

# Construction of *Saccharomyces cerevisiae* KEX2-650 gene expression vector and its introduction into *Escherichia coli* DH5 $\alpha$

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**Abstract.** Adibah DI, Fuad AM, Budiarti S, Pratiwi RD. 2022. Construction of *Saccharomyces cerevisiae* KEX2-650 gene expression vector and its introduction into *Escherichia coli* DH5 $\alpha$ . *Biodiversitas* 23: 4289-4296. Kex2 (EC 3.4.21.61) is a serine protease that binds Ca<sup>2+</sup> molecules and naturally found intracellularly as a transmembrane protein. Kex2 has a unique function, the conversion process of recombinant protein precursor into mature protein. Kex2 from *Saccharomyces cerevisiae* has similar functions also with furin in mammals (about 47% of sequence similarity). To gain advantage, extracellular Kex2 would be highly favorable for this process. This study aimed to construct recombinant Kex2 that could be produced extracellularly in *Pichia pastoris* host through pD902-KEX2-699 vector (synthetic) with FLAG-tag and 6 His-tag by removing most of C-terminal region, including transmembrane domain (TMD) from KEX2 gene sequence. Constructed KEX2 is the KEX2-650 variant with TMD and cytoplasmic domain deletion. The recombinant plasmid was constructed through site-directed mutagenesis using FP-Kex2-699 and RP-Kex2-650 primers, including the *Bam*HI site for plasmid religation. PCR site-directed mutagenesis produces an amplicon DNA with an expected length of 5551 bp. After restriction (*Bam*HI) and religation, the plasmid was reintroduced into *Escherichia coli* DH5 $\alpha$  and obtained 16 colonies. Verification PCR target gene showed that clones number 9 produced an amplicon of the expected length (646 bp). DNA sequencing analysis confirmed that TMD was removed from the gene construct to form the KEX2-650 construct.

**Keywords:** *Escherichia coli* DH5 $\alpha$ , KEX2 gene, site-directed mutagenesis, transmembrane domain

## INTRODUCTION

The *Saccharomyces cerevisiae* Kex2 ATCC 204508/S288c (EC 3.4.21.61) is a protease from the S8 (subtilisin-like peptidase) family and size of about 814 amino acids, deposited in UniPort P13134-1. Kex2 is naturally, intracellularly, especially found in the endoplasmic reticulum and trans-Golgi membrane (Mizuno et al. 1988). Kex2 plays a role in the release of active polypeptides from a precursor protein by cutting the polypeptide chain at specific sites that contains basic amino acid residue pairs of Lys-Arg or Arg-Arg (Mizuno et al. 1988; Bresnahan et al. 1990). Kex2 has post-translational modification characteristics in disulfide bonds and glycosylation, which makes Kex2 protein form a functional structure (Brenner and Fuller 1992). Kex2 is also found in *Pichia pastoris* (UniPort Q5J881) in one family, S8 and it has a similarity of 46.42% with *S. cerevisiae* Kex2. The roles both of *S. cerevisiae* Kex2 and *P. pastoris* Kex2 are to process mating- $\alpha$  factors, remove peptides signal from pre-proteins and secrete mature proteins (Mizuno et al. 1988; Manfredi et al. 2016).

Recombinant Kex2 is widely used in research developments and applications such as in processing preproprotein into mature protein and as a recombinant protein cleavage site to improve the secretory level (Yang

et al. 2013; Zheng et al. 2016; Sun et al. 2019). An example of its application is the maturation of proinsulin glargine into mature glargine molecules, as a protein cleavage site for recombinant bovine lactoferrampin – lactoferricin,  $\alpha$ -galactosidases, and recombinant hG-CSF (Tang et al. 2012; Sreenivas et al. 2015; Zheng et al. 2016; Aggarwal and Mishra 2021; Caballero-Pérez et al. 2021). Kex2 is widely used, especially in the health sector, because Kex2 is homologous to the processing enzyme in mammals (humans), namely furin (EC 3.4.21.75). Furin is also from the S8 family, deposited in UniPort access number P09958-1. Furthermore, Kex2 functions similarly to furin as a pro-hormone processor. Specifically, furin plays a role in cleaving pro- $\beta$ -NGF to produce bioactive NGF, cleaving protein precursor protein, and IGF-1 at the specific site (Bresnahan et al. 1990; Van de Ven et al. 1991). The overall structure of *S. cerevisiae* Kex2 has a similarity of about 41.52% with furin in *Homo sapiens*. It has a hydrophilic TMD and highly charged cytoplasmic tail with two Tyr residues at a distance of 11 residues from TMD. However, the *P. pastoris* Kex2 has only 37.34% of similarity with furin. Higher similarity causes *S. cerevisiae* Kex2 as the selected enzyme to process insulin analog precursors into mature insulin (Van de Ven et al. 1991).

However, to further maximize the unique function of Kex2 in the conversion process such as in the maturation of

the glargine precursor, it is necessary to change the Kex2 expression system to be extracellular. To make recombinant proteins extracellularly expressed, genetic engineering is required primarily by deleting the TMD and C-terminal tail region to increase the amount of Kex2 secretion without affecting protease activity. As with *S. cerevisiae* host cell secretion, Kex2-660 and Kex2-679 have been reported to produce 10 times higher secretion than Kex2-614 (Brenner and Fuller 1992).

The TMD deletion strategy uses vectors constructed in parallel at the BRIN Research Center, namely pD902-KEX2-699 (synthetic) which still has the TMD and has the addition of a recombinant fusion protein FLAG-tag and 6 His-tag. The designed variant of the endopeptidase Kex2 was named KEX2-650 (without TMD). This study aims to construct the truncated variant of the KEX2 gene by optimizing the modification strategy for deletion of the C-terminal region including TMD, using the site-directed mutagenesis method, then introducing it into *Escherichia coli* DH5 $\alpha$  cells for recombinant vector multiplication.

## MATERIALS AND METHODS

### Construction of expression vector of Kex2 endopeptidase variant through PCR site-directed mutagenesis

The plasmid template for PCR-directed mutagenesis was sourced from pD902-KEX2-699 (customized and purchased from ATUM, USA), in which the KEX2-699 was referred to only contained the first 699 amino acids of 814 amino acids in the whole sequence of *S. cerevisiae* Kex2 (UniProt P13134). The first step is that the plasmid template was isolated from *E. coli* DH5 $\alpha$  pD902-KEX2-699 by the alkaline lysis method as described earlier by Hardianto et al. (2015). The size of isolated plasmids was checked through restriction with 10 U/ $\mu$ L of *Sac*I restriction enzyme. Analysis on 0.7% agarose gel electrophoresis was conducted with 70 V for 40 min. The confirmed size of plasmid pD902-KEX2-699 was used as a PCR template. The PCR mixture was prepared following the standard protocol of the KOD-Plus-Mutagenesis Kit (Toyobo, Japan). The primary pairs of directed mutagenesis were FP-Kex2-699 (5'-TAC GGA TCC GAT TAC AAA GAC GAT GAT AAA AGC GCT CAT-3') and RP-Kex2-650 (5'-TGT GGA TCC GAT TGC GGA AGT TTC TAC TCC AAT TGA AAT CGA AG-3'). The PCR was performed in a total volume 50  $\mu$ L, that contained 10 $\times$  iPCR buffer 5  $\mu$ L; 2 mM dNTPs 5  $\mu$ L; 25 mM MgSO $_4$  3  $\mu$ L; 20  $\mu$ M primer FP-Kex2-699 0.5  $\mu$ L; 20  $\mu$ M primer RP-Kex2-650 0.5  $\mu$ L; 1 U/ $\mu$ L KOD-Plus-DNA polymerase 1  $\mu$ L; nuclease-free water 33  $\mu$ L; plasmid pD902-KEX2-650 2  $\mu$ L. PCR verification target gene also includes a positive control PCR reaction with the template using pD902-KEX2-699. PCR amplification was performed according to the protocols of the KOD Plus Mutagenesis Kit (Toyobo, Japan) set as pre-denaturation 2 min at 94°C, then 6 cycles denaturation 10 sec at 98°C, annealing 30 sec at 59.5°C, final extension 90 sec at 68°C, and followed by the hold at 4°C. The PCR products were analyzed with the

electrophoresis of 0.7% agarose gel at 70 V for 40 min, and observation were conducted in Gel Documentation (UV light). The PCR product was added with 20 U/ $\mu$ L of *Dpn*I restriction enzyme, incubated for 1 h at 37°C and purified through gel isolation with Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The analysis was performed on 0.7% agarose gel electrophoresis with 70 V for 40 min, and observation was conducted in Gel Documentation (UV light). Then the samples were digested with 10 U/ $\mu$ L *Bam*HI restriction enzyme for 12 h at 37°C. Samples were analyzed by 0.7% agarose gel electrophoresis at 70 V for 40 min. Then, the restricted sample was purified with Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan).

### Introduction of the expression vector of Kex2 endopeptidase variant into *Escherichia coli* DH5 $\alpha$

Plasmid ligation (pD902-KEX2-650) with T4 polynucleotide kinase obeyed the standard protocol of the KOD Plus Mutagenesis Kit (Toyobo, Japan) and incubated at 4°C for 12 h. Transformation product site-directed mutagenesis recombinant plasmid using heat shock treatment (Bachman 2013). Prepared *E. coli* DH5 $\alpha$  competent cells cultured on Luria-Bertani (LB) medium until the OD $_{600}$  0.25-0.3 and centrifuged at 7,000 rpm at 4°C for 10 min. The pellet was resuspended with CaCl $_2$  + 15% glycerol. Competent cells were taken as much as 50  $\mu$ L and added with 7.5  $\mu$ L site-directed mutagenesis recombinant plasmid, then incubated in the ice for 30 min. Heat shock treatment was conducted by placing the transformation mixture at 42°C for 1 min, then keeping it in the ice for 5 min. After heat shock treatment, the sample was added with 100  $\mu$ L of liquid LB medium and incubated at 37°C, 200 rpm for 1 h. One hundred fifty microliter of sample transformation mixture was spread on LS-LB agar plate with the Zeocin<sup>TM</sup> (Invitrogen, USA) antibiotic 25  $\mu$ g/mL, incubated for 12 h at 37°C. The introduction process included a positive control (*E. coli* DH5 $\alpha$  competent cells on LS-LB agar medium without antibiotics) and negative control (*E. coli* DH5 $\alpha$  competent cells on LS-LB agar medium with Zeocin<sup>TM</sup> antibiotic 25  $\mu$ g/mL).

### Verification of transformants through PCR analysis of target gene

Colonies that grew with *E. coli* morphology according to replica plating were then grown on a liquid LS-LB medium with 25  $\mu$ g/mL of Zeocin<sup>TM</sup> antibiotic to isolation the recombinant plasmid (Lessard 2013). Plasmid isolation was conducted through the alkaline lysis method (Hardianto et al. 2015). The plasmid isolation results were visualized using the 0.7% agarose electrophoresis technique at 70 V for 40 min. The plasmid isolated from each selected transformant colony was used as a template for verification. PCR verification of target gene using primer pairs of target genes FP-Kex2-465 (5'-GAT AGA AAT GTC TAA AAC TTG GGA AAA TGT TAA TGC TCA AAC-3') and RP-6H+3 (5'-TTA GTG GTG ATG ATG GTG ATG AGC G-3'). The PCR was performed in a total volume of 25  $\mu$ L that contained 5 $\times$  Green GoTaq®

Flexi buffer 2.5  $\mu$ L; 25 mM  $MgCl_2$  2.5  $\mu$ L; 10 mM PCR nucleotide mix 1.5  $\mu$ L; 20  $\mu$ M primer FP-Kex2-465 0.5  $\mu$ L; 20  $\mu$ M primer RP-6H+3 0.5  $\mu$ L; 5 U/ $\mu$ L GoTaq® Flexi DNA polymerase 0.25  $\mu$ L; nuclease-free water 16.25  $\mu$ L; plasmid pD902-KEX2-650 1  $\mu$ L. PCR verification target gene also included a positive control PCR reaction with the template using pD902-KEX2-699. PCR amplification was carried out according to the protocols of the Green GoTaq® Flexi Reaction (Promega, USA) set as pre-denaturation of 95°C for 5 min was followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 30 sec, extension at 72°C for 90 sec, final extension at 72°C for 5 min, and followed by the hold at 4°C. The PCR products were analyzed with the electrophoresis of 0.7% agarose gel at 70 V for 40 min, and observations were conducted in Gel Documentation (UV light).

### Verification of transformants through DNA sequencing analysis

Colonies were verified based on the size of the target gene, and then sample preparation was conducted for DNA sequencing. The DNA sequencing analysis was carried out in stages using specific primers FP-Kex2-10 (5'- GAA TTC AAA ACG ATG AAA GTT AGA AAG TAT ATC ACA TTG TGC-3'), FP-Kex2-236 (5'- GAG TCG GTG TTG GTT ATA ACG CAA AAA TCA-3'), and FP-Kex2-465 (5'-GAT AGA AAT GTC TAA AAC TTG GGA AAA TGT TAA TGC TCA AAC-3'). The next step is nucleotide BLAST using the NCBI website <http://www.ncbi.nlm.nih.gov/blast>. Nucleotide BLAST analysis on the results of the sample DNA sequencing, compared with the reference sequence (KEX2-699).

## RESULTS AND DISCUSSION

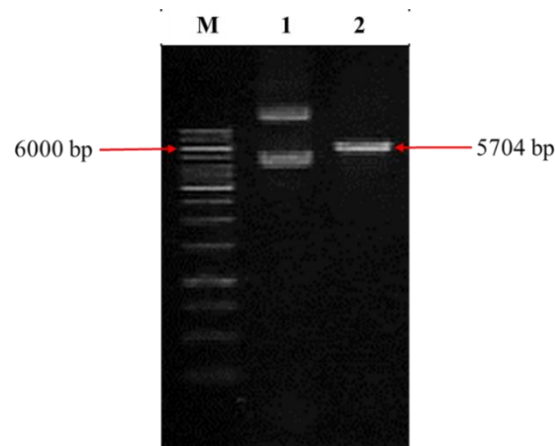
### Construction of expression vector of Kex2 endopeptidase variant through PCR site-directed mutagenesis

The construction was carried out using PCR site-directed mutagenesis technique, using a recombinant plasmid template pD902-KEX2-699 isolated from the recombinant *E. coli* DH5 $\alpha$  pD902-KEX2-699. The pD902-KEX2-699 plasmid isolation used the alkaline lysis method. Analysis of the isolated plasmid pD902-KEX2-699 showed that DNA bands were visualized as circular conformation. Plasmid pD902-KEX2-699, which was digested with the *Sac*I restriction enzyme, aimed to confirm the size of the linear plasmid. The results showed that the size of the linear, conformational DNA band was about 5704 bp

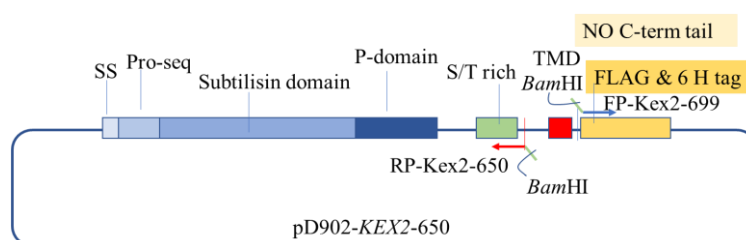
(Figure 1). The size of the pD902-KEX2-699 plasmid was 5704 bp, which was a construction of the KEX2 endopeptidase expression vector which still had a TMD.

Then, the isolated plasmid pD902-KEX2-699 was used as a PCR site-directed mutagenesis template to construct the KEX2-650 structure. Construction of KEX2-650 through PCR site-directed mutagenesis was performed using primary pairs FP-Kex2-699 and RP-Kex2-650. Primer FP-Kex2-699 amplified the frontal sequence of the FLAG-tag, and primer RP-Kex2-650 amplified the frontal sequence of the TMD (Figure 2).

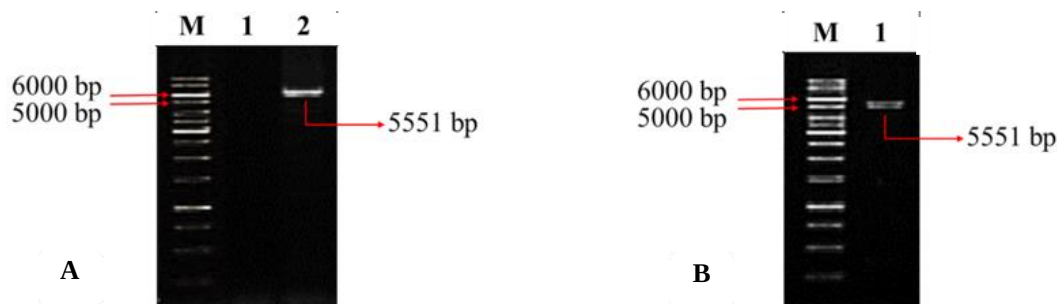
PCR site-directed mutagenesis used primer pairs FP-Kex2-699 and RP-Kex2-650, the optimal temperature was obtained at 59.5°C. Results from PCR site-directed mutagenesis showed that pD902-KEX2-650 visualized a DNA band measuring about 5551 bp (Figure 3.A). The size of the pD902-KEX2-650 visualization was in line with theoretical calculations, and the calculation of PCR site-directed mutagenesis used primer pairs FP-Kex2-699 and RP-Kex2-650, which worked without amplifying the TMD site of 153 bp. The PCR mutagenesis product was added with *Dpn*I enzyme and incubated at 37°C for 1 h. The PCR product was further purified by gel isolation technique using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The agarose gel concentration used to purify was 0.7% with 70 V for 40 min. The results of sample purification with the gel isolation technique showed that each visualized construction of a single DNA band was clean of impurities, relatively thick, and had the same size as before purification (Figure 3.B).



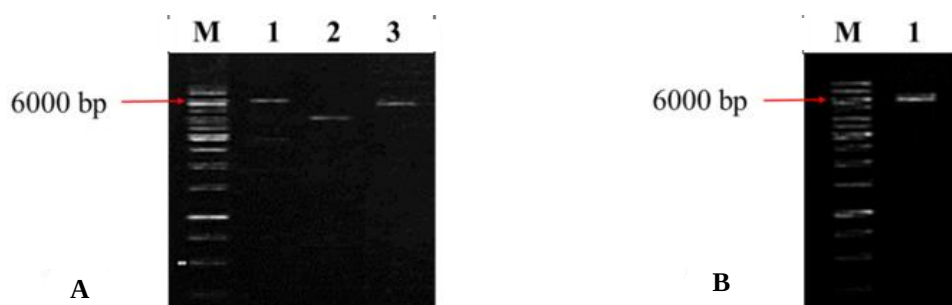
**Figure 1.** Electroforegram of pD902-KEX2-699 plasmid isolation. Line M = 1 kb DNA marker, line 1 = pD902-KEX2-699 plasmid, line 2 = pD902-KEX2-699 plasmid digested with *Sac*I



**Figure 2.** pD902-KEX2-650 site-directed mutagenesis PCR construction scheme



**Figure 3.** Electroforegram of amplicon for site-directed mutagenesis pD902-KEX2-650. Line M = 1 kb DNA marker, line 1 = KEX2-650 PCR reaction negative control, line 2 = pD902-KEX2-650 amplicon product (A). Electroforegram of amplicon for site-directed mutagenesis pD902-KEX2-650 gel purification. Line M = 1 kb DNA marker, line 1 = purified amplicon of pD902-KEX2-650 (B)



**Figure 4.** Electroforegram of pD902-KEX2-650 plasmid digested with *Bam*HI restriction enzyme. Line M = 1 kb DNA marker, line 1 = control plasmid (pPICZα), line 2 = digested reaction control (pPICZα) with *Bam*HI, line 3 = pD902-KEX2-650 plasmid digested with *Bam*HI (A). Electroforegram of amplicon gel purification for pD902-KEX2-650 digested with *Bam*HI. Line M = 1 kb DNA marker, line 1 = purification of pD902-KEX2-650 digested with *Bam*HI (B)

Afterward, the product of PCR site-directed mutagenesis (pD902-KEX2-650), which was purified, was restricted with the restriction enzyme *Bam*HI by including a restriction control. Restriction control using pPICZα plasmid visualized a linear DNA band, proved that the restriction enzyme *Bam*HI had worked optimally. It also represents the effectiveness of the restriction process of *Bam*HI on the pD902-KEX2-650 (Figure 4.A). pD902-KEX2-650, which had been restricted with *Bam*HI, was purified using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). The purification results showed that each construction visualized a single DNA band, and the DNA band was clean of impurities and relatively thick (Figure 4.B).

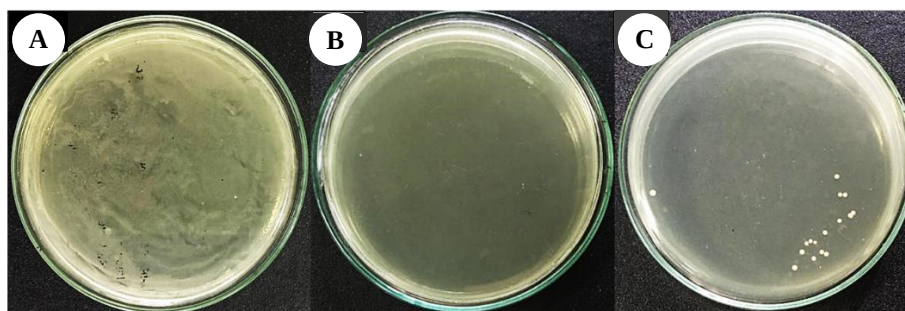
#### Introduction of the expression vector of Kex2 endopeptidase variant into *Escherichia coli* DH5α

Plasmid pD902-KEX2-650 had been digested with the *Bam*HI restriction enzyme and purified, then religated and introduced into *E. coli* DH5α cells, which was carried out simultaneously with transformation control. The results showed that the positive control in *E. coli* DH5α competent cells could maintain high viability in the medium without antibiotics (Figure 5.A). While in the negative control, *E. coli* DH5α cells could not grow in the medium containing the Zeocin™ antibiotic 25 µg/mL (Figure 5.B). The introduction of the expression vector pD902-KEX2-650 obtained 16 transformant single colonies (Figure 5.C).

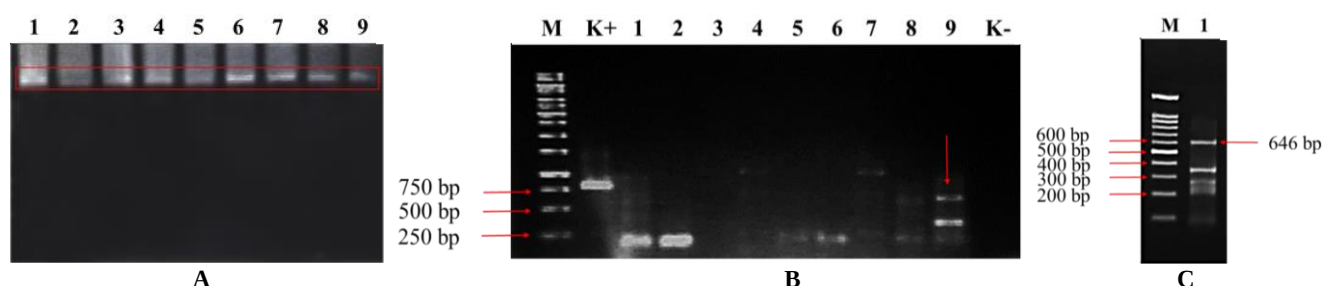
#### Verification of transformants through PCR analysis of target gene

Several single colonies of transformants that grew fast (12 h incubation), had suitable morphology and grew quite large on LS-LB media added with 25 µg/mL Zeocin™ antibiotic, were selected for plasmid isolation and analysis. A total of 9 single transformant colonies were selected for further plasmid isolation using the base lysis method. The results of plasmid isolation showed the same DNA bands at high enough concentrations (Figure 6.A).

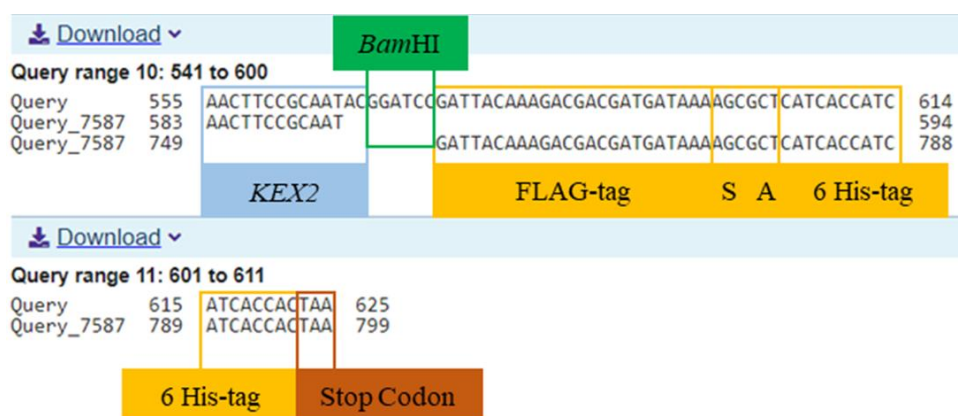
The selected plasmids from isolated transformants were verified by PCR analysis of the target gene using primer pairs FP-Kex2-465 and RP-6H+3. The PCR results of the target gene showed positive control using the plasmid pD902-KEX2-699 as a template and visualized the DNA band at about 799 bp. The negative control used nuclease-free water as a template. The result showed that there was no visualization of the DNA band, meaning that the reaction and PCR process of the target gene were running optimally without any contaminants (Figure 6.B). The sample plasmid pD902-KEX2-650 colony number 9 visualized DNA bands at the appropriate size, which was about 646 bp (Figures 6.B and 6.C). Samples from colonies 1, 2, 4, 5, 6, 7 and 8 which visualized DNA bands were not at the target size. While, sample colony number 3 did not visualize DNA bands (Figure 6.B).



**Figure 5.** Plate of transformation culture. Plate of positive control transformation culture (A), plate of negative control transformation culture (B), plate of pD902-*KEX2*-650 plasmid transformation culture (C)



**Figure 6.** A. Electroforegram of pD902-*KEX2*-650 plasmid isolation. B. Electroforegram of amplicon PCR gene target construct *KEX2*-650 at selection plasmid. Line M = 1 kb DNA marker (1  $\mu$ L), line K+ = PCR reaction positive control, line 1 = colony 1, line 2 = colony 2, line 3 = colony 3, line 4 = colony 4, line 5 = colony 5, line 6 = colony 6, line 7 = colony 7, line 8 = colony 8, line 9 = colony 9, line K- = PCR reaction negative control. C. Electroforegram of amplicon PCR gene target construct *KEX2*-650 at pD902-*KEX2*-650 plasmid number 9 for DNA sequencing. Line M = 100 bp DNA marker, line 1 = pD902-*KEX2*-650 plasmid number 9



**Figure 7.** Alignment DNA sequencing analysis of the selected *KEX2*-650 construct confirmed TMD had been deleted

### Verification of transformants through DNA sequencing analysis and BLAST

After plasmid pD902-*KEX2*-650 number 9 was verified by PCR of the target gene, DNA sequencing was carried out by pre-treatment purification of the isolation of the target gene at a size of 646 bp. The DNA sequencing analysis results showed that the chromatogram pattern was quite good and could be read up to 625 bp covering the tip of the *KEX2* gene up to the 6 His-tag section. The DNA sequencing results of pD902-*KEX2*-650 samples were then carried out nucleotide BLAST with pD902-*KEX2*-699 (native). The BLAST results showed that the construction of *KEX2*-650 was successfully carried out, which was

proved by the successful deletion of the TMD sites (Figure 7).

### Discussion

The plasmid sequence pD902-*KEX2*-699 used as a template was is a synthetic gene *KEX2*, with a sequence referring to *KEX2* from *S. cerevisiae* (EC 3.4.21.61). The synthetic Kex2 protein sequence was 100% similar, but the DNA sequence only had a similarity of 75.97%. The *KEX2*syn gene was modified by adding the FLAG-tag, serine, alanine 6 His-tag and a stop codon at the 3'-terminal of the *KEX2* gene. The FLAG-tag and the 6 His-tag in the construct play a role in detecting and purifying

recombinant proteins to facilitate further research (Zhao et al. 2013; Han et al. 2017). Serine and alanine acts as linkers that encode a unique restriction site for fusion with the *KEX2syn* gene. Specifically, the stop codon is located after the 6 His-tag sequences, because the 3'-terminal of the target gene diffuses directly with the FLAG-tag and 6 His-tag sequences. The selection of pD902 as a cloning vector was because it has a pro-peptide, which is already in some host cells' protein expression, such as *P. pastoris*. In addition, pD902 has a PAOX1 component which is an alcohol oxidase 1 promoter gene that acts on the methanol assimilation pathway to catalyze the oxidation of formaldehyde (Shen et al. 2016).

The site-directed mutagenesis method has been widely used in genetic engineering without re-cloning the recombinant vector (Hallak et al. 2017; Rodiansyah et al. 2019). This procedure offers advantages over conventional cloning, which has a higher failure probability and PCR material consumption (Trehan et al. 2016). In previous studies, modification of recombinant vectors was applied to a minimum alteration, for instance, the substitution of a single base pair or deletion of a specific sequence by adding three base pairs of the stop codon (Hariyatun et al. 2014; Rodiansyah et al. 2019). However in our study, a more extended sequence containing 153 bp, has been successfully deleted using the site-directed mutagenesis. Suitability of the PCR condition and quality of enzymes is the crucial factor in site-directed mutagenesis (Castorena-Torres et al. 2016). Moreover, a relatively small plasmid (~6000 bp) was used in the recent study. For significantly longer vector or plasmid, which is more than 10,000 bp, a modified site-directed mutagenesis procedure might be needed, such as REPLACR mutagenesis, Quick Change mutagenesis, URMAC, or LFEAP (Munteanu et al. 2012; Trehan et al. 2016; Hallak et al. 2017; Zeng et al. 2018). By exploring the site-directed mutagenesis method, variations of protein gene constructs with multiple domains, such as Kex2 were implemented. This step is very important to accelerate the study of the effect of these domains on protein expression and bioactivity levels.

TMD has been known to influence the level of protein expression in all organisms, including yeast (Singh and Mittal 2016). It synchronizes with membrane protein or known as hydrophobic matching, which depends on the length of TMD and lipid bilayer thickness of the membrane protein (Kondrashov et al. 2022). Therefore, TMD deletion has been known to increase the soluble recombinant protein level (Brenner and Fuller 1992). *KEX2-650* endopeptidase variant (without a TMD) was constructed by PCR site-directed mutagenesis method. The primer pairs used in directed mutagenesis were specifically designed to amplify the frontal sequence of the FLAG-tag (FP-Kex2-699) and to amplify the frontal sequence of the TMD (RP-Kex2-650), thus the TMD is not amplified. The two PCR mutagenesis primer sequences used the addition of restriction sites *Bam*HI (5'-GGATCC-3') to facilitate the relegation process and increase the chances of successful site-directional mutagenesis (Castorena-Torres et al. 2016). The optimization of PCR annealing temperature aims to optimize the PCR reaction process, by attaching primers to

the target DNA and producing PCR products in optimal quantities (Kumari and Amaravathi 2016). The addition of the *Dpn*I enzyme to the product of PCR site-directed mutagenesis has the function to cut plasmid DNA which is used as a PCR template (parental) especially on methylated adenine bases, but the PCR product of mutated DNA remains intact (Johnston et al. 2013). Purification of PCR product mutagenesis using gel isolation technique aims to obtain pD902-*KEX2-650* mutant plasmid according to the target size, which is free from DNA impurities or other compounds. Purification using the gel isolation technique was performed by trapping the target DNA on the membrane column via covalent bonds. The purified PCR product mutagenesis was digested with the *Bam*HI restriction enzyme aimed to make the 5' and 3'-terminal of the plasmid cohesive so that the relegation process was more accessible. Plasmid pPICZα was selected as a restriction control because plasmid pPICZα has a *Bam*HI cleavage site (Sallada et al. 2019). The sequence of the *Bam*HI restriction site on the plasmid pPICZα is the same as the restriction site on the pD902-*KEX2-650* construction. The restriction enzyme *Bam*HI recognizes specific sequence GGATCC at GATC sticky ends. It was reported that *Bam*HI activity depends on the existence of divalent metal, the amount of flanking base pairs, and the length of oligonucleotide substrate (Tong et al. 2014). The use of agarose gel with a concentration of 0.7% at the analysis plasmid, PCR product, and restriction product because agarose gel 0.7% can separate DNA targets in the molecular weight range of 0.8 – 10 kb (Sambrook and Russell 2001).

Restricted and purified PCR products were religated with ligation enzymes that catalyze the formation of phosphodiester bonds between 3'OH and 5'PO<sub>4</sub> in nucleic acid residues (Lohman et al. 2014). Religation aims to make the plasmid pD902-*KEX2-650* circular and can be introduced into *E. coli* DH5α cells. The ligation product was successfully transformed into *E. coli* DH5α cells and produced transformant colonies. It tended to maintain plasmid pD902-*KEX2-650* on selection media containing the Zeocin<sup>TM</sup> antibiotic, an antibiotic that breaks DNA double strands. Zeocin<sup>TM</sup> was selected as the antibiotic used in the transformant selection medium because the pD902 vector contains the M\_Zeocin-r\_ site, which has the *Sh ble* gene (*Streptoalloteichus hindustanus bleomycin*). The *Sh ble* gene plays the role of a selection marker that encodes Zeocin<sup>TM</sup> in antibiotic-resistant protein so that *E. coli* DH5α cells that grow on the selected medium were only *E. coli* DH5α cells containing the pD902 plasmid with the M\_Zeocin-r\_ site. LS-LB agar was chosen as the selection medium because the antibiotics used will be inactivated if they are in conditions with high acidity alkalinity (pH 7.5), and high salt concentrations (Lin et al. 2017). The positive control transformation of *E. coli* DH5α which grew well in a low salt medium and without Zeocin<sup>TM</sup> antibiotic, indicates that the *E. coli* DH5α cells used had a good growth ability as competent cells in the introduction of pD902-*KEX2-650*. In the negative transformation control, it was seen that *E. coli* DH5α did not grow in a low-salt medium with 25 ng/μL Zeocin<sup>TM</sup>

antibiotics. This shows that the *E. coli* DH5 $\alpha$  cells used were sensitive to Zeocin<sup>TM</sup> antibiotics, and Zeocin<sup>TM</sup> antibiotics with a concentration of 25 ng/ $\mu$ L could be used as selection antibiotics for *E. coli* DH5 $\alpha$  competent cells. The number of transformant colonies obtained was influenced by the efficiency of the transformation process (Rahimzadeh et al. 2016). In addition, Chan and colleagues explained that technical factors in transformations, i.e., volume ratios in resuspension, the volume of transformation aliquot, growth media and plasmid affect the transformation efficiency (Chan et al. 2013).

The transformant colony was selected on LS-LB selection media with the addition of the Zeocin<sup>TM</sup> antibiotic (25 ng/ $\mu$ L). The aim is to test and re-select growing colonies which contain the target plasmid only with a specific antibiotic (Haon et al. 2015). Colonies selection was carried out by observing the growing colonies' morphology and the speed of the colony's growth ability (12 h). Colonies with *E. coli* morphology and good growth ability, were isolated from plasmids through the alkaline lysis method for further research. The results of plasmid isolation showed that the plasmid DNA band was similar to circular conformation, and the concentration was sufficient.

Then the plasmids obtained from the selected transformant colonies, were verified through PCR of the target gene (*KEX2*-650). It aims to determine the success of directed mutagenesis through the size of the amplicon obtained from the PCR process using primer pairs of the target gene (FP-Kex2-699 and RP-6H+3) which focuses on the back of deleted gene construction. The visualization of amplicon size was as expected, 646 bp. The size of the *KEX2*-650 gene amplified the amino acids starting at position 465 to the 6 His-tag site with a deletion of the TMD site. The other transformant samples which did not visualize DNA bands at the target size probably happened because the religation and transformation processes were not optimal, resulting in transformant colonies containing gene target *KEX2*-650. Thus, an unspecific attachment of the primer on other similar sequences on the DNA template was found. The analysis of DNA sequencing results also showed that the TMD had been successfully deleted, and there was a restriction site in the form of *Bam*HI. It indicates that directed mutagenesis had succeeded in meeting the target. The results of the nucleotide BLAST of the sample sequence pD902-*KEX2*-650 with pD902-*KEX2*-699 (native) confirmed that the template sequence *KEX2*-699 has successfully deleted TMD by conducting a site-directed mutagenesis technique and the result of the deletion of the TMD is called *KEX2*-650.

In conclusion PCR site-directed mutagenesis process using the plasmid template pD902-*KEX2*-699 with specific primer pairs FP-Kex2-699 and RP-Kex2-650 has been successfully carried out. Results site-directed mutagenesis obtained a DNA amplicon product with the expected length of about 5551 bp (pD902-*KEX2*-650). The transformation results obtained 16 single colonies of transformants. PCR verification of the target gene using specific primers (FP-Kex2-465 and RP-6H+3) showed that pD902-*KEX2*-650 transformant colony number 9 was confirmed by the size of the appropriate amplicon. The resulting amplicon target

gene is about 646 bp, including the *KEX2* gene starting amino acids at position 465 to 6 His-tag, without TMD site. The results of DNA sequencing analysis of the selected *KEX2*-650 construction number 9 obtained a reasonably good chromatogram and can be read up to 625 bp. The nucleotide BLAST results on *KEX2*-650 alignment with *KEX2*-699 (reference sequence) confirmed that the transmembrane domain had been successfully deleted from the gene construct to form the *KEX2*-650 construct.

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