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Evolutionary engineering of yeast for closed-circulating ethanol fermentation in PDMS membrane bioreactor

Wenwu Ding, Zeyi Xiao*, Xiaoyu Tang, Kewang Deng, Shengwei Fu, Yandong Jiang, Lin Yuan

Department of Chemical Process Equipment, School of Chemical Engineering, Sichuan University, Chengdu 610065, China

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ABSTRACT

Based on evolutionary engineering, a regimen of step transfer cultivation was designed to breed a yeast strain that adapted the process of closed-circulating ethanol fermentation in PDMS membrane bioreactor. The kinetic parameters of the fermentation such as ethanol concentration in the broth, cell concentration and death ratio, ethanol productivity and specific ethanol production rate behaved the same tendency in varied tests. During the fermentation with the screened strain S232, the glucose utilization, ethanol productivity, ethanol yield on glucose, ethanol yield on biomass and specific ethanol production rate were 3.81 g/L/h, 1.63 g/L/h, 0.427 g/g, 194.3 g/g and 0.385 g/g/h, and had elevation of 7.32%, 10.13%, 2.64%, 14.97% and 19.57% over those of fermentation with the parent strain S3, respectively. These results indicated the screened yeast strain had mutated during the fermentation. The yeast strains bred by the regimen not only adapted to the closed-circulating fermentation process but also could be used for general fermentation process.

1. Introduction

Due to the diminishing fossil fuel reserves, fuel ethanol as a renewable energy resource has attracted much more attentions. However, in industrial practice, the yeast was exposed to numerous environmental stresses including incremental amount of sugar source, metabolized and nonmetabolized inhibitors, secondary metabolite, higher temperature, oxidation, hyperosmotic and ionic stress, and so on [1,2]. These factors made the traditional fermentation encounter some limitations, such as higher product inhibition, lower feed stock conversion and higher energy consumption [3–5]. Therefore, lots of improved measures have been presented and researched, for example, using very high gravity fermentation technology [6–10], breeding and application of the ethanol tolerant yeast [11–13] or thermotolerant yeast [14–19] and extraction of ethanol in situ [20–22]. As one of the new fermentation technologies, ethanol fermentation in membrane bioreactor has been extensively researched because of its higher productivity, easier controls, lower ethanol inhibition and less pollution [23–26]. However, the yeast strains used in the membrane bioreactor also need to have a desirable trait of resistance to multiple stresses because of the adverse fermentation environment caused by the closed-circulating system. As a consequence of the complicated and often unknown molecular basis of even individual stress resistances, multiple-stress resistance would be difficult to engineer rationally by genetic means, and empirical process engineering has been largely exploited [2]. An approach of continuous evolution procedures referred to as evolutionary engineering is one of the alternatives, which relies on the use of an appropriate selection pressure towards a desired phenotype in continuously growing cultures [2]. A number of recent studies on the evolutionary engineering of yeast have been conducted to isolate particular yeast strains [2,27–34] or to study the mechanism of the adaptive mutation [35,36]. Adaptive mutation is a process that could relieve the selective pressure whether or not other nonselected mutations are also produced during nonlethal selections [37]. Adaptive mutation may rise under adverse conditions, and could increase the mutation rates.

Based on the evolutionary engineering, different experiment regimens had been designed and adopted in the studies, and different results were obtained because of the different purposes of breeding strains. In Wisselink's study, an evolved strain was obtained orderly by flask, 30 days solid plates, 40 days chemostat and 20 times batch cultivation. The evolved yeast strain could ferment mixtures of glucose, xylose and arabinose, and the ethanol yield on total sugar reached 0.43 g/g [32]. Guimarães et al. obtained an evolved recombinant mainly by 41 days flask cultivation and 3 times batch fermentation, more than 120 generations propagation. The evolved recombinant could consume lactose twofold faster and produced 30% more ethanol than the original recombinant [31].

* Corresponding author. Tel.: +86 28 85401057; fax: +86 28 85401057.
E-mail address: mgch@scu.edu.cn (Z. Xiao).

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McBryde et al. bred the yeast strain under the selective pressures only by batch fermentation. The mutants that had altered production of metabolites were isolated by two strains after approximately 350 and 250 generations, respectively [28]. Besides, many studies also cultivated the evolved yeast strain with chemostat mainly [29,30,36]. Sonderegger and Sauer obtained the mutant which could ferment xylose after 266 days breeding, 460 generations propagation [30]. In Ferea's study, the mutant was obtained after 250 generations [36], and Pitkänen et al. obtained two mutants after a cultivation of 390 and 550 h, respectively [29].

In this work, in order to screen a yeast strain that is suitable to the process of closed-circulating ethanol fermentation in the PDMS membrane bioreactor, a regimen of step transfer cultivation was designed based on the evolutionary engineering, and a series of experiments were carried out to study the performance of the screened yeast strains.

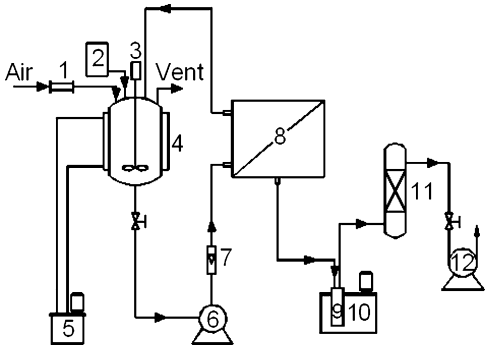

Fig. 1. Schematic apparatus of closed-circulating ethanol fermentation in membrane bioreactor. (1) Air filtration; (2) feed slot; (3) stirrer; (4) fermentor; (5) temperature control unit; (6) pump; (7) rotameter; (8) membrane module; (9) cold trap; (10) refrigerator; (11) desiccator; (12) vacuum pump.

2. Materials and methods

2.1. Initial yeast strain isolation

Four wild yeast strains were isolated from the rotten fruits (grape and pear) by streaking on the agar plates which contained in the medium (w/v): yeast extract 1%, peptone 2%, glucose 2%, agar 1%, ethanol 5% (v/v). The strains were preserved on the YPD agar medium which contained (w/v): yeast extract 1%, peptone 2%, glucose 2%, agar 1%. The yeast strains were respectively applied to inoculate 20 mL YPD medium, and then the medium was incubated for 12 h to prepare inoculums. In order to compare the fermentation abilities of the strains, a 5 mL inoculum was used to inoculate 80 mL ferment medium (FM) in the 250 mL conical flasks containing in g/L: glucose 100 or 200; yeast extract 8.0; $(NH_4)_2SO_4$ 5.0; KH_2PO_4 1.5; $MgSO_4{\cdot}7H_2O$ 0.55; $CaCl_2$ 0.15. The flasks were incubated for 24 h or 48 h at 35 °C on the orbital shaker. Moreover, the fermentation with every strain was conducted with 3 parallel samples.

The yeast strain showing highest fermentation abilities was then selected for the subsequent fermentation. A 5 mL inoculum of the selected strain prepared as described above was used to inoculate 80 mL YPD medium in the flask to determine its growth performance at 35 °C by detecting the cell concentration with microscope every 2 h. In order to determine the fermentation performance of the strain concretely, the inoculated FM was incubated at 35 °C on the conical shaker. The glucose and the cell concentration were detected every 4 h, and the ethanol concentration was determined at the termination of the fermentation. All the above tests were also conducted with 3 parallel samples.

2.2. Step transfer cultivation and mutant isolation

2.2.1. Closed-circulating fermentation in PDMS membrane

The closed-circulating fermentation system was mainly constructed with fermentor, membrane module and cold trap as shown in Fig. 1. The volume of the fermentor was 5 L. The membrane module was in plate frame design with the membrane housed between stainless-steel compartments, providing membrane area of 0.08 m^2. The membrane was a composite polydimethylsilicon (PDMS) membrane with a dense PDMS skin layer of 8 μm and a microporous polyamide (PA) supporting layer of 120 μm. The membrane had higher ethanol flux and selectivity, and could offer superior separation performance of recovering ethanol from fermentation broth [38,39].

To prepare the seed culture, a 40 mL YPD medium was inoculated with the selected yeast strain and incubated for 12 h at 35 °C on the orbital shaker, and then the medium volume was amplified 11 times with YPD medium and continually incubated for 12 h at the same conditions.

The fermentation which medium was FM or doubled components of FM was operated under aerobic conditions for 4 h after the fermentor was inoculated with 10% (v/v) active inoculum. The volume of the fermentation medium was about 4 L. The glucose concentration in the broth was fluctuating at 20–70 g/L with feeding the glucose into the broth regularly and intermittently. When the ethanol concentration in the broth reached about 60 g/L, the pervaporation was started to separate ethanol from the broth by circulating the broth through the membrane module and fermentor. The pervaporation was running in the day but stopped at night. At downstream of the membrane, an absolute pressure of 5000 Pa was maintained by a vacuum pump, and a cold trap immersed in a refrigerator of −30 °C was used for condensing and collecting the permeation vapor.

2.2.2. Mutant isolation from the residual broth

After continuous operation over 500 h in the membrane bioreactor, the yeast in the broth was isolated by streaking on the residual broth agar plates. The composition of the residual broth agar medium (RBAM) was 2% (w/v) glucose, 2% (w/v) agar and residual broth which removed cells by centrifuging at 4000 rpm for 20 min.

In order to select a yeast strain that had best fermentation performance, all of the isolated strains were used to inoculate the residual broth medium (RBM) containing 200 or 150 g/L glucose in the conical flasks, then the flasks were incubated at 35 °C for about 48 h on the orbital shaker, and the fermentation with each strain was conducted with 2 parallel samples.

2.2.3. Transferring the selected strain to next round fermentation

The relative superior yeast strain was selected according to the cultivation on the orbital shaker, and next fermentation in membrane bioreactor was conducted with the selected yeast strain.

2.3. Assay

For the fermentation in the conical flasks, the glucose concentration was tested with the saccharimeter (WYT-P). For the continuous fermentation, the ethanol concentration and the glucose concentration were measured by density meter (DMA4500, Anton Paar, Austria). The ethanol concentration in the permeation condensate at downstream of the membrane was measured directly. The fermentation broth sample was first centrifuged and distilled. The distillate was tested for ethanol concentration, and distillation residue was tested for glucose concentration. The cells obtained from the broth sample was dried at 100 °C and weighed with