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High-level expression of biotin ligase BirA from *Escherichia coli* K12 in *Pichia pastoris* KM71

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ABSTRACT

BirA is a biotin ligase from *Escherichia coli* that has been widely used in studying the interaction between avidin or streptavidin with its avi-tagged substrates, in which BirA adds a biotin molecular to a 15-amino acid acceptor peptide that is also known as avi-tag. The present study was aimed at obtaining high-level of BirA biotin ligase expression in *Pichia pastoris* KM71. In this study, the DNA coding sequence of BirA was amplified from *E. coli* K12. This novel gene was cloned into a lab-derived pHBM905A vector and transformed into *P. pastoris* KM71 for secretary expression. The expression of BirA was then identified by SDS–PAGE and Western blot analysis. The yield of the BirA reached approximately 103.5 mg/L after a 6-d induction in shake flasks, and 583.1 mg/L after 144 h of induction in 5 L fermenters. The expression levels obtained in this study were much higher than those currently reported, in which the heterologous expression of BirA with thioredoxin and MBP fusions in *E. coli* were 24.7 and 27.6 mg/L, respectively. The recombinant BirA exhibited high activity of catalyzing biotinylation *in vitro* with specific activity of 2291 U/μL. To the best of our knowledge, this is the first report of high-level BirA expression in *P. pastoris* KM71.

INTRODUCTION

Pichia pastoris is a type of yeast that uses methanol as its sole source of carbon and energy. Due to the absence of native plasmids in methanol-based yeasts, recombinants that carry foreign genes must be integrated into the chromosomes to achieve foreign gene expression. The advantage of integrated expression lies in maintaining the stability of the foreign gene and generating multiple copies of the gene so that the high expression of the foreign gene can be achieved. *P. pastoris*, as a host for expression, has been extensively studied and has industrially produced recombinant proteins. In addition, *Pichia pastoris* which can be fermented using inexpensive synthetic media can achieve more cost-effective mass production of heterologous proteins than shake-flask cultures that traditionally use enriched complex media. *P. pastoris* GS115 is one of the majorly used host strains, in which large amounts of proteins have been successfully expressed (Daly and Hearn, 2005). However, there are still some heterologous proteins that cannot be expressed well in *P. pastoris* GS115, while switching to *P. pastoris* KM71 as the expression strain successfully solved the problem (Charoenrat et al., 2013; Huiming et al., 2011).

Biotin is an indispensable organic molecule for the interaction between avi-tagged substrate and avidin or streptavidin. BirA is an biotin ligase from *E. coli* that can site-specifically catalyze the attachment of biotin to the lycine side chain with a 15-amino acid peptide acceptor (Beckett et al., 1999; Fairhead and Howarth, 2015), which is known as biotinylation. Botinylation have been used for easy detection, immobilization and purification in

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many fields. And previous studies have demonstrated that the biotinylation process happen more efficient *in vitro* (Yifeng and Rui, 2012).

N utilization substance protein A (NusA) is one of the most commonly used protein tagging labels for enhancing the yield and solubility of recombinant proteins (Cabrita et al., 2006; Vu et al., 2016; Sun et al., 2012). A fusion-tag efficiency evaluating assay indicated that seven out of eight tested large molecular proteins had been efficiently expressed at high solubility by NusA-tag, while only four were expressed with other tags such as thioredoxin (TRX), small ubiquitin-like modifier (SUMO), maltose- binding protein (MBP), glutathione S-transferase (GST) etc. (Guo et al., 2011; Goulas et al., 2014; Wang and Wang, 2006). In order to increase the yield and solubility of recombinant proteins BirA, we fused a tagged molecule NusA to the N-terminus of the protein BirA and reconstructed a new clone plasmid named NusA/pET-23a based on the vector pET-23a and then integrated the fusion gene to the expression vecor pHBM905A for expression.

In this study, the NusA fused BirA was expressed in *P. pastoris* KM71 by high-density fermentation, which significantly enhanced the expression quantity. The NusA fusion tag was cleaved from BirA by tobacco etch virus (TEV) protease, and the BirA ligase was subsequently separated from the carrier using HisTrap HP column. The identification of the recombinant BirA was confirmed by SDS-PAGE and the Western blot assay. Moreover, the biotinylation activity of the recombinant BirA was evaluated by laser scanning confocal microscope (LSCM) and an anti-biotin western blot assay.

MATERIALS AND METHODS

Strains, plasmids and media

P. pastoris KM71, *E. coli* strain XL10-gold, DH5α, Rosettle blue and the vector pET -23a were purchased from Invitrogen (USA). The expression vector pHBM905A is stored in our laboratory (Zhang et al., 2009). Luria-Bertani (LB) medium was prepared for the cultivation of *E. coli* as described in the manual of molecular cloning (Farris and Edwards, 2001). Minimal dextrose (MD), buffered minimal glycerol (BMGY) and buffered minimal methanol (BMMY) medium were prepared as described in the instructions of the *P. pastoris* expression manual from Invitrogen (USA).

Reagents

All oligonucleotides were synthesized at Sangon Biotech (Shanghai). Pfu DNA polymerase, Type II restriction endonucleases, calf intestinal alkaline phosphatase (CIP), T4 DNA ligase and DNA marker were obtained from Thermo Scientific (USA). Tris, glycine, SDS, 30% acrylamide/bis, and protein standards were purchased from Bio-Rad. Isopropyl β-D-1-thiogalactopyranoside (IPTG) and adenosine 5′-triphosphate (ATP) disodium salt hydrate (A7699-10G) were purchased from Gold Biotechnology and Sigma, respectively. HisSep Ni-NTA Agarose Resin was purchased from Qiagen. HisTrap HP column (5 mL) was purchased from GE Healthcare. Immunopure D-biotin and Zeba spin desalting column (0.5 mL) were obtained from Pierce and Thermo Scientific, respectively. The anti-6× His and anti-biotin monoclonal antibody was purchased from California Bioscience (USA). All other chemicals used in this study were of the highest purity commercially available.

Construction of fusion protein plasmid for expression in *P. pastoris*KM71

For NusA fusion expression construction (Figure 1A), the sequence encoding NusA was inserted into the modified commercial vector pET-23a. The sequence encoding NusA was polymerase chain reaction (PCR) amplified with primer 1 (GGGAGCTCATGAACAAAGAAATTTTGGCTGTAGTTG AAGCCG) and primer 2 (GGCCTAGGATTCGGGCAGCCATAATCAGTGCTCCG GCTTTTTCGTCGGTCAAC) using *E. coli* strain DH5α genome as the template and inserting the *BamH*I and *Xho*I double digested PCR product into similarly digested pET-23a (Guo et al., 2011). The recombinant plasmid was named NusA/pET-23a. The gene encoding BirA was PCR amplified with primer 3 (GG CATATG AAGGATAACACCGTGCCACTGA) and primer 4 (GGGTCGACTTAGTGATGGTGGTGATGATGATGATGT TTTTCTGCACTACGCAGGGATATTTCAC) (contain a 6×His tag sequence) using *E. coli* strain DH5α genome as the template (Etemadzadeh et al., 2015; Qing et al., 2005). The forward primer contains the XhoI restriction site and the sequence encoding the TEV protease cleavage site, while the reverse primer contains the *Nde*I restriction site. The PCR amplified BirA gene was then digested with *Xho*I and *Nde*I and ligated into NusA/pET-23a digested with the same enzymes (Vincze et al., 2003). The resulting plasmid was a recombinant plasmid of the NusA fusion, designated as NusA-BirA/pET-23a, allowing target protein expression (Figure 1A).

Recombinants were initially identified by PCR amplification, and then plasmid diagnostic restriction digestion and DNA sequencing again verified the presence and identity of the inserts in both constructs.

For the *P. pastoris* expression, the full-length fusion gene was amplified with primer 5 (GTCAATGAACAAAGAAATTTTGGCTGTAGTTGAAGCC G) and Primer 6 (GGCCATTAGTGATGGTGGTGATGATGTTTTTCTGCAC TACGCAGGGATATTTCAC). Then, the amplified product

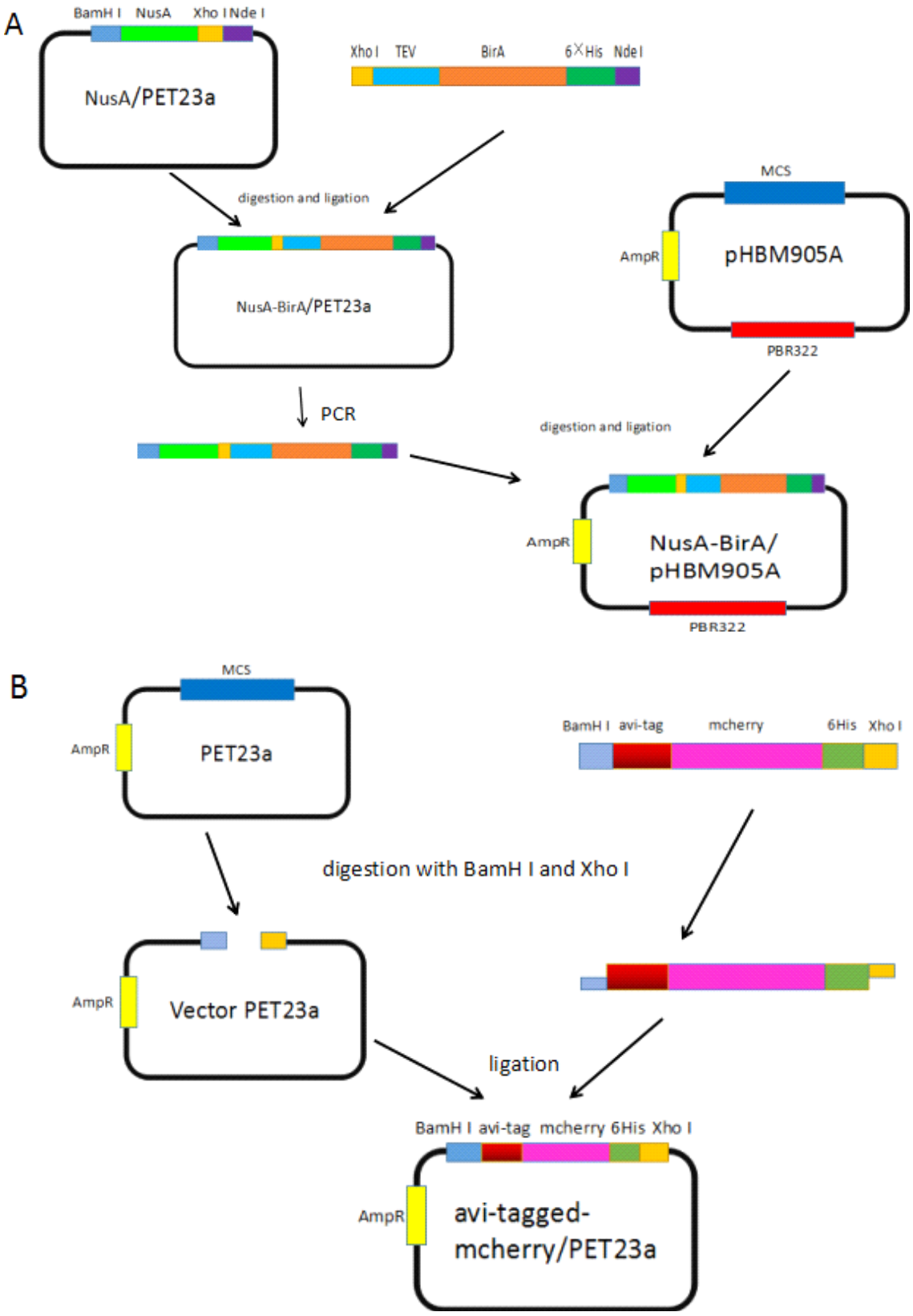

Figure 1. Schematic representation of two constructs in which the target protein is expressed as a NusA fusion protein (A) and the avi-tag substrate with a 6 × His-tag (B).

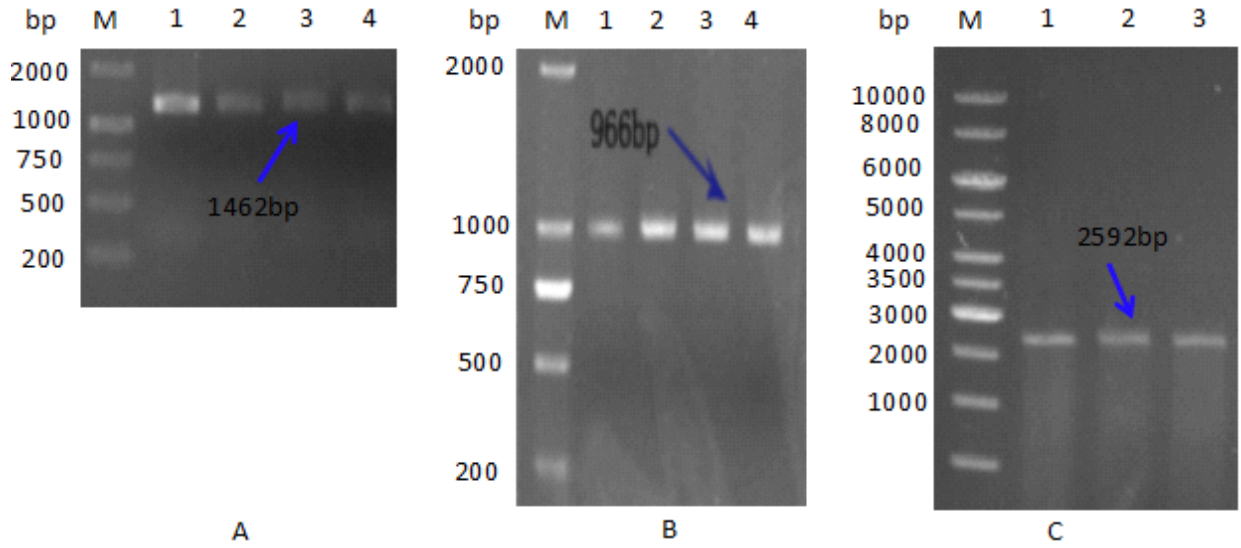

Figure 2. A.M, DNA molecular weight markers (the size of each band was indicated on the left). Lane 1-4:PCR product of NusA tag with prime1 and 2. **B**. M, DNA molecular weight markers (the size of each band was indicated on the left). Lane 1-4: PCR product of BirA with primer 3 and 4. **C**. M, DNA molecular weight markers (the size of each band was indicated on the left). Lane 1-3: PCR product of NusA-BirA with primer 5 and 6.

was treated with T4 DNA polymerase supplemented with 1 mMdTTP to form overhangs compatible with the sticky ends of the pHBM905A vector digested with *Cpo*I and *Not*I (Zhang et al., 2009; Aslanidis and Jong, 1990). These fragments were ligated with T4 DNA ligase and transformed into *E. coli* XL10-gold to generate circular recombinant plasmids. And then the circular recombinant plasmid (10 µg) was linearized with *Sal*I and transformed into *P. pastoris* KM71 via electroporation (4 kΩ, 50 µF, 400 V) using the Life Technologies Cell Porator (USA). The positive transformants were selected on MD plates and identified by colony PCR. The PCR results of the corresponding sequences mentioned previously are shown in Figure 2.

Shaking flask expression of BirA in *P. pastoris* KM71

Recombinant *P. pastoris* KM71, which incorporated the gene of interest NusA-BirA in its genome, was incubated in 100 mL of BMGY. After an additional 2 days at 28°C on a shaker, until approximately OD_{600} of culture reached 20, all cells were collected by centrifugation under sterile conditions and transferred to 50 mL BMMY medium for further culture at 28°C shaker. 250 microliters of 1% (v/v) methanol was added every 24 h to induce the expression of the target protein BirA. Approximately 1 ml of cell culture was collected every 24 h and centrifuged at 8,000×g for 5 min to remove the cell-conditioned supernatant. After 144 h, aliquots (20 µL) of supernatants stored daily were loaded onto a 12% (w/v) polyacrylamide gel and subjected to SDS-PAGE followed by staining with Coomassie brilliant blue G-250 (Schägger, 2006). The total protein concentration in each supernatant was measured using a Micro-BCA protein assay reagent (Pierce, USA).

High-density fermentation expression of BirA in *P. pastoris* KM71

The fermentation process for BirA was performed according to the *Pichia* Fermentation Process Guidelines of Invitrogen. In this study, high-cell density fed-batch cultivation of the recombinant *P. pastoris* KM71 was carried out in a-l BioFlo 2000 fermenter (New Brunswick Scientific, USA). The stock of the transformed *P. pastoris* strain was recovered on a MD plate. The positive colony strain was inoculated into a flask containing 200 mL of yeast extract-peptone-dextrose (YPD) medium and incubated at 28°C and 250 rpm for approximately 20 h, and the OD_{600} reached 2-6; these colonies were used as the primary seeds. And then 200 mL of YPD medium was transferred into 2 L basal salts medium (BSM) medium (85% H_3PO_4 26.7 mL/L, $CaSO_4$ 0.93 g/L, K_2SO_4 18.2 g/L, $MgSO_4 \cdot 7H_2O$ 14.9 g/L, KOH 4.13 g/L, glycerol 40.0 g/L) with 4.35 mL/L PTM1 trace salts and 2.0 g/L histidine. The PTM1 trace salts in 1 L contained $CuSO_4 \cdot 5H_2O$ 6.0 g, KI 0.08 g, $MnSO_4 \cdot H_2O$ 3.0 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, H_3BO_3 0.02 g, $ZnCl_2$ 20.0 g, $FeCl_3$ 13.7 g, $CoCl_2 \cdot 6H_2O$ 0.9 g, H_2SO_4 5.0 mL, and biotin 0.2 g. After the carbon source was exhausted, which was indicated by a sharp

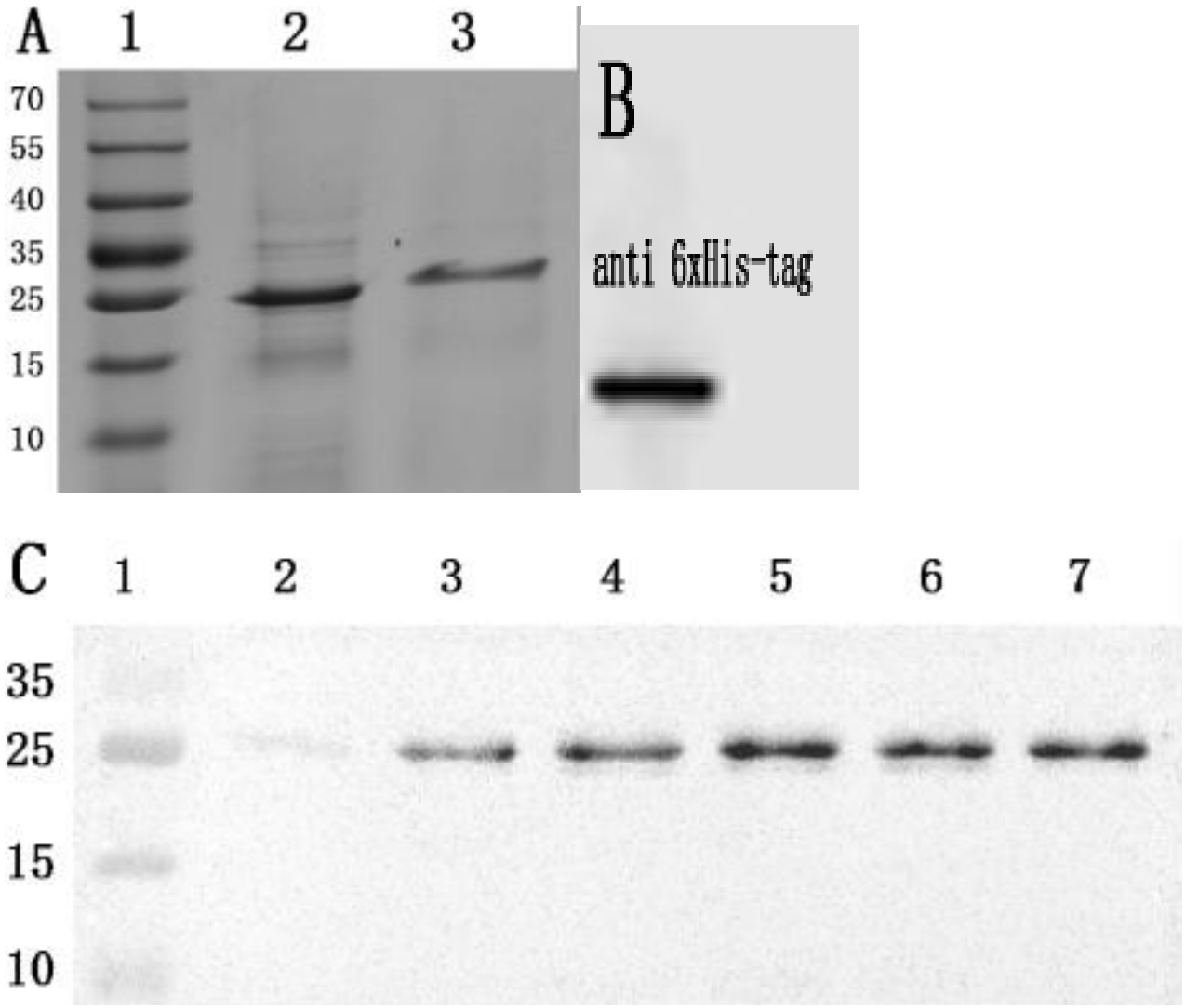

Figure 3. A, SDS-PAGE analysis of substrate with an avi-tag at the N-terminus of protein. Lane 1: protein standards; lane 2: unbiotinylated substrate (avi-taggdmcherry); lane 3: substrate that was biotinylated with a certain amount of the expressed and purified BirA ligase. **B,** The substrate containing an avi-tag was identified by an anti 6xHis-tag western. **C,** The result of streptavidin-HRP anti-biotin western blot. lane 1: The position of the control protein standards that was symbolized by special fluorescent pen; lane 2-7: western blot of the biotinylatedavi-tagged substrate (free biotin left in the solution was removed using a Zeba spin desalting column) adding different amount of BirA ligase (0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 μM of recombinant BirA was added to 30 μM of substrate, respectively).

increase in the dissolved oxygen tension (DOT), 12 mL of PTM1 trace salts/L with 50% (v/v) glycerol was supplied continuously. When the OD_{600} of the cell culture reached ~300 or the cell density reached about 220 g/L, methanol with 12 mL of PTM1 trace salts/L was added to induce the expression of BirA. Methanol was added at a rate of about 1.30 g/h and gradually increasing to 2.80 g/h after 24 h in the production stage. The pH and temperature of the system were decreased to 6.5 and 30°C, respectively. Approximately 50 mL of cell culture was collected every 12 h for SDS-PAGE analysis.

Expression of avi-tag substrate in *E. coli* Rosttle blue

We previously made a construct for producing avi-tagged target protein expressed as a mcherry fusion fluorescent protein whose molecular weight is 25.7 kDa (Figures 3A and 2) and show red when expressed in *E. coli* Rosttle blue by shaking flask. Avi-tag sequence was added at the N-terminus of mcherry, and 6×His tag was added at the C-terminus of mcherry as showed in Figure 1B. This construct allows C-terminal fusion of 6×His-tagged mcherry expression by introducing a stop codon at the

end of the 6×His tag coding sequence. When the expression strain reached an OD_{600} of 0.6-0.8 at 37°C on a 250 rpm shaker, 1 mM IPTG was added to induce expression of this avi-labeled mcherry on a 250 rpm shaker at 18°C. As a result, the protein was found to be highly soluble and purified by Ni^{2+}-affinity chromatography. The avi-labeled mcherry was used as a BirA catalyzed *in vitro* biotinylated substrate.

Purification of the recombinant fusion BirA

To purify the protein of interest, about 300 mL of the fermentation supernatant after 144 h of methanol induction was centrifuged at 8000 × g for 5 min to remove the cells. The supernatants were collected and dialyzed against buffer A (20 mMTris-HCl; 5 mM Na_2EDTA; pH 6.8) using a Millipore 10 kDa cut off membrane device at 4°C to remove ions and salts and then shake with Ni-NTA resin at 4°C for 3 h. After two washes with low concentrations of 25 mM imidazole, the protein was eluted with cell lysis buffer containing 10% glycerol and 300 mM imidazole to remove other proteins and small peptides as described in the Ni-NTA Resin Handbook by TransGen Biotechnology (China) (Etemadzadeh et al., 2015; Moser et al., 2014). The final, relatively pure sample was concentrated and stored in 15 mL biotin ligase storage buffer (50 mM imidazole, 50 mMNaCl, 5% glycerol, 5 mMTris-HCl, pH 6.8).

Enzyme activity assay

Definition of Enzyme Unit: 1 unit of enzyme can biotinylate 1 pmol of polypeptide substrate in a reaction mixture containing 38 µM of polypeptide substrate within 30 min at 30°C. The biological activity of recombinant biotin ligase was evaluated by applying amounts of enzyme to 38 µM avi-tagged substrate in the presence of buffer A (0.5 M Bicine, pH 8.3) and buffer B (100 mM ATP, 100 mMMgOAc, 500 µM D-Biotin). The biotinylation reaction was allowed to proceed at 30°C in 30 min (Cull and Schatz, 2000).We previously made a bacterial surface display construct for confirming the BirA catalyzed *in vitro* biotinylation (Min et al., 2011). It was approached by made the expression of streptavidin on the outer membrane of *E. coli* bacterial cells via a domain called sf-GFP by using auto-display technology. The biotin-binding activity of the auto-displayed streptavidin was estimated after treatment with fluorescently labeled biotin by LSCM which directly certificate the activity of the recombinant BirA.

RESULTS

Shaking flask expression of BirA in *P. pastoris* KM71

BirA contains 321 amino acids, folded as a monomeric protein of 36.3 kDa. The recombinant plasmid pHBM905A-BirA was electroporated into *P. pastoris* KM71 after linearization. Recombinant BirA was then expressed using positive transformants selected from MD plates and verified by whole-cell PCR using primers 3 and 4. The expressed proteins were mainly present in the soluble portion and can be purified to a high degree using Ni^{2+}-affinity and size-exclusion chromatography. The yield of the recombinant BirA in the supernatant reached the highest of ~103.5 mg/L after induced for 6 days with methanol, followed by a decline along with the increasing induction time (Figure 4A). The BirA yield obtained in our study is around 4-fold higher than those of previously reported Yifeng and Rui (2012).

High-density fermentation expression of NusA-BirA fusion protein in *P. pastoris* KM71

The molecular weight of NusA-BirA fusion protein is presumed to be 89.3 kDa. The recombinant plasmid pHBM905A/NusA-BirA was electroporated into *P. pastoris* KM71 after linearization, and the positive transformant was selected from the MD plate and identified by whole-cell PCR using primers 5 and 6. Then the recombinant strain was inoculated into a 5 L fermenter for high-density fermentation. The fermentation supernatant was collected and assayed for detecting the expression of NusA-BirA fusion protein. First, a western blot using anti-6×His antibody confirmed that the major band of approximately 89.3 kDa was NusA-BirA (Figure 5C). The results shown in Figure 5A represent the supernatants collected from the fermentation supernatant. After methanol induction, cell wet weight increased from 165 to 434 g/L. As shown in Figure 5A, the expression yield of NusA-BirA reached a maximum concentration of ~ 583.1 mg/L after 6 days of induction, followed by an increase in induction time with no change in the expression yield. As it was presented in our studies, the yield of the recombinant NusA-fusion protein from the high-density fermentation was approximately 2-fold higher than the BirA without NusA-tag expressed from shaking flask, and is approximately 8-fold higher than the previously reported (Yifeng and Rui, 2012). The expression of fusion BirA was identified by adding a certain amount of supernatant (20 µL) onto a 12% (w/v) polyacrylamide gel for SDS-PAGE, followed by staining with Coomassie Brilliant Blue G-250.

Target protein release and isolation

Through a TEV protease cleavage site located in the linker between NusA and BirA in the NusA-BirA fusion protein, the NusA was cleaved off the fusion complex with TEV protease at a mass ratio of 100:1 (that is, for 1 mg of purified fusion, 10 µg of protease was added). The

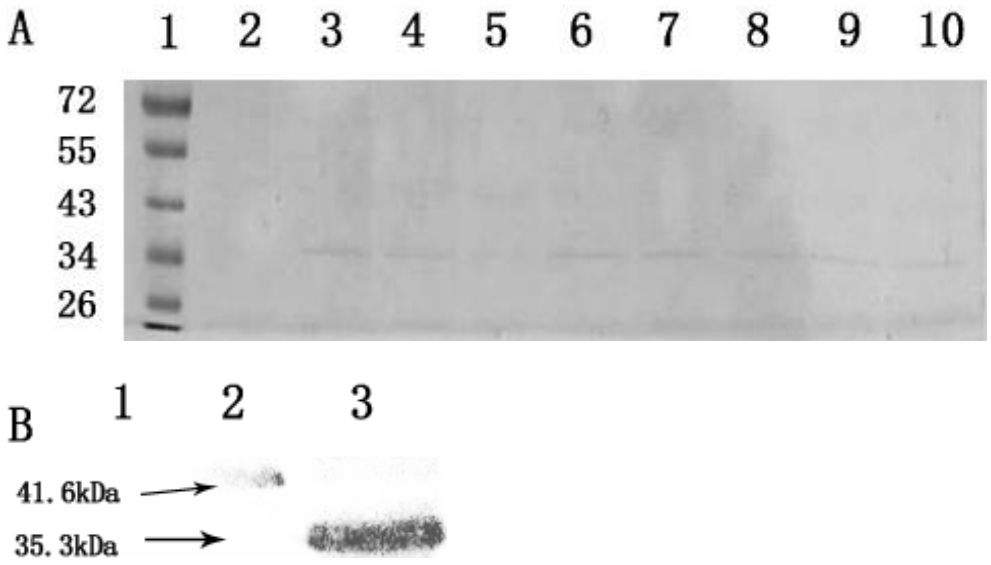

Figure 4.A, SDS-PAGE analysis of BirA ligase secreted in the cell culture supernatant.Lane 1: Protein standards; Lane 2: negative control (*P. pastoris* bearing the pHBM905A vector); lane 3-10: shaking flask supernatants collected from 1 day to 8 day after induced by methanol. **B,** Anti-His-tag Western blot of BirA. lane 1: negative control; lane 2: positive control (Pro K from *P. pastoris GS115* with a 6×His-tag); lane 3: BirA ligase from the shaking flask supernatant.

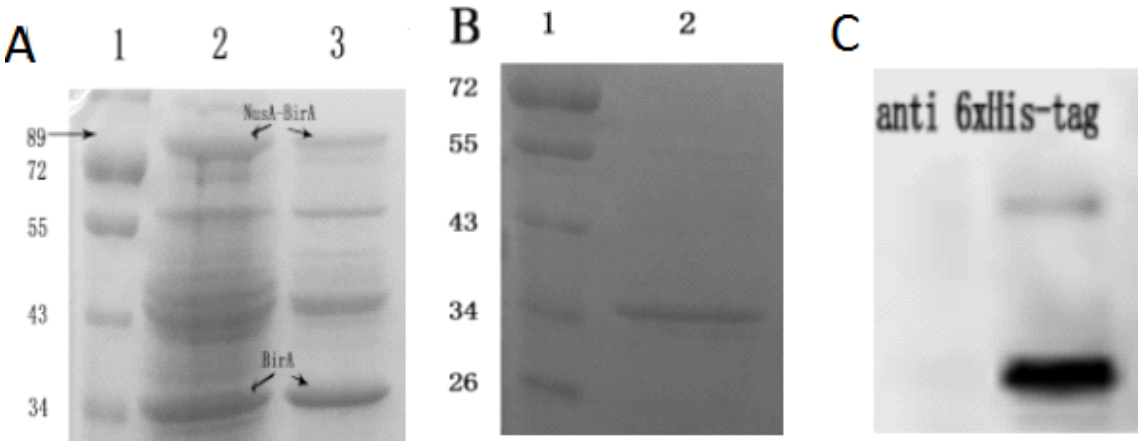

Figure 5. A, SDS-PAGE analysis of BirA ligase from the fermentation supernatant 84 h later after induced by fed-batch methanol. Lane 1: protein standards. Lane 2: primary samples from the fermentation supernatant; lane 3: the fusion protein purified with Ni^{2}+-affinity chromatography. The bands between 89 and 35 kDa may be due to the various degradation of the NusA-tag. **B,** SDS-PAGE analysis of BirA ligase from the fermentation supernatant after TEV protease cleaved. Lane 1: protein standards; lane 2: cleavaged and purified samples. **C,** anti-His-tag Western blot of the cleavaged and purified protein.

cleavage reaction was proceeded at 30°C for 30 min containing 10% TEV buffer (500 mMTris-HCl, pH 8.0; 5 mM EDTA; 10 mM DTT) (Kapust et al., 2001). After TEV protease cleavage, the reaction mixture was subjected to a HisTrap HP column (5 mL), and the unbound fraction containing recombinant BirA was collected and stored in BirA storage buffer containing 50% glycerol at -20°C until use (Li and Sousa, 2012). The result are shown in Figure 5B.

Enzyme activity assay

As shown in Figure 5,in the presence of buffer A (0.5 M

Bicine, pH 8.3) and buffer B (100 mM ATP, 100 mM MgOAc, 500 µM D-biotin), different amounts of enzyme were applied to 38 µM avi-tagged substrate mcherry (in cell lysis buffer, pH 8.0) to assess the biological activity of recombinant BirA. The reaction was allowed to proceed at 30°C for 30 min (Cull and Schatz, 2000). To circumvent the difficulties of confirming the activity, a sf-GFP-fused streptavidin was expressed and surface displayed on the outer membrane of *E. coli* strain BL21 (DE3). The streptavidin is a tetrameric molecule that have an extremely high affinity for biotin with biotin binding sites at each subunit (Moser et al., 2014; Schmitz, 2002; Sau-Ching and Sui-Lam, 2005). Sf-GFP-fused-streptavidin displayed on the cell surface can detect biotinylated protein avi-tagged mcherry. At last, biotinylation was visually confirmed by LSCM (Schumacher and Joachim, 2012). Before applying sample to the glass slide, free biotin left in the solution was removed using a Zeba spin desalting column (0.5 mL). The result is shown in Figure 6.

DISCUSSION

We know that *E. coli* is an intracellular protein expression system. At present, BirA induced in *E. coli* Rosettle blue cells mainly exists in the form of insoluble inclusion bodies in the culture conditions at 37°C. If the protein is not attached affinity tag, it is difficult to be obtained with high purity. Fusion expression usually increases the solubility of the protein and provides affinity for the purification of the protein in order to obtain a purer protein. In addition, no studies have reported the heterologous expression of biotin ligase in *P. pastoris*.

In our study, we reported the development of a superior method for the expression of a high quantity of recombinant NusA-fused BirA ligase. The fusion proteins can be efficiently cleaved by TEV protease at engineered sites to release the BirA ligase. The liberated target protein can be effectively separated from the carriers using HisTrap HP column (Li and Sousa, 2012). We made use of the efficiently *P. pastoris* secretory expression system to express the NusA-tagged BirA and the expression yield was as high as ~583.1 mg/L, which finally gave a yield of cleaved and purified BirA of 191.3 mg/L. Compared with other expression method, including the previously reported BirA ligase purification with thioredoxin and MBP fusion tags, which exhibited the protein yields of 24.7 and 27.6 mg/L, respectively, the BirA protein yield reported here was a huge boost. This study also indicated that the yield of BirA ligase expressed with a NusA-tag (583.1 mg/L) was extremely higher than that of the un-tagged BirA (103.5 mg/L) with a concentration ratio of approximately 5:1. The purified fusion protein has been used to catalyze the *in vitro* biotinylation of avi-tag-bearing substrate (avi-tagged mcherry) at an optimal condition of 10% buffer A and buffer B mentioned previously in 30°C for 30 min,which was indicated by the anti-biotin western blot of the biotinlatedavi-tagged mcherry using streptavidin-HRP nomoclonal antibody and LSCM.

At the beginning of our experiments attempts, the expression of BirA ligase was carried out in the yeast strain GS115 rather than KM71, but no target protein existing in the culture supernatant was detected with the shaking flask fermentation. The additions of soluble tag such as MBP, Trx, SUMO, GST made very limited improvements. As we all know, *P. Pastoris* uses methanol as a carbon source, the catalytic enzyme is an ethanol oxidase, and methanol is metabolized to formaldehyde and hydrogen peroxide under the action of AOX. There are two kinds of AOX in *P. pastoris*, named AOX1 and AOX2, respectively. The affinity of AOX1 to O_2 is very weak, so *P. pastoris* needs to produce a large amount of the enzyme to compensate for the metabolism of methanol. When methanol was the sole carbon source, AOX1 played a major role, and AOX produced more than 30% of total soluble protein. The phenotype of this strain is Mut^+ and GS115 belongs to this category. The amino acid sequence of AOX2 is similar to that of AOX1, and about 97% is homologous sequence. When there is no AOX1 in the body, the use of AOX2 can also decompose methanol. This phenotype is called Mut^s, and KM71 is such a strain. It can be seen that the demand of two strains for methanol is not the same. Therefore, when inducing expression, we must control the concentration of methanol, so as to facilitate the growth of the best way of the strain and maintain its vitality. More importantly, when the KM71 strain induces expression, the yeast inoculum concentration should be increased (Zhang et al. 2001). So switching to KM71, we found that the BirA ligase was successfully expressed at a concentration of 103.5 mg/L in the supernatant. We also tested the protein tags mentioned above in GS115, and find only the NusA-tag could improve the yield of BirA ligase to be 583.1 mg/L in the supernatant.

The results presented in our study suggested that the *P. pastoris*KM71 expression system might be a suitable choice for high levels of expression of various types of proteins as a complementary approach to *P. pastoris*GS115 and could offer a more facile and efficient method to obtain pure BirA ligase. The produced recombinant BirA ligase is highly active in catalyzing *in vitro* biotinylation. The change in the color of the cells by the combination of the green-emitting sf-GFP displayed on the cell surface with the cherry-red mcherry observed by the LSCM confirmed the activity of the biotin protein ligase.

In conclusion, we developed a protocol for producing highly pure BirA ligase in relatively large amount in *P. pastoris* KM71. This recombinant ligase is enzymatically active and can be used for *in vitro* biotinylation of avi-

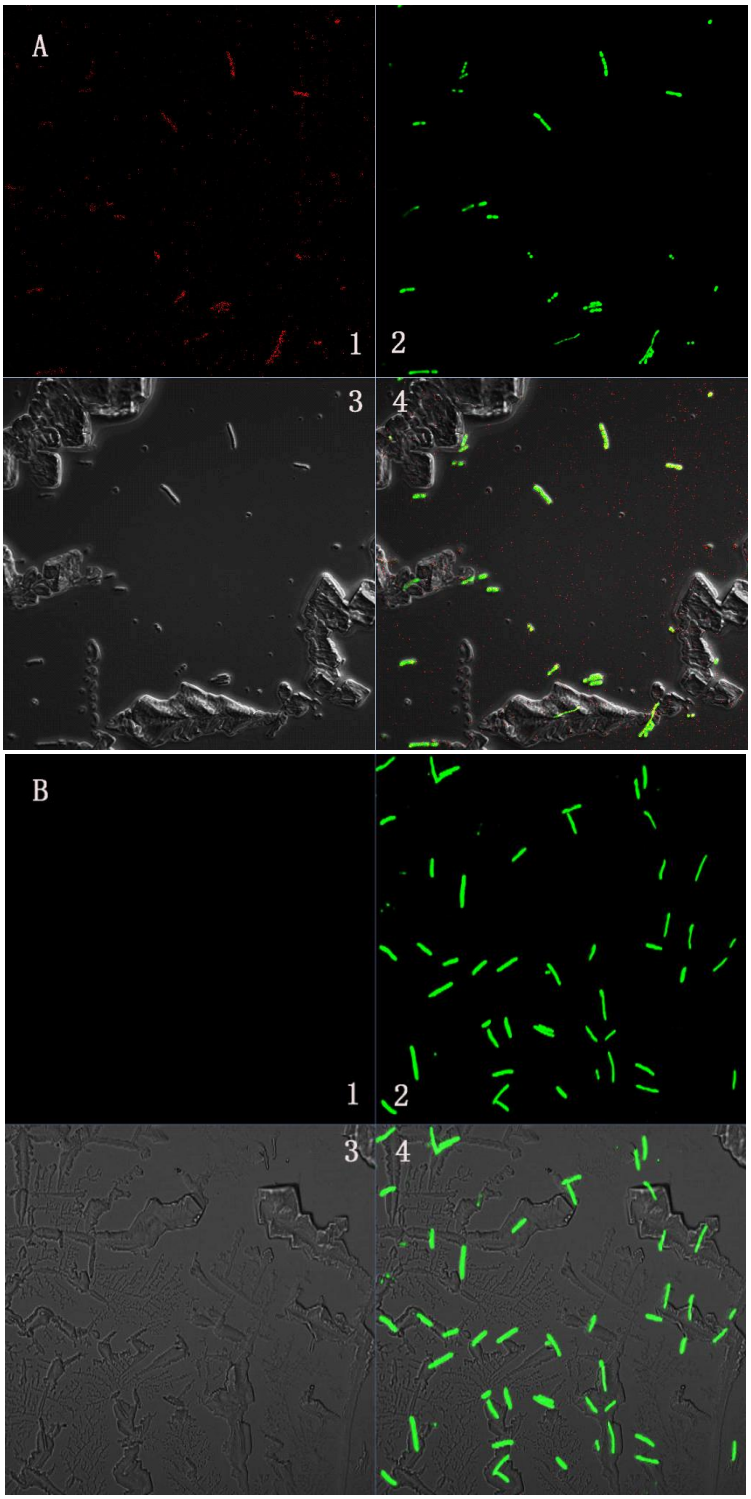

Figure 6. A, Visual identification of the *E. coli* cell with sf-GFP-streptavidin on the surface of out membrane by LSCM. 1, The *E. coli* cell with sf-GFP-streptavidin on the surface of the outer menbrane incubated with biotinylated substrate (biotinylatedavi-tagged mcherry); 2, the *E. coli* cell before incubating with biotinylatedavi-tagged substrate; 3, the negative control; 4, the image overlay of picture 1 and picture 4. **B,** The LSCM of *E. coli* cell incubating with avi-tagged substrate that was not biotinylated because none expressed BirA was added to the reaction mixture.

tagged substrates for various purposes.

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