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# Recombinant Production of Hydrophobin DewA in *Pichia pastoris* and Determination of Its Functions

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**ABSTRACT**
Hydrophobins, found around the aerial hyphae and reproductive structures of fungi, are small amphipathic surfactant proteins. Due to these amphipathic properties, they can change the properties of the surfaces they are on. Thanks to these properties, hydrophilic surfaces can be obtained by imparting hydrophobic properties.. However, the low production amounts in the studies conducted to date limit biotechnological studies. In the study, pPICZα-A vector was used for high protein expression, since its restriction maps, gene functions and intracellular replication mechanisms are known. P. pastoris was used for recombinant production due to its low amount of endogenous protein production, especially capable of performing posttranslational modifications. codon optimization was first performed after obtaining the gene sequence of the DewA protein from GenBank (Uniprot ID: P52750). Firstly, the secretion signal sequence region of this sequence and the stop codon of the gene were removed. Then, the cutting region of restriction enzymes (EcoRI and XbaI) that did not cut the resulting sequence was added to the sequence. After it was synthesized commercially, it was produced recombinantly by plasmid isolation, enzyme cutting reaction and ligation processes. It was then transferred to P.pastoris by electroporation method. Optimization of methanol induction was carried out at 0.5%, 1% and 1.5%. Protein isolation was performed by taking samples every 24 hours during the incubation. After protein isolation, the surface properties on teflon and glass surfaces were examined. The optimal culture condition for DewA expression was obtained at 1% methanol concentration as 77 mg/L in 96 hour. Recombinant DewA has been proven to change the surface characteristics on the teflon and glass surfaces. Conclusions: In the study, the DewA protein of A.nidulans was cloned into the pPICZα-A vector and recombinantly produced in the P.pastoris X-33 strain for the first time.

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## Introduction

Hydrophobins are found around the aerial hyphae and reproductive structures of fungi. They are small amphipathic surfactant proteins consisting of about 100-150 amino acids [1]. They provide their stable structure by containing 4 disulfide bonds (C1-C6, C2-C5, C3-C4 and C7-C8) and characteristically eight conserved cysteine amino acids [2]. Because they have amphipathic properties, they form a monolayer layer at hydrophilic-hydrophobic interfaces.[3] For example, they self-assemble at hydrophilic-hydrophobic interfaces such as water-air, water-oil, and water-hydrophobic solids. Because of these properties, hydrophobins play an important role in many industrial applications such as surface coatings, biosensors, self-cleaning surfaces [3,4,5]. In biological processes, various organic surfaces can be covered by proteins in seconds. Thanks to these properties, hydrophobins are widely used in fields such as regenerative medicine and tissue engineering [6]. Studies on coating proteins on surfaces have shown that the adhesion of proteins to a substrate or surface can be controlled by temperature, ionic strength, and buffer compositions [7]. In addition, adhesion and surface persistence are closely related to the size, structural stability and composition of the protein. Small and rigid proteins are preferred for surface applications as they are not prone to conformational changes after surface absorption [6,7].

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Eukaryotic and prokaryotic organisms are used in the production of recombinant proteins[8]. However, *P.pastoris*, which belongs to the Fungi kingdom, Ascomycota phylum, Saccharomycetes class, Saccharomcetales order, is an oval-shaped and single-celled eukaryotic creature with a diameter of 1-5 μm and a length of 5-30 μm [9], ease of application, low It is widely preferred in laboratory research due to its costly production, post-translational modifications such as glycosylation, methylation, proteolytic degradation, and low amount of endogenous protein production [10]. In addition, methylotrophic yeast is frequently used in genetic engineering and is a useful system for the efficient expression of heterologous proteins from milligram to gram [10].

There are two strong promoter genes, AOX and PGAP, that regulate protein production in *P.pastoris* [11]. PGAP provides high product yields in very short processing times [12]. However, the most preferred genes for these systems are the AOX genes. Thanks to the AOX1 gene, the transcription of foreign proteins can be controlled and efficiency is quite high [10]. When selecting vectors for protein expression, pPICZ and pGAPZ vectors are suitable. However, pPICZα-A/B/C vectors are more suitable for high throughput and are designed for simple cloning and selection, high level protein expression, and rapid detection and purification of recombinant protein. In addition, the most successful systems for the efficient production of heterologous proteins in studies carried out so far are expression systems in the presence of methanol [13].

Our study aimed to clone and produce the *Dew*A protein of *Aspergillus nidulans*, which has a high contact angle on the surface coatings, into the pPICZα-A vector by *P. pastoris* X-33 strain under the control of the AOX1 promoter. It also sheds light on the biotechnological use of recombinant *Dew*A.

## Material and Methods

### Strain, plasmids, and reagents

To be used in the study, pPICZα A and *P.pastoris* strain X-33 were obtained from the High Technology and Research Center. Plasmid Isolation Kit, Gel Extraction Kit, Genomic DNA Isolation Kit and 2X EcoTaq Master Mix were obtained from EcoTech Biotechnology; Restriction enzymes (*Xba*I, *Eco*RI and *Sac*I) and T4 DNA ligase were obtained from Thermo Fisher Scientific.

### Construction Design, Cloning and Transformation

The amino acid sequence of *Dew*A (UniProt ID: P52750) was retrieved from UniProt (https://www.uniprot.org/). First, the secretion signal sequence and the stop codon of the *Dew*A gene were removed. Then, restriction enzymes that did not cut the obtained sequence were detected using the bioinformatics program (http://www.restrictionmapper.org/), and *Eco*RI and *Xba*I cutting sites were added to the sequence. Additionally, codon optimization was performed using the GENEWIZ tool. The sequence information of *Dew*A (Genbank ID: OQ721986) is given as supplementary material. The resulting sequence was synthesized by a commercial company in the pGSI vector containing the Amp resistance gene for selection. The pGSI plasmid containing the *Dew*A gene was transferred to *E.coli* cells by heat shock method. *Dew*A and linear pPICZαA vector were ligated with T4 ligase enzyme. The obtained ligation product (*Dew*A-pPICZαA) was transferred to *E.coli* cells by heat shock method. Positive colonies were then determined by PCR with primer 3'AOX (GCAAAATGGCATTCCTGACATCC) and α-signal factor (TACTATTGCCAGCATTGCTGC). *Dew*A-pPICZαA was linearized by setting up a cutting reaction with the *Sac*I restriction endonuclease enzyme. The purification of the obtained linear recombinant plasmid was performed by phenol-chloroform-isopropyl alcohol (25:24:1) method. (Fig 1 shows the ligation product design of DewA-pPICZαA protein)

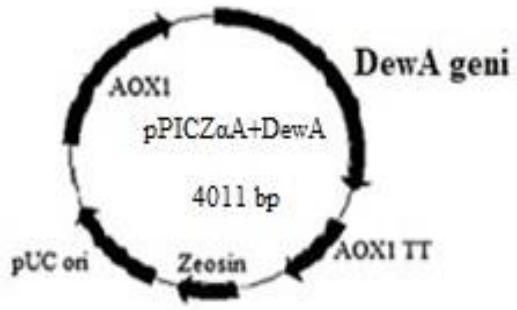


**Fig 1** Design of DewA-pPICZαA

For the electroporation, 80 µl of component cells and approximately 15 µg of linear plasmid were mixed in a sterile PCR tube, then transferred to a 0.2 cm cold electroporation cuvette and incubated on ice for 5-6 minute. Electroporation was carried out by placing the cuvette in the Gene Pulser Xcell Electroporation Systems. Immediately after the electroporation process, 1 ml of 1M cold sorbitol was added to the cuvette and taken into a sterile 15 ml falcon tube. Then, incubated at 30°C for 2 hour on a shaker at 120 rpm, 50-200 µl was taken and spread on YPDS (yeast extract peptone dextrose medium containing sorbitol) agar containing 100µg/ml zeocin and incubated at 30°C for 10 days. Transformants were selected by genomic PCR using 5'AOX and 3'AOX primers. A lineralized pPICZαA vector was used as a negative control.

**Recombinant production and purification of *Dew*A**

Positive transformants (Fig 2) were inoculated into 25 ml of BMGY (buffered glycerol-complex medium) prepared for recombinant protein production and incubated at 150 rpm at 30°C for 24 hour. After the incubation, the cells were centrifuged at 3000 rpm for 5 minute and the supernatant was removed. The remaining pellet was dissolved in 3 ml BMYY (Buffered Glycerol-complex Medium) to remove glycerol, and then centrifuged again under the same conditions. After centrifugation, the supernatant was removed and the pellet was dissolved in 1 ml BMYY medium, then transferred to 100 ml BMMY medium prepared for protein production and incubated for 120 h at 30°C, 150 rpm. Methanol induction was optimized at 0.5%, 1% and 1.5% concentrations. During the incubation period, 2 ml of sample was taken every 24 h and centrifuged after $OD_{600}$ measurement, and the pellet and culture filtrate was stored at -80°C until used for protein isolation.

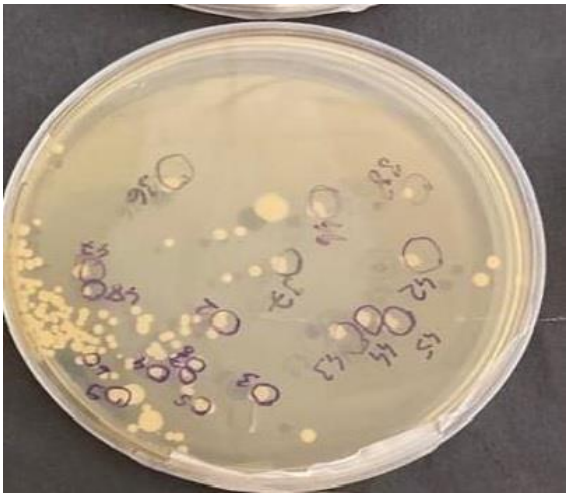

**Fig 2** Positive isolates obtained after electroporation

The isolation of recombinant *Dew*A from the culture filtrate was performed according to the protocol for class 1 hydrophobins by [14]. Briefly, 20 ml of methanol, 5 ml of chloroform, and 15 ml of $dH_2O$ were added into 50 ml of falcon tube, and 5 ml of culture filtrate was added, then mixed well and centrifuged at 10000 g for 25 minute. At the end of the period, the upper phase was removed and 5 ml of methanol was added to the remaining phase and mixed thoroughly. Then, the supernatant was removed by centrifugation at 10000g for 15 minute and the pellet was dried. The dried pellet was suspended by adding 200 µl of TFA. Then, TFA was evaporated under nitrogen gas and 100 µl of 6 M guanidine hydrochloride was added and the proteins were dissolved. Protein concentration was determined by the Bradford Assay [14].

**Analyzes for recombinant proteins**

**SDS-PAGE and Silver Staining**

Proteins were visualized on SDS-PAGE gel by silver staining technique. The resulting image was taken with a Bio-Rad gel imaging system. The silver staining technique was performed according to the protocol recommended by Lee et al [15].

According to this protocol; After the electrophoresis process, the gel was removed from the glass plates in order to visualize the proteins, and was kept in the fixation solution at +4 °C for 1 hour, and then washed in a horizontal shaker with $dH_2O$ for approximately 1 hour. At the end of the period, $dH_2O$ was removed and sensitizer solution was added to denature the proteins and incubated on a shaker for 1 minute. Then, after washing with $dH_2O$ in a shaker for 10 minutes 3 times, the $dH_2O$ was removed and placed in 0.1% silver solution. After washing in a shaker with $dH_2O$ 3 times for 10 minutes to remove silver particles from the environment, the gel was transferred to a new tank, Developer solution was added and waited until the image

was taken. After the image was taken, the deleloper solution was removed from the tank, $dH_2O$ was added to the gel, and washing was carried out in a shaker. Finally, the reaction was terminated by adding 0.5% acetic acid to the gel and photographed with the Bio-Rad gel imaging system.
Preparation of the solutions used;

- Gel fixation solution: 40 ml Ethanol, 10 ml Acetic Acid mixture was completed with distilled water until the total volume was 100 ml.
- Sensitizer solution: 0.04 g Sodium thiosulfate dissolved in 200 ml distilled water.
- Silver staining solution: The mixture of 0.2 g Silver nitrate and 0.02% Formaldehyde was completed with distilled water until the total volume was 200 ml.
- Developer/developer solution: The mixture of 7.5 g Sodium carbonate and 0.05% Formaldehyde was completed with distilled water until the total volume was 250 ml.

**Coating of *Dew*A protein on glass and teflon surfaces**

In the coating experiments, glass was preferred as the hydrophilic surface and teflon as the hydrophobic surface. Teflon and glass pieces (1x1 cm) were passed through 70% alcohol and treated with flame, then sterilized in an autoclave at 120°C and prepared for coating experiments. 100 µl of *Dew*A solution was taken and coating buffer (50 mM Tris/HCl, 1 mM $CaCl_2$, pH 8) was added so that the total volume was 1.2 ml [14]. After the glass and teflon pieces to be coated were taken into the 16-well plate, a control group was included for each sample and 250 µl of coating solution and incubated at 80°C.

**Stability tests of coated surfaces**

The stability of the coated surfaces was determined by hot SDS and UV application. For SDS stability, After the coated glass and teflon surfaces were exposed to 1% SDS for 60 min at 80°C, contact angles were measured and interpreted compared to the untreated control group. For UV stability, the contact angles were measured after the coated glass and teflon surfaces were exposed to UV light for 30, 60 and 90 minute and interpreted compared to the untreated control group [14].

**Contact angle measurement**

The contact angles of the coated glass and teflon surfaces were measured by the evaluation of the photographs taken in the system shown in Figure 5, prepared using a tilted stereo microscope suggested by [16] with Drop Snake Analysis in the ImageJ program. For this purpose, 2 µl of pure water was dropped on each of the coated surfaces with the help of a micropipette. Then, the photos of the drops were taken and transferred to the computer and the contact angles were calculated.

## Results and Discussion

**Construction of recombinant vector for the *P. pastoris* expression system**

The pGSI-*Dew*A plasmid, which is approximately 3,268 bp long, was isolated from *E.coli* cells and restriction enzyme analysis with *Eco*RI and *Xba*I demonstrated that double digestion of plasmid an expected DNA fragment (*Dew*A gene, 411 bp) and vector backbone. The *Dew*A gene was cut from the gel and purified with the Ecotech Gel-PCR Purification Kit according to the protocol recommended by the manufacturer. The pPICZa-A plasmid was also subjected to the digestion reaction with the same enzymes. The pPICZα-A plasmid and *Dew*A gene were ligated with T4 ligase and the resulting product of approximately 4011 bp was confirmed by agarose gel (Fig 3).

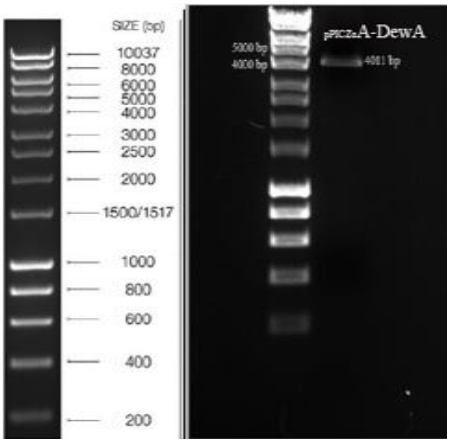


**Fig 3** The pPICZa-A plasmid and the DewA gene were ligated with T4 ligase, resulting in a product of approximately 4011 bp

pPICZαA-*Dew*A plasmid was linearized with *Sac*I enzyme and was transferred to *P. pastoris* X-33 cells by electroporation. After incubation on a zeocin-containing plate for ten days, recombinant colonies were screened by PCR using AOX primers. At this stage, it is expected that the band containing the *Dew*A gene with a size of approximately 920 bp and a control band with a size of 2200 bp in which the AOX region in the genome of *P.pastoris* is amplified. Then, for sequence verification, the target band was cut from the gel and purified with the Ecotech PCR-Gel purification kit according to the protocol recommended by the manufacturer. The resulting sequence reads were combined into a single sequence and compared with the codon optimized sequence to determine whether there was a mutation.

**Recombinant Production and Purification of *Dew*A**

*Dew*A protein was produced by induction at 3 different methanol concentrations (0.5%, 1% and 1.5%) in studies with selected recombinant isolate in BMYY medium shaken culture. During the production, the $OD_{600}$ value was measured by taking a sample every 24 hour until the 120$^{th}$ hour. For protein isolation, samples were taken every 24 hours and production amounts were determined. When Fig 4a is examined, protein production increased from the 24$^{th}$ hour to the 120$^{th}$ hour at 0.5% methanol concentration. However, the production seems to have reached its maximum at 96$^{th}$ hour at 1% and 1.5% methanol concentrations. In addition, protein production at 0.5% methanol concentration is approximately two times lower than at 1% and 1.5% methanol concentrations. When 1% and 1.5% methanol concentrations were evaluated among themselves, there was no statistically significant difference between protein production, especially at 96$^{th}$ and 120$^{th}$ hour ($p<0.05$). The highest production yield was reached at 1% methanol concentration at the 96$^{th}$ hour incubation period (77.42mg/L).

Proteins obtained at 3 different methanol concentrations at 96 hour were visualized on SDS-PAGE gel (Fig 4b) . Here, the *Dew*A protein is approximately 13 kDa, and the c-myc epitope region and His-Taq from the pPICZα-A vector were added to this protein at 2.5 kDa. Therefore, the recombinant *Dew*A protein is expected to give bands in the gel corresponding to approximately 15 kDa.

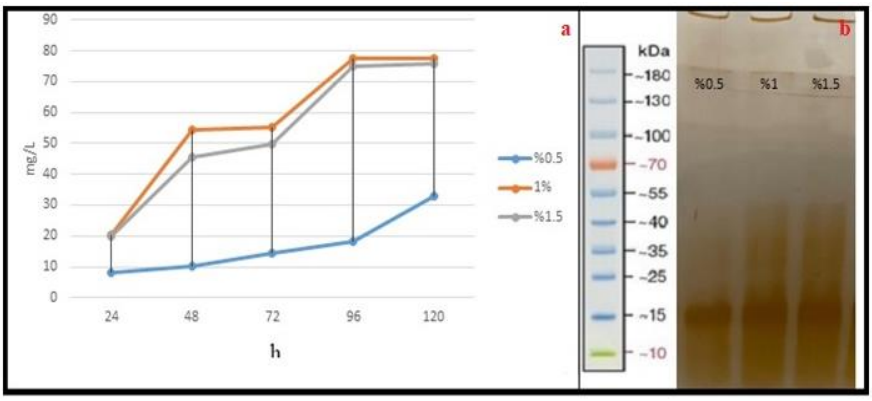


**Fig 4 a:** Amounts of protein produced at different methanol concentrations (mg/L) **b:** Protein bands obtained after 96 hour of incubation of three different methanol concentrations

***Dew*A protein coating on some surfaces and determination of contact angles**

In the coating experiments, glass was preferred as the hydrophilic surface and teflon as the hydrophobic surface. As a result of the measurement of contact angles, the angle determined as 6.174° on the glass surfaces (fig 5a) reached 70.125±5.18° after 100 µg *Dew*A protein coating (fig 5c). On the other hand, teflon surface angle, which was determined as 132.878° (fig 5b), decreased to 114.814±7.25° (fig 5d). As expected, the *Dew*A protein changed the characteristic of the surface.

In addition, stability tests of the coated surfaces were carried out to determine the durability of the recombinantly produced protein and its permanence on the surfaces. While the contact angle on the glass surfaces before the application was 70.125° in the experimental groups in which hot SDS was applied; after 60 minute of application, it was determined as 69.250±6.35°. On teflon surfaces, it was 114.814° before the application; it was measured as 123.388±10.72° after the application. The UV stability of the coated surfaces was measured after exposure to UV light for 30, 60 and 90 minute and interpreted compared to the untreated control group. While the contact angle before application on glass surfaces is 70.125°; as a result of 30 minute of UV application, it was determined as 7.25±1.5°. There was a statistical difference between the contact angle obtained and the contact angle of the glass surface before protein coating ($p<0.05$). On teflon surfaces, it is 114.814° before the application; After 30, 60 and 90 minute of UV application, the contact angle was measured

as 120.704±11.15°. The statistical difference between the UV applications at different times and the control contact angles was not found significant. Interestingly, according to the results obtained, the protein was not degraded by UV application on teflon surfaces and preserved its activity.

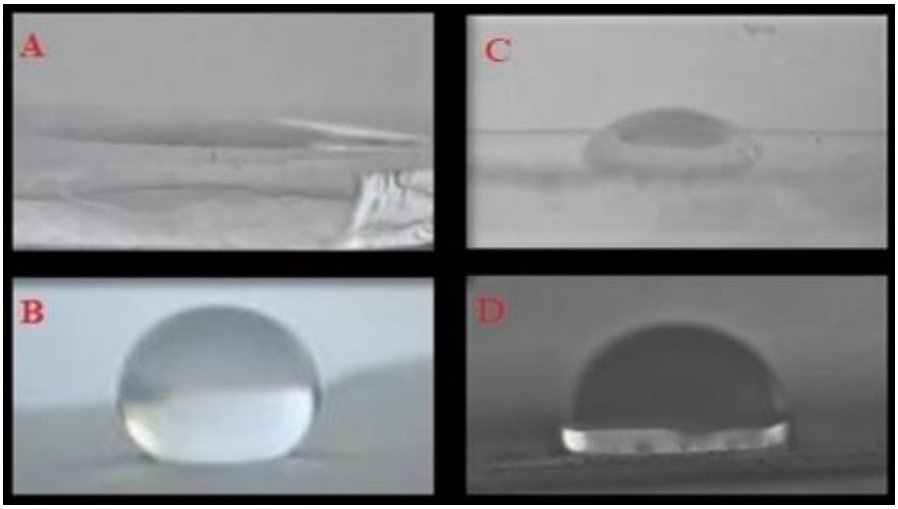


**Fig 5 a**: Glass surface before application, **b:** Teflon surface before application, **c:** Glass surface after application, **d:** Teflon surface after application

*A. nidulans* has six hydrophobins located on the conidiospore surface. Most of these hydrophobins belong to class I, potentially being used for highly stable surface functionalization. *Dew*A contributes to spore hydrophobicity and has so far been identified as the first choice hydrophobin from *A. nidulans* for biosynthetic surface modification.[ 17,18,19,20] *Dew*A from *A. nidulans* and other hydrophobins have been shown to be effective in increasing the hydrophobicity of glass surface coatings. In addition, *Dew*A protein layers showed high resistance to water, ethanol, detergent and temperature treatments than class I hydrophobin layers [21,22]. The angle determined as 6.174° on glass surfaces in our research findings reached 70.125±5.18° coating 100 µg *Dew*A protein. On the other hand, teflon surface angle, which was determined as 132.878°, decreased to 114.814±7.25°. While the contact angle on the glass surfaces before the application was 70.125° in the experimental groups in which hot SDS was applied; after 60 minute of application, it was determined as 69.250±6.35°. On teflon surfaces, it was 114.814° before the application; it was measured as 123.388±10.72° after the application [14]. İn their *Dew*A protein coating experiment, after 16 hour of incubation at 80 °C, the contact angle for the glass surface was 72.9±9.48° before the application of 1% SDS and the contact angle after the application was 70.3±4.03°; for teflon surface, the contact angle was 50.8±4.81° before 1% SDS application, and 79.2±13.2° after 1% SDS application. The same researchers could not explain the angle changes in these results and left them uninterpreted [14]. According to our research findings, we can explain the angle change on teflon and glass surfaces as the hydrophobic region of the *Dew*A protein with amphipathic character caused a change in the surface adhesion behavior as a result of SDS application. It is known that the adhesion and persistence of hydrophobins to surfaces will vary with protein size, structural stability, temperature, ionic strength and buffer composition [14,23]. In addition, small and hard proteins are preferred in surface applications because they are not prone to conformational changes after surface coating due to the disulfide bonds in their structures [24,25,26]. The *Dew*A protein we used in this study is one of the small and hard hydrophobins that do not allow conformational changes in parallel with the literature. The high stability of the *Dew*A protein in SDS application, especially on glass surfaces, is probably due to the disulfide bonds it contains.

In UV applications, *Dew*A protein remained intact and its activity was preserved by UV application on teflon surfaces [27,28]. In this study, *Dew*A degradation, which occurs after 30 minute, especially on glass surfaces, can be explained by the damage of the disulfide bonds that form the rigid structure of the protein under the influence of UV. In addition, it is known that UV stimulation of tryptophan and tyrosine side chains in proteins damages the structure of disulfide bridges. In our study, protein coating was successfully performed on both glass and teflon surfaces. UV treatment was applied to both surfaces. While there was no protein degradation in the coating on teflon surfaces and the activity was preserved, protein degradation occurred in the protein coating on glass surfaces after 30 minute and the activity could not be preserved. This deformation on glass surfaces can be explained by the damage of UV-induced disulfide bridges.

While no change was detected in the amount and conformation of the proteins coated on the glass surface with SDS application, the contact angle measured on the teflon surface increased. Recombinantly produced *Dew*A protein is a class I protein and is insoluble in solutions containing surfactants such as SDS. Therefore, it is not surprising that the contact angle does not change according to the results obtained from the glass surfaces. In a study with *P. pastoris*, hydrophobin was produced at a concentration of 0.5% methanol at 90 hour at 300 mg/L under the control of the AOX1 promoter in the HFBI hydrophobin pPICZα-A vector from *Trichoderma reesei*. However, this protein is included in class II hydrophobins [29]. Another class I hydrophobin protein such as *Dew*A is *Rod*A and *Rod*B; It was also produced in *P. pastoris* under the AOX promoter and ultimately produced at a concentration of 0.5% methanol at the 120$^{th}$ hour in amounts of 21 mg/L and 24mg/L. respectively [28]. In our study, the highest efficiency production of *Dew*A protein, which is a class 1 hydrophobin, was obtained as 77.42 mg/L at 1% methanol concentration at 96$^{th}$ hour. It is clear that the production of hydrophobins using recombinant methods is more effective than the production of natural strains by traditional methods. However, except for some recombinant strains, the hydrophobin production capacity of most fungi is below 150 mg/L [5,30]. Although current production methods seem sufficient for small-scale applications such as medical applications, biosensors and drug formulations [3,30], production efficiency should be increased for larger industrial applications [31]. The main strategies used to increase the production and yield of hydrophobins are to increase the expression of hydrophobins using wild strains and recombinant DNA technology [32]. In particular, the fact that some wild strains do not secrete hydrophobin into the culture medium makes large-scale production difficult [3]. The strategy to increase yield in studies with wild strains is usually the optimization of the culture. For example, using different carbon sources and nitrogen sources, hydrophobin production from *Ceratocystsis ulmi* was increased 5 times more than the control group [33]. The type of culture is also important, some researchers have emphasized that solid-state fermentation can be an effective alternative to submerged culture in the production of hydrophobin [34]. While culture optimization is required to increase yield according to previous studies, although high efficiency production was achieved without culture optimization in our research, culture optimization can be made in our future studies.

## Conclusion

In this study, we aimed to produce recombinant class I hydrophobin *Dew*A *from Aspergillus nidulans*, which was determined to have a high contact angle in the literature. As a result of optimizations, the most suitable culture condition for *Dew*A expression was obtained at 77 mg/L 1% methanol concentration in 96 hours. Recombinant *Dew*A has been proven to change surface properties on teflon and glass surfaces. It showed a strong hydrophobic character, especially on the glass surface. These results demonstrated that recombinant *Dew*A may have biotechnological applications. This information will be used in future studies to investigate the detailed reasons for the different surface characteristics of the *Dew*A protein on glass and teflon surfaces, to determine whether the production process optimized by performing it on the Erlen scale can be optimized by transferring it to the fermenter scale, to evaluate the effect of different carbon and nitrogen sources in recombinant production and to determine the production efficiency and results. It sheds light on studies on cost. Additionally, in this study, the *Dew*A protein of A.nidulans was cloned into the pPICZa-A vector and produced recombinantly in *P. pastoris* X-33 strain for the first time.

**Abbreviations**

AOX, alcohol oxidase; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; YPDS, Yeast Extract Peptone Dextrose Medium containing sorbitol; BMYY, buffered complex medium containing methanol; BMGY, buffered complex medium containing glycerol.

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**Data Availability statement**

All data generated and analyzed during this study are included in this published article.

**Compliance with ethical standards**

**Conflict of interest / Çıkar çatışması**

The author declare no conflict of interest.

**Ethical standards**

This article does not include any studies with human participants or animals by any of the authors.

**Authors' contributions**

AT and SÖ formed the outline of the study and AT played an active role in all study steps. MT played a role in the analysis of the data and AY played a role in the cloning steps. All authors read and approved the manuscript.

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