Current Research Journal of Biological Sciences 8(1): 10-14, 2016
DOI: 10.19026/crjbs.8.2330
ISSN: 2041-076X, e-ISSN: 2041-0778

Submitted: September 9, 2015 Accepted: September 25, 2015 Published: January 20, 2016

Research Article

Screening of Ethanol-Tolerant Fusants of *Saccharomyces cerevisiae* and *Pichia stipitis* from Protoplast Fusion

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Abstract: Bioethanol is gaining increasing attention as a clean and renewable fuel because of its major environmental benefits. Efficient bioethanol fermentation requires the selection of superior strains that are capable of ethanol stress tolerance. Yeast can produce ethanol, thereby reflecting its intrinsic ability to tolerate ethanol. This study focuses on ethanol tolerance enhancement of *Saccharomyces cerevisiae* for ethanol production improvement through protoplast fusion. *S. cerevisiae* and *P. stipitis* fusants (ATCC 58785), which can ferment xylose, were isolated. The ability of these isolates tolerate ethanol was investigated by allowing the strains to grow in different ethanol concentrations. Results showed the ability of the fusantsto have an average tolerance to ethanol when compared with the parent strains and fermented glucose in the presence of 6% ethanol. By contrast, the parent strains *S. cerevisiae* and *P. stipitis* showed ethanol tolerances of 8 and 4%, respectively. Fusant formation was confirmed by the increased DNA content. This outcome suggests that multiple fusions had occurred and the genetic stability of fusants indicates that F24 and F18 are genetically stable and suitable for industrial production.

Keywords: Ethanol tolerant, *Pichia stipitis*, protoplast fusion, *Saccharomyces cerevisiae*

INTRODUCTION

Ethanol has become increasingly important because of its role as an alternative renewable energy source (Sarkar *et al*., 2012). In accordance with the different types of renewable feedstock for ethanol production, bioethanol can be derived from sugar cane, corn, wheat, cassava (first generation), (Lennartsson *et al*., 2014), biomass (second generation) and algal biomass (third generation) (Baeyens *et al*., 2015). During industrial fermentation for ethanol production, yeasts are compulsorily exposed to stress factors, such as increased ethanol concentration, high temperature, by-product toxicity and osmotic pressure (Baeyens *et al*., 2015). Among these factors, ethanol is considered to be the major effect for decreased ethanol production.The high ethanol tolerance of *S. cerevisiae* is closely associated with ethanol productivity and cell viability (Zhang *et al*., 2013). The capacity of *S. cerevisiae* to tolerate stress critical characteristics of ethanol resulted from profound phenotypic variation between yeast strains (Wimalasena *et al*., 2014). When ethanol concentrations exceeded 8%, membrane fluidity increased and membrane integrity decreased because of the phospholipid in the lipid bilayer of cell membranes and organelles (Zhao and Bai, 2009). Ethanol also results in defects in yeast metabolism and energy supply through repression of the glucose transport system (Ma and Liu, 2010). Successful fermentations that use yeasts that are tolerant to high concentrations of glucose and ethanol have been reported in the industry. These cellular features are important because of high gravity fermentations, which lead to high sugar concentrations, at the beginning of fermentation; in addition, these features are important because of high ethanol concentration at the end of fermentation (Haggran and Abo-Sereih, 2014).

Extensive efforts have been made to gain insights into this matter for improvement ethanol fermentation using *S. cerevisiae*; such improvement includes enhancement of ethanol tolerant cells, screening of ethanol tolerant mutants and alteration of nutritional conditions (GuoLi *et al*., 2014). With the use of repetitive protoplast fusion, genomic recombination of several starter strains was obtained from mutagenesis (Zhao and Bai, 2009). Moreover, after three rounds of protoplast fusion and High-Energy Pulse Electron beam (HEPE), the *S. cerevisiae* strain developed high ethanol tolerance and resistance to high temperatures (Zhang *et al*., 2012). The growth defect in ethanol-tolerant yeast improved. In this process, the yeast was mutagenized using protoplast fusion of *S. cerevisiae* NR1 to enhance ethanol production from sugarcane molasses; moreover, sugarcane molasses was used to obtain ethanol-tolerant mutant UVNR56 and displayed

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significantly higher viability and improved ethanol tolerance in 15% ethanol (Thammasittirong *et al*., 2013). The present study aims to develop a yeast strain with improved ethanol tolerance and the ability to utilize the main sugar groups from the degradation of lignocellulose biomass. Through protoplast fusion, the two yeast strains, namely, *P. stipitis* and *S. cerevisiae*, were fused to obtain fusants with xylose-utilizing ability and higher ethanol tolerance.

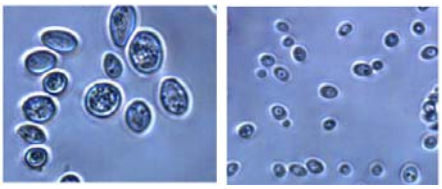
MATERIALS AND METHODS

Yeast strains: Two yeast strains were used in this study. *Saccharomyces cerevisiae* was obtained from the Industrial Biotechnology Research Laboratory of Universiti Sains Malaysia and *Pichia stipitis* (ATCC 58785) was obtained from American Type Culture Collection (Fig. 1). *S. cerevisiae* was maintained at 4°C in a medium that contained 5 g/L yeast extract, 3 g/L peptone, 20 g/L glucose and 20 g/L agar. *P. stipits* was maintained at 4°C in a medium that contained 5 g/L yeast extract, 3 g/L peptone, 20 g/Lxylose and 20 g/L agar. The inoculation medium of *S. cerevisiae* (YPD) medium contained 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose. The seed culture was prepared through transfer of a loop full of cells to 50 mL medium in a 250 mL Erlenmeyer flask on a rotary shaker at 30°C and shaken at 150 rpm for 24 h. Batch culture of *P. stiptis* was prepared in a 250 mL flask that contained 50 mL medium incubated at 30°C and shaken at 170 rpm. The culture broth contained YPX medium (the same ingredient as YPD except D-xylose was used instead of D-glucose) at pH 5.

Protoplast fusion: Protoplast fusion of cells, *S.cerevisiae* and *P. stipitis* (ATCC 58785), was achieved using Zymolase 20T (from *Arthrobactor luteus*, 200 unit/mL, Kirin Brewery Company Chuo-ku, Tokyo, Japan). The method described by Pérez-Través *et al*. (2012) was used.

Selection of glucose-xylose utilization fusants: Fusants that showed growth on a YPD agar or YPX agar plate through 24 h inoculation of yeast culture were selected to determine ethanol tolerance.

Determination of ethanol tolerance: Approximately 100 mL of growth mediumYPD was inoculated with a loop of *S. cerevisiae* strain at 30°C. Subsequently, this medium was shaken on a shaker at 180 rpm to obtain an initial cell density of 1×10^7 cells/mL. Approximately 10 mL of 24 h-old was prepared with constant shaking at 150 rpm. After incubation, samples were obtained every 12 h. Similar experiments were conducted for yeast strains, *P. stipitis* and fusants. To select the tolerant fusant, the ethanol tolerance of parent yeast



(a) (b)

Fig. 1: (a) *S. cerevisiae* and (b) *P. stipitis* (ATCC 58785) - 40×1.25 magnification

strains and fusant strains was determined spectrophotometrically at 600 nm after 48 h incubation through measurement of growth in the presence of exogenously added ethanol.

DNA extraction and estimation: Genomic DNA of *P. stipitis* and *S. cerevisiae* and six selected yeast hybrids were extracted by using AMRESCO's Yeast Genomic DNA Purification Kit (AMRESCO, LLCOhio USA) and conformed in gel electrophoresis. A spectrophotometer was used to quantify the DNA.

RESULTS

Ethanol tolerance of *S. cerevisiae* and *P. stipitis*: According to the presumption that ethanol-tolerant yeast strains would promote ethanol yields, several studies have focused on ethanol tolerance of ethanol-producing yeasts (Thammasittirong *et al*., 2013). The ethanol tolerance of each strain was studied by allowing the yeast to grow in liquid YPG that has increased ethanol concentrations (0 to 10%). *S. cerevisiae* strain tolerated a slightly higher percentage of 8% ethanol. Figure 2 shows that higher biomass production is observed with increased *S. cerevisiae* grow that ethanol concentrations from 0 to 4%. A sharp decrease in growth was observed at concentrations above 8%. A similar result was reported by Osho (2005). Three *S. cerevisiae* strains among 17 wine yeasts, which were isolated from cashew apple juice fermentation, were selected for ethanol and sugar tolerance in the presence of 9% ethanol. Sevda and Rodrigues (2011) reported that biomass production for *S. cerevisiae* NCIM 3095 and NCIM 3287 is higher at ethanol concentrations from 0 to 4%. An ethanol concentration of 4% does not have much effect on yeast growth. When the initial ethanol concentration is increased to 8%, biomass production is reduced by approximately 200%. Similar results were reported by Tikka *et al*. (2013). Seven strains of *S. cerevisiae*, which were obtained from different fruit sources, were screened for ethanol tolerance. In addition, a range of tolerance levels between 7 and 12% was observed in all the strains. Meanwhile, Wai *et al*. (2008) reported that the *S. cerevisiae* (KY1 and KY3) strain, which tolerated up to 15% ethanol in the medium and the *S. cerevisiae* (KY2) strain, which tolerated up to 20% ethanol, led to

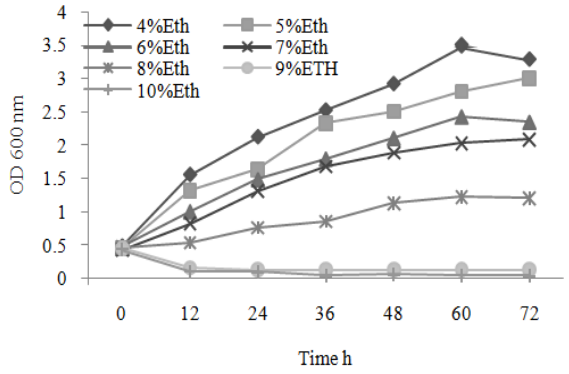


Fig. 2: Growth of *S. cerevisiae* at different ethanol concentration

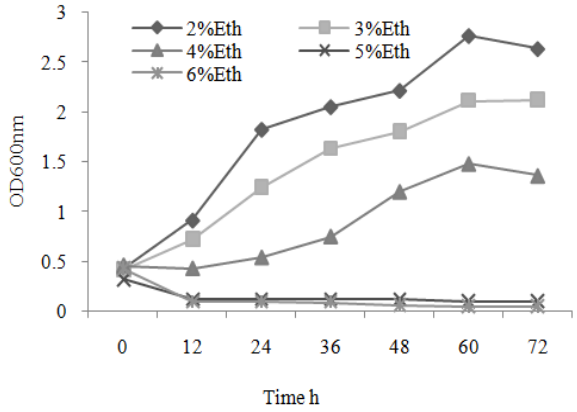


Fig. 3: Growth of *P. stipitis*at different ethanol concentration

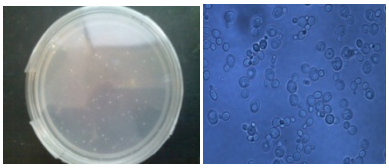


Fig. 4: -a Regeneration plate showed visible regenerated colonies emerged; -b fusants cells 40×1.25 magnification

Fig. 5: Screening of fusant growth in glucose and xylose in media

maximum ethanol production over a long incubation period. Ethanol concentration of up to 4% does not have a significant effect on yeast growth. However, at 10% exogenous ethanol concentration, reduced viability was observed even after 12 h of incubation. Figure 3 shows that when ethanol concentration was increased to 4% and higher, the *P. stipitis* growth was acutely inhibited for higher ethanol concentration. *S. cerevisiae* showed better tolerance than *P. stipitis* ATCC58785. *S. cerevisiae* can survive exogenous ethanol added at 8% concentration. It remained viable after 72 h of incubation, whereas *P. stipitis*can survive only 60 h of incubation in the medium with 4% ethanol (Fig. 3).

Isolation of fusants: *S. cerevisiae* and *P. stipitis* ATCC58785 strains were fused through protoplast fusion. After cell fusion, visible regenerated colonies that emerged in the regeneration media plate were assayed for the desired fusant, which exhibited enhanced ethanol tolerance (Fig. 4). Fast-grown colonies were selected for shake flask analysis to estimate their ethanol tolerance individually. Twenty-four candidate fusants showed xylose utilization (Fig. 5) and their ethanol tolerance was subsequently evaluated.

Screening of ethanol tolerant fusants: Ethanol generally inhibits growth and is toxic to cells that induce cell viability reduction and inhibition of both yeast growth andseveral cellular metabolic pathways. Thus, ethanol-tolerant yeast strains are beneficial to high fermentation efficiency. This behavior is also observed in yeast fusant strains. The obtained results for the effect of ethanol on fusant growth after 48 h of incubation are shown in Table 1. A difference in ethanol tolerance was observed among yeast strains and fusants. At concentrations above 6%, reduced growth was observed with optical density values. Another fusant showed similar ethanol tolerance and tolerated a maximum of 5% ethanol concentration, which was followed by a large decline in growth. The effect of ethanol on growth is indicated in Table 1. Deference in ethanol tolerance was shown among yeast strains and fusants. In line with the constant decline in growth, ethanol concentration, which is responsible for a sharp decline in growth, is considered to indicate an ethanol-tolerant strain (Ali and Khan, 2014). *S. cerevisiae* strains showed the highest ethanol tolerance over other strains, *P. stipitis* and the fusants. The *P. stipitis* strain was able to tolerate a maximum ethanol concentration of 4%. However, the optical density values of growth decreased exponentially above this concentration. Fusant strains, i.e., F4, F12, F18, F22 and F24, were able to tolerate a maximum ethanol concentration of 6%. Among the 60 regenerated fusant yeast screened for xylose utilization, only 25 were able to grow in YPX. These fusants will be screened for ethanol tolerance. Five of the regenerated yeasts fusants, namely, F4, F12, F18, F22 and F24, showed significantly improved tolerance to 6% ethanol

Table 1: Ethanol tolerance of parent strains and fusants

		(Absorbance at 600 nm after 48 h of incubation) Ethanol concentration% (v/v)								
	Strains	0	3	4	5	6	7	8	9	10
1	*S.cerevisiae*	2.43	2.23	2.5	2.1	1.86	1.74	1.56	0.53	0.06
2	*P.stipitis*	1.48	1.20	1.13	0.14	0.08	0.02	0.00	0.00	0.00
3	F1	2.32	2.07	1.90	0.54	0.32	0.06	0.00	0.00	0.00
4	F2	1.54	1.31	0.98	0.22	0.05	0.02	0.00	0.00	0.00
5	F3	1.32	1.20	0.89	0.03	0.0	0.00	0.00	0.00	0.00
6	F4	2.03	2.10	1.65	1.31	1.09	0.43	0.22	0.21	0.08
7	F5	1.32	1.33	0.82	0.12	0.12	0.00	0.00	0.00	0.00
8	F6	1.87	1.64	0.99	0.02	0.00	0.00	0.00	0.00	0.00
9	F7	1.23	1.54	1.04	0.03	0.04	0.00	0.00	0.00	0.00
10	F8	1.45	1.21	1.97	1.33	1.09	0.84	0.32	0.00	0.00
11	F9	1.78	1.32	0.66	0.32	0.03	0.00	0.00	0.00	0.00
12	F10	1.98	1.43	0.45	0.54	0.02	0.01	0.01	0.00	0.00
13	F11	1.66	1.56	0.99	0.21	0.98	0.00	0.00	0.00	0.00
14	F12	1.65	1.44	0.97	0.25	0.19	0.04	0.02	0.00	0.00
15	F13	1.43	1.32	0.98	0.28	0.08	0.01	0.02	0.00	0.00
16	F14	1.01	1.70	0.94	0.31	0.04	0.01	0.02	0.00	0.00
17	F15	2.02	1.99	1.53	0.28	0.12	0.04	0.02	0.02	0.00
18	F16	1.49	1.44	0.98	0.32	0.03	0.04	0.01	0.05	0.00
19	F18	1.73	1.54	1.11	1.00	1.03	0.34	0.21	0.03	0.00
20	F19	1.55	1.23	0.88	0.28	0.03	0.02	0.01	0.00	0.00
21	F20	1.38	1.43	1.02	0.64	0.08	0.11	0.02	0.00	0.00
22	F21	1.34	1.54	0.97	0.32	0.21	0.01	0.00	0.00	0.00
23	F24	2.21	1.76	1.34	0.98	0.72	0.13	0.09	0.06	0.02
24	F23	0.78	0.30	0.43	0.06	0.02	0.01	0.01	0.00	0.00
25	F40	1.89	1.90	1.42	1.06	0.98	0.23	0.21	0.05	0.01

Mean and were calculated from three replicates of experiments for each strain

Table 2: DNA content of the parental and the selected fusants

Strain	DNA ng/µL
S.cerevisiae	389.54±14.76
P.stipitis	417.18±19.21
F4	442.27±16.03
F12	519.21± 13.03
F18	532.54±12.65
F22	512.23 ±12.32
F24	563.38±19.06

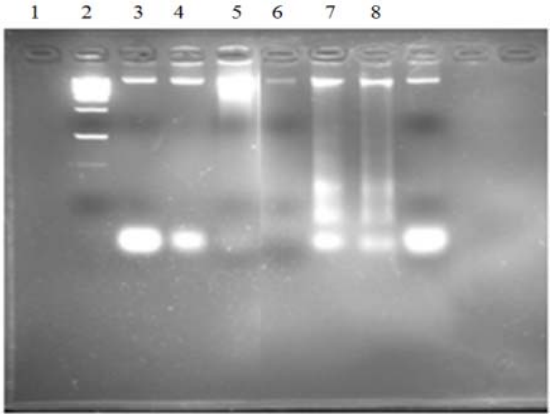


Fig. 6: Confirm DNA extracted on gel electrophoresis; 1: lader; 2: *S. cerevisiae*; 3: *P. stipitis*; 4-8 fusant F4, F12, F18, F22 and F24 resepctivly

concentration compared with the tolerance of the parent strains. Only three fusant yeast strains (i.e., F6, F21 and F23) have low ethanol tolerance (2% to 4%). ANOVA showed a significant difference in Re and Pa yields at $p<0.05$, according to Dujon *et al*. (2004).

DNA contents: Genomic DNA of *P. stipitis* and *S. cerevisiae* and the five selected yeast hybrids were extracted using by AMRESCO's Yeast Genomic DNA Purification Kit and were conformed through gel electrophoresis (Fig. 6). The DNA concentrations of *S.cerevisiae*, *P. stipitis*, F4, F12, F18, F22 and F24 strains were estimated (Table 2), the genomic size (Mb) of *S. cerevisiae* and *P. stipitis* were12.1 and 15.4 Mb, respectively. The DNA concentrations of *S. cerevisiae*, *P. stipitis*, F4, F12, F18, F22 and F24 strains were estimated (Table 2) in accordance with the absence of significant differences between the strains ($p<0.05$). The results indicated that fusant formation was confirmed by the increased DNA content. Thus, multiple fusions occurred. Moreover, addition of the whole chromosomes of parent strains did not occur through the protoplast fusion.

Genetic stability of the fusants: The genetic stability of fusants for 35 generations was determined and the ethanol tolerance and utilization of glucose and xylose were tested. After every 10 generations, a sample growth was plated and random colonies were selected from each strain. All generations showed similar tolerance and utilization as the initial strain. Thus, F24 and F18 are genetically stable and appropriate for industrial application.

CONCLUSION

The ethanol-producing ability of yeast reflects its intrinsic ability to tolerate ethanol. Protoplast fusion was employed to enhance the ethanol tolerance of yeasts. The results of this study suggested that protoplast fusion was an effective strategy to screen *S. cerevisiae* and *P. stipitis* fusants. Fusant strains, F4, F12, F18, F22 and F24, were able to tolerate a

maximum of 6% ethanol concentration in media. Fusant formation was confirmed by the increased DNA content; this increase indicates that multiple fusions occurred. Analysis of the genetic stability of fusants suggested that F24 and F18 are genetically stable. These two fusants have important properties that are beneficial in industrial ethanol production.

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