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Genetic Engineering of *Saccharomyces Cerevisiae* Using A Novel Approach Global Transcription Machinery Engineering for Enhancing Bioethanol Production

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***Abstract* — The purpose of our study was investigate from effect new mutations of *Saccharomyces cerevisiae* which possessing the ability to produce and enhance ethanol yield and characterized by ethanol tolerance which had been obtained by using the new protocol has been developed in our previous study. By molecular biology methods, such as Error-prone PCR, global transcription machinery engineering, DNA hybridization, etc. were used to construct an initial transcription factor and screen the target specific phenotype for obtaining mutations *SPT15-Mu* genes from the *Saccharomyces cerevisiae* R-control strain; which have an ability to produce and enhance ethanol yield and characterized by ethanol tolerance. The results showed more than 80 mutant genes were obtained with *SPT15* gene that diversely expressed when utilized $MnCl_2$. Mutants recorded the highest ethanol production by using 3 % $MnCl_2$ with improving the yield rate to 60.24 % compared to R-control strain. In conclusions, there are many vehicles that produced by metabolism resulting by the fermentation process; which are considered work as stress factors for yeast, leading to weaken their activities, and eventually cause cell loss. In our study, we managed to get random mutagenesis-Super; which were characterized by a high mutation rate, and simultaneously be stable in front of a lot of large compounds of metabolites produced by the fermentation process. Even be able to grow better than wild-type strain.**

***Keywords* — gTME, *Saccharomyces Cerevisiae*, *SPT15*, Ethanol Tolerance, Error-Prone PCR, Ethanol Production**

I. INTRODUCTION

The requirements of the *S. cerevisiae* to produce a high level of ethanol, is the ability to withstand elevated concentration of ethanol and sugar containing in the fermentation medium. These cellular characteristics are important because very high gravity (VHG) fermentations increases high sugar concentrations (and thus increase the level of osmotic pressure) at the beginning of the process; whereas at the end of batch fermentation, the ethanol concentration will be high which is common among bioethanol production [1-3]. The gTME is a novel technique developed by Alper and Gregory ten years ago aiming to obtain new metabolic phenotypes in microorganisms in a fast and accurate manner. Additionally, it can be used to improve traits in more complex organisms. Moreover, it provides complex phenotypes that are not readily accessible by traditional methods [4].

The gTME was a successful technique in solution to difficulties of metabolic and genetic engineering during the production of new metabolic phenotypes that are functional and optimized [3]. The fundamental principle of the gTME technique is to re-program the whole transcriptome profile of a cell through editing of the TATA binding protein (TBP) encoded by *SPT15* and/or *TAF23* and obtaining of mutants with specific phenotypic changes [3,5]. The gTME uses molecular biology methods such as error-prone PCR (Ep-PCR), DNA shuffling, etc. to construct an initial transcription factor and screen the target phenotype to obtain the enhanced metabolic flux or bacteria with specific phenotype. It produces most of the functional genes, while RNA polymerase II transcription efficiency is determined by initiation of transcription factors and promoter binding protein, and one of the first transcription factors in *S. cerevisiae* is TBP. The *SPT15* and *TAF23* are related to the promoter region of TBP capacity changes and affects the efficiency of gene expression [6,7].

In this study, the SPT15 and TAF23 mutant genes was amplified from the non-mutated diploid cells, and a new mutant S. cerevisiae strain (harboring mutated genes) of the type diploid were screened using gTME method. As indicated by some previous studies, more than one hundred genes were found to be diversely expressed when was utilized this technique [3,8].Therefore, gTME is considered as a method which helps to get complex phenotypes that are not readily accessible by traditional methods. It is commonly utilized in the production of many enzymes and chemical compounds, and most importantly ethanol, which enters in the cosmetic and pharmaceutical industry, through which can be employed for other genes via an iterative procedure comprising recombinant generation [9]. This study aims to improve of S. cerevisiae strain through generating new mutations of specific genes using the cellular engineering technique which relies on minor change(s) at interior proteins by using (Ep-PCR). It showed a significant role in regulating and generating a library of mutations of S. cerevisiae strain with high yield of Ethanol using high-throughput From that reason, this technique is

considered as a new method of diversity at the transcriptional engineering level.

In our study, we have been modified the idea of protocol Error-Prone polymerase chain reaction to making more efficient, then used Global Transcription Machinery Engineering (gTME) technique to obtain for construct stable mutations of Saccharomyces cerevisiae. This leads to possess the ability to produce and enhance ethanol yield and characterized by ethanol tolerance**.**

II. MATERIALS AND METHODS

A. Microbial strains and culture media

The *S. cerevisiae* strain was used for genetic manipulation and cultured in basic medium yeast extract peptone dextrose broth (YEPD) medium; this medium was used for routine growth of yeast strains at 30 °C and contained 10 g/l yeast extract, 20 g/l Bacto-peptone, and 20 g/l glucose. The *Escherichia coli* JM109 (*E. coli*) was used as a host for plasmid construction. Moreover, *E. coli* JM109 was grown in super optimal broth (SOB) containing 5 g/l yeast extract, 20 g/l Bacto-peptone, 0.95 g/l $MgCl_2$, 0.186 g/l KCl, and 0.5 g/l NaCl was used before spreading on solid media after transformation and cultured at 37 °C. Solid media contained 2 % agarfor both types of mediums. Respective antibiotics [Ampicillin (Amp) 100 mg/l, kanamycin (Kan) 35 mg/l] were added to maintain the plasmids [10]. For screening, the medium (YEPDG) containing 10 g/l yeast extract, 20 g/l Bacto-peptone, 20 g/l glucose; Glycosid-418, (sulfate salt, Sangon Biotech, China) (G418) resistance gene was added with the final concentration (250 and 350 µg/ml), respectively [11]. The Fermentation medium was YEPDT: 10 g/l yeast extract, 20 g/l Bacto-peptone, 20 g/l glucose, and 0.2 g/l Thiamine. Incubation conditions were standardized on the rotary shaker at 30 °C with 200 rpm. All the media contained glucose as sole carbon source. The strains utilized as a part of this study are shown in Table 1.

B. Reagents

For detection and isolation of yeast gDNA fragments, Mini-DNA fragment Rapid Kit (BioSCi Biotech Co., LTD, Hangzhou, China) was used. Plasmid DNA was extracted by using a Plasmid Mini-Preps Kit (BioSCi Biotech Co., LTD, Hangzhou, China). The (dGTP, dNTP, dCTP, and dTTP) both of individually and rTaq DNA polymerase enzymes were purchased from (TaKaRa, Japan). Enzyme (2×Pfu PCR Mix) was purchased from (BIO SCI Biotech Co., LTD, HANGZHOU, CHINA). Therestriction enzymes, T4 DNA ligase and other enzymes were purchased from Thermo Scientific. Agarose gel purification of DNA fragments was performed using the TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa, Japan).The vector (pMD19-T) purchased from (TaKaRa, Japan). Finally, genes sequencing were by Sangon Biotech, Shanghai Co., Ltd, China.

C. Studies to verify stability and similarity for SPT15-Mu and TAF23-Mu genes to survive after transformation of into S. cerevisiae

After transformation of the yeast; 20 colonies were selected for the screening as a random mutation. In order to ensure the stability test of each colony, 100 µl were plated on YEPD solid medium in triplicate with different concentration of Kanamycin. The colonies which characterized by good viability among the grown colonies were transferred on YEPD containing G418 (250 µg/ml) and incubated at 30 °C until single colonies appeared. After that, 5 big and single colonies were picked up and re-grown on a fresh YEPDG medium containing G418 (350 µg/ml); then incubated at 30 °C for 2days. Afterward, the resistant colonies were streaked out [12,13]. Finally, colony PCR was performed to verify whether the mutations were successful. The PCR products were loaded on 1.5 % Agarose and visualized to make sure the criteria of the stable genes selection.

C. Investigation of Ethanol tolerance of S. cerevisiae mutant strains

In order to test the ability of the 102 mutations in this study which harboring *SPT15* genes. The ethanol tolerance test was carried out by spot assay in duplicate onto YEPD plates containing Kan 35 mg/l within various concentration of ethanol 1, 3, 5, 7, 10 and 15 % (v/v) respectively. The colonies forming ability and viability in high level of ethanol were monitored after incubation at 30 °C for 3days [14].

E. The concentration of ethanol and glucose by SPT15-Mu aerobic fermentation

Fermentation was carried out in 500 ml conical flasks containing 50 ml fermentation medium as followed: 20 g/l glucose, 10 g/l yeast extract, 20 g/l Bacto-peptone and 0.2 g/l thiamine (vitamin B1) [15,16] and 5 ml of inoculum were added. The mixture was cultured at 30 °C for 3 days.

F. High performance liquid chromatography analysis

The broth samples were centrifuged at 12,000 rpm for 20 min then were filtered through a 0.22 µm filter. The appropriately diluted supernatant by 10 % of Trichloro acetic acid (TDA) (1:1) was used for the product analysis. The concentrations of the samples were estimated by high-performance liquid chromatography (HPLC) analysis, with a Shodex RI SUGER SH-1011 HPLC column (7 µm, 8 I.D. × 300 mm) (Showa Denko Co., Ltd., K.K., Japan). The column temperature was heated at 50 °C with 0.01 M H2SO4 as the mobile phase and flow rate 0.8 ml/min. The concentrations were subsequently detected with a refractive index detector with 285 nm wave length (HITACHI High Technologies Co., Ltd., Tokyo, Japan) Model (CM 5110/5210/5310/5430/5450) to estimate the percentage of both glucose and ethanol in the samples.

The HPLC analysis was performed by isocratic condition using 1 % Sulfuric acid in water (v/v) as mobile phase. The product was eluted around 22 minutes for a total running time 30 minutes. All HPLC data were analyzed with EZChrom Elite Version 3.3.2 SP2 Chromatography Data System, Agilent Tech software.

The percentage of the ethanol yield production between the mutant strains and control strain was estimated by using the following equation:

$PRI= [(EP_2/EP_1)*100]-100$ (1)

Where, The PRI: percentage of the rate of increase; EP1: Ethanol production for mutant strains; EP2: Ethanol production for control strain.

Table 1: Microbial strains and plasmids used in this study

Strains / Plasmids	Relevant characteristics	Source / reference
Plasmids		
pYX212	$Amp^{R,}$ TPI promoter	This study
pYX212-kan-*SPT15-Mu*	pYX212 with *SPT15* mutant gene	This study
pYX212-kan-*TAF23-Mu*	pYX212 with *TAF23* mutant gene	This study
pMD19-T vector	$Amp^{R,}$ clone vector	TaKaRa,Japan
Strains		
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (Lac-proAB) F'[traD36 proAB$^+$ lacI^q lacZM15]*	Stratagene
S. cerevisiae Haploid strain	Wild-type (*MAT*- α) CICC 1374	China Center of Industrial Culture Collection (CICC)
S. cerevisiae Haploid strain	Wild-type (*MAT*- a) CICC 31144	
*Mutant Strains**		
S. cerevisiae R-Control strain	Diploid strain	This study
R-M20C1-P3	Ep-PCR process with 3% $MnCl_2$	This study
R-M3C49-P10	Ep-PCR process with 10% $MnCl_2$	This study
R-M17C4-P20	Ep-PCR process with 20% $MnCl_2$	This study

Amp, Ampicillin; Kan, kanamycin
*High bioethanol-producing mutant strains from each concentration

III. Results and Discussions

A. *Studies to verify stability and similarity for SPT15-Mu and TAF23-Mu genes to survive after transformation of into S. cerevisiae*

Both *SPT15* and *TAF23* genes were ligated with PYX212 vector individually by using T4 DNA ligase. The resultant after the processes of ligation was named PYX212-*SPT15-Mu* and PYX212-*TAF23-Mu*, respectively.

Herein, 5 replicates of the 6 *TAF23* mutants were tested for the stability to G418 resistance gene, however they weren't able to resist and grow on the concentration 250 µg/ml of G418. In addition, 5 replicates of *SPT15* mutations by a total number of 250 were selected, and their stability tests were examined. These mutations were subjected for the stability to G418 resistance gene at two different concentrations 250 µg/ml, and 350 µg/ml respectively [17], the obtained results were used as an indicator for those strains with high stability to the resistance gene (Fig. 1, Table 2). Subsequently, the normal PCR was conducted for these strains in order to amplify the successful resistance genes with concentrations of G418 to identify the extent of their activities and the stability for those mutations.

Afterthought, we were able to elect the best 102 mutants with *SPT15* gene of the total selected 250 mutants as shown in Table 2. It was shown that a total of 14, 24, 37 and 27 mutations were successfully elected at 1, 3, 10 and 20 % of $MnCl_2$, respectively.

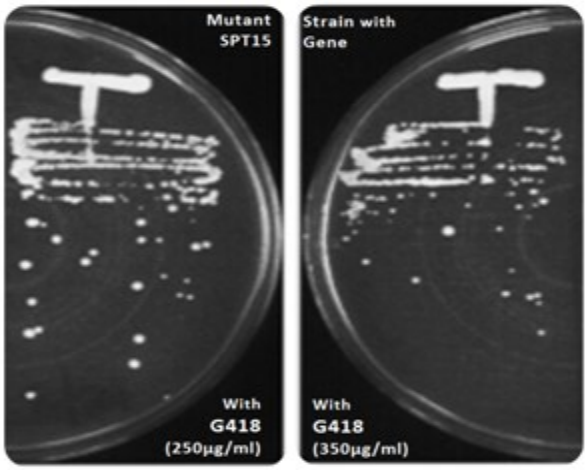

Figure 1: Mutant strain *S. cerevisiae* at morphological forming and test the stability under stress of G418 resistance (250µg/ml, 350µg/ml), respectively

G418 resistance could simplify the selection of stable transfected cell lines that reflected the characteristics of pYX212-*SPT15-Mu* and pYX212-*TAF23-Mu* at the expression of the stabilized genes which were not lost over time. The higher concentrations of G418 (350 µg/ml) enhanced the stability of mutant colonies at adverse pressure conditions that produced higher copy of mutations number. These results are similar to the finding reported by [22].

Table 2: Summarize the tests of stability and similarity

Strains	Colonies selected for stabilitytests	Grown on YEPD with G418 (250 µg/ml)	Grown on YEPD with G418 (350 µg/ml)	After Collected DNA & Normal PCR	Successful mutation	M.C.S**	
R-M5-P1	C2 C3 C7 C11 C15	C2 C3 C7 C11 C15	C2 C3 - C11 C15	C2 C3 - - C15	C2,C3, C15	C2,C3	
R-M7-P1	C1 C2 C3 C9 C13	C1 -* C3 C9 -	C1 - C3 C9 -	C1 - C3 - -	C1, C3	C1, C3	
R-M10-P1	C2 C5 C12 C22 C24	C2 C5 C12 C22 C24	C2 C5 C12 C22 C24	C2 C5 C12 C22 C24	C2,C5,	C2,C5,C12 C22,C23,	

R-M12-P1	C1	C2	C3	C5	C10	C1	C2	C3	C5	C10	C1	C2	C3	C5	-	C1	C2	C3	C5	-	C12,C22, C24 C1,C2, C3,C5	C24 C1,C2,C3, C5
Total Mutant colonies which were selected with 1 % $MnCl_2$ concentration																					14 Mutant colonies	
R-M1-P3	C1	C4	C6	C7	C8	C1	C4	-	C7	C8	C1	C4	-	-	C8	C1	C4	-	-	C8	C1,C4,C8	C1
R-M4-P3	C1	C2	C3	C4	C5	C1	-	C3	C4	C5	C1	-	-	C4	C5	C1	-	-	-	C5	C1,C5	C1,C5
R-M5-P3	C1	C2	C3	C4	C6	C1	-	C3	C4	C6	C1	-	C3	C4	-	C1	-	C3	C4	-	C1,C3,C4	C1,C3,C4
R-M7-P3	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	-	C3	C4	C5	C1	-	C3	C4	C5	C1,C3,C4 ,C5	C1,C4
R-M8-P3	C4	C6	C7	C8	C9	C4	C6	-	C8	C9	C4	C6	-	C8	-	C4	-	-	-	-	C4	C4
R-M10-P3	C2	C4	C5	C7	C8	C2	C4	C5	C7	C8	-	C4	C5	-	C8	-	-	-	-	-	A.M**	A.M
R-M12-P3	C1	C3	C4	C8	C9	C1	C3	C4	C8	C9	-	-	C4	C8	C9	-	-	C4	C8	C9	C4,C8,C9	C4
R-M14-P3	C1	C2	C6	C7	C8	C1	C2	C6	C7	-	-	C2	C6	C7	-	-	C2	C6	C7	-	C2,C6,C7	C2
R-M15-P3	C1	C2	C3	C4	C7	C1	C2	C3	-	C7	C1	C2	C3	-	C7	C1	C2	C3	-	-	C1,C2,C3	C1,C2,C3
R-M16-P3	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1,C2,C4, C5	C1,C2,C4, C5
R-M17-P3	C2	C3	C4	C7	C9	C2	C3	C4	C7	C9	-	C3	C4	C7	-	-	C3	C4	-	-	C3,C4	C3,C4
R-M18-P3	C1	C2	C3	C4	C6	C1	C2	C3	C4	C6	C1	-	C3	C4	C6	C1	-	C3	C4	-	C1,C3,C4	C3,C4
R-M19-P3	C1	C3	C5	C7	C8	C1	C3	C5	C7	-	-	C3	C5	C7	-	-	C3	-	-	-	C3	C3
R-M20-P3	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	C1	-	C3	C4	-	C1	-	C3	-	-	C1, C3	C1
Total Mutant colonies which were selected with 3 % $MnCl_2$ concentration																					24 Mutant colonies	
R-M1a-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	C1	C2	C3	-	-	C1	C2	-	-	-	C1,C2	C1
R-M2a-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	-	C2	-	C4	-	-	C2	-	C4	-	C2,C4	C2,C4
R-M1b-P10	C1	C2	C3	C4	C5	C1	-	C3	C4	C5	C1	-	C3	C4	C5	C1	-	-	C4	C5	C1,C4,C5	C1,C4
R-M2b-P10	C1	C6	C7	C9	C10	C1	C6	C7	C9	C10	C1	C6	C7	C9	C10	C1	C6	C7	-	C10	C1,C6,C7, C10	C6
R-M3-P10	C45	C46	C47	C48	C49	C45	C46	C47	C48	C49	C45	C46	C47	C48	C49	C45	C46	C47	C48	C49	C45,C46, C47 C48,C49	C45,C46, C49
R-M4-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	C1	C2	C3	C4	-	C1	C2	C3	C4	-	C1,C2,C3, C4	C1,C2,C3
R-M5-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	C1	-	C3	C4	-	C1	-	C3	-	-	C1,C3	C1,C3
R-M6-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	C1,C2,C3, C4	C2,C3
R-M8-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	C1	C2	C3	C4	-	C1	-	C3	C4	-	C1,C2,C3, C4	C1,C3,C4
R-M9-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	-	C4	C5	C1	C2	-	C4	-	C1,C2,,C4	C2,C4
R-M10-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	C1	C2	C3	C4	-	C1,C2, C3,C4	C1,C2,C3 ,C4
R-M14-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	-	C2	C3	C4	C5	-	C2	C3	C4	-	C2,C3,C4	C2,C3,C4
R-M15-P10	C1	C2	C3	C4	C5	C1	C2	C3	-	C5	C1	C2	C3	-	-	C1	-	C3	-	-	C1,C2,C3	C1,C3
R-M18-P10	C1	C2	C3	C4	C5	C1	C2	C3	-	C5	C1	C2	C3	-	C5	C1	C2	C3	-	C5	C1,C2, C3,C5	C1,C3,C5
R-M19-P10	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	C1	C2	C3	C4	-	C1,C2, C3,C4	C1,C2, C3,C4
Total Mutant colonies which were selected with 10 % $MnCl_2$ concentration																					37 Mutant colonies	
R-M2-p20	C1	C2	C3	C4	C5	-	C2	C3	C4	C5	-	C2	C3	C4	C5	-	-	C3	C4	C5	C3,C4,C5	C4,C5
R-M4-p20	C1	C2	C3	C4	C5	-	C2	C3	-	C5	-	-	C3	-	C5	-	-	-	-	C5	A.M***	A.M
R-M5-p20	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	C4	-	C1	-	C3	C4	-	C1,C3,C4	C1
R-M6-p20	C1	C2	C3	C5	C8	C1	C2	C3	C5	C8	C1	C2	C3	C5	C8	C1	C2	C3	C5	-	C1,C2,C3 C5	C1,C2,C5
R-M8-p20	C1	C2	C3	C4	C5	C1	-	C3	C4	-	-	-	C3	C4	-	-	-	C3	C4	-	C3,C4	C3,C4
R-M9-p20	C1	C2	C3	C4	C5	C1	C2	-	C4	C5	C1	C2	-	C4	-	C1	C2	-	-	-	C1,C2	C1,C2
R-M10-p20	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	-	C2	C3	C4	C5	-	C2	C3	C4	C5	C2,C3, C4,C5	C2,C3
R-M12-p20	C1	C2	C3	C8	C9	C1	C2	C3	C8	C9	-	C2	C3	C8	C9	-	-	-	C8	C9	C8,C9	C8,C9
R-M13-p20	C1	C2	C3	C4	C5	C1	C2	-	C4	C5	C1	C2	-	-	C5	C1	C2	-	-	C5	C1,C2,C5	C2
R-M14-p20	C1	C2	C3	C5	C6	-	C2	C3	C5	C6	-	-	C3	C5	C6	-	-	-	C5	C6	C5,C6	C5,C6
R-M15-p20	C1	C2	C3	C4	C5	C1	C2	-	C4	-	-	C2	-	C4	-	-	C2	-	-	-	C2	C2
R-M16-p20	C1	C2	C3	C4	C5	C1	-	C3	-	-	C1	-	-	-	-	C1	-	-	-	-	C1	C1
R-M17-p20	C1	C2	C3	C4	C5	-	-	-	C4	C5	-	-	-	C4	-	-	-	-	C4	-	C4	C4
R-M18-p20	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	-	C2	C3	C4	C5	-	C2	C3	C4	-	C2,C3,C4	C3,C4
R-M19-p20	C1	C2	C3	C4	C5	C1	C2	-	C4	C5	C1	-	-	C4	C5	C1	-	-	C4	C5	C1,C4,C5	C4,C5
R-M20-p20	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C1	C2	C3	-	C5	C1	-	C3	-	C5	C1,C3,C5	C1,C5
Total Mutant colonies which were selected with 20 % $MnCl_2$ concentration																					27 Mutant colonies	

Where: (C1, C2, C3,….. etc.) referring to numbering of colony *Loss of colony growth in this test; ** Mutant colonies which were selected; * Absent mutations**

B. Test of Ethanol tolerance for mutant strains

This genetic screen uses a strain that expresses the mutated versions of the protein. Thus, permits the identification of dominant mutations that lead to novel function to improve ethanol production. These colonies which carrying the new mutants *SPT15* gene when compared with control strain, showed slight variations in the improvement of colonies formation within the media containing 1, 3, 5 and 7 % (v/v) of ethanol. Conversely, it was found that the formation and composition of the colonies mutant strains in the presence of 10 % ethanol were weak compared to the low concentrations, while the media containing 15 % (v/v) ethanol didn't show any growth of colonies (Fig. 2).

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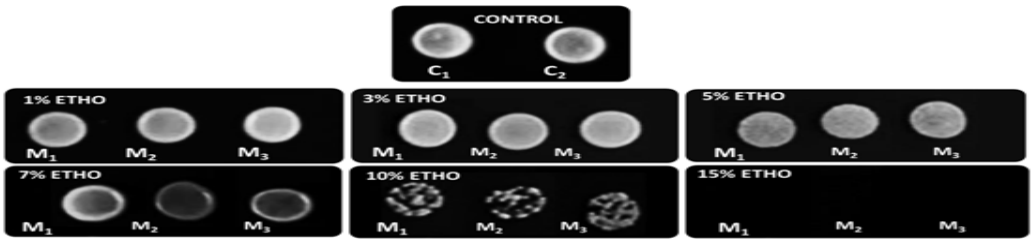

Figure 2: Susceptibility of ethanol tolerance of *S. cerevisiae* mutant strains with various levels of ethanol concentrations (1, 3, 5, 7, 10 and 15%) on YEPD media containing Kan 35mg/l. Where (C_1, C_2) refers to the numbering of control colonies. Moreover, (M1): R-M20C1-P3; (M2): R-M3C49-P10; (M3): R-M17C4-P20
, mutations colonies

C. The concentration of ethanol and glucose by SPT15-Mu aerobic fermentation

In order to estimate the efficiency of the different mutants for the production of ethanol, high performance liquid chromatography was used in this study. During the aerobic fermentation processes, the production of ethanol in the yeast expressing *SPT15* increased by 5.91 %, while the glucose decreased by 0.19 % compared to the control strain. With the addition of 3 % $MnCl_2$, all the mutants showed higher ethanol production from 14.11 to 15.72 % compared to the control which produced 9.8 % ethanol, and the other used mutants with 1, 10, and 20 % concentrations of $MnCl_2$ (Table 3, 4). The R-M20C1-P3 mutant strain reported the highest ethanol production by yielding 15.72 % with a 0.47 % reduction in glucose content. While, by using 1 % of $MnCl_2$, the production of ethanol was less than the other mutants resulted by using the other concentrations.

Table 3: Summarizes mutant's efficiency for the production of ethanol; which were obtained from strains which carrying for mutant gene SPT15, after the process of aerobic fermentation for them.

Control	1 % $MnCl_2$		3 % $MnCl_2$		10 % $MnCl_2$		20 % $MnCl_2$	
ETHO g/l	Mutant Strains	ETHO g/l	Mutant Strains	ETHO g/l	Mutant Strains	ETHO g/l	Mutant Strains	ETHO g/l
9.81	R-M7C3-P1	12.65	R-M20C1-P3	15.72	R-M17C4-P20	13.49	R-M3C49-P10	14.04
	R-M12C1-P1	12.64	R-M16C4-P3	15.47	R-M9C2-P20	13.46	R-M18C5-P10	14.01
	R-M12C3-P1	12.57	R-M4C1-P3	15.47	R-M20C1-P20	13.42	R-M3C46-P10	14.01
	R-M12C5-P1	12.48	R-M12C4-P3	15.32	R-M14C5-P20	13.41	R-M6C2-P10	13.99
	R-M5C3-P1	12.48	R-M18C4-P3	15.29	R-M16C1-P20	13.36	R-M4C1-P10	13.98
	R-M12C2-P1	12.39	R-M5C1-P3	15.21	R-M10C2-P20	13.35	R-M5C1-P10	13.95
	R-M7C1-P1	12.34	R-M18C3-P3	14.83	R-M2C4-P20	13.28	R-M14C4-P10	13.94
	R-M10C12-P1	12.34	R-M17C3-P3	14.75	R-M18C3-P20	13.27	R-M19C1-P10	13.94
	R-M5C2-P1	12.30	R-M8C4-P3	14.74	R-M12C8-P20	13.25	R-M19C3-P10	13.93
	R-M10C24-P1	11.97	R-M4C5-P3	14.68	R-M6C2-P20	13.23	R-M10C4-P10	13.92
	R-M10C2-P1	11.70	R-M15C2-P3	14.66	R-M19C1-P20	13.19	R-M9C4-P10	13.90
	R-M10C5-P1	11.69	R-M16C5-P3	14.65	R-M6C5-P20	13.19	R-M10C2-P10	13.89
	R-M10C23-P1	11.62	R-M16C2-P3	14.63	R-M20C2-P20	13.15	R-M19C2-P10	13.89
	R-M10C22-P1	11.02	R-M16C1-P3	14.40	R-M6C8-P20	13.11	R-M10C1-P10	13.82
	R-M10C10-P1	10.84	R-M19C3-P3	14.35	R-M10C3-P20	13.11	R-M9C2-P10	13.81
			R-M17C4-P3	14.33	R-M19C5-P20	13.02	R-M8C4-P10	13.77
			R-M1C1-P3	14.29	R-M6C1-P20	13.02	R-M4C2-P10	13.76
			R-M7C1-P3	14.13	R-M5C1-P20	12.99	R-M18C3-P10	13.72
			R-M7C4-P3	14.12	R-M8C3-P20	12.92	R-M15C3-P10	13.71
			R-M14C2-P3	14.11	R-M15C2-P20	12.90	R-M14C2-P10	13.70
					R-M18C4-P20	12.85	R-M1''BC1-P10	13.68
					R-M14C6-P20	12.79	R-M1'C1-P10	13.67
					R-M9C1-P20	12.78	R-M19C4-P10	13.67
					R-M12C9-P20	12.78	R-M2'C2-P10	13.65
					R-M2C5-P20	12.66	R-M8C1-P10	13.64
							R-M4C3-P10	13.56
							R-M18C1-P10	13.54
							R-M1''B C4-P10	13.52
							R-M14C3-P10	13.52
							R-M6C3-P10	13.51
							R-M3C45-P10	13.50
							R-M2''C6-P10	13.50
							R-M8 C3-P10	13.49
							R-M15C1-P10	12.37
Total Control		15 mutants Strain		20 mutants Strain		25 mutants Strain		34 mutants Strain

Table 4: Summarizes mutant's efficiency for the residual of glucose; which were obtained from strains which carrying for mutant gene SPT15, after the process of aerobic fermentation for them.

Control	1 % $MnCl_2$		3 % $MnCl_2$		10 % $MnCl_2$		20 % $MnCl_2$	
Residual GLU g/l	Mutant Strains	Residual GLU g/l	Mutant Strains	Residual GLU g/l	Mutant Strains	Residual GLU g/l	Mutant Strains	Residual GLU g/l
0.28	R-M7C3-P1	0.39	R-M20C1-P3	0.47	R-M17C4-P20	0.41	R-M3C49-P10	0.38
	R-M12C1-P1	0.33	R-M16C4-P3	0.38	R-M9C2-P20	0.44	R-M18C5-P10	0.43
	R-M12C3-P1	0.40	R-M4C1-P3	0.38	R-M20C1-P20	0.37	R-M3C46-P10	0.37
	R-M12C5-P1	0.33	R-M12C4-P3	0.35	R-M14C5-P20	0.31	R-M6C2-P10	0.41
	R-M5C3-P1	0.44	R-M18C4-P3	0.41	R-M16C1-P20	0.44	R-M4C1-P10	0.41
	R-M12C2-P1	0.31	R-M5C1-P3	0.34	R-M10C2-P20	0.44	R-M5C1-P10	0.35
	R-M7C1-P1	0.37	R-M18C3-P3	0.43	R-M2C4-P20	0.41	R-M14C4-P10	0.41
	R-M10C12-P1	0.30	R-M17C3-P3	0.44	R-M18C3-P20	0.43	R-M19C1-P10	0.41
	R-M5C2-P1	0.34	R-M8C4-P3	0.39	R-M12C8-P20	0.29	R-M19C3-P10	0.35
	R-M10C24-P1	0.34	R-M4C5-P3	0.47	R-M6C2-P20	0.42	R-M10C4-P10	0.40
	R-M10C2-P1	0.37	R-M15C2-P3	0.36	R-M19C1-P20	0.37	R-M9C4-P10	0.37
	R-M10C5-P1	0.37	R-M16C5-P3	0.19	R-M6C5-P20	0.42	R-M10C2-P10	0.43
	R-M10C23-P1	0.28	R-M16C2-P3	0.39	R-M20C2-P20	0.39	R-M19C2-P10	0.33
	R-M10C22-P1	0.31	R-M16C1-P3	0.20	R-M6C8-P20	0.43	R-M10C1-P10	0.40
	R-M10C10-P1	0.30	R-M19C3-P3	0.39	R-M10C3-P20	0.42	R-M9C2-P10	0.44
			R-M17C4-P3	0.29	R-M19C5-P20	0.38	R-M8C4-P10	0.39
			R-M1C1-P3	0.37	R-M6C1-P20	0.32	R-M4C2-P10	0.42
			R-M7C1-P3	0.39	R-M5C1-P20	0.34	R-M18C3-P10	0.38
			R-M7C4-P3	0.39	R-M8C3-P20	0.32	R-M15C3-P10	0.39
			R-M14C2-P3	0.21	R-M15C2-P20	0.39	R-M14C2-P10	0.34
					R-M18C4-P20	0.35	R-M1''BC1-P10	0.44
					R-M14C6-P20	0.35	R-M1'C1-P10	0.44
					R-M9C1-P20	0.35	R-M19C4-P10	0.39
					R-M12C9-P20	0.37	R-M2'C2-P10	0.38
					R-M2C5-P20	0.35	R-M8C1-P10	0.38
							R-M4C3-P10	0.38
							R-M18C1-P10	0.38
							R-M1''B C4-P10	0.39
							R-M14C3-P10	0.44
							R-M6C3-P10	0.39
							R-M3C45-P10	0.38
							R-M2''C6-P10	0.36
							R-M8 C3-P10	0.42
							R-M15C1-P10	0.34
Total Control	15 mutants Strain		20 mutants Strain		25 mutants Strain		34 mutants Strain	

IV. Conclusion

In conclusion, the genetic results revealed that the gTME technique could be an effective approach for construction of novel mutants under various external stresses. The gTME technique to *S. cerevisiae* has been performed to adapt its attitude towards higher concentrations of ethanol. All the examined mutants showed much better tolerance toward ethanol stress as compared to the control. The mutants resulted by using 3 % of $MnCl_2$ in the process of Ep-PCR recorded the highest ethanol production.

V. Future Prospects

In a prospective study, we seek to establish a basis for future industrial applications, through integrating the *SPT15* mutant alleles of two new mutant strains into the chromosomes, to enhance ethanol tolerance and survive within high concentration ethanol media.

APPENDIX

ABBREVIATION:

gTME	Global Transcription Machinery Engineering
E. coli	*Escherichia coli*
S. cerevisiae	*Saccharomyces cerevisiae*
VHG	very high gravity
Amp	Ampicillin
Kan	kanamycin
G418	Glycosid-418 resistance gene
LB	Luria-Bertani medium
SOB	Super optimal broth medium
YEPD	Yeast extract peptone dextrose broth medium
TBP	TATA-binding protein
gDNA	genomic DNA
OD	Optical density
FDB	fast digest buffer
PCR	polymerase chain reaction
Ep-PCR	Error prone polymerase chain reaction
MAT- α	*MAT*-alpha
GDNC	Genome Database and the National Center
HPLC	High liquid performance chromatography
MCS	Mutant colonies which were selected
AM	Absent mutations
ETHO	Ethanol
GLU	Glucose
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese chloride

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REFERENCES

[1] Thatipamala R, Rohani S, Hill G (1992) Effects of high product and substrate inhibitions on the kinetics and biomass and product yields during ethanol batch fermentation. Biotechnology and bioengineering 40 (2):289-297.

[2] Bai F, Chen L, Zhang Z, Anderson W, Moo-Young M (2004) Continuous ethanol production and evaluation of yeast cell lysis and viability loss under very high gravity medium conditions. Journal of Biotechnology 110 (3):287-293.

[3] Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G (2006) Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314 (5805):1565-1568.

[4] Goryanin I, Hodgman TC, Selkov E (1999) Mathematical simulation and analysis of cellular metabolism and regulation. Bioinformatics 15 (9):749-758.

[5] An J, Kwon H, Kim E, Lee YM, Ko HJ, Park H, Choi I-G, Kim S, Kim KH, Kim W, Choi W (2015) Tolerance to acetic acid is improved by mutations of the TATA-binding protein gene. Environmental Microbiology 17 (3):656-669.

[6] Green M, Sambrook J, Sambrook J (2012) Molecular cloning: a laboratory manual 4 edition Cold Spring Harbor Laboratory Press. New York.

[7] Kim S, Kim J, Song JH, Jung YH, Choi I-S, Choi W, Park Y-C, Seo J-H, Kim KH (2016) Elucidation of ethanol tolerance mechanisms in *Saccharomyces cerevisiae* by global metabolite profiling. Biotechnology Journal 11 (9):1221-1229.

[8] Laluce C, Schenberg ACG, Gallardo JCM, Coradello LFC, Pombeiro-Sponchiado SR (2012) Advances and Developments in Strategies to Improve Strains of Saccharomyces cerevisiae and Processes to Obtain the Lignocellulosic Ethanol-A Review. Applied Biochemistry and Biotechnology 166 (8):1908-1926.

[9] Labrou NE (2010) Random mutagenesis methods for in vitro directed enzyme evolution. Current Protein and Peptide Science 11 (1):91-100.

[10] Liu SP, Liu RX, El-Rotail AAMM, Ding ZY, Gu ZH, Zhang L, Shi GY (2014) Heterologous pathway for the production of L-phenylglycine from glucose by E. coli. Journal of Biotechnology 186:91-97.

[11] Wang Y, Shi WL, Liu XY, Shen Y, Bao XM, Bai FW, Qu YB (2004) Establishment of a xylose metabolic pathway in an industrial strain of Saccharomyces cerevisiae. Biotechnology Letters 26 (11):885-890.

[12] Guo Z-p, Zhang L, Ding Z-y, Wang Z-X, Shi G-Y (2011) Improving ethanol productivity by modification of glycolytic redox factor generation in glycerol-3-phosphate dehydrogenase mutants of an industrial ethanol yeast. Journal of Industrial Microbiology & Biotechnology 38 (8):935-943.

[13] Zhang L, Tang Y, Guo Z-p, Ding Z-y, Shi G-y (2011) Improving the ethanol yield by reducing glycerol formation using cofactor regulation in Saccharomyces cerevisiae. Biotechnology Letters 33 (7):1375-1380.

[14] Kasavi C, Eraslan S, Arga KY, Oner ET, Kirdar B (2014) A system based network approach to ethanol tolerance in Saccharomyces cerevisiae. Bmc Systems Biology 8.

[15] Kotarska K, Czupryński B, Kłosowski G (2006) Effect of various activators on the course of alcoholic fermentation. Journal of food engineering 77 (4):965-971.

[16] Xu GQ, Hua Q, Duan NJ, Liu LM, Chen J (2012) Regulation of thiamine synthesis in Saccharomyces cerevisiae for improved pyruvate production. Yeast 29 (6):209-217.

[17] Walker ME, Gardner JM, Vystavelova A, McBryde C, Lopes MD, Jiranek V (2003) Application of the reuseable, KanMX selectable marker to industrial yeast: construction and evaluation of heterothallic wine strains of Saccharomyces cerevisiae, possessing minimal foreign DNA sequences. Fems Yeast Research 4 (3):339-347.

[18] Tan F, Wu B, Dai L, Qin H, Shui Z, Wang J, Zhu Q, Hu G, He M (2016) Using global transcription machinery engineering (gTME) to improve ethanol tolerance of Zymomonas mobilis. Microbial Cell Factories 15.

[19] Pavlov AR, Pavlova NV, Kozyavkin SA, Slesarev AI (2004) Recent developments in the optimization of thermostable DNA polymerases for efficient applications. Trends in biotechnology 22 (5):253-260.

[20] Wang T-W, Zhu H, Ma X-Y, Zhang T, Ma Y-S, Wei D-Z (2006) Mutant library construction in directed molecular evolution. Molecular biotechnology 34 (1):55-68

[21] Wang Y, Shi W-L, Liu X-Y, Shen Y, Bao X-M, Bai F-W, Qu Y-B (2004) Establishment of a xylose metabolic pathway in an industrial strain of Saccharomyces cerevisiae. Biotechnology letters 26 (11):885-890.

[22] Tan F, Wu B, Dai L, Qin H, Shui Z, Wang J, Zhu Q, Hu G, He M (2016) Using global transcription machinery engineering (gTME) to improve ethanol tolerance of Zymomonas mobilis. Microbial cell factories 15 (1):1.

[23] Liu H, Yan M, Lai C, Xu L, Ouyang P (2010) gTME for Improved Xylose Fermentation of Saccharomyces cerevisiae. Applied Biochemistry and Biotechnology 160 (2):574-582.

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