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-AGα and expression the protein in the cell-surface of Pichia pastoris GS115. Both vector pJ912-AGα and araA gene was digested with SalI and Kpn2I restriction enzymes then was ligated. The ligation solution had been successfully introduced into Escherichia coli DH5α. Vector pJ912-AGα-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 transformaned cell emit green fluorescence derived from the interaction of functional His6 protein and rabbit polyclonal to 6×His tag® 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

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 of 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 Saccharomyces 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 multicity 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 α-agglutinin which is displayed on P. pastoris cell-surface [39]. Many proteins have been expressed in the surface cell of P. pastoris, including Lipase 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 α-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 DH5α 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 µg/mL zeocin, and incubated overnight at 37°C. After transformation, each colony was cultured into 2 mL LSLB medium containing 25 µg/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 OD600 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 µL 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 µg purified plasmid was digested with 100 U of SacI at 37°C overnight. A 80 µL GS115 cells were then mixed with approximately 5-10 µg 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 µF, 200 Ω, and 4.3 ms. Immediately after the pulse, 150 µL 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 µL 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, and 2% dextrose plus 2% agar in plates), and supplemented with 1 M sorbitol and 100 µg/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’-GGTCGACATGCAATCACCATCACCACCATCACATGCTGCTATTACGTCCTTATGAAATTTGG-3’ (contains SacI restriction site at the 5’-end and polyhistidine (6×His) tag) and PPAI_R: 5’-GTCACGCGGAGCCCGCCGCCAAAATACCTTATCATCCATC-3’ (contains Kpn2I 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 SacI and Kpn2I, 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 DH5α

The pJ912-Aga-araA was used for transformation of E. coli DH5α by heat shock method. The transformation mix was spread on LSLB agar medium containing 25 µg/mL zeocin, and incubated overnight at 37°C. After transformation, each colony was cultured into 2 mL LSLB medium containing 25 µg/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].
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/mL 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 KH₂PO₄ at pH 6.0, 1.34% (w/v) yeast nitrogen base without amino acids, 4x10⁷% (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 OD₆₀₀ of 3. The cells were then harvested by centrifugation at 3000xg 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 OD₆₀₀ 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 Mutual 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 5000xg at 4°C. Pellets were resuspended in 200 µL of TBS and 3 µg of specific, FITC-conjugated rabbit polyclonal antibody to His₉ 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 Axiom 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™ 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 sample was then vortexed for 10 min at room temperature. This washing step was repeated two times. The magnetic beads were then resuspended in 150 µL of binding buffer and detected using either cultural immunofluorescence techniques as described previously.

I. Extraction and Analysis of cell surface protein

Cell cultures were collected by centrifugation and washed with buffer A (20 mM Tris-HCl pH 7.5, 20 mM NaCl, and 5 mM MgCl₂). Washed cells were incubated with Celicic® Cte2 (Novozymes, Krogshoejvej, Bagsvaerd, Denmark) in 100 mM sodium acetate buffer, pH 5.2 at room temperature for 24h by gentle 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 Laemmli 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. steaothermophilus. The marine bacterial strain G. steaothermophilus 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 lower viscosity of the substrate [11]. Previous study of L-AI from G. steaothermophilus (GSAl) found that GSAl 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 hindustannus, 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 α factor signal peptide, for production secreted recombinant protein, and AGa gene encoding α-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

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 KpnI 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 DH5α cells and cultured in LSLB media containing zeocin 25 μg/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.

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. 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 KpnI; Lane 4: pJ912-AGa digested with SalI and KpnI.

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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, α 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. S’AOXI: promotor for alcohol oxidase gene; α-factor: S. cerevisiae-derived secretion signal sequence; His: polyhistidine tag; araA: gene encoding L-arabinose isomerase; Flagean: enterokinase restriction site; AGa: C-terminal half of AGa gene; Stop: stop codon; AOXI TT: translation terminator sequence.](image)

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

*P. pastoris*-compatible vectors are designed for homologous integration into AOXI 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 AOXI 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 *SacI* (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*.

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.](image)

Some expression vector for *Pichia* can increase the number of gene copies in *P. pastoris*, so that the amount if 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 were selected from single colonies growing on YPD agar medium containing 100, 200, 500, and 1000 μg/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 μg/mL. Assuming that the *Sh ble* gene is incorporated in the same ratio as the AOXI TT sequence, an estimated 1 copy (minimum) of the gene *Sh ble* zeocin resistance is required for grow at 100 μg/mL zeocin, 4 copies at 500 μg/mL, 9 copies at 1000 μg/mL and clones with as many as 17 copies of gene are found from medium with highest antibiotic concentration of 2000 μg/mL [51]. Colony PCR was conducted toward the transformants to verify if the expression cassette had been integrated into the AOXI gene (Fig. 10).

![Fig. 9 Screening of genetically stable transformed yeast cells on zeocin plates containing various concentration of zeocin. A: 0 μg/mL (as control); B: 100 μg/mL; C: 200 μg/mL; D: 500 μg/mL; E: 1000 μg/mL.](image)

![Fig. 10 PCR colony analysis of *P. pastoris* transformant.](image)

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 detect the protein on the cell surface, it was confirmed by immunofluorescence labeling of transformed cells and then analyzed by fluorescence microscopy. The observed fluorescence in the cell surface indicated that hexa-His and the desired protein were localized and displayed on the cell surface. The functionality 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\textsubscript{6} tag (Abcam). The FITC fluorescence signal was detected at the cell surface from the His-tag labeling of the fusion protein, confirming successful membrane localization of fusion protein.

To confirm the protein was expressed on the cell surface of P. pastoris, it was detected by immunoafrican technique using Pure Proteome\textsuperscript{TM} Nickel Magnetic Beads combine with FITC-conjugated rabbit polyclonal antibody to His\textsubscript{6} 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 fluorescent at the P. pastoris recombinant cells on surface of Pure Proteome\textsuperscript{TM} 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.

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 α-Agglutinin located at the C-terminal end of L-AI.

IV. CONCLUSIONS

The fusion gene araA has been successfully incorporated into the vector pJ912-AGa 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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