Extracellular Production and Purification of the β-glucanase in *Pichia pastoris* Expression System

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Abstract

In this study, *Rhizomucor miehei* β-1,3-1,4-glucanase gene was expressed under the regulation of *AOX1* promoter in the *Pichia pastoris* expression system, and the clone providing the highest production level was determined. The codon-optimized gene was ligated into expression vector pPICZαA and transferred into competent *P. pastoris* KM71H cells. Thirty transformants were selected from plates containing different concentrations of zeocin and cultured in test tubes for protein production. Glucanase enzyme activities in supernatant samples were measured and ten clones showing the highest glucanase activity were determined. The proteins in supernatant samples were also analyzed by SDS-PAGE and the glucanase enzyme was observed in 2 bands, approximately 34 and 38 kDa. Protein production was performed for 72 hours in 200 mL induction medium (BMMY) with the clone providing the highest glucanase enzyme production and the recombinant glucanase enzyme was purified by Ni-NTA affinity chromatography. After purification, it was determined that the analyzed clone reached 79.6 mg/L glucanase production level. SDS-PAGE analysis of samples from each step of the purification procedure showed that the two protein bands also observed in the supernatant samples represent glucanase enzyme.

Keywords: β-glucanase, *Pichia pastoris*, *Rhizomucor miehei*, Protein purification

β-glukanaz Enziminin *Pichia pastoris* Ekspresyon Sisteminde Hücre Dışı Üretimi ve Saflaştırılması

Öz

Bu çalışmada, *Rhizomucor miehei* β-1,3-1,4-glukanaz geni, *Pichia pastoris* ekspresyon sisteminde AOX1 promotorunun regülasyonu altında ifade edilmiş ve en yüksek üretim seviyesini sağlayan klon belirlenmiştir. Kodon optimize edilmiş gen, pPICZαA ekspresyon vektörüne bağlanmış ve kompetent *P. pastoris* KM71H hücrelerine aktarılmıştır. Farklı konsantrasyonlarda zeosin içeren plakalardan otuz transformant seçilerek klonlar protein üretimi için test tüplerinde kültüre alınmıştır. Süpernatant örneklerinde glukanaz enzim aktivitesi ölçülmüş ve en yüksek glukanaz aktivitesi gösteren on klon belirlenmiştir. Ayrıca süpernatant örneklerindeki proteinler SDS-PAGE ile analiz edilmiş ve glukanaz enzimi, yaklaşık 34 ve 38 kDa olmak üzere 2 bant halinde gözlenmiştir. En yüksek glukanaz enzim üretimine sahip klon ile 200 mL indüksiyon ortamında (BMMY) 72 saat boyunca protein üretimi gerçekleştirilmiş ve rekombinant glukanaz enzimi Ni-NTA afinite kromatografisi ile saflaştırılmıştır. Saflaştırma işleminden sonra analiz edilen klonun 79,6 mg/L glukanaz üretim seviyesine ulaştığı belirlenmiştir. Saflaştırma işleminin her aşamasından alınan örneklerin SDS-PAGE analizi, süpernatant örneklerinde de gözlenmiş olan iki protein bandının glukanaz enzimini temsil ettiğini göstermiştir.

Anahtar Kelimeler: β-glukanaz, *Pichia pastoris*, *Rhizomucor miehei*, Protein saflaştırma

1. Introduction

The β-glucanase enzymes that hydrolyze β-glucan are generally divided into 4 subclasses. β-glucanases are named for their mechanism of action on the β-glucan substrate; β-1,4-glucanase

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(EC 3.2.1.4, cellulase), β-1,3(4)-glucanase (EC 3.2.1.6), β-1,3-glucanase (EC 3.2.1.39, laminarinase) and β-1,3-1,4-glucanase (EC 3.2.1.73, lichenase). Among these enzymes, lichenases known as β-1,3-1,4-glucanases have the highest substrate specificity (Yang et al., 2014).

Enzyme preparations are used in a variety of processes in modern food processing methods, such as for oil extraction from seeds, juice extraction and purification, and to increase feed yield in the feed industry. The breakdown of the β-glucan chains is an important step in each of these processes (Bhat and Bhat, 1997). It is technically and economically more advantageous to obtain enzymes, which can also be obtained from plant and animal sources, from microbial sources. *Bacillus* species are the most commonly used bacteria for the production of β-1,3-1,4-glucanases for industrial purposes (Planas, 2000). Fungal glucanases also have a high potential for use in industrial processes for various purposes (Celestino et al., 2006; Mathlouthi et al., 2002; Chaari et al., 2015). Studies on biotechnology and recombinant DNA technology increase the benefit from microbial resources as well as make it possible to improve the technical properties of these enzymes by using genetic engineering techniques (Khan et al., 2016).

Prokaryotic expression systems are often the first choice in recombinant protein production as they are easy and inexpensive. However, some problems may arise during protein production in eukaryotic prokaryotic systems, such as the production of the protein being unstable or unable to perform its biological activity. The yeast intracellular environment is better suited for the correct folding of eukaryotic proteins compared to prokaryotic systems (Cregg et al., 1987). As eukaryotic expression systems, yeasts can perform the post-translational biochemical reactions (such as disulfide bond formation, glycosylation) required for eukaryotic proteins. Additionally, their genetic manipulation is as easy as bacteria. All these reasons make yeast advantageous for the production of eukaryotic proteins (Cregg, 2007).

The methylotrophic yeast *P. pastoris*, which was used as a host system in our study, was developed for the production of single-cell protein in the 1970s because it can reach very high cell density in fermenter conditions. In recent years, it is an excellent host, which has been widely used especially in the industrial field for recombinant protein production (Daly and Hearn, 2005; Jungo et al., 2007; Baghban et al., 2018). *P. pastoris* grows rapidly on inexpensive media like bacteria. In addition, since it is a eukaryotic organism, its intracellular environment is suitable for folding eukaryotic proteins and is capable of performing post-translational modifications such as proteolytic processes, disulfide bridge formation and glycosylation. (Lin-Cereghino and Cregg, 2000).

In the literature, it has been shown that higher protein production levels per unit are achived by codon optimization compared to the native sequence. Huang et al., (2008) reported an approximately 2-fold (mg/L) increase in *Fibrobacter succinogenes* glucanase production in *P. pastoris* with codon-optimized sequence. The glucanase gene from *Bacillus licheniformis* was expressed in *P. pastoris* with codon optimization, and an approximately 10-fold (mg/L) increase was reported compared to codon non-optimized expression (Teng et al., 2007). It is clear from previous studies that codon optimization of the glucanase gene (Luang et al., 2010; Pham et al., 2011) and also others (Wang et al., 2015; Qiao et al., 2010; Karaoğlan and Erden-Karaoğlan, 2020) has a significant effect on the expression level of *P. pastoris.*

R. miehei glucanase gene was first isolated by cDNA synthesis and its gene sequence was revealed (Tang et al., 2012). Also, optimization of the solid-state fermentation was performed for glucanase production in *R. miehei* strain, and carbon source, nitrogen source, pH and temperature optimization was performed to achieve the highest production level in *R. miehei*

strain (Yang et al., 2014). In this study, it was aimed to clone the *R. miehei* glucanase gene under the *AOX1* promoter as codon-optimized in the *P. pastoris* expression system, to select the clone with the highest production level and purification of the produced enzyme by affinity chromatography.

2. Material and Methods

2.1. Material

2.1.1. Strains, Expression vectors and Media

Escherichia coli XL1-Blue was used for sub-cloning and *P. pastoris* KM71H (*aox1::ARG4*) strain was used for the protein expression. The pPICZαA plasmid available in our laboratory was used as an expression vector for the methanol-inducible extracellular protein production.

E. coli XL1-Blue cells were grown on LB Miller Broth (1% tryptone, 0.5% yeast extract and 1.0% NaCl), LB Miller Agar (1% tryptone, 0.5% yeast extract, 1.0% NaCl and 1.5% Agar), LB Lennox Broth (1% tryptone, 0.5% yeast extract and 0.5% NaCl), LB Lennox Agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% Agar). *P. pastoris* KM71H cells were grown on YPD Broth (1% yeast extract, 2% peptone, 2% dextrose) and YPD Agar (1% yeast extract, 2% peptone, 2% dextrose and 1.5% Agar) media. Selection of the transformants were done on media supplemented with Zeocin at a concentration of 25 μg/mL for *E. coli* and 100 μg/mL for *P. pastoris*.

Recombinant protein production studies were carried out under shake-flask conditions. BMGY medium (2% peptone, 1% yeast extract, 2% glycerol, 1.34% YNB, $4 \times 10\text{-}5\%$ biotin and 100 mM phosphate buffer pH 6.0) was used for the growth of the cells in the cell accumulation phase. BMMY (2% peptone, 1% yeast extract, 1% methanol, 1.34% YNB, $4 \times 10\text{-}5\%$ biotin and 100 mM phosphate buffer pH 6.0) media were used in the induction phase for the recombinant protein production. Culture media preparation for cloning and protein expression studies were performed in accordance with the instructions manual of Pichia Expression Kit (Invitrogen, Carlsbad, CA, USA).

2.2. Methods

2.2.1. Construction of the expression vector

R. miehei glucanase (JQ088103) gene was used as glucanase (β-1,3-1,4-glucanase) gene source. The gene was synthesized commercially as codon-optimized based on *P. pastoris* codon usage and provided in the pUC57 cloning vector.

The pUC57-GLCNase vector containing the codon-optimized glucanase gene was digested with *Xho*I and *Xba*I enzymes and the glucanase gene (957 bp) was isolated from agarose gel using the Gel Extraction Kit (Fermentas, Thermo Scientific, USA). The obtained gene was ligated into pPICZαA to enable methanol inducible extracellular protein production at the same restriction enzyme regions and pPICZαA-GLCNase expression vector was obtained. Schematic representation of the constructed expression vector was given in Figure 1.
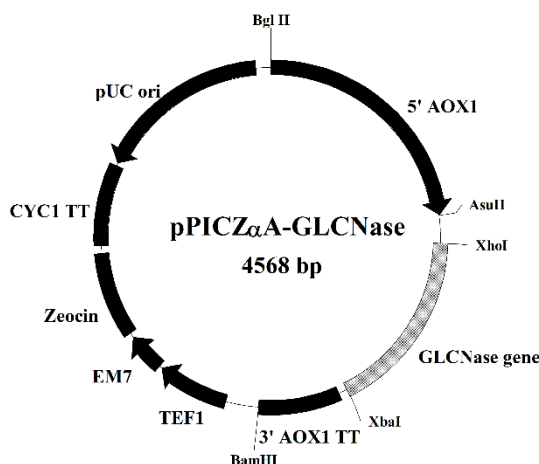
Figure 1. Schematic representation of the constructed expression vector pPICZαA-GLCNase

2.2.2. Integration of the expression cassette into the *P. pastoris* genome

Competent *P. pastoris* KM71H cells were prepared according to the lithium-acetate method. The expression vector obtained was linearized with the restriction enzyme *Pme*I, and the vector was transformed into competent *P. pastoris* KM71H cells by electroporation (Bio-rad electroporator, CA, USA). Transformants were selected from the YPD plate containing different zeocin concentrations (100, 500, 1000 µg/mL) after three days of incubation at 30°C (Wu and Letchworth, 2004).

2.2.3. Selection of the clones for the recombinant glucanase production

Thirty colonies of different sizes, growing on plates with different zeocin concentrations, were selected and transferred to YPD broth. The clones were analyzed in test tubes for preliminary testing to determine the expression strength (ability to produce glucanase). First, the cells were grown in BMGY medium for about 18 hours, then the cells were harvested by centrifugation at 1200 *g* for 5 min. and transferred to BMMY medium, which is a protein production medium. Protein production was continued for 72 hours and the OD value of the culture and enzyme activity in the supernatant were measured from the samples taken at regular intervals. Proteins in the supernatant samples were analyzed by SDS-PAGE (Consort bvba, Turnhout, Belgium).

2.2.4. Determination of the glucanase enzyme activity

Glucanase enzyme activity was determined using the DNSA (3,5-dinitrosalicylic acid) method by measuring the reducing sugar released from β-glucan (Miller, 1959; Bailey, 1988; Tang et al., 2012). The standard curve was generated with glucose solutions prepared at different concentrations (1-10 µmol). One unit of glucanase activity was defined as the amount of enzyme required to produce 1µmol of reducing sugar in 1 minute under experimental conditions (50°C, 50 mM citrate buffer pH 6.0).

2.2.5. SDS-PAGE analysis

The supernatant samples taken during the fermentation and the samples taken from the purification steps were mixed with 4x SDS gel loading buffer and denatured at 70°C for 10 minutes. The prepared samples were run in a 5% polyacrylamide loading gel and 10% polyacrylamide separation gel in 1xTGS buffer (0.025 M Tris base, 0.192 M Glycine, 0.1% SDS, pH 8.3) at 100 V for 90 minutes to separate proteins. The gel was then stained with

Coomassie Brilliant Blue dye and the proteins were visualized after the dye was removed (Karaoğlan and Erden-Karaoğlan, 2020).

2.2.6. Recombinant glucanase production in shake flasks

The clone with the highest glucanase enzyme production was inoculated from the stock culture into test tubes containing 5 mL of YPD medium and incubated at 250 rpm for 24 hours at 28°C in orbital shaker (Jeio Tech, Geumcheon-gu, Seoul, Korea). This culture was used as an inoculum for a 2L flask containing 200 mL of BMGY medium with an initial cell density of OD_{600nm}= 0.1. Incubation was continued at 28°C for 18 hours and the cells collected by centrifugation at 1200 g for 5 min. were transferred to 200 mL of BMMY medium for the enzyme production, and the fermentation was carried out at 250 rpm for 72 hours. At the end of the fermentation period, the culture was centrifuged at 10000 g for 5 min. Approximately 200 mL of the supernatant sample was collected. The sample collected was stored at +4°C for use in the enzyme activity analysis and affinity chromatography purification.

2.2.7. Purification of the recombinant glucanase enzyme by affinity chromatography (Ni-NTA)

The histidine-tagged glucanase enzyme was purified from the supernatant sample using Ni-NTA resin (Pierce, Thermo Scientific, USA). For this purpose, proteins in the supernatant sample were first collected by precipitation with 80% saturated ammonium sulfate and resuspended in 20 mL of 1xPBS (20 mM potassium phosphate pH 7.4, 300 mM NaCl, 10 mM imidazole). The mixture was dialyzed against 1xPBS buffer to remove the ammonium salt. The resulting sample was added 4 mL of nickel resin standardized with 1xPBS buffer. The mixture was shaken gently at room temperature for 30 minutes, and then the mixture was passed through the resin retention column to collect the resin to which the protein was attached. The resin obtained was washed 3 times with 1xPBS containing 25 mM imidazole, and then the protein (glucanase) attached to the resin was treated 3 times with 1xPBS elution buffer containing different concentrations of imidazole (100, 200 and 400 mM). Samples obtained from each step of the purification procedure were analyzed for determination of the quality of purification by SDS-PAGE analysis (Karaoğlan and Erden-Karaoğlan, 2020). The total amount of protein in the elution samples was measured using the Coomassie Bradford Plus Protein Assay Kit following the kit protocol.

3. Results and Discussion

3.1. Construction of the expression vector

The pUC57-GLCNase vector containing the codon-optimized glucanase gene was digested with *Xho*I-*Xba*I and after separation of the sample by gel electrophoresis, the GLCNase gene (957 bp) was isolated from agarose gel (Figure 2A-2B). The pPICZαA expression vector was digested using the same restriction endonuclease enzymes, and the GLCNase gene was ligated into the resulting linear expression vector. The ligation mixture was transferred to competent *E. coli* XL1-Blue cells and selection was made on LB Lennox agar medium containing zeocin. Three colonies growing on the medium containing antibiotics were selected and the expression vector was confirmed by restriction analysis with *Hind*III (Figure 2C). The confirmed expression vector was linearized with *Mss*I restriction endonuclease and transferred to the competent *P. pastoris* KM71H cells by electroporation.
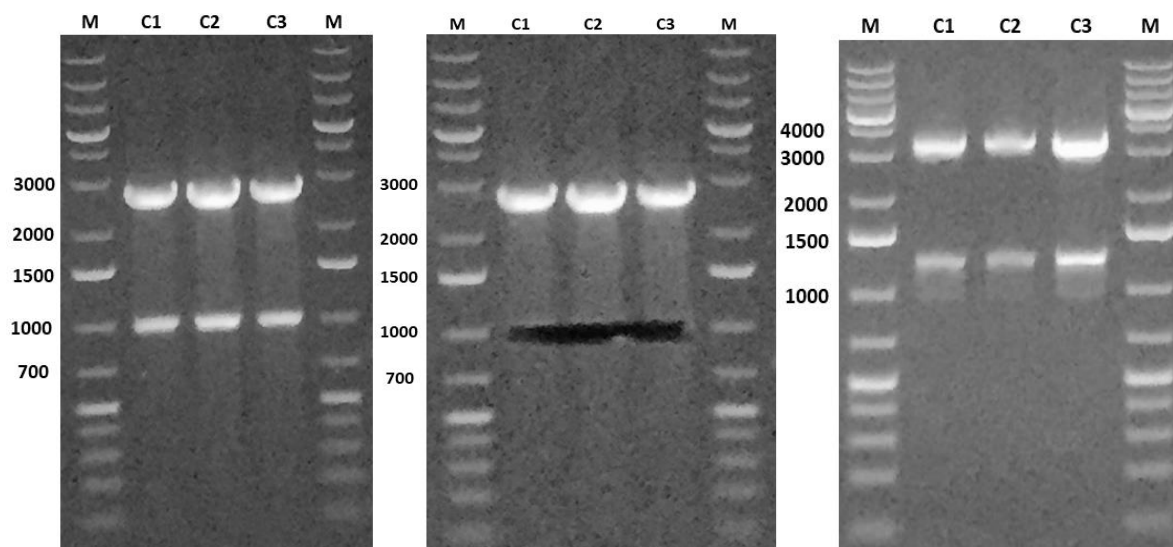
Figure 2. Recovery of the GLCNase gene from the pUC57-GLCNase vector, extraction from the gel and control of the resulting pPICZαA-GLCNase vector by restriction analysis

3.2. Expression of the Glucanase Gene in *P. pastoris*

After the transformation of the linearized expression vector with the Lithium-acetate method, 30 colonies of different morphology and size were first grown overnight in YPD medium at 28ºC. A 100 µL of the grown cells were inoculated into test tubes containing 2 mL BMGY medium and incubated for 18 hours at 28°C and 250 rpm. At the end of the incubation, the cells were harvested and resuspended in 2 mL BMMY medium for the induction phase at the same conditions. The induction phase was continued for 72 hours and methanol was added to the culture for induction at every 24 hours to a final concentration of 1%. At the end of this phase, OD values of the cultures were measured, and the supernatant samples were collected for glucanase enzyme activity analysis. The enzyme activity results obtained were presented in Figure 3. Due to the different cell densities of each culture, the activity values were calculated by normalizing the cell density (OD value) in order to avoid errors that may arise from the difference in cell density and the results were given as relative enzyme activity values.
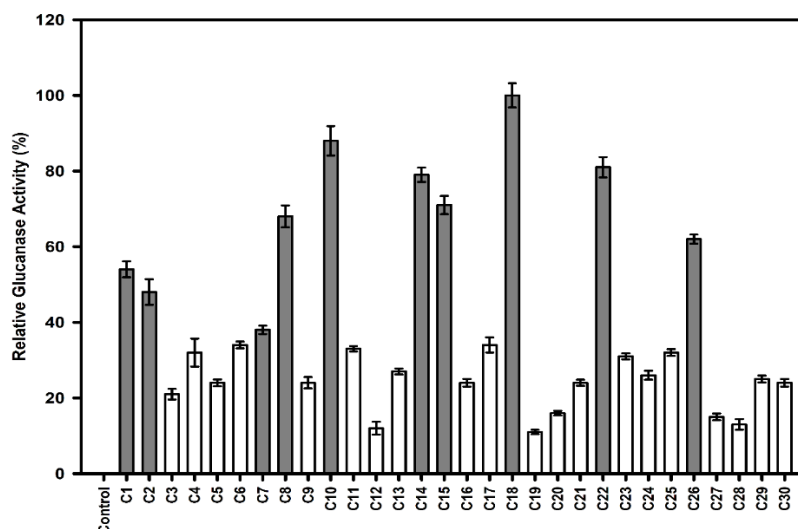

Figure 3. Relative glucanase enzyme activity results of selected clones

After the enzyme activity measurement, the proteins in the supernatant samples of 10 clones that showed the highest glucanase activity were analyzed by SDS-PAGE analysis. The supernatant samples normalized to cell density were loaded on SDS-PAGE gel and run at 100V for 90 minutes. The sample from the *P. pastoris* KM71H culture that was not transformed with the expression vector and grown under the same fermentation conditions was used as a control. The gel image of SDS-PAGE analysis made on the supernatant samples was given in Figure 4. The results obtained showed that the 2 protein bands in the supernatant samples of the clones were different compared to the control strain. In the purification analysis by Ni-NTA affinity chromatography, it was revealed that the histidine-labeled glucanase enzyme was obtained as 2 protein bands and both proteins were glucanase. However, it was observed that the glucanase enzyme with a theoretical molecular weight of approximately 34 kDa was produced in 2 bands, approximately 34 and 38 kDa. A similar situation was reported in the study by Huang et al. (2018) and they showed that GFP (Green fluorescence protein) production was seen as 2 protein bands in SDS-PAGE analysis. They explained that the observation of the same protein in two bands of different sizes may be due to the 9 kDa signal sequence remaining unprocessed during extracellular secretion. However, considering the protein marker in our study, it is clear that there is a difference of less than 9 kDa between the two protein bands. In another study, alginate lyase enzyme was produced in *P. pastoris* and similarly, 2 protein bands, 32 and 35 kDa, were determined in SDS-PAGE analysis after purification (Li et al., 2018). They reported that the low molecular weight (32 kDa) of the bands seen after purification was the non-glycosylated form, the high molecular weight (35 kDa) form was the glycosylated form and the 3 kDa difference was due to the different degree of glycosylation. The difference in molecular weight of 4 kDa in our study is thought to be due to the different glycosylation degrees of the enzyme during post-translational modifications.
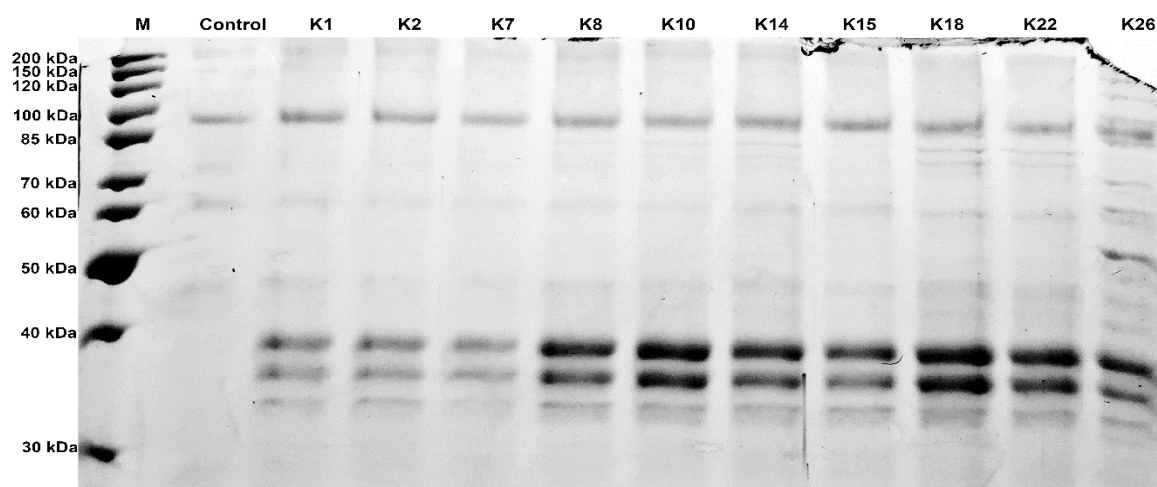

Figure 4. Analysis of glucanase enzyme production in selected clones by SDS-PAGE

3.3. Purification of the recombinant glucanase by Ni-NTA affinity chromatography

Protein production study was carried out with K18 clone, which showed the highest glucanase enzyme activity, under shake-flask conditions. For this purpose, cells grown up to approximately 8-10 OD values in 200 mL BMGY medium in a 2 L flask were centrifuged and transferred to 200 mL BMMY medium and induction was performed for 72 hours at 28°C and 250 rpm. Induction was made by adding methanol to a final concentration of 1% at every 12 hours. At the end of the incubation, the supernatant was collected by centrifugation and 200 mL of supernatant sample was obtained. Proteins in the obtained supernatant sample were

precipitated with 80% saturated ammonium sulfate and the precipitate sample was dissolved in 20 mL 1xPBS buffer. The concentrated enzyme solution obtained was dialyzed overnight against 1xPBS buffer in a snake-skin dialysis bag with a cut-off value of 3 kDa and ammonium salts were removed. The dialyzed sample was mixed with 1x PBS buffer containing 10 mM imidazole and loaded on the chromatography column containing Ni-NTA resin and eluted with different imidazole concentrations in accordance with the purification protocol. SDS-PAGE image of the samples taken from the purification steps is given in Figure 5.

Elutions obtained after purification were collected in the same tube and dialyzed against 1xPBS buffer. By measuring the protein concentration of the sample obtained after dialysis, the production level per liter was determined under shake flask conditions. It was observed that the clone with the highest production reached the production level of 79.6 mg/L. To our knowledge, this is the first study on recombinant production of *R. miehei* glucanase enzyme in *P. pastoris*. In a previous study, the production of this enzyme was carried out in the microorganism, which is the gene source, and according to the result obtained after purification, a production level of 36 mg/L was recorded (Tang et al., 2012). In our study, a 2.2-fold increase in production was achieved with recombinant protein production.
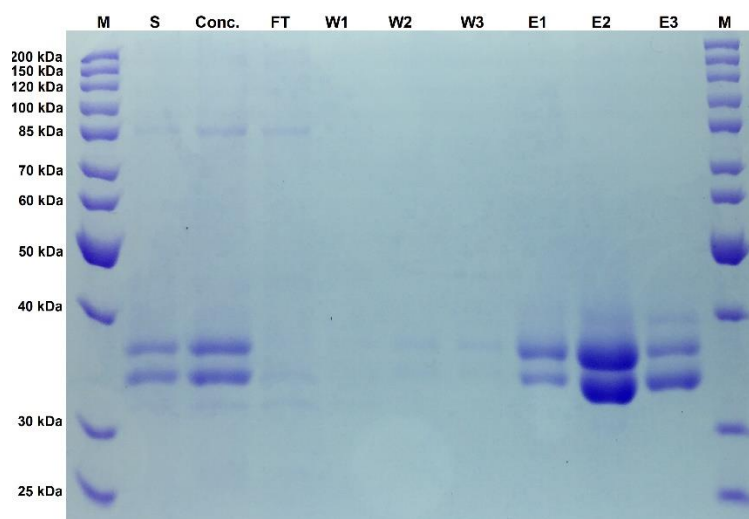

Figure 5. SDS-PAGE image of samples taken from the purification steps of glucanase by Ni-NTA affinity chromatography.

4. Conclusion

In this study, it was aimed to produce the glucanase enzyme from *R. miehei* recombinantly. Within the scope of the study, methanol-induced extracellular glucanase production under the *AOX1* promoter in *P. pastoris* with the gene sequence optimized according to the *P. pastoris* expression system was performed. Thirty protein producing clones were screened and the clone with the highest production level was determined. Subsequently, protein production was carried out at 2 L in shake flask conditions and recombinant glucanase was purified by Ni-NTA affinity chromatography. At the end of the study, a clone providing recombinant production for glucanase enzyme was successfully obtained. In future studies, the production level and yield can be increased with multi-copy clones and optimization of production conditions.

5. Acknowledgement

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