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Selection of Polygalacturonase-Secreting *Saccharomyces cerevisiae* Mutants from Saudi Arabia

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The study was aimed at increasing the production of polygalacturonase (PG) in Saccharomyces cerevisiae by the process of mutation. A high PG-secreting strain of S. crerevisiae was exposed to different doses of diethyl sulfate (DES) and surviving colonies were screened for PG activity by plate assay method. Survival rate and mutation frequency were recorded under different treatments. Many selections obtained in the study showed PG activity in excess of the starting strain and the wild type reference strain. PCR analysis of the mutants revealed unique markers in some of the selections which corroborated well with their respective PG activity. The study augments the relevance of mutagenesis for strain improvement in fungi for production of useful industrial enzymes.

Key words: Yeasts, *Saccharomyces cerevisiae*, Mutation, Diethyl sulfate, RAPD-PCR.

Microorganisms play a major role in food industry, primarily because of their ability to produce valuable organic compounds as a result of cellular metabolism. Enzyme polygalacturonase (PG) is one such compound which can degrade pectin component of plant cell wall by hydrolysis of glycosidic bonds. Pectins are a carbohydrate polymers consisting mainly of galacturonic acid units connected by glycosidic linkages. Polygalactronase is produced by many fungi and bacteria. This enzyme is used in the food industry in many ways, like for extraction and filtration of fruit juice[8,3].

Saccharomyces cerevisiae is yeast of extensive industrial application. It is used widely for production of alcohol, organic acids and bakery items. Besides, it is also used in genetic research because many cellular processes such as replication, genetic recombination etc. are conserved between the yeasts and larger eukaryotes. Its relatively small genome can be manipulated and analyzed readily. Strains of *S. cerevisiae* are known to produce polygalacturonase[17] but their production capacity varies greatly[5,6].

In this study we have attempted to induce mutations using diethyl sulfate (DES) and compare the PG level of mutants with standard strains and a local isolate. RAPD analysis was done to verify the mutations.

MATERIAL AND METHODS

A PG-producing strain Saccharomyces cerevisiae MB1, isolated in our lab earlier, was used for induction of mutation and for subsequent comparison of PG activity while, *S. cerevisiae* 1389 (wild type) was generously provided by Professor Villa of University of Santiago. All strains were grown on YPD (1% yeast extract, 2% peptone, 2% dextrose) broth and YPD agar.

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For induction of mutation, *S. cerevisiae* MB1 was multiplied in YPD broth overnight. Subsequently, 25ml aliquots supplemented with 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM DES were grown at 30°C for 3 days. Aliquot without DES was maintained as control. After three days, the cultures were scored for growth of cells. Survival rate in each treatment was calculated for each treatment according to the procedures of[12, 14]. Pure cultures were developed from a large number of surviving colonies. To screen for mutants, individual colonies along with control *S. cerevisiae* MB1, and wild type *S. cerevisiae* 1389 were tested for PG activity and PCR analysis.

PG activity was detected by plate assay method[15,16] using ruthenium red dye on yeast nitrogen base medium (YNB). After incubation at 30 °C for 3days, enzyme activity was scored on the basis of color development around the colonies on a 1-5 scale in ascending order, the brightest being labeled as 5 and the dimmest as 1. Observations were recorded on eight plates and means with standard deviation were calculated for each colony. Mutation rate was estimated as frequency of colonies showing variation in mean color score as compared to the control strain, *S. cerevisiae* MB1 in the total number of colonies tested for a dose of the mutagen.

For performing PCR analysis, DNA was extracted according to the procedure of Harju9 . Yeast cultures were shaken in YPD broth (1% yeast extract, 2% peptone, and 2% dextrose) overnight at 30°C. Colony clumps were suspended in 200?l extraction buffer (2% Triton-x, 1% SDS, 100mM NaCl, 10mM Tris-HCl, pH 8.0, and 1mM EDTA) and tubes were incubated in water bath at 65°C for 2-3 minutes. After short vortexing (30 sec), 200?l chloroform-isoamyl alcohol (24:1) was added and tubes were centrifuged for 2 minutes at 12,000 rpm. Supernatant was transferred to fresh tubes and precipitation of DNA was done with 400?l ice-cold ethanol. After centrifugation for 5 minutes at 12,000 rpm, pellet was washed with 70% alcohol and was resuspended in 50?l TE buffer. The content of genomic DNA was verified by 260/280 ratio with a UV spectrophotometer.

PCR procedures were performed using Actin primers (Promega) in a thermocycler (MasterCycler®, Eppendorf). Reaction mix (25-µl) consisted of 20ng genomic DNA, 100µM dNTPs, 1mM MgCl2, 2.5?l 10x PCR buffer (10mM Tris-HCl, 50mM KCl, 1.5mM MgCl2, pH 8.3), 0.2µM of each primer pair and 1 U Taq DNA polymerase (Invitrogen). Final volume of the reaction was made up with distilled water. Cycling conditions were: initial denaturation at 95°C for 3 min, followed by 30 cycles, each consisting of 95°C for 50 s, annealing temperature of 58°C for 50 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. Amplification products were separated on 1.2% agarose gel submerged in Tris-acetate-EDTA buffer. Ethidium bromide (10ng/ml) was added to the molten agarose before gelling as a stain. DNA Profiles were visualized under UV in UVIBand system (UVTec, Cambridge).

RESULTS AND DISCUSSION

Survival rate of the yeast cells under different treatments has been presented in Fig. 1. It is noticeable that survival declined regularly with increasing concentration of DES. The survival decreased to 8.9% at the highest concentration of 3mM as compared to 98.2% in control. Similar

Table 1. Markers expressed by selected *S. cerevisae* mutants (Sel: selection)

Strain	Marker										
M	100	200	300	350	400	500	600	700	800	Total brand	Total bp
Wild Type	0	1	1	1	0	1	0	1	0	5	2050
S.c MBI	0	1	1	0	0	1	0	1	0	4	1700
Sel1	0	1	1	0	1	1	0	1	0	5	2100
Sel 2	0	1	1	0	1	1	0	1	0	5	2100
Sel 3	0	1	1	0	0	1	0	1	1	5	2500
Sel 4	0	1	1	0	0	1	1	1	0	5	2300
Sel 5	0	1	1	0	0	0	0	0	0	2	500

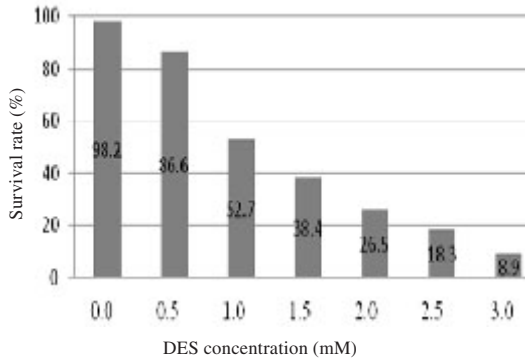

Fig. 1. Survival of *S. cerevisiae* cultures treated with different doses of DES

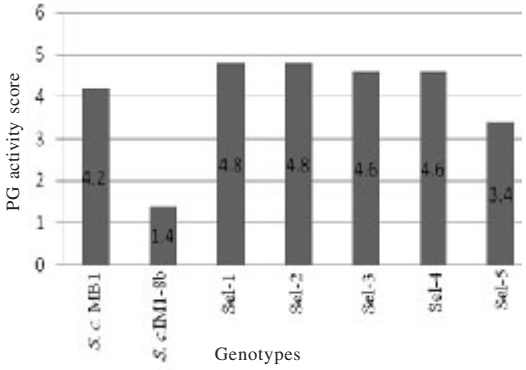

Fig. 2. PG activity of selected strains after mutagenic treatment (S. c: *S. cerevisiae*, Sel: selection)

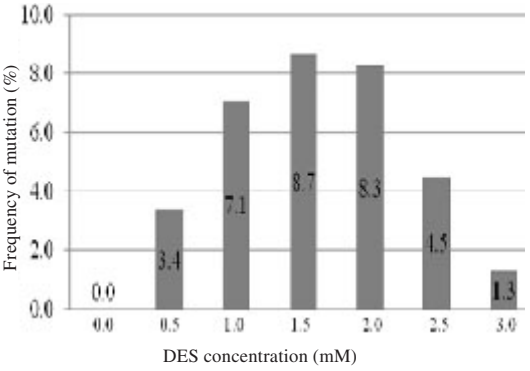

Fig. 3. Frequency of mutation in different concentrations of DES

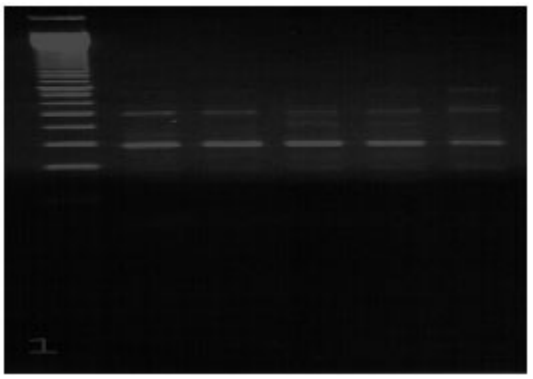

Fig. 4. PCR markers using actin primers: Lanes, 1: marker, 2: S. cerevisiae- wild type, 3: S. cerevisiae MB1, 4: Selection-1, 5: Selection-2, and 6: Selection-3

effects of chemical mutagens have been reported by Zambare[22] while working with Trichoderma reesei. DES is known as a highly potent mutagen, frequently used for induction of mutations in plant, insect and microbial cells. Besides being mutagenic, DES is toxic to most cell types and the toxic potential depends on the target genome and the treatment conditions[4,13].

Several colonies derived from DES-treated cultures showed PG activity at variance with the reference strains and were considered as mutants. Strain *S. cerevisiae* MB1 used for initiation of mutagenesis in this study was selected earlier in our lab and is a high PG-secreting genotype while, *S. cerevisiae* IM1-8b (wild type) is a low PG strain. Activity scores of some selected mutants have been depicted in Fig 2. Four selections have consistently shown higher scores (4.8±0.05; 4.6±0.08) as compared to the better reference (4.2±0.05). One of the selections (Sel-5) showed considerably reduced PG activity score of 3.4±0.06. It might be a case of deleterious or negative mutation. Zambare[22] has reported up to 2.6 fold increase in protease production by several Trichoderma reesei selections after treatment with N-methyl-N-nitro-N-nitrosoguanidine (NTG). Still higher (3.5 fold) protease activity was reported by Wang[21] in NTG mutants of Bacillus pumilus. Ryden[18] reported an increase of 3-10 folds in Staphylococcus aureus mutant generated by NTG. In similar studies, improvement in production of cellulase, chitanse[7] , lipase[2], and lactic acid and glucoamylase[20] has also been reported by induction of mutations in different fungi. Solaiman19 obtained several Bacillus mutants expressing enhanced production of alkaline protease. These reports suggest that mutagenesis can be successfully utilized for enhancing the

production of useful secondary metabolites including industrial enzymes by fungi and bacteria.

Frequency of mutation, presumed at par with the rate of mutation, has been depicted in Fig 3. Highest and similar frequencies were recorded in 1.0-2.0 mM concentration of DES. At the highest tested dose of 3.0mM which severely affected the survival also (Fig 1), only one mutant was noticed out of 72 colonies tested. Hopwood10 suggested that a dose causing highest mortality in cultures is best suited for strain improvement as the few survivors would undergo more extensive mutations which may lead to enhancement in their productivity.

DNA markers generated by PCR amplification have been depicted in Fig-4 and enumerated in Table 1. It is noticeable that our previous selection *S. cerevisiae* MB1, the basic genotype used for induction of mutations in this study, lacks a 350 bp band present in the wild type strain. This genetic difference may be responsible for variant nature of this selection in terms of polygalacturinase production. Subsequent selections derived from this genotype during the present study also lack this marker in concurrence with the basic genetic material. Selections 1 and 2 both show a unique marker of 400 bp which may explain their similar PG activity (Fig 2). This marker may also be used for identification and selection of high PG genotypes of *S. cerevisiae* in future studies. Selections 3 and 4 have expressed unique markers of 800 and 600 bp respectively. This genetic variation might be the reason behind their altered PG activity as compared to the starting genotype and the other selections. Selection 5 shows loss of markers at 500 and 700 bp level. This deficiency might, presumably, be the cause of severely reduced PG activity of this mutant as compared to the reference genotype and other selections. Mutations in the genome of a fungus are known to drastically alter the phenotypes including production of secondary metabolites[1].

The present study has resulted in selection of stable mutants capable of producing high levels of polygalacturonase for applications in many biotechnological processes. These findings highlight the utility of mutagenesis for strain improvement in fungi with regard to production of useful industrial enzymes.

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