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Enhancement of Sophorolipid Production of *Wickerhamiella domercqiae* var. *sophorolipid* CGMCC 1576 by Low-Energy Ion Beam Implantation

Hui Li · Xiaojing Ma · Lingjian Shao · Jing Shen · Xin Song

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Abstract To meet the increasing demands of sophorolipids as biosurfactants and bioactive compounds, it is necessary to obtain higher and more specific sophorolipid-producing strains. One sophorolipid-producing strain, *Wickerhamiella domercqiae* var. *sophorolipid* CGMCC 1576 (Y_{2A}), was mutated by low-energy nitrogen ion beam implantation. Eighteen mutants produced 20 % more sophorolipids than the wild strain, and one mutant, N3-18, produced the highest yield of sophorolipids, 104 g/l, in a shaking flask, which increased by 84.71 % than the wild strain, and further elevated to 135 g/l in a 5-l bioreactor. High performance liquid chromatography analysis showed that the composition of every sophorolipid mixture from different strains was similar, while the contents of most components from mutants were higher than that from the wild strain. Two mutants, N1-32 and N3-18, produced more acidic sophorolipid components; three lactonic sophorolipid molecules with good anticancer activities were greatly enhanced in several mutants, especially monoacetylated lactonic sophorolipid with a C18 monounsaturated fatty acid, which were enhanced by 153 and 211 % in strains N1-32 and N3-18. Low-energy nitrogen ion beam implantation was efficient for obtaining a variety of high and specific sophorolipid-producing mutants to be applied in food, cosmetic, environmental, and pharmaceutical sectors.

Keywords Sophorolipids · Nitrogen ion beam implantation · Mutation breeding · Composition of sophorolipids

H. Li · X. Ma · L. Shao · J. Shen · X. Song (✉)
State Key Laboratory of Microbial Technology, Shandong University, Shan Da Nan Road 27, Jinan, Shandong, China
e-mail: songx@sdu.edu.cn

H. Li
e-mail: sdulihui@gmail.com

X. Ma
e-mail: maxiaojing1011@hotmail.com

L. Shao
e-mail: shaolj1982@163.com

J. Shen
e-mail: shenjing1997@gmail.com

X. Song
National Glycoengineering Research Centre, Shandong University, Shan Da Nan Road 27, Jinan, Shandong, China

Introduction

Sophorolipids (SLs) are important biosurfactants synthesized by a selected number of non-pathogenic yeast species. They are amphiphilic molecules composed of a hydrophobic hydroxyl-fatty acid tail and a hydrophilic carbohydrate head, sophorose [1]. Natural sophorolipids produced by yeast cultivation are a mixture containing up to 20 sophorolipid molecules and they can be classified as lactonic and acidic types based on lactonization or not [2, 3]. Due to their biodegradability, low toxicity, good emulsification ability, and tolerance to temperature and pH variations, sophorolipids can be widely applied as emulsifiers in food [4], cosmetics [5], cleaning [6], and environmental industries [7, 8]. Besides their surface activities, recently, sophorolipids were much reported to have good biological activities, such as antimicrobial [9, 10] and anticancer activities [11–14] and the activity in improving sepsis survival [15], which will lead to their new applications in the pharmaceutical industry as high-value medicines. However, low sophorolipid yield of some strains rendered its industrial application, such as *Rhodotorula bogoriensis* [16, 17] which synthesized unique sophorolipid molecule. To meet the demands of extensive application, the cost of sophorolipid production needs to be greatly decreased, and finding an efficient mutation method to enhance sophorolipid yield is therefore becoming really necessary.

Furthermore, lactonic sophorolipids generally exhibit better biological activities [14], and acidic sophorolipids have better foam formation capacity and solubility [18]. Therefore, the strains that can produce more lactonic sophorolipids or more acidic sophorolipids have their respective importance and can be applied in different industrial sectors.

Low-energy ion implantation is an emerging mutagenesis technique which was first reported to induce mutagenesis in rice [19], and then was applied in the mutation breeding of microorganisms in 1992. Since then, more than 30 strains that produced various products including organic acids [20], enzymes [21, 22], vitamins [23], antibiotics [24] and amino acids [25] have been successfully mutated to obtain mutants of better production abilities by the ion implantation method. For example, the cyclodextrin glucanotransferase-producing strain *Bacillus alcalophilus* was treated with nitrogen ion beam implantation. After several rounds of mutation, a mutant was obtained and its enzyme activity was above 6,000 U/ml, which was 2-fold higher than that of the wild strain [22]. Low-energy ion implantation was also used to mutation breeding of the strain *Bacillus* sp. S65 which produced chitosanase and one mutant, s65F5, was isolated. The production of chitosanase in s65F5 reached 25 U/ml while the production of chitosanase in S65 was only 4.1 U/ml [21]. However, little information on the breeding of high sophorolipid-producing strains by low-energy ion implantation was available.

A strain of *Wickerhamiella domercqiae* var. *sophorolipid* CGMCC 1576 (Y_{2A}), which is capable of producing sophorolipids, was isolated from an oil-containing wastewater sample by our lab and preserved in China General Microbiological Culture Collection Center (CGMCC). In the present research, the strain Y_{2A} was mutated by low-energy nitrogen ion beam implantation method to obtain some mutants that have improved or different abilities for sophorolipid production.

Materials and Methods

Microorganism and Media

The yeast strain, *W. domercqiae* var. *sophorolipid* CGMCC 1576 (Y_{2A}) was isolated from an oil-containing wastewater sample by our laboratory and preserved in CGMCC. YEPD medium contained (grams per liter): glucose 20.0, peptone 20.0, and yeast extract 10.0.

The solid medium was supplemented with 2.0 % agar. The cultivation medium consisted of (grams per liter) glucose 80.0, rapeseed oil 60.0 (*v*/*v*), yeast extract 3.0, KH_2PO_4 1.0, $Na_2HPO_4 \cdot 12H_2O$ 1.0, and $MgSO_4 \cdot 7H_2O$ 0.5.

Ion Implantation Equipment

The nitrogen ion implantation sources were produced by an ion beam implanting instrument IBBe device designed by ASIPP (Chinese Academy of Science, Institute of Plasma Physics, Hefei City, Anhui Province, China).

Effect of the Addition of Glycerol on Cell Survival

The cells on the YEPD slant media were activated at 30 °C by transferring to 50 ml YEPD liquid media in a 300-ml flask. The activated cells were suspended and diluted to 10^7 cells/ml with normal saline containing different concentrations of glycerol. A 100-μl cell suspension was spread in a sterilized empty petri dish to form a single-cell layer and desiccated by sterile air of clean bench.

The dried cells were washed out from the plates with sterilized normal saline. The cell suspension was diluted, spread onto three YEPD agar plates, and incubated at 30 °C for 72 h. The control group was the same as the experimental groups except that the cells were washed out immediately after they were spread as a single-cell layer in a sterilized empty petri dish. The colonies on different plates were counted and an optimal concentration of glycerol was chosen.

Low-Energy Ion Implantation

The single-cell film was prepared by the above procedure. The dishes spread with the single-cell film were implanted by nitrogen ions with the energy of 15 keV and different doses ranging from 1.5×10^{15} to 5.25×10^{15} ions/cm^2. The pulse implantation technique was used and the parameters were as follows: a pulse time of 5 s and an interval time of 30 s; each pulse contained 30 U and the dose of each unit was 2.5×10^{13} ions/cm^2; the beam current was 400 μA and the operating pressure in the target chamber was about 10^{-2} Pa. Every two plates as one group (one experimental and one control) was placed in the target chamber and the experimental one was exposed to nitrogen ion implantation while the control one was unexposed to avoid the vacuum effect on cell survival. The cells in every plate, including the experimental and control plates, were washed out and diluted with sterilized normal saline and then spread onto three YEPD agar plates and incubated for 72 h at 30 °C. The numbers of colonies were counted. The survival of the strain was calculated.

Screening of High Sophorolipid-Producing Mutants

The colonies on YEPD agar plates were randomly selected. The selected strains were inoculated to YEPD agar slant. One loopful of inoculum from the slant was transferred to a 300-ml flask containing 50 ml cultivation medium and incubated for 168 h at 200 rpm, 30 °C, and then the sophorolipid production was determined.

Determination of Sophorolipid Production

To determine total sophorolipid content, 35 ml of ethanol was added to a 5-ml cultivation broth to dissolve both acidic and lactonic sophorolipids, and then after centrifugation at

10,000 rpm for 30 min, the supernatant was distilled to remove ethanol via vacuumed rotary evaporation at 50 °C, and then the viscous remnant was washed with hexane to remove residual oil and lyophilized. Finally, the dry powder was weighed. The lactonic sophorolipids were extracted by ethyl acetate, and then the organic phase was distilled via vacuumed evaporation to remove ethyl acetate, and following the removal of residual oil and lyophilization, the obtained lactonic sophorolipid dry powder was weighed.

$$\text{The content of acidic sopholipids} = \text{total sopholipids} - \text{lactonic sophorolipids}$$

Stability of Sophorolipid Production of High Sophorolipid-Producing Strains

The high sophorolipid-producing strains were passaged five times and then were cultivated for the production of sophorolipids.

Sophorolipid Compositional Analysis

Two milliliters of high performance liquid chromatography (HPLC) grade acetonitrile was added to 1 ml cultivation broth and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected after the mixture was dried at 50 °C. The total sophorolipids were obtained and redissolved in HPLC grade acetonitrile. The components of total sophorolipids were analyzed by HPLC with a 4.6-μm column of Venusil MP-C18 (250 mm×4.6 mm, Agela Technologies Inc., USA). Acetonitrile/water was used as the mobile phase by an acetonitrile gradient from 40 to 90 % in 40 min and at a flow rate of 1.0 ml/min. The injection volume was 20 μl and the eluent was monitored with a UV detector at 207 nm.

Production of Sophorolipids in a 5-l Bioreactor

The mutant strains that produced high sophorolipid concentrations were cultivated in a 5-l bioreactor containing 3-l cultivation medium at 30 °C, 500 rpm with an aeration rate of 0.8 vvm. Sixty milliliter per liter of rapeseed oil was fed at 120 h. pH was determined by a pH meter. Biomass was weighed by the following process: 5 ml cultivation broth and 5 ml *n*-butanol–ethanol–chloroform organic solvent (*v*/*v*, 10:10:1) were mixed and then centrifuged at 6,000 rpm for 10 min; the pellets were washed twice by distilled water and dried at 50 °C to a constant weight. The content of the sophorolipids was analyzed as described above.

Results

Effect of the Addition of Glycerol in Normal Saline on the Survival of the Strain Y_{2A}

Glycerol was chosen as a protective agent in this study. Glycerol of different concentrations (0, 5, and 10 %) was added to normal saline. The effect of glycerol on the survival of Y_{2A} was shown in Table 1. The addition of glycerol could improve survival of strain Y_{2A}. When the concentration of glycerol was 5 %, the survival of strain Y_{2A} was 51.56 % while the survival was 6.22 and 42.22 % when the concentration of glycerol was 0 and 10 %, respectively. The concentration of glycerol was selected as 5 %.

Table 1 Effect of the addition of glycerol in normal saline on the survival of strain Y_{2A}

Drying time (min)	Concentration of glycerol in normal saline (%)	Average colony number	Survival (%)
0	0	225	100
20	0	14	6.22
20	5	116	51.56
20	10	85	42.22

Survival Curve and Selection of Mutation Dose

In the present study, nitrogen ions were used as the ion sources, the energy was 15 keV, and doses ranged from 1.5×10^{15} to 5.25×10^{15} ions/cm^2. The relationship between the survival and the dose of nitrogen ions is shown in Fig. 1. The shape of the survival curve showed a "saddle" shape, that is, with the increase of the dose of nitrogen ion energy, the survival presented a fall–rise–fall pattern. When the doses were elevated from 0 to 2.25×10^{15} ions/cm^2, the survival of strain Y_{2A} was decreased greatly from 100 to 0.8 %; when the doses were increased from 2.25×10^{15} to 3.00×10^{15} ions/cm^2, the survival increased from 0.8 to 3.58 %; with doses from 3.00×10^{15} to 5.25×10^{15} ions/cm^2, the survival decreased from 3.58 to 0.02 %. According to the survival curve of Y_{2A}, the mutation dose was selected as 3×10^{15} ions/cm^2.

Screening of High Sophorolipid-Producing Strains and their Heredity Stability

About 114 strains were selected out from YEPD agar plates after the wild strain had been treated by nitrogen ion implantation. The sophorolipid production from 18 strains increased by more than 20 % compared with that of the wild strain (Fig. 2a). Especially the sophorolipid production of strain N3-18 reached 104 g/l at 200 rpm, 30 °C in a shaking flask, which increased 84.71 % than that of the wild strain.

The lactonic sophorolipids from 18 strains increased by 100 % more than that of the wild strain. Strain N1-14 produced 1.48-fold lactonic sophorolipids than that of the parent strain. For the production of acidic sophorolipids, five strains that produced acidic sophorolipids 30 % more than

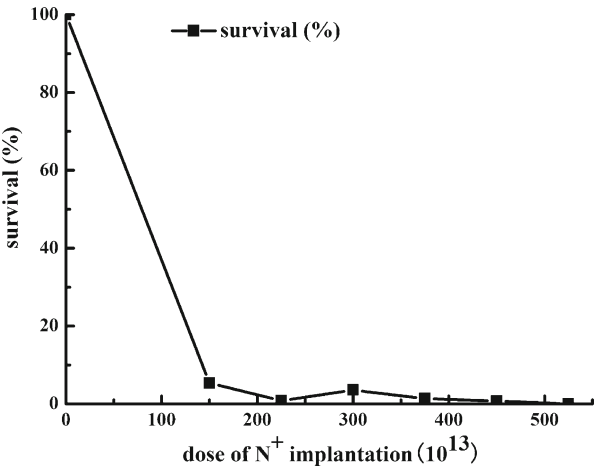

Fig. 1 The survival curve of *W. domercqiae* after nitrogen ion beam implantation treatment

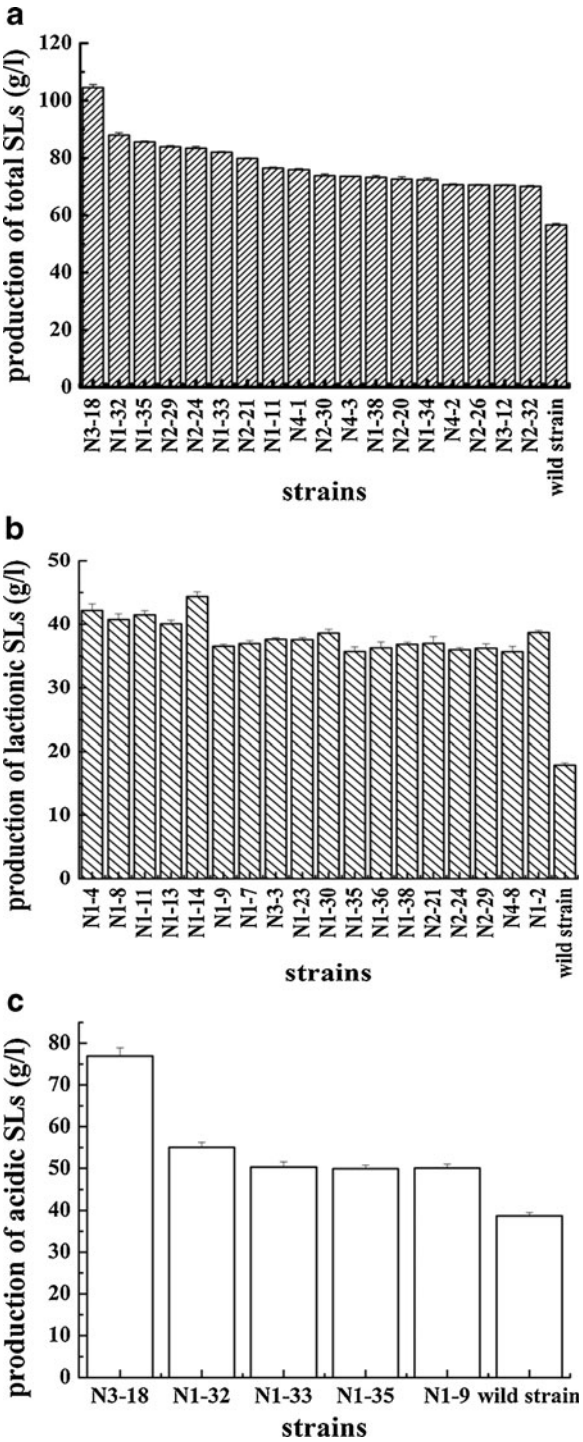

Fig. 2 The sophorolipid production of screened strains: **a** production of total sophorolipids, **b** production of lactonic sophorolipids, **c** production of acidic sophorolipids

Table 2 The sophorolipid production of different mutants before and after passage

Strains	Total sophorolipids (g/l)		Lactonic sophorolipids (g/l)		Acidic sophorolipids (g/l)	
	Pre-passage	Post-passage	Pre-passage	Post-passage	Pre-passage	Post-passage
N3-18	104.49	101.39	27.56	33.06	76.93	68.33
N1-32	88.01	72.39	32.99	33.95	55.02	38.44
N1-30	66.33	79.70	38.61	30.96	27.72	48.74
N1-35	85.61	74.13	35.72	41.52	49.89	32.61
N1-34	72.44	70.04	32.51	32.39	39.93	37.65
N1-38	73.24	93.17	36.86	29.51	36.38	63.66
N1-36	67.80	75.63	36.31	29.18	31.49	46.45
N1-33	82.02	76.85	31.71	30.77	50.31	46.08

that of the wild strain were obtained, and the production of acidic sophorolipids by strain N3-18 was the highest, which increased by 1.98-fold than that of the wild strain (Fig. 2b, c).

Several mutated strains were selected out and passaged five generations, and then the content of sophorolipids was determined after cultivation. As shown in Table 2, the production of lactonic sophorolipids of each mutant was very stable but the production of acidic sophorolipids showed a little fluctuation which resulted in the variation of the total sophorolipid production. But the range of fluctuation of sophorolipid production was limited to 20 %. These results suggested that these mutant strains have stable abilities for producing sophorolipids.

Analysis on Sophorolipid Composition Produced by Different Strains

The compositions of sophorolipids produced by wild and five mutant strains were analyzed by HPLC (Fig. 3, Tables 3 and 4). There were about 20 molecules in the sophorolipid mixture, and the composition of every sophorolipid mixture produced by different strains was similar. However, the composition of peak areas of most components from mutants was greater than that from the wild strain, and the total peak area of the sophorolipids from all mutants was much larger than that of the original strain, which meant that sophorolipid production of all mutants increased a lot. The total peak area of sophorolipids from strain N3-18 was the largest among all strains. According to our previous work [3, 14], the structures of some components have been identified. Peaks 3, 7, 8, 12, and 13 were acidic sophorolipids, and their areas in sophorolipids from mutants, especially from strain N1-32 and strain N3-18, were much higher than that from the wild strain. For peak 3, an unacetylated acidic SL molecule with a C18 diunsaturated fatty acid (C18:2 NASL), the area was 44.22×10^5 and 46.79×10^5 mAU s in sophorolipids from strains N1-32 and N3-18, respectively, which were both much higher than that from the wild strain (9.84×10^5 mAU s). For peak 7, a monoacetylated acidic SL with a C18 diunsaturated fatty acid (C18:2 MASL), the area in sophorolipids from strains N1-32 and N3-18 was increased by 387 and 401 % than that from the wild strain. The structures of peaks 8, 12, and 13 were monoacetylated acidic SL with a C18 monounsaturated fatty acid (C18:1 MASL), diacetylated acidic SL with a C18 diunsaturated fatty acid (C18:2 DASL), and diacetylated acidic SL with a C18 monounsaturated fatty acid (C18:1 DASL), respectively, and their areas in SLs from all mutants were also increased a lot. Besides the increase in production of acidic sophorolipids, the production of three main lactonic SL molecules were also greatly increased. The area of monoacetylated lactonic SL with a C18 monounsaturated fatty acid (peak 17, C18:1

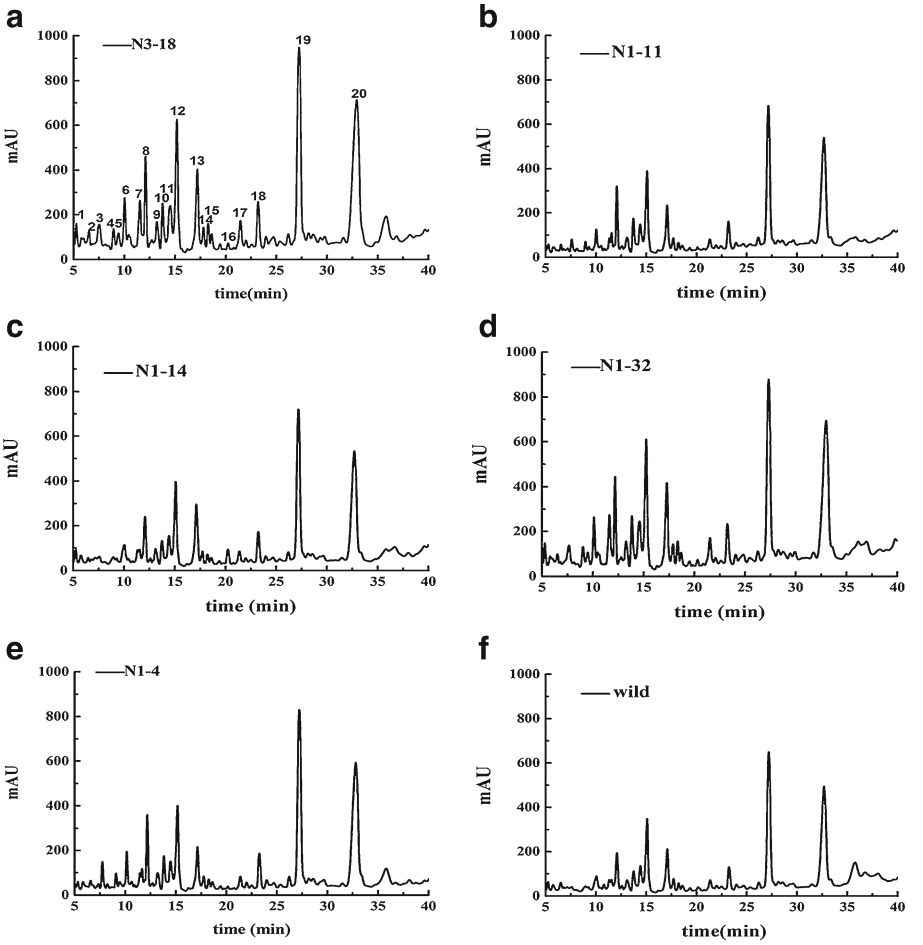

Fig. 3 HPLC analysis of sophorolipid composition produced by *Wickerhamiella domercqiae* wild strain and 5 mutants. (**a**) Sophorolipids from strain N3-18; (**b**) Sophorolipids from strain N1-11; (**c**) Sophorolipids from strain N1-14; (**d**) Sophorolipids from strain N1-32; (**e**) Sophorolipids from strain N1-4; (**f**) Sophorolipids from wild strain

MLSL) was enhanced a little in SLs from strains N1-11, N1-4, and N1-14 but enhanced by 153 and 211 % in SLs from strains N1-32 and N3-18 than that from the wild strain. Diacetylated lactonic SL with a C18 diunsaturated fatty acid (peak 19, C18:2 DLSL) increased by 45.3 %, 65.9 % in N1-32 and diacetylated lactonic SL with a C18 monounsaturated fatty acid (peak 20, C18:1 DLSL) by 71.6 %, and 105.4 % in N3-18, respectively.

Although the composition of the sophorolipids from the wild strain and mutants was consistent, the relative content of each main sophorolipid component from different strains showed great differences. Compared with the wild strain, the area percentages of peaks 3, 5, 9, 10, and 18 for strain N1-11 were enhanced while those of the other several peaks were reduced or unchanged. For strain N1-4, the area percentages of peaks 3, 5, 6, 8, 9, 10, 11, 18, 19, and 20 were increased. Especially the area percentage of the two components (peaks 19 and 20) reached 12.35 and 14.07 while they were 10.65 and 11.05 in the wild strain, and this was quite different from the other strains. For sophorolipids from strain N1-14, the changes

Table 3 The peak area of main sophorolipid component produced by different strains

No.	RT (min)	Area of each SL component ($\times 10^5$ mAU s)					
		Wild	N1-11	N1-4	N1-14	N1-32	N3-18
1	5.24	9.98	9.79	9.74	16.00	21.94	26.42
2	6.5	11.52	7.68	10.30	11.00	18.31	26.37
3	7.58	9.84	12.53	21.19	15.49	44.22	46.79
4	8.97	10.88	9.58	12.04	15.54	20.48	22.58
5	9.25	6.22	8.10	11.43	9.58	19.07	23.97
6	10.05	22.36	21.91	29.95	29.08	40.02	45.41
7	11.33	10.66	11.48	12.77	14.44	50.17	53.41
8	12.09	33.23	38.00	43.23	40.42	54.61	60.53
9	13.09	13.02	17.52	20.02	17.36	27.04	33.55
10	13.75	19.11	27.66	26.13	22.39	38.77	39.70
11	14.43	25.61	27.84	31.34	31.60	54.88	59.34
12	15.09	63.04	68.94	72.58	72.91	106.76	120.78
13	17.08	39.54	41.83	38.69	59.45	77.61	81.59
14	17.71	10.02	11.06	10.35	11.04	17.24	19.91
15	18.22	5.55	5.91	7.14	7.89	16.05	19.52
16	20.15	4.13	3.86	1.93	12.61	4.14	7.78
17	21.36	10.14	11.60	10.92	13.83	25.67	31.58
18	23.23	17.90	23.01	26.48	26.68	32.87	42.42
19	27.17	141.79	153.29	192.61	167.22	206.04	243.35
20	32.67	147.12	165.06	219.37	170.99	244.03	302.18
Area of total SLs		611.67	676.65	808.22	765.50	1,119.95	1,307.18

of its components were different from strain N1-11 because most of them were increased except peaks 2, 10, 12, 14, and 20, and the relative content of peak 16 was increased by 161 % while it was very low in sophorolipids produced by other strains; the ratio of peak 3 and peak 7 to total sophorolipids changed from 0.74 and 0.80 in the original strain to 1.83 and 2.08 in strain N1-32, respectively, and contents of peaks 1, 5, 9, etc. were also enhanced in strain N1-32; there were also some peaks whose concentration increased from strain N3-18 such as peaks 1, 2, and 3. From the above results, we could conclude that the sophorolipids produced by different strains had nearly all the same components but different component contents.

Fed-Batch Cultivation for Sophorolipid Production in a 5-l Bioreactor

Cultivation of sophorolipids in a 5-l bioreactor by strain N3-18 was carried out. The parameters in cultivation were presented in Fig. 4. Cell growth started after a very short lag time and reached a maximum at about 70 h when the biomass was approximately 16.5 g/l. The consumption of glucose began at the early phase of the cultivation and largely supported cell growth and sophorolipid production. pH value was dropped from 5.76 to 2.67 during the cultivation process. Synthesis of sophorolipid started when the yeast cells entered the stationary phase. The production of total sophorolipids was elevated to 135 g/l and the production of sophorolipids in the organic and aqueous phases was 44 and 91 g/l, which were much higher than those of the wild strain.

Table 4 The relative content of different sophorolipid components from different strains

No.	RT (min)	Content of each SL component (%)					
		Wild	N1-11	N1-4	N1-14	N1-32	N3-18
1	5.24	0.75	0.62	0.62	1.03	0.91	0.95
2	6.50	0.87	0.49	0.66	0.70	0.76	0.95
3	7.58	0.74	0.79	1.36	1.00	1.83	1.68
4	8.97	0.82	0.61	0.77	1.00	0.85	0.81
5	9.25	0.47	0.51	0.73	0.62	0.79	0.86
6	10.05	1.68	1.39	1.92	1.87	1.66	1.63
7	11.33	0.80	0.73	0.82	0.93	2.08	1.92
8	12.09	2.50	2.41	2.77	2.60	2.26	2.18
9	13.09	0.98	1.11	1.28	1.12	1.12	1.21
10	13.75	1.44	1.75	1.68	1.44	1.61	1.43
11	14.43	1.92	1.76	2.01	2.03	2.27	2.13
12	15.09	4.74	4.37	4.65	4.69	4.42	4.34
13	17.08	2.97	2.65	2.48	3.82	3.21	2.93
14	17.71	0.75	0.70	0.66	0.71	0.71	0.72
15	18.22	0.42	0.37	0.46	0.51	0.66	0.70
16	20.15	0.31	0.24	0.12	0.81	0.17	0.28
17	21.36	0.76	0.74	0.70	0.89	1.06	1.14
18	23.23	1.34	1.46	1.70	1.72	1.36	1.52
19	27.17	10.65	9.72	12.35	10.75	8.53	8.75
20	32.67	11.05	10.46	14.07	11.00	10.11	10.86

Discussion

Low-energy ion implantation is an emerging mutagenesis technique which was first reported to induce mutagenesis in rice [19], and then was applied in the mutation breeding of microorganisms in 1992. Since then, more than 30 strains that produced various products including organic acids [20], enzymes [21, 22], vitamins [23], antibiotics [24], and amino acids [25] have been successfully mutated to obtain mutants of better production abilities by the ion implantation method. In the present study, this technique was used in the breeding of high sophorolipid-producing strains to decrease the cost of sophorolipid production.

Glycerol was chosen as a protective agent. The survival of cells could rapidly decrease during drying [26], and the rapid evaporation of water inside the cell under vacuum caused the instantaneous decline of the intracellular temperature and the formation of ice crystals in the cell, and this had a negative impact on cell survival. Glycerol could act as a protective agent, and it could permeate through the cell, led to the decline of freezing point, and then prevented the formation of intracellular ice crystals, and at the same time, glycerol resulted in the increase of boiling point, and consequently, intracellular water was more difficult to evaporate [27]. When the concentration of glycerol was 5 %, the survival of the strain was the highest.

The mutagenic effect of ion beam implantation on Y_{2A} was studied. Nitrogen ions were used as the ion sources and the energy was 15 keV and doses ranged from 1.5×10^{15} to 5.25×10^{15} ions/cm^2. The shape of the survival curve showed a characteristic

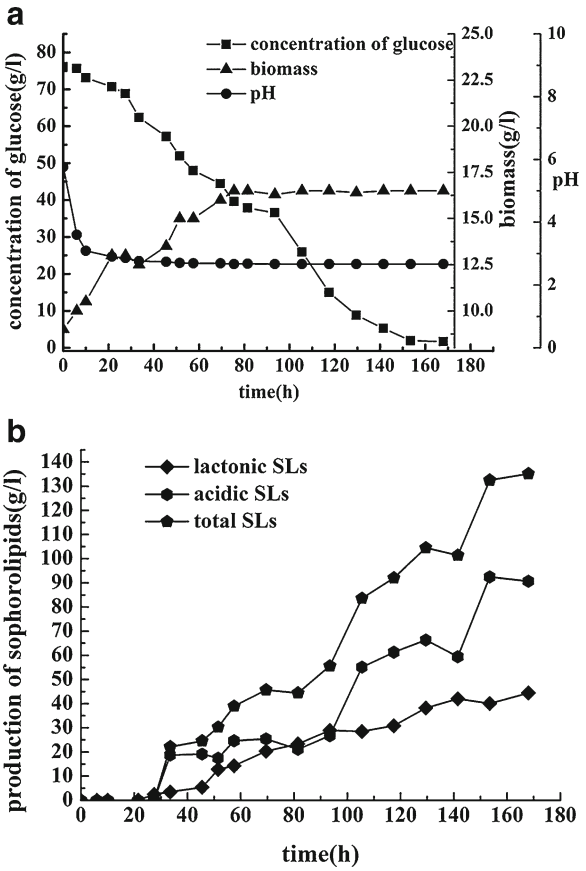

Fig. 4 **a** Sophorolipid production by N3-18 by fed-batch cultivation: time course of cell growth, residual glucose, residual rapeseed oils, and pH changes. **b** Sophorolipid production by N3-18 by fed-batch cultivation: time course of sophorolipid production

"saddle" shape, that is, with the increase of the dose of nitrogen ion energy, the survival presented a fall–rise–fall pattern. Shafique et al. [28] found that when *Alternaria tenuissima* FCBP-252 was exposed to UV light, the percentage of survivors showed a gradual decline with the elongation of UV exposure time. Munakata et al. [29] revealed the exponential relationship between the fractional survivals of five types of *Bacillus subtilis* spores and the amount of exposure (coulomb per kilogram) after irradiation with monochromatic soft X-rays (0.1–0.6 nm) of synchrotron radiation. Significantly, the "saddle" curve was different from the survival curve after UV or X-ray irradiation which reflected the unique mutagenic effect and mechanism of ion beam implantation. The proposed mechanism of ion beam implantation was involved in energy deposition, charge neutralization and exchange, momentum transfer, and mass deposition effect [19, 30, 31]. Low dosage of nitrogen ions only incurred etching damage on the cell surface and no penetration to the cell interior, which resulted in a higher survival. With the increase of dosage, etching damage on the cell surface caused by the momentum transfer effect also increased at this time, and the nitrogen ion energy then deposited in the cells and thus generated massive free radicals which attacked the intracellular biomacromolecules to cause the decrease of survival.

When the dosage continued to increase to a certain value, the collision cascade resulted in vacancies in the genetic substance and some repair mechanisms of the cell such as SOS were activated by part of the vacancies in single-strand DNA breaches and the survival rose temporarily. When the dose further increased, charge deposition changed the electric charge of the cell surface, injured the DNA, and induced gene mutation, and the serious damage caused by energy deposition, momentum transfer, and mass deposition effects could not be repaired by the cells, which would lead to another drop of survival. The positive mutation induced by low-energy ion implantation had a close relationship with implantation dose and was the highest when the survival was in the saddle part [21, 32]. The positive mutation reached 39.61 % after *Streptomyces griseovariabilis* GAAS2507 was mutated with energy of 20 keV and dose of 2.34×10^{15} ions/cm^2 [32]. According to the survival curve of Y_{2A}, the mutation dose was selected as 3×10^{15} ions/cm^2.

After mutation of Y_{2A}, several mutants with higher sophorolipid production were obtained. Especially one mutant, N3-18, produced the highest yield of sophorolipids, 104 g/l in a 300-ml shaking flask, which increased by 84.71 % than the wild strain, and elevated to 135 g/l in a 5-l bioreactor. The theoretical pathway of the formation of sophorolipids involved many enzymes such as cytochrome P450 monooxygenases [33], UDP-glucosyltransferase [34, 35], and acetyltransferase [36]. The enhancement of the activity of each enzyme might lead to the increase in sophorolipid production. So the high sophorolipid-producing strains mutated with low-energy ion beam implantation might have different mutations in genome. However, because the genetic background of the strain was not clear, the specific mutation could not be identified now.

Analysis results on the composition of sophorolipids and the relative content of each component produced by wild and mutant strains using HPLC showed that the composition of every sophorolipid produced by different strains was similar. However, the contents of most components from mutants were higher than that from the wild strain. Especially two mutant strains, N1-32 and N3-18, which produced more acidic sophorolipid components were obtained. Since these acidic sophorolipid components have good emulsification ability, their production enhancement from mutants could be beneficial to their applications in food, cosmetic, cleaning, and environmental industries. Three lactonic sophorolipid molecules with good anticancer activities were enhanced a lot in several mutants: C18:1 MLSL was enhanced by 153 and 211 % in SLs from strains N1-32 and N3-18 than that from the wild strain, C18:2 DLSL and C18:1 DLSL were increased by 45. and 65.9 % in N1-32 and 71.6 %, 105.4 % in N3-18, respectively. The diacetylated lactonic SL with a C18 monounsaturated fatty acid (peak 19) had a strong cytotoxic effect on four human cancer cell lines such as H7402 (human hepatoma cell line), HL60 (human acute promyelocytic leukemia cell line), A549 (human lung adenocarcinoma cell line), and K562 (human chronic myelogenous leukemia cell line) [11]. The three components could inhibit the proliferation of human esophageal cancer cell KYSE 109 and KYSE 450 [14]. The increase of production of these lactonic sophorolipids with good anticancer activities from mutants was of great importance for their applications in the pharmaceutical sector. The relative content of each main sophorolipid component from different strains showed great differences and this also showed the specificity of each mutant in sophorolipid production. In conclusion, nitrogen ion beam implantation was demonstrated as an efficient and potential technique in the mutation breeding of sophorolipid-producing strains, which will expand the application of this technique.

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