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Extracellular Expression of Recombinant Kex2 Protease in *Pichia pastoris* BG10

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Abstract: Kex2 is a ~67 kD protease in yeast and fungi that specifically digest at -Lys-Arg- or -Arg-Arg-. Because of the same cleaving site, Kex2 is the potential to be applied in protein engineering, for instance, for protein purification. In the recent study, the *Saccharomyces cerevisiae KEX2* gene was designed and constructed using pD902 and then integrated with the genome of *Pichia pastoris* BG10. Under *AOX1* promoter control, Kex2 was expressed with methanol induction and found in extracellular fractions. The recombinant Kex2 was successfully confirmed with a western blot using a monoclonal anti-His-tag antibody and LC/MS/MS.

Keywords: Kex2 protease; *AOX1* promoter; extracellular expression; methanol induction; *Pichia pastoris* BG10.

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1. Introduction

Kex2 is a ~67 kD protease, which is produced by yeast and has specific cleaving activity at amino acid sequence -Lys-Arg- or -Arg-Arg-. It is naturally used for endogenous protein secretion, pre-protein maturation, and the mating-α factors process [1–3]. Kex2 also plays a role in fungal infection in humans, caused by *Cryophonectria parasitica*, *Candida albicans*, and *C. glabrata* [4].

In recent years, protease enzymes have been commonly applied in recombinant protein purification, for instance, to separate proteins of interest from non-target protein fusion, e.g., tagging proteins [5]. However, the utilization of Kex2 for purification of the recombinant proteins has not been as popular as other proteases, such as TEV protease, thrombin, carboxypeptidase A and B, enterokinase (EK), DAPase, and factor Xa [6]. Kex2 has been applied for insulin glargine maturation and other protein such as hen egg white lysozyme purification *in vivo* through the co-expression process in *P. pastoris* [7–10].

Co-expression with various heterologous proteins or mutations in the gene encoding Kex2 in host cells has been reported [6,11]. However, this technique is considered a complex *in vivo* process, including expression optimization of the heterologous protein and the Kex2

itself [8]. In addition, the *in vivo* cleavage mechanism by Kex2 is undergone in the Golgi body leading to a more complicated mechanism compared with other proteases which are active in the cell membrane [12]. Kex2 cleavage site had been designed at the terminal carboxy of a cassette construction of an insulin precursor, which requires the Kex2 for the insulin precursor maturation *in vitro* [13]. Therefore, it needs to be expressed as a single recombinant protease and then used to purify other proteins.

In this study, the *KEX2* gene encodes 699 amino acids of Kex2 (namely *KEX2-699*, part of 814 amino acids Kex2 in UniPort P13134-1) from *S. cerevisiae* ATCC 204508/ S288c (EC 3.4.21.61) was constructed to vector pD902 and then expressed in *P. pastoris* BG10. It was successfully secreted to extracellular fraction and positively detected with a monoclonal anti-His-tag antibody blotted to 6×His-tag at the N-terminal of the Kex2. Moreover, a long fragment of the amino acid sequence of the recombinant Kex2 was aligned with the original sequence from *S. cerevisiae*.

2. Materials and Methods

2.1. Strain, vectors, media, and chemicals.

The *Escherichia coli* DH5α was used for routine cloning and propagation of plasmids. The *P. pastoris* BG10 wild-type (WT) strain was purchased from ATUM Inc. (USA) and used for the expression of Kex2. The expression vector was pD902 from ATUM Inc. (USA). Yeast nitrogen base (YNB), D-sorbitol, D-biotin, tryptone, and yeast extract were purchased from HiMedia. Presto™ Mini Plasmid Kit and DNA purification kit were purchased from GeneAid, and restriction enzyme (*Sac*I) and a DNA polymerase for PCR reaction were purchased from Thermo Scientific. Primer synthesis and DNA sequencing services were provided by First Base (Singapore). Zeocin was purchased from Invivogen (CA, USA).

Luria broth (LB: 1% peptone, 0.5% yeast extract, and 1% NaCl) or Luria agar (LA: LB and 1.5% agar) was used for culturing *E. coli*, and low salt LB (LSLB: 1% peptone, 0.5% yeast extract, 0.5% NaCl, and 25 µL/mL zeocin) was used for *E. coli* growth. We used yeast extract peptone dextrose-sorbitol agar (YPDS agar: 1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar) for the transformant *P. pastoris* selection. Yeast extract peptone-dextrose (YPD: 1% yeast extract, 2% peptone, and 2% dextrose) and YPD agar (YPD and 2% agar) were used for routine growth and sub-culturing of *P. pastoris* strain. We used a buffered glycerol-complex medium (BMGY: 1% yeast extract, 2% peptone, 0.34% YNB, 4×10−5% biotin, 1% glycerol, and 100 mM potassium phosphate buffer pH 6.0) for the *P. pastoris* pre-induction growth medium. For *P. pastoris* induction medium, we used buffered methanol-complex medium (BMMY: 1% yeast extract, 2% peptone, 0.34% YNB, 4×10−5% biotin, and 100 mM potassium phosphate buffer pH 6.0).

2.2. Plasmid and gene construct.

Kex2 protein sequence was derived from *S. cerevisiae* S288c *KEX2* gene with GenBank accession number NP014161 (or NM001183076). The *KEX2* recombinant gene contains the first 699 amino acids out of 814 amino acid residues of the full-length Kex2 sequence, including its natural signal peptide at the N-terminal and the transmembrane domain at the C-terminal. The *KEX2* gene excludes the last 115 amino acids at the C-terminal, but a 6×His-tag sequence was added with a short linker peptide as a protein recognition and purification tool. This *KEX2* recombinant gene was made synthetically by ATUM Inc. (USA) and was codon-

optimized for expression in *P. pastoris*. The gene was placed under an inducible promoter *AOX1* in the plasmid named pD902-Kex2-699 (Figure 1).

2.3. Transformation of E. coli DH5α using recombinant plasmid pD902-Kex2-699.

The plasmid pD902-Kex2-699 was transformed into *E. coli* DH5α according to the general protocol of molecular biology by heat shock [14]. *E. coli* transformants were selected on LS-LB agar medium containing zeocin (25 µg/mL). The plasmid of selected transformants was propagated in LS-LB medium and purified using Presto™ Mini Plasmid Kit (GeneAid).

2.4. Transformation of P. pastoris using recombinant plasmid pD902-Kex2-699.

The recombinant plasmid pD902-Kex2-699 was linearized with *Sac*I restriction enzyme (Thermo Scientific) and used for the transformation of auxotrophic *P. pastoris* strain BG10 (WT) from ATUM Inc., USA. The transformation was carried out by electroporation of freshly grown cells in a 0.2 cm cuvette. The pulse was delivered by Gene Pulser (Bio-Rad Laboratories Inc., USA) at 2000 V, 25 µF, and 200 Ω. The electroporated cells were allowed to recover for 1 h in 1 M sorbitol at 30 °C and then 1 h in YPD at 30 ºC. After the secondary recovery, the culture was spread on YPDS agar plates. The transformants were screened on YPD plates containing progressively increasing concentrations of zeocin (500-3000 µg/mL) to identify multicopy clones. Several viable clones at 3000 µg/mL zeocin concentration were selected for productivity check in small-scale induction experiments.

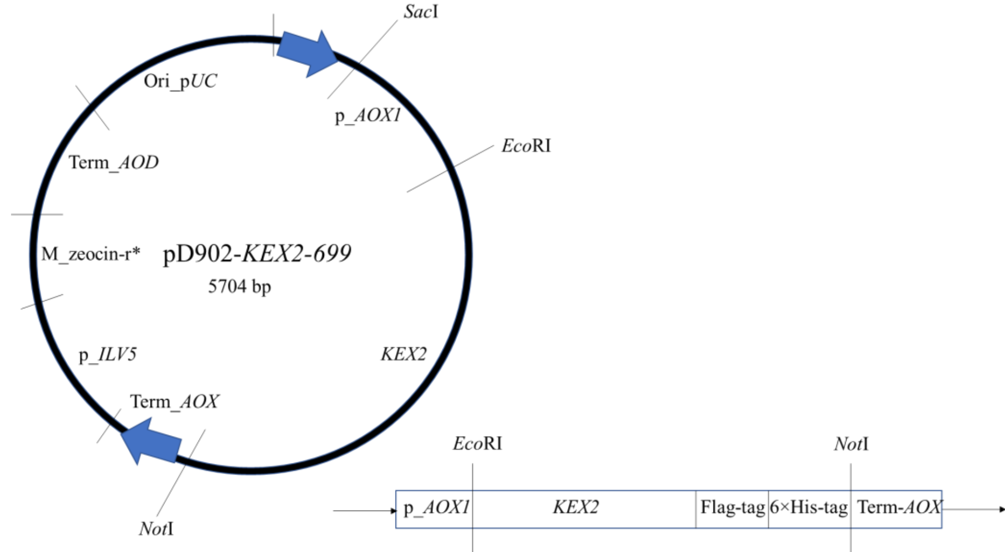


Figure 1. Plasmid map and gene construct. *KEX2-699* was designed using pD902 plasmid and equipped with Flag-tag and 6×His-tag at the N-terminal.

2.5. Expression and characterization of the recombinant Kex2.

Seven individual transformant colonies were chosen to evaluate recombinant protein expression according to the manual instructions of the *Pichia* expression kit (Invitrogen). Transformed or non-transformant *P. pastoris* were cultivated in 2 mL of YPD medium (with and without zeocin, respectively) and incubated at 30 °C and 250 rpm for 24 h. These cultures were used to inoculate 25 mL of BMGY production medium. Cultures were incubated at 30 °C and 250 rpm for 24 h. The cells were harvested aseptically by centrifugation before being transferred into a 25 mL BMMY induction medium with OD_{600} = 1.0. Cultures were incubated at 30 °C and 250 rpm for 96 h while methanol was added at 1% (v/v) into the culture every 24

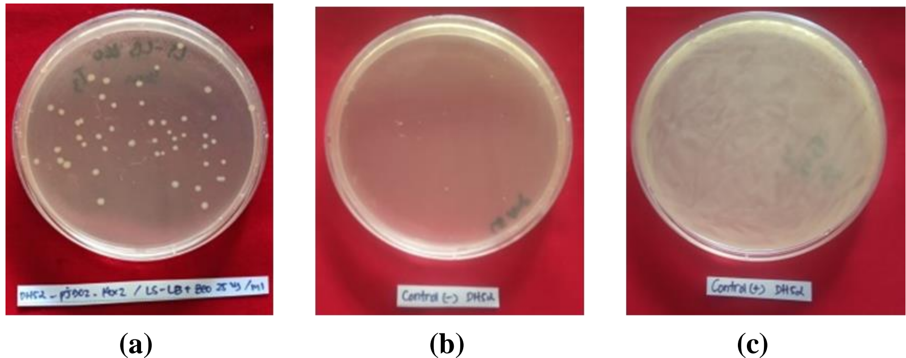
h. Cultures were harvested by centrifugation (10,000 rpm; 15 min; and 4 °C) [15]. The supernatant was concentrated using trichloroacetic acid (TCA) to the final concentration of 10% and incubated at cold temperature (4-10 °C) for at least overnight and then washed with acetone. The pellet was then solubilized in 6×SDS-PAGE loading buffer and then incubated at a cold temperature for at least overnight. Before SDS-PAGE, each sample was denatured at 95-100 °C for 15 min.

For western blot, the SDS-PAGE gel was immediately transferred onto nitrocellulose membrane (Biorad) by the electro-running system. The membrane was blocked with 10 % skim milk in TBS for 2 h at cold temperature, then washed three times with TBS-10% Tween 20 each for 15 min; and 2 × 5 min. The membrane was incubated with 1:3500 dilution of monoclonal anti-His-tag antibody (Santa Cruz) in 10% skim milk in TBS for 1 h and washed three times with TBS-10% Tween 20. The membrane was incubated with the secondary anti-mouse IgG-alkaline phosphatase conjugate (Thermo Scientific) in 10% skim milk and also washed three times with TBS-10% Tween 20. Detection was carried out using 1 Step NBT/BCIP substrate solution (Thermo Scientific).

3. Results and Discussion

3.1. Transformation of E. coli DH5α using recombinant plasmid pD902-Kex2-699 and plasmid preparation for transformation of P. pastoris.

Zeocin was used for transformant selection as the plasmid pD902 owns the zeocin resistance gene (Figure 1). This antibiotic has been widely used in yeast transformant selection and gene evolution [16,17]. A number of transformants spread on three selection agar LSLB plates enriched with zeocin 25 µg/mL were obtained (Figure 2a). Non-transformant *E. coli* DH5α appropriately grew on LSLB without antibiotics; meanwhile, no non-transformant colony was grown on LSLB with antibiotics, meaning that the transformation process was free from contaminants (Figure 2b, Figure 2c).



(a) **(b)** **(c)**

Figure 2. *E. coli* DH5α transformants harboring recombinant plasmid pD902-Kex2-699. **(a)** a single colony of transformants on the selection agar plate. **(b)** negative control or *E. coli* DH5α on LSLB with zeocin). **(c)** positive control or *E. coli* DH5α on LSLB without zeocin.

Twenty transformants were randomly selected for plasmid isolation. Figure 3a shows the difference in plasmid migration on agarose 0.7% due to variation of plasmid structure [18]. Bands higher than 1 kb were concerned as the host or *E. coli* DH5α genome [19]. A single band at approximately 5706 bp was obtained after *Sac*I digestion toward the plasmid representing the theoretical size of the recombinant plasmid (Figure 3b).

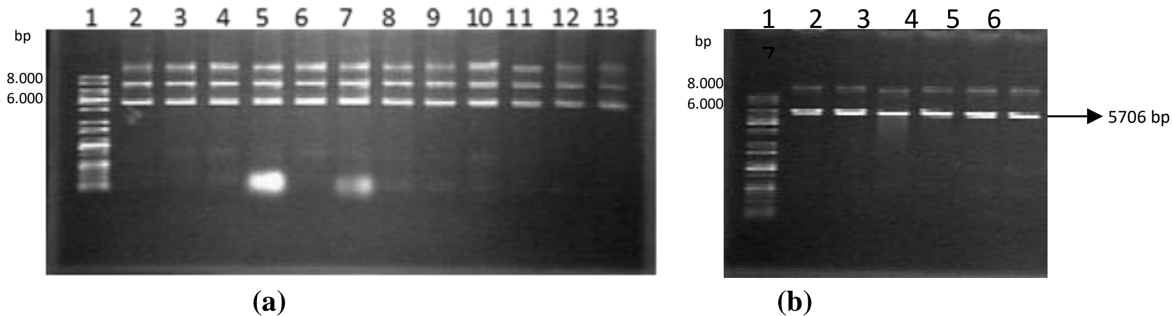


Figure 3. Electropherogram of the pD902-Kex2-699 recombinant plasmid isolated from the transformant *E. coli* DH5. (**a**) uncut plasmid; (**b**) plasmid cut with *Sac*I.

3.2. Transformation of P. pastoris using recombinant plasmid pD902-Kex2-699

The recombinant plasmid pD902-Kex2-699 was linearized at the promoter 5' *AOX1* region with *Sac*I and integrated into the *AOX1* region of the *P. pastoris* genome [20]. Similar to the transformation of *E. coli* DH5α using recombinant plasmid, a number of transformants *P. pastoris* single colonies were obtained on YPD + zeocin 25 µg/mL + ampicillin 100 µg/mL, whereas no colonies were found on negative control and vice versa (Figure 4a, Figure 4b, Figure 4c). The resistance of transformants was tested on YPD agar containing zeocin in gradient concentration from 0.5 - 3 mg/mL in order to obtain those transformants harboring multicopy genes [21,22] (Figure 4d, Figure 4e, Figure 4f).

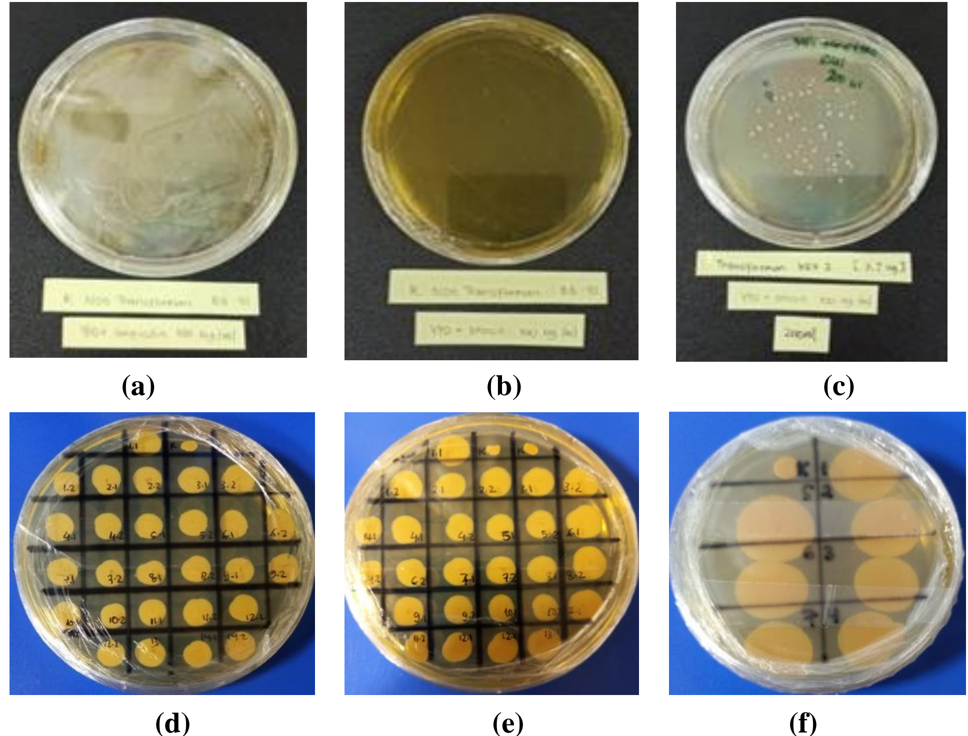
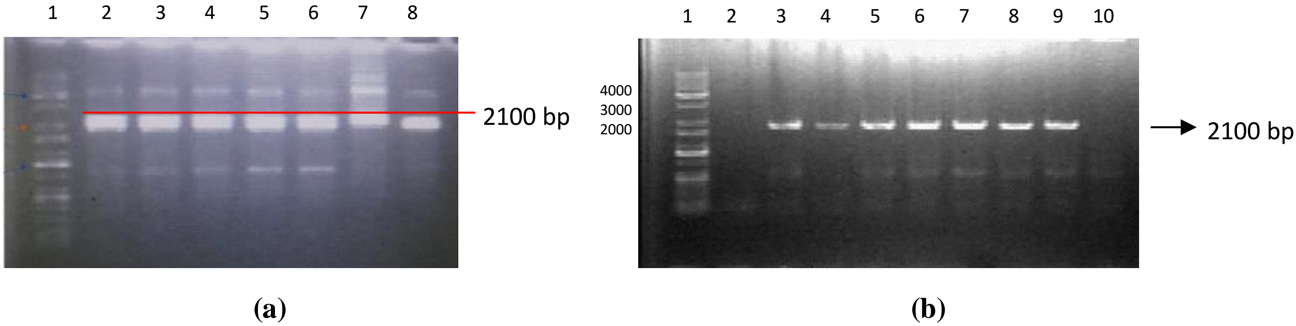


Figure 4. The *P. pastoris* BG10-pD902-Kex2-699 on YPD selection agar. (**a**) positive control or *P. pastoris* BG10 on YPD without antibiotics. (**b**) negative control or *P. pastoris* BG10 on YPD with zeocin 100 µg/ml and ampicillin 100 µg/ml. (**c**) transformant *P. pastoris* BG10-pD902-Kex2-699 on YPD with zeocin 100 µg/ml and ampicillin 100 µg/ml. (**d**) transformant *P. pastoris* BG10-pD902-Kex2-699 on YPD with zeocin 1 mg/ml and ampicillin 100 µg/ml; (**e**) transformant *P. pastoris* BG10-pD902-Kex2-699 on YPD with zeocin 2 mg/ml and ampicillin 100 µg/ml; (**f**) transformant *P. pastoris* BG10-pD902-Kex2-699 on YPD with zeocin 3 mg/ml and ampicillin 100 µg/ml.

3.3. Transformant molecular verification using PCR and DNA sequencing.

The transformant genome was amplified using a pair of *AOX1* primers and *KEX2* insert gene primers. Figure 5a shows the amplicon of *AOX1* primers. A band showed the genome of the transformants containing the insert gene-integrated *AOX1* gene with a size of approximately 2100 bp. Both transformant and non-transformant genomes also owned the indigenous *AOX1* gene with almost similar size, resulting in a band at approximately 2100 bp. Yet, this indigenous *AOX1* gene's band was slightly lower than the insert gene-integrated *AOX1* gene's band. To further confirm that the transformant successfully contained the *KEX2* gene, PCR was conducted using the inserted gene primers. Figure 5b depicts that the inserted gene was only found in the transformant's PCR product.



(a) **(b)**

Figure 5. Molecular verification of *P. pastoris* BG10-pD902-Kex2-699. **(a)** verification using *AOX1* primers. 1. Ladder; 2-7. Transformant genome; 8. Recombinant plasmid; and 9. Non-transformant genome. **(b)** verification using *KEX2* insert gene primers. 1. Ladder; 2. PCR negative control; 3-9. Transformant genome; and 10. Non-transformant genome.

Two transformant genomes with positive PCR products were selected for Sanger DNA sequencing. Because the length of the *KEX2* gene reached more than 2000 bp, sequencing using walking primers were required to decrease mismatches [23]. In our experiment, three forward and reverse primers were designed for the Sanger DNA sequencing. All targeted bases were correctly read and showed no mutation (data not mentioned).

3.4. Expression and characterization of the recombinant Kex2.

Seven *P. pastoris* transformants harboring the *KEX2* gene were used in the protein expression study. Concentrated supernatant from each colony was analyzed with SDS PAGE. All of the transformants expressed a distinctive band compared with non-transformant *P. pastoris* at approximately 77 kDa (Figure 6). In another previously reported study, soluble Kex2 (*ss*Kex2p) from *P. pastoris* strain GS115 was also found as a single band at approximately 80 kDa, almost similar to our protein of interest [24]. Concentrated supernatant from transformant number 02 was then identified and confirmed against monoclonal anti-His-tag antibody and by LC/MS/MS. Figure 7 exhibits that 20% of the obtained peptide sequence was aligned with that of Kex2p Kexin from *Saccharomyces sp (S. boulardii)*, the original gene of the Kex2 designed in *S. cerevisiae (strain ATCC 204508/S288c)* and detected as sufficiently long fragments [25].

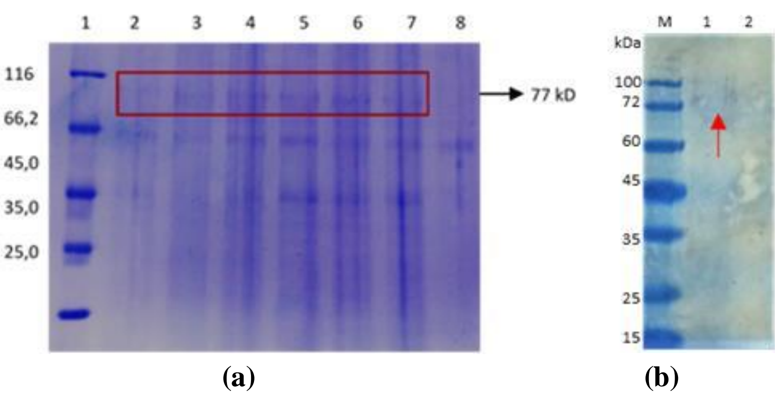


Figure 6. Electropherogram of extracellular protein from Kex2-699 production in *P. pastoris*. (**a**) SDS-PAGE analysis. 1. Protein marker; 2-7. Transformants; 8. Non-transformant (NT) *P. pastoris* BG10. (**b**) western blot analysis against monoclonal anti-His-tag antibody. 1. Protein marker; 2. Transformant; 3. NT *P. pastoris* BG10.

1 MKVRKYITLC FWWAFSTSAL VSSQQIPLKD HTSRQYFAVE SNETLSRLEE
51 MHPNWKYEHD VRGLPNHYVF SKELLKLGKR SSLEELQGDN NDHILSVHDL
101 FPRNDLFKRL PVPAPPMDSS LLPVK**EAEDK LSINDPLFER** QWHLVNPSFP
151 GSDINVLDLW YNNITGAGVV AAIVDDGLDY ENEDLKDNFC AEGSWDFNDN
201 TNLPKPRLSD DYHGTRCAGE IAAKKGNNFC GVGVGYNAKI SGIRILSGDI
251 TTEDEAASLI YGLDVNDIYS CSWGPADDGR **HLQGPSDLVK K**ALVKGVTEG
301 R**DSKGAIYVF ASGNGGTR**GD NCNYDGYTNS IYSITIGAID HKDLHPPYSE
351 GCSAVMAVTY SSGSGEYIHS SDINGRCSNS HGGTSAAAPL AAGVYTLLLE
401 ANPNLTWR**DV QYLSILSAVG LEKNADGDWR DSAMGK**KYSH R**YGFGKIDAH**
451 **KLIEMSK**TWE NVNAQTWFYL PTLYVSQSTN STEETLESVI TISEK**SLQDA**
501 **NFKRIEHVTV TVDIDTEIRG TTTVDLISPA GIISNLGVVR PR**DVSSEGFK
551 DWTFMSVAHW GENGVGDWKI KVK**TTENGHR IDFHSWRLKL FGESIDSSKT**
601 **ETFVFGNDK**E EVEPAATEST VSQYSASSTS ISISATSTSS ISIGVETSAI
651 PQTTTASTDP DSDPNTPKKL SSPRQAMHYF LTIFLIGATF LVLYFMFFMK
701 SRRRIRRSRA ETYEFDIIDT DSEYDSTLDN GTSGITEPEE VEDFDFDLSD
751 EDHLASLSSS ENGDAEHTID SVLTNENPFS DPIKQKFPND ANAESASNKL
801 QELQPDVPPS SGRS

Figure 7. The peptide sequence of the recombinant Kex2-699 that aligned with that of Kex2p Kexin from *S. boulardii* resulted in matched peptides shown in bold red.

In nature, Kex2 is expressed in the late Golgi compartment of yeast such as *S. cerevisae*, *C. albicans*, and *P. pastoris* to play a role in its specific substrate maturation, glycosylation pathway, and in retaining cell wall integrity [26]. Expressing the Kex2 as a soluble recombinant protein is quite complicated. It was reported that the Kex2 gene from *C. albicans* could not be expressed in *P. pastoris* [27]. On the contrary, in our finding, the soluble recombinant *S. cerevisiae* Kex2 was successfully expressed in *P. pastoris.*

P. pastoris is the second most widely used host to express heterologous proteins after *E. coli*. It is superior to *E. coli* in terms of eucaryotic properties, such as glycosylation post-translation, and more convenient for soluble proteins [28,29]. Compared with another eucaryotic expression host, *P. pastoris* resulted in glyoxal oxidase in higher bioactivity than that expressed in *Trichoderma reseei* [30]. It is also reported as a prospective expression host for industrial scale because of the capability to grow rapidly in high density using the low-cost medium, for instance, residual molasses [29].

P. pastoris BG10, used in the current study, is a methylotrophic yeast that metabolizes methanol. The concentration of methanol and preculture density are crucial parameters that need to be optimized for the highest level expression of the target protein [31]. In our preliminary study, methanol 0.25, 0.5, 1, and 2 % were applied, in which induction with 1% exhibited the highest soluble Kex-2 expression level (data not mentioned).

In this study, the gene encoding the first 699 amino acids of *S. cerevisiae* was inserted into the expression plasmid pD902. This expression system utilized the amino acids 1 – 23 of the original *S. cerevisiae*'s Kex2 as signal peptides to obtain soluble Kex2 in extracellular fraction. Harboring the transmembrane domain (TMD), a protein that interacts with membrane protein, at amino acid 679 – 699, the Kex2 was still completely secreted to the extracellular as evidenced by positive blotting against antibody anti-His-tag, which had been previously designed at the C-terminal after the TMD [32]. However, improving the expression level of the soluble Kex2 might be increased by using other expression vectors and by adding another signal peptide aside from the signal peptide of the Kex2 gene itself or by a combination of other signal peptides and promoters [33,34].

4. Conclusions

The gene of recombinant Kex2, which originated from *S. cerevisiae* has been successfully expressed and secreted to the extracellular of *P. pastoris* BG10 by methanol induction. The protein showed a long peptide fragment aligned with the original peptide sequence and positively blotted with a monoclonal anti-His-tag antibody.

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Conflicts of Interest

The authors declare no conflict of interest.

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