

HYPEROSMOTIC STRESS TOLERANCE OF TRANSCRIPTION ACTIVATOR Msn2-
OVEREXPRESSION STRAIN AND PROLINE-NO SYNTHESIS STRAIN OF *Saccharomyces*
cerevisiae IN VERY HIGH GRAVITY BIOETHANOL FERMENTATION

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ABSTRACT

The aim of this study was to assess the tolerance of two modified commercial *Saccharomyces cerevisiae* Ethanol Red, Pro1(I150T)/Mpr1(K63R) and MSN2-OP in hyperosmotic stress and to measure the kinetic parameters of the best yeast from both of them in the production of bioethanol in very high gravity ethanol fermentation. The results show that Pro1(I150T)/Mpr1(K63R) yeast strain is more tolerant to hyperosmotic stress than MSN2-OP. The Pro1(I150T)/Mpr1(K63R) yeast strain is tolerant up to 70% sucrose and 13% ethanol, while the MSN2-OP yeast strain has been inhibited by 70% sucrose and 11% ethanol. The Pro1(I150T)/Mpr1(K63R) yeast strain produces about $14.6 \pm 0.3\%$ (w/w) ethanol for 48 h fermentation with final residual sugar about 9.43% and $83.9 \pm 0.6\%$ substrate consumption efficiency.

Keywords: bioethanol, hyperosmotic stress, very high gravity fermentation

INTRODUCTION

Bioethanol is one of alternatives and renewable energy that is made from any biological materials which are readily available in nature, such as sugary and starchy materials, and also lignocellulosic biomass. Most bioethanol industries use sugary material like sugar cane or sugar beet in the form of juice or molasses as their raw material. Due to high concentration that sugar in molasses or juice makes, the industry has to dilute them until 15% to 20% (w/v) of total sugar to form a suitable growth condition for yeast and produce about 10% to 12% (v/v) ethanol. The diluting process is not only water and energy-wasting but also time consuming. Therefore, in order to make this process more efficient with low cost consumption and saving the processed water, it should implement potential energy saving, productivity and efficiency-maximizing technologies. One of the technologies is Very High Gravity (VHG) fermentation.

VHG increases ethanol productivity through fermenting medium containing sugar in exceeding of 250 g/l in order to achieve more than 15% (v/v) ethanol. However, that high concentration of sugar and ethanol accumulated in the media makes yeast cell is exposed to hyperosmotic stress. Hyperosmotic stress has been shown to induce Reactive Oxygen Species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radical and then oxidative stress is possibly imposed on cells indirectly (Giannattasio *et al.*, 2012).

A way to make yeast cell more tolerant to hyperosmotic stress is by activating the adaptation mechanism through genetically modified technique, like induction of genes involved in the synthesis and transport of osmolytes/ osmoprotectant, up-regulated the antioxidant enzyme to scavenge ROS (Brocker *et al.*, 2012), and up-regulated transcription factor/activator of stress induced genes. A study has constructed recombinant/self-cloned diploid baker's yeast with enhanced proline as osmoprotectant and synthesis of stress-induced Nitric Oxide (NO) which is associated with novel antioxidant mechanism by expressing Pro1(I150T) and Mpr1(F65L) genes (Sasano *et al.*, 2012a).

Another mechanism in response to hyperosmotic stress is over expression/up-regulated transcription factor/activator of stress-induced genes. An industrial Ethanol Red *Saccharomyces cerevisiae* with enhanced/overexpressed transcription factor Msn2 is tolerant to oxidative stress by reducing intracellular ROS level and enhancing the initial rate of fermentation when exposed to furfural as a lignocellulosic biomass fermentation inhibitor (Sasano *et al.*, 2012b). It is also used for industrial baker's yeast and it makes them tolerance to freeze thaw stress and has higher intracellular trehalose level (Sasano *et al.*, 2012c).

Based on that finding, we assessed and tested the tolerance of those two kinds of engineered *Saccharomyces cerevisiae*, Pro1(I150 T)/Mpr1 (K63R) and MSN2-OP in hyperosmotic stress

(high sugar and ethanol media) and measured the kinetic parameters of the most tolerant yeast in very high gravity ethanol fermentation.

MATERIALS AND METHODS

Yeast Strains: The strains used in this research were three strains of industrial/Ethanol Red (ER) *Saccharomyces cerevisiae* from Nara Institute of Science and Technology – Japan, namely ER wild type (unmodified yeast), ER Pro1(I150T)/Mpr1(K63R) and ER MSN2-OP. ER Pro1(I150T)/Mpr1(K63R) strain is a strain which has been modified in the 150th amino acid from isoleucine to threonine in *PRO1* gene and it has also been modified in the 63rd amino acid from lysine to arginine. While the MSN2-OP yeast strain is a strain which is inserted with a plasmid that carries *MSN2* gene with a constitutive promoter.

Media: Yeast cells were grown in YPD (Yeast Dextrose Peptone) medium (2% glucose, 2% Bacto™ peptone [Difco Laboratories, France], and 1% yeast extract), a synthetic minimal SD (Synthetic Defined) medium (2% glucose, 0.67% Bacto™ yeast nitrogen base without amino acids [Difco Laboratories, MI, USA]) and 2% agar was added to solidify medium.

High Sucrose Tolerance Test: This tolerance test conducted by spot test method in three YPD agar plates containing 50%, 60%, and 70% sucrose. Some μ l of yeast's subculture with initial $OD_{600} = 1$ was diluted until 10^{-4} . And then 2 μ l of samples from each dilution ($10^0 - 10^{-4}$) were spotted onto YPD containing 50%, 60%, 70% sucrose agar media and incubated at 30°C for 2-3 days. Every spot of yeast colony on YPD containing sucrose plate were counted and analyzed statistically. Each strain of yeast was tested in duplicate from two different single colonies.

Ethanol Stress Tolerance Test: This tolerance test conducted by spot test method in four YPD plates containing 11%, 12%, 13%, and 14% ethanol agar media. Some μ l of yeast's subculture with initial $OD_{600} = 1$ was diluted until 10^{-4} . And then 2 μ l of samples from each dilution ($10^0 - 10^{-4}$) were spotted onto YPD containing 11%, 12%, 13%, and 14% ethanol agar media and incubated at 30°C for 2-6 days. Every spot of yeast colony on YPD containing ethanol plate was counted and analyzed statistically. Each strain of yeast tested in duplicate from two different single colonies.

Cultivation and Kinetic Parameter Analysis:

The cultivation was done in 300 ml YPD containing 30% sucrose media, inoculated with

pre-culture of yeast with initial $OD_{600} = 0.25$, and incubated at 30°C on rotary shaker about 200 rpm for 48 h anaerobically. The culture was taken every 3 hours for 9 times and then it was taken every 6 hours for 4 times. All of the samples were used for biomass concentration analysis using UV-VIS spectrophotometer, ethanol concentration measurement using gas chromatography GC-17A Shimadzu LT-04-044 with FID as a detector and nitrogen as a mobile phase. Total sugar was analyzed by Anthrone method (Pons *et al.*, 1981). All data were used for kinetic parameters analysis including X_{max} (maximum of biomass in dry weight), P_{max} (maximum of ethanol production), μ_{max} (maximum of specific growth rate), yield of biomass per substrate consumption ($Y_{x/s}$), yield of product per biomass and substrate consumption ($Y_{p/s}$, $Y_{p/x}$) and also the substrate consumption efficiency. This cultivation and kinetic parameter were tested duplicate from two single different colonies of ER Pro1(I150T)/Mpr1(K63R) yeast strain and all the data were analyzed statistically.

RESULTS AND DISCUSSION

High Sucrose and Ethanol Stress Tolerance:

Hyperosmotic stress can be caused by high concentration of sugar and ethanol in the media. The high sugar concentration leads high water flux out of the cell resulting in cell shrinkage, reduction of cell volume, and loss of turgor. It also affects the concentration of protein, mitochondria depolarization, DNA damage, and cell cycle arrest which is affecting their growth, possibly by blocking the cell cycle at G1 or G2/M. High concentration of ethanol accumulated in the culture media inhibits cell growth, reducing cell viability and leading to cell death, and reduces ethanol fermentation rate and final yield (Ma and Liu, 2012). As a result, the process of fermentation is inhibited immediately once yeast growth is arrested by osmotic stress (Bai and Zhao, 2012).

In order to know the hyperosmotic stress tolerance of some genetically modified *Saccharomyces cerevisiae* in very high gravity ethanol fermentation, the growth inhibition of each strain of yeast was tested by counting the amount of cell when they were in exponential phase by spot test method. The tolerant yeast was the yeast with good growth and was not facing growth inhibition. In this study, the spot tests were conducted in YPD agar media containing 50% up to 70% sucrose and 11% up to 14% ethanol. The results of spot test are shown in *Fig. 1* and *Table 1*.

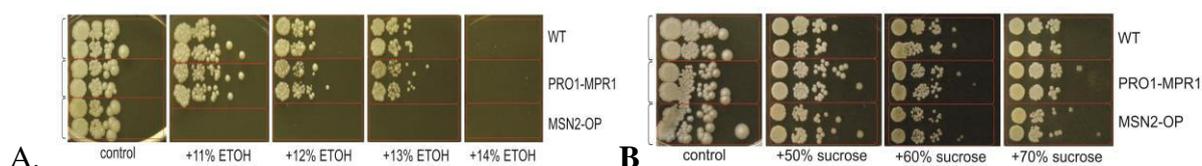


Fig 1: Spot test of 3 strains of yeast [WT = unmodified yeast, PRO1-MPR1 = Pro1(I150T)/Mpr1(K63R), MSN2-OP] in YPD agar containing (A) 50-70% sucrose and (B) 11-14% ethanol.

Table 1. Total colony of *Saccharomyces cerevisiae* in spot test ($\times 10^5$ CFU/ml)

Strain	Sucrose				Ethanol					
	control	50%	60%	70%	control	11%	12%	13%	14%	
Unmodified	4.0 \pm 2.1	5.3 \pm 0.4	5.3 \pm 0.4	5.3 \pm 3.1	4.3 \pm 1.8	2.5 \pm 1.4	1.2 \pm 0.2	1.2 \pm 0.3	0	
Modified	PM	5.5 \pm 0.7	4.8 \pm 1.0	4.8 \pm 1.0	5.3 \pm 2.5	3.0 \pm 0.0	4.8 \pm 2.5	2.9 \pm 2.3	1.9 \pm 2.1	0
	MS	4.0 \pm 0.7	4.3 \pm 0.4	4.3 \pm 0.4	1.3 \pm 0.4	1.1 \pm 0.1	0	0	0	0

*PM = Pro1(I150T)/Mpr1(K63R); MS = MSN2-OP

The growth of yeast in the spot test shows that MSN2-OP yeast strain has been inhibited in the media containing 70% sucrose and 11% ethanol. However, the Pro1(I150T)/Mpr1(K63R) yeast strain can grow in the media that contains up to 70% sucrose and 13% ethanol. It is proved by amount of yeast total colony (Table 1). So, it can be concluded that the Pro1(I150T)/Mpr1(K63R) yeast strain has higher tolerance to high sucrose and ethanol than MSN2-OP yeast strain. But if it is compared with the unmodified yeast in the tolerance of high sucrose, Pro1(I150T)/Mpr1(K63R) yeast strain is not significantly different. But in the media containing 13% ethanol, unmodified yeast has been inhibited and the Pro1(I150T)/Mpr1(K63R) yeast strain has higher tolerance than the unmodified strain.

It is probably because the Pro1(I150T)/Mpr1(K63R) has two mechanisms in stress response, osmoprotectant and antioxidant mechanism which can regulate ROS level that can damage nucleic acids, lipids and proteins and another cellular component. The osmoprotectant mechanism of Pro1(I150T)/Mpr1(K63R) yeast strain comes from proline accumulation [Pro1(I150T)]. Proline is known as osmoprotectant amino acid which can scavenge of ROS that prevents water flux out of the cell (Takagi, 2008).

Other stress response mechanism in Pro1(I150T)/Mpr1(K63R) yeast strain comes from mutated-MPR1 gene which is a novel anti-oxidant enzyme N-acetyltransferase Mpr1 with enhance its activity and stability [Mpr1(K63R)]. The N-acetyltransferase Mpr1 enzyme leads to stress-induced nitric oxide (NO) synthesis. In mammals, NO as signaling molecule is involved in stress tolerance through the activation of soluble guanylate cyclase and the post transcriptional modification such as S-nitrosylation (Sun *et al.*,

2006). Antioxidant enzyme N-acetyltransferase Mpr1 is shown to decrease the intracellular ROS levels when yeast cells are exposed to ethanol (Du and Takagi, 2007; Zhang *et al.*, 2012).

On the other hand, MSN2-OP yeast strain which accumulates transcription factor Msn2 is not more tolerant to hyperosmotic stress than Pro1(I150T)/Mpr1(K63R) and unmodified yeast strain. It was probably due to quantitative imbalance of the expression level between Msn2 and Msn4 in which Msn2 usually forms a heterodimer with Msn4 when cells are exposed to such stresses (Sasano *et al.*, 2012c).

Cultivation and Kinetic Parameter Analysis:

The most tolerant yeast and the optimum amount of sugar in media from previous test were used in cultivation and kinetic parameter analysis to know the characteristic in the formation of bioethanol. In this cultivation test, Pro1(I150T)/Mpr1(K63R) is cultivated to YPD media containing 30% (w/v) sucrose but the result of total sugar analysis shows that the YPD+30% sucrose media contains of 58.7% (w/v) total sugar in which the total sugar of YPD itself is 35.2% (w/v). The result of Pro1(I150T)/Mpr1(K63R) yeast strain in cultivation and kinetic parameter analysis is shown in Fig 2 and Table 2.

From the growth, residual total sugar, and ethanol production curve, the Pro1(I150T)/Mpr1(K63R) yeast strain has three phases of growth. The first is lag phase from 0-3 h. The second are logarithmic/exponential phase from 3-18 h which is the phase with high growth rate and reaches the maximum biomass at the point of 18 h. The third one is stationary and decreasing phase from 18-48 h in which the growth of yeast cells stopped and there is no increasing biomass value, although the ethanol concentration in the media still increased

and reached a maximum ethanol production on the point of 48 h. It is about 146 g/L or about 14.6% (w/w) with residual sugar about 94.4 g/L or about 9.4%.

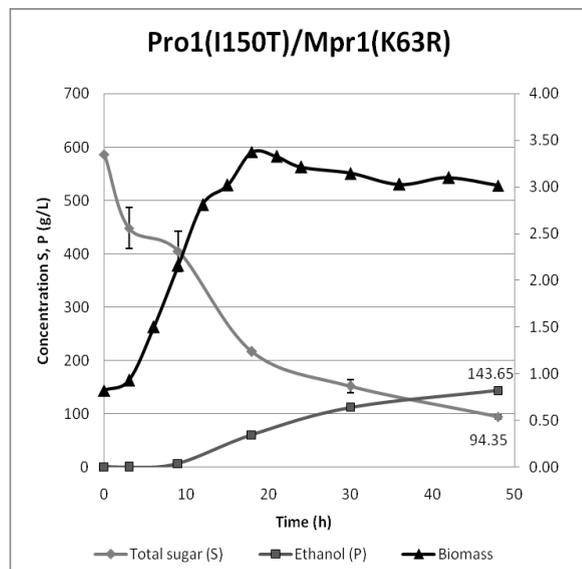


Fig 2. Residual total sugar, ethanol production, and growth curve of Pro1(I150T)/Mpr1(K63R) yeast strain.

Table 2: The amount of residual total sugar, ethanol production, and biomass of Pro1(I150T)/Mpr1(K63R) yeast strain.

Time	Total Sugar (g/L)	Biomass (g/L)	Ethanol (g/L)
0	586.70 ± 0.00	2.28 ± 0.00	0.60 ± 0.57
3	448.38 ± 38.86	2.54 ± 0.09	0.12 ± 0.00
9	405.00 ± 37.76	8.66 ± 0.08	7.15 ± 0.07
18	217.10 ± 0.57	29.25 ± 2.90	61.00 ± 1.41
30	152.05 ± 12.37	23.27 ± 2.76	109.80 ± 0.00
48	94.35 ± 3.75	20.46 ± 0.24	131.30 ± 14.14

Based on the growth data, some kinetic parameters of Pro1(I150T)/Mpr1(K63R) yeast strain was determined. The values of them were 0.05 g biomass/g substrate for $Y_{x/s}$, 0.3 g ethanol/g substrate for $Y_{p/s}$, 4.1 ± 0.4 g ethanol/g biomass for $Y_{p/x}$, 0.17/hour for μ_{max} , 32.5 ± 1.7 g/L for X_{max} , 146 ± 3.3 g/L for P_{max} , and 83.9 ± 0.6% for substrate consumption efficiency.

Rapid cultivation and high final ethanol concentration are crucial in the ethanol industry. From the result of this research, the ethanol concentration that is produced by Pro1(I150T)/Mpr1(K63R) yeast strain for 48 hours was high enough and efficient in substrate consumption but at the end of cultivation, the residual sugar on the media is still high. In another research, the maximum ethanol concentration that was produced from a media containing 300 g/L sugar by *Saccharomyces cerevisiae* was about 137

g/L with final residual sugar about 4.7 g/L and 32 ± 0.3% of cell viability (Zhang *et al.*, 2012). The cultivation of media containing 320 g/L glucose by Ethanol Red yeast about 7 days showed a result of ethanol concentration about 149.7 ± 2.94 g/L with $Y_{p/s}$ about 0.49 ± 0.01 g ethanol/g substrate (Snoek *et al.*, 2015). The cultivation of media containing 15-17% of total sugar in molasses by mutated Ethanol Red yeast for 16 hours at 32 ± 2°C showed a result of ethanol concentration that was produced about 8.8% (v/v) (Mukhtar *et al.*, 2010). Another research using Ethanol Red yeast in thick sugar beet juice media, produced ethanol concentration about 14.2 ± 0.4% (v/v) (Dziugan *et al.*, 2013).

CONCLUSION

The results of this study show that Pro1(I150T)/Mpr1(K63R) yeast strain is more tolerant to high sugar and ethanol concentration than MSN2-OP yeast strain. The maximum ethanol that Pro1(I150T)/Mpr1(K63R) produces in YPD+ 30% sucrose media is about 14.6 ± 0.3% (w/w) for 48 h fermentation with final residual sugar about 9.4% and 83.9 ± 0.6% substrate consumption efficiency. The Pro1(I150T)/Mpr1(K63R) yeast strain is recommended to be used in bioethanol industry to increase the ethanol production.

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