Heterologous expression of a novel linoleic acid isomerase BBI, and effect of fusion tags on its performance

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Functional proteins with the ability to isomerise free linoleic acid (LA) to conjugated linoleic acid (CLA) are termed linoleic acid isomerases (LAI). BBI is a novel LAI from Bifidobacterium breve with unique advantages in the production of a single CLA isomer; however, its complex membrane-bound form hampers over-expression of the protein in its natural host. To overcome this challenge, heterologous expression of BBI in Pichia pastoris was studied. Further, to investigate the influence of His-tags on the heterologous expression of BBI, three P. pastoris recombinant strains carrying either a C-terminal His-tag, an N-terminal His-tag, or none were constructed. The expression of recombinant proteins was analysed by dot and western blotting, and the enzyme activity was determined by GC-MS. The results show that the three P. pastoris recombinant strains successfully expressed the recombinant protein and had LAI activity. Compared with those BBIs without a His-tag or carrying a His-tag on the C-terminus, the BBI carrying an N-terminal His-tag had reduced expression and enzyme activity and that was also explained by the protein modelling analysis. Moreover, this study highlights the advantages of using P. pastoris for BBI expression to achieve efficient production of c9, t11-CLA monomers; the highest conversion rate of the substrate LA was over 80%, resulting in the production of 0.81 mg of c9, r11-CLA per mg of crude enzyme.

Key points

Expression of BBI in Pichia pastoris shows a high linoleic acid isomerase activity
N-terminal His-tag negatively affects the expression and activity of BBI
P. pastoris pPink system offers a good platform to achieve efficient production of c9, t11-CLA

1. Introduction

Conjugated linoleic acid (CLA) is a collective term for a group of octadecadienoic acids with a conjugated double bond (Yang et al., 2017). It is recognized as a prebiotic by the International Scientific Association of Probiotics and Prebiotics (ISAPP) (Gibson et al., 2017). Among CLA, two of the most physiologically active isomers are c9, t11-CLA, and r10, c12-CLA, both are widely used in medicine and food industries (Yang et al., 2017). Nutritional scientists have discovered that there are functional differences between different CLA isomers and that the presence of unsuitable isomers may cause health hazards (Yang et al., 2015). For this reason, it is essential to obtain pure single CLA isomers by bioconversion.

Although many natural microorganisms have the ability to convert LA into CLA isomers, certain inherent defects limit their use in nutritional health and in the food industry (Salsinha et al., 2018). For example, Propionibacterium acnes has outstanding t10, c12-CLA production capabilities but this strain is potentially pathogenic (Liaonchanka et al., 2006). In addition, c9, t11-CLA production is distributed in Lactobacillus plantarum and bacteria from the genus Bifidobacterium (Yang et al., 2017). Among studied bifidobacteria, B. breve has shown the strongest CLA isomer production capacity. Several strains, like B. breve CCFM683 (Gao et al., 2020) and LMC 017 (Chung et al., 2008),...
have conversion efficiencies of even more than 90% (substrate: 0.5 mg/mL LA), clearly higher than that of other CLA-producers such as *L. plantarum* ZS2058 that has a conversion rate of about 50% (Yang et al., 2017).

Recently, the gene sequence of BBI, the linoleic acid isomerase (LAI) from the high CLA-producer *B. breve* CCFM683, was reported; BBI was shown to be a novel membrane-bound protein (Gao, 2020). Compared to *L. plantarum*, which produces ε9, t11-CLA via a three-component enzyme system, CCFM683 can produce ε9, t11-CLA (more than 80% of its total CLA products) via BBI in a one-step reaction without producing 10-HOE as an intermediate molecule (Chen et al., 2016; Gao et al., 2019). Hence, when synthesising CLA monomers, the usage of *B. breve* CCFM683 and BBI is recommended. However, the procedure still faces many challenges. First, CLA-production by *B. breve* occurs during the growth phase of the strain, and the culture conditions of *B. breve* CLA-producers are harsh and costly (Mei et al., 2021); second, the bioconversion of LA to CLA by *B. breve* involves a stress-defence reaction, making it difficult to increase the substrate concentration further (Senizza et al., 2020). Moreover, due to the complex transmembrane structure of BBI (with nine transmembrane regions), only low levels of expression may occur in the original strain, making it difficult to obtain large amounts of BBI directly from its natural host. Together, these factors heavily limit the application of BBI in the commercial production of ε9, t11-CLA monomers. Even though, heterologous expression of proteins based on the genetic engineering is a widespread method to obtain bioactive proteins (Huo et al., 2019), there is still a lack of relevant information on BBI.

*Pichia pastoris*, one of the most important hosts in yeast expression systems, has several advantages for the heterologous expression of target proteins, including high biomass production, which has been shown to be beneficial for protein translation and folding (Juturu and Wu, 2018; Karbalaei et al., 2020). Additionally, previous studies have shown that *P. pastoris* is a favourable choice to produce membrane proteins: more than three hundred membrane proteins have been efficiently expressed using this system (Karbalaei et al., 2020). In this work, to achieve an efficient expression of BBI, *P. pastoris* was chosen as the host organism for its heterologous expression. Both the expression and activity levels of the protein were tested to assess the effectiveness of the *P. pastoris* expression system for this bioactive protein. Affinity tags are often used as effective tools to label the N- or C-termini of target proteins to facilitate purification, characterization, and detection of their expression levels. His-tags containing 6 to 10 consecutive histidines are the most widely used affinity tags (Young et al., 2012). In addition, it has been reported that 87% of heterologously expressed membrane proteins had a His-tag as their affinity tags (Lee and Altenberg, 2005). Therefore, in this study, three different recombinant strains were constructed to investigate the effect of a His-tag on the expression and activity of BBI proteins in *P. pastoris*.

2. Materials and methods

2.1. Chemicals

DNA restriction endonucleases were purchased from Thermo Fisher Scientific (Walham, MA, USA). KOD-plus DNA polymerase was purchased from Toyobo (Shanghai, China). DNA T4 ligase was purchased from Takara (Dalian, China). Plasmid extraction, DNA extraction, and PCR products were purchased from Shanghai TaKaRa Biotechnology (Beijing, China). Yeast nitrogen base without amino acids (YNB), biotin, and pentadecanoic acid and LA standards (purity ≥99%) were purchased from Sigma-Aldrich (USA).

2.2. Strains, plasmid, and culture conditions

*Escherichia coli* DH5α, used to preserve constructed plasmids, was provided by the Culture Collection of Food Microorganisms (CCFM), Jiangnan University. *PichiaPink* Strain 2 and the plasmid pPink-HC-3CZHEK (Wang et al., 2018) were purchased from Invitrogen. *E. coli* and *P. pastoris* strains were incubated at 37 °C and 28 °C, respectively, strictly following the yeast protocol from the manual (Thermo Fisher Scientific). YPD medium (1% yeast extract, 2% peptone from fish, and 2% glucose) was used to activate *P. pastoris*. PAD medium (1.34% YNB, 0.125% CSM-ADE, 0.0005% biotin, and 2% glucose) was used for transformation selection. BMGY (1% yeast extract, 2% fish meal peptone, 100 mM phosphate buffer, 1.34% YNB, 0.0004% biotin, and 1% glycero) and BMMY (BMGY with 0.5% methanol instead of 1% glycerol) media were used for cultivation and induction of *P. pastoris*, respectively. All yeast transformants were induced by methanol at 28 °C for 24 h. *B. breve* CCFM683 was sub-cultured twice (1.5% inoculum) in mMRS broth (De Man, Rogosa, and Sharpe [MRS] broth supplemented with 0.05% cysteine) in an anaerobic environment (Electrotek 400 TG, West Yorkshire, UK) at 37 °C for 20 h, and then used as a seed solution. Finally, it was inoculated in 200 mL of mMRS broth to collect log-phase cells.

2.3. Recombinant strain construction

Using the bbi sequence from *B. breve* CCFM683, the gene was optimized for *P. pastoris* codon preference using the GenScript OptimumGene™ system (https://www.genscript.com/tools/gensmart-codon-optimization). The optimized gene was named obbi (GenBank: OM158463); it was synthesised by the GenScript Biotechnology company (Nanjing, China). The expression plasmid pPink-HC-3CZHEK was then used to construct three different recombinant expression vectors: one without a His-tag, one with a His-tag at the N-terminal end of the target protein, and one with a His-tag at the C-terminal end of the target protein. The digestion sites and primers used are shown in Table S1. pPink-HC-3CZHEK and the amplified target gene were double digested protein. The digestion sites and primers used are shown in Table S1. After verifying the recombinant plasmids by electrophoresis and sequencing, they were linearized and correspondingly transformed into the host organism *PichiaPink Strain 2*. Plasmid construction steps and small-scale expression determination were done as described previously (Chen et al., 2013). Universal primers (A0X-F/CYC-R) were used for PCR validation of all recombinant strains. Conditions for PCR were as follows: pre-denaturation at 95 °C for 3 min; 95 °C for 30 s, 55 °C for 30 s, extension at 72 °C for 60 s, 30 cycles; extension at 72 °C for 5 min.

2.4. Preparation of crude enzyme solution

*B. breve* and *P. pastoris* cultures were centrifuged at 8000×g for 3 min. After removing the supernatants, cell pellets were resuspended in a Tris buffer (0.137 M NaCl, 7.0 mM KH₂PO₄ and 2.5 mM KH₂PO₄) and centrifuged once again. Cells were washed three times. Equal volumes of Tris buffer and 0.5 mM acid-washed glass beads were then added to the pellets and cells were crushed using a tissue crusher with an oscillation frequency of 65 Hz and an oscillation time of 30 s, and letting samples stand on an ice bath for 30 s; samples were subjected to 10 crushing cycles each. All samples were then measured for total protein concentration using a commercially available BCA kit (Beyotime Biotechnology Ltd., Shanghai, China).

2.5. Dot blot and Western blot analyses

For dot blotting, the crude enzyme solution prepared above was added to 5 × loading buffer, treated in a metal bath at 95 °C for 10 min, cooled, and centrifuged at 3000×g for 3 min. Protein samples (2 μL) were simultaneously spotted on nitrocellulose membranes, allowed to air dry, and shaken in the presence of a blocking solution ( skim milk powder) for 1 h. After blocking, the membranes were washed three
times with TBST for approximately 5 min per wash, immersed in primary antibody (6 × -His Tag monoclonal antibody, Invitrogen, USA), and incubated overnight at 4 °C. After washing, the membranes were transferred to secondary antibody (HRP-Goat Anti-Mouse IgG, Jackson ImmunoResearch, USA) solution and incubated for 1 h. Membranes were then saturated with chemiluminescent developing solution and photographed.

For western blotting, 30 μg of protein samples were electrophoresed; first, in an upper concentrated gel at a constant pressure of 80 V for about 30 min and then ran through a lower separated gel at a constant pressure of 120 V. Proteins were then electrically transferred (constant current 300 mA for about 1 h) from the gel to a PVDF membrane. The membrane was treated as those used for dot blotting after blocking.

2.6. Enzyme activity assay

Crude enzyme 1 mg samples were mixed with 0.1, 0.5, 1.0, or 2.0 mg/mL LA (stock solution 30 mg/mL LA), and 100 μL of C15:0 (2 mg/mL) as internal standard, and left to react at 37 °C with shaking (200 r/min) for 1 h. After the reaction, fatty acid determination was carried out using the diazomethane methylation method (Coakley et al., 2010). Briefly, samples were mixed with 1 mL isopropanol and shaken thoroughly for 30 s, then mixed with 2 mL of hexane and shaken again. The supernatant (the hexane layer containing lipids) was aspirated into a clean lipid extraction bottle, blown dry under nitrogen, and resuspended in 400 μL of methanol and 40 μL of diazomethane (for methylation). Sample yellow colour was maintained for 15 min before being blown dry under nitrogen again. Samples were then resuspended in 1 mL of hexane and transferred into gas-phase vials for gas chromatography analysis.

A gas chromatograph (Shimadzu GC, 2010 plus) coupled with a Rtx-wax (30 m × 0.25 mm × 0.25 μm) gas phase column and a mass spectrometer (Ultra QP2010) were used for GC-MS analysis under previously described conditions (Gao et al., 2020). The ratio of the peak area of the target to the internal standard (known concentration) was used to calculate both the concentration of LA and CLA (Gao et al., 2020). Then, conversion rates of LA to CLA, used as indicators of LAI activity, were calculated using the following equation:

\[ \text{LA conversion rate (\%) = } \frac{C_{\text{CLA}}}{C_{\text{CLA}} + C_{\text{LA}}} \times 100 \]

2.7. Modelling and alignment of recombinant BBI

It was necessary for de novo modelling as the structure of the BBI protein was unknown and its homologues or similar (>30%) structures were not queried in existing databases, including NCBI and PDB. Modeling of BBI proteins was performed by RoseTTAFold, a deep learning-based structure prediction method (https://www.rosettacommons.org/software/servers#rosie). The constructed protein models were quality checked for usability through Ramachandran plots (Colovos and Yeates, 1993) and confidence scores (by RoseTTAFold) based on the Global Distance Test (GDT) function. Finally, the RMSD scoring function (by PyMOL) was used to assess the similarity of different protein models to quantify the effect of tag position on BBI structure; PyMOL (http://www.pymol.org) provided a visual image to capture protein structure conformational changes.

2.8. Statistical analysis

GraphPad Prism 8.0 was used for graphing, and SPSS 24.0, for statistical analysis of the data. The results are expressed as means ± SD, statistical significance was set at P < 0.05.

3. Results

3.1. Construction and screening validation of recombinant P. pastoris strains

As shown in Fig. S1, the constructed expression vectors, namely pPink-obbi, pPink-His-obbi, and pPink-obbi-His, were verified by PCR; the theoretical sizes of the amplified genes from the three recombinant vectors were 1704 bp, 1707 bp, and 1701 bp, respectively. The results showed that two transformants (No. 2 and No. 7) carried the recombinant vector pPink-obbi, and two carried (No. 7 and No. 8) the recombinant vector PPink-His-obbi. Among all the strains recovered from transformation with the recombinant vector PPink-obbi-His, all but one (No. 7) carried the construction. These results indicates that the target genes had been successfully introduced into the three different recombinant expression vectors. Constructions were further verified through sequencing.

Successfully constructed expression vectors were linearized using restriction endonucleases and transformed into the host strain P. pastoris. White transformants were selected from the screening medium for genomic validation (Fig. S2). Amplification through PCR resulted in fragments with the expected sizes, indicating that the three different recombinant strains Pichia-pPink-obbi, Pichia-pPink-His-obbi and Pichia-pPink-obbi-His were successfully constructed.

3.2. Analysis of protein expression and enzyme activity levels in recombinant P. pastoris

In order to increase the efficiency of external DNA integration into the P. pastoris genome, the linearisation of the cloning vector is necessary, before inserting the vector into receptor cells by electroporation. The entered linear DNA is integrated into the cell genome via the crossover recombination process, and consequently, a recombinant strain is formed. Although in most cases only single crossover occurs in the genome, multiple insertions can still occur (1–10% chance) (Karbalaei et al., 2020; Macauley-Patrick et al., 2005). It makes a variation between clones, since suitable multiple insertions are widely considered to facilitate the expression level of recombinant proteins (Shen et al., 2020; Yang and Zhang, 2018). Therefore, to cover the potential effects of the above phenomenon, 48 transformants from three types of recombinant strains were selected for subsequent tests in this study.

Protein expression was induced in transformant strains Pichia-pPink-obbi, Pichia-pPink-His-obbi, and Pichia-pPink-obbi-His and screened by dot blotting, GC-MS (Figs. 1 and 2). It’s worth noting that all of the recombinant organisms studied generated a single CLA isomer: c9, t11-CLA (Fig. S3). As shown in Fig. 1(a), Pichia-pPink-obbi strains showed different activities, being transformants Nos. 3 and 13 (B3 and B13, respectively) those with the highest LAI activity, with LA conversion rates of 71.16% and 73.24%, respectively. Among Pichia-pPink-His-obbi strains, transformants Nos. 1 and 6 (HB1 and HB6, respectively) showed two transformants (No. 2 and No. 7) carried the recombinant vector pPink-His-obbi-His and screened by dot blotting, GC-MS (Figs. 1 and 2). It’s worth noting that all of the recombinant organisms studied generated a single CLA isomer: c9, t11-CLA (Fig. S3). As shown in Fig. 1(a), Pichia-pPink-obbi strains showed different activities, being transformants Nos. 3 and 13 (B3 and B13, respectively) those with the highest LAI activity, with LA conversion rates of 71.16% and 73.24%, respectively. Among Pichia-pPink-His-obbi strains, transformants Nos. 1 and 6 (HB1 and HB6, respectively) showed the highest LAI activity (Fig. 1(b)), with LA conversion rates of 14.68% and 16.55%, respectively. In addition, Pichia-pPink-obbi-His transformants Nos. 2, 8, and 11 (BH2, BH8, and BH11, respectively) showed the highest protein expression among the strains of this group, having LA transformation rates of 78.79%, 80.86%, and 81.53%, respectively (Fig. 1(c)). Despite the variation observed among these transformants, the average conversion rates obtained from the 16 transformants of each recombinant strain clearly exhibited a fact that the His-tag on the N-terminal had a negative consequence on the activity of BBIs (Fig. 1(d)).
with different tag positions were screened for protein expression levels (Fig. 3(a)). Comparing the expression levels between strains carrying His-tags at their C- or N-termini, it was determined that Pichia-pPink-obbi-His BH11 and Pichia-pPink-His-obbi HB6 had the highest expression among the strains in their corresponding groups. As the expression levels of Pichia-pPink-obbi strains cannot be determined by Western blot, they could not be used for comparison in this experiment. According to the grey-scale analysis of protein expression, BH11:HB6 expression is approximately 2.29:1, suggesting that, compared with the His-tag on the C-terminus, the His-tag on the N-terminus has an adverse effect on the expression of the BBI protein. These experiments show that the addition of a His-tag can affect the expression of a protein, and that the effect may vary according to its position on the protein.

3.4. Effect of His-tags on the enzymatic activity of BBI

The activity of the recombinant proteins with different tags was measured (Fig. 3(b)). The highest LA conversion rates, by group of transformants, were achieved by Pichia-pPink-obbi-His BH11, Pichia-pPink-His-obbi HB6, and Pichia-pPink-obbi B13. The B13: BH11:HB6 proportion in LA conversion rates was approximately 4.43:4.93:1. Noteworthy, the differences in relative activity between the recombinant strains was much greater than their protein expression differences. The above results indicate that, compared to the C-terminal His-tag, the N-terminal His-tag not only reduced protein expression, but also reduced enzyme activity. Although the His-tag is small, different tag positions may have different effects on the enzyme active centre, leading to changes in the catalytic activity of the enzyme. To facilitate an in-depth study of BBI, the recombinant strain Pichia-pPink-obbi-His BH11 was selected for subsequent experiments.

3.5. Effect of His-tags on the protein structure of BBI

Confidence scores (Table 1) and Ramachandran plots (Fig. 4(a)) showed the high quality of the constructed protein models. Briefly, all models had confidence levels greater than 0.7 and the proportion of amino acid residues located in the most popular regions exceeded 90%. Furthermore, the RMSD value is a popular similarity metric in structural
bioinformatics. Once the conformation between the two proteins was fully aligned, the RMSD value was shown to be 0. As shown in Table 1 and Fig. 4(b and c), the RMSD value between Model_BH and Model_B was 0.696 Å (<1.054 Å, between Model_HB and Model_B), indicating that the N-terminal fusion His-tag produced a more obvious conformational change to the BBI protein. More importantly, since the function of a protein is closely related to its own structure, it is reasonable to speculate that this conformational change is a potential factor in the reduced activity of the N-terminal recombinant protein.

3.6. Comparative analysis of CLA production by P. pastoris transformants and the original host strain

To measure the advantages of CLA production by P. pastoris, strain BH11 was selected for comparative analysis with the original host strain B. breve CCFM683. As shown in Fig. 5, P. pastoris BH11 showed significant advantages over B. breve CCFM683 in the production of c9, t11-CLA in the crude enzyme reaction system. For instance, at a substrate (LA) concentration of 0.1 mg/mL, the crude enzyme from P. pastoris BH11 achieved a conversion rate of 98% compared to the approximately 1% conversion achieved by B. breve CCFM683 crude enzyme. Interestingly, as the substrate concentration increased, a decreasing trend in LA conversion rates was observed for both host-derived crude enzymes. Nevertheless, at an LA concentration of 1.0 mg/mL, the percentage of conversion of the crude enzyme from P. pastoris BH11 was still over 80%, reaching a c9, t11-CLA concentration of 0.81 mg/mL (Fig. 5(a)), much higher than that reached by the crude enzyme from the original strain. Furthermore, at an LA concentration of 2.0 mg/mL, a slight increase in c9, t11-CLA concentration was observed when using the crude enzyme from P. pastoris BH11, but also a decrease from 81% to 40% in LA conversion. This suggested that high concentrations of either the substrate or the product might inhibit the reaction.

4. Discussion

As one of the most important CLA isomers, c9, t11-CLA is of great interest to food and nutrition scientists because of its multiple health-promoting functions (Yang et al., 2015). Although BBI, the novel LAI from B. breve CCFM683, has been considered a promising enzyme for the commercial production of this isomer, its availability in sufficient quantities remains a challenge. THMM-based predictions (Fig. S4) show that BBI is a membrane-bound protein with nine transmembrane regions. Because membrane proteins require a proper balance between translation, folding, and transport to ensure that functional proteins are ultimately anchored to the membrane (Foot et al., 2017), BBI is difficult to express in large quantities in natural strains.

In this work, P. pastoris was selected as a heterologous expression system to improve BBI expression because this yeast has frequently proven a good host for expressing complex membrane-bound proteins. As a methylotrophic yeast, P. pastoris combines the eukaryotic expression system for protein processing, folding, and post-translational modification with special traits, such as ease of operation, relatively fast expression times, low cost, and high expression levels, like those of the prokaryotic expression systems (Karbalaei et al., 2020). For example, P. pastoris has been shown an optimal host for the expression of the membrane-bound protein cytochrome P450, and can support the needs of this class of membrane proteins during expression, including specific membrane-anchored conformations, complex co-factors, and redox chaperones (Byrne, 2015; Hausjell et al., 2020). In addition, water channel AQP family members, ion channel protein K2P4.1 (Brohawn et al., 2012), P-glycoprotein transporters (Bai et al., 2011), and G protein-coupled receptors (Hino et al., 2012; Singh et al., 2012) with a seven-fold transmembrane structure are expressed in high amounts in P. pastoris; the system has been further used in the elucidation of their enzyme activities and crystal structures.

However, the successful expression of exogenous proteins in the P. pastoris system is influenced by a diversity of factors, including the optimization of genetic codons, the selection of transformants, and the fusion of affinity tags (Oberg et al., 2011; Young et al., 2012). For example, codon optimization and the usage of high-expression yeast strains carefully prepared for transformation have been confirmed effective strategies for maximizing membrane protein hAQP family yields in P. pastoris (Oberg et al., 2011). Similar to the results of the
present study, hAQP expression and activity levels in transformants from three different types of recombinant strains were significantly different. Thus, a rational cloning strategy including the screening of high expression transformants is central for achieving a high expression of the target protein.

As the most widely and successfully used affinity protein tags, it is generally assumed that His-tags have little or no effect on the function or structure of target proteins. However, this hypothesis is not always true.

Fig. 4. Modelling and alignment of recombinant BBI proteins. Ramachandran plot (a) was used to determine the plausibility of the protein models and alignment diagram (b,c) showed the effect of extra tags on the protein structure of BBIIs. Model_B, BBI without a His-tag; Model_HB, BBI with a N-terminal His-tag; Model_BH, BBI with a C-terminal His-tag. In Ramachandran plot s, most favoured regions shown in red and disallowed regions shown in white.

Fig. 5. Comparative analysis of CLA production by crude enzymes isolated from P. pastoris BH11 (a) and B. breve CCFM683 (b). The left vertical axis shows the change in LA conversion rate at different substrate concentrations (lines); the right vertical axis shows the concentration of 9, 11-CLA produced at different substrate concentrations (bars).
For instance, \textit{Halomonas elongata}-derived water channel proteins containing a His affinity tag have been suggested to better desalinate the water entering the cell than channel proteins without it (Yilmaz et al., 2020). Additionally, an undisrupted poly-histidine tag could act as a weak competitive inhibitor of peptide substrates, affecting \textit{Pseudomonas aeruginosa} N-acetyltransferase (GNAT) activity by altering the substrate binding site (Majorek et al., 2014). Intriguingly, more and more studies suggest that His-tags have a bidirectional effect on the properties of recombinant proteins, including crystal structure, stability, solubility, catalytic activity, kinetic constants, and substrate selectivity (Booth et al., 2018; Carson et al., 2007; Majorek et al., 2014). Nevertheless, the mechanism whereby His-tag affects the heterologous expression of target proteins has rarely been clarified.

Notably, the effect of His-tags on different target proteins varies from protein to protein and is closely related to their attachment position. For example, some studies showed that a C-terminal His-tag did not affect the expression or substrate transport of multidrug resistance protein 1 (MRP1), but an N-terminal affinity tag reduced MRP1 expression (Lee and Altenberg, 2003). Additionally, Rothberg et al. describe that a C-terminal His-tag is an obstacle for the correct folding of the molybdenum enzyme YedY (Rothberg et al., 2011). In contrast, a C-terminal His-tag was reported to exhibit a positive effect on the catalytic activity of the recombinant transaminase GαBT (Meng et al., 2020). The activity of GαBT with a C-terminal His-tag was approximately 2.2-fold higher than that of GαBT with an N-terminal His-tag and similar to that of native GαBT, which is consistent with our observations. The LA conversion activity of high expression recombinant strains with either an untagged BBI or a C-terminal His-tagged BBI were almost equal, whereas the BBI expression and bio-activity levels of high expression recombinant strains with the N-terminal His-tag represent only 43.67% (HB6: BH11 = 1:2.29) and 20.280% (HB6:HB11 = 1:4.93) of the expression and activity observed in recombinant strains with a C-terminal His-tag, respectively. Undoubtedly, these results emphasized that the location of the His-tag is a critical factor in the expression and biological activity of BBI proteins; the attachment of the His-tag at the C-terminus of BBI was more advantageous for protein expression and purification than that of the N-terminus.

In addition, the process of CLA production by \textit{Bifidobacterium} using BBI is considered as a defense mechanism in response to LA stress (Mei et al., 2021). Hence, a reasonable hypothesis is that the BBI active site is located in the N-terminal portion of the exposed extracellular (Fig. 54). The data from protein modelling also supported these conclusions. Compared to the C-terminal, the BBI with the N-terminal His-tag exhibited less structural similarity to the native BBI, which implied that the N-terminal fused His-tag caused more conformational changes in the BBI, which in turn had a detrimental effect.

Our results highlight the advantages of using \textit{P. pastoris} for BBI expression to achieve efficient production of c9, r11-CLA monomers. At a concentration of 1.0 mg/mL LA, 1 mg of the crude enzyme produced 0.81 mg/mL of c9, r11-CLA, a conversion rate of over 80%, much higher than that of the original host source. To our knowledge, this is the highest yield reported regarding the production of c9, r11-CLA by a single enzyme (in vitro). The exact BBI content in \textit{P. pastoris} BH11 and \textit{B. breve} CCFM6893 could not be accurately determined due to the lack of reliable antibodies. However, data suggest that \textit{P. pastoris} is an efficient expression system for the expression of BBI and the production of c9, r11-CLA.

5. Conclusion

In conclusion, this study characterized some parameters of the heterologous expression of the novel LAI BBI in \textit{P. pastoris} and determined the detrimental effect of the N-terminal His-tag on its expression and activity. The results on protein expression levels and functional activity strongly support the notion that \textit{P. pastoris} is an efficient expression system for CLA-producing proteins with multiple transmembrane structures. These results provide a solid foundation for expressing BBI proteins at a large scale; this will also be helpful for further studies on the crystal structure of the protein. The \textit{P. pastoris}-based BBI high expression system may constitute a good platform for the selective production of CLA monomers.

\section*{CRediT authorship contribution statement}

Yongchao Mei, Xiuxing Li, Bo Yang, Hao Zhang, and Haiqin Chen conceived and designed research. Yongchao Mei and Xiuxing Li conducted experiments. Jianxin Zhao and Wei Chen contributed reagents and analytical tools. Yongchao Mei, Xiuxing Li, and Haiqin Chen analyzed data and wrote the manuscript. All authors read and approved the manuscript.

\section*{Declaration of competing interest}

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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\section*{Appendix A. Supplementary data}

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