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Induction of Thermotolerance in *Saccharomyces cerevisiae* Strain(s) using Different Mutation Methods

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Abstract: The present study aimed to induce heat tolerant mutants in haploid *Saccharomyces cerevisiae* 5a FAIII strain by using different mutation methods. Three different mutation induction experiments were carried out to produce genetically stable heat tolerant *Saccharomyces cerevisiae* strains. The haploid strain 5a of the *S. cerevisiae* FAIII that has the highest growth rate was used throughout these experiments. Three thermo-tolerant mutants named as 5a1, 5a2 and 5a3 were obtained using *N*-methyl *N*-nitro *N*-nitrosoguanidine (MNNG), combination of ultra-violet radiation and hydroxylamine (UV+HA) treatments and spontaneous mutation, respectively. The mutants could tolerate 42 and 42.5°C but they couldn't survive extended period of time at 43°C. The growth rate of the mutants indicated that they exhibited well growth at 42°C, while they exhibited slow growth rate at 42.5°C. Although (MNNG) induced thermotolerance mutation at high frequency, it was found that mutants induced following synergistic effect of (UV and HA) exhibited more stability. On the other hand, the spontaneous mutant exhibited the best growth rate at high temperature.

Key words: *Saccharomyces cerevisiae*, thermotolerance, random mutagenesis, yeast extract/peptone/dextrose, rich medium, ultraviolet radiation, heat shock proteins

INTRODUCTION

The optimum growth temperature of most industrial yeast strains ranges from 30 to 39°C. Raising the temperature to 42°C is a major factor limiting the industrial uses of these strains for many reasons; the protein synthesis and respiratory activity were decreased. Moreover, the destabilization of microbial strains was observed at temperature above the optimum growth temperature (Jaenicke *et al*., 1991). The accumulation of misfolded proteins in the cell at high temperature may cause entry into a nonproliferating, heat-shocked state. This drawback may be overcome by selection of mutants that synthesis protein at relatively high temperature (Trotter *et al*., 2001).

Induction of stable thermotolerant mutants were previously investigated (Sreekumar and Basappa, 1991). The importance of isolation thermotolerance yeast strain has been recognized (Whitaker and Blatt, 1991).

Mutation can be either spontaneous or induced; arise because of changes in the base sequence of the nucleic acid of organism's genome (Madigan and Martinko, 2006). Genotoxic agents, such as ultraviolet (UV) light, ionizing radiation, or chemicals can be randomly induced DNA lesions in the genome. *N*-methyl *N*-nitro *N*-nitrosoguanidine (MNNG) is an extremely potent mutagen in microorganisms. It induces transition, transversion, large deletions or rearrangements (Barbour *et al*., 2006). It also induced base pair substitutions mutations (Myung and Kolodner, 2003). MNNG is the mutagenic agent commonly used for inducing mutation in yeast (Mitchel and Morrisom, 1987; Altboum *et al*., 1990). Recent study focused on the improvement of *Saccharomyces cerevisiae* through random mutagenesis to enhance the production of β-d-fructofuranosidase (FFase). The isolate with

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the highest activity was subjected to ultraviolet (UV) radiation and mutagenesis using *N*-methyl *N*-nitro *N*-nitrosoguanidine (MNNG), the mutant *S. cerevisiae* UME-2 was found to be a hyperproducer of FFase (Ul-Haq *et al*., 2008).

Another method of mutation using combination of ultraviolet radiation (UV) and hydroxylamine (HA) was performed by Popova *et al*. (1979). The toxicity of HA is due to that it disrupts many metabolic processes, it inhibits amino acid accepting ability of t-RNA and the synthesis of protein and it also interferes with the action of variety of enzymes (Mcclain *et al*., 2004). HA induces, whereas UV may cause inhibitory effect on DNA replication and the process of transcription transition (Madigan and Martinko, 2006). UV irradiation induces mitotic crossing over, mitotic gene conversion and reverse mutations in *Saccharomyces cerevisiae* (Hannan and Nasim, 1978; Meira *et al*., 1992). UV may also induce damage in centromere DNA of yeast (Capiaghi *el al*., 2004). The factor responsible for the lesion's mutagenicity by UV was previously studied by Lawrence *et al*. (1993), who suggested that cyclobutane dimmers may be responsible for most of mutation.

Therefore, the present study aimed to induce heat tolerant mutants in haploid *Saccharomyces cerevisiae* 5a FAIII strain by using different mutation methods. The isolated mutants might be of great importance in many industrial applications.

MATERIALS AND METHODS

Yeast Strain

Saccharomyces cerevisiae FAIII haploid strain obtained from microbial genetics lab. NRC, Egypt by tetrad dissection of diploid wild type *Saccharomyces cerevisiae* FAIII strain. Yeast strain was preserved in 15% glycerol at -65°C as described by Maniatis *et al*. (1982).

Mutation Using *N*-Methyl-*N*-Nitro *N*-Nitrosoguanidine (MNNG)

The general procedure was described previously by Mayer and Legator (1970). The complete medium (Yeast extract/peptone/dextrose (YPD)-rich medium) used for cultivation and fermentation of the haploid strain of *Saccharomyces cerevisiae* FAIII (5a) contained the following constituents (g L^{-1}): glucose (20), peptone (20), yeast extract (10), pH 5.3 (Ito *et al*., 1983). Cultivation was done using 500 mL Erlenmeyer flasks containing 100 mL medium. Cell suspension of this haploid strain was incubated for 18 h then transferred to complete broth for 24 h. Cells were harvested and washed twice in saline solution containing (g L^{-1}): 0.9 NaCl, by centrifugation at 6000 rpm for 20 min. The cell pellet was suspended in saline solution. Its percent of transmission was determined at 620 nm using UV-120-01 Shimadzu-spectrophotometer.

MNNG solution was freshly prepared for each experiment; appropriate dilutions were prepared in saline solution. Then, 9 mL of the cell suspension containing ≈ 5×10^7 mL^{-1} cells was incubated with 1 mL of diluted MNNG solution to each final concentration of 1, 2.5, 5, 7.5, 10 and 12.5 μg mL^{-1}. Treatments were continued for 1 h at 30°C on rotary shaker. The agitation rate was kept at 200 rpm, 5 min before the end of each treatment, the cell suspension was centrifuged and at exactly 1 h, the solution was decanted and the cells were washed twice in saline. About 0.1 mL of suitable dilution from each treatment was spread on YPD plates incubated at either 30 or 43°C for 2-3 days to determine the survival percentages and mutation percentages, respectively. Fifty colonies from each treatment were used to determine the mutation percentages at 42, 42.5 and 43°C.

Mutation Using Combination of Hydroxylamine (HA) and Ultraviolet Radiation

The general procedure was carried out as described by Popova *et al*. (1979). An 18 h culture of *S. cerevisiae* FAIII 5a was washed off with sterile distilled water and allowed to stand for 2 h. The cells were harvested by centrifugation at 1300 rpm for 5 min to obtain cell precipitate which was

washed with phosphate buffer pH 5.8. Solution of 0.4 M of hydroxylamine (HA) in the same phosphate buffer was added to the washed cell precipitation in an amount such that the number of cells was 10^7-10^8 mL^{-1} and the mixture was incubated for 1 h at 30°C. Diluting the mixture in 2% acetone in saline solution eliminated the mutagenic action of HA.

Cells treated with HA were washed twice with sterile distilled water and subjected to the influence of (UV) rays. For this purpose, sterile distilled water was added to a washed cell precipitate in an amount such that the number of cells was 10^7-10^8 mL^{-1}. Cell suspension was transferred to a petri dish and irradiated with UV rays for 10 sec. The radiation source consisted of two lamps in parallel. The dose of irradiation in this case was 320 erg mm^{-2}. The treated cell suspension was inoculated into 250 mL Erlenmeyer flasks containing 50 mL of complete mineral medium, which contained (g L^{-1}): $(NH_4)_2SO_4$ (2), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4$ (0.5), KCl (0.5), yeast extract (10), glucose (20) (Popova *et al*., 1979). Culture of treated cells was incubated at 39°C for 48 h. The control consisted of cell suspension cultured under the same conditions without treatment with mutagens. 0.1 mL of suitable dilution from the control and the treated organisms was spread on to YPD medium and incubated for 48 h at 30 and 42°C to determine survival and mutation percentages, respectively.

Auto selection was performed for 160 h with gradual rising of temperature from 39 to 43°C. Loopfull from the treated cells at each raising temperature was spread on YPD plates and tested for their resistance to each temperature by incubation at 30°C for 72 h.

Spontaneous Mutation

A loopfull of the haploid strain *S. cerevisiae* FAIII was inoculated into YPD broth and incubated at 42°C for 2 days, then, transferred to 30°C for 72 h. Loopfull from the resistant culture were inoculated again into YPD broth and incubated at 42°C for 2 days and then transferred to 30°C. This procedure was repeated several times to obtain any resistant colonies that result spontaneously. The resistant colonies were selected and cultivated another time at 42 and 42.5°C for 72 h on YPD plates (Madigan and Martinko, 2006).

Determination of Yeast Growth

The yeast growth was determined by inoculating each of the yeast strains in 500 mL Erlenmeyer flasks containing 100 mL YPD broth, followed by cultivation at 42 and 42.5°C for 72 h under static condition. Then samples were removed and the yeast growth was determined either by OD measurements at 620 nm using UV-120-01 Shimadzu-spectrophotometer or by direct plate count of yeast cells.

RESULTS

Three different mutation experiments using *N*-methyl *N*-nitro *N*-nitrosoguanidine (MNNG) combination effect of ultra violet radiation and hydroxylamine (UV+HA) and spontaneous mutation were carried out to produce stable heat tolerance *Saccharomyces cerevisiae* strain.

Mutation Induction Using (MNNG)

The survival percentages and mutation frequencies as a function of different concentrations of (MNNG) 1, 2.5, 5, 7.5, 10 and 12.5 μg mL^{-1} were determined (Table 1).

Results obtained indicated that the survival percentages of *S. cerevisiae* were highly affected by (MNNG) concentrations. The survival percentages at 30°C decreased gradually with increasing (MNNG) concentrations. No growth was found at any treatment after one-week incubation at 43°C. On the other hand, the mutation percentages were variable at 42 and 42.5°C. At 42°C, the maximum

Table 1: Effect of different *N*-methyl *N*-nitro *N*-nitrosoguanidine (MNNG) concentration on survival and mutation percentages of *Saccharomyces cerevisiae* FAIII 5a, significance level ($p<0.05$)

Treatments (mg mL^{-1})	Survival (%) 30°C	Mutation (%) 42°C	42.5°C
0.0	100.0	0	0
1.0	85.0	28	14
2.5	81.7	76	12
5.0	66.7	80	14
7.5	51.7	80	6
10.0	40.0	76	10
12.5	25.0	72	12

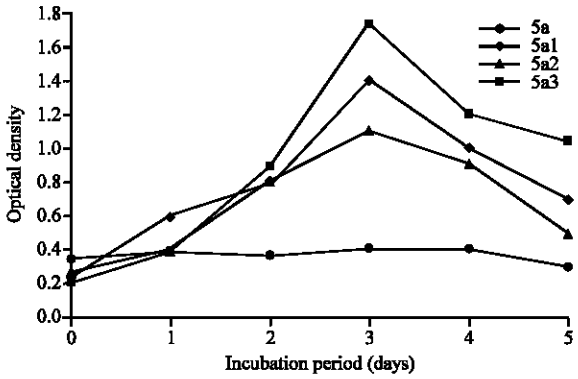

Fig. 1: Growth behavior of *Saccharomyces cerevisiae* wild type and its mutants at 42°C, where g (h) is the generation time (h) ($p<0.05$)

yield of mutation was 80% obtained following treatments with both 5 and 7.5 μg MNNG mL^{-1}. At 42.5°C the maximum yield of mutation was 14% obtained following treatment with both 1 and 5 μg MNNG mL^{-1}. These results indicated that the treatments with MNNG had an effect on tolerance of *S. cerevisiae* FAIII haploid strain at 42 and 42.5°C. The mutation percentages at 42 and 42.5°C are highly significant ($p<0.001$) (Fig. 1).

Mutation Induction Using Hydroxylamine (HA) and Ultraviolet Radiation (UV)

The method of auto selection (selection with continuous culturing) was used to isolate thermo tolerant yeast mutants. The survival percentage of the yeast cells, after the combined influence of the mutagens (HA+UV), was 15% at 30°C, but no viable cells was obtained at 42°C. Auto selection was performed for 160 h with gradual rising of temperature from 39 to 43°C. The results showed that only one isolate (5a2) out of all tested strains resisted 43°C.

Growth Behavior of Heat Tolerant *Saccharomyces cerevisiae* Mutants

This study examined the effect of relatively high temperature (42 and 42.5°C) on the growth rate of *S. cerevisiae* FAIII mutants; 5a1, 5a2 and 5a3 compared to their wild-type parental strain (5a).

The growth rate of *S. cerevisiae* FAIII mutants compared to its parental strain was estimated under similar culture conditions and at similar temperature (42°C). The results in Fig. 1 showed that *S. cerevisiae* wild type 5a exhibited very slow growth rate, however the growth rate of the three mutants 5a1, 5a2 and 5a3 increases within time to reach maximum around 3 days then decreased to near the original inoculum level except the growth rate of spontaneous mutant 5a3 which remained relatively constant after 3 days of incubation period. It also showed that the mutant 5a3 can duplicate within 2 h approximately, whereas mutant 5a1 and 5a2 required 3.35 and 4.16 h, respectively.

Table 2: The heat stability of the heat tolerant mutants at 42°C

Strain	No. of tested colonies	No. of heat stable colonies	Heat stability (%)
Saccharomyces cerevisiae 5a1	100	78	78
Saccharomyces cerevisiae 5a2	100	98	98
Saccharomyces cerevisiae 5a3	100	80	80

S. cerevisiae 5a1: Mutant obtained following MNNG treatment, *S. cerevisiae* 5a2: Mutant obtained following (UV+ HA), *S. cerevisiae* 5a3: Spontaneous mutant

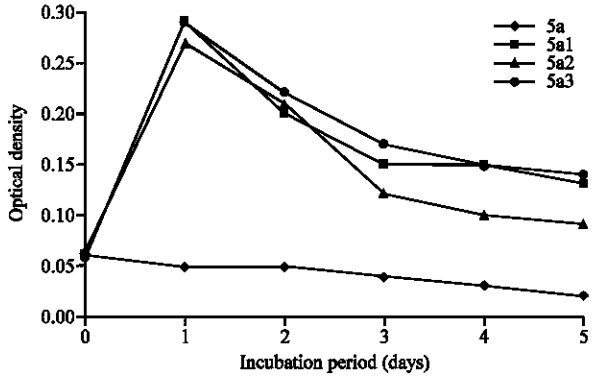

Fig. 2: Growth behavior of *Saccharomyces cerevisiae* wild type and its mutants at 42.5°C ($p<0.05$)

The growth rate of *S. cerevisiae* FAIII 5a wild type and its mutants was also estimated at 42.5°C. The results showed in Fig. 2 is entirely different from that obtained at 42°C. The three mutants 5a1, 5a2 and 5a3 behave alike and they exhibited slow growth rate. The population size peaked around 1 day. However, wild type 5a exhibited no growth. The values of each mutant at 42°C and at 42.5°C are differing significantly at $p<0.05$.

Heat Stability of Yeast Mutants

The three mutants strains; 5a1, 5a2 and 5a3 were tested for heat stability (Table 2) by spreading 0.1 mL from appropriate dilution of the tested strain on YPD medium and incubated it at 30°C for 48 h. Then one hundred of the resulted colonies were tested for their ability to grow at 42°C after 72 h.

DISCUSSION

In the present study an attempted was carried out to isolate *Saccharomyces cerevisiae* mutants, which tolerate industrial culture conditions (high temperature). To this goal, *Saccharomyces cerevisiae* FAIII 5a, which has the highest growth rate at 41°C, was used.

Induction of *S. cerevisiae* mutant able to tolerate temperature higher than 37°C was adopted using mutation experiments either by using *N*-methyl- *N*-nitro- *N*- nitrosoguanidine (MNNG) or combination of ultra-violet radiation and hydroxylamine (UV+HA). A spontaneous mutation was also performed.

The survival percentages of *S. cerevisiae* obtained after MNNG treatments which ranging from (25-85%). Most efficient mutagenesis occurs when a mutagen confers a high frequency of mutations with low lethality, in the range of 10 to 50% survival (Barbour *et al*., 2006). Similar results were found for *S. cerevisiae* by Calderson and Cerda-Olmedo (1982).

The mutants obtained following MNNG treatments resist both 42 and 42.5°C with high frequency. But only one mutant 5a1 out of large tested number (fifty colonies) could survive extend period of time at 43°C.

From the previous results, it has been possible to induce thermotolerance mutants by using the method described by Mayer and Legator (1970), Myung and Kolodner (2003). Similarly, the induction of thermotolerant organism using MNNG was supported by Sreekumar and Bassappa (1991), who found that MNNG induce stable thermotolerant mutants of *Zymomonas mobilis*.

Another method of mutation induction using synergistic interaction between ultra-violet radiation and hydroxylamine was performed. The method used was similar to that described for *Hansenula anomala* stain T-327 (Popova *et al*., 1979). The previous observation demonstrated that radiation increased the mutagenic effectiveness of hydroxylamine (Mitchel and Morrison, 1987). Present results correlated with those obtained by Unaldi *et al*. (2002) and Sridhar *et al*. (2002) indicating that UV mutagenesis can be used for improving thermotolerance and ethanol tolerance in yeast strains. However, the current results revealed that the survival percentage was 15% following combination of UV-radiation (320 ergs/mn for 10 sec) and HA (0.4 M) treatment and the mutation frequency were very low. The low frequency of survival and mutation may be due to the high toxicity of HA rather than its mutagenic effect. The toxicity of HA explained as it disrupts many metabolic processes, it inhibits the amino acid accepting ability of tRNA and the synthesis of protein and interferes with the action of variety of enzyme (Mcclain *et al*., 2004). The low mutation frequency might also be explained as the thermotolerance character is controlled by large number of genes such as, *CDC*25 gene (Geymonat *et al*., 1998), *CDC*28 gene and a yeast heat shock transcription factor (Hsf1) (Zarzov *et al*., 1997), the *HSP104* gene and the *RAS* genes which is largely responsible for induced thermotolerance in yeast (Piper, 1993; Shama *et al*., 1998). Moreover, for Thermus thermophilus, a positive correlation was observed between the growth temperature of the cells and the presence of 2-thioribothymidine in position 54 of tRNA (s^2T54). The presence of a thiol group on ribothymidine leads to an increase in the melting temperature of *T. thermophilus* $tRNA_1^{Ile}$ Interestingly, inactivation of the *T. Thermophilus trmI* gene results in a thermosensitive phenotype (growth defect at 80°C), which suggests a role of the N^1-methylation of tRNA adenosine-58 in adaptation of life to extreme temperatures (Droogmans *et al*., 2003). These genes are distributed on all chromosomes of the cell and the ability of mutagenic agent to induce transition, transversion and deletion give a chance for genes to react with each other and give the character (Lillo *et al*., 1997). It appears that the cellular response to heat shock is the sum of the separate responses to individual heat induced defects, of which we can identify misfolded proteins and cell surface stress (Trotter *et al*., 2001).

The growth curve of *S. cerevisiae* wild type and mutants indicated that the spontaneous mutant exhibited the best growth rate at 42°C over 5 days of incubation period rather than other induced mutants. These findings might be explained as the survival (cell viability) was affected as a function of chemical and physical mutagens. These results gain insight about the best method for producing thermotolerance strain was spontaneous mutation. This mutation called adaptive mutations which are generated by mechanisms activated by the organism itself in order to survive a particular stress condition, while the spontaneous rate of mutation is very low (Madigan and Martinko, 2006). In contrast to this finding, the stability test for thermotolerance mutants revealed that the mutant of *S. cerevisiae* induced after treatment with combination of hydroxylamine and UV radiation, showed high stability relative to that produced by other agent.

Thus it was concluded that the three methods used here are very promising for isolating thermotolerance haploid strain of *S. cerevisiae* which tolerate 42.5°C and they also seemed to be very stable since the strains obtained might be of great importance in many industrial applications as fodder protein production, ethanol production, agricultural and industrial wastes degradative enzyme production and others.

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