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Adaptive Evolution of *Saccharomyces cerevisiae* in a Continuous and Closed Circulating Fermentation (CCCF) System Coupled with PDMS Membrane Pervaporation

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Abstract As an efficient means of strain improvement, adaptive evolution is a technique with great potential. Long-term cultivation of *Saccharomyces cerevisiae* was performed in a polydimethylsiloxane membrane bioreactor system which was constructed by coupling the fermentation with pervaporation. A parent strain was subjected to three rounds of fermentation–screening–transfer procedure lasting 1,500 h in a continuous and closed circulating fermentation (CCCF) system, and its 600-generation descendant S33 was screened. In shaking flask culture test, the selected strain S33 from the third round showed great superiority over the parent strain in the residual broth medium, with the ethanol yield and specific ethanol productivity increasing by 34.5 and 34.7 %, respectively. In the long-term CCCF test, the fermentation performance of the descendant strain in the third round was higher than that of its parent strain in the second round. These results show the potential of this novel adaptive evolution approach in optimization of yeast strains.

Keywords PDMS membrane bioreactor · *Saccharomyces cerevisiae* · Closed circulating fermentation · Adaptive evolution

Introduction

Because of the gradual depletion of crude oil and the environmental deterioration, it is urgent to develop alternatives that are both renewable and environment friendly. Bioethanol, produced from renewable biomass such as sugar and starch at present and maybe lignocellulose or microalgae in the future, is believed to be one of these alternatives [1–4]. As reported, the fuel ethanol production capacity of China reached 1.94 million tons by 2008, which will be 19–50 million tons by 2020 as predicted [5]. *Saccharomyces cerevisiae*, as a

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biocatalyst of fermentation, plays a significant role in ethanol production [6]. A slight improvement of microbial strains may result in great yield increase and cost reduction.

Currently, numerous studies on strain screening methods have been reported, such as protoplast fusion [7, 8], genetic engineering [9–11], genome shuffling [12–14], adaptive evolution [15, 16], and even combinations of these methods [17–19]. Although the genomics age and the associated genome-wide analytical technologies provide further impetus for rational approaches, genetic engineering of more complex cellular systems or cellular systems that are not fully understood remains a challenge. Besides, the use of recombinant techniques to optimize strains intended for ethanol production is not favored by some consumer groups [20].

Adaptive evolution is an approach that has been widely used in the development of optimized yeast. By this technique, an organism is subjected to serial or continuous cultivation for many generations under conditions to which it is not optimally adapted. In the presence of such an environmental stress, an increase in fitter variants occurs because of natural selection. This method is relatively simple and convenient compared with other techniques, with the advantage of not necessarily requiring genetic modification of strains or precise knowledge of genes especially when the mechanism is not clear [15]. Many investigations have demonstrated the success of evolutionary engineering in improving ethanol production [15, 16, 18, 21].

As known, the high ethanol concentration established within the fermentation system strongly inhibits cell growth and viability, limiting fermentation productivity and ethanol yield [22, 23]. Pervaporation has been extensively combined with fermentation to remove organic compounds [24–27]. Compared with traditional fermentation, the system of continuous fermentation coupled with pervaporation has many advantages: the hydrophobic membrane can remove ethanol in situ and then greatly relieve the ethanol inhibition; the continuous and closed circulating fermentation could increase cell density and result in greatly improved volumetric productivity; the increased volumetric productivity not only reduces capital costs of ferment vessels, but also benefits the environment by reducing the volume of residue; and recovery of a concentrated ethanol vapor requires less distillation capacity and thus energy consumption. O'Brien et al. [28] compared the costs of the fermentation–pervaporation process with a conventional batch fermentation process, and the results indicated that with modest improvement in either pervaporation flux or selectivity, such a system could be cost competitive.

Though the adaptive evolution and continuous fermentation/pervaporation has been widely applied respectively, to our knowledge, no report on screening strains through the continuous fermentation/pervaporation bioreactor system was published. A continuous and closed circulating fermentation (CCCF) of 500 h (21 days) had been achieved in the previous work [29]. It was surprising to find that the cell viability was still 30 % after 500 h fermentation, so superior strains may be obtained from the residual broth by cultivating the isolated strains many times in the prolonged CCCF system. From the perspective of heredity, this special operation mode generates a gradually worse fermentation condition and may affect the evolution of yeast. In this study, we followed the fermentation–screening–transfer procedure three times to acclimate and evolve the *S. cerevisiae*, expecting to obtain a robust mutant strain adapting the CCCF system.

Materials and Methods

Yeast and Medium

The original strain used in the first round of CCCF was thermostable alcohol-active dry yeast (ADY) provided by Angel Yeast Corporation, which was widely used for industrial ethanol

production in China. Prior to being inoculated into the fermentor, ADY was dissolved in water at the temperature of 35–40 °C (1 gL^{-1}) and rehydrated for 15–20 min.

The original strain used in the second round of fermentation was S14, which was isolated from the residual broth of the first round of 500 h CCCF, by the means of the following "strain screening procedure."

The original strain used in the third round of fermentation was S24, which was isolated from the residual broth of the second round of 500 h CCCF, with the same procedure of the previous round of experiment.

In order to test the performance of the parent and isolated strains in different fermentation circumstances, two sorts of medium were used in this study: nutrient medium (NM, liquid and solid) and residue medium (RM, liquid and solid).

Composition of liquid NM (in gram per liter) is as follows: glucose, 100; yeast extract, 8; $(NH_4)_2SO_4$, 5; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.55; and $CaCl_2$, 0.15. The components were dissolved in distilled water. Composition of solid NM (in gram per liter) is as follows: glucose, 10; yeast extract, 5; peptone, 10; and agar, 20. The components were dissolved in distilled water. Liquid RM is composed of: 50 gL^{-1} glucose was dissolved in the 500-h fermentation residual broth. Composition of solid RM (in gram per liter) is as follows: glucose, 10; $(NH_4)_2SO_4$, 5; KH_2PO_4, 1.5; and agar, 20. The components were dissolved in the 500-h fermentation residual broth.

All the media were autoclaved at 121 °C for 20 min. RM was adjusted to pH 6.5 with 1 M NaOH.

Membrane and Module

The pervaporation membrane used in the experiment was a composite polydimethylsiloxane (PDMS) membrane prepared in our lab. The detailed structure of the module and preparation process of the membrane had been fully described in the previous work [30–32].

CCCF System

A schematic diagram of the CCCF system was presented in Fig. 1. The system was mainly comprised of fermentation unit, pervaporation unit, and collection unit. A broth of 5 L was

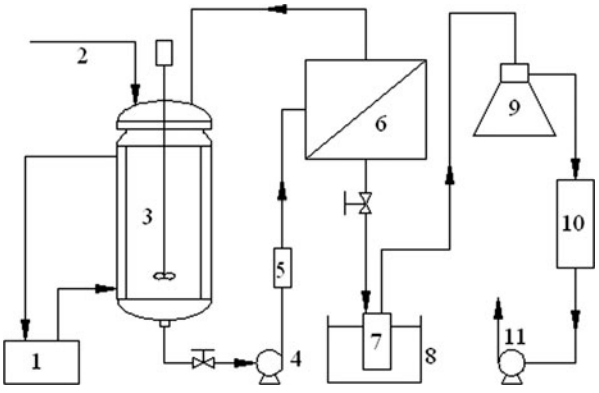

Fig. 1 Schematic experimental setup. *1* Thermostat. *2* Feed inlet. *3* Fermentor. *4* Circulating pump. *5* Rotameter. *6* Membrane module. *7* Cold trap. *8* Refrigerator. *9* Vacuum reservoir. *10* Desiccator. *11* Vacuum pump

formed by inoculating the seed liquid of 0.5 L into liquid NM of 4.5 L in the fermentor. At the primary phase of fermentation, sterile air was sparged to the bioreactor for 4 h, in order to promote yeast cell growth in an aerobic environment. Fermentation broth was adjusted to pH4.5±0.5 with dilute ammonia water and the fermentation temperature was maintained at 35±1 °C by a thermostat. With the fermentation going on, glucose solution or glucose powder was added regularly into the broth to maintain the glucose concentration and the broth volume balance. When the ethanol concentration in the broth was close to 5 % (*w*/*w*), membrane pervaporation was started and then fermentation and separation of ethanol were coupled. Fermentation broth was circulated through the membrane upstream and the fermentor by a feed pump, at a flow rate of 110 $L h^{-1}$. The absolute pressure at the downstream was maintained at 40 mbar by a vacuum pump. Permeation vapor released at the membrane downstream was collected in cold traps immersed in a refrigerator with temperature of −30 °C. The fermentation operation with pervaporation coupling was running on 8:00 a.m.–7:00 p.m. everyday, while the fermentation operation without pervaporation was running for the rest hours of each day. After 21 days (500 h), when cell viability was below 30 %, ethanol concentration fell to about 3 % (*w*/*w*), and glucose consumption decreased drastically, we chose terminating the fermentation operation.

Strain Screening Procedure

Plate culture The isolation of strain was done by the traditional dilution-plate procedure. After centrifugation and washing, the yeast sludge deposited from the residual broth after 500 h fermentation was aseptically inoculated to a 250-mL Erlenmeyer flask with 150 mL sterile water, by means of a transferring loop. After gradient dilution, samples of yeast suspension of different concentration were spread on plates, respectively. The plate medium used for first two rounds was NM, while that for third round was RM. After streaking, those plates were put into a vacuum incubator and incubated at 35 °C for 2–3 days. As the control strain, the parent ADY was cultured similarly.

Tube Culture Several selected single-colony populations from plate mediums were inoculated into tubes filled with 20 mL sterile NM. Then, they were transferred to a shaker incubator to cultivate at 35 °C for 20 h. Each strain was marked as S suffixed two numbers indicating experiment round and sample (e.g., S21 means sample 1 at experiment round 2). Meanwhile, ADY was cultured the same way in the control experiment.

Shaking Flask Culture Yeast suspension from the tube culture was inoculated into a 250-mL Erlenmeyer flask with 80 mL sterile fermentation medium (NM was used in first two rounds, while RM in the third round) and incubated in a rotary shaker at 35 °C. After fermentation for 24 h, the dry cell weight, ethanol concentration, and residual glucose concentration were measured accurately. Each sample strain was performed in triplicate and the average value was obtained finally.

On the basis of the yeast generations, two assumptions were proposed: *S. cerevisiae* in this work followed the propagation rules of the traditional yeast and the generation time did not change much as fermentation proceeding. To compromise the effect of gradually changed fermentation circumstance, we defined the generation time in this work as 2.5 h. Therefore, 500 h fermentation duration of each round means the parent yeast propagated about 200 generations.

As a descendant of 200 generations of ADY, a preferable strain was screened from the fermentation broth then transferred and inoculated into next fermentation and the same

process was conducted as previous round. The fermentation–screening–transfer procedure was repeated three times until a 600-generation descendant was obtained.

Assay

Cell was counted with a blood-counting chamber and the viable yeast cell was identified by methylene blue staining. Cell concentration was determined in terms of dry cell weight, by weighing the deposit of the broth after centrifugation at 4,500 rpm for 20 min, washing with the distilled water, and heat drying in an oven at 100 °C. And the supernatant after centrifugation was distilled to separate ethanol from the broth. The concentrations of ethanol in the distillate and glucose in the distillation residue were determined with a density meter DMA 4500 (Anton Paar, Austria), and the glucose analysis was verified with a fully automatic biochemistry analyzer AU400 (Olympus, Japan). Ethanol yield was calculated as ethanol produced in gram per liter divided by glucose utilized in gram per liter and is expressed as in gram per gram. Specific ethanol productivity was calculated as ethanol produced in gram per liter per hour divided by cell concentration in gram per liter and is expressed as hour. Specific utilization rate and specific growth rate were calculated similarly.

Results

Characteristics of Shaking Flask Culture

In order to screen superior strains that adapt the special PDMS bioreactor fermentation system, experiments were designed to run three rounds, with each round including 500 h CCCF and the corresponding screening procedure. After the first round of 500 h CCCF, the metabolic profiles of ADY and isolated strains from shaking flask culture were shown in Fig. 2. The fermentation profiles of the parent strain and other six sample strains, such as ethanol yield, specific growth rate, specific ethanol productivity, and specific glucose utilization rate, did not have much difference.

A selected single-colony representative strain S14 was transferred, propagated, and inoculated into next fermentation, after the second round of 500 h CCCF, the metabolic profiles of ADY and isolated strains from shaking flask culture were presented in Fig. 3. Similarly, it was also the case for fermentation performance of all the strains when cultured in NM. It seems that S24 was slightly superior to other strains. For example, the ethanol yield of S24 was 0.433 $g\,g^{-1}$, while the mean value of the six sample strains was 0.415 $g\,g^{-1}$.

S24 was selected and transferred in the same way as previous procedure, and after the third round of 500 h CCCF and conventional screening, the metabolic profiles of ADY and isolated strains from shaking flask culture were presented in Fig. 4. The parent ADY and other sample strains showed remarkable difference in both ethanol yield and specific ethanol productivity, although the specific growth rate and specific glucose utilization rate were almost at the same level. Take S33 as an example, the ethanol yield and specific ethanol productivity were higher than ADY by 34.5 and 34.7 %, respectively, noting that the medium of the shaking flask culture used in this round was prepared with the residual broth after 500 h CCCF.

The results suggest that the isolated strains could behave superiorly in the medium prepared with residual broth over in the nutrient medium. S33, as a descendant of about 600 generations of ADY, has ethanol yield and specific ethanol productivity which were much higher than its parent, which indicated that the flux of carbon (glucose) through the

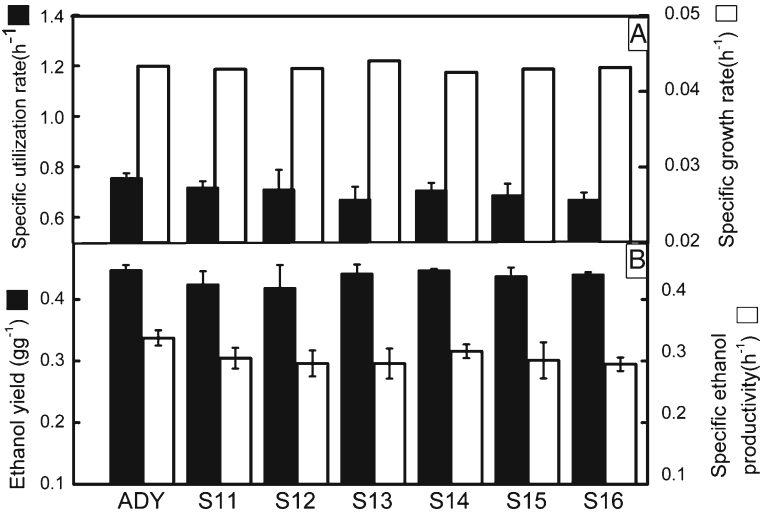

Fig. 2 Metabolic profiles of the parent strain ADY and the isolated six sample strains from the first round when culturing in shaking flask. **a** Specific utilization rate (*solid bars*) and specific growth rate (*open bars*). **b** Ethanol yield (*solid bars*) and specific ethanol productivity (*open bars*). All the strains were incubated in NM at 35 °C for 24 h. The *error bars* (except the specific growth rate) represent the standard error of the triplicate experiments

fermentative pathway occurred at a higher level. This means that the descendant has much better fermentability than its parent in this special fermentation circumstance.

As shown in figures, the specific utilization rate was almost stable in each round of shaking flask culture. However, compared with the first two rounds (0.70 and 0.63 h^{-1}, respectively) of

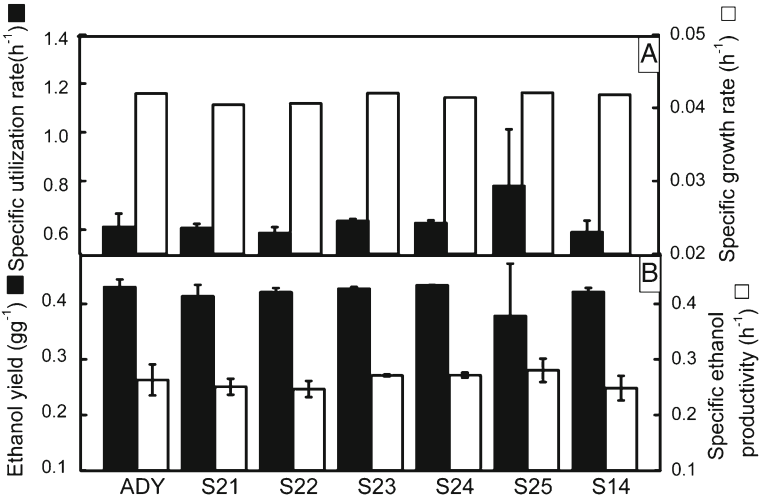

Fig. 3 Metabolic profiles of the parent strain ADY, the selected strain S14 from the first round, and the isolated five sample strains from the second round when culturing in a shaking flask. **a** Specific growth rate (*solid bars*) and specific utilization rate (*open bars*). **b** Ethanol yield (*solid bars*) and specific ethanol productivity (*open bars*). All the strains were incubated in NM at 35 °C for 24 h. The *error bars* (except the specific growth rate) represent the standard error of the triplicate experiments

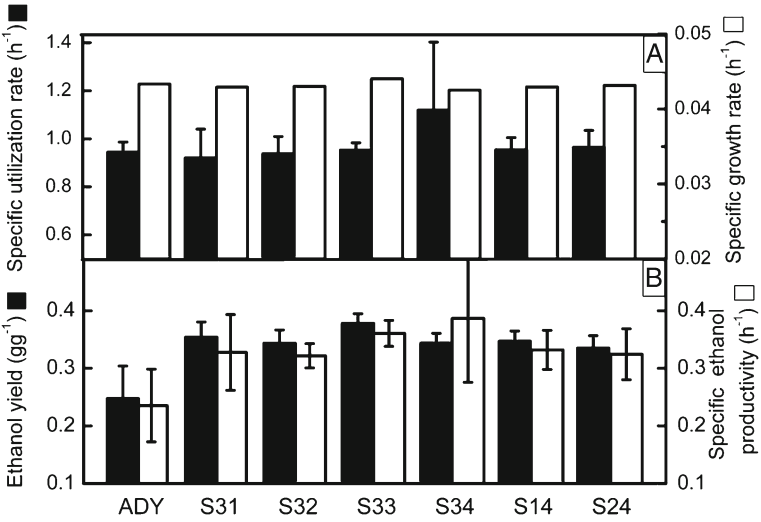

Fig. 4 Metabolic profiles of the parent strain ADY, the selected strain S14 from the first round, the preferable strain S24 from the second round, and the isolated four sample strains from the third round when culturing in shaking flask. **a** Specific utilization rate (*solid bars*) and specific growth rate (*open bars*). **b** Ethanol yield (*solid bars*) and specific ethanol productivity (*open bars*). All the strains were incubated in RM which was prepared with 500 h fermentation residual broth at 35 °C for 24 h. The *error bars* (except the specific growth rate) represent the standard error of the triplicate experiments

fermentation under NM, the mean specific glucose utilization rate of the third round under RM ($0.97\ h^{-1}$) was much higher. It also provides evidence to the conclusion that the descendants had stronger adaptation than its parent in the severe environment. Besides, from Figs. 2, 3, and 4, it is not hard to find that the mean ethanol yield of the sample strains of the third round ($0.34\ g\,g^{-1}$) was lower than those of the first two rounds (0.44 and $0.42\ g\,g^{-1}$) due to the decreased cell concentrations, but the mean specific ethanol productivity of the sample strains of the third round ($0.33\ h^{-1}$) was relatively higher than that of the first two rounds (0.31 and $0.26\ h^{-1}$). That also shows that the individual fermentation ability of descendants was better in the gradually deteriorated fermentation circumstance.

In addition, as shown in Fig. 4, all the sample strains had superior ethanol yield and specific ethanol productivity over the parent ADY, indicating that the descendants were homogenous and able to perform steady fermentabilities in the same condition.

Characteristics of the CCCF

The fermentation profiles of the second and third rounds, when fermentation was coupling pervaporation on 8:00 a.m.–7:00 p.m. everyday, were demonstrated in Fig. 5. Each point in the curve represents the mean value of the four samples withdrawn every day during the 500-h CCCF. All the values were calculated after pervaporation has started and therefore only 19 points were on each curve. Both the specific ethanol productivity and specific glucose utilization rate of the strain in the third round were much higher than those in the second round, noting the strain of the third round was descendant of the second round. This also indicates the individual cell of the descendants has stronger ferment capability than its parent in the 500-h CCCF.

As shown in Fig. 5, fermentation performances of both rounds showed a trend of decrease first and then an increase, as fermentation going on. That might be attributed

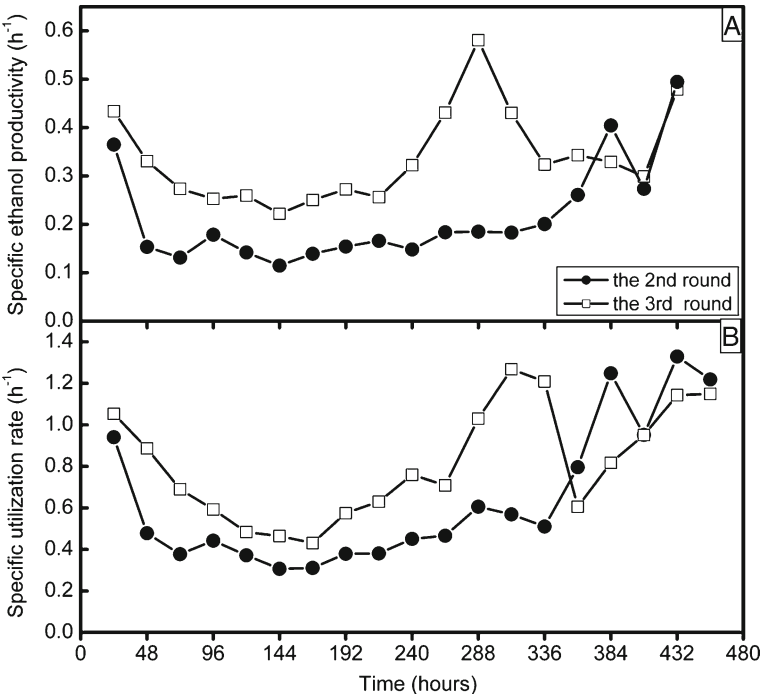

Fig. 5 Specific ethanol productivity (**a**) and specific utilization rate (**b**) of the second round (*filled circle*) and the third round (*blank square*) from 8:00 a.m. to 7:00 p.m. when fermentation was coupling pervaporation

to the response of the strains to the stress induced by the changing fermentation environment. Efficient stress responses are very important for yeast to survive under stressful environment.

The fermentation profiles of the two rounds from 7:00 p.m. to 8:00 a.m. of the next day, when fermentation was not coupling pervaporation, were presented in Fig. 6. It also indicates that the strain of the third round was stronger than that of the second round in this operation mode, with higher specific ethanol productivity and specific glucose utilization rate. Besides, the fermentation abilities were increasing consistently as fermentation was proceeding, which also implies that the descendants demonstrated better fermentability during exposure to the stress induced by the gradually deteriorating environment.

As shown in Fig. 5, both the specific ethanol productivity and specific utilization rate had a descending stage at the early phase, which were very different from those of Fig. 6. The difference should be due to the different operation modes, but the mechanism for interpretation remains unknown to us and needs more research. Besides, compared with the two figures, the average fermentabilities when pervaporation was on were higher than those when pervaporation was off, especially at the early phase, which showed the advantages of pervaporation in the ethanol production process.

The time course profile of the dry cell weight, which indicated growth behaviors of the strains, was shown in Fig. 7. Pervaporation of the second round started after 72 h fermentation when the ethanol concentration reached 7.45 % (*w*/*w*) and the cell growth inhibition had been emerging, while pervaporation of the third round began after 48 h fermentation

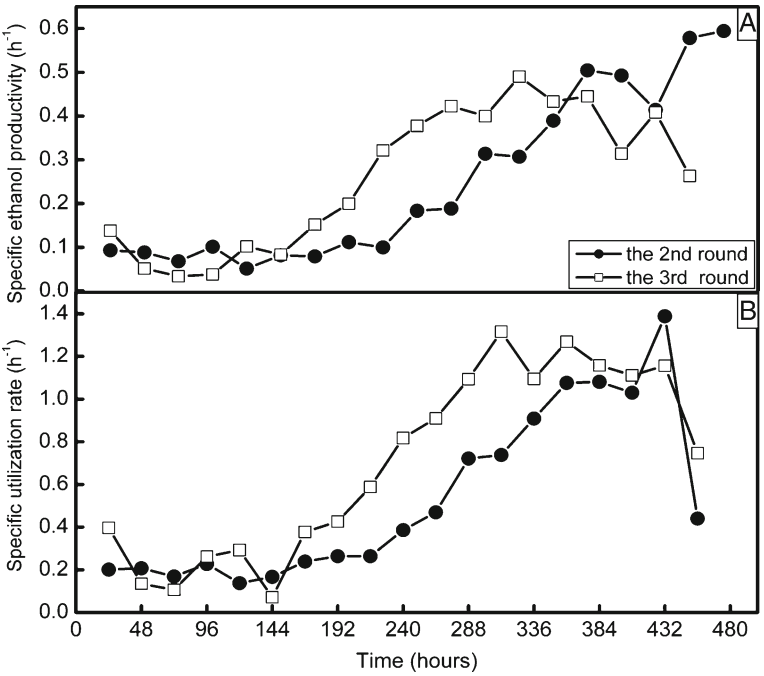

Fig. 6 Specific ethanol productivity (**a**) and specific utilization rate (**b**) of the second round (*filled circle*) and the third round (*blank square*) from 7:00 p.m. to 8:00 a.m. of the next day when fermentation was not coupling pervaporation

when the ethanol concentration reached 4.92 % (*w*/*w*), which was below the ethanol inhibition point.

As shown in Fig. 7, the growth profile of the third round increased smoothly for the first 8 days because ethanol was removed instantaneously. Conversely, the growth profile of the second round had two obvious inflection points, which could be considered as a direct evidence of pervaporation alleviating ethanol inhibition.

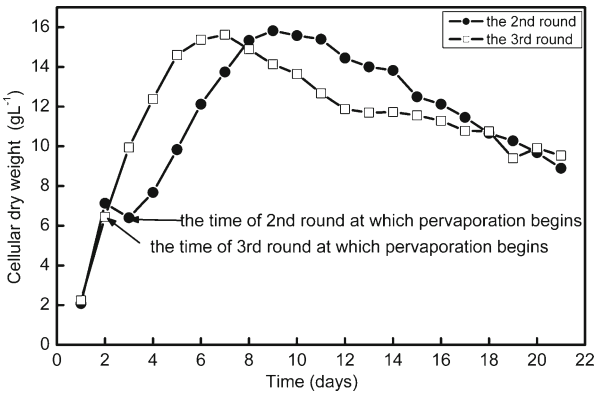

Fig. 7 Growth profiles of the second round (*filled circle*) and the third round (*blank square*) for 500 h CCCF in the PDMS membrane bioreactor

Discussion

In the prolonged CCCF system, with only glucose addition and ethanol removal, some metabolites released by the microorganism, such as glycerol, acetic acid, and lipids, which could not permeate the membrane, would accumulate in the broth. The terminal fermentation broth of the third round was detected to contain 1.03 % (*w*/*w*) ethanol, 5.7 % (*w*/*w*) glycerol, and other acids and lipids of certain concentration. Accumulation of inhibitory products in fermentation broth might affect cell growth and survival, glucose and amino acid transporation, membrane fluidity, and consequently fermentation capability [33]. Thus, it was desirable to obtain a robust strain suitable for the PDMS composite membrane bioreactor system. In this work, taking the gradually deteriorating broth as a stressor, through natural selection of about 600 generations, the phenotypes of yeast strains were tending to adapt the deteriorated condition of this PDMS membrane bioreactor system (Fig. 4).

As reported in many literatures, selective pressure may increase the rates of spontaneous mutation. In this work, unlike chemical mutagenesis, UV radiation, or other physical and chemical approaches, the gradually deteriorated fermentation broth provides a stressful environment including multiple effects: oxygen sparging during aerobic fermentation or culture, inhibition of ethanol and high concentrations of glucose, accumulation of metabolites which could not permeate the membrane, and extended fermentation process. As reported, the acquisition of stress tolerance reveals that yeast has an inherent ability to improve its response to stress [23, 34]. From the results of shaking flask culture, the descendants showed greatly improved fermentability than their parent in the residual broth medium, with the ethanol yield and specific ethanol productivity increasing by 34.5 and 34.7 %, respectively. It seems that this result would prove the hypothesis suggested by McBryde [15]: the evolved strains dominating the culture after extended exposure to these stresses would be better able to complete fermentation during exposure to one or more of these parameters.

On the other hand, as described by Zeyl [35], the fitness of both haploids and diploids increased linearly with generation number. The fitness of haploids with large populations after 2,000 generations was 80 % higher than the original ones. Besides, apart from the difference caused by ploidy, the rates of adaptation in large population were much higher than those in small population [35]. In those cultures accomplished by us, *S. cerevisiae* with the average population size of 4.06×10^8 cells mL^{-1} was consistent with the conception of large population provided by Zeyl [35]. It might be hypothesized that descendants of 600 generations through acclimatization in the prolonged CCCF system could adapt the environment rapidly. Moreover, if we further conducted a culture lasting ten rounds of CCCF and screened a mutant, the screened descendant would behave higher fermentation performance.

Evolutionary engineering has been widely used in strain screening due to its advantages over other complicated screening techniques. For example, an evolution strategy for improving the utilization of mixtures of three or more substrates via consecutive batch cultivation in media with alternating sugar compositions greatly improved the kinetics of anaerobic fermentation of the *S. cerevisiae* strain and furthermore resulted in a 40 % reduction in the time of complete fermentation [21]. A similar process of adaptation had been attempted with a recombinant strain, which resulted in a considerable improvement of the lactose fermentation phenotype [18]. The evolved strain consumed lactose twofold faster through a serial transfer and dilution in lactose medium, which illustrates the usefulness of simple evolutionary engineering approaches in strain improvement. In another research, both the chemically mutagenized mutants CM1 and spontaneous mutants SM1 had increased acclimation and growth rates than the parent [19], which may provide evidence to the effectiveness of evolution from a strain without gene engineering or mutagenesis. In

addition, evolutionary engineering can be also used in the improvement of multiple stress-tolerant properties of *S. cerevisiae* [36].

To date, pervaporation has been investigated widely to separate the organics from fermentation broth as well. Cho and Hwang [37] studied the integration of continuous fermentation and membrane separation, which increased ethanol productivity by 10–20 %. Ding et al. [24] coupled an immobilized cell fermentation system with pervaporation; the ethanol concentration in the broth was maintained at about 4 % (*w*/*w*) while that in the permeation was 23.1 % (*w*/*w*). In another work, the productivity of fermentation of acetone, butanol, and ethanol by pervaporation through supported ionic liquid membrane was increased, and the bacteria died from high concentration of butanol after pervaporation was switched off [38].

However, the strategy of adaptive evolution with continuous fermentation/pervaporation system to isolate a mutant has not been described previously. In addition to the advantages of adaptive evolution and pervaporation, the specific CCCF system in the PDMS membrane bioreactor, which was different from the general batch fermentation system nor traditional continuous fermentation system with dilution, was simple process and easy to operate. From our experimental results, it seems that long-term CCCF in the PDMS membrane bioreactor could be a promising adaptive evolutionary method.

Conclusions

After repeating the fermentation–screening–transfer procedure three times, a descendant of 600 generations could adapt the worse fermentation circumstance. It showed great improvement over the parent in the residual broth medium, with the ethanol yield and specific ethanol productivity increasing greatly. Moreover, as descendant of the strain in the second round, the fermentation profiles of strain in the third round were always higher than those of its parent in the 500-h CCCF. Our findings indicate the feasibility of screening superior strains through acclimation in the long CCCF system, and it might also be applicable to other microorganisms used in ethanol production.

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Conflict of interest The authors have declared no conflict of interest.

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