Biotech Studies 33(2), 119-126
https://doi.org/10.38042/biotechstudies.1601283

PROOF
RESEARCH PAPER

BIOTECH STUDIES

Cloning of glucoamylase gene from *Aspergillus niger* and its expression in *Pichia pastoris*

Meryem Damla Ozdemır Alkıs[1], Dilek Gokturk[1]*, Osman Gulnaz[2], Mehmet Inan[3,4]

[1]Adana Alparslan Türkeş Science and Technology University, Faculty of Engineering, Department of Bioengineering, Adana, Türkiye.
[2]Cukurova University, Faculty of Education, Department of Mathematics and Sciences Education, Adana, Türkiye.
[3]Akdeniz University, Department of Food Engineering, Antalya, Türkiye
[4]İzmir Biomedicine and Genome Center, İzmir, Türkiye.

How to cite:
Ozdemir Alkıs M. D., Gokturk, D., Gulnaz, O., & Inan, M. (2024). Cloning of glucoamylase gene from *Aspergillus niger* and its expression in *Pichia pastoris*. *Biotech Studies*, *33*(*2*), 119-126.https://doi.org/10.38042/biotechstudies.1601283

Article History
Received 14 February 2024
Accepted 12 November 2024
First Online 11 December 2024

Corresponding Author
Tel.: +90 543 917 70 44
E-mail: dilekgokturk@gmail.com

Keywords
Aspergillus niger
Enzyme activity
Glucoamylase
Industrial enzyme
Pichia pastoris
Recombinant DNA technology

Abstract

Glucoamylase (1,4-α-glucosidase) is a crucial commercial enzyme responsible for the conversion of starch, glycogen, and oligosaccharides into D-glucose through the hydrolysis of their non-reducing terminal glycosidic bonds. While many microorganisms, including bacteria, yeasts, and fungi, can produce glucoamylase, fungal glucoamylase is the preferred choice for industrial applications. The goal of this study was to produce the glucoamylase enzyme recombinantly in *Pichia pastoris*. To achieve this, the *glaA* gene from *Aspergillus niger*, responsible for encoding the glucoamylase enzyme, was cloned into a plasmid (pGAPZα-A) under the control of the GAP promoter and subsequently transferred into *P. pastoris*. The gene was verified through sequence analysis, while the effectiveness of transfection was validated using colony PCR and enzyme activity assays. The results demonstrated that the recombinant *P. pastoris* strain successfully secreted a substantial amount of glucoamylase (307.05 mg/L). The activity of the recombinant enzyme was measured at 79 U/mL.min. The enzyme exhibited robust activity over a broad range of temperatures (50-80°C) and various pH levels (pH 5-10), retaining 92-60% of its maximum activity. In conclusion, this study highlights the potential for laboratory-scale production of the glucoamylase enzyme, crucial for various industries, from a cost-effective and easily cultivable recombinant yeast strain, *P. pastoris.*

Introduction

Glucoamylase (GA), also known as 1,4-α-D-glucanglucohydrolase (EC 3.2.1.3), is an exoenzyme with a pivotal role in catalyzing the hydrolysis of glycosidic bonds within various substrates such as glycogen, starch, and oligosaccharides. Specifically, it targets the non-reducing ends of substances such as glycogen, starch, and oligosaccharides, cleaving these bonds and releasing D-glucose as a product. All sorts of α -glycosidic linkages between two glucosyl components, including α-(1→4) and α-(1→6) glycosidic linkages, can be broken down by GA, with the exception of the α-α-trehalose bond (Aehle, 2007; Sauer et al., 2000). This enzymatic activity has significant industrial relevance and comes in second place globally in terms of sales and distribution (Li et al., 2017). The importance of GA to industry stems from its function in starch hydrolysis. Starch has a significant role in industry, particularly in the food, textile, pharmaceutical, and paper industries. The food industry primarily utilizes starch to manufacture bioethanol, glucose, high-fructose syrups, and other

biochemicals. Since the 1960s, GA has been utilized in the industry for the purpose of processing starch, and this is one of the major breakthroughs. Because starch hydrolysis was previously accomplished using inorganic acids before GA. However, enzymatic hydrolysis offered several benefits over acidic hydrolysis, including higher efficiency, lower energy consumption, and lower cost (Christakopoulos & Topakas, 2012; Hua et al., 2014; Satyanarayana et al., 2004).

Most bacteria, yeasts, and fungi have the ability to produce GA in a variety of forms, but fungal GA is particularly significant for industry (Bagheri et al., 2014; Li et al., 2017). The filamentous fungi are excellent providers of GA due to their high production capacity, thermostability, and stability over a broad pH range. A filamentous fungus called *Aspergillus niger* has been identified as the source of the first GA. Due to its great activity at neutral pH levels (3.5 to 5) and thermostability (optimally active at 50-60°C), the GA obtained from *A. niger* is primarily preferred in industry, notably in starch processing (Bagheri et al., 2014; Karim et al., 2016; Li et al., 2017; Norouzian et al., 2006; Sauer et al., 2000).

This study aimed to clone the GA encoding gene (*glaA*) from *A. niger* to *P. pastoris*. Recombinant technology was applied since recombinant enzymes could more economically and efficiently meet the demands in this field (Longoni et al., 2015). GA can be produced from fungi by different methods, but fungi grow slowly and are challenging to manage in the fermenter due to their complicated morphology (Adrio & Demain, 2010; Norouzian et al., 2006; Wucherpfennig et al., 2011). Additionally, there are significant drawbacks to using a fungus-like *A. niger* to produce homologous overexpressed proteins, such as the restricted types of plasmid vectors that may be employed and their inability to be sustained over an extended period. In other words, *A. niger* can generate a significant amount of GA, which is suitable for the industry, but producing GA from *A. niger* as a heterologous in a particular host will be more effective for industrial processes (Fleißner & Dersch, 2010; Storms et al., 2005). Herein, *P. pastoris* is a commonly used and very prospering host (Karakaş et al., 2010).

Materials And Methods

Strains, plasmids, and culture media

In this study, the *glaA* gene sourced from *A. niger* (NRRL3, ATCC 9029, CBS 120.49) was chosen as the target for cloning. For this purpose, mycelia from *A. niger* were cultured in Luria-Bertani (LB broth) medium (Merck, cat no:110285) containing 4 g/L starch at 37°C for propagation. The expression of the glucoamylase enzyme was achieved using *P. pastoris* X33, and the expression vector pGAPZα-A. *Escherichia coli* (DH5α), chemically competent, was used for plasmid proliferation and cultured in LB Lennox medium containing 25 µg/mL of the antibiotic Zeocin (Invivogen, cat no: ant-zn-05) at 37°C. *P. pastoris* was cultured in YPD medium (Sigma-Aldrich, cat no: Y1375) at 30°C with orbital shaking (250 rpm). For the GA activity assay, cells were cultured in starch added M9 minimal medium (M9 salts (64 g/L $Na_2HPO4.7H_2O$, 15 g/L KH_2PO_4, 2,5 g/L NaCl, 5 g/L NH_4Cl), 1M $MgSO_4$, 1M $CaCl_2$).

Cloning of *glaA* and sequencing

The total RNA of *A. niger* was obtained using the Total RNA Purification Kit (Sigma, GenElute). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™). Polymerase chain reaction (PCR) was conducted using the High Fidelity PCR Kit (Roche) in which the cDNA was employed as the template (primers: 5'GGCTGAAGCTGAATTCATGTCGTTCCGATCTCTACTCGC 3' (sense), 5'GAGTTTTTGTTCTAGACTACCGCCAGGTGTCAGTCACC3' (antisense)). The PCR product containing the *glaA* gene was recovered from an agarose gel (0.8%) using a proper kit (Thermo Scientific™, GeneJET). EcoRI and NotI restriction enzymes were used to digest PCR products and the PGAPZαA carrier plasmid. Subsequently, a ligation reaction utilizing the Rapid DNA Ligation Kit (Thermo Scientific™) and plasmid isolation utilizing the Maxiprep Kit (Thermo Scientific™, GeneJET) were performed. The DNA amount was determined using a Qubit Fluorometer (Invitrogen). Sequence analyses of isolated plasmids were performed (by Sentegen Biotech) to confirm the absence of any potential mutations that might have occurred during PCR.

Transformation and expression of the *glaA* in *Pichia pastoris*

The plasmid was linearized with the restriction enzyme XmaJI within the GAP promoter and purified using a PCR purification kit (Qiagen, MinElute) before transformation. Transformation into *P. pastoris* (X33) was conducted via electroporation (Bio-Rad, Gene Pulser II) following the manufacturer's protocols. Yeast cells were then cultured on agar (YPD with Zeocin (100 µg/mL)). After 48 h of growth, the clones were collected, and colony PCR was performed as described in the manual of the PCR kit (Sigma, CORET). PCR conditions are initial denaturation at 95°C for 30 s, denaturation at 95°C for 30 s (35 cycles), annealing at 60°C for 30 s (35 cycles), extension at 68°C for 2.5 min (35 cycles), and final extension at 68°C for 5 min.

Starch agar plate test

To determine if the transfected gene (*glaA*) had starch hydrolyzing activity, the starch agar test was carried out. First, minimal medium agar plates with starch were made. To compare the transfected and normal cells, the transfected cells were cultured on one side of the plate and the normal yeast cells were cultured on the other side. After three days of cell incubation, iodine vapor was used to stain the plates.

Protein quantitation

Protein quantitation was performed using the supernatants of 72-h cultured recombinant yeast and normal yeast, as well as *A. niger* (NRRL3) cell cultures. The assay was conducted using the BCA Protein Assay Kit (Thermo Scientific™, Pierce).

Enzyme purification

The GA enzyme purification from the supernatant was carried out through ammonium sulfate precipitation, following the protocol of "Bulk Precipitation of Proteins by Ammonium Sulfate" (Simpson, 2006). The precipitated enzyme was dissolved in phosphate-buffered saline (PBS) (Merck, pH 7.4). The enzyme was separated from ammonium sulfate using a centrifugal filter system (Merk-Amicon) and collected in 500 µL of PBS.

Glucoamylase enzyme activity assay

Enzyme activity was estimated using a dinitrosalicylic acid (DNS) assay (Bernfeld, 1955) measuring the change in optical density at 540 nM (Shimadzu UV–VIS, UV mini-1240). A calibration curve was plotted using the optical densities of standard glucose solutions (20, 40, 60, 80, and 100 µg/mL). The enzyme was diluted 1/100 with PBS, and this diluted enzyme was used in all enzyme activity assays, with all assays conducted in triplicate. Enzyme activity (U/mL.min) was calculated using the calibration curve. In the specified test conditions, a single unit of GA enzyme activity was defined as the amount of enzyme capable of releasing 1 µmol of glucose per minute. The relationship between enzyme activity, temperature, and time was investigated at 25°C, 34°C, and 55°C for 15, 30, and 60 minutes. 500 µL of 10 g/L starch solution was treated with 500 µL of 1/100 diluted enzyme. The thermostability of the enzyme was determined by incubating 500 µL of 1/100 diluted enzyme with 500 µL of 10 g/L starch solution for 60 min at various temperatures (40°C, 50°C, 60°C, 70°C, and 80°C). The effect of pH on enzyme activity was measured using a citrate buffer (0,1 M). A 500 µL solution of 10 g/L starch was treated with 500 µL of 1/100 diluted enzyme. The enzymatic reactions were conducted at various pH levels (5, 6, 7, 8, 9, and 10) for 60 min.

Statistical analyses

All assays were performed in triplicate. The enzyme activity was calculated from three replicates, and the results were consistent. The standard deviation was extracted using Excel with previously obtained averages. The data were subjected to analysis of variance, and the means were compared between groups at the $p \leq 0.05$ level with ANOVA, One-Way and post-hoc Tukey test.

Results

Verification of cloning by colony PCR

In the initial stage, colony PCR was performed to confirm the effectiveness of cloning. Colony PCR was carried out using *E. coli* cells containing the recombinant plasmid. The results indicated that the empty plasmid had a size of approximately 500 bp, whereas the plasmid containing *glaA* was 2410 bp in size (Figure 1a). Additionally, to determine whether yeast cells successfully received the recombinant plasmid DNA, a colony PCR analysis was conducted. The gel image displayed a PCR product size of 2410 bp, providing clear evidence of successful transformation (Figure 1b).

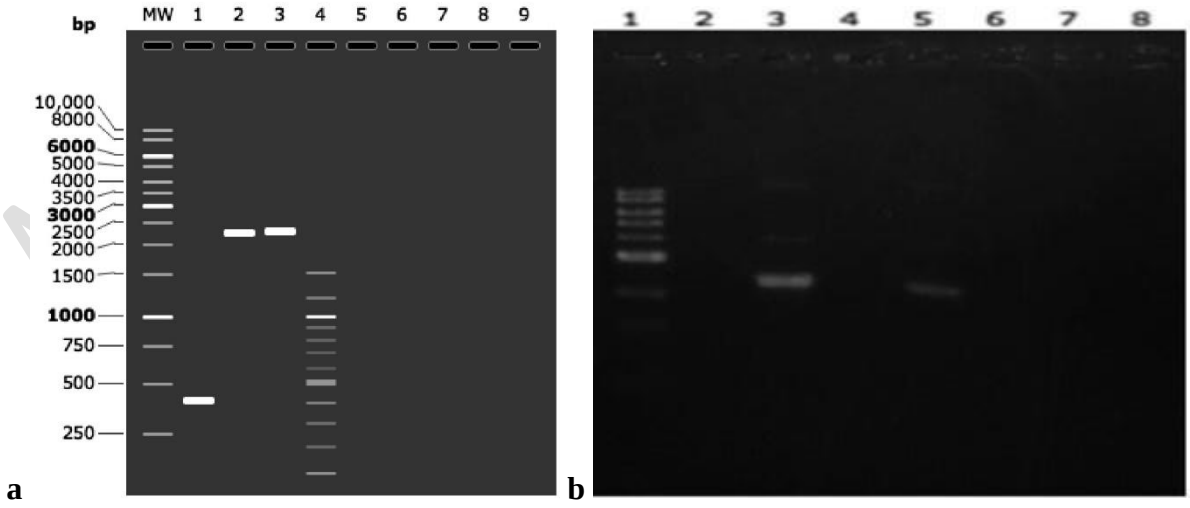

Figure 1. a. Colony PCR was made with *E.coli* cells containing recombinant plasmid. In the first well, PGAPZαA (the empty plasmid) is found; In the 2. and 3. well recombinant PGAPZαA (plasmid + glucoamylase) is found. **b.** The gel image of colony PCR after transfection. In the first well, 1kb DNA ladder (GeneRulerTM Thermo Scientific, Cat. No. SM0311) is found; In the third well the PCR product in which recombinant PGAPZαA plasmid was used as the template is found; In the fifth well the PCR product in which recombinant yeast cell lysate was used as the template is found (The PCR product size is between 2000 and 2500 bp).

Starch agar plate test

Glucoamylase is a type of exoamylase that breaks the α-(1→4) and α-(1→6) glycosidic linkages found in starch (Li et al., 2017). The starch polymers are bound by the iodine, resulting in a blue-black color (Pfister et al., 2016; Xiao et al., 2006). When GA hydrolyzes the starch on the plate, which is secreted by recombinant *P. pastoris* cells, iodine vapor treatment causes the starch-containing portions to become discolored. In Figure 2. a, part B of the iodine vapor-treated plate displayed a significant blue-black color staining, whereas part A remained clear (unstained). This result clearly demonstrated that, while host cells cannot digest starch by secreting GA, recombinant cells can.

Protein quantitation assay

The protein concentrations in the liquid phase of transformed and untransformed yeast and *A. niger* cultures were determined using a standard curve. The quantity of protein released by untransformed P. pastoris, recombinant P. pastoris, and A. niger cells was found to be zero, 307.05325 g/mL and 361.033 g/mL respectively (Figure 2.b).

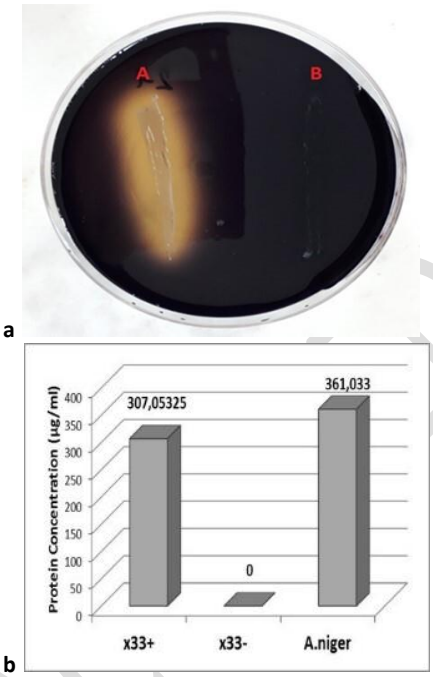

Figure 2. a. Starch agar plates stained with iodine vapor. A. Recombinant yeast cells, B. Normal yeast cells (the color didn't change as a result of the breakdown of starch by GA produced by recombinant yeast cells). **b.** The amount of protein found in the supernatants of recombinant ($X33^{+}$), untransformed *P. pastoris* ($X33^{-}$), and *A. niger* which were the source organism cultures.

Determination of enzyme activity

Enzyme activity was assessed through a DNS (3,5-dinitrosalicylic acid) assay and evaluated under different circumstances. The DNS assay results showed that 71.2 mg/L of glucose was released per minute. Since a single unit of GA enzyme activity is defined as the amount of enzyme that can release 1 µmol of glucose per minute, the enzyme activity was calculated as 79 U/mL.

The enzyme activities at room temperature (25°C), culture temperature (34°C), and industrial temperature (55°C) were remarkably similar when the enzyme was treated with a starch solution (10 g/L) for 60, 30, and 15 min (Figure 3). Enzyme activities at room and industrial temperatures were similar and not much lower than at culture temperatures. The optimal enzyme activity of the recombinant GA obtained in this study was recorded at 40°C, whereas the enzyme activity at 55°C and 60°C was 92% and 88% of its optimal activity (Figure 4). The enzyme activity of recombinant GA was also investigated at different pH levels (Figure 5). The highest enzyme activity was recorded at pH 7, and the enzyme activity at pH 5 and 6 had 90% and 97% of the highest activity, respectively. It was 68% of its maximum activity at pH 10.

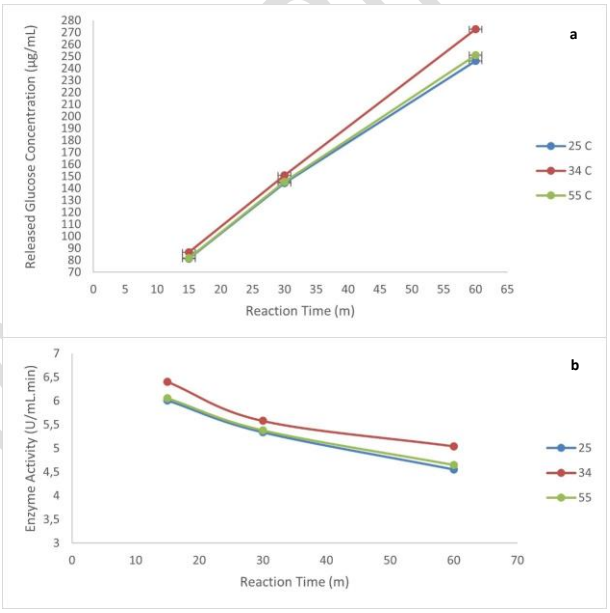

Figure 3. The relation of (a) released glucose and (b) enzyme activity with temperature and time. (Blue: 25°C, Red: 34°C, Green: 55°C) ($p<0.05$).

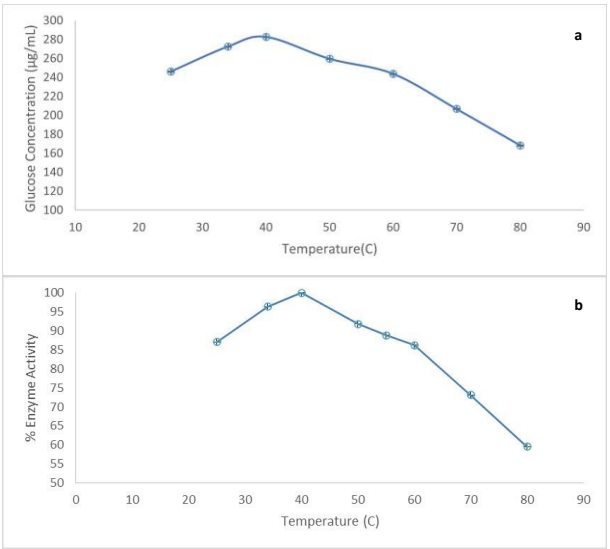

Figure 4. The relation between enzyme activity percent and temperature. The enzyme was incubated with 10 g/L soluble starch at different temperatures for 1 hour. Released glucose concentration (a) and enzyme activity (b) were calculated ($p<0.05$).

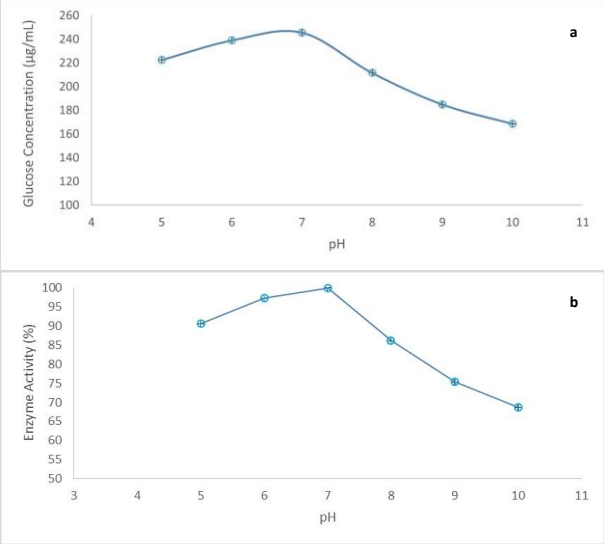

Figure 5. The relation between (a) released glucose and (b) enzyme activity (%) with pH ($p<0.05$).

Discussion

Pichia pastoris has gained considerable attention as an effective system for expressing recombinant proteins (Pan et al., 2022). Studies in the literature have demonstrated that *P. pastoris* cells exhibit a tendency to secrete relatively modest amounts of endogenous proteins, which is one of the characteristics rendering them suitable hosts for recombinant protein production (Burgard et al., 2020; Macauley-Patrick et al., 2005). This indicates that yeast cells do not normally synthesize extracellular proteins under these conditions. It can be assumed that the quantity of protein released from recombinant yeast cells is equivalent to the quantity of GA enzyme secreted. *A. niger* can produce a variety of extracellular proteins depending on the source of carbon present in the medium, with varying amounts (Lu et al., 2010). When *A. niger* is grown in a medium that contains just starch as a carbon source, GA represents more than 50% of its extracellular proteome. (Kwon et al., 2012). The levels of extracellular protein released by *A. niger* (361.033 g/mL), and recombinant yeast (307.05325 g/mL) were quite similar. The difference is likely due to the different exoproteins of *A. niger,* and the GA enzyme level of the transformed *P. pastoris* in this study is thought to be nearly as high as or possibly higher than that of the donor organism.

No studies have been found in the literature regarding the cloning of the GA gene region from *A. niger* into *P. pastoris*. However, there is a study reporting the cloning of the GA gene region from *Aspergillus awamori*, which has been shown to be identical to that of *A. niger.* The results indicate that recombinant glucoamylase produced in *P. pastoris* exhibits enzymatic, chemical, and physicochemical properties that are very similar to those of the source organism (Fierobe et al., 1997). Additionally, several studies have been published on GA derived recombinantly from *P. pastoris* using different source organisms. (Table 1). It was reported that GA enzyme activity obtained from recombinant *P. pastoris* cells transfected with the GA encoding gene from *Bispora sp.* (MEY-1) reached 34.1 U/mL (Hua et al., 2014). Recombinant *P. pastoris* cells cloned with the *Chaetomium thermophilum* GA gene produced a GA activity of 16.73 U/mL (Chen et al., 2007). The cDNA that encodes glucoamylase GA from the thermophilic fungus *Thermomyces lanuginosus* was cloned into *P. pastoris* yielding an activity of approximately 7.4 U/ml (Thorsen et al., 2006). In a study aiming to increase the secretion of GA in *P. pastoris*, GA from *Rhizopus oryzae* was cloned into *P. pastoris* by combining it with a modified signal peptide and a gene encoding a protein associated with secretory vesicles. As a result, the GA activity obtained from systems containing different combinations and various copy numbers of the gene was minimum 0.126 U/mL and maximum 12.619 U/mL (Liu et al., 2005). The activities of GA secreted by recombinant *P. pastoris*, which was cloned with the GA gene from *Rasamsonia emersonii* and *Talaromyces leycettanus*, were 38.6 U/mL and 9.2 U/mL, respectively (Tong et al., 2022). These examples from the literature suggest that the GA enzyme activity obtained in this study (79 U/mL) was notably higher.

GA is a highly demanded and main enzyme for industrial processes involving starch (Li et al., 2017). It is involved in fermentation processes such as alcohol and vinegar, in the production processes of vitamins, antibiotics, and organic acids whose substrate is glucose, together with cellulose in the recycling of straw and other agricultural wastes, and in the de-inking process in paper recycling (Kumar & Satyanarayana, 2009; Pasin et al., 2024; Zong et al., 2022). Recently, GA used in industry primarily sourced from filamentous

Table 1. Enzyme activities of recombinant glucoamylases expressed in *Pichia pastoris*

Source	Host	Enzyme Activity	Reference
Thermomyces lanuginosus	*P. pastoris*	7,4 U/mL	(Thorsen et al., 2006)
Chaetomium thermophilum	*P. pastoris*	16.73 U/mL	(Chen et al., 2007)
Bispora sp. MEY-1	*P. pastoris*	34.1 U/mL	(Hua et al., 2014)
Aspergillus flavus (NSH9)	*P. pastoris*	8.24 U/mL	(Karim et al., 2016)
Penicillium oxalicum	*P. pastoris*	81.2 U/mg	(Xu et al., 2016)
Rasamsonia emersonii	*P. pastoris*	38.6 U/mL	(Tong et al., 2022)
Talaromyces leycettanus		9.2 U/mL	

fungi such as *Aspergillus niger*, *Aspergillus awamori,* and *Rhizopus niveus* (Bagheri et al., 2014; Tong et al., 2021). In the literature, it is reported that the ideal temperature for GA obtained from *A. niger* and various other filamentous fungi is between 40-60°C, although the majority are below 55°C (Kumar & Satyanarayana, 2009; Pandey, 1995; Paszczvdski & Miedziak, 1982). However, GA enzyme is used at temperatures above 55°C in processes that hydrolyze starch, such as bioethanol and glucose syrup production, and at temperatures between 40-50°C in paper recycling processes (James & Lee, 1997; Pasin et al., 2024). Hence, if the obtained GA is thermostable, it will be more suitable for industry as it will not lose its activity at high temperatures. The recombinant GA enzyme obtained in this study preserved 92% of its optimum activity at 55°C and even showed more than half of its optimum activity at 80°C. This will be highly favorable regarding use in industrial applications. The commercially available GA derived from *A. niger* is normally active at acidic pH levels (Norouzian et al., 2006). The liquefaction stage of starch conversion processes occurs at pH 5.5-6 (Hua et al., 2014). After liquefaction, the liquefied starch is processed with GA to produce glucose syrup, high conversion syrups, or substrates for alcohol fermentation. During these processes pH will vary from 4.5 to 5.5 (James & Lee, 1997). In addition, in the paper recycling process, which is another usage area of GA, the enzyme should be stable between pH 4-6 (Pasin et al., 2024). The enzyme activity of the recombinant GA produced in this work was significantly high at acidic pH levels and was active across a wide pH range (5-10). Thus, it is considered that the recombinant GA obtained in this study is suitable for industrial use.

Within the scope of this study, we successfully conducted the molecular cloning and expression of the glucoamylase gene (*glaA*) derived from *A. niger* in *P. pastoris*. The transformation of *P. pastoris* with the recombinant plasmid was not only efficient but also resulted in recombinant yeast cells that demonstrated a remarkable capability to secrete GA, comparable to the original *A. niger* organism.

One of the key findings of our study was the high activity level of the recombinant GA, reaching 79 U/mL.min. This level of activity suggests that the recombinant enzyme holds great promise for industrial applications. Moreover, we assessed the enzyme's performance across a range of temperatures and pH levels, revealing its versatility and suitability for various industrial processes.

Furthermore, the recombinant *P. pastoris* used in this study showed the potential to produce a substantial quantity of industrially relevant GA. This finding implies that large-scale production of this industrially significant enzyme can be achieved more cost-effectively and with higher yields using recombinant *P. pastoris*.

Conclusion

In conclusion, this research underscores the potential of recombinant glucoamylase-expressing *P. pastoris* as a valuable tool for industrial enzyme production. This approach offers a more economical and efficient means of obtaining glucoamylase on a large scale, with broad industrial applications.

Funding Information

Work supported by the Scientific Projects Unit of Adana Science and Technology University under project numbers 16103002 and 16103004.

Author Contributions

MDOA: Formal analysis, Investigation, Writing - Original Draft, Visualization DG: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision OG: Methodology, Resources, Writing - Review & Editing MI: Validation, Resources, Writing - Review & Editing

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Dr. Zeynep YÜCE from the Department of Medical Biology, Faculty of Medicine, Dokuz Eylül University for her kind laboratory and material support in colony PCR and protein quantitation experiments.

References

Adrio, J. L., & Demain, A. L. (2010). Recombinant Organisms For Production of İndustrial Products. *Bioengineered Bugs*, *1*(2), 116–131. https://doi.org/10.4161/bbug.1.2.10484

Aehle, W. (2007). *Enzymes in Industry: Production and Applications, 3rd Edition* (W. Aehle, Ed.; third). WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Bagheri, A., Khodarahmi, R., & Mostafaie, A. (2014). Purification and Biochemical Characterisation of Glucoamylase from A Newly İsolated Aspergillus Niger: Relation to Starch Processing. *Food Chemistry*, *161*, 270–278. https://doi.org/10.1016/j.foodchem.2014.03.095

Bernfeld, P. (1955). Amylases, α and β. In *Methods in Enzymology* (Vol. 1, pp. 149–158). https://doi.org/10.1016/0076-6879(55)01021-5

Burgard, J., Grünwald-Gruber, C., Altmann, F., Zanghellini, J., Valli, M., Mattanovich, D., & Gasser, B. (2020). The secretome of Pichia pastoris in fed-batch cultivations is largely independent of the carbon source but changes quantitatively over cultivation time. *Microbial Biotechnology*, *13*(2), 479–494. https://doi.org/10.1111/1751-7915.13499

Chen, J., Zhang, Y.-Q., Zhao, C.-Q., Li, A.-N., Zhou, Q.-X., & Li, D.-C. (2007). Cloning of a gene encoding thermostable glucoamylase from Chaetomium thermophilum and its expression in Pichia pastoris. *Journal of Applied Microbiology*, *103*(6), 2277–2284. https://doi.org/10.1111/j.1365-672.2007.03475.x

Christakopoulos, P., & Topakas, E. (2012). Editorial Note: Advances in Enzymology and Enzyme Engineering. *Computational and Structural Biotechnology Journal*, *2*(3), 1. https://doi.org/10.5936/csbj.201209001

Fierobe, H. P., Mirgorodskaya, E., Frandsen, T. P., Roepstorff, P., & Svensson, B. (1997). Overexpression and characterization of *Aspergillus awamori* wild-type and mutant glucoamylase secreted by the methylotrophic yeast Pichia pastoris: comparison with wild-type recombinant glucoamylase produced using Saccharomyces cerevisiae and Asperg. *Protein Expression and Purification*, *9*(2), 159–170. https://doi.org/10.1006/prep.1996.0689

Fleißner, A., & Dersch, P. (2010). Expression and Export: Recombinant Protein Production Systems for Aspergillus. *Applied Microbiology and Biotechnology*, *87*(4), 1255–1270. https://doi.org/10.1007/s00253-010-2672-6

Hua, H., Luo, H., Bai, Y., Wang, K., Niu, C., Huang, H., Shi, P., Wang, C., Yang, P., & Yao, B. (2014). A Thermostable Glucoamylase from Bispora sp. MEY-1 with Stability over a Broad pH Range and Significant Starch Hydrolysis Capacity. *PLoS ONE*, *9*(11), e113581. https://doi.org/10.1371/journal.pone.0113581

James, J. A., & Lee, B. H. (1997). Glucoamylases: Microbial Sources, Industrial Applications and Molecular Biology — A Review. *Journal of Food Biochemistry*, *21*(6), 1–52. https://doi.org/10.1111/j.1745-4514.1997.tb00223.x

Karakaş, B., Inan, M., & Certel, M. (2010). Expression and characterization of Bacillus subtilis PY22 α-amylase in Pichia pastoris. *Journal of Molecular Catalysis B: Enzymatic*, *64*(3–4), 129–134. https://doi.org/10.1016/j.molcatb.2009.07.006

Karim, K. M. R., Husaini, A., Hossain, M. A., Sing, N. N., Mohd Sinang, F., Hussain, M. H. M., & Roslan, H. A. (2016). Heterologous, Expression, and Characterization of Thermostable Glucoamylase Derived from Aspergillus flavus NSH9 in Pichia pastoris. *BioMed Research International*, *2016*. https://doi.org/10.1155/2016/5962028

Kumar, P., & Satyanarayana, T. (2009). Microbial glucoamylases: characteristics and applications. *Critical Reviews in Biotechnology*, *29*(3), 225–255. https://doi.org/10.1080/07388550903136076

Kwon, M. J., Jørgensen, T. R., Nitsche, B. M., Arentshorst, M., Park, J., Ram, A. F. J., & Meyer, V. (2012). The transcriptomic fingerprint of glucoamylase over-expression in Aspergillus niger. *BMC Genomics*, *13*(1). https://doi.org/10.1186/1471-2164-13-701

Li, Z., Ji, K., Dong, W., Ye, X., Wu, J., Zhou, J., Wang, F., Chen, Q., Fu, L., Li, S., Huang, Y., & Cui, Z. (2017). Cloning, heterologous expression, and enzymatic characterization of a novel glucoamylase GlucaM from Corallococcus sp. strain EGB. *Protein Expression and Purification*, *129*(June), 122–127. https://doi.org/10.1016/j.pep.2015.06.009

Liu, S. H., Chou, W. I., Sheu, C. C., & Chang, M. D. T. (2005). Improved secretory production of glucoamylase in Pichia pastoris by combination of genetic manipulations. *Biochemical and Biophysical Research Communications*, *326*(4), 817–824. https://doi.org/10.1016/J.BBRC.2004.11.112

Longoni, P., Leelavathi, S., Doria, E., Reddy, V. S., & Cella, R. (2015). Production by Tobacco Transplastomic Plants of Recombinant Fungal and Bacterial Cell-Wall Degrading Enzymes to Be Used for Cellulosic Biomass Saccharification. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/289759

Lu, X., Sun, J., Nimtz, M., Wissing, J., Zeng, A.-P., & Rinas, U. (2010). The intra- and extracellular proteome of Aspergillus niger growing on defined medium with xylose or maltose as carbon substrate. *Microbial Cell Factories*, *9*, 23. https://doi.org/10.1186/1475-2859-9-23

Macauley-Patrick, S., Fazenda, M. L., McNeil, B., & Harvey, L. M. (2005). Heterologous protein production using the Pichia pastoris expression system. *Yeast*, *22*(4), 249–270. https://doi.org/10.1002/yea.1208

Norouzian, D., Akbarzadeh, A., Scharer, J. M., & Moo Young, M. (2006). Fungal glucoamylases. *Biotechnology Advances*, *24*(1), 80–85. https://doi.org/10.1016/j.biotechadv.2005.06.003

Pan, Y., Yang, J., Wu, J., Yang, L., & Fang, H. (2022). Current advances of Pichia pastoris as cell factories for production of recombinant proteins. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.1059777

Pandey, A. (1995). Glucoamylase Research: An Overview. *Starch - Stärke*, *47*(11), 439–445. https://doi.org/10.1002/star.19950471108

Pasin, T. M., Betini, J. H. A., de Lucas, R. C., & Polizeli, M. de L. T. de M. (2024). Biochemical characterization of an acid-thermostable glucoamylase from *Aspergillus japonicus* with potential application in

the paper bio-deinking. *Biotechnology Progress*, *40*(1). https://doi.org/10.1002/btpr.3384

Paszczvdski, A., & Miedziak, I. (1982). *A simple method of affinity chromatography for the purification of glucoamylase obtained from Aspergilhs niger C*. *149*(1), 63–66.

Pfister, B., Sánchez-Ferrer, A., Diaz, A., Lu, K., Otto, C., Holler, M., Shaik, F. R., Meier, F., Mezzenga, R., & Zeeman, S. C. (2016). Recreating the synthesis of starch granules in yeast. *ELife*, *5*(NOVEMBER2016), 1–29. https://doi.org/10.7554/eLife.15552

Satyanarayana, T., Noorwez, S. M., Kumar, S., Rao, J. L. U. M., Ezhilvannan, M., & Kaur, P. (2004). Development of an ideal starch saccharification process using amylolytic enzymes from thermophiles. *Biochemical Society Transactions*, *32*(Pt 2), 276–278.

Sauer, J., Sigurskjold, B. W., Christensen, U., Frandsen, T. P., Mirgorodskaya, E., Harrison, M., Roepstor, P., & Svensson, B. (2000). Glucoamylase: structure / function relationships, and protein engineering. *Science*, *1543*.

Simpson, R. J. (2006). Bulk Precipitation of Proteins by Ammonium Sulfate. *Cold Spring Harbor Protocols*, *2006*(1), pdb. prot 4308. https://doi.org/10.1101/pdb.prot4308

Storms, R., Zheng, Y., Li, H., Sillaots, S., Martinez-Perez, A., & Tsang, A. (2005). Plasmid vectors for protein production, gene expression and molecular manipulations in Aspergillus niger. *Plasmid*, *53*(3), 191–204. https://doi.org/10.1016/j.plasmid.2004.10.001

Thorsen, T. S., Johnsen, A. H., Josefsen, K., & Jensen, B. (2006). Identification and characterization of glucoamylase from the fungus Thermomyces lanuginosus. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1764*(4), 671–676. https://doi.org/10.1016/J.BBAPAP.2006.01.009

Tong, L., Huang, H., Zheng, J., Wang, X., Bai, Y., Wang, X., Wang, Y., Tu, T., Yao, B., Qin, X., & Luo, H. (2022). Engineering a carbohydrate-binding module to increase the expression level of glucoamylase in Pichia pastoris. *Microbial Cell Factories*, *21*(1), 95. https://doi.org/10.1186/s12934-022-01833-1

Tong, L., Zheng, J., Wang, X., Wang, X., Huang, H., Yang, H., Tu, T., Wang, Y., Bai, Y., Yao, B., Luo, H., & Qin, X. (2021). Improvement of thermostability and catalytic efficiency of glucoamylase from Talaromyces leycettanus JCM12802 via site-directed mutagenesis to enhance industrial saccharification applications. *Biotechnology for Biofuels*, *14*(1), 202. https://doi.org/10.1186/s13068-021-02052-3

Wucherpfennig, T., Hestler, T., & Krull, R. (2011). Morphology engineering - Osmolality and its effect on Aspergillus niger morphology and productivity. *Microbial Cell Factories*, *10*, 1–15. https://doi.org/10.1186/1475-2859-10-58

Xiao, Z., Storms, R., & Tsang, A. (2006). A quantitative starch – iodine method for measuring alpha-amylase and glucoamylase activities. *Analytical Biochemistry*, *362*(MAY 2006), 146–148. https://doi.org/10.1016/j.ab.2006.01.036

Xu, Q.-S., Yan, Y.-S., & Feng, J.-X. (2016). Efficient hydrolysis of raw starch and ethanol fermentation: a novel raw starch-digesting glucoamylase from Penicillium oxalicum. *Biotechnology for Biofuels*, *9*, 216. https://doi.org/10.1186/s13068-016-0636-5

Zong, X., Wen, L., Wang, Y., & Li, L. (2022). Research progress of glucoamylase with industrial potential. *Journal of Food Biochemistry*, *46*(7). https://doi.org/10.1111/jfbc.14099