breaks down starch into G2, G3 and G5 within 5 minutes, while G4 was seen

in small amounts (Table 1 and Figure 1). As the time increased, the amounts of G1, G2 and G3 also increased. G4 observed in small amounts between 15-45 minutes and G5 in 15 minutes. G4 and G5 were not observed at other times. They were broken down into G1, G2 and G3. Therefore, a little amount of G1 and more G2 and G3 were obtained as the main product. G6 and G7 were not observed at any of the experimental times (Table 1 and Figure 1).

Table 1. Time-dependent hydrolysis of soluble starch by mutant *B. subtilis* EBUE 5–3 α–amylase. Absent (–), Increasing relative density images (+, ++, +++, ++++)

Standard Sugars	5 min.	15 min.	30 min.	45 min.	1 h	2 h	3 h
Glucose (G1)	+	+	++	++	++	++	++
Maltose (G2)	+++	++++	++++	++++	++++	++++	++++
Maltotriose (G3)	+++	++++	++++	++++	++++	++++	++++
Maltotetraose (G4)	+	++	+	+	–	–	–
Maltoheptaose (G5)	++	+	–	–	–	–	–
Maltohexaose (G6)	–	–	–	–	–	–	–
Maltoheptaose (G7)	–	–	–	–	–	–	–

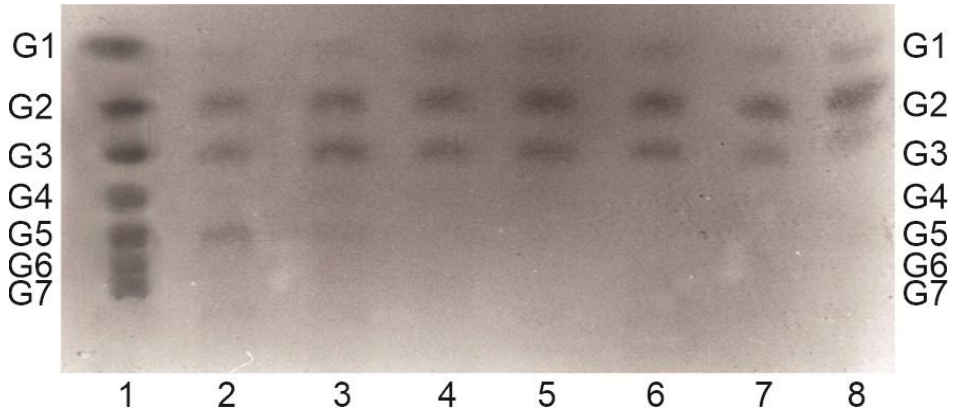

Figure 1. Thin layer chromatographic analysis of main products obtained from starch hydrolysis by mutant EBUE5–3 α–amylase. Standard sugars (1), 5 min. (2), 15 min. (3), 30 min. (4), 45 min. (5), 1 h (6), 2 h (7) and 3 h (8)

Different starch degradation products of α–amylase enzymes obtained from different bacteria and *Bacillus* species were revealed by thin layer chromatography. *Bacillus licheniformis* α–amylase was reported to be the dominant sugars in the 30 minute hydrolysis of 10% soluble starch (cassava, corn, millet and sorghum) as maltose (G2) and glucose (G1) (Adeniran & Abiose, 2011). In another study, *Paecilomyces variotii* ATHUM 8891 α–amylase was incubated in the presence of starch for 8 and 24 h and the hydrolysis products were shown to be mainly maltose and maltotriose (G3) (Apostolidi et al., 2020).

Gligorijević et al. (2014) the hydrolysis products of soluble starch and raw corn of the α-amylases obtained from the *Bacillus* strains they isolated (5B, 12B, 16B, 18 and 24B) were determined by TLC. According to the results they obtained, they reported that most of them produced G3 intensively from soluble starch and hydrolyzed raw corn to G2, G3 and G5.

Bacillus subtilis strain AS01a α–amylase has been reported to convert starch into maltose and glucose as the main hydrolyzed products (Roy et al., 2013).

The first products resulting from both crude and soluble starch hydrolysis by α–amylase from *Geobacillus thermoleovorans* were G2 and G3 (Mehta & Satyanarayana, 2013)

The α-amylase of the marine bacterium *Bacillus* sp. ALSHL3 was reported to degrade soluble starch after 20 h of incubation, with the most concentrated product being G2, followed by G3 and G1 (Vidilaseris et al., 2009). A similar result was noted with the extracellular α-amylase of *B. subtilis* SUH4–2 (AmyE) (Cho et al., 2000). Thin layer chromatography (TLC) analysis of starch hydrolysis from *Aspergillus niger* isolate showed glucose as the predominant product of hydrolysis with small amounts of G2 (Gupta et al., 2010).

The effect of *Pseudomonas stutzeri* AS22 raw enzyme on starch hydrolysis was investigated at different time intervals and only G4 was obtained as the main product after 2 minutes. It was stated that the formation of G4 as the final main product was only seen in *Pseudomonas* strains (Maalej et al., 2014). *Bacillus* sp. GM8901 (Kim et al., 1995) and *B. halodurans* MS-2-5 (Murakami et al., 2008) also had G4–forming amylases.

It has been stated that maltose and maltotriose are the main hydrolysis products of starch hydrolysis by purified amylase produced by *Cryptococcus flavus* (Wanderley et al., 2004).

As a result of 1 hour of *Corallococcus* sp. strain EGB α–amylase enzyme, the presence of oligosaccharides (G4, G5, G6 and G7) as starch hydrolysis products was observed. The main product was G6, while G2 to G5 were found to be minor products (Li et al., 2015).

α–Amylase from Paecilomyces variotii was hydrolyzed by incubation with starch for 0.5–24 h, and the products were mostly G2 and G3, traces of G1 were also observed (Michelin et al., 2010).

In the TLC study conducted with *Saccharomycopsis fibuligera* R64 glucoamylase, it was shown that the starch hydrolysis product was only monosaccharide, namely glucose (G1). It was reported that the intensity of the G1 spot increased with an incubation time of 5 to 45 minutes (Ismaya et al., 2012)

Hydrolysis profiles differ among strains of the same genus or of different genera, as they represent different sources for α–amylase production.

Starches are also characterized by the morphology of the hydrolyzed granules. Scanning Electron Microscopy (SEM) is widely used to study the changing structure in starch granules (Pilling & Smith, 2003). Especially the presence of cracks and fissures in the granules can be observed. SEM images providing higher magnification clearly show the surface topography of the non-hydrolyzed and enzyme-hydrolyzed granules. This allows the understanding of the degradative effect mechanism of enzymes on starch (Chakraborty et al., 2020)

In this study, the 24-hour morphologies of 5 different raw starch granules treated and untreated with mutant *B. subtilis* EBUE 5–3 α–amylase enzyme were examined by SEM. Starch grains untreated with enzyme (Figure 3 A, B, C and D) were compared with starch grains treated with amylase enzyme. As a result of the study, while little fragmentation was observed on potato starch granule surfaces, the most fragmentation was in corn, rice and wheat starches, respectively. It was determined that the enzyme breaks down corn, rice and wheat granules by centripental type fragmentation, i.e. from the surface to the center (Figure A1, B1 and C1), and in potatoes, it breaks down by centrifugal type fragmentation, i.e. by surface abrasion (Figure D1). On the other hand, the classic Swiss cheese model first reported by Smith and Lineback (1976) (Robyt, 2008) was observed in corn granules (Figures A2). While deep holes were observed in wheat, corn and rice granules (Figures C2, A2 and B2), rice and corn granules had a spongy structure due to the fragmentation effect and their surfaces were completely degraded and deep cracks were observed in corn granules (Figures A2). In potato granules, only superficial fragmentation and a spongy structure were determined (Figure D1).

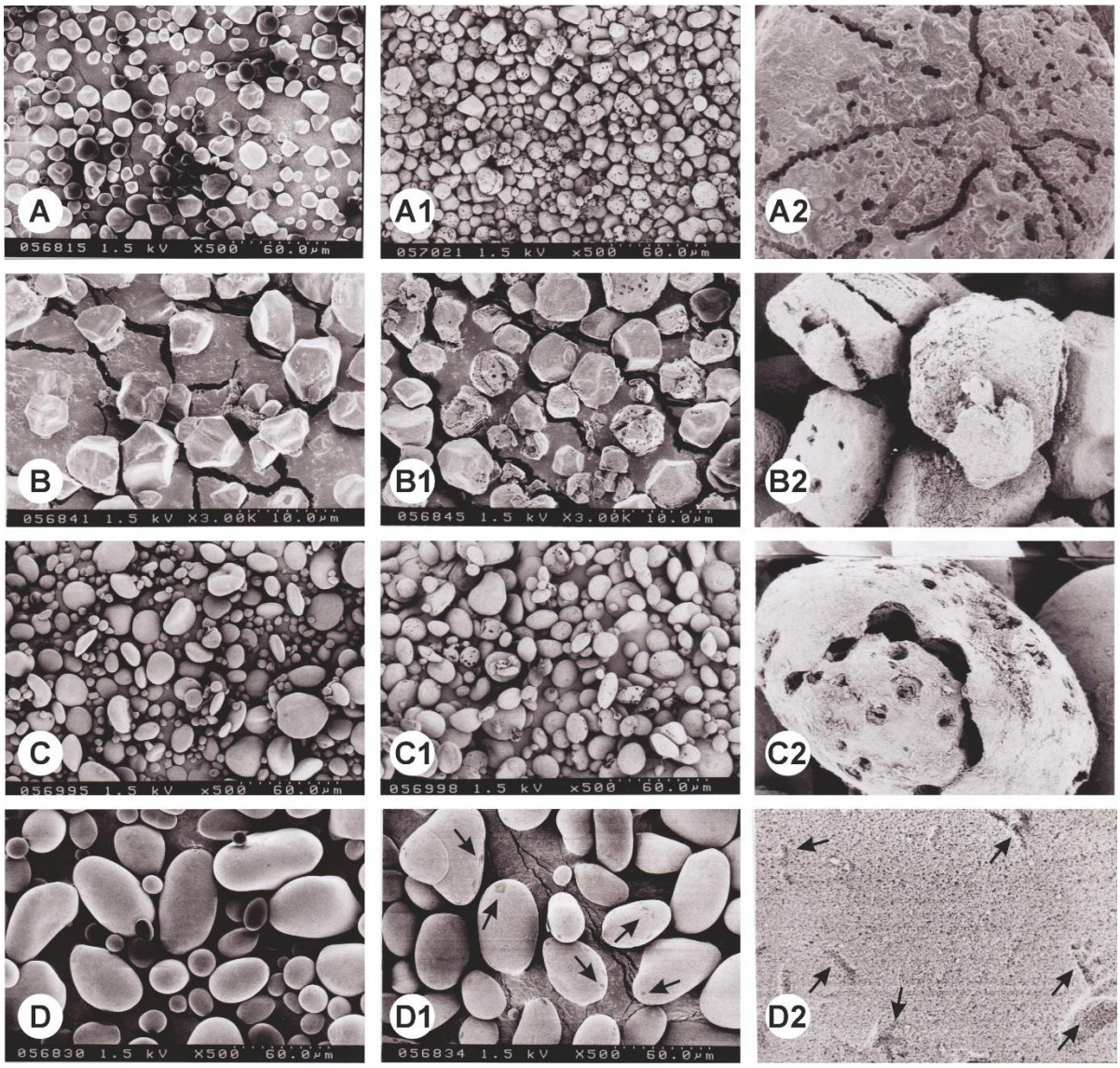

Figure 3. Scanning Electron Microscope images of raw starch granules treated and untreated with mutant *B. subtilis* EBUE 5–3 α–amylase. Micrographs A, B, C and D show untreated starch granules, while A1–2, B1–2, C1–2 and D1–2 shows enzyme-treated starch granules. Corn (A), Rice (B), Wheat (C), Potato (D)

The degradative ability of amylase enzyme on different starch granules has also been reported by different researchers.

In a similar study, Sujka et al. (2006) used a partially purified α–amylase produced from *B. subtilis* for the hydrolysis of corn, potato, wheat and rice starches. α–amylase showed both centripetal ad centrifugal hydrolysis in rice, corn and wheat granules, but centrifugal hydrolysis was detected in potato granules. Helbert et al. (1996) reported a similar result. The ability of α-amylase from *Bacillus* sp. ALSHL3 to degrade rice, corn and cassava starch granules was investigated by SEM. It has been shown that α-amylase degrade different starch granules morphologically differently, thus forming different patterns. It was determined that with the enzyme effect, deep and large holes were formed in rice granules rather than corn granules, and cassava granules were only fragmented at the periphery, but no holes were observed (Vidilaseris et al., 2009).

They reported that the *B. subtilis* strain AS01a α–amylase enzyme formed pits and deep holes on the surface of all raw starches tested, namely wheat, potato and rice, and digested the raw starch very effectively (Roy et al., 2013). In our previous study, it was determined that mutant *B. amyloliqefaciens* α–amylase degraded rice, corn and wheat starches more, whereas potato starches were resistant to

degradation (Demirkan Sarikaya et al., 2005). In a study conducted by Li et al., (2011), the effect of α–amylase on raw starch granules at various incubation time points (12, 24 h) was investigated. In a study conducted by Li et al. (2011), it was stated that after 12 and 24 hours of incubation with starch and α-amylase, there were few cracks on the surface of starch granules as a result of 12-hour hydrolysis, but there was extensive disintegration at the end of 24 hours.

It has been reported that raw potato starch has a surface free of holes and cracks as a result of enzyme treatment, but disintegration occurs over time when high enzyme concentrations are used (Mu et al., 2015). Since there are amorphous regions (pores, cracks) on the starch granules, the enzyme may first attack these regions on the starch surface and hydrolyze the starch. However, it has been reported that granule structure, size and number are important for enzyme attack (Baldwin et al., 1997). In our measurements, it was determined that the grain size of rice was 3 μm, while the grain size of corn, wheat and potato was between 6-15 μm, so it can be concluded that the enzyme attack is greater as the size decreases. Because it was observed that potato starch, which has the largest size, was less hydrolyzed.

Substrate specificity of the enzyme

The ability of mutant *B. subtilis* EBUE 5–3 α–amylase to hydrolyze various raw starch sources was investigated. While the enzyme hydrolyzed potato starch the least, rice was preferred as the best starch. This was followed by corn and wheat, respectively (Figure 2). In electron microscope studies, it was seen that the enzyme degraded rice, corn and wheat starch grains better than potatoes.

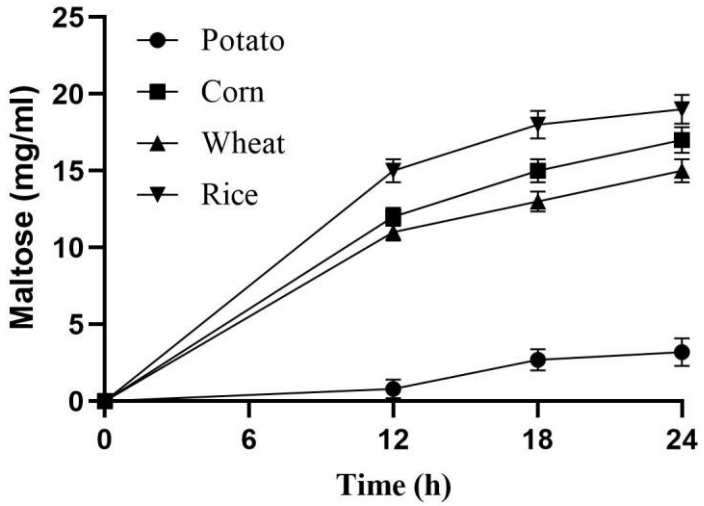

Figure 2. The effect of mutant *B. subtilis* EBUE 5–3 α–amylase on different raw starchs. Release of reducing sugar from starch (conversion to maltose)

CONCLUSION

Knowing the morphological and physico-chemical properties of starch granules provides the basis for studies to be carried out in food science technology. For example, it has been stated that slowly digestible or resistant starch obtained from uncooked foods, whole vegetables, grains and tubers can be used as a suitable carbohydrate source to reduce the risk of various conditions/diseases such as diabetes, obesity and cardiovascular diseases (Chakraborty et al., 2020). Therefore, knowing the affinity of amylases to starch will shed light on the industrial hydrolysis of starch. In this study, knowing which sugars are released from starch by the α–amylase obtained and purified from mutant *B.*

subtilis EBUE 5–3 depending on time and also determining the degradation rates morphologically by electron microscopy may have potential for the application of the enzyme in the starch industry and other biotechnological industry.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

E.Demirkan: The study was planned and designed. E.Demirkan, Baran Enes Guler and Eren Baygın: Collected and analyzed data. E.Demirkan, Baran Enes Guler and Eren Baygın: Wrote and edited the article.

REFERENCES

Adeniran, H. A. & Abiose, S. (2011). Partial purification, characterization and hydrolytic activities of amylases from *Bacillus licheniformis* and *Aspergillus niger* cultured on agricultural residues. *African Journal of Biotechnology*, 11(6), 1465–1477.

Anonymous (2024). Global Starch Market Report,https://www.reportlinker.com.

Apostolidi, M. E., Kalantzi, S., Hatzinikolaou, D. G., Kekos, D. & Mamma, D. (2020). Catalytic and thermodynamic properties of an acidic α–amylase produced by the fungus *Paecilomyces variotii* ATHUM 8891. *3 Biotech*, 10 (7), 311.

Aquino, A. C. M. M., Jorge, J. A., Terenzi, H. F. & Polizeli, M. L. T. M. (2003). Studies on a thermostable α–amylase from the thermophilic fungus *Scytalidium thermophilum*. *Applied Microbiology and Biotechnology*, 61, 323–328.

Baldwin, P. M., Davies, M. C. & Melia, C. D. (1997). Starch granule surface imaging using low-voltage scanning electron microscopy and atomic force microscopy. *International Journal of Biological Macromolecules*. 21(1-2), 103–107.

Barreteau, H., Delattre, C. & Michaud P. (2006). Production of oligosaccharides as promising new food additive generation. *Food Technol Biotechnol*, 44, 323–333.

Berfeld, P. (1955). Amylases, α and β. *Methods in Enzymology*, 1, 149–58.

Chakraborty, I., Pallen, S., Shetty, Y., Roy, N. & Mazumder, N. (2020). Advanced microscopy techniques for revealing molecular structureof starch granules. *Biophysical Reviews*. 12(1), 105–122.

Cho, H. Y., Kim, Y. W., Kim, T. J., Lee, H. S., Kim, D. Y., Kim, J. W., Lee, Y. W., Lee, S. B. & Park, K. H. (2000). Molecular characterization of a dimeric intracellular maltogenic amylase of *Bacillus subtilis* SUH4-2. *Biochimica et Biophysica Acta*, 1478(2), 333–340.

Demirkan Sarikaya E., Mikami, B., Adachi, M., Higasa, T. & Utsumi, S. (2005). α–Amylase from B. amyloliquefaciens: purification, characterization, raw starch degradation and expression in E. coli. *Process Biochemistry*, 40(8), 2629–2636.

Demirkan. E. (2011). Production, purification and characterization of α–amylase by *Bacillus subtilis* and its mutant derivate. *Turkish Journal of Biology*. 35, 705–712.

Gligorijevic, N., Stevanović, N., Lončar, N., Baošić, R., Vujčić, Z. & Božić N. (2014). Thin layer chromatographic comparison of raw and soluble starch hydrolysis patterns of some α–amylases from *Bacillus* sp. isolated in Serbia. *J. Serb. Chem. Soc.* 79(4), 411–420.

Gupta, A., Gautam, N. & Modi, D. J. (2010). Optimization of α–amylase production from free and immobilized cells of *Aspergillus niger*. *E3 Journal of Biotechnology and Pharmaceutical Research*, 1(1), 001–008.

Hamaker, B. R., Tuncil, Y. E. & Shen, X. (2019). Carbohydrates of the kernel. S. O. Serna-Saldivar, (Ed.), *Chemistry and Technology*, (pp. 305–318) Amsterdam: Elsevier.

Helbert, W., Schülein, M. & Henrissat, B. (1996). Electron microscopic investigation of he diffusion of *Bacillus licheniformis* α–amylase into corn starch granules. *International Journal of Biological Macromolecules*. 19(3), 165–169.

Ismaya, W. T., Hasan, K., Subroto, D. N. T. & Soemitro, S. (2012). Chromatography as the major tool in the ıdentification and the structure–function relationship study of amylolytic enzymes from *Saccharomycopsis fibuligera* R64. L. A. Calderon (Ed.), *Chromatography–The most versatile method of chemical analysis*, (pp. 271–294). London: Intech Open.

Jensen, B., Olsen, J. & Allermann, K. (1988). Purification of extracellular amylolytic enzymes from the termophilic fungus *Thermomyces lanuginosus*. *Canadian Journal of Microbiology*, 34, 218–223.

Kim, T. U., Gu, B. G., Jeong, J. Y., Byun, S. M. & Shin, Y. C. (1995). Purification and characterization of a maltotetraoseforming alkaline *α*–amylase fromanalkalophilic *Bacillus* strain, GM8901. *Applied and Environmental Microbiology*, 61(8), 3105–3112.

Laemmli, U. K. (1970). Cleavages of structural proteins during the assembly of the head of Bacteriophage T4. *Nature*, 227, 680–685.

Li, X., Gao, W., Wang, Y., Jiang, Q. & Huang, L. (2011). Granule structural, crystalline, and thermal changes in native Chinese yam starch after hydrolysis with two different enzymes– α–amylase and gluco–amylase. *Starch-Stärke.* 63, 75–82.

Li, Z., Wu, J., Zhang, B., Wang, F., Ye, X., Huang, Y., Huang, Q. & Cui, Z. (2015). AmyM, a novel maltohexaose-forming α–amylase from *Corallococcus* sp. strain EGB. *Applied and Environmental Microbiology*. 81(6), 1977–1987.

Lovšin-Kukman, I., Zelenik-Blatnik, M. & Abram, V. (1998). Quantitative estimation of the action of α–amylase from *Bacillus subtilis* on native corn starch by HPLC and HPTLC. *Eur. Food Res. Technol.* 206, 175–178.

Maalej, H., Ayed, H. B., Ghorbel-Bellaaj, O., Nasri, M. & Hmidet, N. (2014). Production and biochemical characterization of a high maltotetraose (G4) producing amylase from *Pseudomonas stutzeri* AS22. *BioMed Research International*. 156438, 11.

Mehta, D. & Satyanarayana, T. (2013). Biochemical and molecular characterization of recombinant acidic and thermostable raw-starch hydrolysing α–amylase from an extreme thermophile *Geobacillus thermoleovorans*. *Journal of Molecular Catalysis B: Enzymatic*, 85–86, 229–238.

Michelin, M., Silva, T.M., Benassi, V.M., Peixoto-Nogueira, S.C., Moraes, L.A.B., Leão, J.M., Jorge, J.A, Terenzi, H.F., Maria de Lourdes, M., T.M., Polizeli, T.M. (2010). Purification and characterization of a thermostable α–amylase produced by the fungus *Paecilomyces variotii*, Carbohydrate Research. 345, 2348–2353.

Mu, T. H., Zhang, M., Raad, L., Sun, H. N. & Wang, C. (2015). Effect of α-amylase degradation on physicochemical properties of pre–high hydrostatic pressure–treated potato starch. *Plos One*. 10(12), e0143620.

Murakami, S., Nagasaki, K., & Nishimoto, H. (2008). Purification and characterization of five alkaline, thermotolerant, and maltotetraose–producing *α*–amylases from *Bacillus halodurans* MS–2–5, and production of recombinant enzymes in *Escherichia coli*. *Enzyme and Microbial Technology*. 43(4–5), 321–328.

Nakanishi, T., Nomura, K. & Hironori Yoshida, H. (2014). Starch degradation product, food additive containing the starch degradation product, food and drink, drug, and method for producing starch degradation product. URL: https://patents.google.com/patent/JP5507107B2/en (accessed date: September 15, 2024).

Nirmala, M. & Muralikrishna G. (2003). Three alpha-amylases from malted finger millet (Ragi, Eleusine coracana, Indaf–15)-purification and partial characterization. *Phytochemistry*, 62(1), 21-30.

Parker, K., Salas, M. & Nwosu, V. C. (2010). High fructose corn syrup: Production, uses and publichealth concerns. *Biotechnology and Molecular Biology Review*, 5(5), 71–78.

Pawar, R., Jadhav, W., Bhusare, S., Borade, R., Farber, S., Itzkowitz, D. & Domb A. (2008). Polysaccharides as carriers of bioactive agents for medical applications. In natural-based polymers for biomedical applications, *Woodhead Publishing Series in Biomaterials*, 3–53, Publisher: Elsevier.

Pilling, E. & Smith, A.M. (2003). Growth ring formation in the starch granules of potato tubers. *Plant* Physiology, 132(1), 365–371.

Pokhrel, S. (2015). A Review on introduction and applications of starch and its biodegradable polymers. *International Journal of Environment*, 4, 114–125.

Robyt, J. F. & White, B. J. (1987). Biochemical techniques: theory and practice. J. F. Robyt ve B. J. White (Ed.). Wadsworth, Belmont, California, USA: Brooks/Cole Pub. Co.

Robyt, J. F. (2008). Starch: Structure, Properties, Chemistry, and Enzymology. Fraser-Reid, B. O., Tatsuta, K. ve Thiem, J. (Ed.), Glycoscience, (pp. 1437–1472). Berlin: Springer.

Roy, J. K., Borah, A, Mahanta. C. L. & Mukherjee, A. K. (2013). Cloning and overexpression of raw starch digesting α–amylase gene from *Bacillus subtilis* strain AS01a in *Escherichia coli* and application of the purified recombinant α–amylase (AmyBS-I) in raw starch digestion and baking industry. *Journal of Molecular Catalysis B: Enzymatic*, 97, 118–129.

Sarikaya E. (1999). Obtaining α–amylase producing mutants from some *Bacillus* strains using mutagenic agents. *Biotechnology*, 22, 27–34.

Sarikaya, E. & Gurgun, V. (2000). Increase of the α–amylase yield by some *Bacillus* strains. *Turkish Journal of Biology*, 24(2), 299–308.

Sharma, S., Khan, F. G. & Qazi, G. N. (2010). Molecular cloning and characterization of amylase from soil metagenomic library derived from Northwestern Himalayas. *Applied Microbiology and Biotechnology*, 86, 1821–1828.

Stahl, E. (1965). *Thin-Layer chromatography: A laboratory handbook*. Berlin: Springer Science and Business Media.

Sujka, M., Udeh, K. O. & Jamroz J. (2006). α–Amylolysis of native corn, potato, wheat and rice starch granules. *Italian Journal of Food Science*. 18(4), 433–439.

Tako, M., Tamaki, Y., Teruya, T. & Takeda, Y. (2014). The Principles of starch gelatinization and retrogradation. *Food and Nutrition Sciences*, 5(3), 280–291.

Tonkova, A. (2006). Microbial starch converting enzymes of the α–amylase family. R. C. Ray ve O. P. Wards (Ed.), *Microbial Biotechnology in Horticulture*, (pp. 421-472). New Hampshire, USA: Science Publishers.

Vidilaseris, K., Hidayat, K., Retnoningrum, D. S., Nurachman, Z., Noer, A. S. & Natalia D. (2009). Biochemical characterization of a raw starch degrading α–amylase from the Indonesian marine bacterium *Bacillus* sp. ALSHL3. *Biologia*, 64(6), 1047–1052.

Vilpoux, O.F. & Junior J.F.S.S. (2023). Global production and use of starch. M.P. Cereda ve O. François (Ed.), *Starchy Crops Morphology, Extraction, Properties and Applications*, (pp. 43–66). Brazil: Elsevier.

Wanderley, K. J., Torres, F. A. G., Moraes, L. M. P. & Ulhoa, C. J. (2004). Biochemical characterization of α–amylase from the yeast *Cryptococcus flavus*. *FEMS Microbiology Letters*, 231(2), 165–169.

Zhang Y., Rempel C. & Liu Q. (2014). Thermoplastic starch processing and characteristics– a review. *Crit Rev Food Sci Nutr*, 54(10), 1353–1370.