

## Construction and Expression of L-Arabinose Isomerase (L-AI) in Cell- Surface of *Pichia pastoris*

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**Abstract**— *araA* gene encode L-arabinose isomerase (L-AI). It is an enzyme converting D-galactose to D-tagatose. D-tagatose is a hexoketose monosaccharide sweetener, which is an isomer of D-galactose and rarely found in nature. It is a potential sweetener which has low calorie. The aim of this study is to construct *araA* gene in the expression vector pJ912-AGa and expression the protein in the cell-surface of *Pichia pastoris* GS115. Both vector pJ912-AGa and *araA* gene was digested with *SalI* and *Kpn2I* restriction enzymes then was ligated. The ligation solution had been successfully introduced into *Escherichia coli* DH5a. Vektor pJ912-AGa-*araA* was successfully integrated into the genome of *P. pastoris* GS115. Genetically stable transformed cells have been obtained after selection on zeocin medium up to 1000 µg/mL zeocin. We had successfully synthesized L-AI protein in the *P. pastoris* GS115. Observation using fluorescence microscopy has proven that successful transformed cell emit green fluorescence derived from the interaction of functional His6 protein and rabbit polyclonal to 6×His tag<sup>®</sup> and showed that L-AI protein was expressed successfully in cell-surface of *P.pastoris*.

**Keywords**— *araA*; L-Arabinose isomerase; Yeast surface display; *Pichia pastoris*

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### I. INTRODUCTION

D-Tagatose is a hexoketose monosaccharide sweetener, which is an isomer of D-galactose and rarely found in nature [1]. It occurs naturally in small quantities in *Sterculia setigera* gum, and it is also found in dairy products [2], [3]. The sweetness of D-tagatose is 92% of sucrose. This sugar has no cooling effect or aftertaste and is involved in browning reaction. The taste and properties of this sugar are similar to those of sucrose. In addition, it has zero available calory, no laxative effect, and toothfriendly property [4]. Thus, D-tagatose can be used as a low-calorie sweetener in a wide variety of foods, beverages, health foods, and dietary supplements [5].

Recently, there has been great interest in the biological manufacture of D-tagatose from D-galactose. Several enzymes involved in the biotransformation of D-tagatose have been investigated [6]-[9]. L-arabinose isomerase (L-AI) is considered to have the most potential use for D-tagatose production, since it can catalyze the isomerization of D-galactose to D-tagatose and convert L-arabinose to L-ribulose, based on the similarity in configuration of the substrates [10].

Thermophilic L-AI has been reported possessing a catalytic activity for conversion of D-galactose to D-tagatose. Generally, isomerization process performed at high temperature (>70°C) offers several advantages, such as higher conversion yield, faster reaction rate, and lower viscosity of the substrate in the product stream [11]. Many research have reported the thermophile L-AI producing bacteria, i.e. *Bacillus stearothermophilus* US100 [12], *Geobacillus stearothermophilus* [13], *G. thermodenitrificans* [14], *Thermus* sp. [15], *Thermoanaerobacter mathranii* [16], *Bacillus coagulans* [17], *Enterococcus faecium* [18], *Thermotoga maritima* [19], and the acidic L-AI from *Alicyclobacillus acidocaldarius* [20]. Moreover, those of L-AIs had been purified and characterized. L-AI from *G. stearothermophilus* (GSAI) has the highest level of tagatose production and productivity. The production of tagatose is about 230 g/L [21] and the productivity is about 54 g/L/h [22] using a bioreactor containing immobilized GSAI. These results approach commercial production criteria.

Cell surface display allows expression of proteins or peptides on the surface of cells in a stable manner, using the surface proteins of phage [23] [24], bacteria [25] [26], yeast [27-28], or even mammalian cells [29] as anchoring motifs. The first surface display was developed in the mid-

1980s by Smith, who displayed peptides and small proteins on the surface of a bacteriophage [30]. Cell-surface display is a novel technique which is widely used for development a whole cell biocatalyst [31][32]. This system utilize the cell as a carrier for immobilized enzyme [33], i.e. the protein interest which is fused to the cell wall protein, thus the strain developed produces the enzyme as a fused protein to the cell wall [34]. Biocatalyst production via cell-surface display potent to be the most cost-effective method because there is no need for cell disruption, protein purification and enzyme immobilization. In fact, by growing and inducing the host cells, the enzyme will be produced as an immobilized protein on cell-surface and harvested cell could be directly used as biocatalyst. Enzyme-displaying cell may be reused several times as biocatalyst [35].

Yeast cell-surface display system was first described for *Saccaromyces cerevisiae* [36]. Recently, the methylotropic yeast *Pichia pastoris* has also been employed as a host for cell-surface display [37]. The major advantages of *P. pastoris* over *S. cerevisiae* as a cellular host include prevention of hyperglycosylation, integration of multicopy of transforming DNA into genomic DNA and formation of stable transformants, and its higher protein production [38]. The cell-surface display system for *P. pastoris* was first reported in the work of fused *Kluyveromyces* yellow enzyme to the C-terminal half of *S. cerevisiae*  $\alpha$ -agglutinin which is displayed on *P. pastoris* cell-surface [39]. Many proteins have been expressed in the surface cell of *P. pastoris*, including Lipase B from *Candida Antarctica* [40], Lipase from *Rhizopus orizae* [41], mPmRab7 and pVP28 protein [42].

Here in, we constructed a *P. pastoris* cell-surface display system based on *S. cerevisiae*  $\alpha$ -agglutinin cell wall protein and studied cell-surface display of GSAI by *P. pastoris*.

## II. MATERIALS AND METHODS

### A. Strains and growth media

The *E. coli* DH5 $\alpha$  strain (Invitrogen) was used as a host for DNA manipulations. The strain was cultured in low salt Luria Bertani (LSLB) medium (1% tryptone, 0.5% NaCl, and 0.5% yeast extract plus 2% agar in plates) by using 25  $\mu$ g/mL zeocin (Invitrogen) for the selection of transformants. The *P. pastoris* GS115 strain (His4, AOX1) (Invitrogen) was routinely cultured in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose plus 2% agar in plates), and supplemented with 1 M sorbitol and 100  $\mu$ g/mL zeocin for the selection of transformants.

### B. Construction of the expression vector

The complete open reading frame of GSAI coding gene (*araA*) was PCR-amplified by Platinum Tag DNA-polymerase (Invitrogen) using pET21b-GSAI as template. Primers were design according to the sequence of *araA* gene and multiple cloning site of pJ912-*AGa*. The *araA* gene was amplified by PCR using the primers PPAI\_F: 5'-GCGTCGCATGCATCACCATCACCATCACATGCTGTCATTACGTCCTTATGAATTTTGG-3' (contains *SalI* restriction site at the 5'-end and polyhistidine (6 $\times$ His) tag) and PPAI\_R: 5'-GTCACTCCGGACCGCCCCGCCAAAATACTTCATTCCATC-3' (contains *KpnI* restriction site at 5'-end) with the

following programs: initial denaturation for 2 min at 94°C; followed by 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and extension for 2 min at 72°C; and final extension for 5 min at 72°C. The resultant PCR products were digested with *SalI* and *KpnI*, and cloned into pJ912-*AGa* vector by using T4 ligase (Thermo Scientific), respectively. The resultant plasmids were named as pJ912-*AGa-araA*.

### C. Transformation of *E. coli* DH5 $\alpha$

The pJ912-*AGa-araA* was used for transformation of *E. coli* DH5 $\alpha$  by heat shock method. The transformant mix was spread on LSLB agar medium containing 25  $\mu$ g/mL zeocin, and incubated overnight at 37°C. After transformation, each colony was cultured into 2 mL LSLB medium containing 25  $\mu$ g/mL zeocin overnight at 37°C with shaking at 250 rpm. Further, plasmid DNA from each culture was isolated by miniprep technique using QIAprep spin miniprep kit (Qiagen). The authenticity of the recombinant plasmid was confirmed by restriction analysis, PCR analysis and sequencing (1st BASE, Selangor, Malaysia) [43].

### D. Transformation of *P. pastoris* and selection of transformants

Single colony of *P. pastoris* GS115 was cultured into 100 mL YPD medium at 30°C with shaking at 250 rpm until an OD<sub>600</sub> of 1.3. The cells were then centrifuged at 5000 rpm for 5 min at 4°C, and the pellets were washed with 25 mL ice-cold sterile milli-Q water. This washing step was repeated twice. Further, the pellets were resuspended with 200  $\mu$ L ice-cold sterile 1 M sorbitol medium.

The yeast expression library vectors were linearized by *SacI* digestion and used for transformation of *P. pastoris* GS115 by electroporation method described in EasySelect *Pichia* expression kit user manual (Invitrogen). A 20  $\mu$ g purified plasmid was digested with 100 U of *SacI* at 37°C overnight. A 80  $\mu$ L GS115 cells were then mixed with approximately 5-10  $\mu$ g *SacI*-linearized pJ912-*AGa-araA* plasmid, and subsequently transferred to an ice-cold 0.2 cm electroporation cuvette (Bio-Rad, Hercules, California, USA) and incubated on ice for 5 min. Electroporation process was performed by using Genepulser electroporation system (Bio-Rad, Hercules, California, USA) and the manufacture setting for *P. pastoris* was used, i.e. under the following conditions: 1977 V, 25  $\mu$ F, 200  $\Omega$ , and 4.5 ms. Immediately after the pulse, 150  $\mu$ L ice-cold sterile 1.M sorbitol was added to the cuvette, and the solution was then transferred to 1.5 mL tube and incubated for 60 min at 30°C. After that, 100  $\mu$ L YPD medium was added to the tube and incubated for 120 min at 30°C. The cells were plated onto YPDS agar medium (1% yeast extract, 2% peptone, 2% glucose, 2% Bacto agar, and 1 M sorbitol) containing 100  $\mu$ g/ $\mu$ L zeocin in 5 different quantities of culture which are 25, 50, and 100  $\mu$ L. The plates were incubated at 30°C for 4-10 days. Zeocin-resistant clones were picked up and transferred to YPD agar medium containing 200, 500 and 1000  $\mu$ g/mL zeocin for determination of the copy number of integrants. The subsequent comparisons of secreted proteins were only made between transformants with approximately the same copy number as determined by the same concentration range of

drug resistance against zeocin. Zeocin-resistant clones were PCR-screened for integration of the plasmid construction into the yeast genome.

#### E. Purification of chromosomal DNA from *P. pastoris* GS115 transformants

The method which was used to purify the chromosomal DNA from the yeast was based on the smash and grab DNA miniprep method [5]. Colonies of the transformants were replated onto YPD agar medium containing 100 µg/µL zeocin and incubated for 2 days at 30°C. A 5 mm diameter glass beads were washed in 30% HCl, and subsequently milli-Q water, and autoclaved. A breaking buffer, composing of 10 mM Tris buffer at pH 8.0, 1 mM EDTA at pH 8.0, 100 mM NaCl, 1% SDS, and 2% Triton X-100, was prepared. A 10 mL culture of *P. pastoris* GS115\_pJ912-AGa-araA was grown overnight at 30°C, and the cells were then harvested by centrifugation at 5000 rpm for 2 min at room temperature. The cells were placed in an eppendorf tube and resuspended in 200 µL breaking buffer and 200 µL PCI (phenol, chloroform, and isoamyl alcohol), and 0.25 g glass beads were then added. The tube was then vortexed at top speed for 10 min at room temperature. A 200 µL TE buffer (10 mM Tris at pH 8.0 and 1 mM EDTA at pH 8.0) was added, and the tube was vortexed for 10 sec. The tube was then centrifuged at 10.000 rpm for 10 min at room temperature. The aqueous phase was added to a fresh eppendorf tube, and the DNA was precipitated using ethanol precipitation method. The pellets obtained through the precipitation were resuspended in 50 µL DNA/RNase free water.

#### F. Expression of recombinant *P. pastoris* clones

A 100 mL of buffered glycerol-complex medium (BMGY, 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM KH<sub>2</sub>PO<sub>4</sub> at pH 6.0, 1.34% (w/v) yeast nitrogen base without amino acids, 4x10<sup>-5</sup>% (w/v) biotin, and 1% glycerol (w/v)) was inoculated using a single colony in a 250 mL flask. The flask was incubated at 30°C in a shaking incubator at 200 rpm until an OD<sub>600</sub> of 3. The cells were then harvested by centrifugation at 3000×g for 5 min at room temperature, and resuspended in buffered methanol-complex medium (BMMY, the same media as BMGY but 1% methanol (v/v) replaced for glycerol) to an OD<sub>600</sub> of 10. The flask was then covered with 2 layers of sterile gauze, and incubated at 25°C in a shaking incubator at 200 rpm. To maintain induction, 100% methanol was added to the culture to a final concentration of 0.5% every 24 h. *P. pastoris* GS115/His<sup>+</sup>Mut<sup>+</sup> Albumin (Invitrogen) strains were included in expression experiments and used as negative control, respectively. Samples of culture were taken after 72 h and analyzed for expression.

#### G. Immunofluorescence microscopy analysis

Portions (10 µL) of cell cultures were added to 500 µL of TBS (50 mM Tris-HCl, 150 mM NaCl [pH 7.5]) and centrifuged for 3 min at 5000×g at 4°C. Pellets were resuspended in 200 µL of TBS and 3 µg of specific, FITC-conjugated rabbit polyclonal antibody to His<sub>6</sub> tag (Abcam) was added to the suspensions, followed by incubation for 2 h at room temperature with constant shaking at 100 rpm. The cell were then washed with 200 µL of 0.1% TBST and

resuspended in 300 µL of TBS [44]. For immunofluorescence microscopy, slides were prepared from 10 µL of cell suspensions, and observed by Zeiss Axio Imager.Z2 fluorescence microscope (Zeiss, Oberkochen, Germany).

#### H. Immunomagnetic screening analysis

Portions (25 µL) of cell cultures were placed in a microtube 1.5 mL with 25 µL *Pure Proteome*<sup>TM</sup> Nickel Magnetic Beads (Milipore Corporation, Billerica; Massachusetts, USA). After vortexing, the sample was incubated at room temperature rotating slowly for 1 h to allow attachment of *P. pastoris* recombinant to the magnetic beads. Following incubation, the beads were separated from the cell suspension using magnetic particle concentrator. The residual liquid was pipetted off and the beads were washed with binding buffer solution. The sampel was rotated slowly for 10 min at room temperature. This washing step was repeated two times. The magnetic beads were finally resuspended in 150 µL of binding buffer and detected using either cultural immunofluorescence techniques as described previously.

#### I. Extraction and Analysys of cell surface protein

Cell cultures were collected by centrifugation and washed with buffer A (20 mM Tris-HCl pH7.5, 20 mM NaCl, and 5 mM MgCl<sub>2</sub>). Washed cells were incubated with Cellic® Ctec2 (Novozymes, Krogshoejvej, Bagsvaerd, Denmark) in 100 mM sodium acetate buffer, pH 5.2 at room temperature for 24h by gently agitation. Extracted protein were precipitated using Acetone and These precipitated protein were stored at -20°C and prepared for SDS-polyacrylamide gels and western blot analysis.

#### J. SDS-Polyacrylamide Gels

Portions (10 µL) of precipitated protein were added to 10 µL of Laemli buffer and were placed in a 100°C (boiling) water bath for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [45] on 12% polyacrylamide gels.

### III. RESULTS AND DISCUSSION

#### A. PCR and recombinant strain development

L-AI encoding gene (*araA*) from *G. stearothermophilus*. The marine bacterial strain *G. stearothermophilus* isolated from Tanjung api, Poso was found in the sea around a mountain. This bacterium lives at high temperature, so that it has potency to produce a thermophile L-AI [46]. Generally, isomerization is performed at high temperature, so that thermophile L-AI is suitable for this process. Isomerization at high temperature offers several advantages, such as higher conversion yield, faster reaction rate, and lover viscosity of the substrate [11]. Previous study of L-AI from *G. stearothermophilus* (GSAI) found that GSAI is suitable for commercial production of D-tagatose because it has high conversion of D-galactose to D-tagatose [21, 22].

*araA* was cloned using PCR technique. The primers were designed base on the sequence of *araA*, and containing restriction-enzyme sites at the end of encoding sequence for insertion into expression vector pJ912-AGa. To obtain highly stable expression strain, expression vectors are

usually integrated into the genome of *P. pastoris* [47]. *P. pastoris* has the following main advantages: first, extremely high yield of intercellular protein; second, very high levels of secretion into an almost protein-free medium; third, ease of fermentation to high cell density; and fourth, genetic stability and scale-up without loss of yield [48]. In this study, we used pJ912-AGa as - expression vector. The pJ912-AGa encoding *Sh ble* gene from *Streptoalloteichus hindustanus*, coding for a zeocin resistance protein. Zeocin can be used for selection in *E. coli* and *P. pastoris*. This vector is based on strong, methanol inducible AOX1 promoter and terminator spaced by a multiple cloning site for cloning of the gene of interest. Targeted integration of this plasmid into the AOX1 genomic locus is promoted by linearization of the vector within the AOX1 promoter region. Nonetheless, linearization at the AOX1 terminator is also an option [49]. A pUC origin of replication in this vector enables plasmid replication and maintenance in *E. coli*. This vector is also available with  $\alpha$  factor signal peptide, for production secreted recombinant protein, and AGa gene encoding  $\alpha$ -agglutinin for anchoring protein on the cell-surface (DNA 2.0). There are many advantages with anchoring protein on the cell surface, in which protein are genetically displayed on the cell surface, are easy reproduction of the displayed biocatalysts and easy separation of product from catalyst.

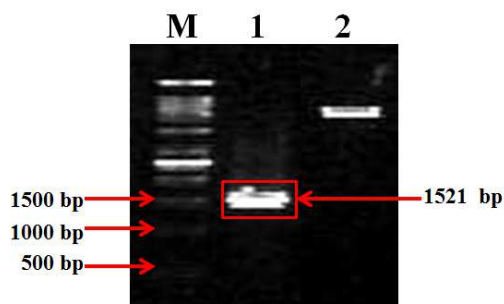


Fig. 1 Result of isolation and PCR amplification on *araA* gene. Lane M: Marker; Lane 1: Amplification of *araA* gene; 2: pET21b-GSAI

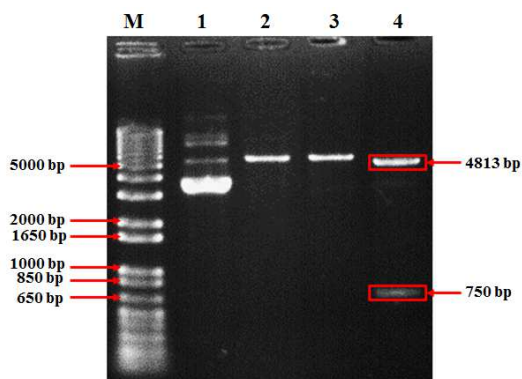


Fig. 2 Restriction analysis of pJ912-AGa. Lane M: Marker, Lane 1: isolation of pJ912-AGa. Lane 2: pJ912-AGa digested with *SalI*. Lane 3: pJ912-AGa digested with *Kpn2I*. Lane 4: pJ912-AGa digested with *SalI* and *Kpn2I*.

Plasmid construction was performed to obtain the recombinant plasmid carrying *araA* gene. The *araA* gene was first amplified using pET21b-GSAI as template and result showed only single band was estimated size of 1521 bp (Fig. 1). The expression vector was prepared by digestion of pJ912-AGa using *SalI* and *Kpn2I* restriction enzymes (Fig. 2). The *araA* gene was also digested using the same enzymes

(figure not shown). *araA* gene was inserted in pJ912-AGa vector. Then the construct was subsequently transformed into competent *E. coli* DH5 $\alpha$  cells and cultured in LSLB media containing zeocin 25  $\mu$ g/mL. Insertion of *araA* gene into vector pJ912-AGa resulted in around 25 colonies (Fig. 3). In addition, some colonies as positive control were obtained. The resultant colonies were evaluated for the true insert size by two different enzymatic digestions, PCR on colony extracted plasmids and DNA sequencing analyses.

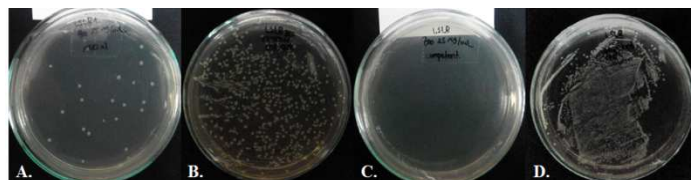


Fig. 3 *E. coli* transformant colonies. A: *E. coli* with pJ912-AGa-*araA*; B: *E. coli* with pJ912-AGa uncut; C: control negativ; D: control positive

The recombinant plasmid obtained was named pJ912-AGa-*araA*. Restriction analysis was carried out determine the actual size of expression vector and insert DNA. Fig. 4 showed restriction of recombinant plasmid using *SalI* and *NcoI* enzymes, resulting DNA bands with size of 6334 bp with single restriction, 3412 bp and 2992 bp in double restrictions (Fig. 4) which corresponded to the theoretical size of insert DNA and expression vector.

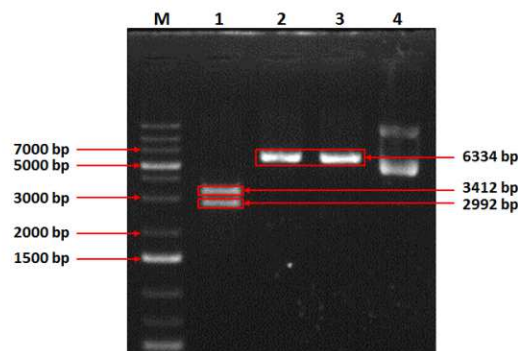


Fig. 4 Restriction analyses of pJ912-AGa-*araA*. Line M: Marker; Line 1: pJ912-AGa-*araA* restriction using *SalI* and *NcoI*; Line 2: pJ912-AGa-*araA* restriction using *SalI*; Line 3: pJ912-AGa-*araA* restriction using *NcoI*; Line 4: pJ912-AGa-*araA* uncut.

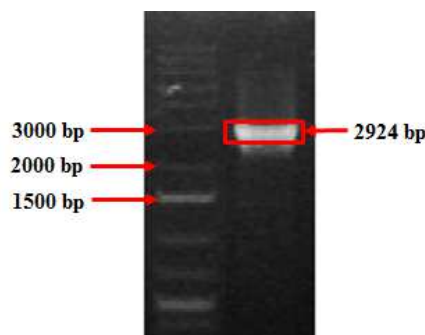


Fig. 5 PCR analysis of pJ912-AGa-*araA* using AOXI primers.

The PCR analysis was perform using AOXI primers. These primers were used to determine the construct of gene within the pJ912-AGa plasmid. Thus, the PCR product would consist of AOX promoter,  $\alpha$  factor signal peptide, and *araA* gene. Fig. 5 showed a DNA band of approximately



2924 bp, which corresponds to the theoretical size of desirable fragment. Based on DNA sequencing analysis (data not shown), there was no mutation in DNA encoding L-AI. After all of analyses conducted, it could be concluded that the recombinant plasmid was successfully constructed (Fig 6.).



Fig. 6 Schematic description of gene fusion construct pJ912-AGa-araA plasmid. 5'AOXI: promoter for alcohol oxidase gene;  $\alpha$ -factor: *S. cerevisiae*-derived secretion signal sequence; His<sub>6</sub>: polyhistidine tag; araA: gene encoding L-arabinose isomerase; FlagTag: enterokinase restriction site; AGa: C-terminal half of AGa gene; Stop: stop codon; AOXI TT: translation terminator sequence.

### B. Transformation of *P. pastoris* GS115 with pJ912-AGa-araA

*P. pastoris*-compatible vectors are designed for homologous integration into AOX1 locus. Linear DNA can generate stable transformants of *P. pastoris* via integration or homologous recombination between the transforming DNA and region of homology within the genome [50]. Recombinant plasmid is integrated into the genome of *P. pastoris* via the mechanism of homologous recombination by utilizing the AOX1 promoter sequence similarity between *P. pastoris* genome and vector pJ912-AGa. Therefore, before transformation of yeast cells for protein production, restriction mapping was carried out by using restriction enzyme *Sac*I (Fig. 7). For creating a stable recombinant, homologous regions between pJ912-AGa-araA and yeast genome were applied. Recombinant plasmid linearization process is one of important things in the transformation of *P. pastoris* because linearized recombinant plasmid can stimulate the recombinant plasmid recombination when plasmid is integrated into the genome of *P. pastoris*.

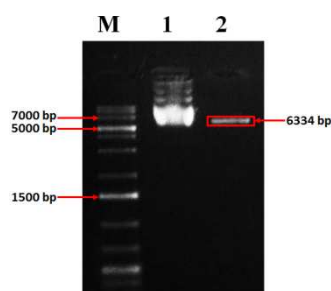


Fig. 7 Restriction analysis of pJ912-AGa-araA. Lane M: Marker; Lane 1: pJ912-AGa-araA uncut; Lane 2: pJ912-AGa digested with *Sac*I.

The linear recombinant plasmid was transformed into yeast cells by electroporation, so that the recombinant plasmid could be stably integrated into the yeast genome and express the protein. The principle of electroporation method is to use an electric shock to enlarge the pores of the cell membrane, thus increasing membrane permeability. An electrical signal will induce enlargement of the membrane pores, so that the molecules of DNA can enter the cell. The transformation process yielded 107 individuals transformed

colonies (Fig. 8). Cell-growth state, cell density, incubation time, medium used influence the transformation efficiency.

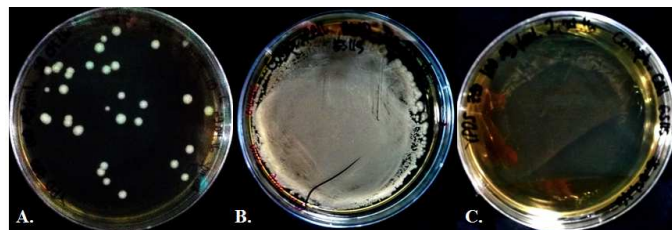


Fig. 8 *P. pastoris* transformant cells. A: *P. pastoris* with pJ912-AGa-araA; B: control positive; C: control negative

Some expression vector for *Pichia* can increase the number of gene copies in *P. pastoris*, so that the amount of expressed protein will be higher. The pJ912-AGa vector also carries zeocin resistance gene, so that the selection of transformants carrying multiple copies of integrated vector can be conducted. Genetic stability analysis was selected from single colonies growing on YPD agar medium containing 100, 200, 500, and 1000  $\mu$ g/mL zeocin, respectively (Fig. 9). YPD agar medium without zeocin was also used as control. Fig. 6 showed that all colonies look stable in medium with zeocin up to 1000  $\mu$ g/mL. Assuming that the *Sh ble* gene is incorporated in the same ratio as the AOX1 TT sequence, an estimated 1 copy (minimum) of the gene *Sh ble* zeocin resistance is required for growth at 100  $\mu$ g/mL zeocin, 4 copies at 500  $\mu$ g/mL, 9 copies at 1000  $\mu$ g/mL and clones with as many as 17 copies of gene are found from medium with highest antibiotic concentration of 2000  $\mu$ g/mL [51]. Colony PCR was conducted toward the transformants to verify if the expression cassette had been integrated into the AOX1 gene (Fig. 10)

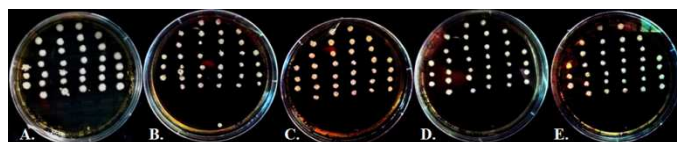


Fig. 9 Screening of genetically stable transformed yeast cells on zeocin plates containing various concentration of zeocin. A: 0  $\mu$ g/mL (as control); B: 100  $\mu$ g/mL; C: 200  $\mu$ g/mL; D: 500  $\mu$ g/mL; E: 1000  $\mu$ g/mL.

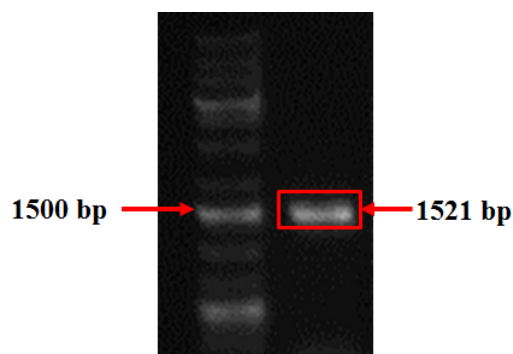


Fig. 10 PCR colony analysis of *P. pastoris* transformant

Observation under fluorescence microscopy revealed that transformed *P. pastoris* cell exhibited green fluorescence at the cell-surface of *P. pastoris* transformants (Fig. 11). The fusion protein was constructed with a hexa-His at the N-terminal of gene. Hexa-His is widely used in production of protein to facilitate purification and detection of the desired

protein [52]. To For detection of the protein on the cell surface, it was confirmed by immunofluorescence labeling of transformed cells and then analyzed by fluorescence microscopy. The observed fluorescene in the cell surface indicated that hexa-His and the desired protein were localized and displayed on the cell surface. The fuctionality of protein L-AI was validated by fluorescence microscopy of the *P. pastoris* transformants. Localization of fusion protein was visualized using FITC (fluorescein isothiocyanate)-conjugated rabbit polyclonal antibody to His<sub>6</sub> tag (Abcam). The FITC fluorescene signal was detected at the cell surface from the His-tag labeling of the fusion protein, confirming successful membrane localization of fusion protein.

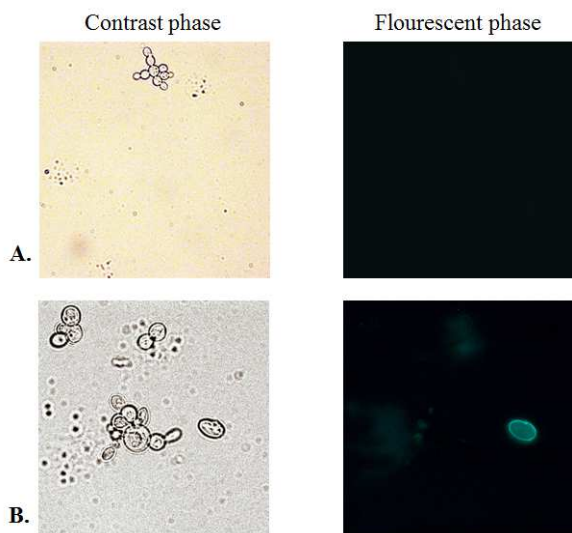


Fig. 11 Confocal microscopy of transformed and non transformed *P. pastoris* cells on the contrast (left) and fluorescent (right) phases. A: non transformed cells as control; B: transformed cells show fluorescene on the surface cell.

To confirm the protein was expressed on the cell surface of *P. pastoris*, it was detected by immuomagnetic technique using *Pure Proteome™ Nickel Magnetic Beads* combine with FITC-conjugated rabbit polyclonal antibody to His<sub>6</sub> tag (Abcam). This magnetic beads can be used to screening and purify polyhistidine-tagged recombinant protein. It has developed para-magnetic affinity media for the purification of recombinant, His-tagged protein based on the well established nickel ion/histidine interaction. Observation under fluorescence microscopy revealed that transformed *P. pastoris* cell attached to the surface of beads and exhibited green fluorescene at the *P. pastoris* recombinant cells on surface of *Pure Proteome™ Nickel Magnetic Beads* (Fig. 12).

Polyacrilamide gel electrophoresis and hybridization analysis support the results of an observational analysis of hexa-His and desired protein under the microscope. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used for analysis of soluble and insoluble protein. Analysis of the protein profile of the cell-free supernatant showed several bands of protein with molecular weights different (Fig. 13). The protein bands are the secreted protein of *P. pastoris* during ongoing overproduction.

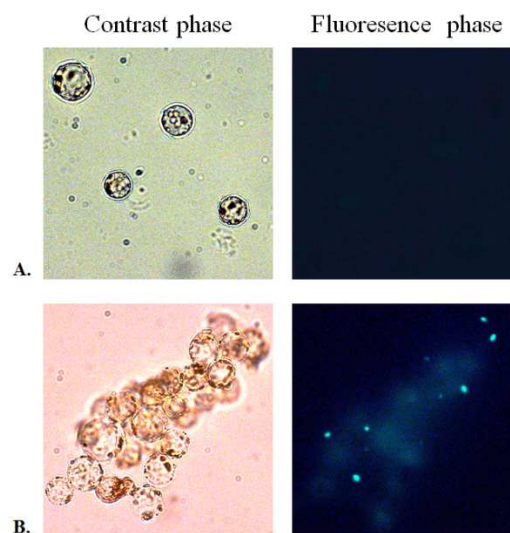


Fig. 12 Microscopic observation of interaction between cells, magnetic beads and antibody fluorescene on the contrast (left) and fluorescent (right) phases. A: non transformed cells as control; B: transformed cells bind to magnetic beads and show fluorescene on the surface cell.

Characterization of protein based on molecular weight using SDS-PAGE indicates that L-AI recombinant has a molecular weight approximately 91 kDa, while the molecular weight of native protein is 56 kDa. Differences in molecular weight due to the additional fragments of His-Tag, Flag-Tag, and  $\alpha$ -Agglutinin located at the C-terminal end of L-AI.

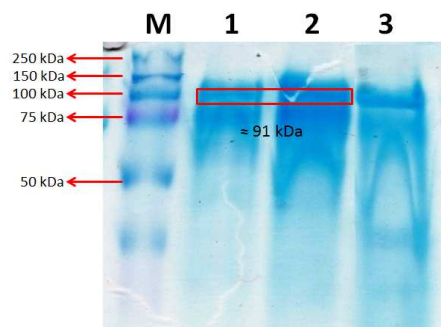


Fig. 13 Analysis of recombinant protein by SDS-PAGE. Lane M: Prestained Protein Standards; Lane 1: Recombinant not induced; Lane 2: Recombinant induced; Lane 3: Wildtype not induced.

#### IV. CONCLUSIONS

The fusion gene *araA* has been successfully incorporated into the vector pJ912-AG $\alpha$  and confirmed by sequencing. The fusion gene was successfully transformed in genome of *P. pastoris*. The fusion protein was expressed on the cell surface of *P. pastoris*.

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