Screening and Mutation of *Saccharomyces cerevisiae* UV-20 with a High Yield of Second Generation Bioethanol and High Tolerance of Temperature, Glucose and Ethanol

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Abstract A wild-type strain was isolated from slightly rotted pears after three rounds of enrichment culture, identified as *Saccharomyces cerevisiae* 3308, and evaluated for its fermentation capability of second generation bioethanol and tolerance of temperature, glucose and ethanol. *S. cerevisiae* 3308 was mutated by using the physical and chemical mutagenesis methods, ultraviolet (UV) and diethyl sulfate (DES), respectively. Positive mutated strains were mainly generated by the treatment of UV, but numerous negative mutations emerged under the treatment of DES. A positive mutated strain, UV-20, produced ethanol from 62.33 ± 1.34 to 122.22 ± 2.80 g/L at 30–45 °C, and had a maximum yield of ethanol at 37 °C. Furthermore, UV-20 produced 121.18 ± 2.51 g/L of second generation bioethanol at 37 °C. Simultaneously, UV-20 exhibited superior tolerance to 50% of glucose and 21% of ethanol. In a conclusion, all of these results indicated that UV-20 has a potential industrial application value.

Keywords Enrichment culture · *Saccharomyces cerevisiae* · Second generation bioethanol · Tolerance of temperature, glucose and ethanol · Ultraviolet

Introduction

Two stupendous challenges, energy demands and environmental concerns, have escalated an extensive attention of second generation bioethanol which is served as the

partial replacement of transportation fuels and is also one of the most representative energy alternatives in the global. In general, yeasts are widely used to produce bioethanol [1–3]. However, it is susceptible for yeasts under stress conditions, such as lack of tolerance to high concentrations of glucose and ethanol, which leads to a sharp decline in ethanol production [4–6]. To avoid these problems, ethanol yield is increased by using different types of yeasts with osmotolerance, thermotolerance, tolerance to toxic compounds, etc. [2, 7, 8].

Generally, yeasts without recombinant cellulase genes can not utilize cellulose to produce ethanol under anaerobic conditions. *Saccharomyces cerevisiae* whose cell surfaces display recombinant cellulase genes can efficiently convert cellulose into second generation bioethanol. Optimum temperature of most cellulases usually is 40–65 °C, but it is suitable for *S. cerevisiae* to grow and produce ethanol at 25–30 °C [9–11]. Therefore, it is not efficient to produce bioethanol due to the mismatch of cellulase activity temperature and *S. cerevisiae* fermentation temperature. To overcome this problem, the traditional mutagenesis methods of ultraviolet (UV) and diethyl sulfate (DES) were used in this work to look for *S. cerevisiae*s with a high yield of second generation bioethanol and high tolerance of temperature, glucose and ethanol.

Materials and Methods

Materials and Strain

Slightly rotted pears were purchased from a local fruit market in Nanchang City, Jiangxi Province, China, and incubated at room temperature for several days. Wheat bran was purchased on the internet, and other chemical reagents were made in China.

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Penicillium oxalicum 16 was isolated from soil of Nanchang and deposited in the China Center for Type Culture Collection (CCTCC) under AF2015017 [12], and used to produce the hydrolase for saccharification of wheat bran.

Media

The enrichment medium (g/L) contained 3 yeast extract, 5 peptone, 200 glucose, 3 (NH₄)₂SO₄, 2 urea, and 2 KH₂PO₄. The screened plate (g/L) contained 3 yeast extract, 5 peptone, 500 glucose, 3 (NH₄)₂SO₄, 2 urea and 2 KH₂PO₄. The ethanol production medium (g/L) contained 200 glucose, 3 yeast extract, 7 peptone, 3 (NH₄)₂SO₄, and 2 KH₂PO₄.

Determination of Glucose Content

The concentration of glucose from hydrolysis of wheat bran with the cellulase of *P. oxalicum* 16 was assayed by Glucose assay kit (Robio, Shanghai, China) according to a manufacturer's instruction.

Determination of Lethality

Lethality was calculated as following equation

$$\text{Lethality (\%)} = \frac{(\text{numbers of untreated plate colonies} - \text{numbers of treated plate colonies})}{\text{numbers of untreated plate colonies}} \times 100\%.$$

Determination of Ethanol Yield

About forty milliliter of fermentation broths was injected on Beer Analyzer (Anton Paar, Austria) at 20 °C and ethanol yield was calculated as following equation:

$$\text{Ethanol yield (g/L)} = \text{ethanol content \% (v/v)} \times 0.789 \text{ g/cm}^3 \times 10^3 \times \text{fermentation broth volume}.$$

Ethanol content % represents volume percentage of ethanol detected on Anton Paar Beer Analyzer at 20 °C and 0.789 g/cm³ is the density of ethanol at 20 °C.

Screening and Morphological Identification of Wild Yeasts

Slightly rotted pears were placed at room temperature for several days, and then added with sterilized physiological saline. Ten milliliter of liquid from rotted pears was

inoculated in the enrichment medium under static culture for 2 days at 45 °C. After 2 days' culture, ten milliliter of liquid from the first enrichment medium continued to be inoculated in the enrichment medium under static culture for 2 days at 45 °C, and then ten milliliter of liquid from the second enrichment medium was carried out as described above. The third enrichment medium was diluted as 10⁻¹–10⁻⁵ times. One hundred microliter of 10⁻³, 10⁻⁴ and 10⁻⁵ diluent were spread on the screened plate at 45 °C for 3 days, respectively, and then single colony with big sizes was picked out from the screened plate. All colonies were inoculated into 50 mL of the ethanol production medium at 45 °C for 3 days. Screened yeasts were further cultured for the second screening at 250-mL flask filled with 100 mL of the ethanol production medium for 2 days at 45 °C in order to reject false positive yeasts and to obtain yeasts with a high production level of ethanol. In addition, wild yeasts were observed by microscopy for morphological identification of wild yeasts.

Molecular Identification of Wild Yeast

Total DNA of wild yeasts was extracted by using nucleic acid purification kit (Tiangen, China). There were two primers, NL-1 and NL-4, 5'-GCATATCAATAAGCGG

AGGAAAAG-3' and 5'-GGTCCGTGTTTCAAGACGG-3', respectively [13]. Partial sequence of 26S rDNA from yeasts was amplified by polymerase chain reaction (PCR) and performed in 100 µL of reaction volume containing 100 ng of yeast DNA, 50 pmol of each primer, and 5 U of ExTaq DNA polymerase (TakaRa, Japan). Cycling conditions of PCR were 94 °C for 5 min, followed by 25 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 40 s, with a final extension of 10 min at 72 °C.

Preparation of Separated Single Spores

Wild yeast with the highest ethanol yield was inoculated in YPD plate at 45 °C for 4 days. Its spores scaled off from YPD plate were placed into 250-mL flask filled with 20 mL physiological saline and with about 30 sterile glass beads (diameter 90 mm), and then they were shaken at 30 °C and 160 rpm for 30 min. After it, separated single

spores from cell suspension were obtained by cotton filtration.

Mutagenesis of UV and DES

Cell suspension with separated single spores placed in empty plates was exposed to UV light (30 W) with a distance of 20 cm for 30–90 s by magnetically stirring, respectively, kept static for 30 min in the dark, diluted to 10^{-10} – 10^{-5} times, and spread on the screened plate containing 50% glucose at 45 °C for 3 days.

A DES solution was made of 0.5 mL of DES and 4.5 mL of 95% ethanol. The separated spore suspension was dealt with by 0.2–1.0% (V/V) of DES solution at 30 °C for 20–60 min, respectively. The reaction was stopped by adding 5 mL of 0.5 mol/L sodium thiosulfate, and diluted to 10^{-10} – 10^{-5} times, and spread on the screened plate containing 50% glucose at 45 °C for 3 days.

Determination of Optimal Temperature of Ethanol Production

Mutated yeast with the highest ethanol yield was cultured at 30–45 °C for 3 days in order to get optimal temperature of ethanol production, and ethanol yield was determined as described above. Every data was the average of three determinations and vertical error bars represent standard deviation.

Enzymatic Hydrolysis of Wheat Bran and Production of Second Generation Bioethanol

Wheat bran was hydrolyzed by the cellulase of *P. oxalicum* 16 at 50 °C for 96 h, and hydrolysate was concentrated by using vacuum freeze-drying, and stored at –20 °C for further use. Concentration of glucose from the hydrolysate was determined as described above.

Mutated yeast with the highest ethanol yield produced bioethanol in the medium (g/L) containing 200 glucose from hydrolysis of wheat bran with cellulase of *P. oxalicum* 16, 3 yeast extract, 7 peptone, 3 (NH₄)₂SO₄, and 2 KH₂PO₄ at 37 °C for 3 days, its content was determined as described above.

Tolerance to High Concentration of Glucose and Ethanol

For determining glucose tolerance, the mutated yeast with the highest bioethanol yield was inoculated into the ethanol production medium plate containing 30–55% of glucose at 37 °C for 3 days. For determining ethanol tolerance, mutated yeast with the highest ethanol yield was inoculated into ethanol production medium containing 18–22% of

ethanol at 37 °C for 3 days. Because the living yeast cells have a reductive reductase which makes methylene blue colorless, the method of methylene blue staining was used to determine tolerance of glucose and ethanol.

Results and Discussion

Screening, Morphological and Molecular Identification of No. 20 Strain

Forty wild-type strains were randomly selected to produce ethanol. Ethanol yields of these strains were displayed in Fig. 1, and the yield of the No. 20 strain with the highest yield produced 63.25 g/L of ethanol, stating that its fermentation capability of ethanol was the most excellent among those strains.

According to morphological characteristics, the No. 20 strain was identified [14, 15]. As shown in Fig. 2, the strain appeared like pseudohypha, euhyphae, and sausage-like individual cells with budding, spherical, oval, and columnar. Furthermore, the strain showed milky white and smelled of wine, indicating that it may be *S. cerevisiae*.

However, morphological characteristics of microorganism may be changed with alteration of medium components, and result in a wrong classification. Molecular biotechnology gradually has played a great role in strain classification. Thus, molecular identification was carried out. The primers, NL-1 and NL-4, were used to amplify the part sequence of 26 s rDNA by PCR [16]. As seen in Fig. 3, the part sequence possessed an about 600-bp fragment, and showed 100% of identity with *S. cerevisiae* from NCBI.

On the basis of the above morphological and molecular identification, the No. 20 strain was definitely identified as *S. cerevisiae* and named as *S. cerevisiae* 3308.

DES Mutation of *S. cerevisiae* 3308

S. cerevisiae 3308 with the most excellent ability of ethanol production was mutagenized by DES reagents. Nineteen mutants were randomly selected to produce ethanol in this work. As shown in Fig. 4, the ethanol yields of the nineteen strains were fairly low compared with the wild-type, and the highest yields of ethanol only was 33 g/L, stating that all of these strains treated by DES were negative mutations. Generally, DES is a strong mutagenesis method, but its effect is not satisfying [17]. It is a possible reason that DES leads to the alkylation of guanine N-7, makes guanine fall off from DNA strand and produces negative mutation of numerous ethanol production-related genes. The other reason may be associated with alteration of silent biosynthetic pathways, and result in thorough

Fig. 1 Ethanol yields from the screened yeasts **Fig. 2** Morphology of the screened strain with the highest yield of ethanol

reduction of ethanol production [18]. However, more mechanism of negative mutations is complex and we can not completely understand it.

UV Mutagenesis of *S. cerevisiae* 3308

UV mutagenesis is one of the most effective and reliable methods on improving a strain's tolerance ability of glucose and ethanol [19]. Theoretically, when a lethal rate of eukaryotes was about 75%, positive mutagenesis would be more easily obtained. *S. cerevisiae* 3308 was irradiated by

UV for 30, 60 and 90 s, respectively, and its lethality was about 70% for 60 s. Hence, positive mutagenesis would be more easily obtained for 60 s.

Twenty six mutants were randomly selected to ferment ethanol. As shown in Fig. 5, these mutants exhibited obvious differences in ethanol production. Positive and negative mutagenesis rates were 38.46 and 61.54% (data not shown), respectively, which can be attributed to that enhancement of single nucleotide repair mechanism of negative mutant DNA and reduction of effective mutant genes. It was obvious that the method of UV mutagenesis was a more effective method than that of DES.

As shown in Fig. 5, the *S. cerevisiae* 3308 mutant named as UV-20 with the highest yield of ethanol produced about 90 g/L at 45 °C, and its ethanol yield increased by 42.85% compared with *S. cerevisiae* 3308, indicating that its capability of ethanol fermentation was the most excellent among those mutants.

Fermentation Temperature Optimization of UV-20

Optimization of fermentation temperature plays a significant role in increasing yield of ethanol, since temperature is one of the important factors in microbial fermentation [20]. As exhibited in Table 1, UV-20 produced ethanol from 62.33 ± 1.34 to 122.22 ± 2.80 g/L at 30–45 °C, and had a maximum yield of ethanol at 37 °C. Furthermore, UV-20

Fig. 3 Phylogenetic tree constructed by using neighbor-joining method based on the partial sequences of objective strain 26S rDNA D₁ domain

Fig. 4 Ethanol yields of mutants treated by DES

produced 121.18 ± 2.51 g/L of second generation bioethanol at 37 °C (data not shown).

As shown in Table 1, *S. cerevisiae* generally produced ethanol with the highest yields at 25–30 °C [11, 21–28], but UV-20 at 37 °C. Although Ma.Jesús Torija etc. obtained a yeast with the optimal ethanol fermentation

temperature at 35 °C, its ethanol yield was lower than UV-20 [29]. Notably, yeasts with high fermentation temperatures have a number of benefits, such as simultaneous saccharification and co-fermentation, and reduction in energy costs and bacterial contamination [30, 31].

Fig. 5 Ethanol yields of mutants treated by UV **Table 1** Comparison of ethanol yield, and ethanol and glucose tolerance

Strains	Temperature (°C)	Ethanol yield (g/L)	Tolerance		References
			Glucose (%)	Ethanol (%)	
MTCC 174	28	20.00	–	–	[21]
<i>S. cerevisiae</i> ZM1-5	40	18.79	–	–	[22]
Hybrid H1	30	147.54	32	–	[23]
<i>S. cerevisiae</i>	–	13.34	–	–	[24]
<i>S. cerevisiae</i> EC-1118	30	43.86	–	–	[25]
<i>S. cerevisiae</i>	–	–	–	1.8	[26]
C4377	–	–	30	17	[11]
<i>Saccharomyces</i>	–	–	–	24	[27]
Mutant M1	–	–	–	10	[28]
UV-20	30	62.33 ± 1.34	–	–	This work
	35	96.10 ± 1.70	–	–	This work
	37	122.22 ± 2.80	50	21	This work
	40	99.18 ± 1.77	–	–	This work
	45	90.18 ± 1.42	–	–	This work

Evaluation of Tolerance of UV-20 to Glucose and Ethanol

For better application in industries, it should take some measures to solve complex challenges in substrate and product inhibition during ethanol fermentation. As shown in Fig. 6, UV-20 grew well in the ethanol production

medium containing 50% glucose, indicating that mutant performs supernormal tolerance to high concentration of glucose. High concentration of ethanol produces toxicity on yeast, inhibits growth of yeast, and reduces ethanol yield. However, as shown in Fig. 7, UV-20 cultured for 3 days did well in the ethanol production medium containing 21% (v/v) ethanol, and did not show any loss of

Fig. 6 Morphology of UV-20 grown in the ethanol production medium containing 50% glucose

Fig. 7 Morphology of UV-20 grown in the ethanol production medium containing 21% ethanol

living yeast cell. As shown in Table 1, 50% of glucose and 21% of ethanol were higher than other studies.

Conclusion

S. cerevisiae 3308 with a high yield of ethanol was obtained by the enrichment culture method at 45 °C, and mutagenized in order to further enhance its excellent properties. It was demonstrated that UV mutation was superior to DES mutation. UV-20, a mutant from treatment of UV, generated more second generation bioethanol and was tolerant to high concentration of glucose and ethanol. Hence, UV-20 has a potential industrial application value.

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