Adıyaman University Journal of Science
https://dergipark.org.tr/en/pub/adyujsci
DergiPark AKADEMİK
ISSN 2147-1630
e-ISSN 2146-586X

Characterization of Amylase from *Bacillus licheniformis* and Application in Desizing Process

Samet KOCABAY[1,*]

[1]*Inonu University, Faculty of Science and Art, Department of Molecular Biology and Genetic, 44050, Malatya, Türkiye*
samet.kocabay@inonu.edu.tr, ORCID: 0000-0002-0120-2910

Received: 13.08.2024 **Accepted:** 25.06.2025 **Published:** 31.12.2025

Abstract

There are more attention on the amylase enzymes in recent biotechnology because of application of them. To provide these needed enzymes, bacterial amylase enzymes are especially focused on. The bacterial strain was identified by sequencing of 16S rRNA coding gene (BM Company, Ankara/Türkiye). After identified amylase production in starchy medium, the enzyme isolated partially, and optimum pH and temperature parameters were characterized. The enzyme was applied to remove starch from starchy fabric. The last product of the enzyme was determined with TLC analysis. Its optimum pH and temperature were 5.0 and 45 ºC, respectively. The potassium/iodine color change showed that enzyme was able to remove starch from fabric due to at least 8 of TEGEWA. Maltose is the last product. The overall results suggest that the extracted crude amylase can be a potential enzyme candidate for textile industries after further characterization in the future.

Keywords: Amylase; *Bacillus licheniformis*; Desizing; Starch; Fabric.

* Corresponding Author DOI: 10.37094/adyujsci.1532994

Bacillus licheniformis Amilazının Karakterizasyonu ve Haşıl Sökme İşleminde Uygulanması

Öz

Son yıllarda biyoteknolojide amilaz enzimlerinin uygulanması nedeniyle üzerinde daha fazla durulmaktadır. İhtiyaç duyulan bu enzimlerin sağlanması için özellikle bakteriyel amilaz enzimleri üzerinde durulmaktadır. Bakteri suşu, 16S rRNA kodlayan genin dizilenmesiyle tanımlanmıştır (BM firması,Ankara Türkiye). Nişastalı ortamda amilaz üretimi belirlendikten sonra enzim kısmen izole edilerek optimum pH ve sıcaklık parametreleri karakterize edildi. Enzim, nişastalı kumaştan nişastayı çıkarmak için uygulandı. Enzimin son ürünü TLC analizi ile belirlendi. Optimum pH ve sıcaklığı sırasıyla 5.0 ve 45 °C olarak kaydedildi. Potasyum/iyot renk değişimi, enzimin en az 8 TEGEWA sayesinde kumaştan nişastayı çıkarabildiğini gösterdi. Maltoz son üründür. Sonuç olarak, elde edilen bulgular ekstrakte edilen ham amilazın gelecekte daha ileri karakterizasyondan sonra tekstil endüstrileri için potansiyel bir enzim adayı olabileceğini göstermektedir.

Anahtar Kelimeler: Amilaz; *Bacillus licheniformis*; Haşıl sökme; Nişasta; Kumaş.

1.Introduction

α-Amylases (endo-1,4-a-D-glucan glucanohydrolase, EC 3.2.1.1) [1] are among the most important class of enzymes and has great importance in biotechnology. The application of amylase expanded to cover many areas from medical, analytical chemistry to starch confectionery (saccharification), food, textiles. Industrial importance of amylases increases interest in the isolation of new bacterial species and the enzyme accounts for about 25% of its trade [2]. Although these enzymes can be isolated from plant, animal and many microbial sources, microbial sources are more preferred to meet the industrial needs. Amylase isolation from microbial sources has advantages than the others such as animal and plant. The microbial enzymes are able to show thermostability that is important in the industry. Many of microorganisms such as *Lactobacillus fermentum, Bacillus amyloliquefaciens, Bacillus licheniformis and Bacillus stearothermophilus*, produce extracellular amylase [3-5].

Starch and cellulose are the most abundant natural polymers in the world. Both glucose formed by different bonding of monomers. Glucose in starch monomers are linked by α-glycosidic bonds, whereas in cellulose, glucose monomers are B- linked by glycosidic bonds. Therefore, these two energy sources, which are important for animals, humans and microorganisms, are hydrolyzed by two different enzymes: starch α-glycoside hydrolases and cellulose is hydrolyzed by β-glycoside hydrolases [6, 7]. Starch is polymer linking of glucose molecules to each other by glycosidic bond. Amylose and amylopectin are two different classes of starch polymers. While the amylose is a straight chain of about 6,000 glucose units linked by α-1,4

glycosidic bond and insoluble in water, amylopectin consists of about $2x10^6$ units has branched chains linked by α-1,6 glycosidic bond and soluble in water [8].

In the textile industry, the amylases are used to remove starch molecule from fabrics. In the weaving of fabrics, the yarns face undesirable situations during weaving. That's why the yarn breakage should be prevented. For this reason, it is tried to give strength to the yarn with a process called as sizing. To this end, a removable protective layer is applied over the yarns. Starch is one of the most used materials in this process because it is cheap, abundant and can be easily removed completely. The process of removin starch used as sizing material is called desizing. Amylases selectively remove starch from fabrics and they do not attack to yarns. The enzymes randomly hydrolyze starch into dextrins which dissolves in water and easily washes away [3, 9, 10].

In this study, we characterized the amylase enzyme from *Bacillus licheniformis* and analyzed potential application of it on the fabric to remove the starch.

2. Material and Methods

Bacillus sp. was used from culture collection of Microbiology Laboratory Course at Department of Molecular Biology and Genetics in Malatya/Türkiye. LB medium (Sigma), starched fabric, desizing fabric was taken from Çalık Denim Textile in Malatya/Türkiye. Aluminum TLC plate (Merck), DNS (dinitrosalicylic acid) (Sigma), the other chemicals are analytical grade.

2.1. Identification of Bacterial Strain

Single bacterial colonies were grown from the overnight culture by using the pour-plate method. One hundred of the colonies were then transferred onto LB agar plates (pH 7.0). The pure cultures were stored at −80 °C. Molecular characterization was performed through outsourcing (BM Company, Ankara/Türkiye). DNA was prepared and used for the amplification of 16S rRNA gene. The sequences obtained from an automatic DNA sequencer were subjected to BLAST analysis and similarities were determined using the National Center of Biotechnology Information databases [11]. An accession number for the sequence was also obtained OP581206.

2.2. Extracellular Amylase Production and Partial Purification

Amylase production of strain was determined on LB agar medium supplemented with 1% starch. Individual colony was inoculated onto the prepared starched LB medium agar plates and incubated at 37 °C overnight. 200 µL Potassium iodine solution (10%) was added onto the agar medium. The zone where the starch molecules digested was visualized as transparent. The bacteria were grown 500 ml LB medium

containing 1% starch for 3 days at 37 °C. It was centrifuged at 4000 rpm for 15 minutes at 4 °C. The supernatant was collected. The crude enzyme was partially purified by ethanol precipitation (1:2 v/v) at -20 °C for 24 hours. It was centrifuged at 4000 rpm for 15 minutes at 4 °C. The supernatant was poured out. The precipitated was lyophilized at -65 °C and used as enzyme source [4].

2.3. Optimum pH

The amylase activity was determined using a pH range, 3.0–10.0, in the following buffer systems (200 mM) at room temperature. Sodium acetate, pH 3.0–5.0; potassium phosphate, pH 6.0–7.0; carbonate, pH 8.0–10.0. The experiment was made triplicate [4].

2.4. Optimum Temperature

In order to investigate the effect of temperature, the assay was conducted separately at 25, 37, 45, 60, 75 and 92 °C. The activity of the enzyme was determined by pre-incubating the enzyme sample at these temperature points for 1 hour at pH 5.0, the activity was recorded using the DNS assay. The experiment was performed in triplicate [4].

2.5. Desizing Progress and DNS Assay

The starchy fabric sample was cut in the dimensions of 5.6 cm X 3 cm (415 mg) for enzymatic treatment and 5.5 cm X 2.7 cm (392 mg) for control group. 10 ml amylase solution with a concentration of 25 mg/mL was prepared at 0.02 M acetat buffer (pH 5.0). The piece of cloth was soaked in the solution. It was kept at 45 °C 5 hours to remove starch from the fabrics. Aliquot samples were taken just before the fabric was immersed in the solution and at various times. The presence of free reducing ends in the respective samples was determined using the dinitrosalicylic acid (DNS) method [4]. The absorbance values were recorded using spectrophotometer (epoch biotech). The samples were run in triplicate. Similar procedures were performed for the negative control. The buffer was used instead of enzyme solution. After 5 hours, it was washed in boiling distilled water for 10 minutes. After 10 minutes in cold distilled water, it was left to dry at room temperature. Weight and size losses of fabric samples were recorded. The fabrics was colored with potassium iodine solution (1% I: 10% KI: H_2O; 1:1:3 v/v). As soon as 20 µL of potassium iodine was poured on the fabrics, the images were captured. Then the results were recorded [10].

2.6. Analysis of Hydrolysis Products by TLC

After reacting 250 µL of 25 mg/mL enzyme solution and 250 µL 1% starch solution under optimum conditions for 24 hours, 30 µL was applied to commercial aluminum TLC plate (Merck, Germany) as a stationary phase. 5 µL 2% glucose and maltose mixture was loaded as standard. A mixture of

butanol:ethanol:water (5:3:2) mobile buffer was used. After waiting for 24 hours for the separation of products, the chromatography paper was dried. After spraying with a sulfuric acid solution (20%) prepared in methanol, it was heated at 120 °C for 20 minutes. The end product of the enzyme was determined [10].

3. Results and Discussion

3.1. 16S rRNA Gene Sequencing

After the detection of the amylase-producing isolate, the genomic characterization results are presented in Fig. 1. According to the obtained phylogenetic tree diagram, it was identified as *Bacillus licheniformis*. The similarity score is 100%.

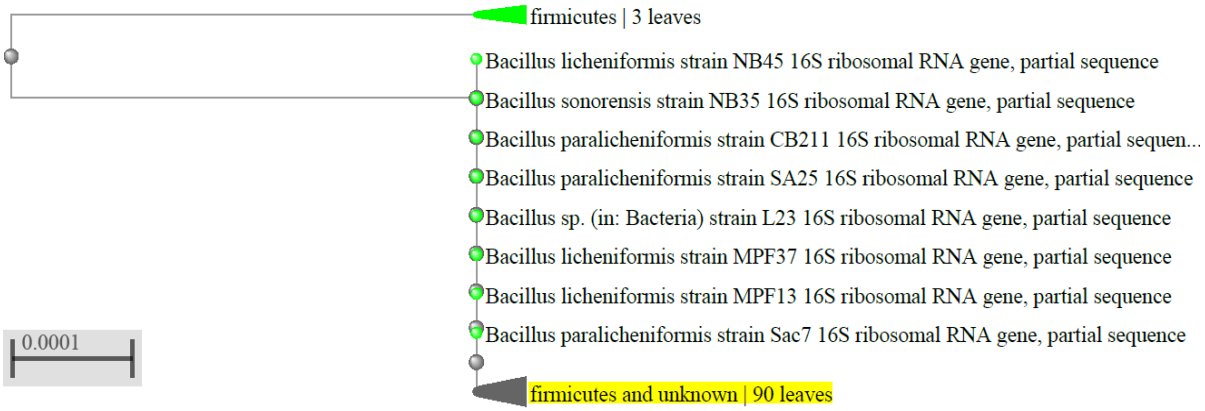

Figure 1: The phylogenetic tree based on partial sequencing of 16S rRNA gene. According to the tree, it belongs to *Bacillus licheniformis*. The scale bar represents 0.0001 substitutions per base position

3.2. Enzyme Production

Bacillus licheniformis was inoculated into the starched culture medium. After overnight, the potassium iodine solution was added on it. Fig. 2 A depicts that the bacterial amylase enzyme activity was determined like transparent. The rest of transparent was purple. Fig. 2 B shows the colony shape of *Bacillus licheniformis*. The production of amylase from *Bacillus sp.* strain was determined in the literature [12-16]. Fig. 2 A explains that there is transparent zone around the colony meaning to having amylase activity. Iodine staining showed the transparent zone around single bacterial colony having amylase production. The dark blue color appears after the iodine reacts with the starch molecules.

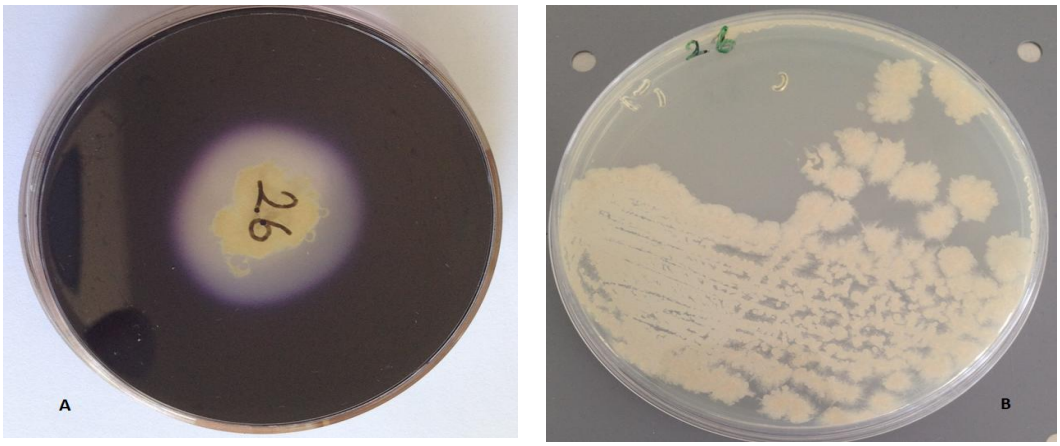

Figure 2: The amylase production (A) and colony shape (B) of *Bacillus licheniformis*

3.3. Optimum pH

The optimum pH was examined with pH 3.5, 4.0, 5.0 in 0.2 M acetate buffer, and 6.0, 7.0, 7.5, 8.0 in 0.2 M phosphate buffer, and 9.0,10.0,10.5 in 0.2 M carbonate buffer, respectively. The Fig. 3 shows that pH 5.0 and 9.0 are optimum for amylolytic activity. The enzyme displays lowest activity at pH 6.0 while The enzyme exhibits relative activity at other pHs. The optimum pH evaluated between the different pH from 3.5 to 10.5. The enzyme showed most activity at pH 5.0 and 9.0 as shown in Fig. 3. The literature explained different amylase enzymes from bacillus had different optimum pH such as pH 10.0 [5], pH 7.0 [13], pH 5.0 [17]. Although amylase from *bacillus subtilis* showed maximal activity at pH 6.0 [18], the interested enzyme indicated lowest activity at pH 6.0. The enzyme had activity at range of pH 3.5 and 10.5.

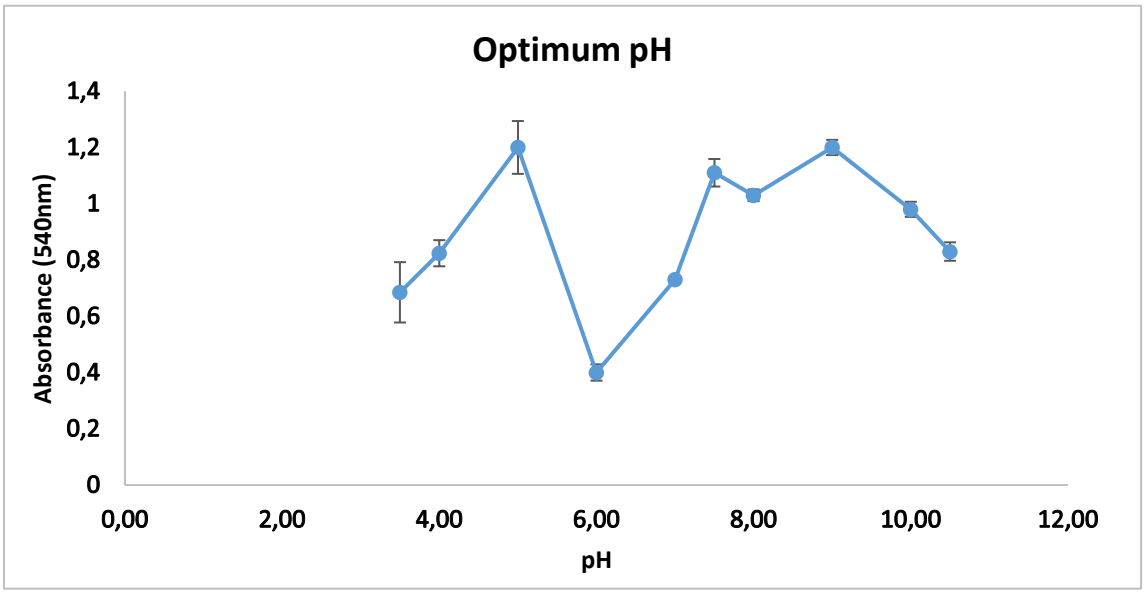

Figure 3: Optimal pH value for the enzymatic activity of extracellular amylase

3.4. Optimum Temperature

The optimum temperature was determined to be 45 °C based on the data presented in Fig. 4. Enzyme activity was assessed at various temperatures, including 25 °C, 37 °C, 45 °C, 60 °C, 75 °C, and 92 °C, to evaluate the thermal stability and catalytic efficiency of the bacterial amylase. Among these, the highest enzymatic activity was observed at 45 °C, indicating it as the optimal temperature for this enzyme under the tested conditions. Different temperature degrees were assayed. The enzyme showed highest activity at 45 °C. Similar results were recorded in work of Ibrahim et.al (2013) [19]. The enzyme had catalytic activity at 92 °C. Different enzyme optimal temperature values ware recorded in the literature. Akkaya et.al (2012) showed the amylase had optimum temperature at 95 °C [5]. This temperature is good for the application of amylase in textile industry. However, low temperature sensitive enzyme is also applicable for the same industry because of its cost effects in electric consuming [20-22]. Chinnamal et. al (2013) In their desizing experiment, the TEGEWA score of the amylase enzyme obtained from the bacteria *Bacillus licheniformis* was determined as 8-9 and the optimum temperature was determined as 60 °C. In addition, the absorbency result was recorded as 12 [16]. The enzyme we used in our study is more advantageous in terms of reducing the cost, as it shows activity at the best 45 °C.

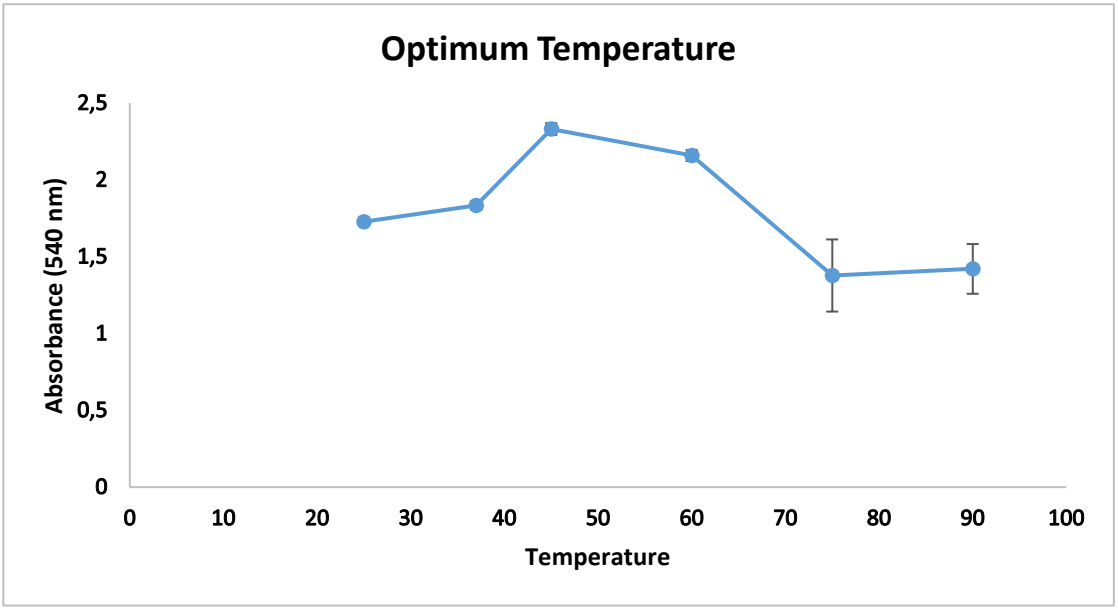

Figure 4: The optimum temperature at which extracellular amylase exhibits maximum activity

3.5. Desizing

Figure 5 explains the enzyme activity on the starched fabric. A shows treated starched fabric without enzyme. B depicts treated starched fabric with 25 mg/mL crude extract enzyme concentration after 5 hours at 45 °C. In Fig. 5, A1, B1 have air dried potassium iodine solution on the fabrics. The desizing activity of

the obtained enzyme is shown in Fig. 5 (B). When the color intensity of the enzyme applied fabric is compared with the color intensity of the fabric without enzyme application, in other words, when Fig. 5 A and B are compared, it can be said that the amount of starch is less in the enzyme applied fabric. This may mean that the applied amylase enzyme is successful in removing starch from the fabric. Fig. 5 A1 and B1 are the results obtained after pouring more marker on the same fabrics and drying in air for a long time. The A1 and B1 results can be interpreted as the result of the marker does not change instantly. When the A1 and B1 results are compared with the TEGEWA score, it can be said that A1 is 1 while B1 is at least 8. When all these findings are considered together, it can mean that the obtained amylase enzyme is applicable in the desizing process. It is ready for the next step for dyeing.

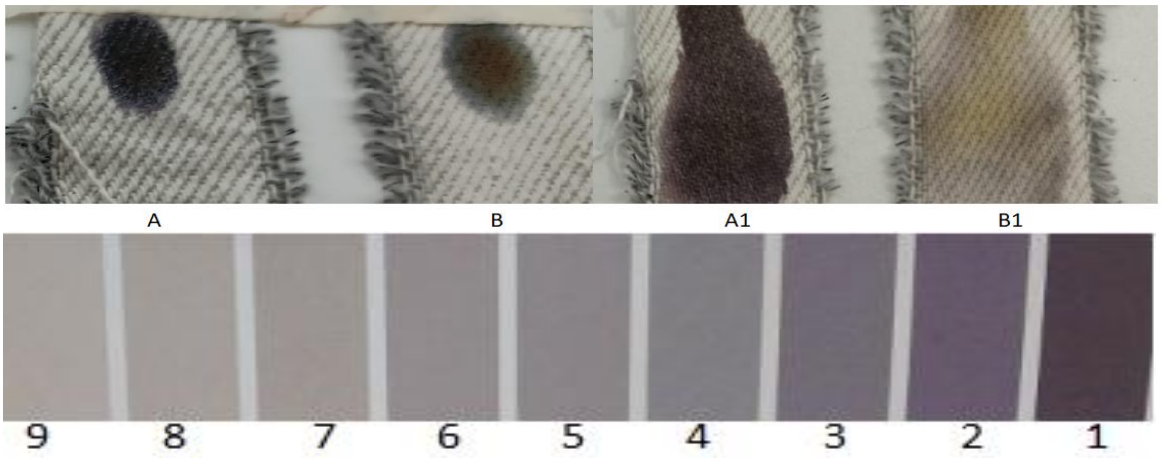

Figure 5: The results obtained 3 minutes after adding 20 µl of 10% potassium iodine solution of the fabric piece treated with 25 mg/ml enzyme concentration after 5 hours at 45 °C. A: treated starched fabric without enzyme, B: treated starched fabric with enzyme. A1, B1 have air dried potassium iodine solution on the fabrics. TEGEWA standard scale from 1 to 9 (https://texcontrol.com.br/produto/escala-de-iodo-violeta-tegewa/)

3.6. DNS Method

The reducing end of starch was determined using the DNS method. Fig. 6, line blue shows amylase activity. After addition of amylase, the absorbance value is 0.642 at 5 minutes. 0,1 and 1 minute later, the absorbance is 0.303, 0.624 respectively. It is observed that the activity of the enzyme on the fabric increases after 5 hours compared to the fabric medium that does not contain the enzyme (Fig. 6 right). Desizing activity of the amylase was measured with DNS method. Removed starch from the fabric has reducing end and is able to determine with DNS reagent. Fig. 6 explains the amylase activity on the fabric during the time. The enzyme activity reached to maximum level at 1 min. Then the activity continued at 5 min. After 5 hours, when the activity of the enzyme is compared with the initial activity, the binding of the reducing ends in the medium with DNS is more than 2 times, while the result is not changed in the control group without enzyme (Fig. 6 right).

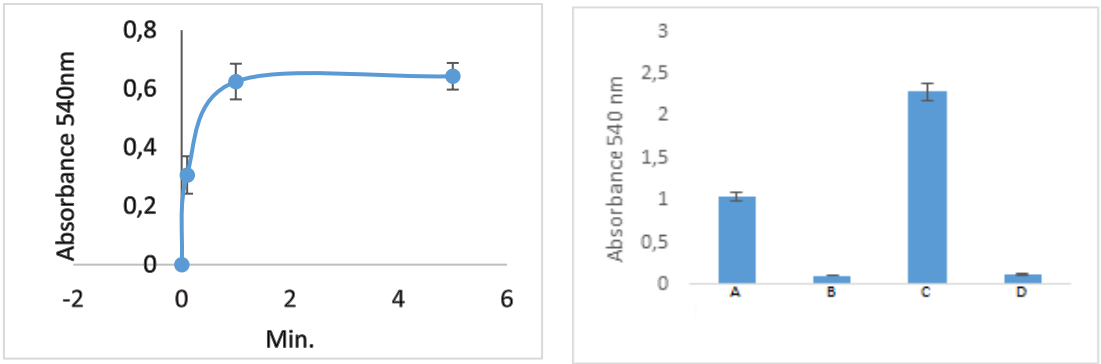

Figure 6: The amylase activity was measurement with DNS method. The reducing end was compared in the enzyme-fabric treatment at initial time and final time (5 hours) (right). A) initial enzyme activity, B) initial enzyme activity without enzyme, C) final enzyme activity, D) final enzyme activity without enzyme

3.7. TLC Experiment

As a result of the chromatography experiment, the end product of the enzyme is maltose, which is shown in Fig. 7.

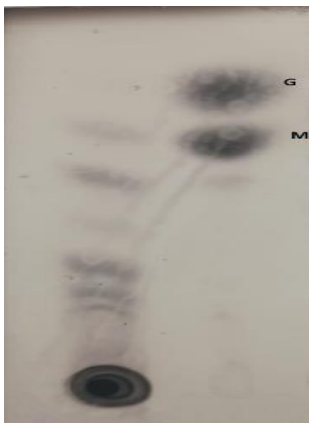

Figure 7: Last product of the amylase determined with TLC experiment. **(**G: Glucose, M: Maltose)

Comparison of fabric information before and after enzyme application for desizing process is given in Table 1. When the initial weight of the fabric and the dried weight after the process are compared, it is found that there is a weight loss of 8.94% in the fabric applied with enzyme, while it is 5.86% in the fabric not applied with enzyme. TEGEWA rating is also determined as 8 and 1, respectively.

Table 1: Comparison of the effect on the fabric with the control group after the enzyme treatment

	With enzyme	**Without enzyme**
Initial weight (mg)	425	392
Final weight (mg)	387	369
Weight loss (%)	8.94	5.86

Initial width (cm)	3	2.7
Final width (cm)	2.5	2.5
Initial length (cm)	5.6	5.5
Final length (cm)	5.1	4.9
TEGEWA rating	8	1
Absorbency test (sec)	5	non

4. Conclusion

The isolated enzyme showed activity optimally at 45 ºC and pH 5.0. It can be used in textile industry thanks to amylase activity. Working at low temperatures is very important in terms of industrial use. Both the factory costs are reduced and the fabric is not adversely affected by high temperature. It is a good candidate for industry application after detailed studies in the future.

Acknowledgement

The author thanks to Malatya Çalık Denim Textile Industry and Trade Inc., for their kind provision of starched and desizing fabrics.

Conflicts of Interest

The author declares that there is no conflict of interest.

References

[1] Arabacı, N., Arıkan, B., *Isolation and characterization of a cold-active, alkaline, detergent stable α-amylase from a novel bacterium Bacillus subtilis N8*, Preparative Biochemistry and Biotechnology, 48(5), 419-426, 2018.

[2] Pandey, P., Nigam, P., Soccol, C.R., Soccol, V.T., Singh, D., Mohan, R., *Advances in microbial amylases*, Biotechnology and Applied Biochemistry, 31(2), 135-152, 2000.

[3] Sen, A., Kapila, R., Chaudhary, S., Nigam, A., *Biotechnological Applications of Microbial Enzymes to Replace Chemicals in the Textile Industry-A Review*, TEXTILE Association, 82(2), 68-73, 2021.

[4] Kocabay, S., Cetinkaya, S., Akkaya, B., Yenidünya, A.F., *Characterization of thermostable β-amylase isozymes from Lactobacillus fermentum*, International Journal of Biological Macromolecules, 93, 195-202, 2016.

[5] Akkaya, B., Yenidunya, A.F., Akkaya, R., *Production and immobilization of a novel thermoalkalophilic extracellular amylase from bacilli isolate*, International Journal of Biological Macromolecules, 50(4), 991-995, 2012.

[6] Ball, S., Guan, H.P., James, M., Myers, A., Keeling, P., Mouille, G., et.al., *From glycogen to amylopectin: a model for the biogenesis of the plant starch granule*, Cell, 86(3), 349-352, 1996.

[7] Mouille, G., Maddelein, M.L., Libessart, N., Talaga, P., Decq, A., Delrue, B., et. al., *Preamylopectin processing: a mandatory step for starch biosynthesis in plants,* The Plant Cell, 8(8), 1353-1366, 1996.

[8] Van Der Maarel, M.J., Van der Veen, B., Uitdehaag, J.C., Leemhuis, H., Dijkhuizen, L., *Properties and applications of starch-converting enzymes of the α-amylase family*, Journal of biotechnology, 94(2), 137-155, 2002.

[9] Hendriksen, H., Pedersen, S., Bisgard-Frantzen, H., *A process for textile warp sizing using enzymatically modified starches*, Patent Application, 1999.

[10] Yahya, S., Sohail, M., Khan, S.A., *Characterization, thermal stabilization and desizing potential of amylase from A. tubingensis SY 1*, The Journal of the Textile Institute, 113(6), 993-1000, 2022.

[11] Ghosh, K., Ray, M., Adak, A., Halder, S.K., Das, A., Jana, A., et. al., Role of probiotic *Lactobacillus fermentum KKL1* in the preparation of a rice based fermented beverage, Bioresource Technology, 188, 161-168, 2015.

[12] Kubrak, O.I., Storey, J.M., Storey, K.B., Lushchak, V.I., *Production and properties of α-amylase from Bacillus sp. BKL20,* Canadian Journal of Microbiology, 56(4), 279-288, 2010.

[13] Elumalai, P., Lim, J.M., Park, Y.J., Cho, M., Shea, P.J., Oh, B.T., *Enhanced amylase production by a Bacillus subtilis strain under blue light-emitting diodes,* Preparative Biochemistry and Biotechnology, 49(2), 143-150, 2019.

[14] Salem,K., Elgharbi, F., Ben Hlima, H., Perduca, M., Sayari, A., Hmida-Sayari, A., *Biochemical characterization and structural insights into the high substrate affinity of a dimeric and Ca^{2+} independent Bacillus subtilis α-amylase*, Biotechnology Progress 36(4), e2964, 2020.

[15] Olufunke, F.O.T., Azeez, I.I., *Purification and characterization of beta-amylase of Bacillus subtilis isolated from Kolanut Weevil*, Journal of Biology and Life Science, 4(1), 68-78, 2012.

[16] Chinnammal, S.K., ArunKumar, K.V., *Production and application of amylase enzyme for biodesizing*, Journal of Environmental Nanotechnology, 2(2), 6-12, 2013.

[17] Bhatt, K., Lal, S., Srinivasan, R., Joshi, B., *Bioconversion of agriculture wastes to produce α-amylase from Bacillus velezensis KB 2216: Purification and characterization,* Biocatalysis and Agricultural Biotechnology, 28, 101703, 2020.

[18] Kiran, S., Kumari, S., Singh, A., Prabha, C., Kumari, S., *Extracellular amylase production under submerged fermentation by Bacillus subtilis RK6*, International Journal of Pharmacy and Biological Sciences, 8(1), 376-383, 2018.

[19] Ibrahim, D., Zhu, H.L., Yusof, N., Hong, L.S., *Bacillus licheniformis BT5. 9 isolated from Changar Hot spring, Malang, Indonesia, as a potential producer of thermostable α-amylase,* Tropical Life Sciences Research, 24(1), 71, 2013.

[20] Naganthran, A., Masomian, M., Rahman, R.N.Z.R.A., Ali, M.S.M., Nooh, H.M., *Improving the efficiency of new automatic dishwashing detergent formulation by addition of thermostable lipase, protease and amylase,* Molecules, 22(9), 1577, 2017.

[21] Bhatt, B.M., Trivedi, U.B., Patel, K.C., *Extremophilic amylases: Microbial production and applications*, Microbial Enzymes: Roles and Applications in Industries, Singapore: Springer Singapore, 185-205, 2020.

[22] Far, B.E., Ahmadi, Y., Khosroshahi, A.Y., Dilmaghani, A., M*icrobial alpha-amylase production: progress, challenges and perspectives*, Advanced Pharmaceutical Bulletin, 10(3), 350, 2020.