Construction of a vector containing a formaldehyde-hyperresistance gene as a selective marker

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*Thanit Koolkoksong[1], Atcha Boonmee[2,3]**

Abstract

The genetically modified *Saccharomyces cerevisiae* strains in a laboratory level normally use antibiotic substance or amino acid analog as a selective marker which are very expensive. In this research, construction of a vector containing a selective marker gene that used cheaper substance was investigated. From the study of the tolerance DNA damaging agents such as formaldehyde, it was found that a hyperresistance gene *SFA* (Hyperresistance to formaldehyde) was responsible for a tolerance of formaldehyde. Thus, the *SFA* gene from *S. cerevisiae* was used as a selective marker in this research. *S. cerevisiae* strain TISTR 5596 was chosen as a source of *SFA* gene since it was able to grow on 4 mM formaldehyde comparing to other strains. After amplification of the *SFA* gene by PCR, the PCR product was 1,160 bp long, corresponding to the length of the gene from NCBI database (gene accession No. X68020). The PCR product of the *SFA* gene was ligated into a plasmid pGBDU-C3 via XhoI/EcoRI site by which the gene was overexpressed under the *ADH1* promoter. The plasmid containing the *SFA* gene was prepared from bacterial cells and further transformed into *S. cerevisiae* TISTR 5596. The obtained yeast transformants showed enhanced tolerance to formaldehyde concentration up to 5.5 mM; however, the untransformed yeast cells could not survive. From this study, it was demonstrated that the selection of transformed yeast cells was more convenient using *SFA* gene as a selective marker.

Keywords: yeast selective marker, SFA, hyperresistance to formaldehyde

[1] *Graduate School, Khon Kaen University, Khon Kaen 40002, Thailand*
[2] *Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*
[3] *The Center of Agricultural Biotechnology for sustainable Economy, Khon Kaen University, Khon Kaen 40002, Thailand.*
**Corresponding author, e-mail: atcha@kku.ac.th*

Introduction

Selections and screenings of *Saccharomyces cerevisiae* transformants in a laboratory normally of all use auxotrophic markers, but there are several drawbacks in using them. Firstly, there is a limitation of the strains one can use. Genomes of wild-type and most industrial strains generally do not contain deletions or mutations on their auxotrophic genes. Furthermore, the mutation can not be introduced easily because of their recessive nature and the polyploidy of many of these yeast strains. Secondly, only a few auxotrophic markers are available e.g. uracil tryptophan or leucine. Lastly, secondary growth effects of a gene disruption can sometimes be masked by the addition of necessary amino acids (Wenzel et al., 1992).

Therefore, other selective markers have been developed. A number of dominant markers, such as resistance to copper or methotrexate, have been reported (Van den berg & Steenama, 1997).

Transformants can be selected with these systems, but usually with much lower efficiency compared to auxotrophic markers (Van den berg & Steenama, 1997). More serious problems may be caused by high background growth, the cost of the selective agent and the adverse effects of some of the compounds such as used phleomycin causes DNA breakage (Van den berg & Steenama, 1997). The isolation and genetic characterization of yeast transformants hyperresistant to formaldehyde (FA) lead to the discovery of the yeast gene *SFA* (sensitivity to FA), which confers hyper-resistance to formaldehyde. The open reading frame of *SFA* is 1158 bp in size and encodes a polypeptide of 386 amino acids (Wehner et al., 1993).

Overexpression of the *SFA* gene leads to enhanced consumption of formaldehyde and protection of DNA from the action of FA or in the repair of FA-induced DNA damage (Chanet et al., 1975). Formaldehyde is a reactive ubiquitous agent known to form nucleophilic adducts with biomolecules, causing DNA-protein and DNA-DNA cross links (Chanet et al., 1976). The concentrations of formaldehyde (FA) of 1 mM or higher are cytostatic or cytotoxic to haploid wild-type cells of *S. cerevisiae* (Chanet & von Borstel, 1979). Formaldehyde is inexpensive: since formaldehyde selection costs only 0.03 baht per plate. Furthermore, it can be completely metabolized (Van den berg & Steenama, 1997).

In this work, we have cloned the *S. cerevisiae SFA* gene, encoding formaldehyde dehydrogenase under the control of *ADH1* promoter. Transformants were tested on their resistance to formaldehyde. We successfully demonstrated the use of formaldehyde resistance for a direct selection with transformants containing this vector construct.

Materials and methods

1. Yeast and bacterial strains

The *S. cerevisiae* strains used in this study were from the collection of Thailand Institute of Scientific and Technological Research (TISTR). *S. cerevisiae* strain TISTR 5339, former name *S. cerevisiae* AM12 was isolated from japanese soil sample and had ability to ferment ethanol at high yield (Limtong et al., 1986). *S. cerevisiae* strain TISTR 5596 was named as *S. cerevisiae* K103 (Nakamura, unpublished) and had a high yield ethanol production (Boonmee, unpublished).

Escherichia coli TOP10 (invitrogen) Genotype: F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ*80lacZ*Δ *M15* Δ*lacX74recA1 ara*Δ*139*Δ(*ara-leu)7697 galU galK rpsL(Str^R) endA1 nupG* was used for transformation and amplification of the vectors.

2. Media and general growth condition

The standard growth medium for the yeast was YMG -medium (yeast extracts 4 g, malt extract 10 g, glucose 4 g, pH 7.2). *E. coli* was grown in LB-medium (tryptone 10 g, yeast extract 5 g and NaCl 10 g) with antibiotics supplemented according to procedures given in Maniatis et al. (1982).

3. Nucleotides

The yeast *E. coli* shuttle vector used in this study was pGBDU-C3 (James et al., 1996).

Oligonucleotides used for cloning were synthesized by DNA technology Labolatory (BIOTEC, Nakonpathom, Thailand). Oligonucleotides used for sequencing were synthesized by EUROGENTEC AIT (Ayer Rajah Cresent, Singapore) and purified by desalting.

Table 1. Oligonucleotides used in this study

Type of use	Name	Sequence (5'to3')	Total base	Tm(^{o}C)
Cloning	FW_*Xho*I_SFA	CCGCCGCTCGAGAAATGTCC GCCGCTACTGTTGG	34	76.7
Cloning	RV_*EcoR*I_SFA	CGCGGAATTCCCAATGGTTA GCTCATCC	28	69.0
Sequencing	SFA(seq_pGBDU_*XhoI*50_FW)	CTAGGGCACATCTGACAG	18	45.2
Sequencing	SFA(seq.SFA_564_FW)	GGGTATCGCCTTTTTGC	17	41.9
Sequencing	SFA(seq_pGBDU_*EcoR*I50_R V)	CAGTTGAAGTGAACTTGC	18	40.6
Sequencing	SFA(seq.SFA_599_RV)	CTCTTAAGACAGCTAATG	18	38.3

4. Enzymes and chemicals

DNA polymerase used in this work was *AccuPrime*^{TM}P*fx* DNA polymerase (Invitrogen, Carlsbad, CA, USA). The restriction enzymes *Eco*RI was purchase from New England Biolabs, while *Xho*I was purchase from MBI fermentas.T4 DNA ligase from New England Biolabs was used to ligate DNA fragments.

5. *Preparation of nucleotides*

Genomic DNA of *S. cerevisiae* TISTR 5596 was extracted by a method according to Maniatis et al. (1982).

For amplification of the *SFA* gene, genomic DNA of *S. cerevisiae* TISTR 5596 was used as a template. According to the manufacture's protocol, *SFA* gene was amplified under a condition given in table 2.

Table 2. PCR protocol

Cycle step	Temperature	Time	Cycle number
Preheating	94 °C	hold (prior to start)	
Initial denatureation	94 °C	2 min	1
Denaturation	94 °C	30 sec	30
Annealing	52 °C	30 sec	30
Extension	68 °C	1 min	30
Final extension	68 °C	10 min	1
	4 °C	hold	

Plasmid DNA was isolated from *E. coli* using Aurum™ plasmid mini kit (Biorad). Analysis of each cloning step was performed by agarose gel electrophoresis described by Maniatis et al. (1982). *E. coli* and yeast were transformed by methods according to Dagert and Ehrlich (1979) and Ito et al. (1983), respectively.

For ligation reaction, only purified DNA fragments were used, either from gel extraction kit or PCR purification kit. The ligation of double strand DNA molecules was done by T4 DNA ligase (Biolabs). According to the enzyme recommendation of the manufacture, 0.1-0.4 pmol DNA and 100-200 unit (0.5-1 μl) of T4 DNA ligase was applied in reaction volume of 20 μl and ligated for 2 hours at room temperature (20-25 °C).

6. Determination of hyperresistance phenotype.

Hyperresistance phenotype of yeast was determined by a yeast drop test. Yeast cell were prepared by inoculating 2-3 yeast colonies into YMG medium and incubated at 30°C, 200 rpm for 16-18 h (OD_{600} should be 3 - 6). The overnight culture was diluted to OD_{600} at 1.0 and further serial diluted from 10^{-1} to 10^{-4}. Cell suspension from each dilution (5μl) was gently dropped on selective agar plates (containing 0 - 5.5 mM formaldehyde) by pipetting. Plates were incubated for 3 to 4 days at 30 °C and the growth of yeast cells was monitored.

7. Analysis of plasmid containing the SFA gene

Plasmid pSFAU was isolated from *E. coli* by alkaline lysis and primarily analyzed by restriction analysis. The clones containing the *SFA* gene insert were further analyzed by sequencing using specific primers shown in Table 1.

Results

1. Selection of yeast strain for SFA gene source

Both ethanol fermenting yeast strains *S. cerevisiae* TISTR 5339 and *S. cerevisiae* TISTR 5596 were tested on their formaldehyde resistance by a drop test on YMG agar plate containing formaldehyde with concentrations ranging from 0 to 4 mM. From the test, *S. cerevisiae* strain TISTR 5596 could grow on agar plate containing 4 mM formaldehyde, while the strain TISTR 5339 could tolerate only 3 mM formaldehyde (Figure 1.).

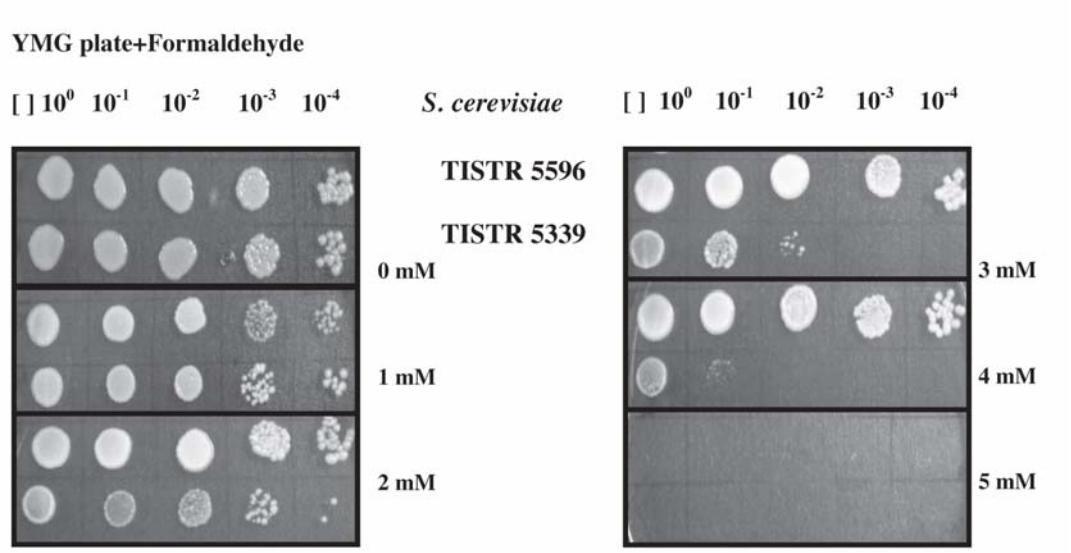

Figure 1. A drop test of *S. cerevisiae* strains on YMG plate containing formaldehyde. [ ] = cell dilution factor

The result showed that *S. cerevisiae* strain TISTR 5596 could tolerate higher concentration of formaldehyde than the strain TISTR 5339. Therefore, we chose *S. cerevisiae* strain TISTR 5596 as a source of SFA gene.

2. Cloning and analysis of the transformants

The *SFA* gene, encoding formaldehyde dehydrogenase (gene accession number X68020, NCBI Genbank) was amplified by polymerase chain reaction from genomic DNA of *S. cerevisiae* TISTR 5596 using the specific primer (FW_*Xho*I_SFA and RV_*Eco*RI_SFA) shown in table 1. A specific 1.1 kb DNA fragment was generated.

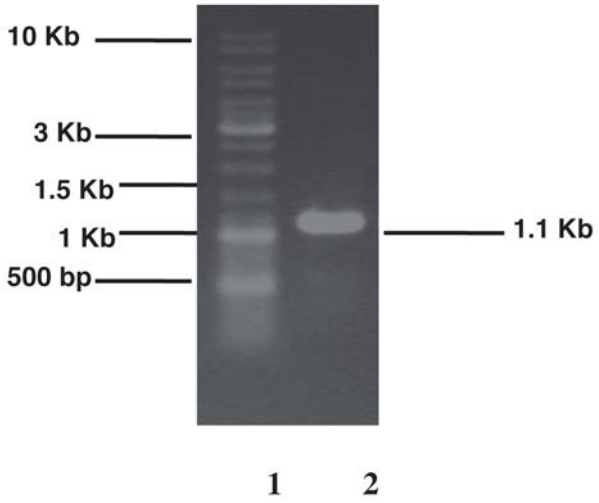

Figure 2. Agarose gel electrophoresis of the PCR product (*SFA* gene), lane 1= GeneRuler^{TM} DNA ladder mix (Fermentas, USA) and lane 2 = PCR product of *SFA* gene.

This DNA fragment was inserted into the *Xho*I/*Eco*RI site of pGBDU-C3, generating a pSFAU vector (Figure 3.). In this vector, the expression of *SFA* gene was under a control of ADH1-Promoter.

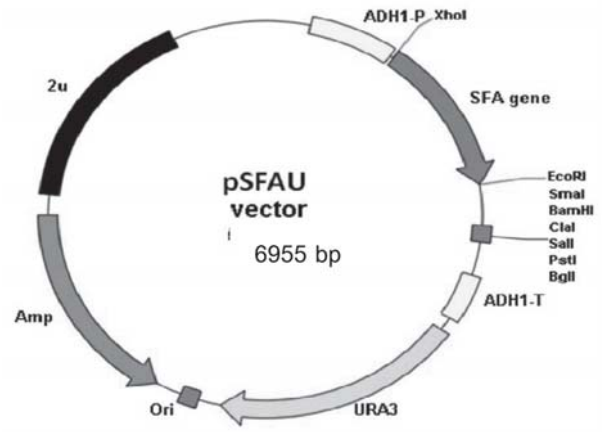

Figure 3. The construct of pSFAU, a formaldehyde-resistance expression vector.

The pSFAU vector was amplified in *E.coli*, sequenced and transformed into *S. cerevisiae* TISTR 5596, by which their formaldehyde resistance ability was increased from 4.0 mM to 5.5 mM (Figure 4).

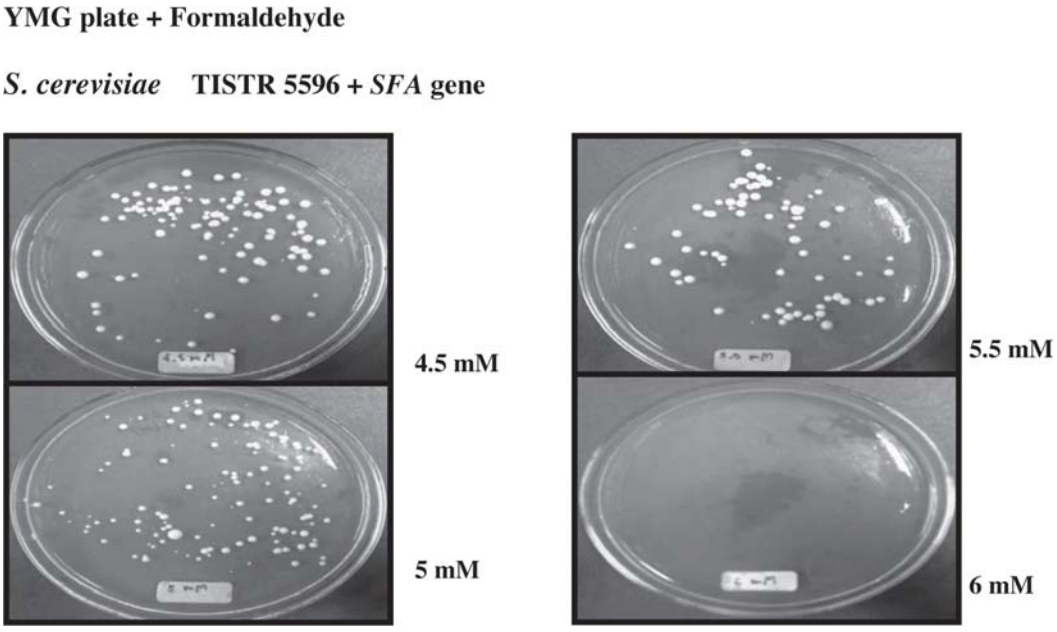

Figure 4. The colony growth of yeast transformant on YMG plate containing formaldehyde.

Discussion

In this study, the yeast strain selected as a host for cloning the recombinant vector was *S. cerevisiae* TISTR 5596. This yeast strain has a flocculant characteristic, which is favored in industrial applications because it provides an effortless removal of yeast cells after fermentation. Furthermore, the initial evaluation of ethanol yield of this yeast strain cultivating in 10g/l glucose was 0.505, which is 99.02 of a theoretical yield (Boonmee, unpublished). Interestingly, this strain primarily showed high tolerance to formaldehyde comparing with a previous described wild type strain, which only tolerate up to 1.5 mM formaldehyde (Wehner et al., 1993).

Sequence of the *SFA* gene amplified from *S. cerevisiae* TISTR 5596 was compared to the *SFA* gene accession number X68020, NCBI Genbank. They showed 100% similarity except that the *SFA* gene in this study contained mutation at two positions. The first mutation was at a nucleotide position 288, changing T to C and resulting thereby a codon GC**T** instead of GC**C**. This mutation was actually a silent mutation since both codons are translated to a same amino acid, namely glycine. Another mutation was found at nucleotide position 831, where C was change to T and therefore causing a codon change from CT**C** to CT**T**. Both codons, again, encodes a same amino acid (leucine). Therefore, both mutations did not lead to a misfunctional enzyme because amino acid sequence in its polypeptide chains remained the same. This occurrence could happen when expressing eukaryotic gene in prokaryote, since they have difference codon usage preference (Hong et al., 2004).

Pathways of formaldehyde degradation in the transformed yeasts could be suggested by the described methanol utilization pathway in methylotropic yeast. In methylotrophic yeasts such as *Hansenula polymorpha*, the enzyme formaldehyde dehydrogenase is part of methanol metabolism. The toxic formaldehyde, which was originated from an oxidize methanol, was changed to S-hydroxymethylglutathione. After being hydrolyzed by S-formylglutathione hydrolase, S-formylglutathione is changed to formic acid, followed by the enzyme formate dehydrogenase, carbon dioxide was formed (Figure 5).

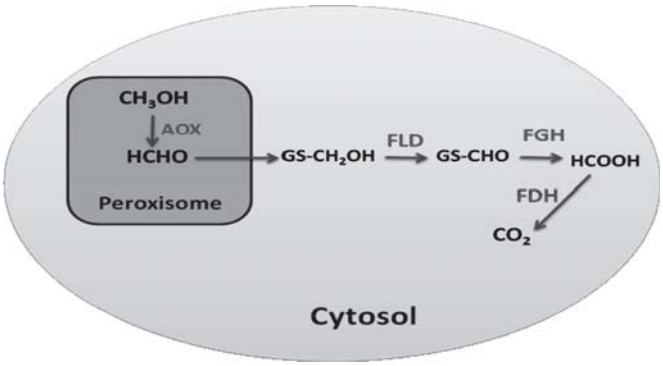

Figure 5. A simplified schematic of methanol utilization pathway in methylotrophic yeasts: The main pathway and enzymes working in the methanol metabolism in methylotrophic yeasts are shown. AOX: alcohol oxidase (EC 1.1.3.13), FLD: formaldehyde dehydrogenase (EC 1.2.1.1), FGH: S-formylglutathione hydrolase (EC 3.1.2.12), FDH: formate dehydrogenase (EC 1.2.1.2) (modified from Hartner & Glieder, 2006).

It is therefore highly likely that *S. cerevisiae* can completely metabolize formaldehyde, yielding carbon dioxide. The biological role of this pathway might be as a safety valve. Formaldehyde and formate are formed during metabolism in yeast cell, amongst others, tetrahydrofolate metabolism and must be broken down before they become toxic (Jones & Fink, 1982). This additional advantage makes this formaldehyde-resistance expression cassette very convenient for selection of yeast transformants.

Summary

We have successfully constructed a cloning vector containing formaldehyde resistance gene as a selective marker. The transformant containing this recombinant vector could resist up to 5.5 mM formaldehyde on agar plate.

This study showed the alternative to select of yeast transformants by using an inexpensive substance.

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