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## Construction of arming *Yarrowia lipolytica* surface-displaying soybean seed coat peroxidase for use as whole-cell biocatalyst

Ji-ming Wang<sup>a</sup>, Chao-ming Wang<sup>b,c</sup>, Xiao Men<sup>a</sup>, Tong-qin Yue<sup>d</sup>, Catherine Madzak<sup>e</sup>,  
Xiao-hua Xiang<sup>f</sup>, Hai-ying Xiang<sup>g</sup>, Hai-bo Zhang<sup>a</sup>

<sup>a</sup> CAS Key Laboratory of Biobased Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 26601, China

<sup>b</sup> CAS Key Laboratory of Animal Models and Human Disease Mechanisms / Key Laboratory of Bioactive Peptides of Yunnan Province, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China

<sup>c</sup> Kunming College of Life Science, University of Chinese Academy of Sciences, Kunming 650204, China

<sup>d</sup> College of Life Science, Qingdao University, Qingdao 266071, China

<sup>e</sup> UMR782 GMPPA, INRA/AgroParisTech/Université Paris-Saclay, 78850 Thiverval-Grignon, France

<sup>f</sup> Hainan Cigar Research Institute, Hainan Provincial Branch of China National Tobacco Corporation, Haikou 571100, China

<sup>g</sup> Yunnan Academy of Tobacco Science, Kunming 650106, China

**Keywords:** *Yarrowia lipolytica*, Immobilization, Cell surface display, Arming yeast, Culture optimization

**Abbreviations:** SBP, soybean seed coat peroxidase; AA, amino acid; GRAS, generally recognized as safe; GPI, glycosylphosphatidylinositol; CLSM, confocal laser scanning microscope; SEM, scanning electron microscopy; ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); EDTA, ethylenediaminetetraacetic acid

### ABSTRACT

Whole-cell biocatalysts could be used in wide-ranging applications. In this study, a new kind of whole-cell biocatalyst was successfully constructed by genetically immobilizing soybean seed coat peroxidase (SBP) on the cell surface of *Yarrowia lipolytica* Po1h, using a new integrative surface display expression vector (pMIZY05). The coding sequence of SBP was optimized and chemically synthesized, then inserted into pMIZY05 to generate expression plasmid pMIZY05-oEp. A DNA fragment corresponding to SBP and selection marker expression cassettes, without bacterial sequences, was released from pMIZY05-oEp by enzyme digestion and used to transform host yeast cells. A transformant (CM11) with a high recombinant SBP activity of 1571.9 U/mL was obtained, and recombinant SBP was proved to be successfully anchored on cell surface by testing the activities of different cellular fractions. After optimization of culture conditions, the recombinant SBP activity of CM11 was increased to 4187.8 U/mL. Afterwards, biochemical properties of the recombinant SBP were determined: optimum catalytic conditions were 37.5°C at pH 3.5, and recombinant SBP exhibited high stability during thermal or acidic treatment. Recombinant activity of cell-displayed SBP was re-examined at optimum temperature and pH, which promoted an increase up to 4432.5 U/mL. To our knowledge, this represents the highest activity ever reported for heterologous expression of SBP. This study also provides a useful strategy for heterologous expression of proteins which could be toxic to intracellular content of host cells.

### 1. Introduction

Soybean seed coat peroxidase (SBP, EC 1.11.1.7) belongs to the family of class III secretory plant peroxidases which are heme-containing glycoproteins and are able to catalyze the oxidation of a broad range of organic and inorganic reductants using H<sub>2</sub>O<sub>2</sub> as electron acceptor. SBP demonstrates extraordinary stability and catalytic properties. The melting temperature (at which free energy change is equal to zero) of SBP is 86°C without Ca<sup>2+</sup> at pH 7.0, and rises to 90.5°C at pH 8.0 with 1mM Ca<sup>2+</sup>, hence much higher than those from other peroxidases [1–3]. SBP retains its catalytic activity over a wide pH range (2.4–12) with an optimum pH of 2.4 using veratryl alcohol as substrate, while pH optimum in the range 5.5–6.4 have also been reported using other substrates [3–7]. Due to its outstanding characteristics, this enzyme is widely used in many fields, such as food additives, medical tests, biosensors, and waste water treatment [8–12]. SBP could be extracted and purified from soybean seed coats [13–15], which usually concerns a series of problems such as difficult storage and transportation of the raw materials and relatively complex purifications. Thus, heterologous microbial production of SBP was considered to be an alternative approach. A gene encoding SBP, named Ep (also known as SPOD4.1, Gene ID: 548068), has been successfully cloned and found to encode a full-length protein of 352 amino acids (AA), including a prepeptide of 26 AA at the N-terminus and a propeptide of 20 AA at the C-terminus which are processed during their trafficking through the secretion pathway [16–18]. The mature of SBP needs post-translational modifications, such as cyclization of the glutamine residue of the N-terminus to pyrrolidone carboxylic acid, glycosylation, and formation of four intramolecular disulfide bonds [16,19]. Bacterial expression systems (e.g. *E. coli*) lacking complete post-translational modification are not suitable for heterologous expression of SBP. When the codon-optimized coding sequence of truncated SBP without its prepeptide was heterologously expressed in *E. coli* BL21(DE3), active recombinant SBP was obtained but exhibited very low activity [20]. Therefore, expression of SBP in a eukaryotic host constituted a preferred strategy. Not only production of SBP but also applications always suffered from some drawbacks related to enzymatic stability and/or reusability. Using enzymatic biocatalysts for cost-effective applications always takes advantage of immobilization to remedy these drawbacks. The classic methods of SBP immobilization bind enzyme to non-biological matrices using some harmful chemical reagents. Recently, an emerging avenue for enzyme immobilization known as cell surface display has obtained much attention due to the emergence of a broad range of biotechnological and industrial applications, including bioconversion, bioremediation and so on [21–23]. Cell surface display immobilizes peptides and proteins on the surface of microbial cells by fusing them with anchoring motives, thus endowing the surface of host cells with new functions. Immobilization of SBP on the yeast surface to construct a whole-cell biocatalyst was expected to be an attracting way. *Yarrowia lipolytica*, a non-conventional yeast with GRAS (Generally Recognized as Safe) status, has aroused great industrial interest and research focus owing to its unique characteristics. It is distinguished by its capacity to grow on n-paraffins, to produce high amounts of organic acids and to secrete naturally several enzymes to the medium [24]. *Y. lipolytica* has been studied as the model organism for general secretion pathway [25] and developed into a successful alternative expression system, especially for the expression/secretion of complex eukaryotic proteins [26]. This yeast is currently being developed as a workhorse for biotechnology, as more and more engineering tools become available, allowing to apply its potential to the biosynthesis of valuable products [27]. In *Y. lipolytica*, display of heterologous peptides or proteins could be achieved by fusing the passenger proteins to the anchoring regions of cell wall proteins, such as YICwp1p and YIPir1p [28,29]. While searching *Y. lipolytica* genome, we recently found that a function-unknown protein, assigned as YALIOF24255p, contained a classical glycosylphosphatidylinositol (GPI) anchoring domain, as predicted by PredGPI algorithm [30]. YALIOF24255p is a 261 AA protein composed of a secretion signal region at its N-terminus, an active region, a region rich in serine and threonine residues, and a

putative GPI anchor-attachment signal. Quite predictably, if a peptide or a protein replaces the active region, it should be anchored on the cell surface. We choose to use the fragment encoding the region rich in serine and threonine residues and the GPI anchor for constructing a new cell surface display vector, based on the zeta-carrying integration vector pINA1317 [31]. Zeta sequence is the long terminal repeat from Ylt1 retrotransposon, which allows the plasmid to integrate either by homology in *Y. lipolytica* strains carrying Ylt1, or by non-homologous recombination in strains devoid of this retrotransposon [31,32]. Our pINA1317-based vectors use the strong growth-phase dependent synthetic hp4d promoter and the coding sequence of extracellular alkaline protease prepeptide (XPR2pre) to drive respectively the expression and secretion of heterologous proteins [32,33]. Furthermore, the bacterial moiety of these vectors can be removed before transformation, generating transformant strains devoid of bacterial DNA (maintenance of GRAS status) and avoiding the spread of antibiotic resistance genes from vectors into the environment [31,33]. In the present work, SBP was heterologously expressed and genetically displayed on the cell surface of *Y. lipolytica* Po1h strain, using a novel cell surface display vector designated as pMIZY05, to construct a whole-cell biocatalyst of which the biochemical properties were investigated after optimization of culture conditions.

## 2. Materials and methods

### 2.1. Strains, plasmids and media

*Y. lipolytica* Po1h was cultivated in yeast-peptone-dextrose (YEPD) medium (10.0 g/L yeast extract, 20.0 g/L peptone, 20.0 g/L dextrose) in a 250-mL shake flask containing 50 mL YEPD broth incubated at 28°C for 18 h. The yeast transformants were selected on synthetic minimal medium plates (YNB-N<sub>5000</sub>: 1.7 g/L yeast nitrogen base without AA and ammonium sulfate, 10.0 g/L dextrose, 5.0 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20.0 g/L agar) at 28°C for 3 days. PPB medium (20.0 g/L sucrose, 1.32 g/L yeast extract, 1.32 g/L NH<sub>4</sub>Cl, 0.32 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.132 g/L MgCl<sub>2</sub>, 0.11 g/L CaCl<sub>2</sub>, 0.34 mg/L thiamine, buffered at pH 6.0 by 20mM citric acid-NaH<sub>2</sub>PO<sub>4</sub> solution) was used to produce recombinant protein. *E. coli* DH5 $\alpha$  used for gene manipulation was grown in 5.0 mL of Luria-Bertani (LB) broth (10.0 g/L tryptone, 5.0 g/L yeast extract, 10.0 g/L NaCl) at 37°C overnight. The strains and the plasmids used are listed in Table 1.

### 2.2. DNA manipulations and transformation

DNA manipulations were carried out using routine methods. Yeast genomic DNA was extracted using Dr. GenTLE™ (from Yeast) High Recovery Kit (Takara). Bacterial plasmid DNA was purified using Monarch™ Plasmid Miniprep Kit (NEB). PCR products were recovered using Monarch™ DNA Gel Extraction Kit (NEB) or Monarch™ PCR & DNA Cleanup Kit (NEB). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer's manuals. PCR amplifications were performed with 20 or 50  $\mu$ L of PCR reaction systems containing 1 $\times$  PrimeSTAR® max premix (Takara), primers 0.5  $\mu$ M each, and 1.0  $\mu$ L of DNA template (about 100 ng). *E. coli* was transformed with plasmid DNA according to Sambrook et al [35]. The *E. coli* transformants were plated out for selection onto LB medium containing 100  $\mu$ g/mL ampicillin or 50  $\mu$ g/mL kanamycin. *Y. lipolytica* was transformed via LiAc/ssDNA/PEG4000 method [36]. The yeast transformants were selected on YNB-N<sub>5000</sub> plates, and incubated at 28°C for 3 days.

### 2.3. Construction of cell surface display plasmids and recombinant strains

A new HindIII cloning site and a slightly modified version of XPR2pre were used to generate pINA1317m from vector pINA1317 using Takara MutanBEST Kit with one pair of primers assigned as 1317m-F and 1317m-RV. DNA manipulation was performed according to the manufacturer's manual. Then, the coding sequence of the region rich in serine and threonine residues and of C-terminal GPI-anchoring domain from the function-unknown cell wall protein YAL10F24255p was amplified with two primers GPI-F and GPI-RV, using genomic DNA of *Y. lipolytica* Po1h strain as template. The amplified DNA fragment was inserted into pINA1317m at KpnI/HindIII sites to generate pMIZY05. The DNA primers used in this study are listed in Table 2. To validate whether the newly constructed vector (pMIZY05) could drive expression and allow surface display of foreign proteins efficiently, a gene encoding eGFP was amplified by PCR from pEGFP-1 using a pair of primers (eGFP-F and eGFP-RV) and cloned in frame into pMIZY05 at SfiI/BamH I sites to generate pMIZY05-eGFP. The insertion of eGFP would form a fusion gene (XPR2pre-eGFP-GPI) on pMIZY05-eGFP. This plasmid was digested with NotI to separate the bacterial moiety from the yeast transformation cassette. This latter, carrying the eGFP coding gene, was separated by agarose gel electrophoresis, recovered by DNA gel extraction kit, and then used to transform *Y. lipolytica* Po1h. The positive transformants were grown at 28 °C in 50 mL of YPD medium in 250 mL-flask for 96 h. The green fluorescent proteins on the transformants cell surface were observed under confocal laser

scanning microscope (CLSM, Olympus Fluo-View™ FV1000, Japan) at 488 nm with a magnification of 400 × and photographed. Cells of *Y. lipolytica* Po1h carrying the yeast expression cassette from a pMIZY05 empty vector were used as controls.

The coding sequence of mature SBP (oEp, GenBank accession ID: MK288832), optimized for *Y. lipolytica* codon bias, was chemically synthesized and inserted into a cloning vector to generate pUC57s-oEp by GenScript. The sequence of the optimized gene is given in Supplementary materials. The optimized oEp gene was inserted into pMIZY05 at SfiI/BamHI sites to generate pMIZY05-oEp, of which yeast cassette was used to transform *Y. lipolytica* Po1h using the method described in the eGFP expression section. The cell morphologies of *Y. lipolytica* Po1h and the transformants were investigated and compared using cells cultured for 120 h with scanning electron microscopy (SEM). The cell samples were prepared according to the method described by Galabova et al. [37]. Digital images were acquired using an S-4800 scanning electron microscope (Hitachi, Ltd., HQ, Japan) at an instrumental magnification of 10,000 ×.

#### 2.4. Assay of recombinant enzyme activity

The colonies of the host strain and the transformants were streaked on selective plates and incubated in 250-mL flasks containing 50.0 mL of PPB medium at 28 °C with shaking at 180 rpm for 48 h. Then, 1.0mM hemin was added to the cultures to a final concentration of 8.0 μM, and the cultures continued to grow for another 72 h. The pH of the cultures was adjusted to 6.0 with an ammonium hydroxide solution at intervals of 24 h. The cultures were harvested and used as the crude enzyme. The activities of the recombinant SBP were assayed using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method [3,20]. The reaction mixture contained 0.1 mL crude enzyme, 0.5mM ABTS and 1.0mM H<sub>2</sub>O<sub>2</sub> in 0.1M phosphate buffer (pH 6.0) in a total volume of 2.0 mL. A reaction mixture prepared with water, rather than with recombinant SBP, was used as the control. The reaction mixtures were incubated at 30°C for 3 min. The changes of the optical density at 420 nm were recorded using a Cary 50 UV-vis spectrophotometer (Agilent Tech. Inc, USA). All measurements were performed in triplicate. One unit of enzyme was defined as the amount producing 1.0 μM of oxidized ABTS per minute at 30°C. The activity of the recombinant SBP was calculated using the equation as follows. A transformant assigned as CM11 was found to produce the highest recombinant SBP activity and was chosen for further experiments.

$$\text{Activity}(U/mL) = \frac{N \times \Delta A_{420} \times V_S \times 10^3}{\epsilon \times t \times L \times V_E}$$

N: dilution factor of the crude enzyme; ΔA<sub>420</sub>: absorbance increase of the reaction mixture at 420 nm; ε: 36,000 L/M·cm, molar extinction coefficient of oxidized ABTS at 420 nm; t: 3.0 min, reaction time; L: 0.1 cm, optical path of the cuvette; V<sub>E</sub>: 0.1 mL, volume of crude enzyme in the reaction mixture; V<sub>S</sub>: 2.0 mL, final volume of reaction mixture; N: dilution factor of the crude enzyme; ΔA<sub>420</sub>: absorbance increase of the reaction mixture at 420 nm; ε: 36,000 L/M·cm, molar extinction coefficient of oxidized ABTS at 420 nm; t: 3.0 min, reaction time; L: 0.1 cm, optical path of the cuvette; V<sub>E</sub>: 0.1 mL, volume of crude enzyme in the reaction mixture; V<sub>S</sub>: 2.0 mL, final volume of reaction mixture [20].

In order to confirm that recombinant SBP was anchored to the cell wall, the recombinant SBP activities of different fractions of the culture were determined. The total activity was calculated as the sum of the activity in the culture and the activity in the cytoplasm. To determine the activity in the cytoplasm, protoplasts were prepared from CM11 cells using Lyticase (Tiangen) digestion and were broken by sonic oscillator Q125 (Qsonica, USA). For the activity of intact cells, the cells of CM11 transformant were washed twice in a physiological saline solution and resuspended in the same volume.

#### 2.5. Optimization of the culture conditions

The effects of the culture conditions on recombinant SBP activity, including culture time, carbon source, nitrogen source, initial pH of the medium, hemin concentration and inoculate age, were investigated, using a one factor at-a-time method. Later experiments could then use the best parameters screened in the former tests. The optimization of

culture conditions was achieved in PPB medium, an industrial medium for enzyme production in *Y. lipolytica* [33]. All experiments were carried out in 250-mL Erlenmeyer flasks containing 50 mL of medium, in triplicate. The best condition of each variable for recombinant SBP production was determined by comparing enzymatic activities.

#### 2.5.1. Culture time

Before medium optimization, the time course of the growth and enzyme production of transformant CM11 was determined to find the time point at which the highest enzyme activity was obtained.

#### 2.5.2. Carbon source

Dextrose, fructose, glycerol or sucrose, at the same concentration of 20 g/L, were separately used as carbon source in PPB medium.

#### 2.5.3. Nitrogen source

10 g/L yeast extract, 8.86 g/L tryptone, 9.38 g/L beef extract powder, 2.42 g/L urea, 4.30 g/L  $\text{NH}_4\text{Cl}$  or 5.31 g/L  $(\text{NH}_4)_2\text{SO}_4$  were separately used to replace the original nitrogen source in PPB medium. The nitrogen sources listed above contained equal molarities of nitrogen element.

#### 2.5.4. Initial pH of the medium

The initial pH of PPB medium was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0 or 8.0.

#### 2.5.5. Hemin concentration

The effect of hemin on enzyme production was investigated by adding different concentrations of hemin (0, 2, 4, 6, 8, 10, 12, or 14  $\mu\text{M}$ ) to the media.

#### 2.5.6. Inoculate age

The seed liquor was cultured for different times including 12, 18, 24, 36 or 48 h and then inoculated into the enzyme producing medium.

### 2.6. Biochemical characterization of the whole-cell biocatalyst

In this study, the recombinant SBP was supposed to be immobilized on the cell wall of *Y. lipolytica*. Generally, the biochemical properties of immobilized enzymes could be different from those of the free ones. A series of experiments related to the catalytic parameters, including optimum temperature, temperature stability, optimum pH, pH stability, effects of metal ions and of ethylenediaminetetraacetic acid (EDTA) were carried out, all of them in triplicate.

#### 2.6.1. Optimum temperature

The optimum temperature of recombinant SBP was determined by comparing the enzyme activities at different temperatures, ranging from 20°C to 90°C, with a gradient of 5°C for 10 min, under the conditions mentioned above.

#### 2.6.2. Temperature stability

The crude enzymes were incubated at different temperatures, ranging from 20°C to 90°C, at intervals of 5°C for 2 h, and the residual enzyme activities were assayed.

#### 2.6.3. Optimum pH

The enzyme activities were measured in buffers at various pHs ranging from 2.0 to 6.0 with gradient of 0.5 at 30°C for 10 min.

#### 2.6.4. pH stability

The crude enzymes were incubated at different pHs ranging from 2.0 to 6.0 for 24 h, and the residue enzyme activities were assayed.

#### 2.6.5. Effects of metal ions and EDTA

5.0mM metal ions ( $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ) or EDTA were added to the reaction solution, respectively. The reaction mixtures were incubated at 30°C for 10 min, and the enzyme activities were determined. The reaction mixtures without additional chemicals were used as controls.

### 3. Results and discussion

### 3.1. Construction of surface display vectors

Cell surface display can be considered as a variant of secretory expression.

In order to express a heterologous protein on the cell surface of *Y. lipolytica*, it is necessary to introduce a highly efficient secretory signal peptide to the heterologous protein. The expression vector pINA1317 contains the coding sequence of the extracellular alkaline protease prepeptide XPR2pre. However, a low bias codon (ACG) which can reduce the translation efficiency and then the secretion signal is present in the 3'-terminal of XPR2pre. This codon overlapped with the first base of Sfil recognition site (GGCCNNNNNGGCC) which was planned to be kept for insertion of SBP. Because other codons for threonine do not contain G at the third base, ACG was mutated to GTG, a higher bias codon for valine structurally similar to threonine, leading to the T11 V mutation and generation of secretory vector pINA1317 m. Besides signal peptide, anchoring domain of a cell wall protein is another essential element for yeast cell surface display. Anchoring domains of several cell wall proteins such as YICwp1p and YIPir1p have been reported for construction of yeast surface display vectors [28,29]. In this study, the coding sequence (330 bp) of the C-terminal part of a function-unknown protein YALIOF24255p was amplified and inserted into pINA1317m to generate the cell surface display vector pMIZY05 (Fig. S1a). It is worth addressing that the N-terminus of the anchoring domain of YALIOF24255p contains a short peptide linker GTGGSGGSTG (a BamHI site was added to the 5'-terminal coding sequence of this peptide), endowing the junction of fusion proteins with a certain degree of flexibility, which has been reported to increase the activities of foreign enzymes [28].

To verify the efficiency of pMIZY05, eGFP was amplified and inserted into pMIZY05 to generate pMIZY05-eGFP which harbored the coding sequence of a fusion protein composing of the site-mutated Xpr2pre (Xpr2prem), eGFP and the C-terminal part of YALIOF24255p (Fig. S1b). pMIZY05-eGFP was expressed in *Y. lipolytica* Po1h and CLSM photographs demonstrated that only the cell wall of the recombinant yeast cells emitted green fluorescence while the controls did not, which indicated that eGFP was successfully anchored on the cell wall of these cells (Fig. 1).

### 3.2. Preparation of *Y. lipolytica* whole-cell biocatalyst

The optimized mature SBP coding sequence oEp was ligated into pMIZY05 to generate pMIZY05-oEp (Fig. S1c), from which a transformation cassette devoid of bacterial DNA was extracted and used for integration into the genome of *Y. lipolytica* Po1h, generating the wholecell biocatalyst. The transformants were separately grown in the enzyme producing PPB medium. Driven by the strong synthetic hp4d promoter, the recombinant SBP production initiated at late log phase and continued during stationary phase [33,38]. Because iron porphyrin, as prosthetic group, was absolutely needed for SBP activity but toxic to host cells at high concentration, a small amount of hemein ( $\mu\text{M}$  grade) must be added to the medium when the cultures reached the stationary phase. The recombinant SBP activity of the transformants was assayed using ABTS, as described in the Method section. One of the transformants, CM11, exhibiting the highest recombinant SBP activity of 1571.9 U/mL in non-optimized PPB medium, was used for further study.

To test whether SBP was immobilized on the cell surface of host yeast, we compared the recombinant SBP activities of CM11 transformant in different fractions of the culture: the total activity of the culture, the activity of intact cells, the activity in cell cytoplasm and the activity in culture supernatant (Table 3). A ratio of 98.4 % of recombinant SBP activity was detected on cell surface, when only a small fraction was found in the cytoplasm or in the supernatant. These results indicate that SBP was successfully immobilized on the cell surface of *Y. lipolytica*.

SBP is an oxidative enzyme which could affect the morphology of the host cells when it is displayed on the cell surface. Consequently, we compared cell morphology between *Y. lipolytica* Po1h and the CM11 transformant using SEM. The cells with SBP anchored on their surface appeared as smooth as the control ones (Fig. 2), which indicated that SBP has no obvious structural effect on the host cells.

A few studies reported the intracellular expression of SBP in *E. coli*, the most widely used expression host for heterologous proteins.

However, the recombinant SBP produced in *E. coli* was insoluble or exhibited very low activity, and active recombinant SBP was obtained only by refolding the enzyme via complex procedures [17,20]. Native SBP contains a lot of posttranslational modifications. While *E. coli* lacks a mature posttranslational maturation system, yeasts especially *Y. lipolytica* possessing complex posttranslational modification system which is similar to those of higher eukaryotic cells and would be a more suitable host for heterologous expression of SBP.

Although SBP is well-known for its excellent stability, it is not enough to deal with the hard conditions during practical applications, such as treatment of phenolic waste waters. To enhance the stability and reusability, a plenty of immobilized SBP biocatalysts had been prepared using different non-biological solid supports with reactively chemical groups [39–41]. The immobilized SBP showed much more stability and could be reused for several times. However, these methods usually use chemical reagents which may denature the enzyme, leading to the loss of enzyme activity. Additionally, time-and-labor-consuming enzyme isolation and purification is needed in these immobilization methods [42].

In the present work, SBP was displayed on the cell surface of *Y. lipolytica* Po1h, which could be considered as another avenue of SBP immobilization, forming a new kind of whole-cell biocatalyst. The yeast cells served as the producer and carrier of SBP. Different from the traditional immobilization of SBP, cell surface display is a mild and natural way of immobilization [43], devoid of complicated and timeconsuming enzyme purification and harmful chemical treatment. Although the process of construction was relatively complex, the wholecell biocatalyst could be cryopreserved for a long time (even for years), regenerated by fermentation at large scale and harvested by centrifuge easily.

### 3.3. Single-factor optimization of the culture conditions

Culture conditions have great effects on the production of recombinant enzymes. To maximize the production of immobilized recombinant SBP from CM11 transformant, an optimization of the culture conditions based on PPB medium was carried out.

#### 3.3.1. Culture time

A proper culture time would allow the yeast to maximize both high biomass accumulation and recombinant enzyme production. To find out when hemin should be added to the medium and when the highest recombinant enzyme activity was achieved, the time courses of both cell growth and recombinant SBP production were determined before culture medium optimization. CM11 transformant reached stationary phase after 30 h, time at which hemin should be added, and recombinant SBP enzyme activity reached its maximum 72 h after inoculation (Fig. 3a).

Subsequently, five important other parameters, including carbon source, nitrogen source, initial pH of the medium, hemin concentration and inoculate age, were explored.

#### 3.3.2. Carbon source

Carbon source is the most important medium component, as it is an energy source and plays a critical role in cell growth and in synthesis of metabolites. Different carbon sources usually have different effects on recombinant SBP production. In this study, four carbon sources (glucose, fructose, glycerol and sucrose), usually used in yeast cultivation, were investigated. The highest recombinant SBP activity was achieved when sucrose was used (Fig. 3b). Sucrose was chosen as the carbon source for recombinant SBP production. It should be figured out that wild *Y. lipolytica* cannot utilize sucrose while genetically engineered Po1h strain contains a heterologous invertase (ScSUC2) expression/secretion cassette integrated into its genome [26,27,34]. Invertase could hydrolyze sucrose to equal molarities of glucose and fructose, which could be metabolized by common strains of *Y. lipolytica*.

#### 3.3.3. Nitrogen source

Like for carbon, the selection of a nitrogen source in the medium also plays a pivotal part in recombinant enzyme production. In this section, the effects of different nitrogen sources (yeast extract, tryptone, beef extract powder, urea,  $\text{NH}_4\text{Cl}$  and  $(\text{NH}_4)_2\text{SO}_4$ ) on SBP production

were investigated. The activities of recombinant SBP were much higher when complex organic nitrogen sources such as yeast extract, tryptone or beef extract powder were used than when using relatively simple nitrogen sources such as urea,  $\text{NH}_4\text{Cl}$  or  $(\text{NH}_4)_2\text{SO}_4$  (Fig. 3c). The highest recombinant SBP activity was obtained with beef extract powder.

#### 3.3.4. Initial pH of the medium

The initial pH of the medium could influence nutrient assimilation, thus affecting both cell growth and recombinant enzyme production. The effects of the initial pH of the medium on recombinant SBP production were examined using different pHs: 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. The highest activity was achieved when the initial pH of the medium was set at 5.0 (Fig. 3d).

#### 3.3.5. Hemin concentration

Hemin is an essential factor for the activity of hemin-containing proteins. The effect of hemin concentration on the yield of immobilized SBP was investigated in this study. It was found that the activity of surface displayed SBP was strongly dependent on the concentration of exogenous hemin. Recombinant SBP activity increased at first with added hemin concentration until a maximum for 8  $\mu\text{M}$  hemin (Fig. 3e). However, when more than 8  $\mu\text{M}$  hemin was added, enzymatic activity started to decrease. It could be speculated that, if a proper concentration of hemin is required for formation of functional SBP, a too high concentration could become toxic to host cells.

#### 3.3.6. Inoculate age

The inoculate age has a great effect on yeast cell fermentation. Enzyme production will take more time if the inoculum is taken too early or too late, while determining the proper inoculate age will help to produce more enzyme in a shorter fermentation time. Different inoculate ages (12, 18, 24, 36 or 48 h) were tested in this study. The highest recombinant SBP activity was achieved when the inoculate was pregrown for 24 h (Fig. 3f).

According to the results mentioned above, our optimized SBP producing medium contained 20.0 g/L sucrose, 9.38 g/L beef extract powder, 0.32 g/L  $\text{KH}_2\text{PO}_4$ , 0.132 g/L  $\text{MgCl}_2$ , 0.11 g/L  $\text{CaCl}_2$ , and 0.34 mg/L thiamine, and was buffered with 20mM citric acid- $\text{NaH}_2\text{PO}_4$  solution at pH 5.0. After 24 h of preculture, the seed culture was inoculated to the modified PPB medium at an inoculum ratio of 2 % and flasks were incubated with shaking at 220 rpm at 30°C for 30 h. Then, 8.0  $\mu\text{M}$  of hemin was added to the culture, and incubation was continued for another 42 h. After optimization, recombinant SBP activity was improved up to 4187.8 U/mL, namely 2.7-fold compared to the initial activity.

### 3.4. Biochemical properties of the whole-cell biocatalyst

Anchored on the cell surface of *Y. lipolytica*, the recombinant SBP may possibly exhibit different catalytic characteristics. The biochemical properties of the whole-cell biocatalyst, such as optimum temperature, temperature stability, optimum pH, pH stability, and effects of metal ions and EDTA, were investigated.

#### 3.4.1. Optimum temperature

The SBP displayed on the yeast cells showed the maximum activity at 37.5°C (Fig. 4a), much lower than that of the native SBP [44], possibly due to physical and chemical changes of the enzyme during cell surface display. It has been reported that the optimum temperature of SBP immobilized on polyaniline decreased [39] while an increase of optimum temperature also has been observed when SBP was immobilized on chitosan mini-spheres attached with a dye [40]. Up to date, a few of enzymes with relatively low optimum temperatures (20–30°C) have been displayed on the cells of *Y. lipolytica*, in which the optimum temperatures were the same or increased compared with their free forms [45–47]. In this study, SBP was anchored on the surface of *Y. lipolytica* which is relatively sensitive to high temperature [27]. At higher temperature, physical or chemical changes may occur that would affect the conformation of the displayed SBP, leading to the change of optimum temperature.



#### 3.4.2. Temperature stability

Another industrially relevant biochemical property of enzymes is their stability. Native SBP, whose  $T_m$  value (midpoint of the thermal unfolding curve) is as high as 85°C, thus displays a remarkable stability at high temperature [48]. After a thermal treatment of 2 h (Fig. 4b), the immobilized SBP displayed a stable activity for temperatures of 25°C to 70°C, while its activity significantly decreased at 70°C to 90°C. However, 710.3 U/mL of residual activity, namely 16 % of the initial activity of crude recombinant enzyme, were still retained after a thermal treatment of 2 h at 80°C, which further verified that the immobilized SBP still showed a significant resistance to high temperatures.

#### 3.4.3. Optimum pH

Ambient pH plays a great role on enzymatic activity. The immobilized recombinant SBP exhibited a high activity over a wide range of pHs (Fig. 4c). Using ABTS as substrate, the optimum pH shifted to 3.5 which is lower compared with the native SBP [3]. This may result from the microenvironment changes from the free form to the anchoring on the cell wall of *Y. lipolytica*, leading to certain ionization state changes of the AA side chains in the active sites of SBP. In terms of effects of immobilization on pH optimum, different results have been reported: showed the same pH-activity profile as that of the free SBP or shifted to lower pH [39,40].

#### 3.4.4. pH stability

Immobilized enzyme stability with pH was investigated by measuring the residual activity after treatment under different pH conditions (pH 2.0–6.0) at 30°C for 24 h. The surface displayed recombinant SBP was stable from pH 2.5 to pH 4.5 and showed a strong resistance to acidic conditions (Fig. 4d).

#### 3.4.5. Effects of metal ions and EDTA

The effects of metal ions or of EDTA on immobilized recombinant SBP were determined. As shown in Table 4,  $Fe^{3+}$ ,  $Cu^{2+}$  and  $Ca^{2+}$  were able to increase the activity of recombinant SBP while EDTA and  $Fe^{2+}$  inhibited it. Especially,  $Fe^{3+}$  allowed an increase the recombinant SBP activity to 135.1 %. In contrast,  $Fe^{2+}$  inhibited it to a level of 46.4 %, and EDTA intensely inhibited activity to only 5.3 %.  $Mn^{2+}$ ,  $Ni^{2+}$ ,  $Co^{2+}$  and  $Mg^{2+}$  showed little effects on recombinant SBP activity.  $Fe^{3+}$  is considered to be necessary for most plant peroxidases because it is involved in the binding of  $H_2O_2$ .  $Ca^{2+}$  is also an essential cofactor for SBP, in order to maintain the conformational integrity of its active site. However, added  $Ca^{2+}$  showed little influence on the activity of recombinant SBP in this study. The reason for this apparent discrepancy may simply be that the 10mM of  $Ca^{2+}$  in modified PPB medium were already saturating for the recombinant SBP.

The biochemical properties of *Y. lipolytica* CM11 whole-cell biocatalyst with SBP anchored on cell surface could be summarized as follows: (1) the recombinant SBP was thermally stable from 20°C to 80°C with optimum temperature at 37.5°C; (2) it was resistant to acidic conditions with optimum pH at 3.5; (3) addition of  $Fe^{3+}$  allowed to increase its activity while EDTA strongly inhibited it. These results indicate that the biochemical properties of cell-displayed SBP differed from those of the native enzyme and also from those of intracellularly expressed SBP. The optimum pH of native SBP is 5.5 using ABTS as substrate [3], while the optimum temperature and pH of the recombinant SBP produced intracellularly in *E. coli* were 85°C and pH 5.0 respectively [20]. In our study, the displayed SBP was immobilized on yeast cell surface, which allows only a lower transferring and conforming degree of freedom. In addition, arming yeast cells were sensitive to higher temperature or lower pH, which may influence the structure of their cell wall, possibly leading to changes in the displayed SBP.

Finally, the activity of *Y. lipolytica* CM11 whole-cell biocatalyst displaying recombinant SBP was re-examined under all optimum conditions (optimized PPB medium and cultivation conditions, with activity assay at 37.5°C and pH 3.5 with 5.0mM  $Fe^{3+}$ ), and was found to have increased up to 4432.5 U/mL, which represents an unequalled level for heterologous expression of SBP.

## 4. Conclusion

In the present study, a new whole-cell biocatalyst with SBP exposed on *Y. lipolytica* cell surface was constructed and exhibited high recombinant activity and stability without obvious negative effect to yeast host cells. A remarkable recombinant SBP activity of 4432.5 U/mL was achieved by this arming yeast after a series of optimizations. To our knowledge, this is the highest recombinant SBP activity reported up to now. The development of this whole-cell biocatalyst avoided harmful chemical reagents. The generated biocatalyst could be easily harvested and regenerated, which would facilitate the downstream process of the recombinant enzyme. Moreover, cell surface displayed SBP could avoid the toxicity of this heterologous enzyme to the cellular components of the host cells, which contributes to improving cell growth, heterologous expression efficiency and hence production and activity of recombinant SBP.

#### Authors' contributions

H.B. Zhang, H.Y. Xiang, and J.M. Wang developed the idea for the study. J.M. Wang and C.M. Wang designed the research, did the literature review, and prepared the manuscript. J.M. Wang, C.M. Wang, X. Men, and X.H. Xiang did the lab work, plasmid construction, strain cultivation, culture optimization and assays of biochemical properties. J.M. Wang and C.M. Wang drafted the initial manuscript together. C. Madzak reviewed and made corrections of the draft. All authors contributed in analyzing the data and writing the draft. All authors read and approved the final manuscript.

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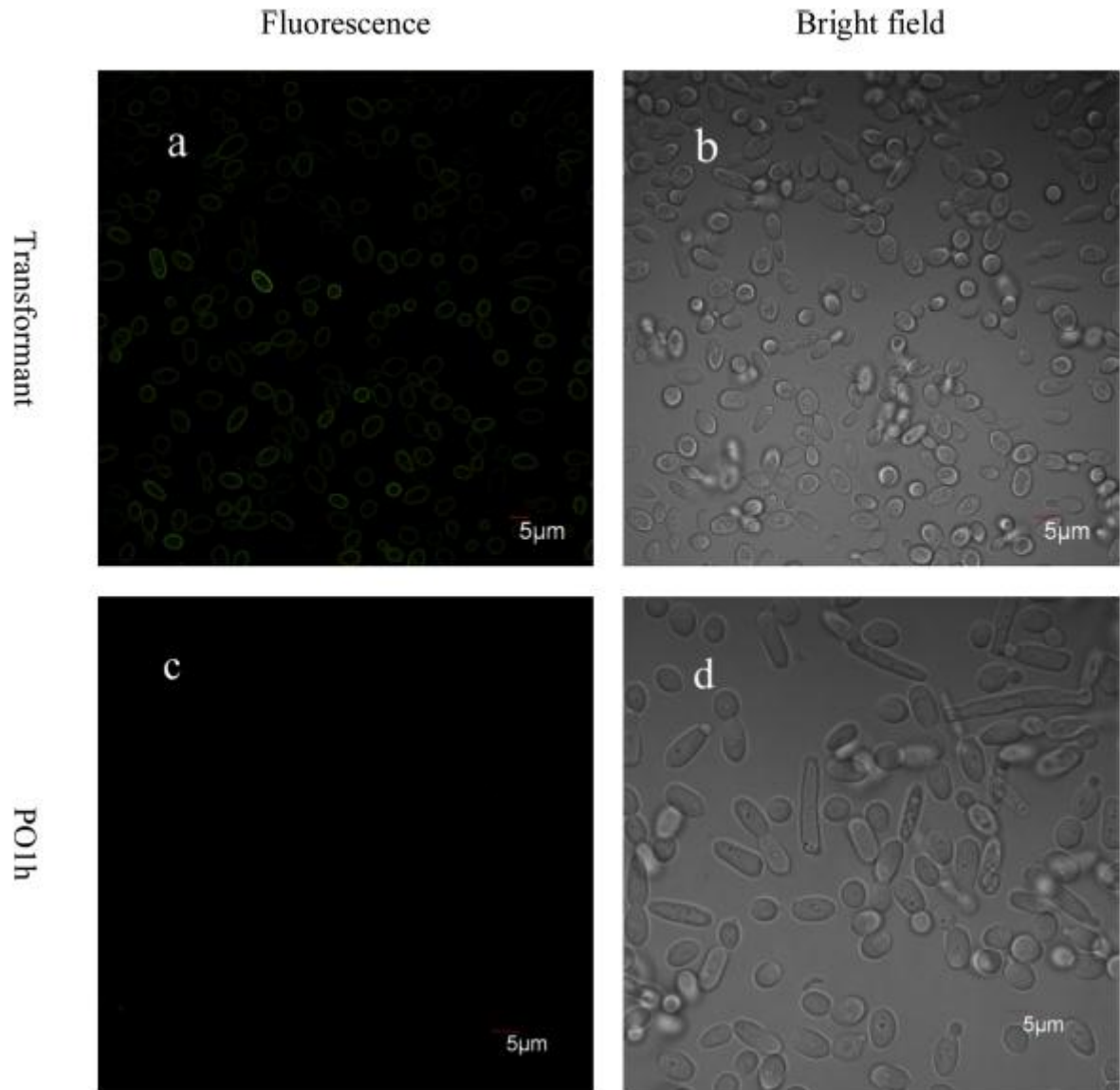


Fig. 1. Photographs of the cells of *Y. lipolytica* captured by CLSM. Fluorescence (a, c) and bright field (b, d) micrographs are depicted. (a, b) The cells transformed by eGFP; (c, d) The cells of *Y. lipolytica* PO1h.

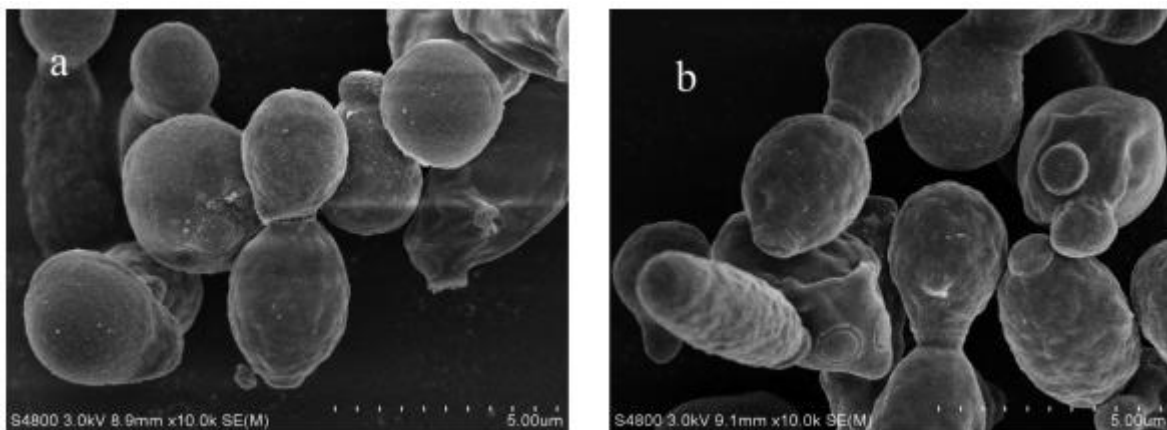


Fig. 2. Scanning electron micrographs of *Yarrowia lipolytica* cells. (a) Cells with SBP anchored on the surface; (b) Control cells.

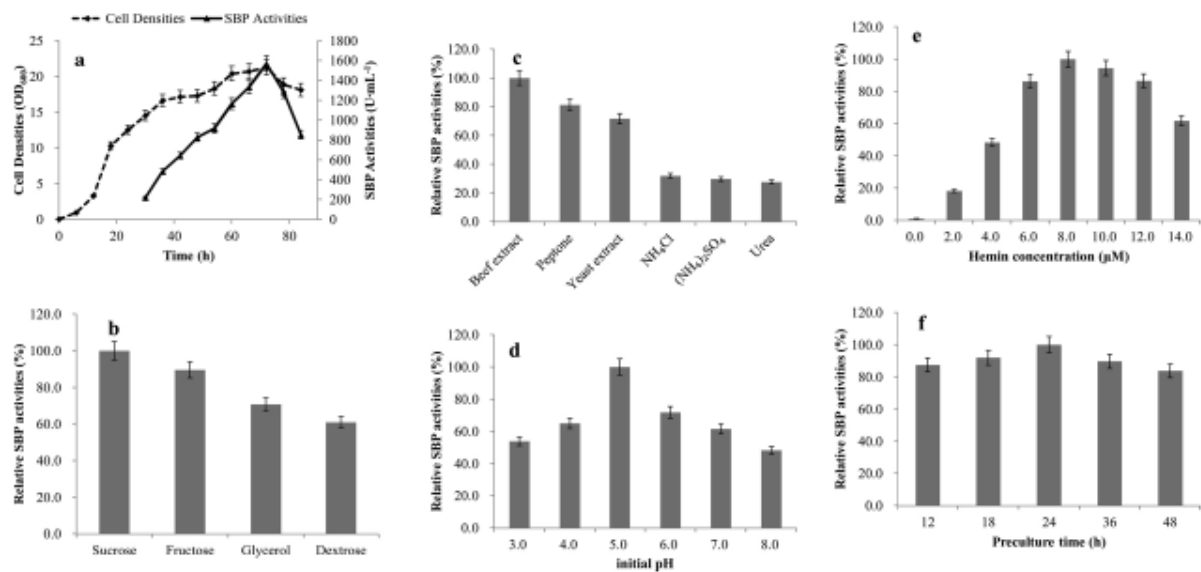


Fig. 3. Optimization of the culture conditions of the whole-cell biocatalyst. (a) Time course profiles of cell growth and recombinant SBP production of transformant CM11; (b) Carbon sources; (c) Nitrogen sources; (d) Initial pH of the medium; (e) Hemin concentrations; (f) Preculture time. Error bars represent standard deviations from biological triplicates.

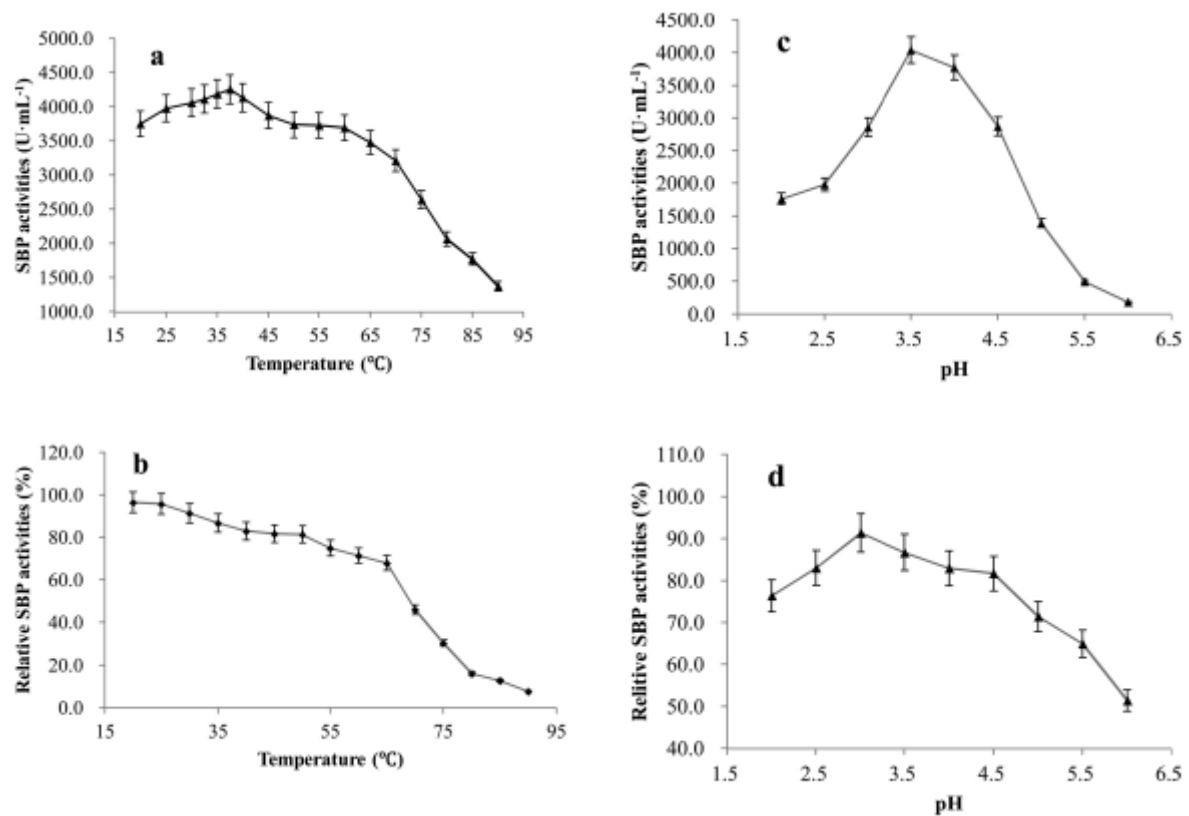


Fig. 4. Effects of temperature and pH on the activity of *Y. lipolytica* whole-cell biocatalyst with SBP anchored on the cell surface. (a) SBP activities at different temperatures; (b) Temperature stability of the whole-cell biocatalyst; (c) SBP activities under different pH conditions; (d) Effects of different pHs on the stability of the whole-cell biocatalyst. Error bars represent standard deviations from biological triplicates.

Table 1

Strains and plasmids used in this study.

Strain/Plasmid	Relevant characteristics	Source
<b>Strains</b>		
<i>Y. lipolytica</i> Po1h	MatA, ura3-302, xpr2-322, xpr1-2, ScSUC2	[34]
<i>E. coli</i> DH5 $\alpha$	F <sup>-</sup> , endA1, glnV44, thi-1, recA1, relA1, gyrA96, deoR, nupG, purB20, $\phi$ 80dlacZ $\Delta$ M15 $\Delta$ (lacZYAargF) U169, hsdR17( <i>r<sub>K</sub></i> -/ <i>m<sub>K</sub></i> ), $\lambda$ -	Invitrogen
<b>Plasmids</b>		
pUC575-oEp	pUC origin; Amp <sup>R</sup> ; oEp	GenScript
pEGFP-1	pUC origin; Amp <sup>R</sup> ; eGFP	Takara
pINA1317	pUC origin; Kan <sup>R</sup> ; P <sub>hp4d</sub> ; XPR2pre	[31]
pINA1317m	pUC origin; Kan <sup>R</sup> ; P <sub>hp4d</sub> ; XPR2prem	This work
pMIZY05	pUC origin; Kan <sup>R</sup> ; P <sub>hp4d</sub> ; XPR2prem; GPI	This work
pMIZY05-eGFP	pUC origin; Kan <sup>R</sup> ; P <sub>hp4d</sub> ; XPR2prem; eGFP; GPI	This work
pMIZY05-oEp	pUC origin; Kan <sup>R</sup> ; P <sub>hp4d</sub> ; XPR2prem; oEp; GPI	This work

Table 2

Primers used in this study<sup>a</sup>.

Primer	Sequence (5'→3')
1317m-F	GTCTTCTGGATCCTCTGGTACCTCTTAAGCTTAATTAACAGATAGTTTGCCGGTGATA
1317m-RV	ATGGCCAGAACGGCCACGAGAATAGTAAAGGCGGTAGCGA
GPI-F	GGTACCGGAGGCTCCGGAGGCTCTACCG
GPI-RV	AAGCTTAGATGAGGAGGCGGCGCAACA
eGFP-F	GGCCGTTCTGGCCATGAGCAAGGGCGAGGAGCTGTCA
eGFP-RV	GGATCCCTTGACAGCTCGTCCATGCCGAGAG

<sup>a</sup> Underlined bases encode the following restriction sites successively: BamHI, KpnI, HindIII, SfiI, KpnI, HindIII, SfiI and BamHI; the site-mutated codon in the coding region of Xpr2 signal peptide is bold and italic.

Table 3

Recombinant SBP activities in different fractions of the CM11 culture.

Component	Total	Intact cells	Cytoplasm	Supernatant
Activity (U/mL)	1571.9	1546.7	6.3	18.9
Percent (%) <sup>a</sup>	100.0	98.4	0.4	1.2

<sup>a</sup> The total activity of the culture defines the 100 % level, which is the sum of the activities of intact cells, cytoplasm and supernatant. Percent of each activity was defined as the ratio to total activity.

Table 4

Effects of metal ions and EDTA on the activity of CM11 whole-cell biocatalyst.

Compounds	Concentration (mM)	Relative activity (%)
Control	0.0	100.0
Fe <sup>2+</sup>	5.0	46.4
Fe <sup>3+</sup>	5.0	135.2
Cu <sup>2+</sup>	5.0	116.2
Mn <sup>2+</sup>	5.0	99.2
Ni <sup>2+</sup>	5.0	97.4
Mg <sup>2+</sup>	5.0	96.6
Co <sup>2+</sup>	5.0	97.4
Ca <sup>2+</sup>	5.0	103.6
EDTA	5.0	5.2