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Recombinant production of chimeric protein Mfp-3-GvpA in yeast *Pichia pastoris*

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ABSTRACT

Mussels secrete protein-based polymers mainly mussel foot proteins (Mfps), enabling them to anchor to various surfaces in a saline, intertidal zone. Using Mfp proteins as novel water-resistant adhesive materials have been suggested for biomedical purposes due to their unique features including large abundance of catechol aligned with amphiphilic and ionic properties. Despite the more promising characteristics of foot proteins, they have not been widely exploited for biomedical or even industrial applications, since farming the mussels are not commercially viable due to ecological issues and their highly territorial nature. Present study aimed to examine engineering of recombinant synthesis of Mfp3 protein fused to another marine based curli protein, gas vesicle protein A(GvpA) in nonpathogenic yeast, *Pichia pastoris,* expression system. To this end, sequences of the Mfp3 and GvpA were extracted from the NCBI database and were inserted into the *pPICZ A* vector in order to obtain an efficient expression level of chimeric protein. Obtained vectors were transformed into *E. coli* TOP10F' for multiplication and then linearized plasmid transformed into *Pichia pastoris for protein expression.* The best expression level obtained after 96 hours' incubation with methanol induction. In conclusion, active recombinant adhesive Mfp3-GvpA fused protein were successfully expressed in *P. pastoris* suggesting potential expression system for future bio-adhesive or any fused protein production. .

1. Introduction

The production of bio adhesives with the required characteristics (biocompatibility, non-toxicity in the human body, and strong, stable adhesion) has long been considered by scientists. One of the most important points in the production of bio adhesives is the resistance of this material in situations similar to the physiological conditions of the human body (humid conditions with low pH) . Actually, every produced bio adhesive has toxic precursors that in many cases lead to tissue rejection or cancer (Amini et al., 2017; Cha et al., 2008; Ferdosian et al., 2017).

Mussel Mytilus edulis has long been of interest to scientists because it has the ability to produce sticky proteins with incredible properties, including resistance to wet conditions and low pH (Even et al., 2008; Zhao and Waite, 2006). Mytilus edulis can produce eight types of different sticky proteins that help each other to produce viscose substances. These proteins include mfp-1, mfp-2, mfp-3, mfp-4, mfp-5,

PCOL-D, and PCOLNG (Danner et al., 2012; Lu et al., 2012).

Mfp-3 and mfp-5 proteins are of particular importance because they have a higher adhesion strength than other proteins produced by *Mytilus edulis*. The high adhesion strength of these proteins is because of their large amount of DOPA (3 and 4 dihydroxyphenylalanine) (Even et al., 2008; Hosseini et al., 2017; Warner and Waite, 1999). As the smallest protein in Mytilus, mfp-3 contains 20-25% of DOPA and a high amount of arginine, which changes to 4-hydroxy-arginine and 2, 4 tryptophan in post-translational (Barclay et al., 2017; Hwang et al., 2012; Yajing Kan et al., 2014; Li et al., 2020). The production of mfp-3 recombinant sticky protein in bacterial hosts has been done by different methods, but research has shown that using curli proteins with the sequence of mussel foot adhesive proteins can increase the adhesion strength (Chao Zhong et al., 2014).

Research on the effect of different pHs on mussel adhesive proteins shows that the adhesion of mussel foot protein in acidic conditions is much higher than in neutral or alkaline conditions. Because under acidic conditions, DOPA is immediately oxidized to dopaquinones (Ezzeldin et al., 2012). In the study conducted by Cereghino et al., bacterial cells were used as hosts, but due to the inability to be modified after the translation of prokaryotic cells, the addition of the tyrosinase enzyme was required to convert all tyrosine to DOPA. The production of recombinant protein in prokaryotic hosts was low and a large amount of culture medium was required to produce protein (Cregg et al., 2000; Damasceno et al., 2012).

2. Materials and Methods

2.1. Sequence selection and design of gene structures

After selecting the sequence of mfp-3, the sequences of GvpA, JS Linker, His-Tag, and antibiotic resistance (Zeocin) were added to the above-mentioned sequence, and the sequence was optimized for expression in the yeast. Then, the sequence was ordered to *Biomatik* company in Canada, and the company synthesized it and put it inside the *pPICZA* vector.

2.2. Transformation of Gene in to the E. coli TOP10F ' cell

The vector was transferred to the competent cell of *E. coli TOP10F' (TOP10F* is a type of *E. coli* bacteria that is very useful for gene transfer). In this study, this cell was prepared from Invitrogen in Luria-Bertani agar medium with zeocin antibiotic (final concentration of 50 µg / mL) by the heat shock method (Froger and Hall, 2007). Then, plasmid was extracted from the bacterial cell with a high concentration using Gen all plasmid extraction kit (Dadgar et al., 2015).

Concentrated plasmids were linearized by Sac1 enzyme by the overnight method (Urdea et al., 1988). The linear gene was then cleaned up by the Gen all cleanup kit to remove salts and RNAs (Dadgar et al., 2015). Afterwards, PCR was performed with primers whose specifications are given in Table 1, and the program is listed in Table 2.

Then, electrophoresis gel (1% agarose) and DNA ladder (100-10kda bp) (SMBIO DM3200) were utilized to confirm the presence of the gene. After staining the gel with DNA STAIN (Gen Bio) for checking the presence of the gene, the gel was transferred to the gel documentation system for the final testing.

2.3. Pichia pastoris competent cells

To prepare the *Pichia pastoris* competent cell, a single clone of *Pichia pastoris* strain gs115 (Invitrogen) was first cultured in 5 ml of YPD MEDIOM medium and placed overnight. When the OD of the desired culture medium reached OD = 600, it showed the ideal growth of yeasts. At this stage, 800 µl of pre-culture sample was introduced into 125 cc of culture medium. After 24 hours, when OD = 0.5 was reached, another 300 µl of pre-culture medium was added after another overnight OD = 1.3.

At this stage, the whole culture medium was centrifuged at 1500 rpm for five minutes at 4 ° C (Eppendorf). Next, the supernatant was discarded, and 15 ml of cold sterile water was added to the remaining sediment. Soft piping was performed to dissolve the sediment well. The centrifuge was performed again at 1500 rpm in five minutes and 4°C; then the supernatant was discarded, and 7.5 ml of cold sterile water was poured and pipetted to dissolve the

precipitate well. Afterwards, the previous centrifuge process was repeated. Next, 1 ml of cold sorbitol (1 M) was poured and pipetted again until the precipitate was completely dissolved and centrifuged. The supernatant was discarded and 250 µl of cold sorbitol was added, pipetted and the samples were transferred to ice. The yeast cell stage was ready to receive a foreign gene (Competent cell) (to prepare the competent cell, the Invitrogen protocol was used (dos Santos et al., 2016; Higgins, 1998).

2.4. Transformation gene

The concentrated plasmid was transferred to the competent cell of the *Pichia pastoris* by electroporation. At this stage, electroporation device (pulsar-Eppendorf) was used (power 2000 watts and time of 500 microseconds). Yeast cells containing possible genes were further examined for five days in YPDS culture medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) with zeocin (100 µg mL-1 g) at 28 Degrees Celsius.(Lin-Cereghino et al., 2005).

2.5. PCR test to prove the existence of the target gene

To confirms the presence of the gene in the yeast, the program of Table 1, gel electrophoresis (1% agarose) and DNA ladder (bp100-10kda) were used. After staining the gel with DNA stain, the gel was transferred to the documentation gel system.

2.6. Yeast cell growth

To evaluate the growth of yeast cells after the addition of methanol in hours 0, 12, 24, 48, 72 and 96, 2 cc was taken from the growing sample. and samples were examined for OD Obviously over time and adding the right amount of methanol. We have observed the logarithmic growth of yeast cells As shown in Figure 10 (Higgins and cregg,1998).

2.7. Protein expression in Pichia pastoris

The Invitrogen protocol was used for gene expression and protein production (Higgins and Cregg, 1998). For this purpose, *Pichia pastoris* was first cultured in Buffered complex glycerol medium (BMGY) as the standard complex medium for *Pichia pastoris* cultivation containing peptone, yeast extract, yeast nitrogen base (YNB), and a phosphate buffer. After 18 hours, the whole culture was centrifuged, and yeast precipitate was transferred to Buffered complex methanol medium (BMMY) as the standard complex medium for *Pichia pastoris* cultivation containing peptone, yeast extract, yeast nitrogen base (YNB), and a phosphate buffer. This culture medium contained methanol. Pure methanol was added to 0.5% of the total culture medium daily because *Pichia pastoris* is a methylotrophic yeast. At 0, 24, 48, 72, and 96, 2 cc of the culture medium was sampled and centrifuged, and the precipitate was kept at a negative 20 degrees Celsius.

2.8. Lysis of Pichia pastoris cells

Precipitate samples collected over five days were lysed by glass bead (acid washed) (size: 0.5 microns). Cell lysis steps: First, 100 microliters were added to each BB buffer (50 mM Sodium phosphate plus 1 mM pmsf and 1 mM EDTA2NA), and 0.5 ml of glycerol to a volume of 100 cc was adjusted for pH (PH = 7.4). Then, glass bead was added to the weight of the precipitate, and the microtubule was placed on a shaker for 30 seconds to break the strong and thick wall of *Pichia pastoris*. After 30 seconds, the microtubes were transferred to the ice for 30 seconds. This cycle was repeated eight times. After centrifugation at 10000 rpm for five minutes, the supernatant was stored. 50 µl was considered for SDS PAGE test and 50 µl for western blot test (Jan et al., 2016).

2.9. SDS PAGE analysis

SDS PAGE test was used to check the presence of protein for this purpose, a loading buffer was added to the sample obtained from the previous step (After boiling the samples, it was added to the SDS cassette). In this test, the bottom gel was 10%, and the top gel was 5%. After the required time (2.5 to 3 hours) and complete opening of the protein ladder, the gel was removed from the SDS-PAGE cassette and stained with Kumasi Blue for 20 minutes. In the SDS-PAGE stage, in addition to the specialized band, many bands may be seen, indicating the presence of yeast proteins. If a specialized band is seen in the right place, the western-blot test

should be used for more specialized examination (Jan et al., 2016).

2.10. Western blot analysis

The western blot test is an accurate test to prove the presence of a specialized protein. In this method, protein bands that are separated from each other on polyacrylamide gel are transferred from the gel to a membrane that can bind and stabilize proteins. In this study, nitrocellulose paper was used because the sequence of the designed target gene used the His-Tag sequence. Anti His-Tag antibody was used to prove the presence of the desired protein in the western blot test. If the desired protein is expressed, a single band will appear next to the ladder protein and in the correct position on the nitrocellulose paper (Mahmood and Yang, 2012).

2.11. Evaluation of protein production in yeast

Because the *pPICZA* vector was used in this test and the *pPICZA* vector is an Intracellular secretion vector, and because we did not have a secretory signal sequence in our gene sequence to check the Intracellular secretion of the vector and prove the absence of the desired protein in the culture medium. The amount of protein produced by yeast was assessed once using supernatant and once using sediment (lysed yeast cell). to evaluate the amount of protein produced, SDS method was used. As shown in Figures 11 and 12, the amount of protein in the culture medium was very small and most of the protein was present in the yeast cell (jan et al., 2016).

2.12. Protein purification

For a large-scale production and preparation of protein mixtures for purification of proteins such as the expression stage, pretreatment was given in the BMGY medium. The precipitate was then transferred to BMMY medium, and 0.5% of the total volume methanol was added to culture medium daily. This process continued for five days, and then the whole culture medium was centrifuged (at 10000 rpm for 10 minutes at 25 ° C). The resulting precipitate was cell lysed with glass bead (0.5 µm, Sigma acid wash) and 3 cc of binding buffer solution. First, a binding buffer solution was added to the precipitate. After dissolving the precipitate, glass bead was added and stirred for 30 seconds, then placed on ice for 30 seconds. This cycle was repeated eight times. After centrifugation (at 10000 rpm for 10 minutes at 4 °C), cell supernatant was isolated and passed through a 0.22 sterile syringe filter (Gøgsig et al., 2012).

Absorption chromatography with nickel resin was used to purify the protein. First, the cell soup was transferred to the chromatographic column with the binding buffer solution (50 mM Na2H2po4 with 300 mM Nacl and 10 mM imidazole was reduced to 100 cc and the pH adjusted) and placed on a shaker with ice for one hour. After this time, specialized proteins were expected to bind to nickel resins due to their His-Tag sequence. Then, the whole solution was passed through the column, and 6 cc of washing buffer solution was added to it. After 10 minutes, this solution was passed through the column and fresh washing buffer solution was added to the column again. After 10 minutes, this solution was also removed. This cycle was performed three times and at the end, 2 cc of solution elution buffer containing high amount of imidazole was added to the column. Since the affinity of imidazole is higher than that of proteins containing His-Tak sequence to nickel resin, it caused the release of mfp-3-GvpA protein. After 20 minutes, when the solution was passed through the column at the exit stage of this solution, the Bradford test was performed, and the solution was removed until the color of the Bradford reagent did not change (Yang et al., 2009).

2.13. SDS page test after purification

The chromatographic solution was transferred to the dialysis bag with kataf 15 to remove imidazole and achieve pure protein. After separating the protein from imidazole, the sample, which only contained the specialized protein, was evaluated by the SDS page. As only the specialized protein was to be present in the resulting solution, we expected to see a single protein band in the correct position. The SDS PAGE test was used to evaluate the single-band position of the protein (In this test, the bottom gel was 10%, and the top gel was 5%). At this stage, the protein marker (10-180 kDa) PM 1500 (Excel Band) was used.

Table 1. Specifications of primers

Application	Primer	Name of Primer	
Confirmation of the presence of genes	3′GACTGGTTCCAATTGACAAGC5′	AOX1 Promoter Forward primer	**1**
	5′GCAAATGGCATTCTGACATCC3′	AOX1 Terminator Reverse primer	**2**

Table 2. Uses of the thermal PCR program

Part	Temperature Section (ºC)	Time	Number of cycles
Start denaturation	95	5 min	1
Denaturation	94	30 Sec	30
Connection	55	30 sec	
Elongation	72	50sec	
End of lengthening	72	7 Min	1

3. Results

Figure1, Structure of Gene mfp3-GvpA

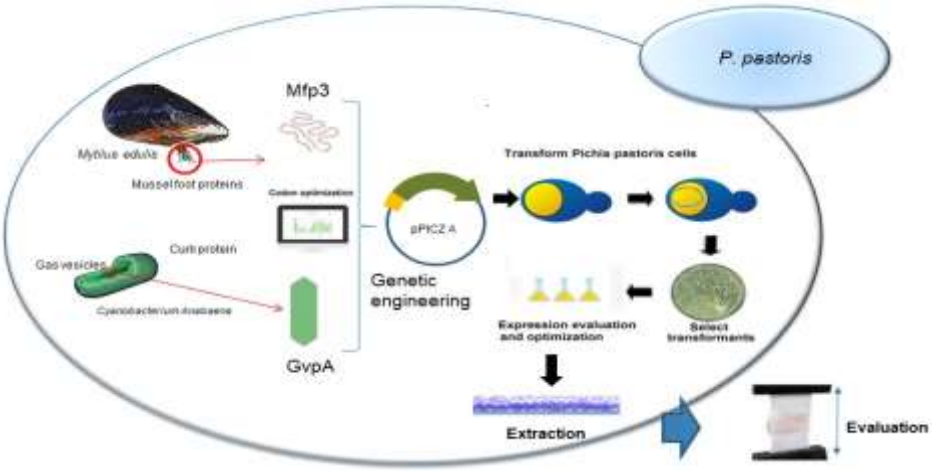


Figure 2. Various steps of the study from design of recombinant vectors to quantitative evaluation of expressed target protein

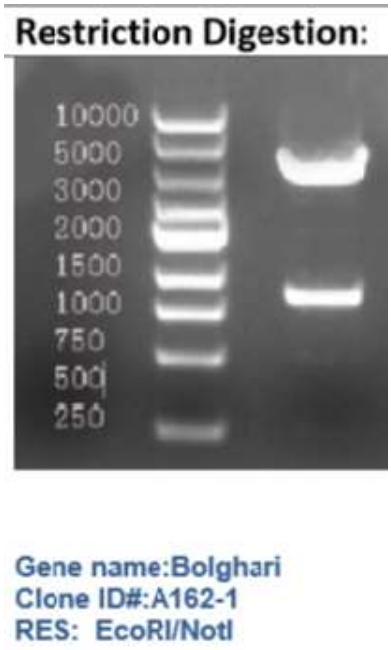


Figure 3. reports a gene MFP3-GvpA, His-Tag, gs linker, and antibiotic resistance sequence (Zeocin) made by *Biomatics* Company and placed in the pPICZA vector.

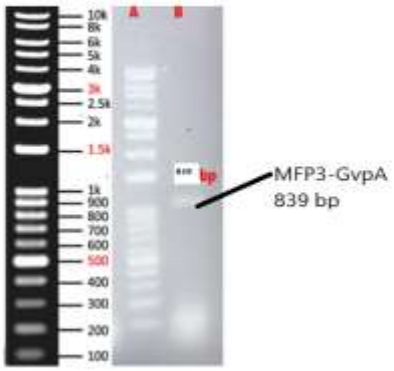


Figure 4. Gene transfer to the competent cell of *E. coli* TOP 10/ and confirmation of the presence of the gene by PCR showing the column a of the leader used in electrophoresis, and column b showing the mfp3-GvpA gene band, observed at 839 bp.

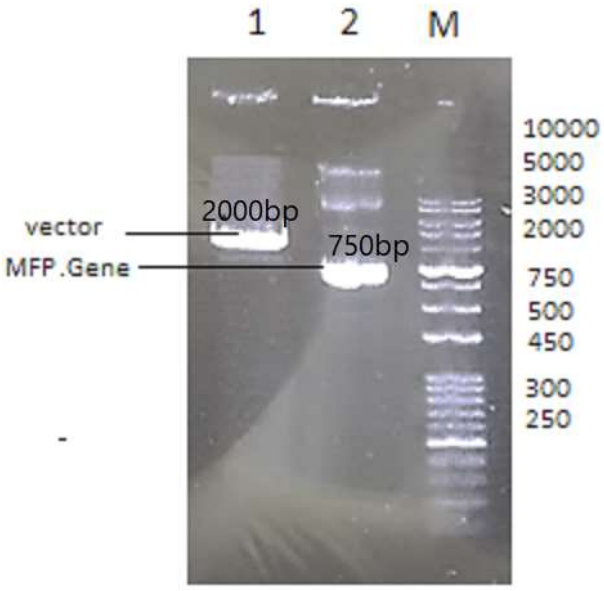


Figure 5. Electrophoresis after enzymatic cut two bands is observed by SAC1 enzyme, the band in the 750 bp region represents the gene and the band seen in the 2000 bp region represents the *pPICZA* vector

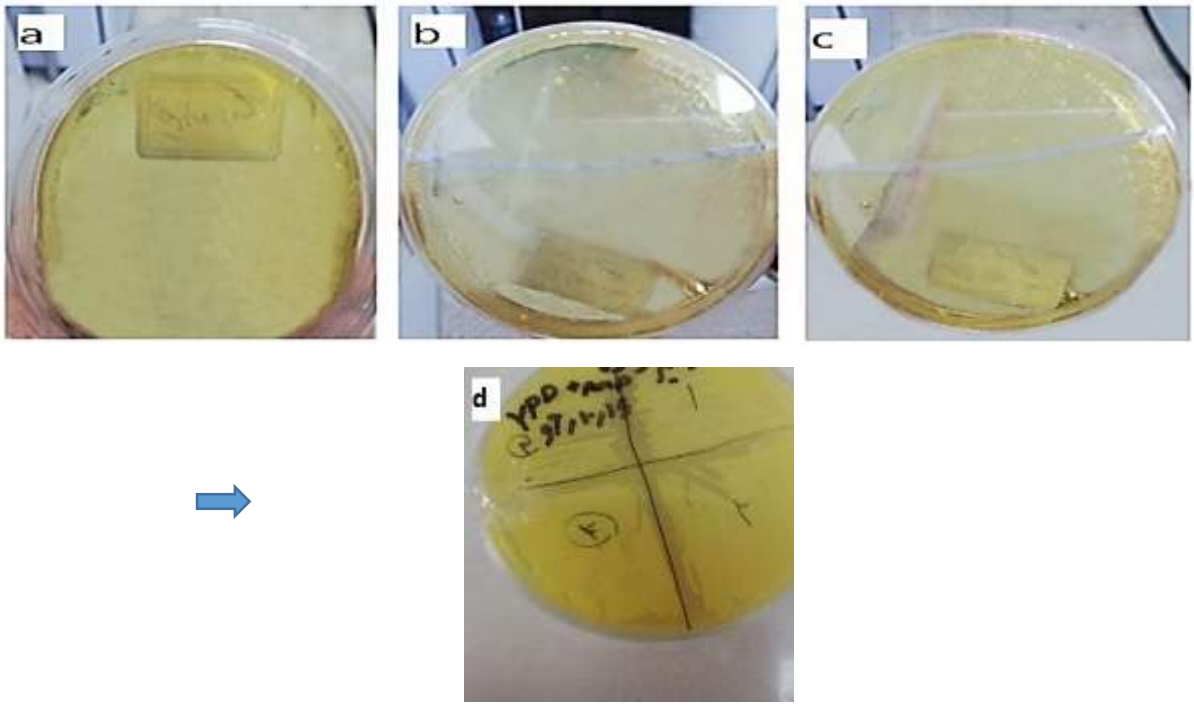


Figure 6. Gene transfer to *Pichia pastoris* strain GS115 by electroporation method a) negative control include cells without target genes and culture medium containing antibiotics b) positive control in this test, 300 microliters of *Pichia pastoris* cell containing target gene was used and incubated in the presence of Zeocin antibiotic for 5 days C) positive control in this test, 280 microliters of *Pichia pastoris* cell containing target gene. Due to the presence of zeocin antibiotic resistance gene, *Pichia pastoris* yeast cells grew in YPD culture medium D) from single colonies in a plate containing YPD culture medium containing zeocin antibiotic matrix was given

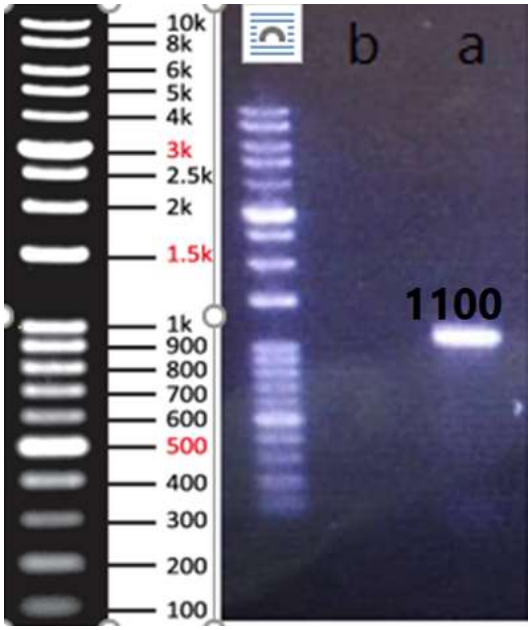


Figure 7. Electrophoresis after mfp3-GvpA gene transfer to *Pichia pastoris*; the colonies grown in the positive control plate were used to evaluate the presence of the gene in the PCR test. shows DNA ladder a) mfp3-GvpA gene observed in 1100K b) mfp3-GvpA gene negative control.

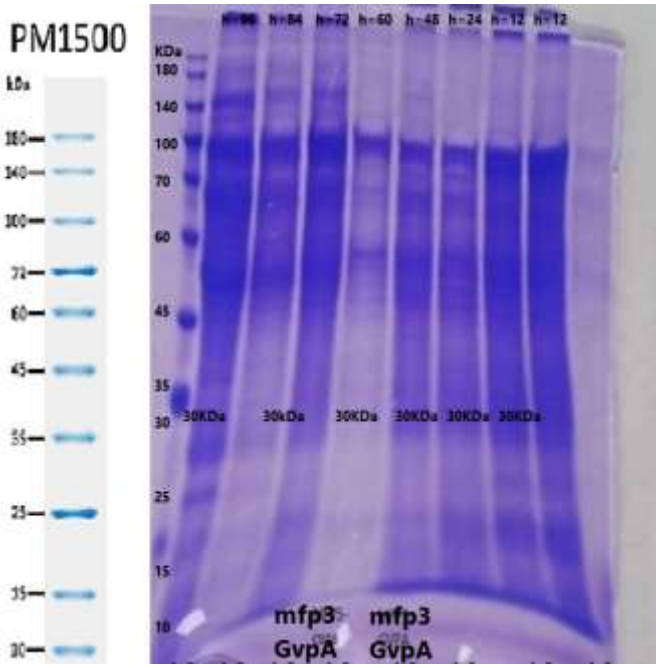


Figure 8. SDS PAGE of mfp3-GvpA protein to show protein expression in *Pichia pastoris* mfp3-GvpA protein was expressed along with other *Pichia pastoris* proteins on different culture days. In this image, the band observed in the range of 30 kDa, which was seen in all hours of sampling from 36, 60, 72, and 96 hours, indicating the possible presence of the mfp3-GvpA protein. Also, the presence of bands in non-specialized sites indicates the expression of *Pichia pastoris* proteins.

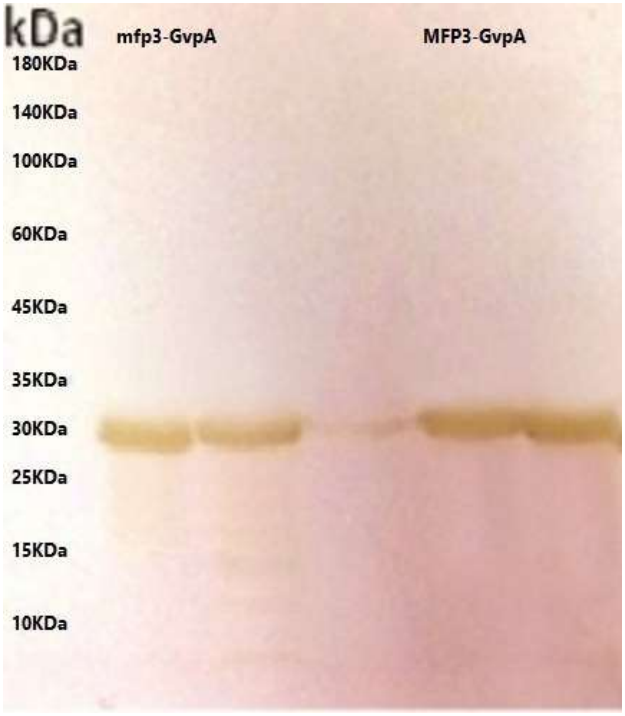
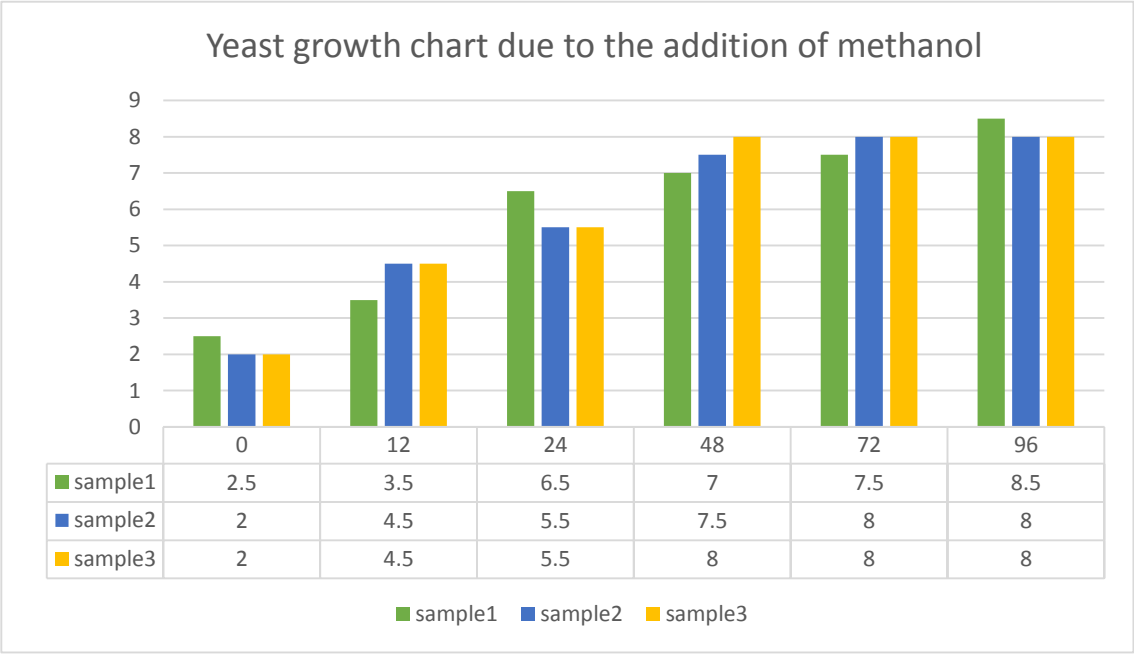


Figure 9. Evaluation of the presence of mfp3-GvpA protein by western blot test; the target protein band was observed in the western blot test with a size of about 30 kDa



	0	12	24	48	72	96
sample1	2.5	3.5	6.5	7	7.5	8.5
sample2	2	4.5	5.5	7.5	8	8
sample3	2	4.5	5.5	8	8	8

Figure10. Growth rate Yeast chart at different hours Add methanol

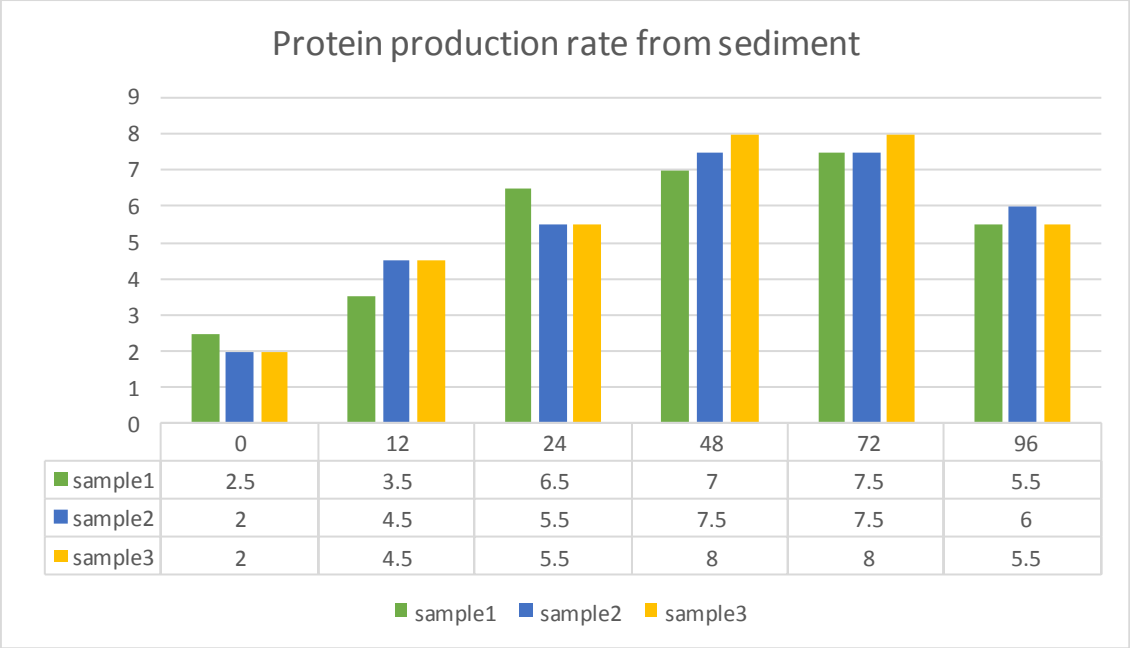


Figure 11. The rate of protein production using residual sediment from yeast cells

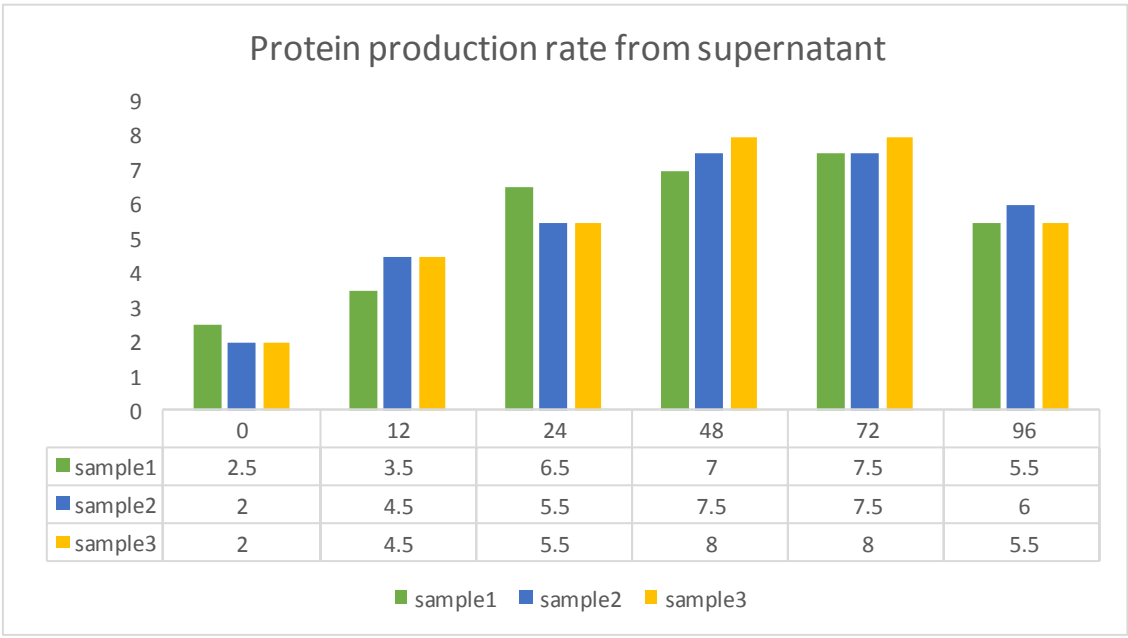


Figure12.The rate of protein production using supernatants given that Vector *pPICZA* is an Intracellular secretion vector . The possibility of protein secretion to the out of cell is very low. This test was performed to prove that the vector is Intracellular secretion .

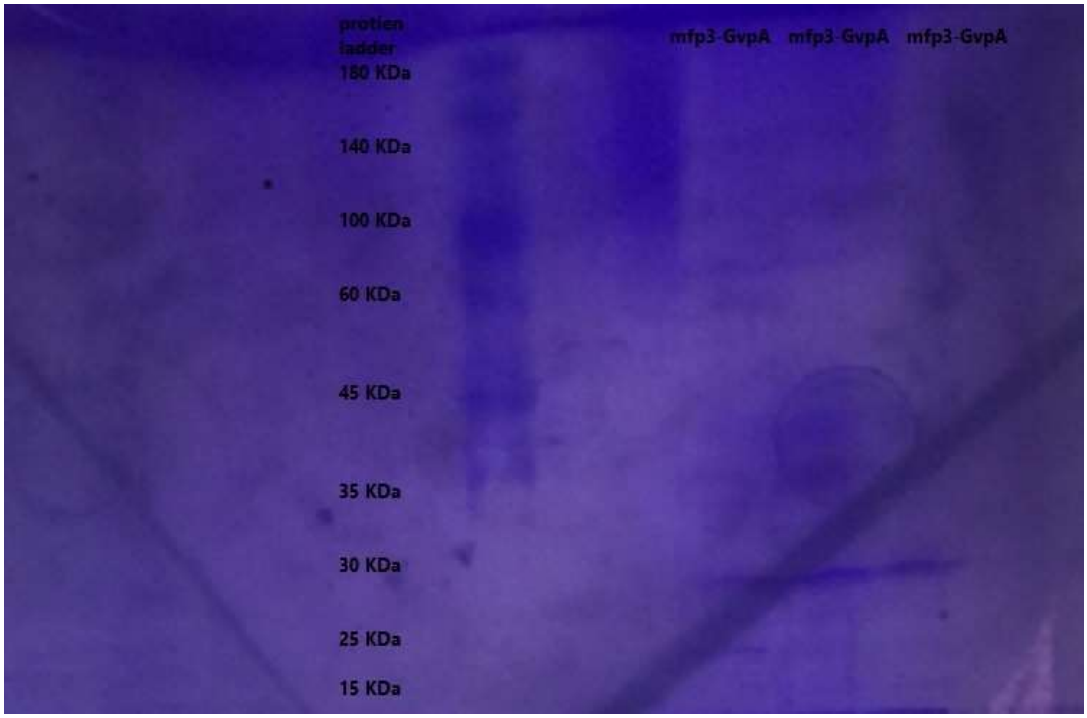


Figure 13. Perform a SDS PAGE test to see a single band that proves the presence of pure protein (MFP-3GVPA) at 30 KDa position, indicating the proper purification of mfp-3-GvpA protein.

4. Discussion

Recombinant protein production was performed using mfp-5 in bacterial host cells (Sun and Waite, 2005; Wang et al., 2019). 6 µg of mfp-1 protein was purified per liter of culture medium and used to produce cell adhesives. The results were better than other cell adhesives, such as cell tak (Hwang et al., 2005).

Mfp-3 protein was used to make a new silicone fiber adhesive that had a high adhesion to dry and wet surfaces but was very difficult and expensive to produce on a large scale (Lin et al., 2007). Mfp-3 protein was examined at different pHs. It was used in the coating of dental implants, but it was found that this recombinant protein had little resistance at the surface of mica, and at pH 5.5. Mfp3 protein was produced by di-tyrosine cross-linking, which was activated despite light rays, but this protein had a toxic effect on cell growth (Yu et al., 2013).

Bio adhesive was produced using mfp-5 dimer. This adhesive had better resistance than the mfp-5 monomer (Jeon et al., 2015).

The adhesion of most of the adhesives produced at low pH was investigated in this study. Overnight protein treatment in the presence of acetic acid at pH 3.5 and 5.5 had a significant effect on protein adhesion and strength. This study showed how cationic ions could increase the strength of mfp-5 to the surface of mica. In nature, mfp-6 has an antioxidant role and provides a good substrate for better adhesion than mfp-5 to the surface. In this study, hydronium ion was used artificially and had a good effect on mfp-5 adhesion (Kan et al., 2014).

Cross-joints in the bioadhesive structure were investigated, indicating increased adhesion and adhesive survival. Among the natural compounds that can be used as cross-linking are compounds that bind to the proteins of primary bacteria and Escherichia coli. The mfp-3-CsgA recombinant protein was able to form a stable β-structure in aqueous solution so that mfp would be present in the CsgA amyloid regions in the center. Successful expression of mussel foot adhesive proteins was evaluated with CsgA. Then, by combining oyster foot proteins with Escherichia coli CsgA amyloid fiber subunit, an

attempt was made to improve the quality and quantity of adhesion of these proteins (Zhong et al., 2014).

Biological wood glue production was performed using recombinant mfps. This adhesive was free of chemicals and very practical (Ferdosian et al., 2017). Zinc and copper were used to increase the adhesion of mfp-5 and mfp-3 proteins. Relatively stronger polymers were produced, and the presence of the amino acid cysteine was shown to improve the adhesion (Forooshani and Lee, 2017).

In this study, the chimeric protein mfp-3-GvpA was expressed in the host cell *Pichia pastoris*, and the amount of protein produced was much higher than that of the bacterial host. The adhesive protein produced was purified. Because previous research has used Ecoli-derived csga curli proteins as a pathogenic bacterium, this study used the Curli protein sequence, which is derived from a non-pathogenic bacterium (Archaebacterium) and can therefore be used, expected not to cause allergies in humans.

Due to the success of the present study in the expression and purification of mfp-3-GvpA adhesive proteins in *Pichia pastoris* yeast cells, performed for the first time in this host, it is recommended that future research focus on these methods to produce protein in larger and higher volumes. Because absorption chromatography was used in this study to purify proteins, it is possible that some proteins will still adhere to nickel particles as they leave the column. Therefore, it is suggested that other purification methods such as ion exchange or HPLC be used to achieve higher concentrations of recombinant protein. The production of recombinant proteins mfp3-GvpA in eukaryotic cells such as CHO or insect cells is also suggested. To evaluate the adhesion of these proteins, the use of living tissues such as cell culture or scratches in mice or rats is also recommended. It is also recommended to get more protein Instead of a vector *pPICZA* use another vector that has a sequence of protein secretion signals out of the cell. Or use the sequence of secretory signals in your gene sequence, because the secretion of protein into the culture medium increases the lifespan of yeast and thus increases the production of the desired protein. Perhaps with this method, the desired protein can be produced on a large scale and in bioreactors with continues method.

Conclusion

The results of the study show that the recombinant adhesive protein mfp3-GvpA is a very good candidate for the production of bio adhesives. It is well expressed in eukaryotic cells of *Pichia pastoris* and does not require the addition of the enzyme tyrosine for expression. This protein can be very cost-effective in large-scale productions.

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Refereces

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