Appl Biochem Biotechnol
DOI 10.1007/s12010-016-2176-7

Enhanced Butanol Production Through Adding Organic Acids and Neutral Red by Newly Isolated Butanol-Tolerant Bacteria

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Received: 25 April 2016 / Accepted: 20 June 2016

Abstract As alternative microorganisms for butanol production with high butanol tolerant and productivity are in high demand, one excellent butanol-tolerant bacterium, S10, was isolated and identified as *Clostridium acetobutylicum* S10. In order to enhance the performance of butanol production, organic acids and neutral red were added during butanol fermentation. Synergistic effects were exhibited in the combinations of organic acids and neutral red to promote butanol production. Consequently, the optimal concentrations of combined acetate, butyrate, and neutral red were determined at sodium acetate 1.61 g/L, sodium butyrate 1.88 g/L, and neutral red 0.79 g/L, respectively, with the butanol yield of 6.09 g/L which was 20.89 % higher than that in control. These results indicated that combination of adding organic acid and neutral red is a potential effective measure to improve butanol production.

Keyword Butanol · Tolerance · *Clostridium acetobutylicum* · Organic acid · Neutral red

Electronic supplementary material The online version of this article (doi:10.1007/s12010-016-2176-7) contains supplementary material, which is available to authorized users.

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Published online: 28 June 2016

Introduction

Shortage of fossil fuel reserves and the increasing environmental problems, such as global warming and climate change, attributed to the use of fossil fuels, has brought more and more attention to the production of biofuels [1]. Biobutanol is a promising alternative to fossil fuels because of its high calorific value, low hydrophilicity and corrosivity, and capability of mixing with gasoline in any ratio compared with traditional biofuel ethanol [2]. Butanol-producing clostridia have received the most concern due to its butanol-producing pathway with high maneuverability and wide variety of substrates (e.g., maize, starch, molasses) [3]. However, an economically feasible biobutanol production process is still a challenge because of the low capability of butanol production [4] and the shortage of butanol-tolerant strains [5].

Low butanol percentage in total solvent is one of the main limitations for butanol production [6], because clostridia synthesize n-butanol along with acetone and ethanol in the ratio 6:3:1 as byproducts [7]. Driving carbon and electron flows toward butanol production are effective measures to enhance butanol production [8]. Some organic acids, such as acetate [9] and butyrate [10], not only are products of butanol-producing clostridia but can also be reutilized to synthesize butanol, which gives them great potential to drive carbon flow to butanol production [11]. In addition, electrons are also necessary in butanol production [12]. Previous reports have demonstrated that the addition of artificial electron carriers including methyl viologen [13] and neutral red [14] could significantly drive electron flow toward butanol, which made butanol percentage of the total solvent exceed 90 %, and acetone almost disappeared in the final solvent composition. Nevertheless, most of these researches have aimed at adding single organic acid or electron carrier to improve biobutanol production, but no information is available on the feasibility of using combined organic acids and electron carrier for metabolic regulation to stimulate butanol production [8].

Therefore, a butanol-producing strain which could grow in high butanol concentration was isolated in this work. In order to improve butanol yield, the possible additive or synergistic interaction of organic acids and neutral red on fermentative butanol production was determined.

Materials and Methods

Isolation of Butanol-Producing Bacteria

Environmental sample was collected from 2 m deep underground in the cornfield of Harbin suburb, Heilongjiang province of China. Briefly, 1 g of environmental sample was diluted in 100-mL glass bottles with 10-mL sterilized water and treated by heat shock at 100 °C for 10 s [15] to enhance the vitality of the spores. The supernatant was transferred at 10 % (*v*/*v*) to 6.5 % sterilized corn mash medium for enrichment, which was carried out anaerobically at 37 °C in 100-mL glass bottles with working volume of 50 mL for 5 days without shaking. A Hungate roll-tube [16] was used for strain purification after enrichment culture for three times. All the bottles and tubes were firstly filled up with high purity nitrogen for the anaerobic condition and sealed, and then media boiled for 20 min were injected. Resazurin with a work concentration of 1 mg/L was used for the anaerobic indicator. Suspensions from the enrichment culture were plated on agar medium (1.5 %, *w*/*v*, agar) containing (per liter) 20 g glucose, 2.5 g KH_2PO_4, 3.0 g Na_2HPO_4, 1.5 g NH_4Cl, 0.4 g $MgSO_4{\cdot}7H_2O$, 3.0 g yeast extract, l mL vitamin solution,

1 mL trace element solution [17], and 0.5 g cysteine, of which pH value was adjusted to 6.5 with 1 M NaOH and 1 M HCl. Vitamin solution contained (per liter) 0.025 g lipoic acid, 0.1 g biotin, 1.75 g niacin , 0.1 g folic acid , 0.025 g thiamine hydrochloride, 0.25 g para aminobenzoic acid, 0.25 g pantothenic acid , 0.005 g riboflavin, and 0.5 g pyridoxine hydrochloride. Trace element solution contained (per liter) 7.5 g $FeSO_4$, 0.35 g $ZnCl_2$, 0.03 g H_3PO_3, 0.5 g $MnCl_2{\cdot}4H_2O$, 0.025 g $CuSO_4{\cdot}5H_2O$, 1.0 g $CoCl_2{\cdot}6H_2O$, 0.25 g $NiCl_2{\cdot}4H_2O$, 0.18 g $Na_2MoO_4{\cdot}H_2O$, 0.075 g $Na_2WO_4{\cdot}2H_2O$, and 0.075 g $Na_2SeO_4{\cdot}5H_2O$. Bacterial colonies were transferred into liquid medium. Repeated plating was done several times until pure cultures were obtained. Butanol (8.0–16.0 g/L) was added into purified cultures to select butanol-tolerant strains. The strain *Clostridium acetobutylicum* ATCC824, purchased from China General Micro-biological Culture Collection Centre (CGMCC), was used as a control.

Strain Identification

16S rDNA technology was used for molecular identification. Bacterial DNA was extracted by using the bacterial DNA kit (Yuanpinghao, Tianjin, China) according to manufacturer instructions. The 16Sr DNA was amplified by PCR technique with a pair of universal primers [18], 27F (AGAGTTTGATCCTGGCTCAG) and 1541R (AAGGAGGTGATCCAGCCGCA). The PCR products were purified by a gel extraction kit (TransGen, Beijing, China) and connected with vector pMD18-T (Takara, Dalian, China). The obtained 16S rDNA sequence was compared to known sequences in the GenBank database by using all nucleotide sequence BLAST. Neighbor-joining tree was constructed in the MEGA 5.05 program with pairwise-deletion option and p-distance model and evaluated by bootstrap resampling with 1000 replicates [19]. The physiological and biochemical characteristics of the isolates were tested according to Bergey's Manual of Determinative Bacteriology [20].

Batch Fermentation Experiments

Isolated strains were cultured in the media containing the following (per liter): 65 g glucose, 2.5 g KH_2PO_4, 3.0 g Na_2HPO_4, 1.5 g NH_4Cl, 0.4 g $MgSO_4$, 3.0 g yeast extract, l mL vitamin solution, 1 mL trace element solution, and 0.5 g cysteine, of which pH value was adjusted to 6.5. The inoculum obtained after 48 h of cultivation was added at 5 % (*v*/*v*). All experiments were carried out in 100-mL glass bottles with 50-mL working volume at 37 °C, 180 rpm/min for 72 h. For the test of evaluation of organic acids and neutral red on butanol production, these substrates were added into the medium separately or combined with each other with the concentration of sodium acetate 1.5 g/L, sodium butyrate 1.5 g/L, and neutral red 0.5 g/L. The groups without any extra additives were used as control. All treatments were performed in triplicate to check data reproducibility.

Optimization of Combined Organic Acids and Neutral Red

Box-Behnken experiments [21] were used to optimize the concentrations of combined sodium acetate (SA, X1), sodium butyrate (SB, X2), and neutral red (NR, X3) with a total of 17 runs and 5 replications. For statistical calculations, the relation between the coded values and real values are described as follows [22]:

$$X_i = (A_i - A_0) \Big/ \Delta A \quad (1)$$

X_i is a coded value of the variable, A_i is the real value of the variable, A_0 is the real value of the center point, and ΔA is the step change of variable. The levels of the variables and the experiment are designed just as shown in Table 1. The butanol yield (BY) was taken as the dependent variable or response of the experiments.

Model (2) was used to determine the relationship between independent variables and response values [23]:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 \quad (2)$$

Y is the predicted response; β_0 is the interception coefficient; β_1, β_2, and β_3 are the linear coefficients; β_{12}, β_{13}, and β_{23} are the interactive coefficients; β_{11}, β_{22}, and β_{33} are the quadratic coefficients; and X_1, X_2, and X_3 are code values of three regulators.

Design-Expert (version 8.0.5.0, Stat-Ease Inc., Minneapolis, USA) software was used for the design and analysis of the data obtained [24].

Analysis Methods

The volatile organic acids and solvent were detected by gas chromatography (4890D, Agilent Cooperation, USA) according to the method described in previous research [24]. Design-Expert (version 8.0.5.0, Stat-Ease Inc., Minneapolis, USA) software was used for the design and analysis of the Box-Behnken experiments. Significant differences were statistically

Table 1 Box-Behnken experiment design and results on metabolic regulation for *C. acetobutylicum* S10

Run	Sodium acetate (g/L)		Sodium butyrate (g/L)		Neutral red (g/L)		Butanol yield (g/L)
	X_1	Code X_1	X_2	Code X_2	X_5	Code X_2	
1	0	−1	1.5	0	0	−1	5.473 ± 0.136
2	1.5	0	3	+1	0	−1	5.456 ± 0.128
3	3	+1	1.5	0	0	−1	5.452 ± 0.315
4	1.5	0	1.5	0	0.5	0	6.083 ± 0.257
5	3	+1	3	+1	0.5	0	5.528 ± 0.240
6	1.5	0	0	−1	1	+1	5.585 ± 0.292
7	0	−1	1.5	0	1	+1	5.757 ± 0.288
8	1.5	0	1.5	0	0.5	0	6.114 ± 0.271
9	0	−1	0	−1	0.5	0	5.503 ± 0.182
10	1.5	0	1.5	0	0.5	0	6.042 ± 0.212
11	1.5	0	1.5	0	0.5	0	6.080 ± 0.206
12	3	+1	1.5	0	1	+1	6.052 ± 0.161
13	1.5	0	3	+1	1	+1	6.056 ± 0.202
14	1.5	0	0	−1	0	−1	5.222 ± 0.212
15	3	+1	0	−1	0.5	0	5.586 ± 0.293
16	0	−1	3	+1	0.5	0	5.836 ± 0.282
17	1.5	0	1.5	0	0.5	0	6.095 ± 0.278

evaluated by using the two-way ANOVA function of the software IBM SPSS Statistics (Version 19.0, International Business Machines Corporation, Armonk, USA).

Results and Discussion

Strain Screening

Eighteen strains isolated from the deep soil of a cornfield were acquired and tested for butanol production capability. Three strains, S2, S10 and N2, had higher butanol yields with acetate, butyrate, acetone, and ethanol as byproducts. As Table 2 shows, strain S10 had the highest butanol yield of 5.040 g/L and butanol tolerance (16 g/L, data not shown), which was even higher than the typical butanol-producing strain *C. acetobutylicum* ATCC824 (4.917 g/L). Although the total acetone-butanol-ethanol (ABE) production of strain S10 (6.617 g/L) was below that of strain ATCC824 (7.546 g/L), the higher butanol yield and butanol content in the total solvent made the strain S10 the final choice for further experiments.

Identification and Characteristics of Strain S10

The physiological and biochemical characteristics of S10 are summarized in Table 3. Consistent with *Clostridium* spp., it was rod-shaped, Gram-positive, strictly anaerobic, spore-forming, and grew in a variety of carbon sources. The 16S rDNA gene sequence (1466 bp) was obtained and deposited at NCBI (accession number KC166235). A phylogenetic tree for strain S10 was constructed based on the similarity analysis of the 16S rDNA analysis (Fig. 1). The closest phylogenetic relative was with *C. acetobutylicum* JCM1419 at a similarity of 99.8 %. This isolate was therefore characterized as *C. acetobutylicum* and named as *C. acetobutylicum* S10.

Effect of Adding Organic Acids and Neutral Red on Butanol Production

Based on the metabolic pathway of solvent-producing *Clostridium*, the butanol was produced from the reutilization of organic acids which are accumulated in the acidogenic phase. Organic acids are therefore considered to have great potential as a substrate for butanol production. In addition, electron carrier like neutral red has been demonstrated that it could alter the electron flan ow and consequently modifies the metabolic flux toward butanol formation in

Table 2 Comparison of fermentation products, yields and butanol tolerance among different strains

Strain	Butanol yield (g/L)	Metabolic byproducts (g/L)				Total ABE production (g/L)	Butanol content in ABE (*w*/w %)
		Acetone	Ethanol	Acetate	Butyrate		
S2	2.080	0.245	1.931	3.041	1.428	4.256	48.87
S10	5.040	1.065	0.512	0.948	1.302	6.617	76.17
N2	1.398	0.058	1.089	2.547	1.591	2.545	54.89
C. acetobutylicum ATCC824	4.917	1.925	0.704	1.213	1.247	7.546	65.16

ABE total productions of acetone, butanol, and ethanol

Table 3 The characteristics of *C. acetobutylicum* reported in Bergey's Manual of Determinative Bacteriology and in this work

Characteristic	Strain S10	*Clostridium acetobutylicum*
Spore	Secondary end	Secondary end
pH	4–8	ND
Temperature	20–40 °C (37 °C Best)	37 °C Best
Gelatin hydrolysis	+	+
Gram stain	+	+
H_2S	+	+
Indole	−	−
Nitrate reduction	−	−
Carbon sources		
Arabinose	+	+
Cellobiose	−	−
Fructose	+	+
Galactose	+	+
Glucose	+	+
Glycerin	−	−
Lactose	+	+
Maltose	+	+
Mannitol	−	d
Salicin	+	+
Starch	+	+
Sucrose	+	+
Xylose	+	+

d weak reaction, *ND* not determined

solventogenic clostridia [25]. In this study, the effects of adding organic acids (acetic acid and butyric acid) and electron carrier (neutral red) separately or in combination on butanol fermentation by the newly isolated strain S10 were investigated. As shown in Fig. 2, sodium acetate, sodium butyrate, and neutral red all had positive effects on butanol production. When sodium acetate, sodium butyrate, and neutral red were added separately, the butanol yield increased 3.15 %, 9.37 %, and 10.04 %, respectively. In view of the pathway of butanol-producing *Clostridium*, butyrate is able to convert to butyryl-CoA as the precursor of butanol [8]. So adding butyrate is conducive to butanol production. Moreover, butyryl-CoA can also be synthetized from acetyl-CoA and one of its substrates is acetate [8], which may lead to the positive effect of acetate on butanol production. Apart from this, the addition of organic acid enhanced the buffering capacity of the medium, resulting in protection against premature growth inhibition due to low pH values. In the butanol fermentation, it was reported that levels of undissociated acids and at certain pH value in the medium correlated with the initiation of butanol formation. The substantial accumulation of organic acids and too low pH value (below 4.5) may harm the cell and lead to the reduction of output [26]. In this study, the final pH value of the medium without any extra additives fell to about 4.10 ± 0.04, which affected the production of the solvent seriously. Although, the alkalinity of sodium salts may also lead to the increase of pH value and the initial addition would result in the delay of the solvent

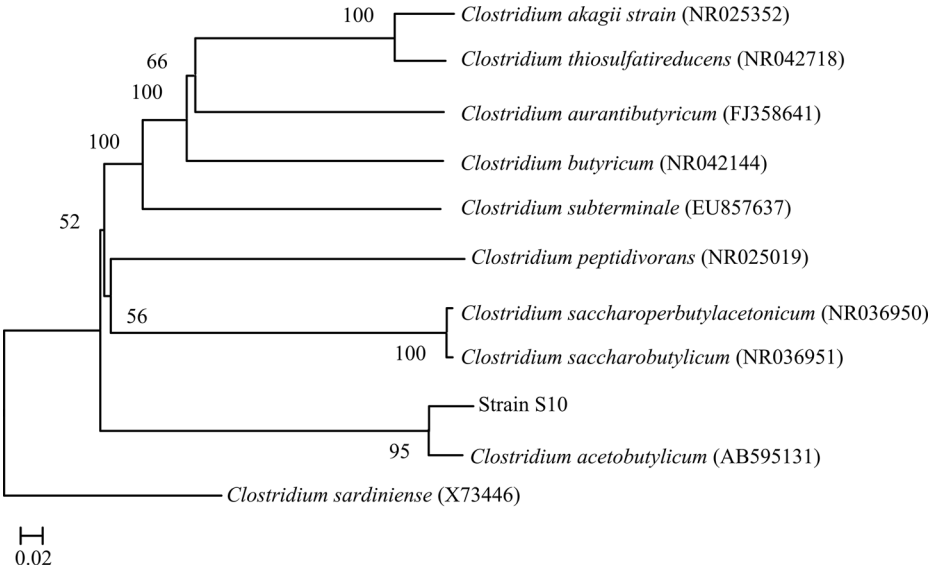

Fig. 1 Phylogenetic tree by neighbor-joining method showing the relationships between strain S10 and relational strains based on 16S rDNA nucleotide blast. The staff means 2 % sequence divergence

production. To avoid these negative effects, the optimal adding time needs to be determined. Based on the growth curve, the time of the 16th hour was the beginning of the stationary growth phase of bacteria (Supplementary Fig. 1). At this time, the cultivation environment started to be unsuitable and adding additives at this moment had the least negative effect on the fermentation. Through comparing the effect of adding additives at the beginning of the fermentation (0 h), the logarithmic phase (7 h), and the beginning of the stationary phase (16 h), the 16th hour was chosen as the final adding time (Supplementary Fig. 2). As Fig. 2 shows, before additives were added, the pH value of the medium lowered to 4.36 ± 0.11, which may damage the cells. But due to the alkalinity of the additives, the pH value increased to about 5.0–5.5, which triggered solventogenesis and resulted in a proper butanol production [27]. However, overmuch sodium salts will also lead to high pH value. When the additive

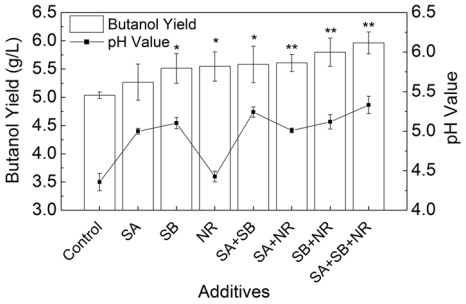

Fig. 2 The effect of extra additives on butanol yields and pH value in different combinations. *SA* represented the sodium acetate, *SB* the sodium butyrate, and *NR* the neutral red. *Asterisks* indicated the difference analysis between the results of test groups and control group (the data of lag phase in Fig. 2a were set as control). *One asterisk* meant difference was significant ($p < 0.05$) and *two* meant difference was very significant ($p < 0.01$)

amounts of sodium acetate and sodium butyrate reached 3.0 g/L, the pH value increased to 5.63 ± 0.12. High pH value may slow down the productivity of butanol and it was necessary for the optimal concentrations of the additives.

On the other hand, the addition of neutral red, a molecule that can partly replace ferredoxin in the oxidoreduction reactions but hardly influenced the pH value (Fig. 2), induced the formation of butanol as a result of altered electron flows [14]. As Fig. 2 shows, higher butanol yields were achieved when organic acids and neutral red were added together. Under the positive effects of the three kinds of additives, the butanol yield reached the maximum value of 5.96 g/L, which was 18.3 % higher than that in the control (without additives). Apparently, synergistic effects were existed in combination of organic acids and neutral red on butanol production. It is therefore speculated that using combination of organic acids and neutral red might be an effective solution to improve butanol production.

Optimization of Combining Organic Acids and Neutral Red

Box-Behnken experiments were applied for optimal concentrations of combined organic acids and neutral red. Based on experimental data (Table 1), one equation was established to describe the relationship between the three variables and the butanol yield in terms of decoded values (Eq. 3):

$$Y = 6.08 + 6.05\mathrm{E}{-3}X_1 + 0.12X_2 + 0.23X_3 - 0.098X_1X_2 + 0.079X_1X_3 + 0.059X_2X_3 - 0.18X_1^2 - 0.29X_2^2 - 0.22X_3^2 \quad (3)$$

Y was the predicted response of butanol yield and X_1, X_2, and X_3 were the coded values of concentrations of sodium acetate, sodium butyrate, and neutral red.

According to Eq. 3, the linear coefficients of X_1, X_2, and X_3 were of positive value, indicating positive effects of these parameters on butanol yield. In addition, the values of coefficients of X_2 and X_3 were far larger than that of X_1, which suggested that sodium butyrate and neutral red had greater effects butanol yield than sodium acetate did. Fisher's statistical test for analysis of variance (ANOVA) in Table 4 also supported this consequence. The linear coefficients of X_2 and X_3 were all significant at the 5 % significance level while that of X_1 was not ($p > 0.05$). Therefore, the linear effects of sodium butyrate (X_2) and neutral red (X_3) were the most influential factors. In addition, the quadratic coefficients of X_1, X_2, and X_3 were below zero, indicating negative effects of these parameters on butanol yield. At last, all of the other coefficients had slight effects on butanol yield, as indicated by the large P values. Based on the ANOVA of this model in Table 4, the value of Prob > F′ less than 0.05 was considered to be significant. Moreover, the model did not present lack-of-fit (Prob > F less than 0.05) and had a high determination coefficient ($R^2 = 0.960$). Therefore, Eq. (3) could describe the results in this test properly.

Figure 3 showed the response surfaces and corresponding contour plots with butanol yield as the response. These figures demonstrated the relative effects of two variables on the butanol yield with the third kept constant. In Fig. 3a, butanol yield generally increased to a peak with the increase in the concentration of sodium acetate and sodium butyrate, and then decreased with the further increase of sodium acetate and sodium butyrate. Figure 3b, c showed the similar tendency to Fig.3a. Nevertheless, when neutral red was added at a low concentration, the auxo action of the organic acids with increasing concentrations on butanol production was not significant, especially for sodium acetate, while with the increase of neutral red (above

Table 4 ANOVA for response surface quadratic model on metabolic regulation producing butanol by *C. acetobutylicum* S10

Source	Sum of squares	*df*	Mean square	F-Value	*p* value Prob > F
Model	1.38	9	0.15	18.64	0.0004
X_1	0.00	1	0.00	0.04	0.8559
X_2	0.12	1	0.12	14.50	0.0066
X_3	0.43	1	0.43	51.69	0.0002
X_1X_2	0.04	1	0.04	4.64	0.0682
X_1X_3	0.02	1	0.02	3.02	0.1261
X_2X_3	0.01	1	0.01	1.70	0.2332
X_1^2	0.14	1	0.14	17.04	0.0044
X_2^2	0.35	1	0.35	41.93	0.0003
X_3^2	0.20	1	0.20	23.91	0.0018
Residual	0.06	7	0.01		
lack of fit	0.05	3	0.02	26.30	0.0043
Cor. Total	1.44	16			

Coefficient of determination (R^2) of $Y = 0.960$

0.4 g/L), the positive effects were displayed sharply along with the increase of acetate and butyrate until they exceeded the optimal value. Each two-dimensional contour plot (Fig. 3) showed a symmetrical mound shape with an axis of symmetry parallel to the diagonal, indicating the great interactive effect between every two additives. Based on Eq. (3), the optimal coded values of X_1, X_2, and X_3 were confirmed to be 0.073, 0.253, and 0.570, respectively. Correspondingly, it can be obtained that the optimal concentrations of these three variables at maximum point of the model were 1.61 g/L sodium acetate, 1.88 g/L sodium butyrate, and 0.79 g/L neutral red, respectively, by using Eq. (1). The maximum predicted value of butanol yield was 6.161 g/L.

Under above optimal concentrations, the solvent yields of S10 were 6.093 g/L butanol, 0.635 g/L ethanol, and 0.038 g/L acetone. The value of butanol yield had excellent correlation with the predicted value from the model by using significant difference analysis ($p < 0.05$), which confirmed the validity of the model and the existence of the optimal point [28].

The total ABE yields after optimization was 6.766 g/L, and this data had no significant change compared with that before optimization (6.617 g/L). But, the butanol yield was 20.89 % higher than the control (5.040 g/L) and the butanol percentage of total solvent reached 90.05 %, which much more exceeded the value in traditional butanol fermentation (60 %) [7]. The above results demonstrated that although the combination of organic acids and neutral red failed to promote the ABE production effectively, it changed the composition proportion of the total solvents. Through changing the carbon flows (acetic acid and butyric acid) [8] and electron flows (neutral red) [14], more source and energy were used for the synthesis of butanol. However, overmuch organic acids or salt will change the living environment of bacteria [26]. On the other hand, overmuch electron carrier may also promote the output of ethanol, leading to the decrease of source and energy used for butanol production [25]. By the model in this study, the optimal concentrations of additives were obtained. Under the optimal conditions, higher butanol yield and percentage of total solvent were achieved, which was beneficial to lower the cost on production and separation of butanol from

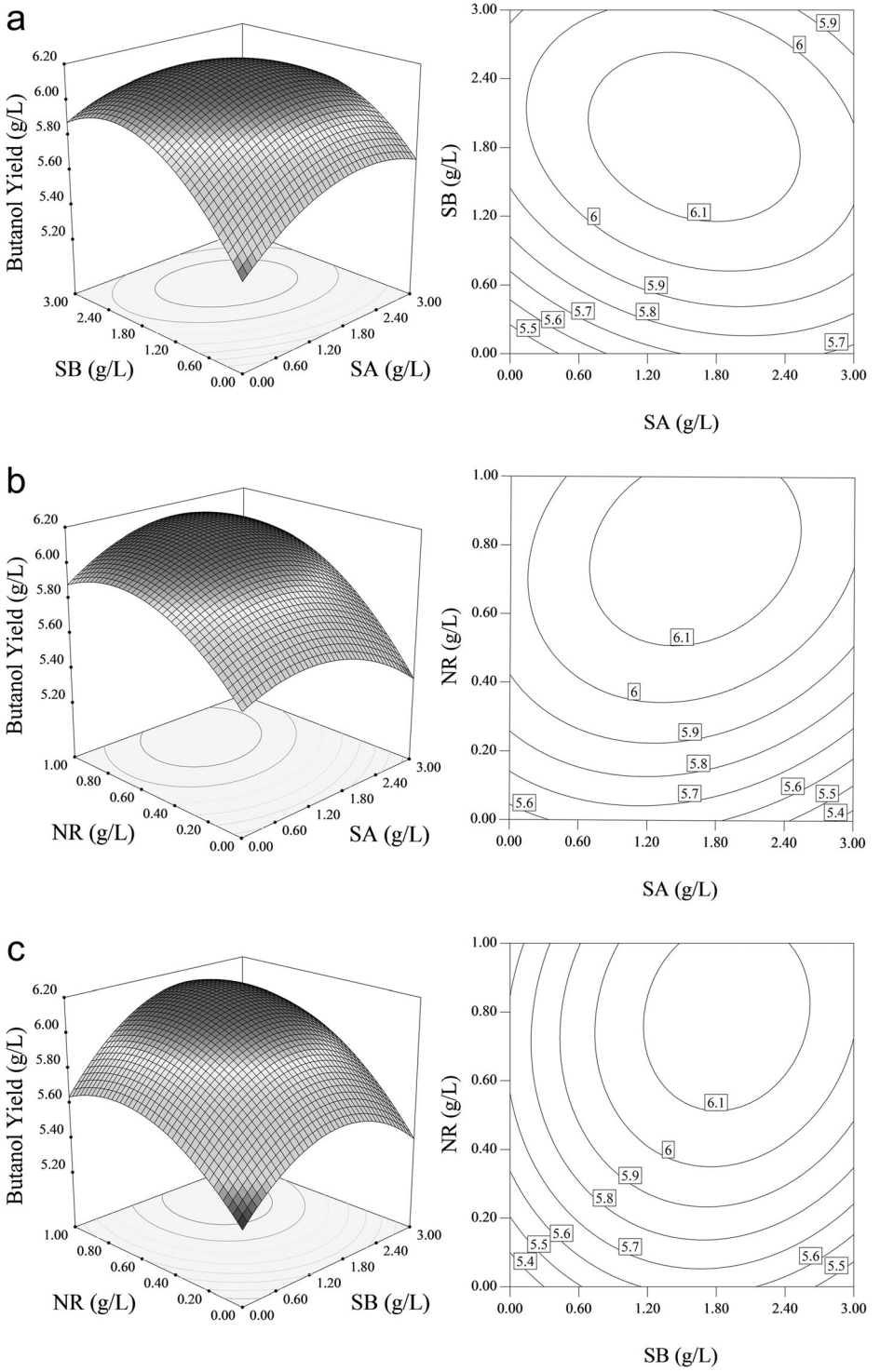
a
Butanol Yield (g/L)
6.20
6.00
5.80
5.60
5.40
5.20
SB (g/L)
SA (g/L)
0.00
0.60
1.20
1.80
2.40
3.00
SB (g/L)
SA (g/L)
5.9
6
6.1
5.8
5.7
5.6
5.5
b
Butanol Yield (g/L)
NR (g/L)
SA (g/L)
1.00
0.80
0.60
0.40
0.20
0.00
6.1
6
5.9
5.8
5.7
5.6
5.5
5.4
c
Butanol Yield (g/L)
NR (g/L)
SB (g/L)
6.1
6
5.9
5.8
5.7
5.6
5.5
5.4

Fig. 3 Response surface plots and corresponding contour plots showing the effects of every two additives on butanol yields by *C. acetobutylicum* S10. **a** Effects of sodium acetate (*SA*) and sodium butyrate (*SB*) under neutral red fixed at 0.79 g/L. **b** Effects of sodium acetate (*SA*) and neutral red (*NR*) under sodium butyrate fixed at 1.88 g/L. **c** Effects of sodium butyrate (*SB*) and neutral red (*NR*) under sodium acetate fixed at 1.61 g/L

mixed solvent. In a word, the results showed that adding additives with suitable concentrations was an effective method to improve butanol production and the strain S10 had excellent utilization potentiality.

Conclusions

One bacterium strain S10 with high butanol tolerance was isolated and identified as *C. acetobutylicum* S10. Results showed that combination of organic acids and neutral red had the most significant on promoting butanol production. Under the optimal concentrations of additives (sodium acetate 1.61 g/L, sodium butyrate 1.88 g/L, and neutral red 0.79 g/L), the butanol yield increased by 20.89 % in comparison with control with a final concentration of 6.093 g/L.

Acknowledgment This research was supported by the National Science and Technology Ministry (No. 2014BAJ21B02), National High Technology Research and Development Program (2011AA10A205), and the "Twelfth Five-Year Plan" National Science and Technology Program on Rural Area (2014BAL02B00) and Fundamental Research Funds for the Central Universities (No. HIT. KISTP. 201424).

Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest between each other.

Human and Animal Rights This article does not contain any studies involving human participants or animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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