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A novel high-throughput and quantitative method based on visible color shifts for screening *Bacillus subtilis* THY-15 for surfactin production

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Abstract A novel chromatic visible screening method using bromothymol blue (BTB) as a color indicator and cetylpyridinium chloride (CPC) as a mediator was constructed to obtain the high titer surfactin-producing strains. The reliability and quantification accuracy of color shift were also confirmed. Regular chromatic responses from faint yellow-green to dark green and bright blue reflected the different ranges of surfactin concentrations. Moreover, the quantitative accuracy of surfactin quantification in the range of 100–500 mg/L was verified by reverse-phase high-performance liquid chromatography (RP-HPLC) using different fermentation supernatant samples. Using this CPC–BTB method, a superior surfactin producer, *Bacillus subtilis* THY-15, was successfully screened. The producer's surfactin (Srf) titer reached 1240 mg/L. RP-HPLC analysis of THY-15 revealed four surfactin isoforms. As identified by amino acid analysis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis, the isoforms of surfactin in fraction 1, 2 and 4 had the same circular peptide sequence of Glu-Leu-Leu-Val-Asp-Leu-Leu but different iso-C_{13}, C_{14} and C_{15} fatty acid chains, but the isoform in fraction 3 possessed a special peptide sequence of Glu-Val-Leu-Leu-Asp-Leu-Val.

Keywords CPC–BTB screening method · Chromatic responses · Surfactin · *Bacillus subtilis* · MALDI-TOF MS/MS

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Introduction

The demand for energy is critical worldwide. Petroleum is one of the most important energy resources, but usually two-third of the oil that located in areas inaccessible to fluids or adhered to sands is left unrecovered after primary and secondary extraction [21]. The tertiary extraction techniques characterized by the utilization of chemicals (polymers and surfactants) of enhanced oil recovery (EOR) methods are highlighted. Biosurfactants from microbes attracted great interests due to many advantages, such as low toxicity, high efficiency, biodegradability and sustainability. Surfactin predominantly produced by the *Bacillus* species [9] is one of the most famous lipopeptides, not only for superior surface activity, but also high stability against temperature extremes [13]. These endow surfactin with great potential in EOR applications that can drastically reduce the usage of chemical surfactants and promote oil exploitation.

However, the industrialization of surfactin is hampered by the lack of high yield producers. *Bacillus subtilis* ATCC 21332, *B. subtilis* S15 and *Bacillus amyloliquefaciens* accumulated 109.5, 125.6, 452.5 mg/L of surfactin, respectively [8]. The *Bacillus velezensis* H3 and *B. subtilis* BS-37 gave a maximum yield of surfactin by 488 mg/L [14] and 585 mg/L [13], respectively. The surfactin titer of wild strains was usually in the range from 100 to 600 mg/L. Thus, screening of high yield strains is still necessary.

Previous studies have reported many screening methods for strains producing biosurfactants. Some methods measure hemolytic activity or interaction with surfactants and dyes, including blood agar plate [31] and cetyltrimethyl ammonium bromide (CTAB) agar plate [22]. Dozens of methods are based on the surface activity directly, including surface tension evaluation [18], drop collapse [1], axisymmetric drop shape analysis profile (ADSA-P)

[24], oil spreading [15], microplate view assay [25] and mist of oil droplets [3]. What's more, there are also some colorimetric methods based on the interaction with polydiacetylene vesicles [34]. Some methods have shortcomings such as poor specificity, demands on specialized equipment and large amount of samples for analysis. Samples cannot be measured in parallel restricts the application to high-throughput screening in a large scale.

Aqueous sulfonephthalein dyes were previously used for detection of quaternary ammonium or anionic chemical surfactants in river or waste water [4, 26, 33]. In this study, bromothymol blue (BTB) was used as a color indicator and cetylpyridinium chloride (CPC) was introduced as a mediator, forming the (CPC–BTB) complex. Afterwards, CPC was competitively caught out from the (CPC–BTB) complex on the basis of the intense binding with surfactin, releasing free molecules of BTB and generating a color shift response. Making use of microplates and the chromatic response, we proposed a novel visible and high-throughput screening method for high yield surfactin producers. The accuracy and reliability of this CPC–BTB method were evaluated, and an optimal strain was identified, which we named *B. subtilis* THY-15. The four surfactin isoforms produced by THY-15 were also identified.

Materials and methods

CPC–BTB reagent, the enrichment medium and the fermentation medium for the strains

The CPC–BTB reagent was obtained by mixing equal volumes of 0.2 mM CPC and 0.2 mM BTB, both dissolved in 0.1 M phosphate-buffered saline (PBS, NaH_2PO_4/Na_2HPO_4, pH 8.0). The enrichment medium for screening of the surfactin producers from the soil/waste water samples was as follows: 2 g/L glucose, 0.2 g/L yeast extract, 0.2 g/L NH_4NO_3, 0.3 g/L KH_2PO_4, 0.5 g/L $Na_2HPO_4{\cdot}12H_2O$, 0.05 g/L $MgSO_4{\cdot}7H_2O$, 5 mg/L $CaCl_2$, 2 mg/L $MnSO_4{\cdot}H_2O$, and 2 mg/L $FeSO_4{\cdot}7H_2O$. The fermentation medium for culturing the screened strain in a 48-well plate was as follows: 60 g/L glucose, 1.0 g/L yeast extract, 25 g/L $NaNO_3$, 0.333 g/L KH_2PO_4, 1.0 g/L $Na_2HPO_4{\cdot}12H_2O$, 0.15 g/L $MgSO_4{\cdot}7H_2O$, 7.5 mg/L $CaCl_2$, 6 mg/L $MnSO_4{\cdot}H_2O$, and 6 mg/L $FeSO_4{\cdot}7H_2O$, pH 7.0.

Optimization of the CPC–BTB method

The volume of CPC–BTB screening reagent was set at a constant 800 μL in a 48-well plate (Nunc™ Surface, Denmark). Different volumes of samples (V_{srf}: 50, 100, 150, 200, 300 and 400 μL) with different surfactin concentrations (C_{srf}: 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg/L) were mixed with the CPC–BTB reagent at 25 °C for 5 min. A color shift was observed after the interaction. The absorption at wavelengths ranging from 400 to 800 nm was scanned, and the optimal absorbance was detected at 600 nm. The pH of the fermentation supernatant was adjusted from 5 to 10 by HCl or NaOH to examine the effect of the different pH on the chromatic response. In a constant volume (100 μL) of fermentation supernatant, the concentration of surfactin (0–1000 mg/L) on the chromatic response of the 800 μL CPC–BTB reagent was also observed.

Screening of the superior surfactin-producing strains using the CPC–BTB method

Sludge and wastewater samples from Shandong Province, China, were used for the screening procedure. The samples were first activated by an appropriate volume of distilled water at 37 °C for 30 min. Then, 1 mL liquid was inoculated into 10 mL enrichment medium and cultured for 24 h at 37 °C, 200 rpm. The culturing broth was diluted on a gradient, spread on LB agar plates (or blood plates) and incubated at 37 °C until clear colonies appeared. Individual colonies (or colonies with transparent circles) were selected to inoculate into 48-well plates containing 800 μL fermentation medium. The plates were incubated at 37 °C for 36 h with 200 rpm shaking. Then, 100 μL fermentation broth was mixed with 800 μL CPC–BTB screening reagent in a 48-well plate at 25 °C for 5 min. Those strains generating obvious visible chromatic shifts from light green to bright blue were selected and the titer of surfactin was determined by absorbance at 600 nm.

Genus identification of the selected optimal strain

The genera of the selected strains were identified by 16S rRNA sequencing amplified by the primer pairs 5′-AGAGTTTGATCCTGGTCAGAACGCT-3′ and 5′-TACGGCTACCTTGTTACGACTTCACCCC-3′. The amplified 16S rRNA gene sequence was compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/). Physiological and biochemical characteristics were further determined at the Institute of Microbiology, Chinese Academy of Sciences, according to the procedures outlined in Bergey's manual of systematic bacteriology (Sneath 1986).

Evaluation of the surfactin producers and preparation of surfactin

The optimized fermentation medium formula: 70 g/L brown sugar, 1.0 g/L yeast extract, 25 g/L $NaNO_3$, 0.333 g/L KH_2PO_4, 1.0 g/L $Na_2HPO_4{\cdot}12H_2O$, 0.15 g/L $MgSO_4{\cdot}7H_2O$, 7.5 mg/L $CaCl_2$, 6 mg/L $MnSO_4{\cdot}H_2O$,

6 mg/L $FeSO_4 \cdot 7H_2O$, pH 7.0. The brown sugar is a kind of crude sucrose product concentrated from sugar cane juice and cheaper than sucrose, containing 94 % sucrose as well as some pigment, phytochemicals and trace metal elements that can promote the cell growth and production [32].

The selected strains were first cultivated in 10 mL LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) at 37 °C for 14 h at 200 rpm. Then, a 5 % seed broth was inoculated into 100 mL optimized fermentation medium in a shaking flask and incubated for 48 h at 37 °C at 200 rpm. The fermentation broth was centrifuged at 12,000×*g* for 5 min before the fermentation supernatant was used to measure the concentration of surfactin by CPC–BTB quantification and reverse-phase high-performance liquid chromatography (RP-HPLC).

The pH of the supernatant was adjusted to 2.0 with 6 M HCl. The samples were then stored overnight at 4 °C. The samples were centrifuged at 12,000×*g* for 20 min to harvest the solid crude lipopeptides, followed by extraction with methanol. The extractions were filtered to remove insoluble impurities, and the methanol was evaporated under vacuum at 50 °C using a rotary evaporator to obtain the crude lipopeptides for subsequent isoform separation, purification and identification of surfactin.

Quantification and isoform separation of surfactin by RP-HPLC

The fermentation supernatant obtained after centrifugation (12,000×*g* for 5 min) was diluted 4 times in an aqueous solution of 1 g/L $NaHCO_3$ and filtered through a 0.2 μm membrane. A 20 μL aliquot was injected into an Inert Sustain C_{18} column (5 μm, 250 × 4.6 mm) in the HPLC system to separate the surfactin isoforms. A surfactin standard (purity ≥98 %, Sigma-Aldrich Trading Co., Ltd., Shanghai, China) was used to confirm the identity of the fractions. The mobile phases were 10 % water and 90 % methanol, containing 0.1 % trifluoroacetic acid (TFA). The total flow rate of the mobile phase was kept at 1.0 mL/min and the chromatograms were detected at 205 nm. The total peak area of four surfactin isoforms was calculated for quantification of the total concentration of surfactin according to the concentration standard curve quantified by Sigma surfactin. The fractions were harvested for subsequent amino acid analysis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis.

Hydrolysis and amino acid identification of the harvested surfactin

The surfactin samples (1.0 mg) were hydrolyzed with 1 mL 6 M HCl at 110 °C for 24 h. The fatty acid residue was extracted three times by 1 mL of ether. Then, the aqueous phase of the hydrolysis solution was dried at 50 °C under vacuum to remove the residual ether and HCl. The dried sample was dissolved in 1 mL of double distilled water.

Eighteen amino acids (Sigma-Aldrich Trading Co., Ltd., Shanghai, China) were used for standard references, and norleucine (Nleu) was added as an internal standard. The pre-column derivatization with phenyl isothiocyanate (PITC) and quantitative analysis on RP-HPLC were executed as previously described [27].

MALDI-TOF MS and MS/MS analysis

MALDI-TOF MS analysis and MALDI-TOF MS/MS collision-induced dissociation (CID) analysis were carried out on a SCIEX 4800Plus Analyzer (Applied Biosystems, Foster City, CA, USA) with a 337 nm nitrogen laser for desorption and ionization. An equal volume of 0.1 % α-cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix. Mass spectra were accumulated over 50 individual laser shots and obtained in the reflector mode at an initial accelerating voltage of 20 kV. The *m/z* values were measured in the range of 800–2000. The collision cell was floated at 2 kV to attain the collision energy of 2 keV. Air, used as collision gas, was introduced at a pressure leading to an attenuation of the precursor ion beam by almost 70 %.

Results

Mechanism and feasibility of the chromatically visible CPC–BTB method for quantitative screening of surfactin producers

Surfactin, a lipopeptide biosurfactant with negative charge, does not directly interact with sulfonephthalein dyes for chromatic response. A novel use of BTB as a color indicator and CPC as a mediator was thus presented for visible chromatic determination of surfactin concentration on the basis of the intense binding of the surfactin with the cationic CPC. The design of the method is schematically illustrated in Fig. 1a. The color indicator BTB was first bound to the mediator CPC, leading to a color shift from dark blue to faint yellow-green. CPC was then competitively caught out by surfactin from the (CPC–BTB) complex, releasing free molecules of BTB and generating a second-time color shift from faint yellow-green to dark green or bright blue. This second-time color shift was not only a qualitative response to the concentration of surfactin, but also could be quantitatively measured by spectrophotometry.

As shown in Fig. 1b, the addition of surfactin into (CPC–BTB) reagent generated a visible color change

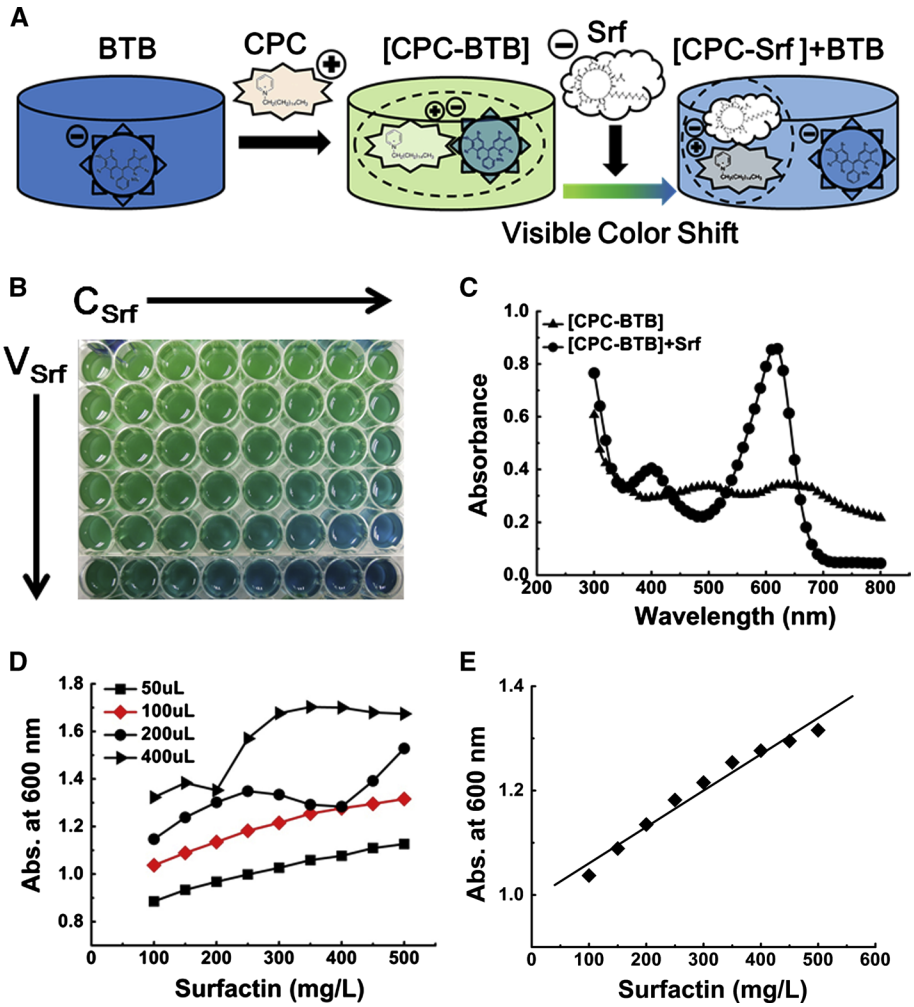

Fig. 1 The establishment of the chromatically visible and quantitative screening method. **a** The mechanism for the chromatic response is described by the following equations: (1) BTB + CPC → [CPC–BTB], (2) [CPC–BTB] + Surfactin → [CPC-Srf] + BTB. **b** Optimization for the reaction ratio between screening reagent and surfactin. The volumes of the standard surfactin solution (V_{srf}) were 50, 100, 150, 200, 300 and 400 μL. The surfactin concentrations (C_{srf}) were 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg/L. **c** Absorbance scanning from 400 to 800 nm showed a significant absorbance recovery between 550 and 650 nm after the addition of surfactin into the [CPC–BTB] reagent. **d** Quantitative relationship of absorbance at 600 nm against the actual surfactin concentration of testing samples. The sample volumes were 50, 100, 200 and 400 μL. **e** The absorbance at 600 nm of the final condition: 800 μL 0.1 mM CPC–BTB (buffered at pH 8.0 by 0.1 M PBS) + 100 μL sample (0–1000 mg/L surfactin). The linear regression for surfactin concentration quantitative analysis was: $A_{600nm} = 7.601 \times 10^{-4} \times$ Srf (mg/L) + 0.9824, $R^2 = 0.9793$ ($100 \leq$ Srf ≤ 500)

due to the competitive binding of surfactin with CPC and formation of the (CPC–Srf) complex, with a significant absorbance peak between 550 and 650 nm, as shown in Fig. 1c. Selecting 600 nm as the optimal wavelength, the molar ratio of surfactin against (CPC–BTB) was optimized and the absorbance at 600 nm was measured under different surfactin concentrations and volumes. The optimal volume of 100 μL sample containing 100–500 mg/L surfactin was added to 800 μL CPC–BTB reagent, as shown in Fig. 1d. The absorbance at 600 nm at this volume presented a satisfactory linear relationship with surfactin concentration within 100–500 mg/L (Fig. 1e).

Visualization reliability and accuracy evaluation of the CPC–BTB method

The pH of fermentation broth can range from 6 to 9, thus it is necessary to ensure that 100 μL blank fermentation mediums of pH 5–10 do not affect the chromatic response. Figure 2a shows no significant color change, confirming the pH stability of the CPC–BTB method when dealing with fermentation supernatant with different pH (5–10). The samples with different surfactin concentrations (0–1000 mg/L) were also tested and satisfactory chromatic visible changes were obtained (Fig. 2b). Based on this result, 41 fermentation broth samples were selected

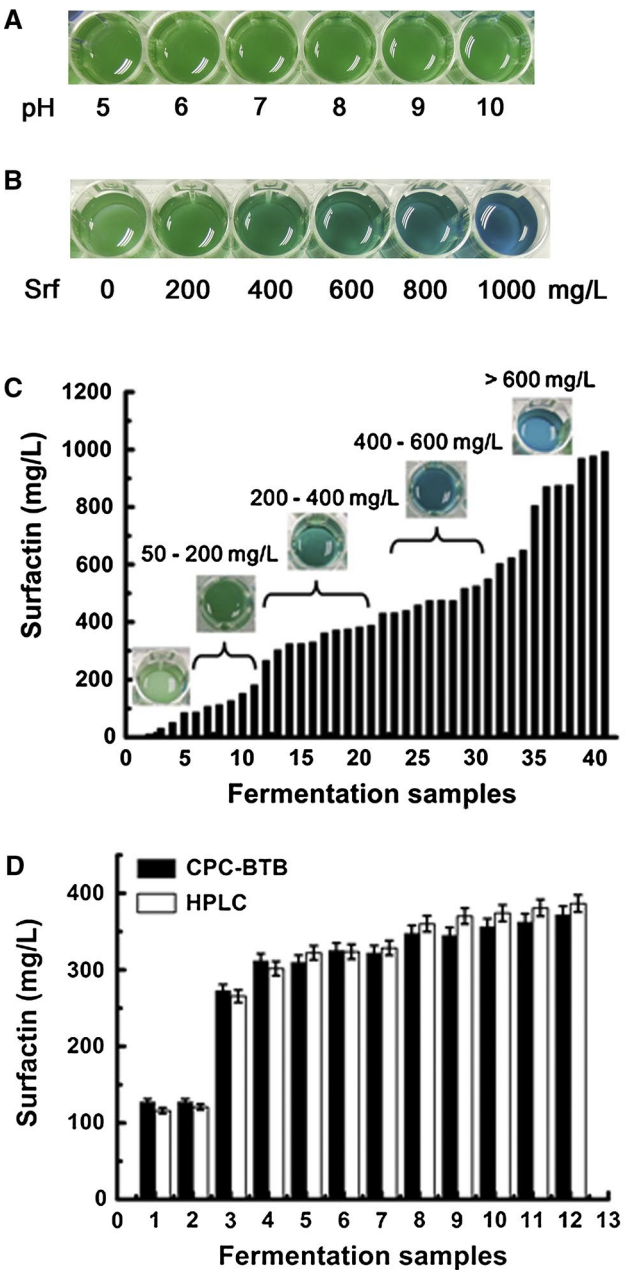

Fig. 2 Accuracy and reliability evaluation of the CPC–BTB method. **a** The pH capacity test of the CPC–BTB method. The pH of 100 μL blank fermentation medium ranges from 5 to 10. **b** The visible color gradient response of blank fermentation broth samples containing 0–1000 mg/L surfactin for the final CPC–BTB method. **c** Accuracy of the quantitation results (100–500 mg/L surfactin) of the fermentation supernatant samples using the CPC–BTB method compared with RP-HPLC. **d** Evaluation of the visualization reliability in the range of 0–1000 mg/L surfactin

for the evaluation of the visualization reliability. A regular chromatic response from faint yellow-green to dark green and bright blue exactly reflected the difference in the surfactin concentrations (0–1000 mg/L), as shown in Fig. 2c. The quantitative accuracy of the CPC–BTB method for surfactin quantification was verified by RP-HPLC using twelve different fermentation supernatant samples with 100–500 mg/L surfactin, as shown in Fig. 2d.

Application of the CPC–BTB method in screening for surfactin producers

Hundreds of individual bacterial colonies from underground soil/water samples were cultured and used to screen for optimal surfactin producers by the CPC–BTB method. All strains with a dark green, slight blue or bright blue chromatic change were selected, and the bright blue strains were specifically highlighted. As shown in Fig. 3a, 9 strains with bright blue response (as marked by the arrows) were particularly selected for CPC–BTB quantification. The results are summarized in Fig. 3b. The strain with the highest surfactin titer, THY-15, was identified by 16S rRNA sequencing. The amplified 16S rRNA sequence was submitted and deposited in the GeneBank database (NCBI accession: KP974276). Comparison of the 16S rRNA sequence of THY-15 in the GenBank database of NCBI showed 99.91 % identity with *B. subtilis* SBRh5 (NCBI accession: HQ443229.1), as revealed by a BLAST search (http://blast.ncbi.nlm.nih.gov). The physiological and biochemical characteristics of THY-15 are listed in Table 1. According to Bergey's manual, this Gram-positive, rod-shaped strain was identified as *B. subtilis*. During the culture of *B. subtilis* THY-15 in 100 mL fermentation medium for 48 h, the cell growth and surfactin titer were measured and illustrated in Fig. 3c and d, respectively. The profiles of cell growth (OD_{600}) and surfactin production were approximately consistent except a period in the mid-log phase. Production of surfactin started in the early logarithmic growth phase, but almost no surfactin accumulation during the mid-log phase when the cells grew rapidly. The second period of predominant surfactin production continued in the late logarithmic and the stationary phase, resulting in another remarkable surfactin accumulation reached a final surfactin titer of 1240 mg/L measured by RP-HPLC.

Fraction separation and isoform quantification of surfactin from *B. subtilis* THY-15

Four major surfactin fractions were identified by RP-HPLC from *B. subtilis* THY-15, corresponding to the surfactin standard from the Sigma Company, as shown in Fig. 4. The proportions of the first and the fourth surfactin fraction of THY-15 were similar to the Sigma standard. The major difference lay in the amount of the second and third fractions. Using RP-HPLC, the concentrated product of each isoform was separated from the 50 g/L surfactin mixture produced by THY-15. The fractions corresponded to 4 types of surfactin isoforms were individually collected for amino acid

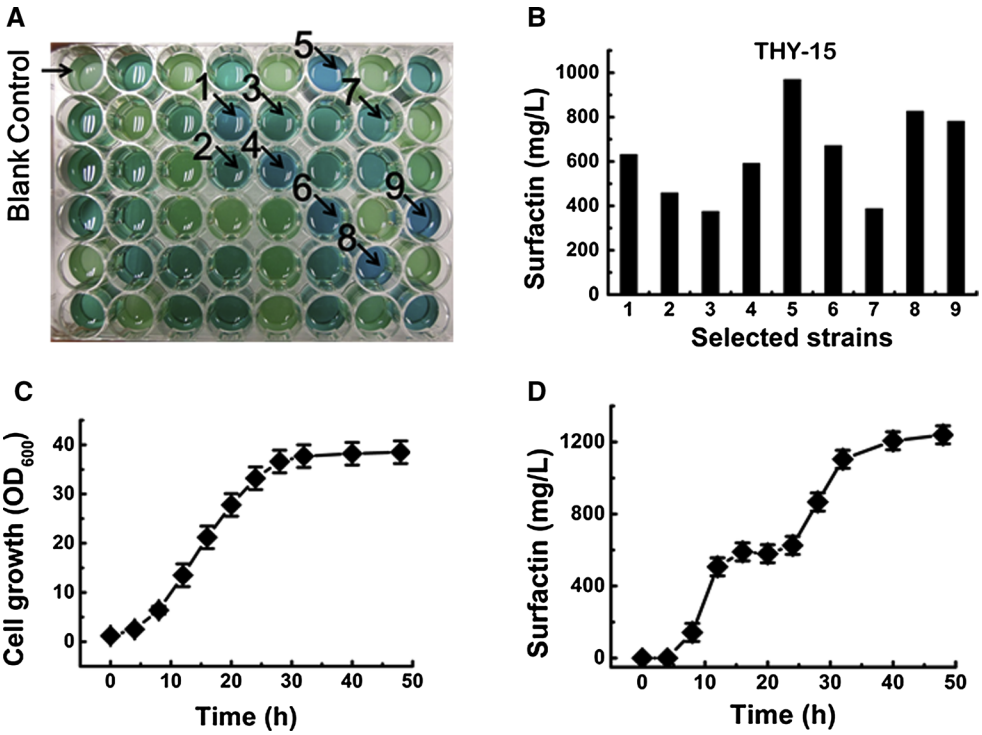

Fig. 3 Application of the CPC–BTB method for screening the high yield surfactin producers. **a** The chromatic visible response of the screening of 9 optimal strains. Conditions: after 48 h fermentation, 100 μL of fermentation broth was added to 800 μL 0.1 mM CPC–BTB reagent in a 48-well plate and incubated at 25 °C for 5 min. **b** The quantity of surfactin in the optimal strains and in the best producer, *B. subtilis* THY-15. **c** The cell growth curve of *B. subtilis* THY-15 in a flask culture with 100 mL fermentation medium. **d** The surfactin production curve of *B. subtilis* THY-15 in a flask culture with 100 mL fermentation medium

Table 1 The physiological characteristics of *Bacillus subtilis* THY-15

Test items	Results	Test items	Results
Gram staining	Positive	Growth at 7 % NaCl	+
Cell shape	Rod	Growth at pH 5.7	+
Cell diameter >1 μm	−	Citrate utilization	+
Spore-forming	+	Casein hydrolysis	+
Catalase	+	Starch hydrolysis	+
Oxidase	+	Acid production from	
Anaerobic growth	−	D-glucose	+
Voges–Proskauer test	+	L-arabinose	+
Methyl red test	+	Xylose	+
Nitrate reduction	+	Mannitol	+
Growth at 50 °C	+	Lactose	−

+, positive; −, negative

analysis and MALDI-TOF mass spectrometry analysis (as shown in Fig. S1 of Supplementary Material).

Amino acid sequence determination of the circular peptide of the THY-15 surfactin

Taking RP-HPLC fraction 4 as an example, the hydrolyzed surfactin products from *B. subtilis* THY-15 were analyzed by HPLC in comparison with amino acid standards (Fig. 5a). The molar ratio of the four amino acids (Asp: Glu: Val: Leu) was 1.05: 1.03: 1.08: 4.2 using Nleu as the internal standard (Fig. 5b).

The molecular mass of the purified surfactin isoform in fraction 4 was determined by MALDI-TOF-MS, with main peaks at *m/z* 1036, 1058 and 1074, which corresponded to the H^+, Na^+ and K^+ adductions of C_{15} surfactin, respectively, as shown in Fig. 6a. The mobile proton model [17] and the double hydrogen transfer (DHT) mechanism [28] provide a solid background for understanding the fragmentation pathways of protonated peptides. Tandem mass spectrometry of the precursor ion results are illustrated in Fig. 6b. The series of b^+ fragment ions at *m/z* 1058 → 945 → 832 ($-H_2O$, 814) → 717 revealed the loss of Leu-Leu-Asp from the C-terminus. Furthermore, another typical series of y^+ fragment ions at *m/z* 707 → 594 → 481 → 382 → 267 suggested the loss of the Leu-Leu-Val-Asp in the middle of the peptide chain. The precursor ion at *m/z* 1058 was the sodium adduct of a surfactin containing a C_{15} β-OH fatty acid, whose peptide sequence was Glu-Leu-Leu-Val-Asp-Leu-Leu, which agreed with the molar ratio of Asp, Glu, Val and Leu as 1.05:1.03:1.08:4.2 by amino acid analysis. The molecular structure of iso-C_{15} surfactin in fraction 4 is shown in Fig. 6c.

MALDI-TOF MS and MS/MS spectra of fraction 1, 2 and 3 were also analyzed, and the results are illustrated in the Supplementary Material. The MS/MS spectra of sodium adducted ions found at *m/z* 1030 and 1044 in fraction 1 and fraction 2 with a difference of 14 Da ($-CH_2-$)

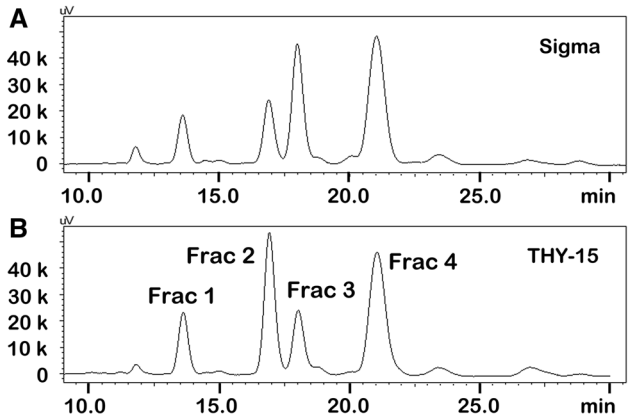

Fig. 4 RP-HPLC chromatograms of surfactin from Sigma and *B. subtilis* THY-15. **a** Chromatogram of the surfactin standard purchased from Sigma. **b** Chromatogram of the surfactin produced by *B. subtilis* THY-15. *Frac* fraction

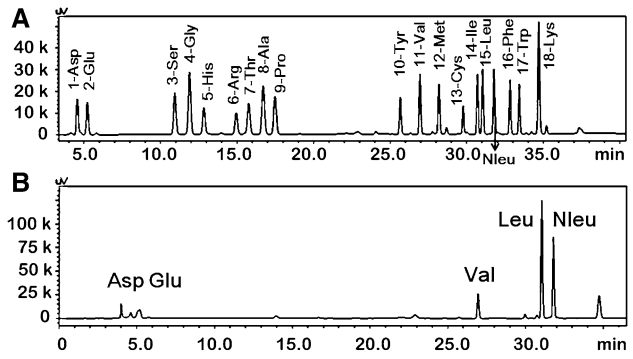

Fig. 5 Amino acid analysis of C_{15} surfactin in fraction 4. **a** Chromatogram of 18 standard amino acids. **b** Chromatogram of the amino acids from the hydrolyzed surfactin in fraction 4 of the HPLC analysis

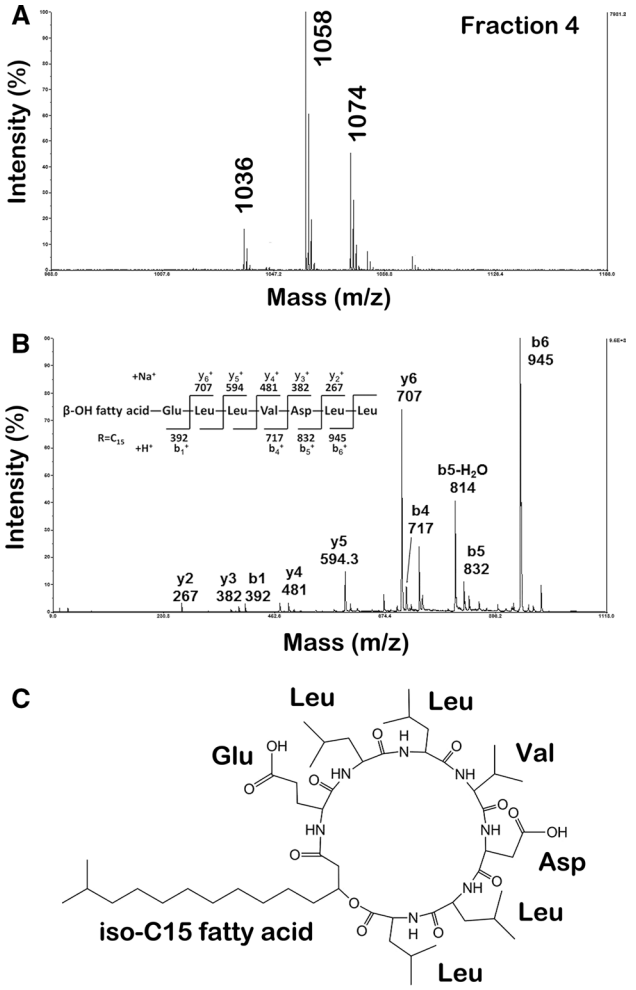

Fig. 6 MALDI-TOF MS and MS/MS analysis of the iso-C_{15} surfactin isoform in fraction 4. **a** MALDI-TOF MS of surfactin in fraction 4, $[M+H]^+$ at *m/z* 1036, $[M+Na]^+$ at *m/z* 1058 and $[M+K]^+$ at *m/z* 1074. **b** MALDI-TOF-MS/MS of the [surfactin + $Na]^+$ precursor at *m/z* 1058 in fraction 4. **c** The molecular structure of surfactin isoform in fraction 4, containing a C_{15}-β-hydroxy fatty acid chain and a Glu-Leu-Leu-Val-Asp-Leu-Leu peptide

were homologs of fraction 4 possessing the same peptide structure but different C_{13} and C_{14} β-OH fatty acids, respectively (details in Supplementary Material Fig. S2 and Fig. S3). The surfactin isoform in fraction 3, which possessed a peptide sequence of Glu-Val-Leu-Leu-Asp-Leu-Val and a C_{14} fatty acid (details in Supplementary Material Fig. S4), was slightly different from the typical surfactin structure in fractions 1, 2 and 4.

Discussion

Surfactin is a valuable new chemical with great potential in EOR and microbial-enhanced oil recovery (MEOR) [11, 12], agriculture [20], environmental bioremediation [2, 16], cosmetics [29] and health care [19]. However, better surfactin producers with high yield are required for its large-scale production and application. An efficient and high-throughput screening method was developed in this work for obtaining a superior surfactin producer. Because the sulfonephthalein dye BTB does not directly interact with the surfactin for a color change, CPC was introduced as a mediator, and a second-time color shift for the visible detection of surfactin was realized.

The CPC–BTB screening method is applied in a 48-well plate (Nunc™ Surface, Denmark) and the fermentation broth can be applied without centrifugation, since the cells have little effect on the chromatic response. The final protocol was as follows. CPC and BTB were dissolved in 0.1 M PBS (NaH_2PO_4/Na_2HPO_4, pH 8.0) in preparation for respective 0.2 mM solution. 20 mL of 0.2 mM CPC was mixed with 20 mL 0.2 mM BTB to get the 0.1 mM

CPC–BTB reagent prior to use. A constant 800 μL of CPC–BTB reagent was added into the 48-well plate. Then, 100 μL testing sample was well-mixed and allowed to sit for 5 min at 25 °C. Those strains generating an obvious visible chromatic response from light green to bright blue (surfactin >400 mg/L) were selected. In addition, for quantitative purpose, centrifugation is necessary to obtain supernatant and the surfactin concentration of samples should be diluted into 100–500 mg/L. The absorbance at 600 nm was measured by the microplate reader.

The CPC–BTB screening method is qualitatively and quantitatively verified to be feasible for high-throughput selection of surfactin producers with accuracy and reliability. In addition, this CPC–BTB method is particularly appropriate for selecting the best positive mutants from a large mutagenesis library during the mutation breeding of a specific strain producing surfactin. It can also be used in combination with other screening methods, such as blood agar plate, to further enhance screening efficiency and throughput.

Applying the CPC–BTB method, an optimal strain, *B. subtilis* THY-15, was obtained with a surfactin titer of 1240 mg/L after 48 h of flask fermentation. This result confirmed the promising potential of the genus *Bacillus* in surfactin production. With the development of diverse genetic modifications, such as promoter engineering of synthetases [13, 23] and modification of specific proteins [10], wild *B. subtilis* THY-15 would also be a satisfactory host for genetic recombination to obtain an ideal scaled-up producer of surfactin.

Surfactin is synthesized by non-ribosomal peptide synthetases (NRPSs) [5], which can generate variants with differences in the fatty acid chain length or amino acid sequence. Identification of the surfactin isoforms can be difficult. In this study, 4 surfactin homologs harvested from *B. subtilis* THY-15 were separated and purified by RP-HPLC. Fraction 1 contributed approximately 11 % to the total and fraction 4 contributed approximately 43 %, similar to the Sigma standard. However, the amounts of the second and third fraction were completely different (14, 27 % for Sigma and 29 %, 14 % for *B. subtilis* THY-15, respectively). The circular peptide sequence of the surfactin isoforms was identified by both amino acid analysis and MALDI-TOF MS/MS. Isoforms of surfactin in fraction 1, 2 and 4 contained the same peptide sequence of Glu-Leu-Leu-Val-Asp-Leu-Leu, but were different in iso-C_{13}, C_{14} and C_{15} fatty acid chains, while the isoform in fraction 3 possessed a special peptide sequence of Glu-Val-Leu-Leu-Asp-Leu-Val.

Recently, many studies with different degrees of success were reported for EOR/MEOR in low permeability dolomite cores [6], oil sand deposits [7] or oilfields in situ [30]. Arising from the special molecular structure, high surface activity and environment-friendly characteristics of surfactin, its large-scale production and wide applications in EOR/MEOR are expected in the near future.

In this study, a chromatically visible and quantitative high-throughput screening method for surfactin producers was constructed. A superior wild-type surfactin producer, *B. subtilis* THY-15, which produced 1240 mg/L surfactin was successfully selected by this novel CPC–BTB method. The producer's surfactin isoforms were individually purified and identified by RP-HPLC and MALDI-TOF MS/MS. *B. subtilis* THY-15 is expected to make a significant contribution to high titer production of surfactin after genetic modification and optimization of fermentation process. These results support the identification of more efficient surfactin producers and promising applications of surfactin in different fields.

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Conflict of interest The authors declare that they have no conflict of interest.

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