Combination of Genetic Manipulation Improved *Saccharomycopsis fibuligera* α-Amylase Secretion by *Pichia pastoris*

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Abstract: *This study assessed the combinations of genetic manipulation; signal peptide modification, gene dosage increment and co-expression of folding component, to increase* Saccharomycopsis fibuligera *R64 α-amylase (*Sfamy*) secretion in* Pichia pastoris. Sfamy *native signal peptide was replaced with modified signal peptide which contained 15 amino acid of mouse salivary α-amylase signal peptide fused to the pro-region of the signal peptide of* Saccharomyces cerevisiae *α-mating factor (α-MF). Increase in gene dosage was identified by screening for* P. pastoris *harboring multicopy of the* Sfamy *gene. Whereas, co-expression of folding component was done by addition of Protein Disulfide Isomerase (PDI). Expression plasmids harboring* Sfamy *containing a modified signal sequence (pPICZA-*MS-Sfamy*) was used to transform* P. pastoris *GS115, and gene dosage increment was screened using zeocin. Effect of PDI co-expression on secretion levels of* Sfamy *was assessed by constructing the pPIC3.5K-Pdi1 plasmid and introducing into* P. pastoris *harboring multicopy of* MS-Sfamy *for expression of* Sfamy. *Signal peptide modification consequently increased* Sfamy *secretion by* P. pastoris *by 3.3-fold compared to the native signal peptide. Gene dosage increment had improved* Sfamy *secretion by 11-fold in* P. pastoris *[*MS-Sfamy*] resistant to 2000 μg/mL zeocin, compared to* P. pastoris *harboring one copy of* WT-Sfamy. *Hence, PDI co-expression increased the secretion of* Sfamy *by 2-fold as compared without PDI co-expression. In summary, the combination of genetic manipulation successfully increased* Sfamy *secretion by 20-fold compared to* P. pastoris *harboring one copy of* WT-Sfamy.

Keywords: Sfamy*; signal peptide; gene dosage; folding; PDI;* Pichia pastoris

■ INTRODUCTION

Saccharomycopsis fibuligera R64 α-amylase (*Sfamy*) is an extracellular enzyme that has the potential to be applied in the industry because it is known to degrade raw starch although it cannot absorb row starch [1-2]. *Sfamy* R64 (GenBank accession no. HQ172905) contains 494 amino acid residues, including the 26 amino acid signal peptides [3]. *Sfamy* R64 has high sequence identity to Aspergillus α-amylases of which three-dimensional structures have been elucidated [4-5].

The use of methylotrophic yeast *Pichia pastoris* is becoming increasingly popular as a host for the expression of recombinant proteins because of its high level of expression. *P. pastoris* is suitable for producing eukaryotic proteins with proper folding and post-translational modification. More than 550 heterologous proteins have been synthesized and produced in this yeast [6-7]. Some examples of heterologous protein secretion by *P. pastoris* show high yields, such as human serum albumin [8], *Rhizopus oryzae* glucoamylase [9], Hen egg lysozyme [10], and mouse gelatin [11]. However, secretion levels of several other proteins are still low [12-13]. Production of *Sfamy* using *P. pastoris* expression system must consider the factors that influence the secretion of heterologous protein; some of which are studied in this research. Among the studied

factors include copy number of genes, type of signal peptides, as well as processing and protein folding in the endoplasmic reticulum (ER) and Golgi bodies [8-9].

Signal peptides generally contain terminal charged amino acid sequence followed by hydrophobic residues and recognition site of signal peptidase enzyme. Signal peptides mediate co-translational translocation of the polypeptide into the ER and are removed by a signal peptidase during polypeptide translocation [7,14-15]. Selection of the signal peptide for secretion of the recombinant protein can affect the secretion level of proteins by the yeast expression system. Human lysozyme secretion was increased 1.8-times by *S. cerevisiae* by increasing the number of hydrophobic residues in the signal sequence [16]. Secretion of α-amylase *Bacillus licheniformis* using *S. cerevisiae* SUC2 signal peptide was increased by 2.7 times as compared to the native signal peptide [15]. Glucoamylase production of *R. oryzae* in *P. pastoris* was also improved through genetic manipulation of glucoamylase signal peptide [9]. Hence, the influence of the signal peptide selection on the secretion level of *Sfamy* by *P. pastoris* needs to be studied. *S. cerevisiae* α-(MF) pre-pro signal sequence is a signal sequence that is often used with a high level of secretion. A number of different signal peptides, including the wild-type signal peptide contained in the heterologous protein, were also recognized by the *P. pastoris* with different secretion rate.

Increasing gene dosage will increase the amount of mRNA transcript, so it will also affect the amount of polypeptide translation product. The increase in gene copy number of protein Hepatitis B surface antigen (HBsAg) increased the expression of HBsAg in *P. pastoris* [17]. Integration of multicopy genes also increased the secretion of insulin precursor in *P. pastoris* from 19 to 250 mg/L [18].

The relationship between protein folding and secretion of protein from reticulum endoplasmic (RE) proves that productivity of eukaryotic expression systems can be maximized by adding the components of protein folding. One of the enzymes that play an important role in protein folding is the Protein Disulfide isomerase (PDI). PDI catalyzes the formation of disulfide bonds in newly synthesized proteins and disulfide bond isomerization of non-native to native form. Several studies have shown that an increase in concentrations of PDI can increase the expression of proteins with disulfide bonds in yeast. It was shown that the expression of parathyroid hormone human in *P. pastoris* was significantly improved through co-expression PDI [19]. PDI is also reported to act as chaperones for co-expression of PDI increased secretion of β-glucosidase of *Pyrococcus furiosus* which has only one cysteine and has no disulfide bonds, in *S. cerevisiae* [20]. *Sfamy* has four disulfide bonds; the co-expression of PDI in *P. pastoris* recombinant can increase the number of properly folded *Sfamy* thus, increasing *Sfamy* secretion.

■ EXPERIMENTAL SECTION

Strain, Vector and Reagent

Escherichia coli TOP10F' for cloning and *P. pastoris* GS115 (*his4*) for expression recombinant protein was purchased from Invitrogen (Carlsbad, Germany). Vectors pPICZA and pPIC3.5K were bought from Invitrogen and pGemT vector acquired from Promega. Recombinant plasmid pPICZαA-*Sfamy* was given from Departement of Chemistry, Institute Technology Bandung [21]. Recombinant plasmid pPICZA-*WT-Sfamy* was obtained from our previous work [3]. Restriction endonuclease enzyme, *Taq* DNA polymerase, and T4 DNA ligase were procured from Thermo Scientific. (Massachusetts, USA). Oligonucleotides for PCR, probe and sequencing DNA were synthesized by Research Biolabs (Singapore).

Procedure

Construction of pPICZA-MS-Sfamy recombinant plasmids

Amplification of *MS-Sfamy* was done using pPICZαA-*Sfamy* as a template using the primer pairs: 5'mspαF: 5'-GAGGAGGTACCATGAAATTCTTCCT GCTGCTTCTCCTCATTGGATTCTGCTGGGCCGCT CCAGTCAACACTAC-3' and 3'Sfa: 5'-GAGGAGGGC CCTGAACAAATGTCAGAAGC-3'. A fragment of ~1700 bp was recovered and ligated into pGEMT cloning vector to result in a recombinant plasmid pGemT-*MS-Sfamy*. Furthermore, recombinant plasmid

was cut with *Kpn*I and *Apa*1, followed by insertion into the same site in pPICZA vector to result in the pPICZA-*MS-Sfamy*. The nucleotide sequence of MS-Sfamy was confirmed by DNA sequencing analysis.

Transformation of P. pastoris

The transformation was carried out using electroporation method according to Faber et al. [22]. *P. pastoris* GS115 competent cell (50 µL) is prepared from the log phase growing culture. The competent cells were mixed with 1-5 µg of *Pme*1 linearized plasmid pPICZA-*WT-Sfamy*, pPICZA-*MS-Sfamy* and pPICZαA-*Sfamy*, respectively. Electroporation was done for ~5 milliseconds at 7.5 kV/cm using BTX electro cell manipulator 600. *P. pastoris* transformants were grown on YPDS medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% bacto agar) plates containing 100 µg/mL zeocin for 3 days at 30 °C.

Selection of Mut⁺/Mut^s transformants

P. pastoris transformants were streaked on MMH plates (minimal methanol medium+ histidine: 1.34% YNB, 4x10^{-5}% biotin, 0.5% methanol, 0.004% histidine) and MDH plates (minimal dextrose medium + histidine: 1.34% YNB, 4x10^{-5}% biotin, 2% dextrose) and grown for 2 days at 30 °C. GS115/His$^+$Mut^s Albumin and GS115/His$^+$Mut$^+$β-gal strains were used as Mut^s and Mut$^+$ control phenotype. The growth ability of these clones was evaluated to select the Mut$^+$ phenotipe.

Identification of transformant P. pastoris *containing multicopy of* Sfamy *genes*

Putative clones harboring multicopy of *MS-Sfamy* and *WT-Sfamy* expression cassette were selected using the zeocin screening procedure. Zeocin resistance clones derived from the initial selection (Mut$^+$ phenotype) were streaked on YPD plates containing 100, 500, 1000, and 2000 µg/mL. After 3 days of incubation at 30 °C, the growth ability of these clones was evaluated in the presence of increasing concentrations of the antibiotic. The clones that are resistant to different concentration of zeocin were analyzed by Southern blot.

Isolation of P. pastoris *genomic DNA*

Genomic DNA was isolated according to standard procedure [23]. *P. pastoris* culture was grown in 5 mL YPD (1% yeast extract, 2% peptone, 2% dextrose) overnight. The pellet was harvested by centrifugation at 10000 g for 2 min. Cells were resuspended in 500 µL $T_{100}E_{50}$ buffer (100 mM Tris-Cl, 50 mM EDTA pH 8) followed by addition of 5 µL β-mercaptoethanol and incubated for 15 min at 37 °C with shaking. Cells were separated by centrifugation at 10,000 *g* for 2 min and resuspended in 1 mL $C_{100}E_{10}$ buffer (100 mM sodium-citrate pH 5 and 10 mM EDTA) followed by centrifugation at 10,000 g for 2 min, then was resuspended in a 300 µL $C_{100}E_{10}$ buffer prior to addition of 1 mg Zymolyase. Cells were incubated at 37 °C for 60 min, followed by addition 15 µL of 10% sodium dodecyl sulfate (SDS). The cells were mixed by inversion then incubated at 65 °C for 10 min. Furthermore, 100 µL of 6 M sodium chloride was added, mixed by inversion, and incubated on ice for 5 min, mixed again and centrifuged at 14,000 g for 3 min. The supernatant that contained DNA was removed to the new tube, prior to the addition of 300 µL isopropanol and mixed by inversion, then centrifuged at 14,000 g for 3 min. The supernatant was carefully decanted. The pellet was washed with 750 µL of 70% ethanol then centrifuged at 14,000 g for 3 min. After dried, DNA was dissolved in 50 µL $T_{10}E_1$ (10 mM Tris-Cl, 1 mM EDTA pH 8) with the addition of 20 µg/mL RNase and incubated for 1 h at 50 °C or overnight at 4 °C. Five microliters of DNA was analyzed in agarose electrophoresis, and 10 µL DNA was used for restriction analysis and Southern Blot.

Southern Blot analysis

Briefly, ten micrograms of genomic DNA were digested with *Eco*R1 at 37 °C for 2 h. The resulting fragments were separated by electrophoresis in 0.8% agarose gel with SPP1/*Eco*R1 (Roche) as DNA marker. Electrophoresis was done by low voltage (4 mA) overnight. DNA was transferred onto Hybond N+ membrane (GE Healthcare) and hybridized with a specific probe for AOX1 promoter. The probe is the PCR product using primer pairs: 5' AOX1P: 5'-GGGCTTGA TTGGAGCTCGCTCATTC-3' and 3' AOX1P: 5'-CGTT TCGAATAATTAGTTGTTTTTT-3', followed by gel purification. Probe DNA (1-3 µg) was dissolved in 14.5 µL water, mixed with 500 ng of DNA marker SPP1-*Eco*R1

and labeled with 2 µL of mixed hexanucleotide (DIG DNA labeling mix) and 1 µL of Klenow polymerase. The mixture was mixed vigorously and incubated overnight at 37 °C. The membrane was hybridized with the probe at 40 °C for 16 h in ECL Gold hybridization solution (GE Healthcare) under continuous rotation. The membrane was further washed twice for 15 min with 50 mL washing buffer (6 M Urea, 0.4% SDS and 0.5x saline-sodium citrate buffer (SSC) at 40 °C, then dried using Whatman paper. The membrane was washed twice for 5 min with 2x SSC at room temperature with rotation and dried using Whatman paper. The membrane was washed for a few minutes with 1x tris-buffered saline with Tween-20 (TBST) (10 mM Tris-Cl pH 8; 150 mM sodium chloride; 0.05% Tween 20), then incubated in blocking buffer (1x TBST; 1% skim milk) for 1 h. Remove the blocking buffer, add 1x TBST and anti-digoxigenin Ap Fab fragment (Roche) with 1:10,000 dilution followed by incubation for 30 min. The blot was rinsed three times with 1x TBST (for 5–10 min) and the remaining buffer was removed by placing the blot between two Whatman papers. Finally, the blot was incubated in 10 mL alkaline phosphatase buffer (100 mM Tris-Cl pH 9.5, 100 mM sodium chloride, 5 mM magnesium chloride) with the addition 200 µL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) as a substrate until the band appeared. Detection was stopped by removing NBT/BCIP. The blot was incubated in 20 mL water for 1–3 h (until the band became dark) and dried using 3 MM Whatman paper.

Expression of Sfamy *by* P. pastoris

P. pastoris transformant was grown in 10 mL of buffered glycerol complex medium (BMGY): 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% YNB, $4x10^{-5}$% biotin, 1% glycerol) with the addition of 2% sorbitol as an additional carbon source [24]. Cells were incubated at 30 °C for 24 h with shaking at 300 g until OD_{600} reached about 8-10. Cells were harvested by centrifugation at 3000 g for 20 min at 25 °C and then resuspended in 25 mL buffered minimal methanol histidine (BMMH) medium (1.34% YNB, $4x10^{-5}$% biotin, 40 mgL^{-1} histidine, 100 mM potassium phosphate pH 6 and 0.75% methanol) before incubated for up to 144 h. Induction was done every 24 h by the addition of methanol to a final concentration of 0.75% in the medium. Sampling was done every 24 h according to the time of induction and after 144 h. The cells were harvested by centrifugation at 3000 g for 20 min at 4 °C. The culture supernatant was analyzed for α-amylase activity and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and western blot analysis

SDS-PAGE was performed under reduced conditions using 10% running gel and 4% stacking gel. Twenty-five microliters of transformant *P. pastoris* culture supernatant were analyzed by SDS-PAGE. Protein bands on the gel were detected by Coomassie brilliant blue staining. Semi-dry western blotting was done according to a previously reported method [25].

Analysis of Sfamy *activity*

α-Amylase activity is assayed by either following the release of reducing sugar by dinitrosalicylic acid (DNS) [26] with glucose as the standard, or the formation of starch-iodine complex (Fuwa method) [27]. For the DNS method, one amylase activity unit is defined as the amount of enzyme that catalyzes the formation of reducing sugar equivalent to 1 µmol of reducing sugar per minute under the assaying conditions. One amylase activity unit in Fuwa's method is defined as a decrease of 10% absorbance at 600 nm of the reaction mixture per mL enzyme used under the assaying conditions.

Integration of Pdi1 gene into P. pastoris *[*MS-Sfamy*] multicopy transformant*

Pdi1 gene of *S. cerevisiae* was amplified by PCR method from the template pGemT-*Pdi1* using 5'BamPDI: 5'-GAGGAGGATCCATGAAGTTTTCTG CTGG-3' and 3'EcoPDI: 5'GAGGAGAATTCCAATTC ATCGTGAATGG-3' primer pairs. PCR product was subcloned into pGemT vector followed by digestion using *Eco*R1 dan *Bam*H1 enzyme, and ligation into the same site on pPIC3.5K vector (Invitrogen) to create pPIC3.5K-*Pdi1*. The nucleotide sequence was verified by sequencing the dideoxy Sanger method. pPIC3.5K-*Pdi1* plasmid was linearized at HIS4 locus with *Bsp*E1 and

used to transform *P. pastoris* [*MS-Sfamy*]. The transformation was done following EasyComp™ method (Invitrogen). *P. pastoris* transformants with His^+ phenotype were selected in MMH (minimal methanol medium+ histidine: 1.34%, YNB, $4x10^{-5}$% biotin, 0.5% methanol, 0.004% histidine) and MM medium (minimal methanol medium: 1.34% YNB, $4x10^{-5}$% biotin, 0.5% methanol). *Sfamy* expressed by recombinant *P. pastoris* harboring *MS-Sfamy* and *Pdi1* genes were analyzed using the procedure described above.

■ RESULTS AND DISCUSSION

Generation of Recombinant *P. pastoris* Containing *WT-Sfamy*, *MS-Sfamy*, and *α-Sfamy*

The secretion level of *Sfamy* by *P. pastoris* was analyzed using three different signal peptides: *Sfamy* with native signal peptide [3], *Sfamy* with α-factor signal peptide [21] and *Sfamy* with modified signal peptide (this research). Modification was done by replacing 19 residues of N-terminal amino acid of the α-factor signal peptide on pPICZαA plasmid with a 15 residue N-terminal amino acid of mouse salivary α-amylase. The scheme of three different *Sfamy* is presented in Fig. 1.

Template to amplify *MS-Sfamy* was pPICZαA-*Sfamy* recombinant [21]. The pPICZαA-*Sfamy* already contains *S. cerevisiae* α-MF signal sequences and *Sfamy*. The modified primer was designed to be attached to the α-MF pro-peptide region and modified at its 5'end (pre-peptide region), whereas the primer reversed was the complement to 3'*Sfamy* (Fig. 1).

PCR with 5'mspαF and 3'Sfa primer pair produced 1.7 kb amplicon of *MS-Sfamy* gene, consisted of 261 bp of modified signal sequences, 1422 bp of *Sfamy* sequence and restriction site sequence *Kpn*I at 5' end and *Apa*I at

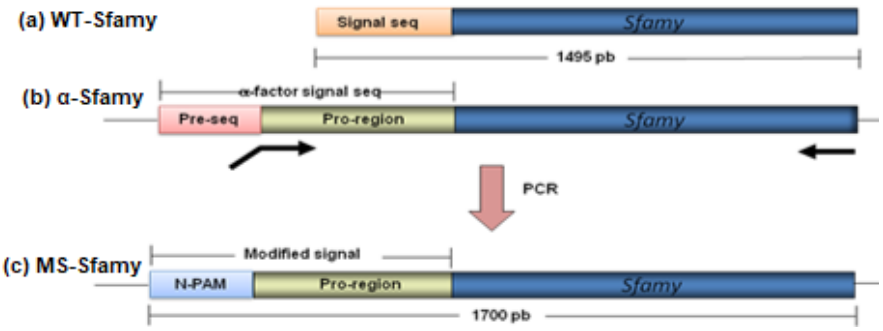

Fig 1. Scheme of *WT-Sfamy*, *α-Sfamy,* and *MS-Sfamy***.** (a) *WT-Sfamy* with native signal sequence. (b) *α-Sfamy* with pre-pro α-Mf signal sequence. (c) *MS-Sfamy* with modified signal sequence. N-PAM: N terminal signal peptide of mouse salivary α-amylase

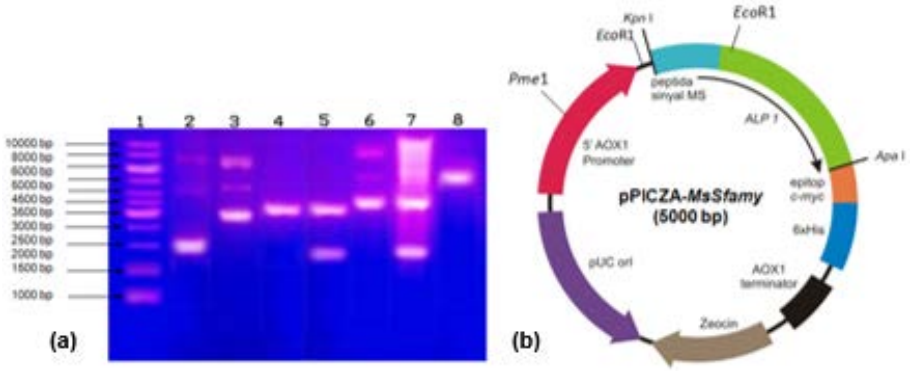

Fig 2. Characterization and map of recombinant plasmid. **(a)**.(1) DNA 1 kb; (2) pPICZA (3.3 kb); (3) pGemT-*MS-Sfamy* (~4.7 kb); (4) pPICZA/*Kpn*1/*Apa*1 (3.3 kb); (5) pGemT-*MS-Sfamy*/*Kpn*1/*Apa*1 (3.0 kb and 1.7 kb); (6) pPICZA-*MS-Sfamy* (5.0 kb); (7) pPICZA-*MS-Sfamy*/*Kpn*1/*Apa*1 (3.3 kb and 1.7 kb); (8) pPICZA-*MS-Sfamy*/*Pme*1 (3.3 kb). **(b)**. Map of pPICZA-*MS-Sfamy* plasmid

the 3' end. *MS-Sfamy* was subcloned in pGemTvector, yielding recombinant plasmid pGemT-*MS-Sfamy* then was ligated to pPICZA vector produced pPICZA-*MS-Sfamy*. Characterizations of the recombinant plasmid are shown in Fig. 2.

Based on the alignment of *MS-Sfamy* and *WT-Sfamy* to *Alp1* sequence (*Alp1* = *S. fibuligera* α-amylase sequence published by Itoh et al. [28], it can be concluded that signal sequence of *MS-Sfamy* had been successfully modified and in accordance with the design (data was not shown). However, five substitution mutations were found in the recombinant *MS-Sfamy* gene, $T_{304}C$, $A_{599}G$, $T_{873}C$, $T_{927}C$ and $T_{1494}C$ (*MS-Sfamy* numbering). The $T_{304}C$ mutation converts the serine amino acid into proline. However, this mutation was not expected to affect the activity of *Sfamy* since the position of mutation is far from the catalytic site. Four other mutations were silent mutations, so they did not alter the sequence of *Sfamy* amino acids. The alignment results also showed the presence of six different *Sfamy S. fibuligera* R64 nucleotides compared to *Alp1* [28], resulting in five different amino acids in *MS-Sfamy* and *WT-Sfamy* compared to ALP1.

P. pastoris was chosen as the host for *Sfamy*'s expression based on the consideration that *P. pastoris* and *S. fibuligera* are both yeasts, so they are thought to have the same codons preference and translational signals. *P. pastoris* has similar codon usage with *S. cerevisiae* [30], but since the *S. fibuligera* genome sequence has not been reported, the degree of resemblance of its codon usage has not been ascertained.

Selection of signal peptides affects the level of protein secretion by *P. pastoris*. Some commonly used signal peptides in the *P. pastoris* expression system are the α-mating factor (α-MF) signal peptide of *S. cerevisiae*, the phosphatase acid (PHO1) signal sequence of *P. pastoris*, and the invertase (SUC2) signal sequence of *S. cerevisiae* [12]. Different signal sequence gives varying results; therefore, signifying that the level of secretion depends greatly on the expressed protein.

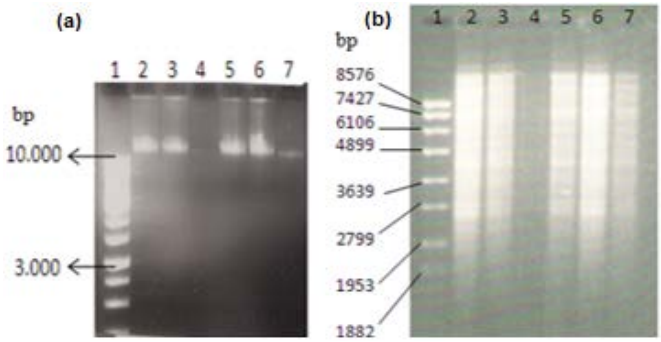

Fig 3. Isolation and restriction of *P. pastoris* genomic DNA by *Eco*R1. (a).Isolated genomic DNA; (1) 1000 bp marker DNA. (2) *P. pastoris* GS115 genome. (3-7) Genome of *P. pastoris* GS115[pPICZA-*MS-Sfamy*] transformants. (b). Result of genomic restriction with *Eco*R1; (1) SPP1/*EcoR*1 marker DNA. (2) *P. pastoris* GS115/*EcoR*1 genome. (3-7) *P. pastoris* GS115[pPICZA-*MS-Sfamy*]/*EcoR*1

Characterization of Multicopy *MS-Sfamy* by Southern Blot

The *P. pastoris* genome was isolated by standard methods [23]. A total of 10-15 µg of genomic DNA was cut with *Eco*R1 enzyme then 5–10% of the cutting reaction mixture was confirmed by electrophoresis using 0.8% agarose gel (Fig. 3).

The DNA bands on the agarose gel (Fig. 3) were transferred to the positively charged nitrocellulose membrane (Hybond N+) using a capillary blotting pile. Hybridization was carried out using a probe with a size of about 700 bp, the amplicon of 5'AOXP/3'AOXP primer pair. The probe will be attached to the AOX1 promoter and the probe is labeled with a hexanucleotide mixture already bound to DIG (digoxigenin).

In a GS115 host cell with no expression cassette, the probe was attached to the AOX1 promoter, so that the band at a position of about 5.5 kb in the GS115 genome was the band containing the AOX1 locus (Fig. 4). These results are consistent with those reported by Liu et al. [9]. In a *P. pastoris* transformants containing one copy of the *MS-Sfamy* gene, this 5.5 kb band was replaced by two bands of about 2 kb in size containing

the AOX1 promoter region (since the MCS still has *Eco*R1 sides) and a 6.1 kb band containing pPICZA-*MS-Sfamy* expression cassette and AOX1 locus (Fig. 4). In a *P. pastoris* transformant containing more than one copy of the integrated *MS-Sfamy* gene, there will be an additional band at 4.7 kb containing pPICZA-*MS-Sfamy* expression cassette minus 292 bases. The diagram illustrating the construction result is shown in Fig. 5.

Expression and *Sfamy* Activity Analysis

Analysis of Sfamy *secretion by* P. pastoris *[*MS-Sfamy-1*], [*α-Sfamy-1*] and [*WT-Sfamy-1*]*

P. pastoris transformants that contained one copy of *MS-Sfamy*, *α-Sfamy,* and *WT-Sfamy* resistant to 500 µg/mL zeocin were used to express Sfamy. Secretion of *MS-Sfamy* and *WT-Sfamy* increased rapidly until 72 h but then decreased until the last sampling at 144 h. At 72 h, the activity of *MS-Sfamy-1* and WT-Sfamy-1 on culture supernatant was 106.69 U/mL and 32.29 U/mL, respectively (Fig. 6). The highest activity of *α-Sfamy* was obtained at the 48 h that was 86.91 U/mL and activity was still detectable high until 144 h. Whereas the α-amylase activity in culture supernatant host GS115 was only 1.9 U/mL, therefore it can be assumed that the *P. pastoris* host did not secrete α-amylase. This result shows that *MS-Sfamy* secretion level was 3.3-times higher than *WT-Sfamy* and 1.2-times higher than *α-Sfamy* (Table 1).

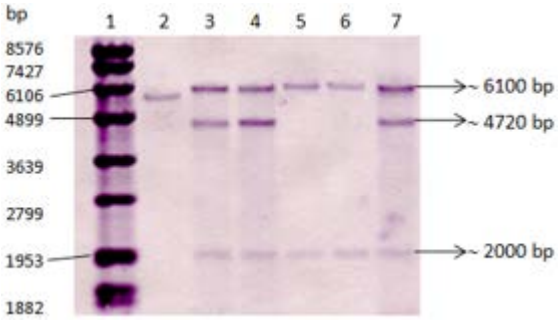

Fig 4. Southern Blot analysis. (1) SPP1/*Eco*R1 Marker DNA. (2) Genome of GS115. (3,4) *P. pastoris* [*MS-Sfamy*-multicopy] resistant to 2000 µg/mL zeocin. (5) *P. pastoris* [*MS-Sfamy*-one copy] (resistant to 500 µg/mL zeocin). (6) *P. pastoris* [*WT-Sfamy*-one copy] (resistant to 500 µg/mL zeocin). (7) *P. pastoris* [*WT-Sfamy*-multicopy] (resistant to 2000 µg/mL zeocin)

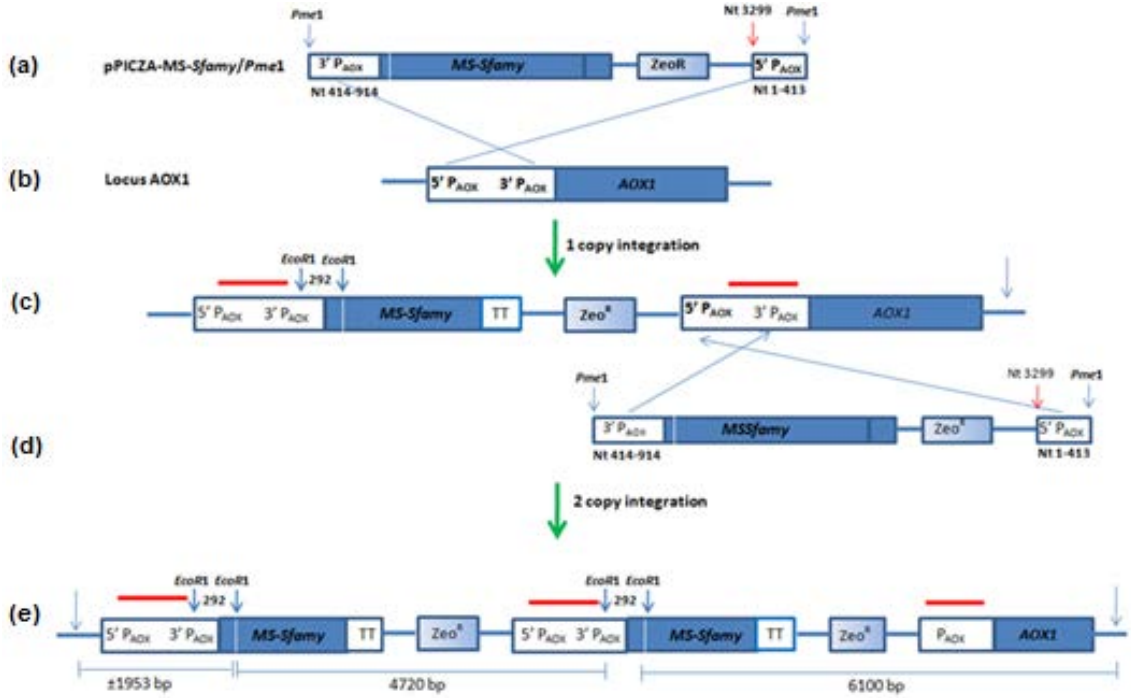

Fig 5. Map of *MS-Sfamy* expression cassette integration into *AOX1* locus. Linearization map of plasmid followed by cross over type insertion. (a) pPICZA-*MS-Sfamy*/*Pme*1 expression cassette. (b) *AOX1* locus. (c) Insertion of one copy expression cassette. (d) pPICZA-*MS-Sfamy*/*Pme*1 expression cassette (e) Insertion of two copy expression cassette

Increase in secretion level of *Sfamy* may be due to modification of the signal peptides improved the translocation efficiency of *Sfamy* by the *P. pastoris* secretory device. The signal peptide serves to direct the protein translocation to the endoplasmic reticulum. The efficiency of protein translocation across the membrane to the lumen of the RE is influenced by the hydrophobicity of the signal peptide.

The use of more hydrophobic signal peptides for *Sfamy* secretions was more efficient than the α-MF and native signal peptides. The α-MF signal peptide is widely used for the secretion of heterologous proteins in *P. pastoris* and gives a high yield. Modification of the α-MF signal peptide which increases its hydrophobicity is thought to increase the interaction of the signal peptide with SRP (signal recognition particles), thereby increasing the efficiency of the *Sfamy* translocation.

The secretion of *MS-Sfamy-1* is 3,3-times higher than *WT-Sfamy-1*. This result was almost the same as those reported by Liu et al. [9] who studied the secretion of *R. oryzae* glucoamylase using the same modified signal peptide and obtained 3.6-times improvement over the native signal peptide. The result was higher than secretion by *B. licheniformis* α-amylase in *P. pastoris* using SUC2 signal peptide reported by Paifer et al. [15].

Comparison of Sfamy *secretion level of* P. pastoris *containing multicopy of* MS-Sfamy, WT-Sfamy, *and* α-Sfamy

Based on Southern Blot analysis, a number of *P. pastoris* transformants containing multicopy of *Sfamy* were obtained. However, the actual amount of *Sfamy* gene contained in the transformants was not determined in this study. The comparison of *Sfamy* secretion level by *P. pastoris* was performed on the basis of resistance to different concentrations of zeocin.

Comparison of *Sfamy* secretion level by three types of recombinant *P. pastoris* [*MS-Sfamy*], [*WT-Sfamy*], and [*α-Sfamy*] that were resistant to 2000 μg/mL zeocin was shown in Fig. 7(a). The highest activity was obtained on *P. pastoris* [*MS-Sfamy*] which was 353.85 U/mL.

There was an increase of *Sfamy* secretion in *P. pastoris* transformants which were resistant to high concentrations of zeocin. The increase in *Sfamy* secretion was summarized in Table 1. Sfamy secretion was increased 11-times in *MS-Sfamy* compared to *WT-Sfamy-1*. The SDS-PAGE analysis in Fig. 7(c) shows that *Sfamy*'s MW between 55–70 kDa, with different band thickness according to the activity difference. These results suggest that the increase of gene doses identified through increased resistance to zeocin antibiotics, increases secretion of *Sfamy* by *P. pastoris*, using either native, α-MF as well as modified signal peptide. However, the use of modified signal peptides gives the highest yield.

The effect of increasing gene copies number increased *Sfamy* secretion by *P. pastoris*, using native, α-MF or modified signal peptide. The increase of *Sfamy* copy number integrated into the *P. pastoris* chromosome will increase the amount of *Sfamy* mRNA

Table 1. Comparison of *Sfamy* secretion by *P. pastoris* [*WT-Sfamy*], [*α-Sfamy*] and [*MS-Sfamy*]

Zeocin (μg/mL)	Increase of secretion level was compared to *P. pastoris* [*WT-Sfamy-1*]		
	WT-Sfamy	*α-Sfamy*	*MS-Sfamy*
500	1.0x	2.3x	3.3x
1000	2.4x	5.2x	4.6x
2000	4.8x	7.9x	**11.0x**

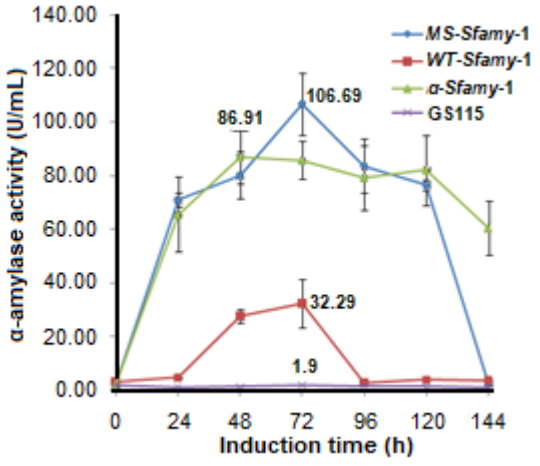

Fig 6. Comparison of α-amylase activity between *MS-Sfamy-1*, *α-Sfamy-1*, *WT-Sfamy-1*, and GS115. Cultures were sampled at an interval of 24 hours and centrifuged to separate the cells from culture supernatant (extracellular fraction). α-amylase activity on the culture supernatant was measured by DNS method

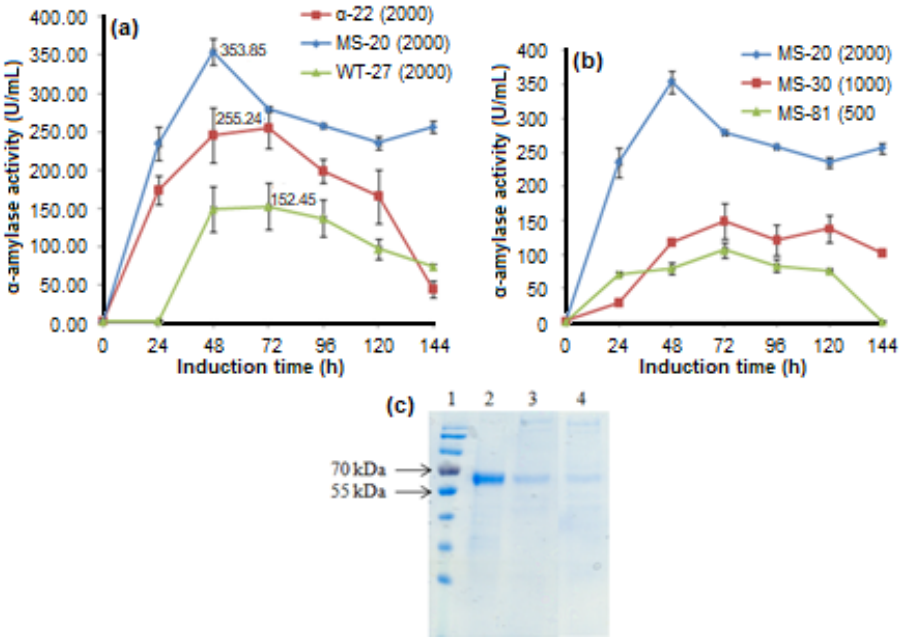

Fig 7. Comparison of *Sfamy* secretion level by *P. pastoris.* (a) *MS-Sfamy* vs. *α-Sfamy* vs. *WT-Sfamy* resistant to 2000 ug/mL zeocin. (b) *MS-Sfamy* resistant to different concentration of zeocin. (c) SDS-PAGE analysis; Lane 1: Protein marker, lane 2: supernatant of *MS-Sfamy* (2000), Lane 3: supernatant of *MS-Sfamy* (1000), lane 4: culture supernatant of *MS-Sfamy* (500)

transcribed, so the number of translated polypeptides will also increase. The increase of *Sfamy* copy number will be in line with the increase in copy number of zeocin resistance gene (*Sh ble* gene) since the overall expression cassette that was digested in the *AOX*1 promoter region was integrated into the *P. pastoris* chromosome at the *AOX*1 locus. An increase of *Sh ble* gene copy number will produce transformants which are resistant to high concentration of zeocin since the *Sh ble* gene is expressed using a constitutive TEF1 promoter (*S. cerevisiae* elongation transcription factor 1). *P. pastoris* transformant which is resistant to high concentration of zeocin is thought to contain a multicopy *Sfamy*. In this study, the integration of multicopy *Sfamy* that was identified through the level of resistance to zeocin proved to increase *Sfamy* secretion.

Multicopy integration in this study was identified by quantitative dot blot method, PCR, Southern blot analysis, and differential hybridization. However, we need a densitometer or software tool that can distinguish the level of band sharpness. The most valid method for determining the copies number of genes is the real-time PCR, which can determine the copy number based on quantitative PCR products. In this research, we identified a number of transformants containing multicopy integrant by Southern Blot method, but the identification of exact copy number was not done. The effect of gene doses was reported based on differences in resistance to zeocin. In the modified signal peptides, it was found that *Sfamy* secretion level by *P. pastoris* resistant to 2000 μg/mL zeocin was 3.3-fold higher as compared to 500 μg/mL zeocin. Therefore, the results obtained were lower than that reported by Liu et al. [9], but in this study, the exact number of integrated copy genes was not determined, so it could not be compared with the seven integrated copy genes reported by Liu et al. [9]. If the exact number of copies of *MS-Sfamy* integrant were determined, then the correlation between *MS-Sfamy* copy and *Sfamy* activity would also be known.

Comparison of Sfamy *intracellular and extracellular activity on* P. pastoris *[*MS-Sfamy*] and [*WT-Sfamy*]*

Determination of *Sfamy* intracellular activity was

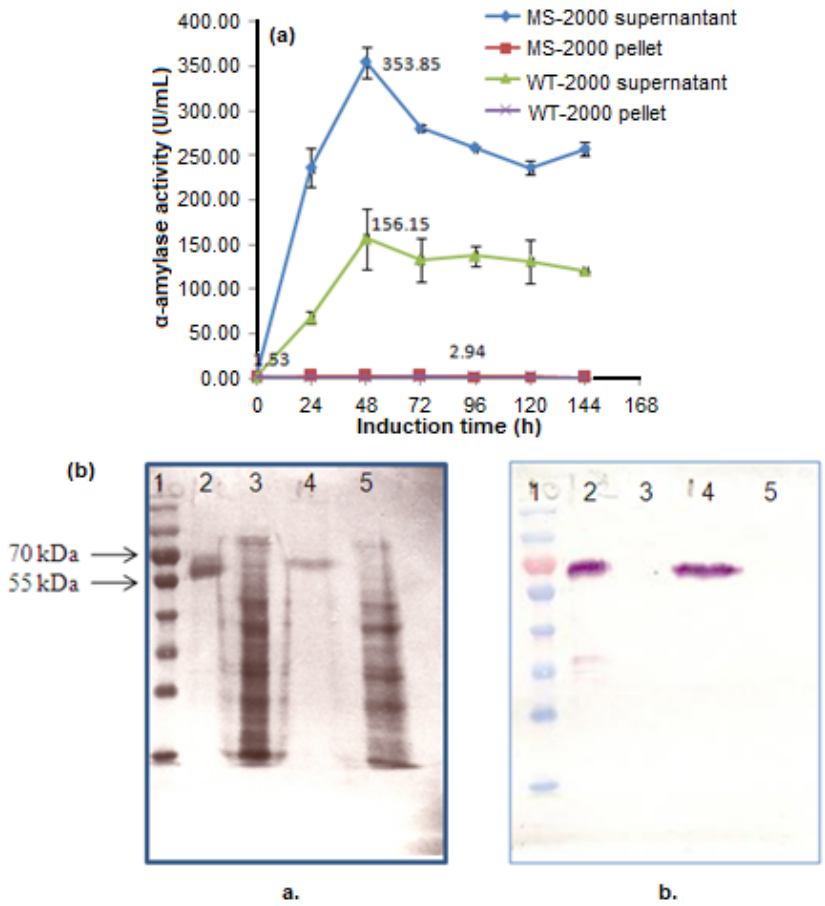

Fig 8. Comparison of Sfamy intracellular and extracellular activity on *P. pastoris* [*MS-Sfamy*] and [*WT-Sfamy*] (a) Comparison of intra and extracellular Sfamy activity in *P. pastoris [MS-Sfamy]* and *[WT-Sfamy]* resistant to 2000 ug/mL zeocin (b) a. SDS-PAGE analysis b. Western Blot analysis using anti-His(C-terminal) antibody; Lane 1: Protein marker, lane 2: culture supernatant of MS-2000, lane 3: pellet of MS-2000, lane 4: culture supernatant of WT-2000, lane 5: pellet of WT-2000

performed to prove that all of *Sfamy* was secreted outside the cells. The cultures used for the analysis of intracellular expression were *P. pastoris* [*MS-Sfamy*] and [*WT-Sfamy*] resistant to 2000 µg/mL zeocin. The results showed that *Sfamy* intracellular activity was much lower than extracellular; that was 2.94 compared to 353.85 U/mL in *P. pastoris*[*MS-Sfamy*] and 1.53 compared to 156.15 U/mL in *P. pastoris* [*WT-Sfamy*] (Fig. 8(a)). This result was also supported by Western Blot analysis which shows the absence of *Sfamy* band on *P. pastoris* pellet (Fig. 8(b)). Based on these results, it was concluded that both signal peptide was recognized by the *P. pastoris* secretory device. *Sfamy* was expressed extracellularly, using both modified and native signal peptides.

Co-expression of PDI in *P. pastoris* [*MS-Sfamy*] and Analysis of *Sfamy* Expression

PDI plays a role in the protein folding through the formation and isomerization of disulfide bond. The folding process is essential in order to assemble proteins into functional protein; those without proper folding will be destroyed by the cell secretion system. Based on structural modeling, Sfamy has four disulfide bonds; therefore the addition of protein folding factors are expected to increase the number of properly folded Sfamy, thus increasing Sfamy secretion.

Construction of pPIC3.5K-Pdi1 expression vector and cloning in E. coli *Top10F'*

The *Pdi1* gene was amplified from recombinant

vector that already contained *Pdi1*. The PCR primer was designed to amplify *Pdi1* with the addition of *Bam*H1 and *Eco*R1 restriction site at the 5 'end and the 3' end, respectively. PCR using 5'BamPDI and 3'EcoPDI primers will produce a ~1569 bp amplicon of the *Pdi1*. The PCR product was purified and ligated to pGemT vector and cloned into *E. coli* TOP10F. White colonies were characterized using the Rapid Disruption method [29] and restriction analysis (data was not shown). Subsequently, *Pdi1* gene was cut off with *Bam*H1 and *Eco*R1 enzyme, purified and ligated to pPIC3.5K vector to produce pPIC3.5K-*Pdi1* recombinant and cloned in *E. coli* TOP10F'. The pPIC3.5K-*Pdi1* recombinant plasmid was isolated by Qiagen Spin Plasmid Miniprep Test Kit (Qiagen) and characterized by sequencing analysis. Based on nucleotides sequence analysis and alignment with *Pdi1* on GeneBank (Acc D00842,1) it was concluded that *Pdi1* gene had successfully cloned and the nucleotides sequence of *Pdi1* gene was the same as in NCBI database (data not shown).

The pPIC3.5K vector is one of the expression vectors that have a gene marker resistant to ampicillin and kanamycin. The expression of Pdi1 is performed under the control of the promoter *AOX1*. The pPIC3.5K also has a HIS4 gene that is useful as a marker for integration to the *P. pastoris* chromosome. The pPIC3.5K is an integration plasmid, where integration can occur at His4 or *AOX1* loci. To obtain insertion at the His4 locus, the plasmid should be cut with *Sal*1 or *Bsp*E1 enzyme. This insertion will result in His^+ and Mut^+ phenotype (Invitrogen).

In this study, integration of Pdi1 occurs at the His4 locus by cutting plasmids using the *Bsp*E1 enzyme because *P. pastoris* [*MS-Sfamy*] genome already contained *MS-Sfamy* gene at the *AOX* locus. Fig. 9 shows the map of pPIC3.5K-*Pdi1* plasmid.

P. pastoris [*MS-Sfamy*] Transformation

The plasmid pPIC3.5K-*Pdi1* was used to transform *P. pastoris* [*MS-Sfamy*] (*P. pastoris* MS-20; His^-, Mut^+). Briefly, 2-5 µg of pPIC3.5K-*Pdi1* was linearized with *Bsp*E1 enzyme. The linearized plasmid was used to transform *P. pastoris* [MS-20] to yield *P. pastoris* [MS-20-*Pdi1*]. The growing *P. pastoris* transformant will have His^+, Mut^+ and geneticin resistance phenotypes. *P. pastoris* transformants then were screened using a selection medium containing histidine and methanol by growing on MM (minimal methanol) and MMH (minimal methanol + Histidine) medium.

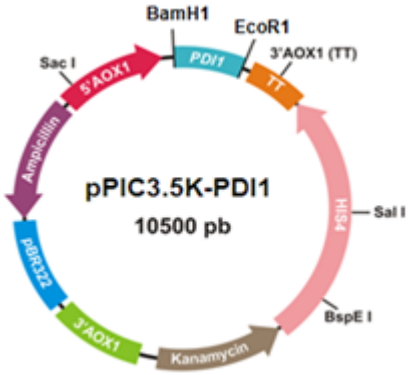

Fig 9. Map of pPIC3.5K-*PDI1* recombinant plasmid

Furthermore, the transformant with His^+ phenotype was screened for resistance to geneticin antibiotics and obtained some transformants with His^+ phenotype and resistant to geneticin. A geneticin resistant transformant screening was performed by transferring a grown transformant on a liquid YPD medium overnight to a solid YPD medium containing 0.25 mg/mL geneticin (Invitrogen). Screening of yeast resistance to geneticin cannot be done directly because the transformed cell takes time to express a number of resistance factors. Yeast grows much more slowly than bacteria; thus direct growth of a medium containing geneticin will cause some of the yeast transformant to die.

Expression and Characterization of *Sfamy* Recombinant

Fig. 10 shows that *Sfamy* activity on *P. pastoris* containing *Pdi1* was 2-times higher than *P. pastoris* which did not contain *Pdi1*. The highest activity of *Sfamy* on *P. pastoris* MS-20-*Pdi1* was obtained at 72 h that was 650.72 U/mL with a protein content of about 1.067 mg/mL, whereas in *P. pastoris* MS-20 the highest *Sfamy* activity was obtained at 48 h corresponding to 353.85 U/mL with a protein content of about 1.069 mg/mL. The higher specific activity of *Sfamy* produced by *P. pastoris* MS-20-*Pdi1* that was 609.85 U/mg compared

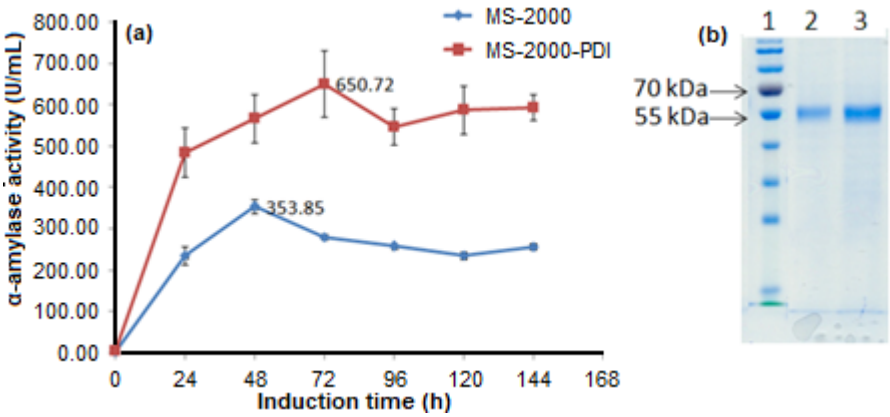

Fig 10. Comparison of Sfamy activity in *P. pastoris* MS-2000 and *P. pastoris* MS-2000-PDI**.** (a) Graph of *Sfamy* activity. (b) SDS-PAGE analysis; lane 1: protein marker, lane 2: Supernatant culture of *P. pastoris* MS-2000, lane 3: supernatant culture of *P. pastoris* MS-2000-PDI1

with *P. pastoris* MS-20 of 331.0 U/mg. SDS-PAGE characterization showed a *Sfamy* band with a molecular weight ± 55 kDa (Fig. 10).

Several studies had reported the effect of PDI co-expression on heterologous protein secretion with different results [19,31-32]. The variations in the obtained results were believed to be influenced by the amount of the disulfide bonds in the heterologous proteins and 3D protein structures. PDI is one of the protein folding factors catalyzing the formation of disulfide bonds between two cysteine residues. *Sfamy* has four disulfide bridges, so it needs some PDI to form the disulfide bond. If the PDI produced by *P. pastoris* is insufficient to form the disulfide bond, then *Sfamy* which has been translocated to the RE will not be folded correctly and will not be removed from the RE. The PDI co-expression in the recombinant *P. pastoris* was seen to increase the number of PDIs in *P. pastoris* so that the number of *Sfamy* that folds correctly will increase. PDI co-expression proved to increase *Sfamy*'s expression by 2-fold compared with non PDI-expression. Overall, a combination of genetic manipulation via the modification of signal peptides, an increase in gene copy number and PDI co-expression had increased *Sfamy* secretion by *P. pastoris* by 20-fold.

■ CONCLUSION

The combination of genetic manipulation, which includes modification of signal peptides, an increase in the number of copy of genes and PDI co-expression had resulted in a 20-fold increase of *Sfamy* secretion by *P. pastoris* over *P. pastoris* [*WT-Sfamy*] one copy.

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