Liu *et al.*
Biotechnology for Biofuels and Bioproducts (2024) 17:133
https://doi.org/10.1186/s13068-024-02581-7

Biotechnology for Biofuels and Bioproducts

METHODOLOGY

Open Access

Production and characterization of novel/chimeric sophorose–rhamnose biosurfactants by introducing heterologous rhamnosyltransferase genes into *Starmerella bombicola*

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Abstract

Glycolipid biosurfactant, sophorolipids (SLs) and rhamnolipids (RLs) can be widely used in agriculture, food and chemical industries. The different physicochemical properties of SLs and RLs, such as hydrophilic lipophilic value (HLB) and critical micelle concentration (CMC), determine they have different application focus. Researchers are still hoping to obtain new glycolipid surfactants with unique surface activities. In this study, we successfully transformed two rhamnosyltransferase genes *rhlA* and *rhlB* from *Pseudomonas aeruginosa* to the sophorolipid-producing *Starmerella bombicola* CGMGG 1576 to obtain a recombinant strain was Sb_{rhlAB}. Two novel components with molecular weight of 554 ($C_{26}H_{50}O_{12}$) and 536 ($C_{26}H_{48}O_{11}$) were identified with the ASB C_{18} column from the fermentation broth of Sb_{rhlAB}, the former was a non-acetylated acidic C14:0 glycolipid containing one glucose and one rhamnose, and the latter was an acidic C14:1 glycolipid containing two rhamnoses. With the Venusil MP C_{18} column, one new glycolipid component was identified as an acidic C18:3 glycolipid with one rhamnose ($C_{24}H_{40}O_7$), which has not been reported before. Our present study demonstrated that novel glycolipids can be synthesized in vivo by reasonable genetic engineering. The results will be helpful to engineer sophorolipid-producing yeast to produce some specific SLs or their derivatives in more rational and controllable way.

Keywords Sophorolipids, Rhamnosyltransferase, Biosurfactants, *Starmerella bombicola*, New glycolipid component

Introduction

Surfactants have been widely applied in many industrial and household chemical sectors. As of 2019, the global surfactant market was 40 billion U.S. dollars and is expected to grow to 50 billion U.S. dollars by 2025 [12], about half of which is used in domestic and laundry detergents that end up in the environment [36]. Biosurfactants are surfactants that synthesized by microorganisms, including bacteria, yeasts using renewable resources or waste through fermentation processes. Biosurfactants are promising alternatives to chemically synthesized surfactants, due to their remarkable surface and

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biological properties, biocompatibility and environmental compatibility [7, 35]. The market for biosurfactants is growing rapidly, especially the demand for new biosurfactants with better activity and unique properties.

As a kind of glycolipid biosurfactant, sophorolipids (SLs) are produced from certain species of non-pathogenic yeast strains and naturally produced as a mixture containing up to 20 acidic and lactonic sophorolipid molecules. Each of these different sophorolipid molecules is composed of a sophorose moiety (hydrophilic part) linked by a glycosidic bond to a long chain hydroxyl fatty acid (lipophilic part). In addition to some small structural modifications, such as acetylation degree of sophorose, acetyl group position in the sophorose moiety, chain length and unsaturation degree of hydroxyl fatty acid, hydroxyl group position in the fatty acid moiety, and lactonization or not, etc. [3, 10, 16]. Same as other biosurfactants, SLs exhibit good surface-active properties, low toxicity, high biodegradability, biocompatibility, and are eco-friendly. SLs also stand out for their high production. Therefore, sophorolipid is the most likely biosurfactant to achieve large-scale application in industry [19, 28]. Recently, besides the good surface activities being well evaluated, the fine bioactivities of SLs have much more been reported, such as their good antibacterial [25, 29], antifungal [9, 39], anti-tumor [8, 20], anti-inflammatory [18], and even anti-HIV activities [26]. More recently, the antibiofilm property of SLs is attracting increasing interest [22, 23]. Natural sophorolipid molecules and their specific derivatives (such as sophorolipid methyl ester, sophorolipid ethyl ester, and so on) presented anticancer effects against pancreatic cancer cells [8]. In our previous studies, a diacetylated lactonic sophorolipid with a C18 monounsaturated fatty acid was purified and the significant inhibitions on four human cancer cell lines H7402 (liver cancer line), A549 (lung cancer line), HL60 and K562 (leukemia line) were observed [4]. The effects of 10 purified sophorolipid molecules of different structures on human esophageal cancer cell lines KYSE 109 and KYSE 450 were also investigated in our previous work. The anti-tumor activities of the 10 SLs molecules showed significant differences, which are probably caused by their structural differences. These differences mainly included acetylation degree of sophorose, unsaturation degree of hydroxyl fatty acid, and lactonization or not [27].

With the increasing demand of biosurfactants that are of better bioactivities and surface-active properties, researchers have tried to make some modifications of the structures of SLs by chemical method and enzymatic catalysis. Commonly, SLs can be deacetylated by alkaline hydrolysis. Azim et al. synthesized several SLs derivatives with amino acids, such as aspartic acid, serine, leucine, phenylalanine, glutamic acid, glycine and their ethyl forms [1]. In the case of enzymatic catalysis, acetylesterase and cutinase can remove the acetyl group of SLs. Some lipases can catalyze the transesterification of the carboxyl terminal of SLs to form the ester derivative of SLs [2, 21], and some other lipases can catalyze acetylation of SLs to form corresponding amino derivatives of SLs [30]. Glycosidase or pectinase can transform SLs into glycolipids by releasing one glucose from the sophorose moiety of SLs [11]. Bolaform biosurfactants were synthesized by an engineered *S. bombicola* with the deletion of acetyltransferase gene and lactone esterase gene [32]. SLs synthesized with waste-cooking oil as a hydrophobic substrate are used to prepare methyl hydroxy branched fatty acids [13]. SL esters with longer chain length and higher acetylation degree of the sophorose moiety were produced, through ring opening of natural lactonic SL with *n*-alkanols of varying chain length under alkaline conditions and lipase-selective acetylation [31].

However, the previously reported modifications to the structures of SLs were performed in vitro, the genetic engineering of SLs-producing strains to produce novel SLs or their derivatives were rarely reported. Some researchers have established genetic manipulation platforms on which some genes have been successfully transformed into *Candida bombicola* by minorly modified Lithium acetate electroporation or electric shock [24, 34]. Because hygromycin B is the only sensitive antibiotic in *C. bombicola* [33], an auxotroph strain of *C. bombicola* without orotidine-5'-monophosphate decarboxylase gene (*ura*3) was screened as a host strain to perform genetic manipulations [34].

Rhamnolipids is another potential biosurfactant that is usually produced by *Pseudomonas aeruginosa*. Among all the biosurfactants, the production yield of rhamnolipids is second only to that of SLs. They have great potentials in petroleum extraction, green agriculture, environmental protection, food processing, cosmetics and medical areas [6, 14]. Two key enzymes Rt1 and Rt2 (rhamosyltransferase I, II) are responsible for the synthesis of rhamnolipids. Rt1 catalyzes the precursors TDP-rhamnose and β-hydroxyalkanic acid (HAAs) to produce monorhamnose rhamnolipids, while Rt2 transfers another TDP-rhamnose onto monorhamnose rhamnolipids to produce dirhamnose rhamnolipids [5]. The enzyme Rt1, a complex enzyme composed of two enzymes RhlA and RhlB, is a rate-limiting enzyme in the synthetic pathway of rhamnolipids. Thereinto, RhlA coded by *rhlA* is responsible for the synthesis of HAAs and RhlB coded by *rhlB* catalyzed TDP-rhamnose and HAAs to produce monorhamnose rhamnolipids.

This work aims to obtain more novel glycolipid biosurfactants with better surface-active properties and bioactivities through modifications of SLs in vivo by genetic

manipulations. In the present study, we expected that the two key genes *rhl*A and *rhlB* of rhamnosyl transferase I (Rtl) be heterologously expressed and function in sophorolipid-producing strain *S. bombicola* to produce novel chimeric glycolipid biosurfactants carrying both rhamnose and sophorose moieties.

Materials and methods

Chemicals and reagents

Acetonitrile, methanol, hexane and heptane were all of chromatographic grade and purchased from TEDIA Company Inc. (Fairfield, USA). Anthrone was purchased from Sigma (St. Louis, USA). Sorbitol and SDS (Shanghai shenggong biological engineering company), hygromycin (Amresco). Antibiotics were purchased from Amresco (Solon, USA). Other reagents used in this study were of analytical grade and purchased from Sangon (Shanghai, China). Rapeseed oil is of edible grade. DNA Ligation Kit Ver. 2.0 (TaKaRa, Dalian Bio Ltd), Plasmid minikit (BioTeke, Beijing Biotechnology Ltd), Kit (Sigma, USA).

Strains, plasmids and culture

S. bombicola was isolated from oily wastewater by our laboratory and was preserved in China General Microbiological Culture Collection Center (CGMCC) numbered CGMCC 1576 and was used as a wild type in this study.

The wild-type *S. bombicola* strain was preserved and activated on yeast extract peptone dextrose (YEPD) medium as described previously [15]. Basic ingredients of fermentation medium contained (g/L): KH_2PO_4 1.0, $Na_2HPO_4{\cdot}12H_2O$ 1.0, $MgSO_4{\cdot}7H_2O$ 0.5, yeast extract 3.0; carbon source 1: rhamnose 20.0 g/L, rapeseed oil 6% (v/v); carbon source 2: rapeseed oil 6% (v/v); carbon source 3: rhamnose 20.0 g/L.

First, the wild-type strain was cultured in liquid YEPD medium at 30 °C and 200 rpm overnight, then 2% (v/v) of cell culture was inoculated into fermentation medium (50 mL in 300 mL shaking flask) and grown at 30 °C, 200 rpm for 168 h.

Escherichia coli DH5α was used as a plasmid cloning host strain and purchased from TransGenBiotech Beijing. The plasmid pRLMG containing hygromycin resistance marker (P*pki* promoter of *Trichoderma reesei* and T*cbh2* (*cellobiohydrolase 2*) terminator of *Penicillium oxalicum*) was constructed and deposited in our laboratory. The plasmid pAJ401, P*gpd* promoter and T*pgk* terminator of *Saccharomyces Cerevisiae* preserved by our laboratory. The plasmid pJDOR4 containing the *rhlABRI* gene (4.46 kb) under its own promoter was donated by Prof. Jianqiang Lin, Shandong University. The plasmid pRLMG containing *rhlA* expression cassette was constructed in this study. The plasmid pRLMG containing *rhlA* and *rhlB* expression cassettes was constructed in this study. Primers used in this study are listed in Supplementary Materials Table S1.

Construction of *rhlA*/*rhlB* expression cassette

The *rhlA and rhlB* expression cassettes contained three components, P*gpd* (promoter of glyceraldehyde 3-phosphate dehydrogenase gene, *gpd*) from *S. bombicola,* coding region fragment of *rhlA/rhlB*, T*pgk* (terminator of phosphoglycerate kinase gene, *pgk*) from *S. cerevisiae*. Amplification of each module was performed by PCR using high fidelity PrimeSTAR HS DNA polymerase. The promoter P*gpd* was created by PCR amplification from the genome DNA of *S. bombicola* using the primers gApgfor and gApgrev (gApgfor and gBpgrev); the terminator T*pgk* was amplified from the template of plasmid pAJ401 using the primers gAppfor and gApprev (gBppfor and gApprev) primers; the coding region fragment of *rhlA/rhlB* was obtained by PCR amplification from the template of plasmid pJDOR4 using the primers gApAfor and gApArev (gBpBfor and gBpBrev). Finally, the upstream/downstream flanking homologous sequences and the above purified working modules were fused together through triple double-joint PCRs [15]. The *rhlA/rhlB* expression cassettes were achieved by nest PCR amplification with gApfor and gAprev (gBpfor and gBprev) primers and *Hind*III, *Bgl*II, *Sal*I sites placed in the upstream region.

Construction of recombinant plasmid pRhlA containing *rhlA* expression cassette

The *rhlA* expression cassette and pRLMG were, respectively, linearized with the restriction enzymes *Hind*III. The pRLMG DNA after digestion was treated with alkaline phosphatase from calf intestine (CIAP) to prevent self-cyclization of linear vector DNA. Appropriate amount of vector DNA was added to a 50 μL system (10 × CIAP Buffer 5 μL add water to 50 μL) for 1 h, extracted twice with phenol/chloroform/isopentanol (v/v, 25:24:1), extracted with chloroform/isopentanol (v/v, 24:1) once. Then the extraction solution was concentrated by ethanol precipitation, dried at room temperature and dissolved in redistilled water for purity validation by agarose gel electrophoresis. The digested plasmid vector and the above *rhlA* gene were mixed by the molar ratio (1:4) and linked together by DNA Ligation Kit Ver. 2.0, then the expression plasmid carrying *rhlA* gene were electrotransformed into *E. coli*. The recombinant plasmid pRhlA containing *rhlA* expression cassette were obtained after plasmid extraction and validation by sequencing.

Construction of recombinant plasmid pRhlAB containing *rhlAB* expression cassette and recombinant strain Sb_{rhlAB}

The *rhlB* expression cassette and the recombinant plasmid pRhlA were, respectively, linearized with the restriction enzymes *Sal*I. The pRhlA DNA after digestion was treated with CIAP according to the above method. After purity validation by agarose gel electrophoresis, the fragment of *rhlB* expression cassette and the dephosphorylated vector were mixed by the molar ratio (6:1–8:1) and linked together by 0.5 ~ 0.8 μL T4 DNA ligase at 16 °C, overnight. As the vector is large, the redistilled water, target gene and vector were added into the system first. The mixture was maintained at 45 °C for 5 min and then placed in an ice bath for 2 min to be cooled to 0 °C, and then buffer and ligase were added to increase the connection efficiency. The expression plasmid carrying *rhlAB* was first transferred into *E. coli* by electroporation. The transformants were singled out from the LB plate supplemented with ampicillin. The recombinant plasmid pRhlAB was obtained after plasmid extraction and validation by colony PCR and sequencing. The obtained plasmid pRhlAB was transferred into wild-type *S. bombicola* by electroporation, the positive transformants were singled out and purified on YPD agar plates supplemented with hygromycin B (500 μg/mL, final concentration). The fragments of *rhlA* and *rhlB* coding regions in the transformants were amplified from the template of the genome DNA of *S. bombicola* using the primers gApAfor/gApArev and gBpBfor/gBpBrev, the *hph* fragment was obtained by PCR amplification with the hphfor/hphrev primers. The positive transformants after validation were obtained and named as Sb_{rhlAB}.

Biomass and residual rhamnose determination of *S. bombicola*

The recombinant strain Sb_{rhlAB} was activated in 50 ml YEPD medium, then 2% (v/v) of the above culture broth was inoculated into three glycolipid-producing media with different carbon sources (carbon source 1: rhamnose 20.0 g/L, rapeseed oil 6% (v/v); carbon source 2: rapeseed oil 6% (v/v); carbon source 3: rhamnose 20.0 g/L) and incubated at 30 °C, 200 rpm for 168 h, respectively. The biomass, residual rhamnose, production of glycolipid and compositions of glycolipids were determined, respectively. The biomass was determined by dry weight. Five milliliters of n-butanol/ethanol/chloroform (10:10:1) was added into fermentation broth of equal volume, resuspended by vortexing, and then centrifuged at 8000 rpm for 10 min, at room temperature. After discarding the supernatant, the cell pellets were washed twice with redistilled water and then dried to a constant weight. The production of glycolipids was determined using a method based on anthrone–sulfuric acid method [17]. 1 mL of the fermentation broth was added with two volumes of ethyl acetate and ethanol, respectively, vortexed, fully extracted and centrifuged (1000 rpm for 10 min) at 30 °C for determination. Because the conversion coefficient between glycolipids and sugar was unknown, the concentration of glycolipids was approximatively expressed in the term of sugar. Residual rhamnose was analyzed by HPLC. The fermentation broth was first analyzed by analytical HPLC (SHIMADZU, Japan) with an Aminex HPX-87H column (7.8 μm × 300 mm, Biorad). Water was used as the mobile phase; the injection volume is 10 μL and the flow rate was 0.5 mL/min. The eluent was monitored at 207 nm using a differential refraction detector. In the meantime, standard solution of 1 mg/mL rhamnose was used for plotting standard curve. The injection volume of standard rhamnose was 2 μL, 4 μL, 6 μL, 8 μL, 10 μL, respectively, each peak area of the rhamnose of different concentrations was analyzed. Then standard curve was plotted by taking standard concentration as abscissa and peak area as ordinate, residual rhamnose in the fermentation broth can be calculated by its peak area based on the standard curve. The results were the mean of three statistical replicates.

Fermentation product analysis and preparation of wild-type and recombinant strains of *S. bombicola* by HPLC

For the analysis of components of SLs in fermentation of wild-type and recombinant strains of *S. bombicola*, 1 mL of fermentation broth was mixed with 2 mL chromatographic grade acetonitrile and centrifuged at 10,000 g for 10 min, the supernatant was transferred to a clean Eppendorf tube, dried at 50 °C, the total SLs was obtained and dissolved in absolute methanol. The components of SLs were analyzed with an ASB C_{18} column (4.6 μm × 250 mm × 4.6 mm, Agela Technologies Inc., USA); acetonitrile/water was used as the mobile phase by a gradient elution at a flow rate of 1 mL/min. The elution procedure was programmed as follows: the acetonitrile concentration was from 15 to 30% (v/v) within 0–10 min; increased to 30–50% (v/v) within 10–20 min; to 50–60% within 20–30 min; to 60–70% within 30–45 min; to 70–90% within 45–50 min; the injection volume was 1 mL, detected at 207 nm by a UV detector. The results were the mean of three statistical replicates.

To separate more hydrophobic glycolipid components, the fermentation product in the fermentation broth was analyzed by analytical HPLC with a Venusil MP C_{18} column at the same time. Fifty milliliter of fermentation broth was extracted with 100 mL of ethyl acetate and centrifuged at 10,000 g for 10 min, the upper organic phase was vacuumed evaporated at 50 °C and then washed twice with *n*-octane to remove residual oils, applied to

vacuumed evaporation at 50 °C again, and the light yellow solid was crude lactonic SLs.

The above crude SLs sample was dissolved in chromatographic grade methanol, the filter liquor after 0.22 μm organic membrane was applied to analytic HPLC with a Venusil MP C_{18} column (4.6 μm × 250 mm × 4.6 mm, Agela Technologies Inc., USA); acetonitrile/water was used as the mobile phase by a gradient elution at a flow rate of 1 mL/min. The elution procedure was programmed as follows: the acetonitrile concentration was from 40% to 60% (v/v) within 0–15 min; to 60–70% within 15–30 min; to 70–90% within 30–40 min and lasted for 5 min; the injection volume was 15 μL, detected at 207 nm by a UV detector. The results were the mean of three statistical replicates. The preparative HPLC was performed with a PrepHT XDB C_{18} column (250 mm × 21.2 mm, Agela Technologies Inc., USA) at a flow rate of 15 mL/min; the injection volume was 500 μL; the elution procedure was programmed as that for analytical HPLC. The collected fractions were concentrated under vacuumed evaporation and then subjected to purity verification after drying at 50 °C. When only one peak was visible and the retention time was the same as that from analytical HPLC. The purified peaks were used for MS analysis.

Structure elucidation of SLs (glycolipids) by mass spectrometry

The structures of SLs were elucidated by mass spectrometry. Positive MS spectra were obtained on Agilent Q-TOF6 510 (Agilent). Positive ESI mass spectra were obtained by MS-1. MS/MS analysis was performed by selecting the desired precursor ion with MS-1 and the ion selected was collided in a collision cell with different collision energy values (13–30 eV) depending on the compound analyzed. N_2 was used as carrier gas. Two microliter of sample was injected into mass spectrometer for analysis.

Results

Construction of *rhlA* expression cassette

Since rhamnosyltransferase A and B are both derived from *P. aeruginosa* SH6, it is better to use the strong promoter from *S. bombicola* itself to express rhamnosyltransferase A and B. Therefore, the promoter of triglyceride dehydrogenase (P*gpd*) from this strain was used as the promoter of two genes, respectively. The terminator T*pgk* of phosphoglycerate kinase gene (*pgk*) from *S. cerevisiae* was used as the terminator. Many restriction enzyme sites are found to be located in the coding sequences of rhamnosyltransferase A and B genes (*rhlA* and *rhlB*), therefore, it is unfavorable to construction expression cassettes using traditional enzyme digestion and ligation way. Therefore, the promoters, coding regions and terminators of fragments were ligated by fusion PCR to obtain expression cassettes of the two genes, respectively.

The *rhlA* expression cassette was first constructed. The three target fragments were amplified by PCR, respectively. The P*gpd* fragment with the size of 1572-bp was obtained by amplification from the genome DNA template of *S. bombicola* and using the primers gApgfor and gApgrev. The 934-bp coding region fragment of *rhlA* gene was obtained by amplification from the template of plasmid pJDOR4 using the primers gApAfor and gApArev. Using plasmid pAJ401 as the template and primers gAppfor and gApprev, the 351-bp *Tpgk* fragment was obtained by PCR amplification (Figure S2 in the Supplementary Material).

The above three fragments were fused by fusion PCR at an equal molar ratio and a 2797-bp fusion product was obtained (Figure S2 in the Supplementary Material). Then, the resulting fusion product was further amplified by nest PCR and introduced *Hind*III restriction site, finally, *rhlA* expression cassette (gAp fragment, 2759-bp) was obtained (Figure S2 in the Supplementary Material). The PCR product was purified and further subjected to sequencing for validation.

Construction of recombinant plasmid pRhlA containing *rhlA* expression cassette

The gAp fragment and plasmid pRLMG was digested by *Hind*III and linked by ligase, then was transferred into *E. coli* DH5α by electroporation. The single colonies were singled out to transfer into the liquid LB medium supplemented with ampicillin and incubated overnight. The positive plasmid was extracted and verified by *Hind*III (Figure S3 in the Supplementary Material). The 2.7-kb fragment which corresponded with the theoretical value was further confirmed by sequencing and named as pRhlA.

Construction of *rhlB* expression cassette

The *rhlB* expression cassette was constructed according to the same method as the construction of *rhlA* expression cassette. The P*gpd* fragment from *S. bombicola*, coding region of *rhlB* and the T*pgk* fragment from *S. cerevisiae* were fused together by double-joint PCRs. The procedure was as follows: the three target fragments were obtained by PCR amplification, respectively. The 1572-bp P*gpd* fragment was amplified from the template of *S. bombicola* genome DNA using the primers gApgfor and gBpgrev. The 1314-bp *rhlB* gene coding region fragment was obtained by amplification from the plasmid pJDOR4 using the primers gBpBfor and gBpBrev. The 339-bp T*pgk* terminator was obtained by PCR amplification from the plasmid pAJ401 using the primers gBppfor and gApprev.

The above three working fragments were fused together through triple double-joint PCRs and the 3165-bp fusion product was obtained. The resulting fusion product was further amplified by nest PCR, finally, *rhlB* expression cassette (gBp fragment, 3110 bp) with *Sal*I restriction site was obtained (Figure S3 in the Supplementary Material). The PCR product was purified and further subjected to sequencing for verification.

Construction of recombinant plasmid pRhlAB containing *rhlA* and *rhlB* expression cassette

The gBp fragment and plasmid pRhlA was digested by *Sal*I and linked by ligase, then was transformed into *E. coli* DH5α by electroporation. The single colonies were transferred to the liquid LB supplemented with ampicillin and incubated overnight. The plasmid was extracted and submitted to digestion via *Sal*I, finally, the 3.1-kb fragments which corresponded with the theoretical value were confirmed to contain gBp fragment (Figures S1 and S3 in the Supplementary Material).

Transformation of recombinant plasmid pRhlAB into *S. bombicola*

The recombinant plasmid pRhlAB was transferred into *S. bombicola* by electroporation. The plasmid was inserted into the *S. bombicola* genome by random insertion, and the positive transformants were singled out and purified on YPD plate supplemented with the hygromycin B marker. Then, *hph* (hygromycin resistance gene), *rhlA*, *rhlB* coding region fragments, were, respectively, obtained from the template of genome of the transformant by PCR amplification. The target band was amplified and confirmed by sequencing (Figure S4 in the Supplementary Material). The obtained recombinant strain was named as Sb_{rhlAB} to distinguish it from the wild-type strain *S. bombicola* (*Sb*).

Fermentation product analysis of recombinant strain *S. bombicola* on different carbon sources

Wild-type strain *Sb* and recombinant strain Sb_{rhl}AB were fermented in the glycolipids-producing medium with rhamnose as the hydrophilic carbon source and rapeseed oil as the hydrophobic carbon source. After the fermentation, the content of residual rhamnose in the fermentation broth was analyzed by HPLC (Aminex HPX-87H column, 7.8 μm × 300 mm, Biorad), and the growth of the wild-type strain *Sb* and recombinant strain Sb_{rhlAB} was determined, as shown in Table S2 in the Supplementary materials. It can be seen that the utilization rate of rhamnose was very low by both wild-type strain and recombinant strains, and the sugar concentration in the glycolipids was determined by anthrone method to analyze the ability of the strains to produce glycolipids. When rhamnose was used as the hydrophilic carbon source, the total glycolipids and lactonic glycolipids obtained by fermentation of the recombinant strain were slightly higher than that of the wild-type strain, but the difference was not significant, which may be due to the low activity of rhamnosyltransferase itself.

To investigate the effects of rhamnose on glycolipids synthesis of wild-type strain *Sb* and recombinant strain Sb_{rhlAB}, the two strains were cultured for 168 h in three different fermentation media using rapeseed oil, rhamnose, rapeseed oil and rhamnose as the carbon sources, respectively. The cell growth and the fermentation products of the two strains were determined.

As shown in Table 1, both wild-type and recombinant strains could grow well with both rhamnose and rapeseed oil or with only rapeseed oil. In the case of complex carbon sources, the biomass of the recombinant strain was much higher than that of wild-type strain *Sb*, while in the presence of rapeseed oil, the growth of Sb_{rhlAB} is similar to that of the wild-type strain; unsurprisingly, they both could not grow with rhamnose as the sole carbon sources. Compared with the fermentation with only rapeseed oil, the presence of rhamnose slightly decreased

Table 1 Fermentation results of *S. bombicola Sb* and Sb_{rhlAB} using three different carbon sources

Strain	Carbon source	Biomass (g/L)	Rhamnose residue (g/L)	Lac glycolipid (mmol/L)	Tot glycolipid (mmol/L)
Sb	R + O[1]	5.00 ± 1.16	20.08 ± 0.17	27.17 ± 0.61	35.32 ± 3.01
Sb_{rhlAB}	R + O	7.00 ± 0.62	20.11 ± 0.24	31.97 ± 0.42	49.44 ± 0.74
Sb	R	0.00	20.15 ± 0.09	0.00	0.00
Sb_{rhlAB}	R	0.00	20.07 ± 0.19	0.00	0.00
Sb	O	6.25 ± 1.08	0.00	22.77 ± 0.58***	66.60 ± 1.66***
Sb_{rhlAB}	O	6.50 ± 0.34	0.00	31.88 ± 0.12***	73.97 ± 0.42***

[1] R + O means rhamnose and rapeseed oil; R means rhamnose; O means rapeseed oil

*** $p < 0.001$

the biomass of the wild-type strain. When rhamnose and rapeseed oil used as the complex carbon source or rapeseed oil as the sole carbon source, the titer of glycolipids produced by the recombinant strain Sb_{rhlAB} was much higher than that by the wild-type strain *Sb*. For wild-type strain, when rapeseed oil was used as the sole carbon source, the production of lactonic glycolipids was lower than that in the presence of rhamnose and rapeseed oil, while the production of total glycolipids was higher in the case of rapeseed oil as the sole carbon source than that under the condition of complex carbon source. For the recombinant strain, there was no difference in the production of lactonic glycolipids under the two conditions, but the production of total glycolipids under the condition of complex carbon source was lower than that in the presence of only rapeseed oil. To sum up, neither the wild-type strain nor the recombinant strain could use rhamnose as a carbon source, and the addition of rhamnose inhibited the synthesis of acidic glycolipids to some extent, and the ability of the recombinant strain to synthesize glycolipids was slightly higher than that of the wild-type strain.

When the complex carbon source of rhamnose and rapeseed oil as well as sole carbon source of rapeseed oil was used for glycolipids fermentation, the compositions analysis of glycolipids was performed with a Venusil MP C_{18} column using HPLC analysis, as shown in Fig. 1 and Table 2. Within the range of the retention

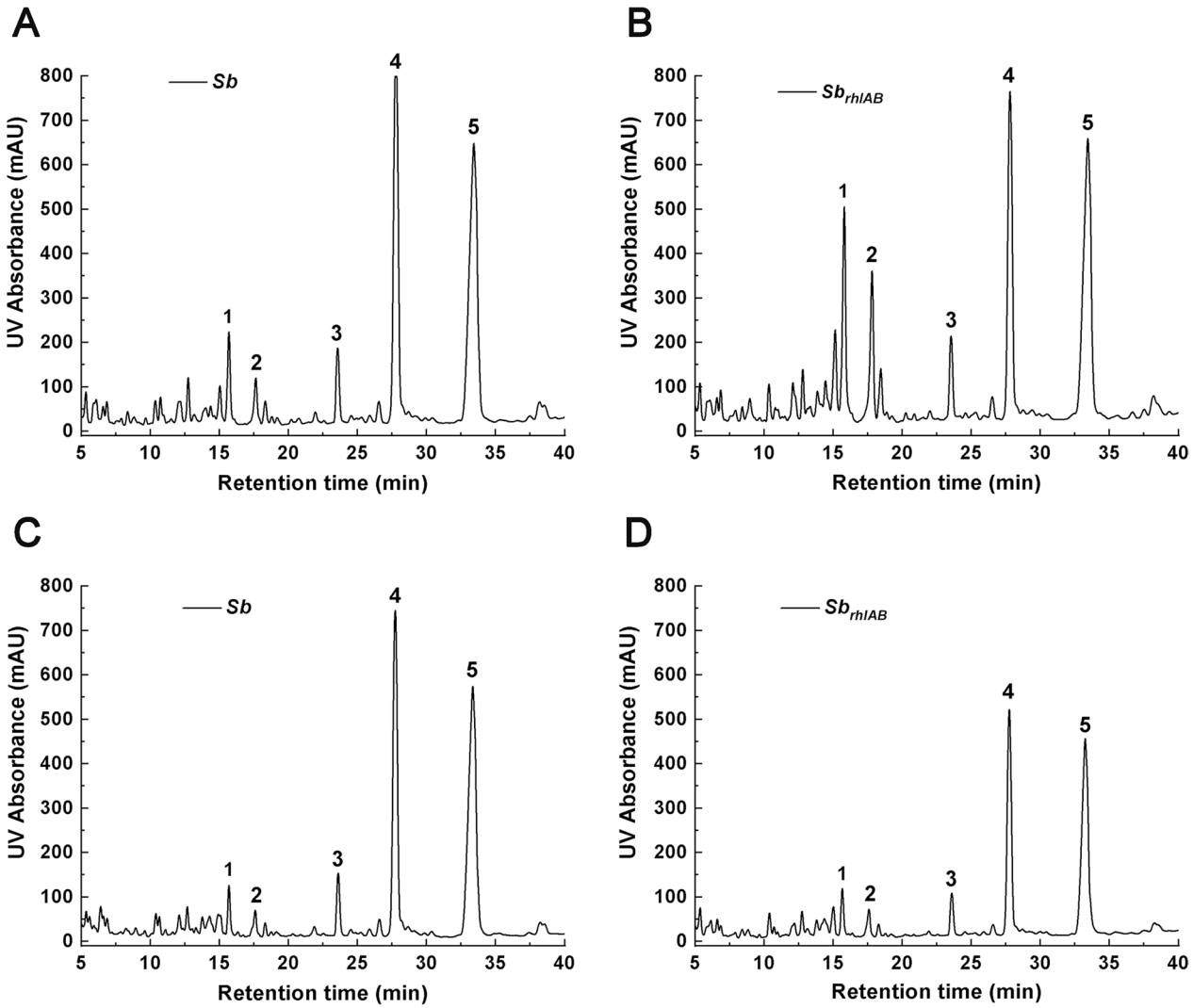

Fig. 1 HPLC chromatogram (Venusil MP C_{18} column) of different crude glycolipids fermented by wild-type strain *Sb* and recombinant strain Sb_{rhlAB}. **A, B** Glycolipids fermented by *Sb* and Sb_{rhlAB} on rhamnose and rapeseed oil, respectively. **C, D** Glycolipids fermented by *Sb* and Sb_{rhlAB} on rapeseed oil, respectively

Table 2 Relative content of different sophorolipid components from strain *Sb* and Sb_{rhlAB} using rhamnose and rapeseed oil or rapeseed oil as carbon sources, respectively

No	RT (min)	Structure	Relative content (%) of each component			
			R + O-*Sb*[1]	R + O-Sb_{rhlAB}	O-*Sb*	O-Sb_{rhlAB}
1	15.79	C18:2 DASL	3.08 ± 0.41	4.53 ± 0.19**	1.49 ± 0.30**	1.71 ± 0.23**
2	17.80	C18:1 DASL	1.79 ± 0.39	3.40 ± 0.57*	1.32 ± 0.26	1.40 ± 0.33
3	23.53	C18:1 MLSL	2.39 ± 0.17	2.32 ± 0.35	2.34 ± 0.81	1.72 ± 0.14**
4	27.80	C18:2 DLSL	16.41 ± 1.93	11.26 ± 1.85*	15.13 ± 0.49	11.45 ± 1.96*
5	33.43	C18:1 DLSL	19.32 ± 1.31	15.78 ± 1.39*	26.38 ± 1.94**	19.29 ± 0.54

[1] R + O-*Sb* means strain *Sb* on rhamnose and rapeseed oil; R + O-Sb_{rhlAB} means strain Sb_{rhlAB} on rhamnose and rapeseed oil; O-*Sb* means strain *Sb* on rapeseed oil; O-Sb_{rhlAB} means strain Sb_{rhlAB} on rapeseed oil

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

time of 10–23 min, in the case of rhamnose and rapeseed oil as compound carbon source, it was found that the proportion of glycolipids produced by recombinant strains Sb_{rhlAB} were significantly higher than that by wild-type strain. The proportion of the two main acidic sophorolipid components (C18:2 and C18:1 DASL) in the recombinant strain Sb_{rhlAB} was higher than that in the wild-type strain, which increased by about 47.1% ± 11.2% and 90.0% ± 8.2%, in the meantime, the proportion of several lactonic SLs decreases, especially C18:2 DLSL decreased by 31.4% ± 2.7%; with rapeseed oil as the sole carbon source, the difference is much smaller between the recombinant and wild-type strains, C18:2 DASL and C18:1 DASL increased by 14.8% ± 6.6% and 6.1% ± 3.5%, respectively, while all the lactonic SLs decreased by 25% (C18:2 DLSL decreased by 24.3% ± 8.6%, C18:1 DLSL decreased by 26.8% ± 2.7%). The above results indicated that the recombinant strain Sb_{rhlAB} can produce more acidic SLs and less lactonic SLs compared with the wild-type strains.

Identification of chimeric glycolipids with both sophorose and rhamnose moieties by HPLC with an ASB C_{18} column

When rhamnose and rapeseed oil were used for glycolipids production of wild-type and recombinant strains, the components of crude glycolipids in the fermentation broth of wild strain *Sb* and recombinant strain Sb_{rhlAB} were analyzed by analytical HPLC. Regarding those different SLs or glycolipids molecules possess different polarity, the components of SLs were separated by two columns suitable for the separation of the compounds with different polarity. The crude glycolipids from wide-type and recombinant strains were first analyzed with an ASB C_{18} column of large polarity and good separation effect (Fig. 2). When the retention time of the recombinant strain was 26 min–33 min (shown in the frame

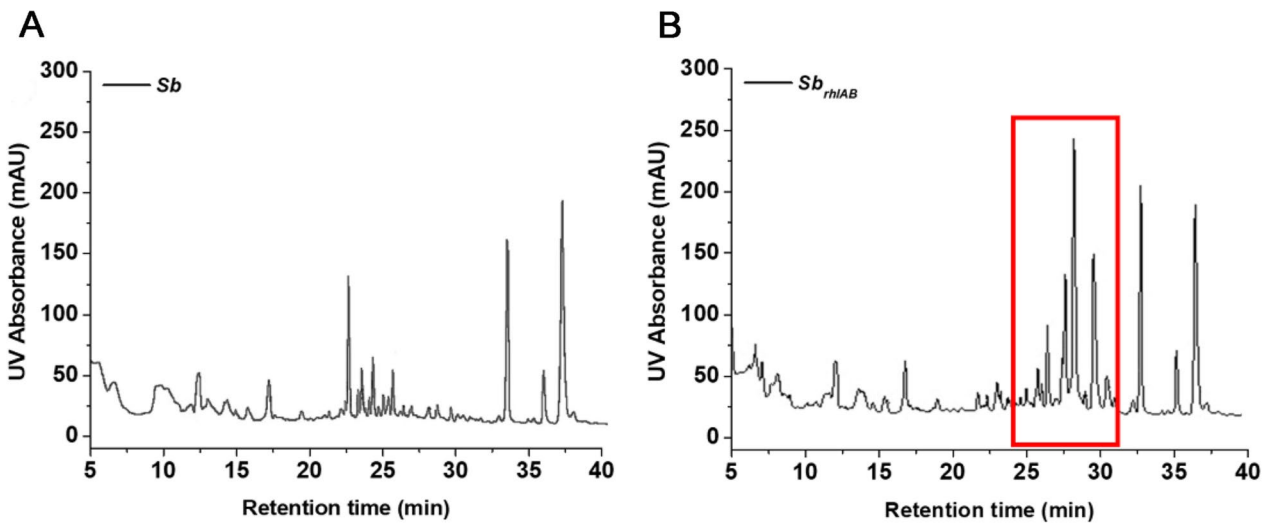

Fig. 2 HPLC chromatogram (ASB C_{18} column) of different crude glycolipids fermented by *Sb* and Sb_{rhlAB} on rhamnose and rapeseed oil. (**A**) and (**B**) Glycolipids fermented by *Sb* and Sb_{rhlAB} on rhamnose and rapeseed oil, respectively. Frame line indicates the differential components from that of wild-type strain

line), the components that were not found in fermentation broth of the wild-type strain appeared in that of recombinant strain, and these compositions were most likely to be new glycolipids containing rhamnose and glucose. HPLC–MS analysis results confirmed that the recombinant strain produced a few specific components compared with wild-type strain (Table 3), molecular weight of two specific components (retention time was at 16.74 min and 17.47 min) was 554 ($C_{26}H_{50}O_{12}$) and 536 ($C_{26}H_{48}O_{11}$), respectively. The former was speculated to be an acidic glycolipid that hydrophilic part includes one rhamnose and one glucose and hydrophobic part includes a non-acetylated C14:0 fatty acid, the latter was speculated to be a glycolipid with a hydrophilic part of two rhamnose molecules, hydrophobic part of C14:1.

Identification of novel glycolipids by HPLC with a venusil MP C_{18} column

In the meantime, the crude glycolipids from wide-type and recombinant strains were applied HPLC analysis with a Venusil MP C_{18} column which is good at the separation of the compounds with larger hydrophobicity.

As shown in Fig. 3 and Table 4, when the retention time was between 10–23 min (shown in the frame line), several components occurred in the fermentation product of Sb_{rhlAB} while were not found in that of the wild-type strain. The components occurred at the retention time of 10–23 min were collected and purified by preparative HPLC with PrepHT XDB C_{18} column by preparative HPLC and 5 components were identified by MS on Agilent Q-TOF6 510 (Agilent).

Discussion

S. bombicola is an excellent sophorolipid-producing yeast and the composition of SLs produced by fermentation of this yeast reaches up to 20. Rhamnolipids is a kind of glycolipid produced by bacterium. Different sophorolipid molecules and rhamnolipids with small modifications on the basic chemical structures exhibited different surface active and biological properties. So far, the information regarding alteration of the structures of rhamnolipids are

Table 3 Specific components of Sb_{rhlAB} on rhamnose and rapeseed oil

RT (min)	MW	Molecular formula	Structure
16.744	554	$C_{26}H_{50}O_{12}$	One non-acetylated glucose and one rhamnose, acidic C14:0 glycolipid
17.048	644	$C_{32}H_{52}O_{13}$	Non-acetylated sophorose, acidic C20:4 SL
17.468	536	$C_{26}H_{48}O_{11}$	Di-rhamnose, acidic C14:1 glycolipid
18.104	556	$C_{26}H_{52}O_{12}$	undefined

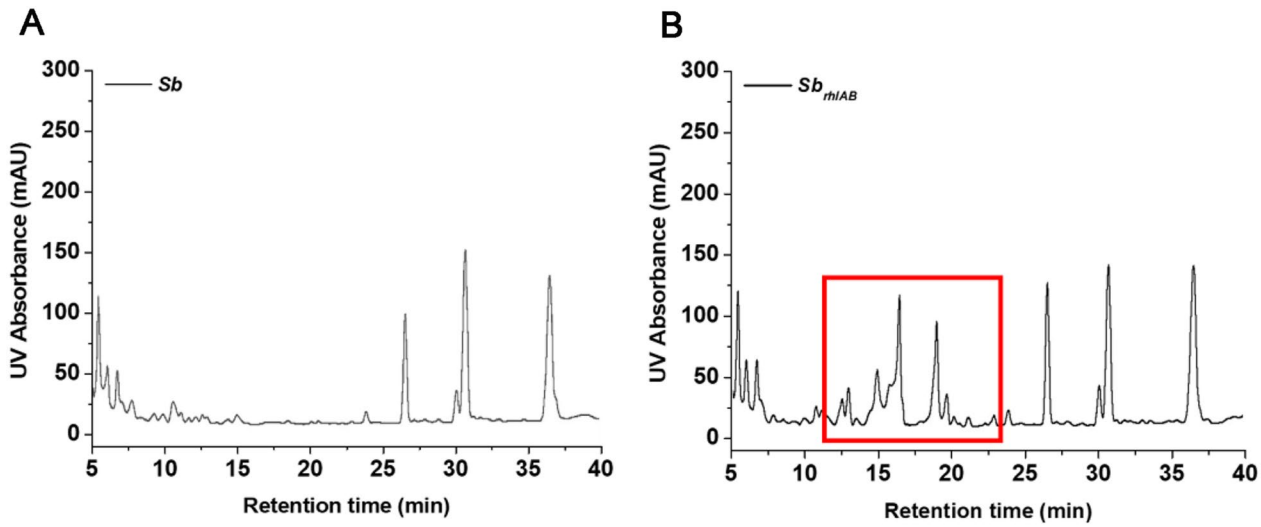

Fig. 3 HPLC chromatogram (Venusil MP C_{18} column) of crude glycolipids fermented by wild-type and recombinant strains of S. bombicola. (**A**) and (**B**) Glycolipids fermented by Sb_{rhlAB} and Sb_{rhlAB} on rhamnose and rapeseed oil. Frame line indicates the differential components from that of wild-type strain

Table 4 Structure elucidation of molecules from Sb_{rhlAB} using rhamnose and rapeseed oil as carbon sources by MS method

RT (min)	MW	Molecular formula	Structure
11.72	664	$C_{32}H_{56}O_{14}$	Mono-acetylated sophorose, acidic C18:1 SL
14.85	704	$C_{34}H_{56}O_{15}$	Di-acetylated sophorose, acidic C18:2 SL
17.65	706	$C_{34}H_{58}O_{15}$	Di-acetylated sophorose, acidic C18:1 SL
19.35	440	$C_{24}H_{40}O_{7}$	One rhamnose, acidic C18:3 glycolipid
20.20	636	$C_{30}H_{52}O_{14}$	Mono-acetylated sophorose, acidic C16:1 SL

unavailable, in the meantime, the previous researches on SLs mainly focused on several aspects, such as key enzymes in the synthetic pathway, the identification of novel SL components, the optimization of fermentation yield, and the applications of SLs [37, 38]. Only very few researches focused on the modifications on the structures of SLs by chemical or enzymatic methods in vitro, to produce sophorolipid derivatives that are expected to have better surface and biological active properties of SLs. However, no attempts have been made to enhance the properties of SLs or rhamnolipids by genetically engineered strains. In this study, we successfully transformed an expression vector with two key rhamnosyltransferase gene *rhlA* and *rhlB* into the SLs-producing wild-type strain *Sb* and a recombinant strain Sb_{rhlAB} of *S. bombicola* CGMCC 1576. Some glycolipid components were found in the recombinant strain Sb_{rhlAB} while not in the wild-type strain. The fermentation products of the recombinant strain Sb_{rhlAB} were analyzed by two different HPLC columns, one column was of larger polarity while another was of larger hydrophobicity. The HPLC and MS analysis results proved that the recombinant strain Sb_{rhlAB} acquired the ability of producing chimeric and novel glycolipids by introducing heterologous rhamnosyltranferases into *S. bombicola*. The resulted recombinant *S. bombicola* strain became a producer of chimeric and new glycolipids with both rhamnose and glucose moieties or with rhamnose besides SLs.

According to the respective synthetic pathways of sophorolipids and rhamnolipids, we speculate the possible synthetic pathways of new glycolipids (Figure S5 in the Supplementary Material), but some of the synthetic pathways are still unknown and need to be further explored.

Glycolipids are generally named by their hydrophilic part, namely, the glycosyl part. For a glycolipid, the length of the carbon chain determines its physicochemical properties, such as abilities to foam and emulsify, and their critical micelle concentration (CMC). For a large class of glycolipid biosurfactants, the difference in the glycosyl part is an important reason for their completely different physical properties. So far, the fatty acid chain of biosurfactants such as sophorolipids and rhamnolipids has been mainly modified to obtain better biological activity and surface activity. Only a few studies have focused on modifying the glycosyl part. Our study showed that the recombinant *S. bombicola* strains can produce novel/chimeric sophorose–rhamnose biosurfactants. The novel glycolipids may have distinct biological and surface activity from sophorolipids and rhamnolipids. Unfortunately, although the new glycolipids containing rhamnose and glucose was inferred by mass spectrometry results in this paper, due to current technical reasons, the yield of the recombinant strains is low, and it is difficult to produce a large amount of the product, so we did not accurately analyze its structure, nor did we measure the specific biological and surface activities. In future experiments, a suitable method of mass preparation should be found to produce enough pure products to accurately analyze the structure of the new types of glycolipids, and determine the corresponding properties to find possible industrial applications.

In general, the synthesis of sophorolipids requires two substrates, hydrophilic carbon sources (such as glucose) and hydrophobic carbon sources (such as rapeseed oil or oleic acid). When only one carbon source (hydrophilic or hydrophobic) is used for SLs production, the yield of sophorolipids is low, and only when two carbon sources are simultaneously used as the substrates, sophorolipids can be synthesized in large quantities. In the fermentation of recombinant strains, we used three carbon sources: rapeseed oil and rhamnose, only rapeseed oil and only rhamnose. The results showed that neither the wild-type strains nor the recombinant strains could use rhamnose as carbon source, but both the recombinant strains and the wild-type strains could grow well with both rhamnose and rapeseed oil or with only rapeseed oil. When rhamnose and rapeseed oil used as the complex carbon source or rapeseed oil as the sole carbon source, the titer of glycolipids produced by the recombinant strain Sb_{rhlAB} was much higher than that by the wild-type strain *Sb*. For wild-type strain, when rapeseed oil was used as the sole carbon source, the production of lactonic glycolipids was lower than that in the presence of rhamnose and rapeseed oil, while the production of total glycolipids

was higher in the case of rapeseed oil as the sole carbon source than that under the condition of complex carbon source. For the recombinant strain, there was no difference in the production of lactonic glycolipids under the two conditions, but the production of total glycolipids under the condition of complex carbon source was lower than that in the presence of only rapeseed oil. To sum up, the addition of rhamnose inhibited the synthesis of acidic glycolipids to some extent, and the ability of the recombinant strain to synthesize glycolipids was slightly higher than that of the wild-type strain. However, when rhamnose was used as a hydrophilic carbon source compared with glucose [16], the biomass and glycolipid production of the strains were significantly reduced.

In conclusion, our present study demonstrated that novel glycolipids can be synthesized in vivo by reasonable genetic engineering. The findings of this study would have implications in engineering glycolipids-producing microorganisms to produce more novel glycolipids with more advantageous properties.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13068-024-02581-7.

Supplementary Material 1.

Acknowledgements
We thank Chengjia Zhang, Nannan Dong, Dr. Jingyao Qu of the State Key Laboratory of Microbial Technology, Shandong University for helping controlling fermentor and analyzing data.

Author contributions
Xin Song conceived and designed research. Mingxin Liu, Tianshuang Tu, Hui Li, conducted experiments. Xin Song and Mingxin Liu analyzed the data. Mingxin Liu and Tianshuang Tu wrote the manuscript. Xin Song revised the manuscript. All authors read and approved the manuscript.

Funding
This work was supported by the National Key Research and Development Project of China (Grant no. 2021YFC2101300), the National Natural Sciences Foundation of China (Grant No. 31971387) and the Major Basic Research Project of Natural Science Foundation of Shandong Province, China (Grant No. ZR2019ZD19).

Availability of data and materials
The data sets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors approved the final manuscript and the submission to the journal.

Competing interests
The authors declare no competing interests.

Received: 22 June 2024 Accepted: 27 October 2024
Published online: 05 November 2024

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