International Journal of Research in Agricultural Sciences
Volume 4, Issue 2, ISSN (Online): 2348 – 3997

Constructing a Library of Stable Mutations for Enhanced Bioethanol Production by *Saccharomyces Cerevisiae* Using Various Molecular Biology Methods

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Date of publication (dd/mm/yyyy): 25/04/2017

***Abstract* — The aim of this study was to investigate the effects of $MnCl_2$ concentration instead of $MgCl_2$ to construct libraries of mutant from Saccharomyces cerevisiae with error-Prone PCR protocol to prepare a novel random mutagenesis characterized by a high mutation rate and stable better than wild-type strain. In our work, the genetic strategies were utilized to construct libraries from *SPT15* and *TAF23* mutant genes. Those genes are basically existed in the haploid (MAT-a [CICC 1374]) and (MAT-α [CICC 31144]) of S. cerevisiae. The diploid mutants were generated after the mating process. Through our novel technique and integration gTME and Error Prone PCR protocol, different concentrations of $MnCl_2$ instead of $MgCl_2$ were used to adjust the technique.The results showed an average 12-fold increasing for mutation rate when addition different concentrations of $MnCl_2$ instead of $MgCl_2$ with using Error prone PCR reaction and traditional enzymes such as rTaq. In addition to, Mutants recorded the highest stability level by using 3 % $MnCl_2$. In conclusions, there have been many previous studies presented methods to get mutations by using PCR Random Mutagenesis Kit for commercial companies, while in this study we adopted the construction of a new protocol using a combination of items to get random mutagenesis-Super; which were characterized by a high mutation rate, and simultaneously be stable better than wild-type strain.**

***Keywords* — *Saccharomyces Cerevisiae*, *SPT15*, Error-Prone PCR, Manganese(II) Chloride, Random Mutants, Gtme**

I. INTRODUCTION

During the last years, *S. cerevisiae* has been studied extensively as a model organism among eukaryotes. Several technical advantages including rapid cell division easy gene manipulation and its ability to be maintained in haploid or diploid form were developed. However, the production process of biofuel faces many challenges; mainly the low tolerance of the microbial host when there is an increase of ethanol concentrations. There are large compounds of metabolites produced by the fermentation process; some are considered deleterious to the growth of *S. cerevisiae* cells such as ethanol and acidic compounds like pyruvic acids and acetic acid [1], others work as stress factors for yeast, leading to weaken their activities, and eventually cause cell loss.

Random mutagenesis is a powerful tool for generating enzymes, proteins, entire metabolic pathways, or even entire genomes with desired or improved properties. This technology is used to evolve genes in vitro through an iterative process consisting of recombinant generation. Coupled with the development of powerful high-throughput screening or selection methods, this technique was successfully used to solve problems in protein engineering. There are many methods to generate genetic diversity by random mutagenesis and to generate combinatorial libraries. This can be achieved by treating DNA or whole bacteria with various chemical mutagens, by passing cloned genes through strains, by Error-Prone PCR (Ep-PCR) mutagenesis, by prone PCR, or by saturation mutagenesis. The presence of either compound helps stabilize mismatched base pairs. Magnesium chloride ($MgCl_2$) will typically be added at a concentration up to 7mM (a 4.7-fold increase from a typical reaction) [2]. In general, manganese chloride ($MnCl_2$) is not typically present in PCR reactions and can be added at a concentration of 0.5mM just prior to thermo-cycling. Whereas this study aims to improve *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 characterized by the highest of stability levels.

II. MATERIALS AND METHODS

A. Culture media and microbial strains

All media was produced using recipes listed below and prepared in distilled water and sterilized by autoclaving at 121°C for 15 min unless stated otherwise. The *E. coli* JM 109 (***E.coli***) strain had been grown in Luria Broth medium **(**LB**)** and Super Optimal Broth (SOB), which was used as a host for plasmid construction. Moreover the yeast strains *S. cerevisiae* it has been cultivated routinely in Yeast Extract Peptone Dextrose Broth medium (YEPD); which was used to obtain the target gene. All media and microbial strains were used in this study listed in Table 1,2 below.

Table 1. All media was used in this study

Reagent	Concentration (g/l)
YEPD Solid Medium	
Yeast Extract	10
Glucose	20
Tryptone	20
Agar	15
YEPD Medium	
Yeast Extract	10
Glucose	20
Tryptone	20
LB Medium	
Yeast Extract	5
NaCl	10
Tryptone	10
SOB Medium	
Yeast Extract	5
Bacto-peptone	20
$MgCl_2$	0.95
KCl	0.19
NaCl	0.50

Table 2. Microbial strains and plasmids used in this study

Strains / Plasmids	Relevant characteristics	Source / reference
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
Plasmids		
pMD19-T vector	$Amp^{R,}$ clone vector	TaKaRa, Japan
pMD19-T-Amp-*SPT15-Mu*	pMD19-T with *SPT15* mutant gene	This study
pMD19-T-Amp-*TAF23-Mu*	pMD19-T with *TAF23* mutant gene	This study
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

Amp, Ampicillin; Kan, kanamycin.

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. Method of Yeast Mating (hybridization of MAT-a and MAT-α)

The *S. cerevisiae* is a single celled eukaryote, which can exist either as haploid (one set of chromosomes) or diploid (two sets of chromosomes). The *S. cerevisiae* contains the endogenous non-mutant genes chromosomal copy of *SPT15* and *TAF23* genes. In this study, two haploids types of industrial ethanol-producing yeast (MAT-a/MAT-α) were mated and genetically manipulated. All parameters were set and subsequent ligation and transformation, to begin the process of mating strains and cloning of *SPT15* and *TAF23* genes were described below in Table 3.

Table 3. The oligonucleotide primers used in this study

Primers	Sequence (5′–3′) [a]
*SPT15-Eco*RI-FW	CCG*GAATTC*ATGGCCGATGAGGAACGTTTAAAGG
*SPT15-Sal*I-RV	CGCTAG*GTCGAC*TCACATTTTTCTAAATTCACTTAGCACA
*TAF23-Nhe*I-RV	ACTCGA*GCTAGCC*TAACGATAAAAGTCTGGGCGACCT
*TAF23-Sal*I-FW	CGCTAG*GTCGAC*ATGGATTTTGAGGAAGATTACGAT
pYX212-FW	GGGCAGCATAATTTAGGAG
pYX212-RV	AGGGATGTAT CGGTCAGTCA
(*MAT-F*)	AGTCACATCAAGATCGTTTATGG
(*MAT*-a)	ACTCCACTTCAAGTAAGAGTTTG
(*MAT-alpha*)	GCACGGAATATGGGACTACTTCG

[a] Restriction sites are in italic and underlined.

For haploid strain MAT-α, a small amount (200µL) of frozen biomass stocked in -80°C was inoculated in YEPD medium without antibiotic and incubated at 30°C for 24 H in the orbital incubator at 100 rpm (TAICANG QIANG LE, CO., LTD., HUANGJING, JIANGSU, CHINA); and for haploid strain (MAT-a) a small amount (400µL) of frozen biomass from stocks was inoculated in YEPD medium without antibiotic also, incubation conditions were at a predetermined temperature and shaker speed at 12 h. Then 200µL of each grown strain was transferred into fresh YEPD medium and incubated at a predetermined temperature at 200 rpm at 12 h. After that, 1ml medium from each culture was transferred to 50 ml YEPD medium and then cultured for two days into a rotary shaker at 30°C to re-grow cultures up to log phase. For mating process, 1ml medium from each strain was transferred into a fresh 50 ml YEPD and then cultured for two days in the rotary shaker at agitation speed of 100 rpm, 30°C for 12 h. Subsequently,

the medium was diluted (2 × 10-3) by using sterile distilled water; and coated onto YEPD plate for one day at 30°C.

Then after the big colonies were picked up to identify positive colonies from conjugation process i.e. converted from haploids to diploids. Thereafter, Colony PCR with amplification mixture was used to verify positive colonies and protocol PCR thermal program for amplification were mentioned in Table 4, 5. Yields were estimated by running 5 µL of the reaction mixture on a 1.5 % Agarose gel with 0.5µg/mL ethidium bromide-stain and then visualized in comparison to known suitable DNA ladder. Image generation electrophoresis was used by Bio-Rad Chemi Doc XRS+ Molecular Imager system with image Lab Scientific Software version 3.0.1. This system consists of Universal Hood II with UV Camera A12C840038, Canada; Inc.

Table 4. Protocol PCR thermal program for amplification the big colonies as shown in the table

Cycles No.	Temperature	Time	Aim of the step
1	95°C	5 min	For initial denaturation
2	95°C	30 Sec	For denaturation step
3	58°C	30 Sec [a]	For annealing step
4	72°C	30 Sec	For elongation step
Go to (2)			29 cycles
5	72°C	10 min	Final extension
6	16°C	(∞)	Forever [b]

[a] For experimental mutagenesis reactions with templates longer than 1 kb, add 1 min of extension time per additional kb.

[b] After the PCR reaction, cool the reactant to 16°C.

D. Protocol of reaction mixture

Prepare reaction mixture consists of all tree Primers (*MAT-F*, *MAT*-a, *MAT*-α) each an equal amount as follows below:

0.5 µl of (*MAT*-F, *MAT*-a, *MAT*-α), 25 µl of 2XTaq Mix and 0.5 µl of template DNA, then fulfill the volume mixtures for tube to 50 µl by distilled water; at pH 7.0.

E. Isolation and extraction of genomic and target genes

The genomic DNA (gDNA) was extracted from a standard haploid strain of *S. cerevisiae* from the protocol of [3] with slight modifications. The extracted amount of gDNA was used as a template to begin the process of cloning of *SPT15* and *TAF23* genes in this study.TATA-binding protein (TBP) subunit of the transcription factor complexes TFIID and TFIIB were used [4]. It plays an enormous role during gene expression in yeast cells [5]. Additionally, much gene regulation takes place by targeting TBP with co-activators and co-repressors, which interact with TBP for gene transcription [6]. Mutations in the TBP confer enhanced tolerance to stress in *S. cerevisiae* from ethanol and glucose [7]. The *SPT15* and *TAF23* were used in this study to improve cellular phenotype for yeast strain with ethanol tolerance. Both genes express via transcription during RNA polymerase II coupled with the association of TBP to general transcription factors and regulators [8,9].

F. Design of primers and vectors

The primers for *SPT15* and *TAF23* were designed by using scientific software Vector NTI Version 11.5; DNAMAN Version 7; Primer Premier 5 Version 5. For premier's preparation, we added distilled water as recorded on the tubes after centrifuged at 12,000 rpm for 5 min, after that the tubes were placed on a vortex device for 40 seconds to ensure mixing and dissolving the premiers; subsequently centrifuged at 12,000 rpm for 10 seconds and finally premiers were stored at -20°C. Oligonucleotide primers were designed based on the pYX212 vector, with restriction sites EcoRI and SalI for *SPT15* gene mutagenesis, while SalI and NheI were used for *TAF23*. For plasmid construction, over expression of genes, and electroporation, standard methods were applied as directed [10,11] See Table 3.

G. Plasmids construction with the target genes

The genome sequence of *S. cerevisiae* and both genes sequences were investigated using the *S. cerevisiae* (GDNC) for Biotechnology Information. All the target genes were randomly mutated, were amplified by standard PCR from the gDNA of *S. cerevisiae* R-Control as a template; by using oligonucleotide primers as shown in Table2. The resulted PCR products were purified by Mini-DNA fragment Rapid Kit. 4.8µl of each target genes were ligated with 0.2 µl vector (pMD19-T) and (pYX212). Both plasmids were individually transformed into *E. coli* JM109 competent cells for multiplication on SOB medium with Amp (100 mg/l) at 37°C for 24 h. On other hand, the fragment of *SPT15*, *TAF23* mutant genes were digested with (*EcoRI*/ *SalI*) and (*SalI*/*NheI*), respectively ligated into similarly digested pYX212 individually using T4 DNA Ligase (Thermo Scientific, CO., LTD, Meridian, USA). Then, these plasmids were extracted and purified by using a Plasmid Mini-Preps Kit and immediately, transformed individually into *S. cerevisiae* as a host organism to induce the expression of the target genes. Finally, the plasmids were sequenced for verification of *TAF23* and *SPT15* mutants. These genes obtained from the previous step were used as a template to initiate DNA amplification using Error-prone PCR technique for next steps and preserved in pMD19-T vector.

H. Construction of mutant genes libraries

In this study, we adopted the protocol established by [2] with some modification in the process of PCR protocol. We used different concentration of $MnCl_2$ instead of $MgCl_2$ to introduce mutation in the sequence of genes using the Ep-PCR amplification process. The latter produced the largest possible number of changes in the gene sequence (low, medium, and high) with strong and stable rate of mutation(s). The procedure was adjusted by adding different amounts ranged between 1 -50 µL of $MnCl_2$ (5 mM), $MgCl_2$ (25 mM), and mixed with dNTPs one by one (The dGTP, dNTP, dCTP, dTTP, and rTaq DNA polymerase enzymes were purchased from TaKaRa, Japan). Then within 100 µL Ep-PCR reaction mixture (mentioned above in Table 5).

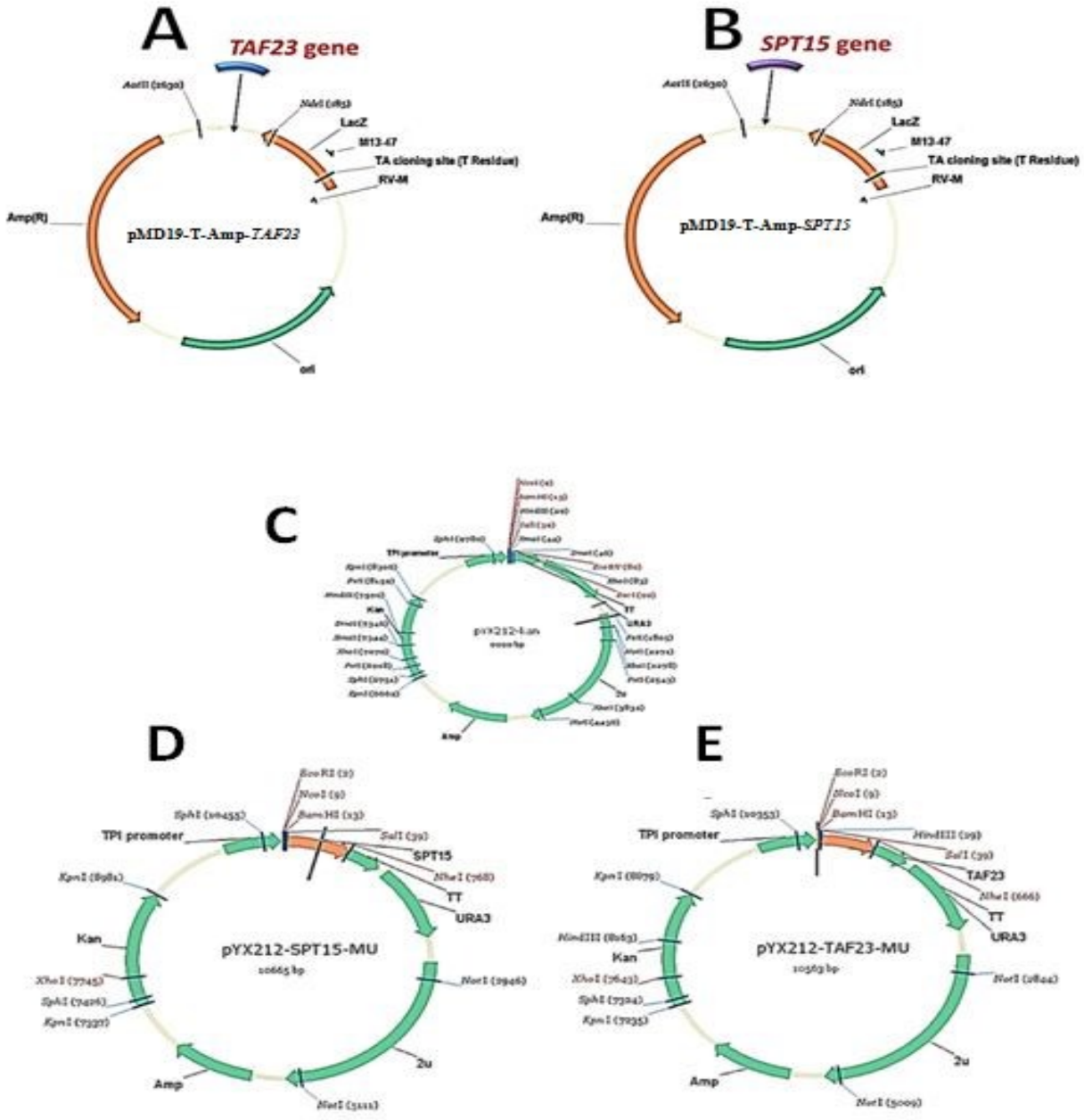

Figure 1: The physical maps of recombinant plasmids**(A)** and **(B)** the physical maps of vector pMD19-T with *TAF23* and *SPT15*, which formed two plasmids pMD19-T-Amp-*SPT15* and pMD19-T-Amp-*TAF23,* respectively. Moreover, (C) The pYX212-kan (empty vector).(D)and(E), the pYX212 with *SPT15-Mu* and *TAF23-Mu*, respectively. The genes are under the R-control of TPI promoter and terminator with *EcoR*I and *Sal*I restriction sites for *SPT15* and *Sal*I and *Nhe*I restriction sites for *TAF23*. (Amp^r) ampicillin resistance gene; (Kan^r) Kanamycin resistance gene; (G418) resistance gene.

Two mutagenic reaction mixtures were prepared, the first mixture contained different amount of $MnCl_2$ (0, 1, 3, 5, 10, 15, 20, 30, 40 and 50 µl) (5 mM), 22 µl of $MgCl_2$ (25 mM), 10 µl of (10 × Taq Buffer without $MgCl_2$), 1 µl (each) PCR primers. 1 µl of Template gDNA, 1 µl of rTaq DNA polymerase, 10 µl of (unbalanced 10XdNTP Mix) as shown below in the section of protocol unbalanced 10XdNTP Mix. On the other hand, the second mixture contained different amount of $Mngl_2$ (0, 1, 3, 5, 10, 15, 20, 30, 40 and 50 µl) (25 mM), 10µl of $MnCl_2$ (5 mM), 10 µl of (10 × Taq Buffer without $MgCl_2$), 1 µl (each) PCR primers, 10 µl of (unbalanced 10XdNTP Mix) as shown below in the section of protocol unbalanced 10XdNTP Mix. Then fulfill the volume mixtures for all tubes to 100 µl by distilled water. After that PCR reaction was continued for 30 cycles.

It is expected that these modifications by using this condition leads to an excess of changes from the Adenine to Guanine and Thymine to Cytosine, which will be a great impact on the error rate [12]. The PCR and Ep-PCR experiments were performed by using the Real-Time PCR Detection System with software CFX Manager 3.0 (Bio-Rad, USA), using rTaq DNA polymerase (TaKaRa, Japan). All PCR products were sequenced to verify the mutation

rate for all the new mutations and to get mutated genes sequence for *SPT15- Mu* and *TAF23-Mu*.

I. Protocol of unbalanced 10XdNTP Mix

Prepare unbalanced 10XdNTP Mix(2'-deoxynucleoside 5'-triphosphate) consists of all four nucleotides (dATP, dCTP, dGTP, dTTP) each at different concentrations as follows below:

2 µl of dATP (0.2mM), 2 µl of dGTP (0.2mM), 10 µl of dCTP (1mM), 10µl of dTTP (1mM) Then fulfill the volume mixtures for all tubes to 100 µl by distilled water; at pH 7.0. Finally, Store at -20 C.

J. Transformation for SPT15-Mu and TAF23-Mu genes into S. cerevisiae

High-efficiency yeast transformation method carried out by [13, 9]with modification. Briefly, 50 µl of plasmid DNA were added to each tube than for fulfilling the volume mixture up to 380 µl with sterile distilled water. Then, heat shock at 42°C for 25 min. Harvest the cells by centrifugation at 5000 rpm for 2 min and remove supernatant. Re-suspend the cells in 1 ml sterile water and mix by inverting the tube up and down. Plate 100 µl of the suspension on solid medium containing G418 (100 µg/ml). The plates were incubated at 30°C for 2days. Furthermore, screening was undertaken to select colony which is ethanol-tolerant and could produce a high yield of ethanol via the tests of stability and similarity.

Table 5. Mutagenesis reactions prepared in total volume 100 µl

Conc. Of $MnCl_2$ (5mM)[b] %	Amount of component of the first mixture (µL)								
	SDW [a]	10XTaq Buffer Without $MgCl_2$	$MnCl_2$ (5mM)	$MgCl_2$ (25mM)	Primer (F)	Primer (R)	Template DNA [C]	10XdNTP Mix	rTaq
0	54	10	0	22	1	1	1	10	1
1	53	10	1	22	1	1	1	10	1
3	51	10	3	22	1	1	1	10	1
5	49	10	5	22	1	1	1	10	1
10	44	10	10	22	1	1	1	10	1
15	39	10	15	22	1	1	1	10	1
20	34	10	20	22	1	1	1	10	1
30	24	10	30	22	1	1	1	10	1
40	14	10	40	22	1	1	1	10	1
50	4	10	50	22	1	1	1	10	1
Conc. Of $MgCl_2$ (25mM) %	Amount of component of the second mixture (µL)								
	SDW	10XTaq Buffer Without $MgCl_2$	$MnCl_2$ (5mM)	$MgCl_2$ (25mM)	Primer (F)	Primer (R)	Template DNA [b]	10XdNTP Mix	rTaq
0	66	10	10	0	1	1	1	10	1
1	65	10	10	1	1	1	1	10	1
3	63	10	10	3	1	1	1	10	1
5	61	10	10	5	1	1	1	10	1
10	56	10	10	10	1	1	1	10	1
15	51	10	10	15	1	1	1	10	1
20	46	10	10	20	1	1	1	10	1
30	36	10	10	30	1	1	1	10	1
40	26	10	10	40	1	1	1	10	1
50	16	10	10	50	1	1	1	10	1

[a] sterile deionized water

[b] 5mM % : [5 µl ($MnCl_2$) + 995 µl (dd.H2O)] prepared in total volume 1 liter

[C] To increase the mutation rate, using the new product of error-prone PCR reaction as template; this will increase the number of mutations per 1000 base pairs.

III. Results and Discussions

A. Hybridization, amplification and construction of pMD19-T-Amp-SPT15 and pMD19-T-Amp-TAF23

The biggest and similar colonies to the wild-type strain of *S. cerevisiae* were chosen for mating process between the haploid strains of *S. cerevisiae MAT-a*, and *MAT-α* an industrial ethanol- producing yeast. Conjunction process resulted a strain of *S. cerevisiae* possesses genetic traits diploid, was named *S. cerevisiae* R-Control (Fig. 2a).The gDNA of (*S. cerevisiae* R-Control) was used as a template for PCR reaction. The clear bands at 723 bp and 621 bp for *SPT15* and *TAF23* genes were identified respectively, as shown at Figure 2b. Construction and ligation of plasmid with genes were resulted clear bands at 3323 bp and 3221 bp for pMD19-T-Amp-*SPT15* and pMD19-T-Amp-*TAF23*, respectively (Fig. 1).

B. Plasmids construction with the target genes

Both *SPT15* and *TAF23* genes were ligated individually

with PYX212 using T4 DNA ligase to construct vector PYX212 with *SPT15* and *TAF23*, respectively. The resultants after ligation processes were named (PYX212-*SPT15-Mu*) and (PYX212-*TAF23-Mu*), respectively. The clear bands at 723 bp and 621 bp for *SPT15* and *TAF23* genes were identified, respectively after the plasmids were digested, as shown in Fig. 3 (a, b).

C. Construction of mutants

Ep-PCR was applied using different concentrations of $MnCl_2$ and $MgCl_2$ (0, 1, 3, 5, 10, 15, 20, 30, 40 and 50 %) for the alteration of each gene sequence. Accordingly, after several rounds, mutants showed between 1 - 2 mutations per 300 bases with $MgCl_2$, whereas 1- 60 mutations per 300 bases with $MnCl_2$. More than 200 mutants were obtained. Based on the great rate of success, our study was focused of 59 mutations; which have been divided as follows: 55 mutant strains with $MnCl_2$ for *SPT15* gene, and 8 mutant strains with $MnCl_2$ for *TAF23*. However 31 mutant strains with $MgCl_2$ for *SPT15* gene, and 6 mutant strains with $Mngl_2$ for *TAF23*.

The chosen mutant strains were classified to three categories; (high, medium, and low mutations) according to the classification previously reported by [14]. Regarding for mutations with $MnCl_2$, 27 mutants were identified as medium rate strains and 23 mutants as high rate strains in *SPT15* gene. Moreover, 8 mutant strains with a low rate of mutations were detected in *TAF23*. In contrast, Regarding the $MgCl_2$, no any mutants were identified as high and medium rate strains with and *SPT15* gene. In contrast, 29 mutant strains with a low rate of mutations were detected in *TAF23* gene (Table 6) and figure4 (a,b).

In details, the obtained mutations from $MnCl_2$ were distributed as follows: For the concentration 1 % of $MnCl_2$ recorded with *SPT15* gene at 75 %, 25 %, and 0 % mutations rate were obtained with a high, medium and low rate, respectively. On the other hand, the concentration of 3 % has shown mutations with a high rate of 38 % and 36 % mutation of medium rate. Whereas the concentration of 10 % of $MnCl_2$: we obtained a high rate by 87 % and medium rate by 13 %. Moreover, the concentration 20 % of $MnCl_2$ shown mutations characterized by a high rate of 6 % and medium rate of 94 %. In contrast, the other concentrations did not record any genetic mutations. Also, the mutations from $MgCl_2$ did not record any mutations with the high or medium rate,only were obtained for low rate mutations with both genes *SPT15* and *TAF23* (See Figure 4).

Table 6. Summary of the successful mutations rate

0 % $MnCl_2$								**0 % $MgCl_2$**							
***SPT15* Gene**				***TAF23* Gene**				***SPT15* Gene**				***TAF23* Gene**			
Strain name	**NC [a]**	**SIM [b]**	**ERM[c]**	**Strain name**	**NC**	**SIM**	**ERM**	**Strain name**	**NC**	**SIM**	**ERM**	**Strain name**	**NC**	**SIM**	**ERM**
R2-Mn	1	99.9	Low	R3-Mn	1	99.9	Low	**ND [d]**				**ND**			
R8-Mn	2	99.7	Low	R6-Mn	1	99.9	Low	Mutants for *SPT15*				Mutants for *TAF23*			
1 % $MnCl_2$								**1 % $MgCl_2$**							
R5-Mn	8	98.9	Medium	R8-Mn	1	99.9	Low	R5-Mg	1	99.9	Low	R8-Mg	1	99.9	Low
R7-Mn	10	98.6	High	R9-Mn	2	99.7	Low	R7-Mg	1	99.9	Low	R9-Mg	1	99.9	Low
R10-Mn	12	98.3	High	R11-Mn	1	99.9	Low	R11-Mg	1	99.9	Low	R11-Mg	1	99.9	Low
R12-Mn	181	75.0	High					R12-Mg	**ND**	100	**ND**				
3 % $MnCl_2$								**3 % $MgCl_2$**							
R1-Mn	5	99.3	Medium	R1-Mn	4	99.5	Low	R1-Mg	**ND**	100	**ND**	R1-Mg	1	99.9	Low
R4-Mn	8	98.9	Medium	R4-Mn	2	99.7	Low	R4-Mg	1	99.9	Low	R4-Mg	**ND**	100	**ND**
R5-Mn	8	98.9	Medium	R10-Mn	1	99.9	Low	R5-Mg	**ND**	100	**ND**	R10-Mg	**ND**	100	**ND**
R6-Mn	**ND**	100	**ND**					R6-Mg	**ND**	100	**ND**				
R7-Mn	10	98.6	High					R7-Mg	1	99.9	Low				
R8-Mn	7	99.0	Medium					R8-Mg	2	99.7	Low				
R10-Mn	12	98.3	High					R10-Mg	1	99.9	Low				
R11-Mn	**ND**	100	**ND**					R11-Mg	**ND**	100	**ND**				
R12-Mn	12	98.3	High					R12-Mg	1	99.9	Low				
R14-Mn	8	98.9	Medium					R14-Mg	**ND**	100	**ND**				
R15-Mn	10	98.6	High					R15-Mg	1	99.9	Low				
R16-Mn	68	90.6	High					R16-Mg	2	99.7	Low				
R17-Mn	7	99.0	Medium					R17-Mg	2	99.7	Low				
R18-Mn	9	98.8	Medium					R18-Mg	2	99.7	Low				
R19-Mn	6	99.2	Medium					R19-Mg	2	99.7	Low				
5 % $MnCl_2$								**5 % $MgCl_2$**							
***SPT15* Gene**				***TAF23* Gene**				***SPT15* Gene**				***TAF23* Gene**			
ND				**ND**				**ND**				**ND**			
Mutants for *SPT15*				Mutants for *TAF23*				Mutants for *SPT15*				Mutants for *TAF23*			

10 % $MnCl_2$							
***SPT15* Gene**				***TAF23* Gene**			
Strain name	**NC**	**SIM**	**ERM**	**Strain name**	**NC**	**SIM**	**ERM**
R1a-Mn	14	98.1	High				
R2a-Mn	39	94.6	High				
R1b-Mn	44	93.9	High				
R2b-Mn	14	98.1	High				
R3a-Mn	45	93.8	High				
R3b-Mn	29	96.0	High				
R4-Mn	9	98.8	Medium	**ND** Mutants for *TAF23* with 10% $MnCl_2$			
R5-Mn	180	75.1	High				
R6-Mn	13	98.2	High				
R8-Mn	14	98.1	High				
R9-Mn	20	97.2	High				
R10-Mn	20	97.2	High				
R14-Mn	17	97.6	High				
R15-Mn	15	97.9	High				
R18-Mn	7	99.0	Medium				
R19-Mn	14	98.1	High				
15 % $MnCl_2$							
R10-Mn	**ND**	100	**ND**				
R12-Mn	2	99.7	Low	**ND** Mutants for *TAF23*			
R14-Mn	2	99.7	Low				
R15-Mn	**ND**	100	**ND**				
20 % $MnCl_2$							
R2-Mn	8	98.9	Medium				
R4-Mn	8	98.9	Medium				
R5-Mn	5	99.3	Medium				
R6-Mn	112	84.5	High				
R8-Mn	6	99.2	Medium				
R9-Mn	8	98.9	Medium				
R10-Mn	6	99.2	Medium	**ND** Mutants for *TAF23* with 20 % $MnCl_2$			
R12-Mn	6	99.2	Medium				
R13-Mn	6	99.2	Medium				
R14-Mn	6	99.2	Medium				
R15-Mn	6	99.2	Medium				
R16-Mn	9	98.8	Medium				
R17-Mn	5	99.3	Medium				
R18-Mn	6	99.2	Medium				
R19-Mn	5	99.3	Medium				
R20-Mn	5	99.3	Medium				
25 % $MnCl_2$							
***SPT15* Gene**				***TAF23* Gene**			
Strain name	**NC**	**SIM**	**ERM**	**Strain name**	**NC**	**SIM**	**ERM**
ND Mutants for *SPT15*				**ND** Mutants for *TAF23*			
30 % $MnCl_2$							
ND Mutants for *SPT15*				**ND** Mutants for *TAF23*			
40% MnCl2							
ND Mutants for *SPT15*				**ND** Mutants for *TAF23*			
50 % $MnCl_2$							
R5-Mn	7	99.0	Medium	**ND** Mutants for *TAF23*			

10 % $MgCl_2$							
***SPT15* Gene**				***TAF23* Gene**			
Strain name	**NC**	**SIM**	**ERM**	**Strain name**	**NC**	**SIM**	**ERM**
R1a-Mg	1	99.9	Low	R4-Mg	1	99.9	Low
R2a-Mg	**ND**	100	**ND**	R19-Mg	1	99.9	Low
R1b-Mg	**ND**	100	**ND**				
R2b-Mg	**ND**	100	**ND**				
R3a-Mg	**ND**	100	**ND**				
R3b-Mg	**ND**	100	**ND**				
R4-Mg	**ND**	100	**ND**				
R5-Mg	**ND**	100	**ND**				
R6-Mg	1	99.9	Low				
R8-Mg	**ND**	100	**ND**				
R9-Mg	1	99.9	Low				
R10-Mg	**ND**	100	**ND**				
R14-Mg	1	99.9	Low				
R15-Mg	**ND**	100	**ND**				
R18-Mg	**ND**	100	**ND**				
R19-Mg	**ND**	100	**ND**				
15 % $MgCl_2$							
R10-Mg							
R12-Mg	**ND** Mutants for *SPT15*			**ND** Mutants for *TAF23*			
R14-Mg							
R15-Mg							
20 % $MgCl_2$							
R2-Mg	**ND**	100	**ND**				
R4-Mg	1	99.9	Low				
R5-Mg	1	99.9	Low				
R6-Mg	**ND**	100	**ND**				
R8-Mg	1	99.9	Low				
R9-Mg	1	99.9	Low				
R10-Mg	1	99.9	Low	**ND** Mutants for *TAF23* with 20 % $MgCl_2$			
R12-Mg	**ND**	100	**ND**				
R13-Mg	1	99.9	Low				
R14-Mg	1	99.9	Low				
R15-Mg	1	99.9	Low				
R16-Mg	0	100	ND				
R17-Mg	1	99.9	Low				
R18-Mg	1	99.9	Low				
R19-Mg	1	99.9	Low				
R20-Mg	0	100	ND				
25 % $MgCl_2$							
***SPT15* Gene**				***TAF23* Gene**			
Strain name	**NC**	**SIM**	**ERM**	**Strain name**	**NC**	**SIM**	**ERM**
ND Mutants for *SPT15*				**ND** Mutants for *TAF23*			
30 % $MgCl_2$							
ND Mutants for *SPT15*				**ND** Mutants for *TAF23*			
40 % $MgCl_2$							
ND Mutants for *SPT15*				**ND** Mutants for *TAF23*			
50 % $MgCl_2$							
R5-Mg	1	99.9	Low	**ND** Mutants for *TAF23*			

[a] Number of changefor molecules DNA from (Adenine to Guanine) and (Thymine to Cytosine); [b] Similarity; [C] ERM: Error rate at mutants; [d] Not detected.

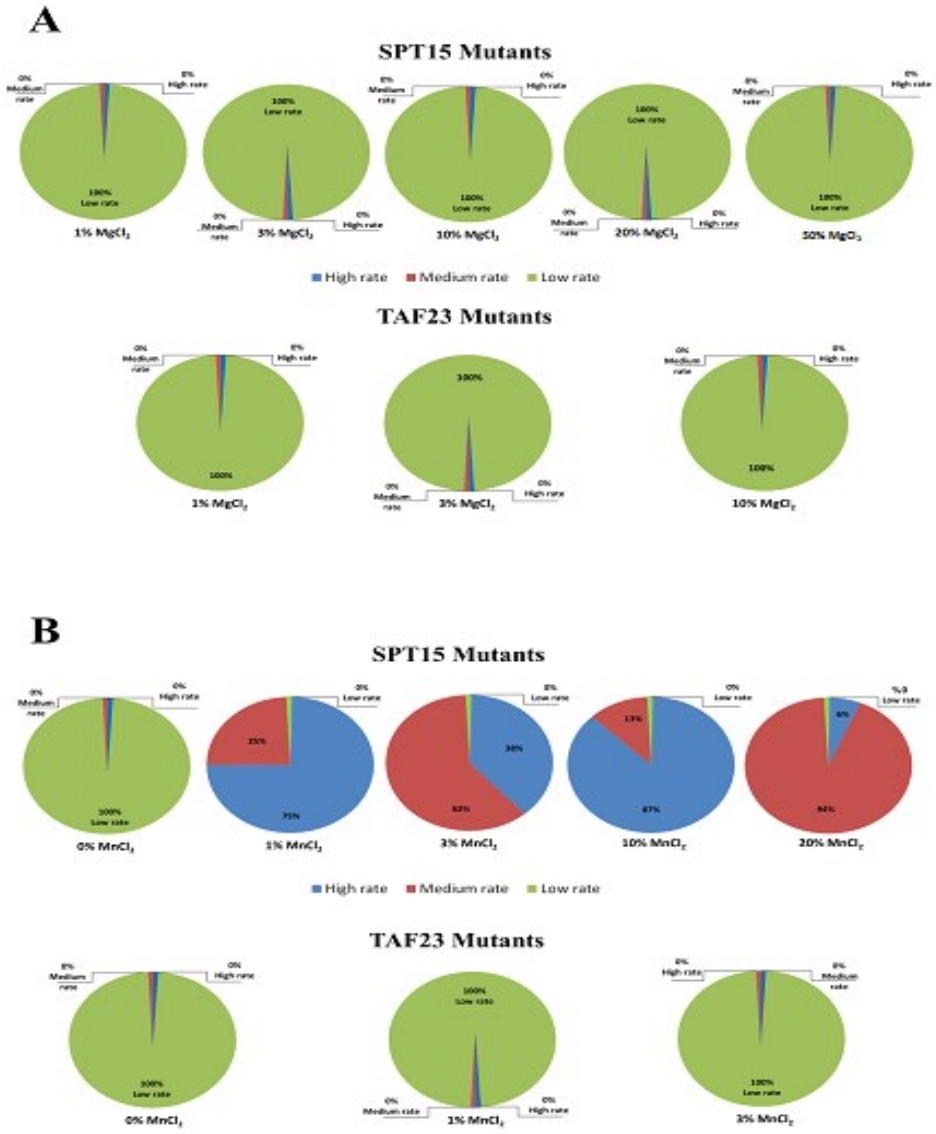

Figure 4:(**A**) and (**B**) summary of the mutations rate for *SPT15* and *TAF23* mutant genes

IV. Conclusion

Ep-PCR protocol generally introduces between 1 and 7 mutations (i.e. single base substitutions) per 1000 bases of template DNA. This is due to an increase in the $MgCl_2$ concentration, the addition of $MnCl_2$, the low template concentration, an unbalanced solution of dNTPs (that is, A,G,C, and T are not all present at the same concentration) and the use of Taq polymerase without an error-checking enzyme. In our study, we have been conducted modification for the Ep-PCR technique by using different $MnCl_2$ concentrations instead of the most applied mixture Ep-PCR reaction $MgCl_2$. in order to verify the effect of construction and formation of mutations with the characteristics of stability. Furthermore, the haploid wild type strains of *S. cerevisiaeMAT*-a, and *MAT*-α has been mated for the creating of new diploid strain *S. cerevisiae* R-Control. Accordingly, after Ep-PCR, mutants showed between 1 - 2 mutations per 300 bases with $MgCl_2$, whereas 1- 60 mutations per 300 bases with $MnCl_2$. gTME strategy and Ep-PCR was used to obtain genetically engineered *S. cerevisiae* expressing mutant genes *SPT15* and *TAF23* encoding TBP. This is a preferred method to give the irregular variations of mutations into a defined segment and specific of DNA; and is based on the amplification of the entire gene(s) using primers that possess the desired changes [15]. This technique was used to create randomized genomic libraries [16,17]; working on the principle that r-Taq polymerase can anneal incompatible base pairs to each other during amplification under imperfect PCR conditions.

V. FUTURE PROSPECTS

In a prospective study, we seek to study of stability and similarity tests for those mutants to enhance ethanol tolerance and survival upon ethanol shock on a rich medium, in addition to surviving after transformation to be able to grow on a large industrial scale. Moreover, we study the estimation of mutant's efficiency for the production of ethanol and glucose.

VI. Appendix

Abbreviation

E. coli	*Escherichia coli*
S. cerevisiae	*Saccharomyces cerevisiae*
Amp	Ampicillin
Kan	kanamycin
LB	Luria-Bertani medium
SOB	super optimal broth medium
YEPD	Yeast extract peptone dextrose broth medium
gDNA	genomic DNA
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
MCS	Mutant colonies which were selected
AM	Absent mutations
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese chloride

Acknowledgments

The authors are grateful to the International Education School at Jiangnan University for financial support throughout the study.

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