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Cell surface display of *Propionibacterium acnes* linoleic acid isomerase by *Pichia pastoris*

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Abstract

Linoleic acid isomerases (LAIs) catalyze the bioconversion of the linoleic acid to conjugated linoleic acids (CLAs). Due to several beneficial nutritional effects of CLA, research on developing biocatalysts and processes for production of CLA is of great interest. The aim of this study was to develop a whole cell biocatalyst based on the Propionibacterium acnes LAI and yeast cell surface display technology. A cell surface display vector (pPDZ) was first constructed using α-factor secretion signal sequence and 3'-half of α-agglutinin from S. cerevisiae for Pichia pastoris. Next, the P. acnes LAI was subcloned into pPDZ, and the resulting vector, pPDZ-LAI, was electroporated into Pichia pastoris. Immunofluorescence microscopy and flow cytometric analysis confirmed the successful display of P. acnes LAI on P. pastoris cell surface. However, upon display of the P. acnes LAI on P. pastoris cell surface, inactivation of the enzyme occurred may be due to the post-translational glycosylations and conformational changes. This is the first study on the cell surface display of a LAI enzyme and results may be helpful in developing strategies for LAI biocatalyst production.

Kewords: Linoleic acid isomerase, conjugated linoleic acid, cell surface display, *Pichia pastoris*

Introduction

Conjugated linoleic acid (CLA) is a collective term used for description of a group of isomers of linoleic acid (18:2 *cis*-9, *cis*-12) with conjugated double bonds. Double bonds in CLA are in conjugated form, i.e., they are not methylene (-CH_2-)-interrupted and may exist in several positional (8, 10; 9, 11; 10, 12; 11, 13) and geometrical (*cis*, *cis*; *cis*, *trans*; *trans*, *cis*; *trans*, *trans*) forms [1]. Interest in production of CLA is increasing due to its beneficial nutritional effects including anticarcinogenic, antidiabetic, immunomodulating, antiadipogenic, antiatherogenic and body protein mass increasing effects. Biological activities of different CLA isomers are different and the most active ones are *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers [2]. *Trans*-10, *cis*-12 CLA is of great interest especially because of its antiadipogenic and body protein mass increasing effects [1, 2].

Linoleic acid isomerases (LAIs) are responsible for bioconversion of linoleic acid to CLA. Two major groups of LAIs have been discovered: 9, 11-isomerases or myosin cross-reactive antigen-like LAIs which produces *cis*/*trans*-9, *trans*-11, and *trans*-10, *cis*-12-isomerases which produces *trans*-10, *cis*-12 CLA [3]. The LAI from *Propionibacterium acnes* is the sole 10, 12-isomerase which its structure and mechanism of action is described [4, 5]. However, no suitable biotechnological strategy has been described for development of a biocatalyst based on *P. acnes* LAI.

Cell surface display is a novel technique which is widely used for development of whole cell biocatalysts. In this way which uses the cell as a carrier for the immobilized enzyme, the protein of interest is fused to the cell wall proteins and a strain is developed which produces the enzyme as a fused protein to the cell surface [6]. Biocatalyst production via cell surface display may 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 cells may be used as biocatalyst, directly. Enzyme-displaying cells may be reused several times as biocatalyst [7].
Yeast cell-surface display system was first described for *S. cerevisiae* [8]. Recently, the methylotrophic yeast *Pichia pastoris* has also been employed as a host for cell surface display [9-16]. The major advantages of *P. pastoris* over *S. cerevisiae* as a cellular host include prevention of hyperglycosylation, integration of multicopies of transforming DNA into genomic DNA and formation of stable transformants and its higher protein productivity [17]. Almost all *Pichia* surface display systems are based on systems developed for *S. cerevisiae*. The surface display system for *P. pastoris* was first reported by Mergler et al. [10]. They fused the *Kluyveromyces* yellow enzyme to the C-terminal half of α-agglutinin from *S. cerevisiae* and displayed it on *P. pastoris* cell surface. Since that, other display systems such as Flo1p [9, 12], Pir1 (protein with internal repeats) [13,14], Sed1 anchor systems [11,15] and a-agglutinin [16] all from *S. cerevisiae* have been developed and applied in cell surface display of proteins by *P. pastoris*.
Here in, we constructed a *Pichia* cell surface display system based on *S. cerevisiae* α-agglutinin cell wall protein and report the first study on the surface display of *P. acnes* LAI by *P. pastoris*.

Materials and methods

Obtaining linoleic acid from sunflower oil
The substrate for linoleic acid isomerase reaction, linoleic acid, was extracted and purified from sunflower oil as described elsewhere [18]. Briefly, sunflower oil was saponified with ethanolic KOH and free fatty acids were then released by acidification with 6 N HCl. Free fatty acids were then extracted to n-hexane and recovered by evaporating the hexane. For linoleic acid purification, free fatty acids, urea and ethanol (with the mass ratio of 1:2.5:7.5) were mixed, heated to 60 °C until formation of a clear homogeneous solution and then stored at 0 °C for 3 hr. The crystallized mixture was vacuum-filtered and the linoleic acid extracted from un-crystallized fraction (where it accumulated) by n-hexane. The hexane was evaporated and linoleic acid with purity of ~ 95 % was obtained.
Construction of *Pichia* cell surface display vector
Genomic DNA of *S. cerevisiae* DBY368 (INVITROGEN, CA, USA) was isolated as described by Hanna and Xiao [19] and used as template for PCR amplification of the 3' half of the open reading frame of the *S. cerevisiae* α-agglutinin gene (*AGα1*, accession number

M28164.1) encoding 320 amino acids and 446 bp of 3'-flanking region (1.4 kp). Primers 5'GAG GTA CCT CCG CCA AAA GCT CTT TTA TC3' and 5'GTG TCT AGA GAT TAT GTT CTT TCT ATT TGA ATG3' (*Kpn I* and *Xba I* restriction sites are underlined, respectively) were designed and the amplification was performed under the following condition: 5 min denaturation at 95 °C, followed by 30 cycles of 1 min at 95 °C, 100 s at 50 °C, 3 min at 72 °C and finally 10 min extension at 72 °C. The resulting DNA fragment was digested with *Kpn I* and *Xba I* and then cloned into pPICZαA (INVITROGEN, CA, USA) to obtain *Pichia* display vector, pPICZα-αAG, which was named pPDZ. The recombinant vector was confirmed by restriction analysis and sequencing (SEQLAB SEQUENCE LABORATORIES, Göttingen, Germany) [20].

Construction of LAI display vector

The complete open reading frame of *P. acnes* LAI (Accession number AAT82791) was PCR-amplified by *pfu* DNA-polymerase using pGEM-PAI (kindly donated by professor Ivo Feussner, Georg-August university Göttingen, Germany) as template. Primers 5'ACC TCG AGA AAA GAA TGT CTA TCT CCA AGG ATTC3' (with *Xho I* restriction site) and 5'ATG GTA CCC ACA AAG AAC CGC GTC AC3' (with *Kpn I* restriction site) were used for amplification of LAI with the following program: 5 min denaturation at 95 °C, followed by 30 cycles of 1 min at 95 °C, 100 s at 50 °C, 3 min at 72 °C and 10 min final extension at 72 °C. The resulting 1.3 kb DNA fragment was then cloned into the pPDZ yielding the LAI display vector pPDZ-LAI. The authenticity of the pPDZ-LAI was confirmed by restriction analysis and sequencing (SEQLAB SEQUENCE LABORATORIES, Göttingen, Germany) [20].

Cloning of LAI display vector into *P. pastoris*

P. pastoris KM71H (INVITROGEN, CA, USA) was transformed according to the instructions of the vendor [21]. Briefly, 20 μg of *BstX I*-linearized plasmid DNA was mixed with 80 μL electrocompetent cells and subjected to electroporation using a Genepulser electroporation system (BIO-RAD LABORATORIES, Hercules, CA) under the following condition: cuvette gap of 2 mm, 2000 V, 25 μF, 200 Ω, 5 ms. Electroporated cells were then plated on YPDS plates (1% yeast extract, 2% peptone, 2% glucose and 1M sorbitol) containing 100 μg/ml zeocin and incubated at 30 °C for 2-4 days. Zeocin-resistant clones were picked up and PCR-screened for integration of the plasmid construct in to the yeast genome [21]. Briefly, yeast genomic DNA was isolated according to Hanna and Xiao [19] and PCR was performed using universal *AOX*1 primers (5′GAC TGG TTC CAA TTG ACA AGC3′ and 5′GCA AAT GGC ATT CTG ACA TCC3′) under the following condition: 5 min denaturation at 95 °C, followed by 30 cycles of 1 min at 95 °C, 100 s at 50 °C, 3 min at 72 °C and 10 min final extension at 72 °C. PCR-positive clones showing 3177 bp band were then tested for the display of LAI on *Pichia* cell surface.

Expressing recombinant *P. pastoris* clones

100 ml of buffered glycerol-complex medium (BMGY, 1 % (w/v) yeast extract, 2 % (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % (w/v) yeast nitrogen base without amino acids, 4 x 10^{-5} % (w/v) biotin, 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 (200 rpm) until an OD_{600} of 3. Cells were then harvested by centrifugation (3000 g, 5 minutes, 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_{600} of 10. The flask

was then covered with 2 layers of sterile gauze and incubated at 25 °C in a shaking incubator (200 rpm). To maintain induction, 100 % methanol was added to the culture to a final concentration of 1.0 % every 24 hours. *P. pastoris* KM71H transformed with pPDZ vector and *P. pastoris* GS115/His^+ Mut^s Albumin (INVITROGEN, CA, USA) strains were included in expression experiments and used as negative and positive control, respectively. Samples of culture were taken after 72 hours and analyzed for expression [21].

Flow cytometric analysis and immunofluoresence microscopy

Preparation of yeast cells for flow cytometric analysis and immunofluoresence microscopy was performed as described elsewhere [22]. Expressed yeast culture samples (100 µl) were centrifuged at 3000 g for 5 minutes at 4 °C and the cells were resuspended in 1 ml phosphate buffer solution (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) and centrifuged as in previous step. Cell pellets were then resuspended in 250 µl of PBS containing 1 mg/ml bovine serum albumin (BSA), and rabbit LAI-specific antiserum (1:50 dilution, kindly donated by Professor Ivo Feussner, Georg-August university Göttingen, Germany). The mixture was then incubated on ice for 30 minutes with occasional mixing. Cells were then removed from the mixture by centrifugation at 3000 rpm for 5 minutes at 4 °C and washed with 1 ml PBS. Cell pellets were then resuspended in PBS containing 1 mg/ml BSA, and 1 µg mouse anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) and incubated on ice for 30 minutes in the dark with occasional mixing. The cells were finally washed two times with 1 ml of PBS and analyzed using a flow cytometer (PARTEC, model PASS2, GmbH). For immunofluoresence microscopy, slides were prepared from 10 µl of cell suspensions and observed by a Nikon Eclipse E200 fluorescence microscope (NIKON INSTRUMENTS Inc., USA).

Biocatalysis of linoleic acid conversion to CLA

Fresh cell pellets of expressed culture were washed with 0.1 M tris-CL pH 7.5 and used as biocatalyst for conversion of LA to CLA. Typically washed cell pellets (100 mg wet weight) were mixed with 100 µl 1 M tris-Cl (pH 7.5), 1 mg LA and 0.2 mg BSA (which was used for dispersing linoleic acid in aqueous solution; final volume of 1 ml) and incubated at 30 °C in a shaking incubator (150 rpm) over night. Fatty acids were then extracted using 2 ml n-hexane and analyzed by gas chromatography [18].

Gas chromatography analysis

Hexane was removed from the organic phase by applying vacuum (0.2 bar abs) at room temperature. Extracted fatty acids were then reacted with 1 ml BF3 in methanol solution (14%, v/v) for 10 min at room temperature and methyl esters were then extracted into n-hexane (2 mL). After washing the hexane phase by water (5 mL) it was dried over anhydrous sodium sulfate, and used for GC analysis [18]. A SHIMADZU 14A gas chromatography (Kyoto, Japan) equipped with flame ionization detector and RT2560 fused silica capillary column (100 m, 0.25 mm id and 0.2 lm film thickness; RESTEK corporation, Bellefonte, PA, USA) was used for analysis of fatty acid methyl esters. Two µl of the sample was injected into the GC at split ratio of 1:50. Injector and detector temperature was 225 and 250 °C, respectively. Column head pressure was 225 KPa and nitrogen was used as carrier gas. The oven temperature was programmed for 3 min at 160 °C, increase to 220 °C at 2 °C/min, and

finally holding at 220 °C for 30 min. A commercial CLA mixture was used as standard for identification of CLA isomers in the samples [18].

Biocatalysis of linoleic acid conversion to CLA by *Escherichia coli*-derived LAI

The LAI was also overproduced in *E. coli* and purified as described elsewhere [18]. The *E. coli*-derived LAI was used as a control for enzymatic activity. For LAI activity assay, 2 mg linoleic acid was mixed with 200 µg of purified enzyme, 0.4 mg BSA and 100 µl 1 M tris-Cl (final volume of 1 ml, pH 7.5). A mixture missing enzyme was prepared as above and used as control reaction. The biocatalysis reactions were performed at 30 °C in a shaking incubator (150 rpm) for one hour. Fatty acids were then extracted from the reaction mixture using 5 ml n-hexane and fatty acid composition was determined by gas chromatography analysis as described in previous section.

Nucleotide Sequence Accession Number

The Genbank accession number of the nucleotide sequence of the 3′-half of the α-agglutinin gene presented in this work is HM132073.

Results and Discussion

Construction of LAI cell surface display vector

In this study a *Pichia* cell surface display vector (pPDZ) was constructed by cloning the 3'-half of α-agglutinin gene (*AGα1*) into pPICZαA downstream to its multiple cloning site (Fig 1A). As it is shown in Fig 1A, it uses alcohol oxidase promoter (*AOX*1) and *S. cerevisiae* α-factor secretion signal sequence and contains 9 different restriction sites in its multiple cloning site which allows convenient cloning of other genes in frame with α-factor secretion signal and 3'-half of α-agglutinin (Fig 1B shows the multiple cloning site of the vector pPDZ). The vector allows attachment of the protein of interest from its C-terminus to the cell surface. Using this vector transformants can be selected based on zeocin resistance. To construct a LAI display vector, the complete open reading frame of *P. acnes* LAI (without stop codon) was subcloned into pPDZ within *Xho I* (5') and *Kpn I* (3') restriction sites downstream to α-factor secretion signal and upstream to the 3'-half of α-agglutinin (Fig 1C). The constructed vector pPDZ-LAI was confirmed by restriction analysis and sequencing (data not shown) and used for transformation of *P. pastoris*.

Cell surface display of LAI

A PCR on genomic DNA of transformants using AOX1 universal primers was used to check the integration of LAI display cassette into yeast genome. Fig 2 shows the result of PCR screening of *Pichia* transformants. Positive clones showing a 3177 bp band corresponding to the sum of the size of 3'-half of α-agglutinin and LAI genes, were then tested for display of LAI on yeast cell surface.

Fig 3 shows the light and immunofluorescence micrograph of induced *P. pastoris* cells. *Pichia* cells expressing LAI at their cell surface exhibited green fluorescence while no fluorescence observed for untransformed cells. This confirms both the successful construction of *Pichia* display vector and successful display of LAI by *P. pastoris*.

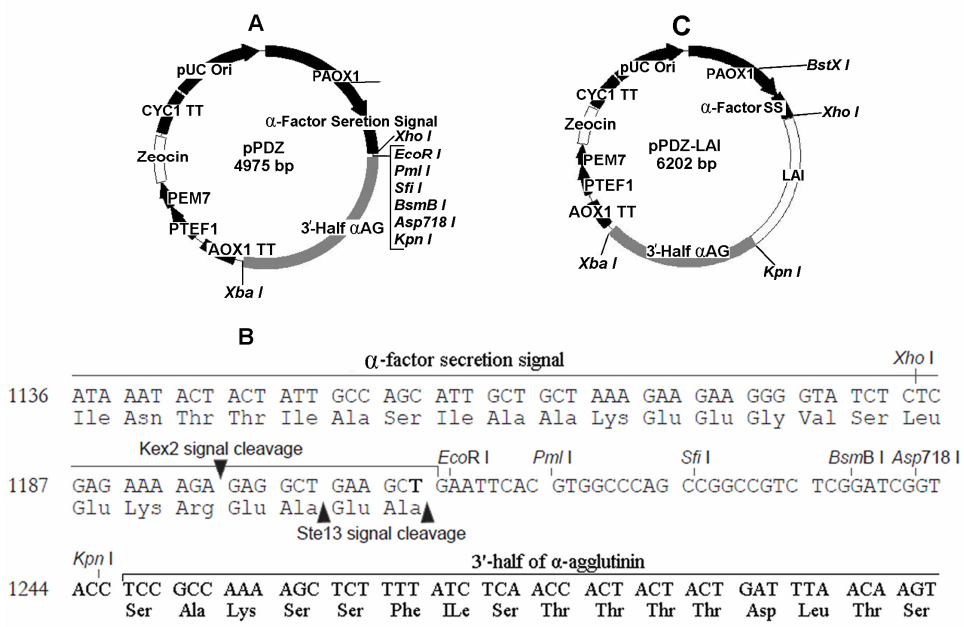

Fig 1. A) Schematic representation of *Pichia* display vector (pPDZ). The 3'-half of *S. cerevisiae* α-agglutinin gene (αAG) was cloned into pPICZαA via *Kpn I* and *Xho I* sites. The vector is under the control of alcohol oxidase (*AOX*1) promoter and transformants can be selected based on zeocin resistance. B) Multiple cloning site of the *Pichia* display vector, pPDZ. To display a protein on *P. pastoris* cell surface, the gene of interest should be cloned in to pPDZ multiple cloning site in frame with α-factor secretion signal (SS) and 3'-half of α-agglutinin gene. The *Xho I* site allows the user to clone the gene of interest in frame with the *Kex2* cleavage site, resulting in expression of the native gene without additional amino acids at the N-terminus. C) Schematic representation of linoleic acid isomerase (LAI) display vector (pPDZ-LAI). The *P. acnes* LAI open reading frame was cloned into pPDZ vector between *Xho I* and *Kpn I* sites in frame with the α-factor secretion signal and 3'-half of α-agglutinin gene.

Cell-surface display of LAI was also confirmed by flow cytometric analysis (Fig 4). The fluorescence histogram of cells carrying pPDZ-LAI cassette showed an apparent shift and increase in the fluorescence peak compared to that of untransformed control cells, indicating cell-surface expression of LAI by *P. pastoris*. Using flow cytometric analysis, about 7-14 % (in different clones) of cells were detected to display *P. acnes* LAI on their surface. The results of immunofluorescence microscopy and flow cytometric analysis confirmed successful display of LAI by *P. pastoris*.

Mergler et al [10] cloned 3'-half of α-agglutinin into pPIC9K downstream to α-factor secretion signal. Using this vector they developed a bisphenol A-absorbing yeast system by expression of the *Kluyveromyces* yellow enzyme on *P. pastoris* cells surface. Based on pPIC9K, Wang et al [23] also developed a vector for heterologous display of proteins on the cell surface of *P. pastoris* using 3'-half of α-agglutinin from *S. cerevisiae*. They confirmed the vector by displaying Enhanced green fluorescence protein (EGFP) on *P. pastoris* cell surface. Using pPICZαA, we constructed a *Pichia* display vector and displayed the *P. acnes* LAI on *P. pastoris* cell surface.

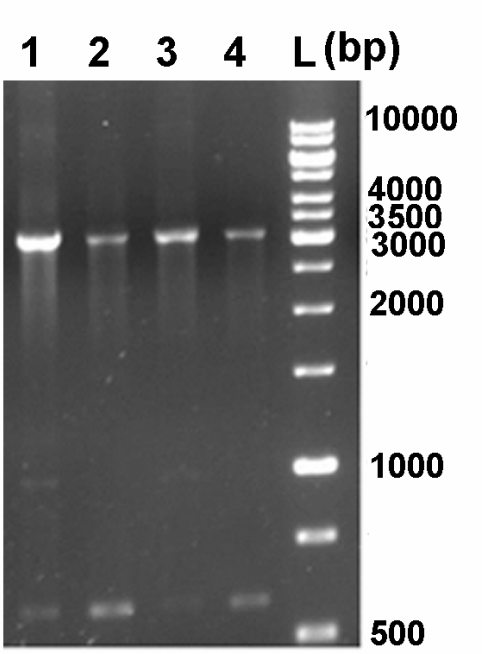

Fig 2. Verification of recombinant *P. pastoris* clones transformed by pPDZ-LAI vector. Genomic DNA from *Pichia* transformants was extracted and then PCR-amplified with *AOX*1 universal primers. The integration of the pPDZ-LAI vector to the genomic DNA was confirmed by amplification of 3177 bp band corresponding to the size of the cloned genes. Lanes 1–4, positive clones; lane 5, Fermentas DNA ladder.

Biocatalysis of LA to CLA

After confirming the successful display of the LAI by *P. pastoris*, induced cells were tested for LAI activity. Only traces of CLA was detected in reaction media catalyzed by LAI-displaying cells while about 7 % *trans*-10, *cis*-12 CLA was produced by the *E. coli*-derived LAI after one hour reaction (Fig 5). This means that the displayed LAI was not in its active form. *P. acnes* LAI is a flavoenzyme and needs flavine adenine dinucleotide (FAD) as cofactor for biocatalysis of the reaction. Knowing this fact, we thought that the enzyme may be inactivated due to its inaccessibility to FAD. However, again no activity was observed in the presence of FAD (data not shown). Even increasing the amount of cells in the reaction media to 5 folds (500 mg wet weight in 1 ml reaction media) had no effect on biocatalyst activity. Some factors may be involved in inactivation of LAI by yeast cell surface display. Fusing *P. acnes* LAI to 3'-half of α-agglutinin probably altered the active conformation of LAI. Furthermore glycosylation by yeast secretion machinery may also interfere in enzymatic activity. In fact, as we reported previously [18], compared to the intracellularly produced LAI (by *E. coli*), due to post-translational glycosylations, the *Pichia*-secreted enzyme had about 23 % increased molecular weight and ten-folds decreased enzymatic activity in biocatalysis of linoleic acid to CLA. Despite its theoretical advantages of cell surface display systems in development of biocatalysts, display of some proteins may lead to inactivated protein. Litzenburger [24] also tried to display HLA-A2 complexes on *S. cerevisiae* cell surface using a-agglutinin system. However, attempts to display a properly folded protein on cell surface were not successful. He related incorrect protein folding to conformational changes because of yeast protein glycosylation.

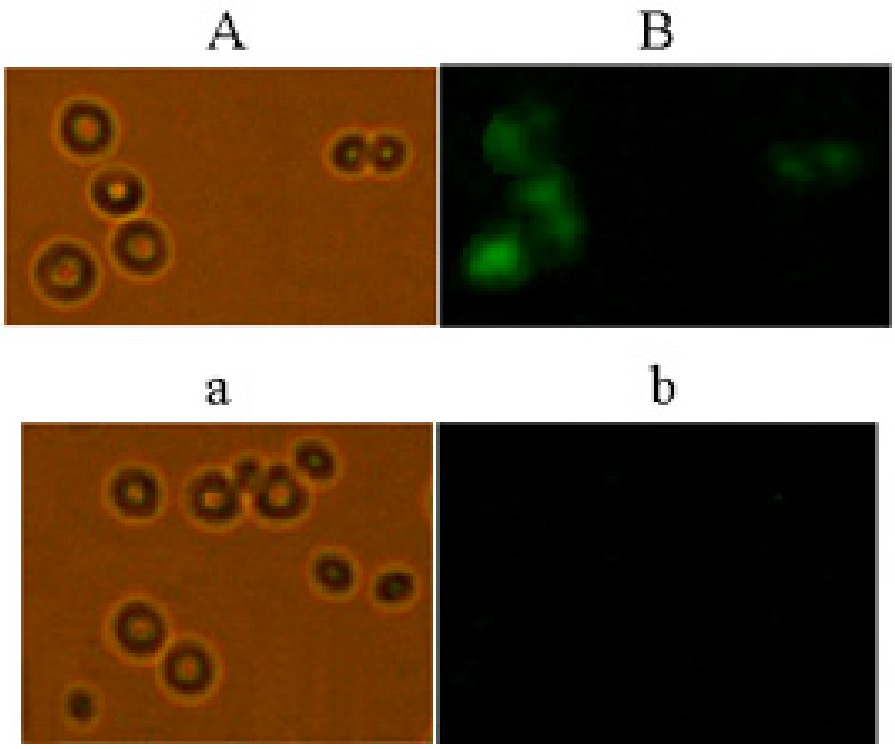

Fig 3. Light (left) and immunofluorescence (right) micrographs of transformed and untransformed *P. pastoris* cells. A and B, micrographs of *P. pastoris* KM71H transformed by pPDZ-LAI; a and b, micrographs of untransformed *P. pastoris* KM71H (control). Compared to untransformed cells, a green fluorescence showing the display of the linoleic acid isomerase can be easily seen.

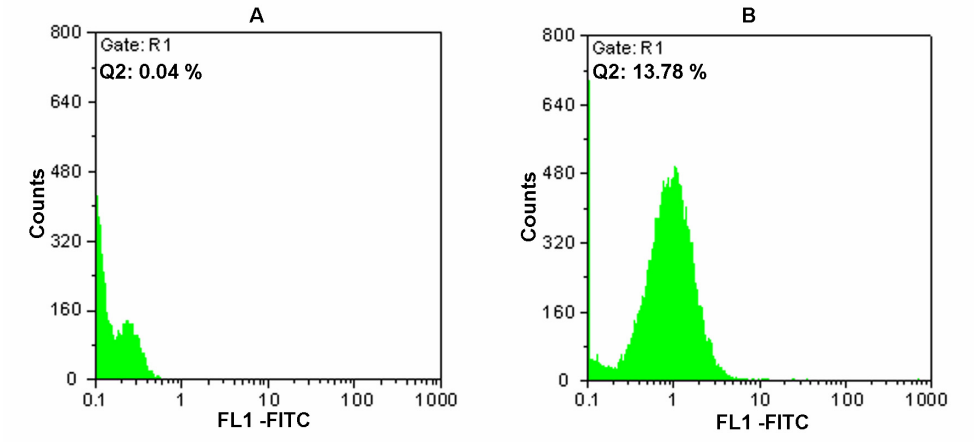

Fig 4. Flow cytometric analysis of transformed and untransformed (control) *P. pastoris* cells. A, untransformed cells; B, cells transformed by pPDZ-LAI and displaying linoleic acid isomerase. Compared to untransformed cells, an increase and a shift in fluorescence of cells displaying linoleic acid isomerase can be clearly seen.

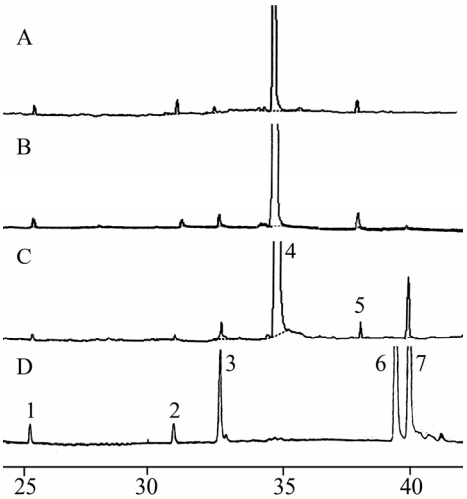

Fig 5. Gas chromatogram of fatty acids extracted from reaction media. A: control reaction (reaction without enzyme or cell); B: reaction catalyzed by linoleic acid isomerase-displaying-*P. pastoris*; C: reaction catalyzed by *E. coli*-derived linoleic acid isomerase; D: conjugated linoleic acid (CLA) standard. 1, palmitic acid; 2, stearic acid; 3, oleic acid; 4, linoleic acid (18:2 *cis*-9, *cis*-12); 5, linolenic acid; 6, CLA (18:2 *cis*-9, *trans*-11); 7, CLA (18:2 *trans*-10, *cis*-12).

The *P. acnes* LAI was previously expressed by *E. coli* [18, 25-27], *Lactococcus lactis* [27] and *S. cerevisiae* [25] as an intracellular protein. We reported the secretory expression of the LAI elsewhere [18]. Till now the most active enzyme is obtained by heterologous expression in *E. coli* host. However, the enzyme tends to form inclusion bodied upon overexpression in *E. coli*. Due to possible glycosylations, the *Pichia*-secreted LAI had much lower activity in CLA production, on the other hand [18]. Herein, we reported the first attempt to develop a biocatalyst based on the cell surface display system. However, more studies are needed to develop a whole cell biocatalyst based on cell surface display systems. The effects of glycosylation on LAI activity is now under study in our laboratory. Application of cell surface display systems that fuses the protein of interest from its N-terminus to cell wall protein, such as Flo1p and a-agglutinin display systems may be another potential strategy for developing a whole cell biocatalyst.

In summary, a *Pichia* cell surface display vector was constructed which uses α-factor secretion signal sequence and 3'-half of α-agglutinin from *S. cerevisiae*. The vector is under the control of *AOX*1 promoter and transformants can be selected based on zeocin resistance. The display of *P. acnes* LAI on *P. pastoris* cell surface confirmed the successful construction of *Pichia* display vector. Despite successful display of *P. acnes* LAI on *P. pastoris* cell surface, no enzymatic activity was observed for the displayed enzyme. This may be attributed to the post-translational glycosylations and conformational changes in the LAI protein structure during its display on cell surface. More studies are needed in this regard to find the exact causes of LAI inactivation during cell surface display.

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