

## Heterologous Expression of $\alpha$ -Amylase from *Saccharomycopsis fibuligera* R64 and Its Tyr401Trp Mutant in *Pichia pastoris*

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$\alpha$ -Amylase from *Saccharomycopsis fibuligera* R64 is a non-adsorbing raw-starch degrading enzyme, a unique characteristic. This character is difficult to explain in the absence of its three-dimensional structure. Here we discuss the expression of  $\alpha$ -amylase from *Saccharomycopsis fibuligera* in *Pichia pastoris* and the effect of site directed mutagenesis on its activity. A model based on the structure of its homologs suggested mutation of codon of Tyr401 into that of a Trp residue. An activity study using whole cells *P. pastoris* showed similar substrate degradation rates by cells carrying either the native or mutant amylase encoding gene. However, the purified enzyme of the mutant strain showed faster starch hydrolysis.

Key words:  $\alpha$ -amylase, *Pichia pastoris*, raw-starch degrading enzyme, *Saccharomycopsis fibuligera* R64, site directed mutagenesis

$\alpha$ -Amylase dari *Saccharomycopsis fibuligera* R64 adalah enzim pendegradasi pati yang tidak teradsorpsi ke pati mentah, suatu karakter yang unik. Karakter yang unik ini sulit untuk dijelaskan karena ketidakadaan struktur tiga dimensinya. Dalam artikel ini ditampilkan ekspresi enzim ini di ragi *Pichia pastoris* dan efek mutagenesis terarah terhadap aktivitas enzim. Model berdasarkan struktur homolognya menyarankan mutasi codon Tir401 menjadi Trp. Uji aktivitas secara langsung menggunakan sel *P. pastoris* menunjukkan sel inang yang membawa gen pengkode enzim natif dan mutan mampu mendegradasi pati, tetapi kemampuan mengikat pati mentahnya tidak membaik. Namun demikian, ketika menggunakan enzim murni, ditemukan bahwa mutan mampu menghidrolisis pati lebih cepat.

Kata kunci:  $\alpha$ -amilase, bioinformatik, enzim pendegradasi pati mentah, mutagenesis terarah, *Saccharomycopsis fibuligera* R64, studi struktural

$\alpha$ -Amylase (1,4- $\alpha$ -D-glucan glucanohydrolase, EC 3.2.1.1) belongs to the hydrolase family of enzymes, which hydrolyze 1,4- $\alpha$ -glycosidic bonds of starch or glycogen in a random manner, producing various lengths of oligosaccharides with reducing glycosidic groups in an  $\alpha$ -configuration (Janecek and Balaz 1992). The enzyme plays an important role in industries that use starch conversion, such as in glucose-syrup production and in the food, paper and textile industries, and in renewable energy development e.g. bioethanol production, or in biomass

degradation (Nielsen and Borchert 2000; van der Maarel *et al.* 2002; Shigechi *et al.* 2004). Biomass degradation requires an enzyme that is able to act on raw starch substrates, a characteristic that recently has been demonstrated for  $\alpha$ -amylase from the yeast *Saccharomycopsis fibuligera* R64 (SfR64) (Hasan *et al.* 2008).

A structure–function study revealed that  $\alpha$ -amylase from SfR64 (Sfamy) can degrade without being adsorbed onto raw starches (Hasan *et al.* 2008). This finding was rather unique because raw starch degrading  $\alpha$ -amylases are usually characterized by the presence of a starch binding domain, i.e. the C domain that interacts with the substrate (Mahovic and Janecek 2006). Structural investigation of raw-starch degrading

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amylolytic enzymes suggested that the capability to bind and to degrade raw-starch substrates is facilitated by a pair of tryptophan and/or of tyrosine residues, respectively (Sorimachi *et al.* 1997). The presence of these structural motifs in SfamyR64 is not yet clear, therefore structural studies of the enzyme is of paramount importance.

Purification of SfamyR64 from *S. fibuligera* is challenging because of the presence of glucoamylase that shares very similar characteristics (Ismaya *et al.* 2012), thereby requiring purification by hydrophobic interaction chromatography with low protein recovery. Heterologous expression of SfamyR64 appears to solve the issues with contamination with the glucoamylase and low protein production. Moreover, overexpression of SfamyR64 in *Pichia pastoris* allows modification of the enzyme by means of gene mutagenesis to obtain a SfamyR64 recombinant with the desired properties. Following this approach, the gene coding for SfamyR64 has successfully been cloned into the heterologous yeast *P. pastoris* KM71H (Natalia *et al.* 2015). Following that success, we overexpressed the enzyme also in *P. pastoris* GS115. Furthermore, the encoding gene was modified, resulting in expression of modified SfamyR64 that demonstrated faster degradation of raw starch substrates. This paper discusses overexpression of SfamyR64 in *P. pastoris* and site directed mutagenesis of codon of Tyr401 was changed into Trp, resulting in a recombinant enzyme that degraded the raw starch substrate better.

## MATERIALS AND METHODS

**Microbial Strain and Plasmids.** *Pichia pastoris* GS115 and *Escherichia coli* TOP10F' were obtained from Invitrogen (Carlsbad, CA – USA). The pPICZA and pGEMT vectors were obtained from Invitrogen (Carlsbad, CA – USA) and Promega (Madison, WI – USA), respectively. Restriction enzymes, Taq DNA polymerase, and T4 DNA ligase were obtained from Fermentas (Vilnius, Lithuania). Primers were synthesized by Research Biolabs (Singapore).

**Cloning and Expression of SfamyR64 in *Pichia pastoris*.** The gene coding for SfamyR64 has previously been cloned into YEp-Secretex for expression of recombinant SfamyR64 in *Saccharomyces cerevisiae* (Ismaya *et al.* 2012). The cloning was performed using the forward and reverse primers designed following the published SfamyHUT7212 sequence (Itoh *et al.* 1987) to result in

expression of the mature SfamyR64 (residues 26-494), and the genomic DNA of *S. fibuligera* R64 as the template. The gene was inserted between the *NdeI* and *XbaI* restriction sites. The YEp-Secretex-SfamyR64 plasmid was employed as the template for cloning into a vector for expression in *P. pastoris* in the current work. The gene coding for SfamyR64 with additional *EcoRI* and *XbaI* restriction sites was amplified by PCR, using GAATTCGTGACTCTATTCAAAGAGAAA and TCTAGACATGAACAATGTCAGAAGC as the forward and reverse primers, respectively. The PCR product was then ligated to the pGEMT vector to result in pGEMT-SfamyR64. Insertion of *SfamyR64* into pGEMT was confirmed by sequencing. The pGEMT-SfamyR64 plasmid was isolated and then digested with *EcoRI* and *XbaI*. The resulting *SfamyR64* fragment was sub-cloned into pPICZA vector at the same restriction sites to result in pPICZA-SfamyR64. This plasmid was then transformed into *P. pastoris* by means of the EasyComp™ method according to the manufacturer's instruction (Invitrogen, Carlsbad, CA – USA). The *P. pastoris* carrying pPICZA-SfamyR64 was screened on yeast peptone dextrose (YPD) plates supplemented with 100 µg mL<sup>-1</sup> zeocin and the overexpressed recombinant enzyme was designated as rSfamyR64.

**Production, Isolation, and Purification of rSfamyR64.** Production of the recombinant enzymes was conducted according to the protocol from Invitrogen (Invitrogen, Carlsbad, CA – USA). Briefly, a single colony of *P. pastoris* was inoculated in 10 mL buffered glycerol complex medium (BMGY) at 28 °C with a constant agitation of 200 rpm. After 18-20 h, the yeast cells were collected by centrifugation at 5 000 g and 4 °C for 10 min. The cells were resuspended in 250 mL buffered minimal methanol-histidine (BMMH) medium and grown at 28 °C at constant agitation of 200 rpm for 3 d with daily methanol supplementation. The recombinant enzyme in the overproduction media was separated from the yeast cells by centrifugation at 10 000 g for 5 min at 4 °C; the supernatant was designated as the crude extract. Enzyme activity was directly measured using this crude extract. The enzyme was recovered from the crude extract by precipitation with ammonium sulphate at 70% saturation (w/v) at 4 °C for 2-3 h with constant stirring. The precipitate was collected by centrifugation at 10 000 g at 4 °C for 10 min, the supernatant was decanted and the precipitate was resuspended in ±10 mL 20 mM potassium phosphate buffer, pH 6.0. The ammonium salts were removed by overnight (15-18 h) dialysis at 4 °C against

±1 L of the same buffer. The ammonium sulphate free fraction was used for the characterization and subsequent analysis.

**Characterization and Protein Analysis of rSfamyR64.** The characterization of the recombinant SfamyR64 was performed as previously reported (Hasan *et al.* 2008; Ismaya *et al.* 2013). Briefly, the enzyme activity was assayed using both soluble and raw starch substrates, based on measuring the formation of a starch – iodine complex (Fuwa 1954) or the production of reducing sugars (Miller 1959). The characterization involved establishing the optimum pH and temperature for reaction, the specific activity, adsorption onto substrates, and enzyme kinetics. All tests performed following procedures used with the wild type enzyme (Hasan *et al.* 2008; Ismaya *et al.* 2013). A qualitative assay was performed on YPD agar plates containing 1% or 2% starch and 0.5% methanol, where the residual starch was visualized with an iodine solution (Fuwa 1954). The expression of the recombinant proteins was also evaluated using an SDS-PAGE analysis.

**Bioinformatics Analysis.** Amino acid sequences of Sfamy analyzed were those of R64 (GenBank: HQ172905) (Ismaya *et al.* 2013), HUT7212 (GenBank: P21567) (Itoh *et al.* 1987), and KZ (GenBank: ADD80242.1) (Hostinová *et al.* 2010). However, only the amino acid sequence of SfamyR64 was employed in the further structural analysis. The amino acid sequences of the A/B and C domains of SfamyR64 were independently subjected to a BLAST analysis (Johnson *et al.* 2008) against the amino acid sequence of any proteins in the database and of proteins with known tertiary structure. *In silico* model of Sfamy was prepared based on the structure of *Aspergillus niger*  $\alpha$ -amylase. The model was built using the program SWISS MODEL, and subsequently checked and validated using the programs LSQKAB from the CCP4 package (Collaborative Computational Project Number 4 1994) and Procheck (Laskowski *et al.* 1993), respectively, and were finally manually evaluated using the program COOT (Emsley *et al.* 2010). The programs Procheck and MolProbity (Chen *et al.* 2010) were employed for validation. The graphic representation of the model was prepared by the program PyMOL (DeLano 2008). Residues important for the binding and degradation of raw starch substrates were evaluated by scanning for tryptophan and tyrosine residues and compared to the structures of  $\alpha$ -amylase from barley (PDB: 1P6W), human saliva (PDB: 1MFV) (Hostinová *et al.* 2010), *Thermoactinomyces vulgaris* (PDB: 1UH4) (Abe *et al.*

2004) and *Bacillus halodurans* (PDB: 2C3H) (Boraston *et al.* 2006).

**Site Directed Mutagenesis of the Gene Coding for Tyrosine to that of Tryptophan.** pPICZ $\alpha$ A-SfamyR64 was used as the template for site-directed mutagenesis, which was carried out using quick-change site-mutagenesis according to the manufacturer's protocol (Stratagene, La Jolla, CA – USA). The mutagenic forward and reverse primers employed were CCAGAAACGCCGCGTT-TGG-CAAGACT CAAGC and GCTTGAGTCTTG-CCA-AACGGCG GCGTTTCTGG, respectively. The PCR conditions included one cycle of one minute at 95 °C, and sixteen cycles of 30 s at 95 °C, one minute at 55 °C, ten minutes at 68 °C, and finally one cycle of five minutes at 68 °C. The PCR product was transformed into *E. coli* TOP10F', which was further screened against low-salt LB containing 25  $\mu$ g mL<sup>-1</sup> Zeocin and 50  $\mu$ g mL<sup>-1</sup> tetracycline. pPICZ $\alpha$ A-SfamyR64 Tyr401Trp was isolated from positive clones and the mutation was confirmed by nucleotide sequencing analysis. The mutated plasmid was then transformed into *P. pastoris* for the overproduction of rSfamyR64 Tyr401Trp, which was designated as mSfamyR64. Production, isolation, purification, and characterization of the Tyr401Trp mutant were performed in the similar manner to rSfamyR64.

## RESULTS

**Cloning and Overexpression of rSfamyR64 in *P. pastoris*.** The gene coding for SfamyR64 has successfully been cloned and overexpressed in *P. pastoris* GS115. *P. pastoris* colonies carrying pPICZ $\alpha$ A-SfamyR64 appeared to grow well on plates supplemented with soluble starch (Fig 1A). Clear zones around colonies indicate starch degradation by rSfamyR64, whereas the wild type yeast itself is unable to perform starch degradation. Furthermore, in expression tests at small scale, *P. pastoris* secreted 25 times more Sfamy than the natural host *S. fibuligera* R64 and six times more than *S. cerevisiae* (Fig 1B).

However, the amylolytic activity of rSfamyR64 decreased during the production of rSfamyR64, particularly after the third day. An SDS-PAGE analysis of rSfamyR64 indicated the appearance of additional protein bands of lower molecular masses, which had not been observed in the first three days (data not shown). This observation suggests that enzyme degradation occurred therefore the enzyme was

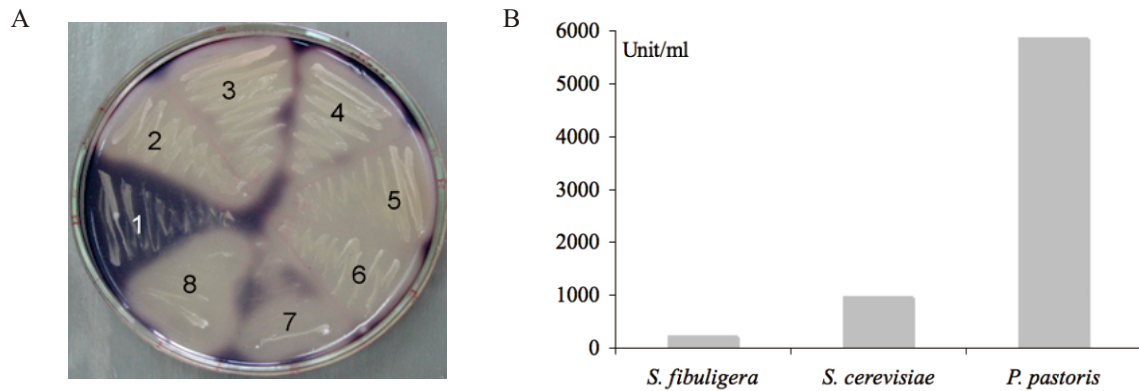


Fig 1 (A) Screening of *Pichia pastoris* colonies that carry SfamyR64 encoding gene: numbered 1 is the untransformed *P. pastoris*. (B) Comparison of SfamyR64 production with different hosts as compared to the natural producer.

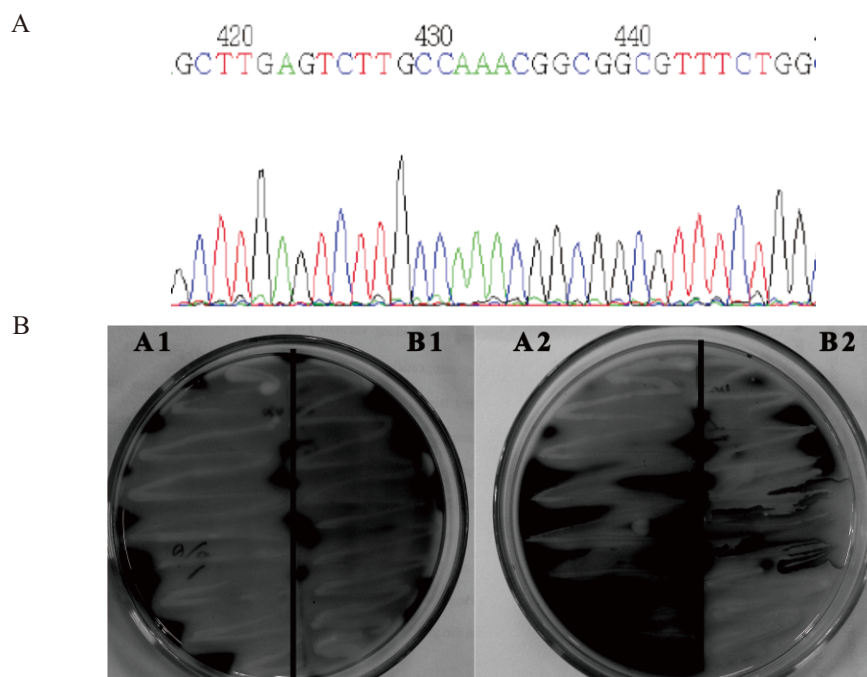


Fig 2 (A) Confirmation of Tyr401Trp mutation by sequencing analysis. (B) Qualitative measurement of activities of rSfamyR64 (A1, B1) and mSfamyR64 (A2, B2) on YTD plates containing 1% (A1, A2) and 10% (B1, B2) soluble starch.

harvested at the third day of fermentation.

The molecular weight of purified rSfamyR64 (at  $\pm 56$  kDa) appeared higher than that of the wild type SfamyR64 (at  $\pm 54$  kDa). Its optimum pH and temperature for activity were 6.0 and 50 °C, respectively. While the optimum temperature is the same as that of the wild type enzyme (from *S. fibuligera* R64), its optimum pH is slightly higher (up from 5.5).

**Site Directed Mutagenesis and Subsequent Cloning and Overexpression of the Mutant.** Mutation of codon for Tyr to that of Trp was confirmed by sequence analysis, which showed the presence of the

codon CCA at the position 430 (Fig 2A) instead of ATA in the anti-sense sequence (tryptophan is coded by TGG while tyrosine is coded by TAT). Further, mSfamyR64 was also expressed in *P. pastoris* at similar amount to rSfamyR64, as suggested by a qualitative enzyme activity assays (Fig 2A). Like rSfamyR64, the amylolytic activity decreased, also after the third day of fermentation. This result had been expected because the mutation was engineered not to alter the structure of the  $\alpha$ -amylase, the enzyme responsible for the amylolytic activity of the *P. pastoris* clone. Thus, mutation of Tyr401Trp has no effect on the amylolytic activity.

**Characteristics of the Tyr401Trp  $\alpha$ -Amylase. In**

order to understand the effect of the mutation at protein level, the activities and kinetics of the purified rSfamyR64 and mSfamyR64 were compared. Interestingly, the activity of mSfamyR64 on both soluble and raw-starch substrates was slightly higher than that of rSfamyR64. The highest activity of mSfamyR64 with soluble and raw-starches were 7.8 and 4.9 U mL<sup>-1</sup>, respectively, about ~10% higher than those of rSfamyR64 (7.1 and 4.5 U mL<sup>-1</sup>, respectively). This 10% increase was not observed during the qualitative amyolytic assay on plates because high expression of SfamyR64 recombinants had masked the assay. The optimum pH and temperature for both recombinant and mutant enzymes were the same.

The  $V_{max}$  values of rSfamyR64 and mSfamyR64 for soluble and raw-starches were identical, i.e. 7.7 and 2.1 U min<sup>-1</sup>, respectively. The  $k_{cat}$  values were also identical 1.1 per min and 0.5 per min for the soluble and raw-starches, respectively. The  $K_M$  of mSfamyR64 for soluble and raw-starches were 14.2 and 2.7 mg mL<sup>-1</sup>, respectively, lower than the  $K_M$  of rSfamyR64 at 15.2 and 2.8 mg mL<sup>-1</sup>, respectively. Consequently, the  $k_{cat}/K_M$  ratios of mSfamyR64 for soluble and raw-starches were slightly better than that of rSfamyR64, which were 0.07 and 0.17 mL (mg.min)<sup>-1</sup>, respectively (as opposed to 0.08 and 0.18 mL (mg.min)<sup>-1</sup>, respectively). This observation suggests that the higher activities of mSfamyR64 can be attributed to a better interaction with the starch substrates. Furthermore, similar to the wild-type SfamyR64, both rSfamyR64 and mSfamyR64 did not adsorb onto raw-starch. Thus, the mutation of a tyrosine residue to tryptophan was insufficient to alter the capability of the enzyme to bind raw starch. Subsequently, this mutation failed to improve the capacity of *P. pastoris* carrying mSfamyR64 to degrade raw starch.

## DISCUSSION

*P. pastoris* has widely been used in the production of recombinant enzymes (Macauley-Patrick *et al.* 2005) because of various advantages over other expression systems, especially its capability to perform post translational modification and high protein expression. Our results also demonstrate the superiority of this expression system over the well know yeast *S. cerevisiae*, and is even much more powerful than the natural producer *S. fibuligera*. This provides an excellent opportunity to perform genetic modification of the enzyme to obtain amyolytic enzymes and/or yeast strains with the desired

properties for applications. Furthermore, proteolytic degradation upon expression of recombinant proteins in *P. pastoris* has often been reported, and, in fact, is a major drawback in the use of the system (Potvin *et al.* 2012). To prevent this degradation, the enzyme was harvested after 3 d of fermentation.

Strains of *S. fibuligera* have been reported to secrete amyolytic enzymes with different thermal stability and raw-starch binding capacity. Amazingly, these variations originate from slight differences in the amino acid sequences in strains R64 (Ismaya *et al.* 2013), HUT7212 (Itoh *et al.* 1987), and KZ (Hostinová *et al.* 2010). Mutations in the amino acid sequence of glucomyR64 remarkably cause the enzyme to adopt both the raw-starch binding capability of HUT7212 and the thermal stability of KZ (Natalia *et al.* 2011). Furthermore, the mutations in the amino acid sequences did not include bulky residues necessary for the binding of starch or raw-starch substrates; therefore, both Sfamys from strains HUT7212 and KZ are likely also a raw-starch degrading but non-adsorbing enzymes.

We have previously shown that SfamyR64 is a raw-starch degrading but non-adsorbing enzyme (Hasan *et al.* 2008). This finding was later confirmed with a similar study performed with SfamyKZ (Hostinová *et al.* 2010); this characteristic appears to be conserved among Sfamys. Structurally,  $\alpha$ -amylase consists of three domains sequentially named A, B and C. The A/B domain is responsible for the catalytic activity (Janecek *et al.* 1997), whilst the C domain is organized separately and is hypothesized to be the substrate binding domain (Janecek and Sevcik 1999; Hasan *et al.* 2008). The model of SfamyR64 (Fig 3) shows the arrangement of domains in the enzyme.

Two binding sites for raw-starch substrate were observed in the structure of glucoamylase from *A. niger* (Sorimachi *et al.* 1997), which involve a rigid pair of tryptophan residues (binding site 1) and a flexible pair of tyrosine residues (binding site 2). Two tryptophan residues in the first binding site are responsible for the binding of raw-starch, whilst one of the tyrosine residues in the second binding site is responsible for guiding linear starch chain to the active site, and is thereby responsible for raw-starch degradation (Penninga *et al.* 1996). In SfamyR64, Tyr401 and Tyr488 have similar positions to serve in carbohydrate binding, but tryptophan does not. As the presence of the two binding sites for the binding of carbohydrate is fundamental (Giardina *et al.* 2001), the absence of the tryptophan residue (the binding site 1)

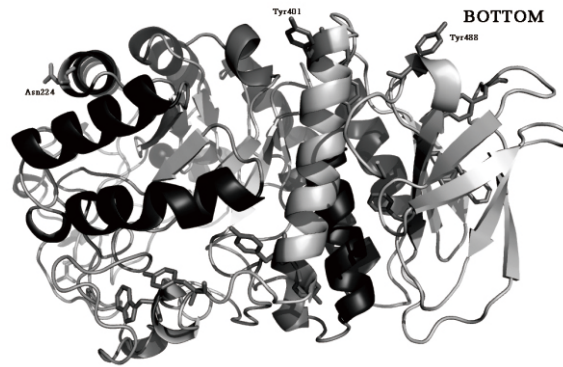


Fig 4 Ribbon representation of the structure of Sfamy R64.

explains the inability of SfamyR64 for adsorption onto raw-starch granules. Therefore, a Tyr401Trp mutant was prepared in order to introduce the capability of raw-starch degradation. Introducing the raw-starch binding capability to carbohydrate acting enzymes is an attractive proposition, because it increases the local concentration of the substrate in the active site, thereby increasing the rate of the starch hydrolysis reaction (Juge *et al.* 2002). This alteration increases the capacity of the enzyme and subsequently the *P. pastoris* clone to degrade raw-starch.

In conclusion, we have successfully produced SfamyR64 in *P. pastoris* and its derivative that is engineered to degrade raw-starch. Although the improvement was not obvious upon testing with whole cells, the engineered enzyme catalyzed the raw-starch degradation at a considerably higher rate. This result initiates further work to obtain *P. pastoris* carrying gene coding for SfamyR64 with the desired properties.

### ACKNOWLEDGMENT

We thank the Indonesian Research Council (DRN) and the Directorate General for Higher Education (DIKTI) of the Ministry of National Education for their financial support.

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