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Identification of Proteins with 2 Dimensional Electrophoresis (2DE) and Mass Spectrometry in Native Strain of *Saccharomyces cerevisiae* with High Yield Bioethanol Production after Random Mutagenesis

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Ethanol is one of the most important fuels. To access an enhanced yield of ethanol and alleviated effect of inhibitor compounds like furfural, a native strain of *Saccharomyces cerevisiae* was mutated by ultra violet radiation. For realizing the enzymes, expression of which, have been changed in mutant strain, a comparative proteome analysis was performed. Mutant strain was resistant against 4 g/l of furfural and its ethanol production yield was 39% more than parent one. Proteome analysis of both strains revealed significant changes in 3 enzymes were involved in ethanol production, glycolysis pathway and antioxidative Stress ,. These enzymes are known to have key roles in metabolism of energy and resistance against environmental stresses. The observed changes in expression of these proteins are vital for effective and purposeful strategies for designing of recombinant strains.

Key words: Bioethanol, Furfural, Mutation, Proteomics, *Saccharomyces cerevisiae.*

Saccharomyces cerevisiae is a common microorganism which is utilized in ethanol fermentation industry. This microorganism has good tolerance against inhibitors, such as furfural. This inhibitor is normally produced in acid hydrolysis processes of lignocellulosic materials to sugars. Quantities of 1 g/l of furfural are reported to be toxic for the yeast[16]. Furfural has been known to create strong inhibition in metabolism and growth rate, as well as the fermentation rate of *S. cerevisiae* under aerobic and anaerobic conditions[17]. The inhibitory effect of furfural on different glycolytic enzymes and dehydrogenases, is also investigated. Dehydrogenases were identified as enzymes most sensitive to furfural[2]. Furfural and Phenolic compounds such as vanillin that are generated by lignin degradation have been shown to inhibit mitochondrial superoxide dismutase (Mn-SOD)[9].

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Growth in the presence stress conditions lead to multigenic responses, that is detectable in proteome structure of *S. cerevisiae*[22]. In this study, random mutagenesis was done by radiation for improvement of the yeast strain. Selection of the mutants after treatment by ultra violet light was done in the presence of furfural. Efficient producers of ethanol in the presence of furfural were chosen for comparative proteome analysis. By 2-DE combined with mass spectrometry (MALDI-TOF/MS), some differently expressed proteins were detected, one of which was of the antioxidative Stress protein, while the others were associated with the ethanol production and glycolysis/gluconeogenesis pathways.

MATERIALS AND METHODS

Microorganism

Saccharomyces cerevisiae T12 (PTCC[1] 5315)[21], a native strain that showed good ethanol production and furfural resistance (up to 2 g/l), was used in this study as parent strain.

Random mutagenesis

Ultra violet treatment and selection of mutants

The yeast cells, grown on Potato Dextrose Agar (PDA) Medium, containing (g/l): glucose, 20; potato, 300, for 24 h, were diluted and transferred to YEPD-agar plates, containing (g/l): yeast extract,10; peptone, 20; dextrose, 20; agar, 15 and freshly distilled furfural 2 g/l; pH 5.5. The plates were mutagenized from a distance of 20 cm by using a Philips™ 30 W germicidal U.V. lamp for 275 seconds[1, 19]. The single colonies which were able to grew on these plates, were transferred to YEPD broth (the same ingredients, as above, without agar) containing 4 g/l of furfural, for 48 h. The survived cells were isolated and kept on YEPD agar medium, containing 1.5 g/l furfural, to prevent the loss of resistance against furfural.

Fermentation

After selecting the most resistant mutant strains, ethanol production experiments were performed. In aerobic phase, the resistant yeast cells were cultured in synthetic medium, containing (g/l): glucose, 190; yeast extract, 10; $(NH_4)_2SO_4$, 1.2; $(NH_4)_2HPO_4$ 0.6; pH 5.3, and placed on rotary-shaker at 150 rev. min^{-1}, 30 °C, for 8 h. After this phase, the cultures were aseptically transferred and filled into 25 ml Bijoux bottles for 40 h to complete the anaerobic/ ethanolic fermentation period[17]. After completion of fermentation, the cells were removed by centrifugation and the supernatant was distillated to separate the ethanol.

Analytical methods

For measurement of ethanol concentration, samples were injected directly into a gas chromatography system (GC-14A, Shimadzu, Japan) with a UV detector and OV17 column (2m×3.1mm) that had been packed with methyl silicon (Thermo Scientific Pierce, USA). The chromatographic conditions were set to: initial temperature, 50°C; final temperature, 90°C; injector temperature, 230°C; nitrogen as the carrier gas, with a flow rate of about 30 ml/min [12].

Sugar analysis in medium

The amount of glucose in the fermentation samples were measured by an enzymatic glucose reagent (Parsazmun, Karaj, Iran) based on glucose oxidase/ peroxidase reaction. The intensity of colored product (quinoneimine) was measured specrophotometrically at 500 nm.

Statistical analysis

For comparing ethanol production and glucose consumption in native and mutant strain ANOVA analysis was performed on the results, using SPSS ver. 16 software (SPSS, Inc. USA), and assuming p- value < 0.05 for significance.

Sample preparation for proteomics analysis

The parent T12 (PTCC 5315) and mutant (Fj) strains of *S. cerevisiae* were grown in PDB medium and incubated on rotary-shaker at 150 rev. min^{-1}, 28 °C, for 20 h, in three replicates. Cells were harvested by centrifugation (2800×g, 4°C, 10 min) in the late mid-exponential phase. The supernatant was removed and the cell pellet was washed by ice-cold deionized water and spun (2800 × g, 4°C, and 10 min). Yeast cell pellets was collected and grounded to fine powder in liquid N_2. The frozen powder was suspended in a lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(cholamidopropyl) dimethylammonio]-1- propanesulfonate (CHAPS), 40 mM Tris-base). Subsequently, a DNase and RNase solution (1% DNase I, 0.25% RNase A, 50 mM $MgCl_2$, 0.5 M Tris–HCl, pH 7.0) was added and incubated on ice. Then, 1 mM phenylmethylsulfonyl fluoride was added and the sample was sonicated briefly[6]. Cell debris was removed by centrifugation at 10000×g for 30 min in 15ºC.

For increasing protein concentration and desalting, TCA- Acetone precipitation was done[13]. The dried proteins were dissolved in determined rehydration buffer (8 M urea, 2% CHAPS, 20 mM dithiothreitol (DTT), 0.5% immobilized pH gradient (IPG) buffer pH 4–7. Protein content was determined according to the Bradford method using BSA as standard[4]. The lysate was either used immediately or aliquoted and kept in -70°C for further use[6].

2-DE

Isoelectric focusing was performed on 17-cm IPG strips (BioRad, USA) with linear pH range of 4-7, which were passively rehydrated overnight by loading the samples, diluted with rehydration buffer containing: 8 M urea, 2% CHAPS, 20 mM DTT, 0.5% immobilized pH gradient (IPG, USA) buffer pH 4–7 and small amounts of bromophenol blue. Isoelectric focusing was carried out using the PROTEAN IEF cell (BioRad, USA) began with linear increase from 0-250 V for 20 min, followed by

linear increase to 10000 V 2.5 h, and remained on 10000 V to achieve total 50,000 Vh.

Then, the focused IPG strips were reduced for 20 min at room temperature in equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT) and subsequently alkylated for 20 min in equilibration buffer containing 5% iodoacetamide instead of DTT at room temperature. The equilibrated strips were placed on top of 12% SDS- PAGE handmade gels and sealed with 1% agarose. The second dimension electrophoresis was performed using a standard Laemmli buffer system[14] at 16 mA/gel for 30 min and 24 mA/gel for the next 5 h at 20°C. After 2-DE, for MS identification, proteins in gel were stained by a modified colloidal Coomassie blue method[5].

Image analysis

The stained gels were scanned at a resolution of 300 dpi using the densitometer GS-800 (BioRad, USA). The quantification of spots intensity and statistical evaluation were done on Coomassie blue stained analytical gels using Image Master 2D Platinum Ver. 6.0 (GE healthcare, USA). Statistical analysis of protein variations was carried out in 2-D gels prepared from three replicates (CV<40%) in each group using the student *t*-test on vol% of matched spots. The statistical significance was assumed less than 0.05 for *p*-values.

MS analysis, database searching

The protein spots were manually cut from colloidal Coomassie blue stained 2-D gels. Gel pieces were washed two times with 50% aqueous acetonitrile containing 25 mM ammonium bicarbonate, then once with acetonitrile and dried in a vacuum concentrator for 20 min. Sequencing-grade, modified porcine trypsin (Promega, UK) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold by adding 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.02 mg/ml gel pieces were rehydrated by adding of trypsin solution, and after 30 min enough 25 mM ammonium bicarbonate solution was added to cover the gel pieces. Digests were incubated overnight at 37°C.

A 1 mL aliquot of each peptide mixture was applied directly to the ground steel MALDI target plate, followed immediately by adding an equal volume of a freshly-prepared solution of 5 mg/mL of 4- hydroxy-α- cyano- cinnamic acid (Sigma, UK) in 50% aqueous acetonitrile, containing 0.1%, trifluoroacetic acid.

Bruker flex Analysis software (version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra. Tandem mass spectral data were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.1), through the Bruker ProteinScape interface (version 2.1). Search criteria included: Enzyme, Trypsin; Fixed modifications, Carbamidomethyl (C); Variable modifications, Oxidation (M); Peptide tolerance, 250 ppm; MS/MS tolerance, 0.5 Da; Instrument, MALDI-TOF/TOF .Statistical confidence limits of 95% was applied for protein identification.

RESULTS

According to survival curve (not shown) after 275 sec of UV treatment, 99.99% of the yeast cells were killed and this time was selected for next step of mutagenesis. After 275 sec of UV treatment on native strain, the mutant cells that grew were exposed to 4 g/l furfural for isolation of resistant mutants. The obtained mutants were compared regarding production of ethanol in the presence of furfural, where the mutant Fj was isolated among many others.

Comparison of ethanol production and sugar utilization in parent and mutant strains

The mutant strain (Fj) was compared to the parent strain (T12) regarding sugar consumption and ethanol production. As shown in Fig.1 and 2, differences in residual glucose concentration and ethanol production in mutant and parent strains, until 24 h are negligible, while after this time, utilization of glucose and production of ethanol in mutant strain was increased in relation to the parent. Comparing the production yield (g ethanol/ g initial glucose) showed that this figure was 39% higher in the mutant than the parent.

Proteome differences examination in native and mutant strain

Native and mutant *Saccharomyces cerevisiae* cells were cultured in the same conditions in three replicates.

Comparative proteomic profiles can help us to understand the reasons for changes in

ethanol efficiency after mutation in native strain. Analytical and preparative 2-D gels of proteins extracted from native and mutant strain were carried out on 17cm IPG strips with linear pH range of 4-7 (Fig. 3) [6].

Four protein spots with predefined scale for significant expressional changes were determined (Fig. 4).

After statistical analysis using t-test, 4 proteins were shown to have been over expressed in mutant strain. Differentially expressed proteins were excised from 2-D gels and identified by MALDI-TOF/TOF mass spectrometry (Table 1). The identified proteins were categorized by their known and/or putative functions into three groups. The cellular proteins were involved in

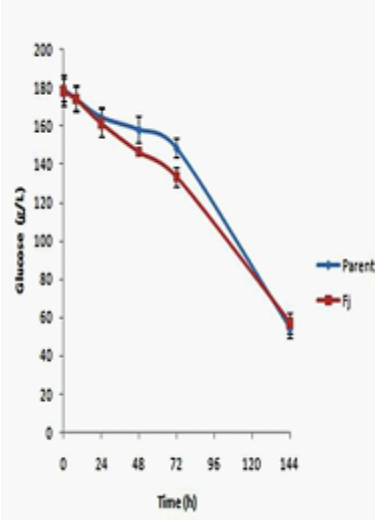

Fig. 1. Comparison of glucose consumption by parent and mutant strains

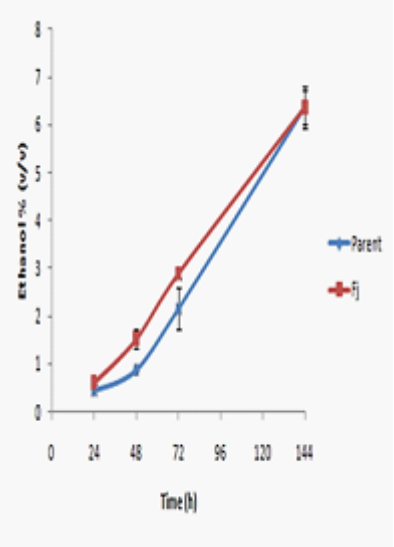

Fig. 2. Comparison of production of ethanol by parent and mutant strains

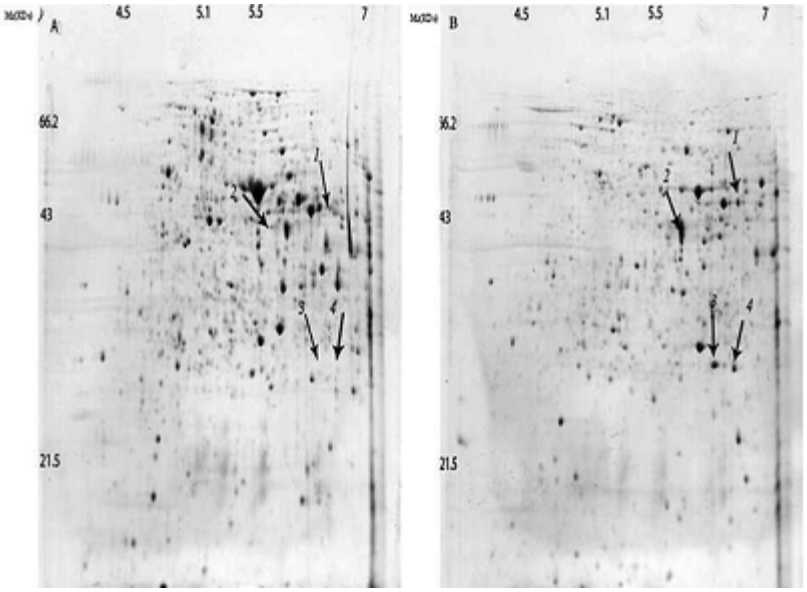

Fig. 3. Colloidal Coomassie blue stained 2-D gels derived from parent (A) and mutant strain(B). Proteins were separated on pH 4-7, 17 cm L IPG strips in the first dimension and SDS-polyacrylamide gel (12%) in the second dimension. (Scanned by 300 x 300 DPI resolution)

Glycolysis pathway, ethanol production pathway and antioxidative stress.

DISCUSSION

After UV treatment on native strain of saccharomyces cerevisea was caused mutant strain was resistant against 4 g/l of furfural and its ethanol production yield was 39% more than parent one For Proteins recognition and identification after mutation and variations consideration in protein pattern in native and mutant strain, a proteomics profiling strategy was utilized (Fig. 3). For extensive variations in protein pattern between native and mutant strain, cell lysis and 2-DE analysis was done repeatedly that final results of protein pattern completely was similar to prior results.

Our findings show that the expression

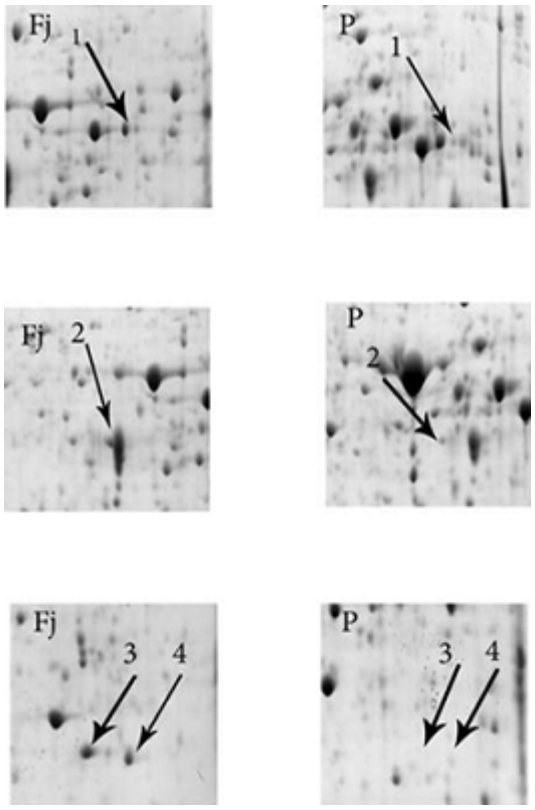

Fig. 4. Highlighted picture of 4 identified protein spots demonstrating significant differences between mutant (Fj) and parent strains' (P) groups (Information of spots is presented in table 1). (Scanned by 300 x 300 DPI resolution)

Table 1. Summary of protein identification, using MALDI-TOF mass spectrometric analysis

Spot no.	Protein name	MOWSE score	Accession no.*	Theoretical Mr/pI	Observed Mr/pI	Sequence coverage (%)	Student -T	Expressional fold change (mutant/parent)
1	Alcohol dehydrogenase I	577	gi|1168350	37.2/6.26	46/6.4	22%	6.63	4.6
2	Fructose 1, 6-bisphosphate aldolase	755	gi|6322790	39.8/5.51	41/5.75	29%	8.62	2.0
3	unnamed protein product homologous to glyceraldehyde-3-phosphate dehydrogenase)	239	gi|50288681	35.95/6.46	28/6.1	8%	11.25	3.7
4	dismutase,Mn superoxide	260	gi|223570	22.7/5.8	28/6.3	13%	39.4	9.6

level of ethanol producing enzyme, alcohol dehydrogenase I (ADH1) was increased upon treatment of cells with UV radiation and screening in the presence of furfural (Table 1 and Fig. 4). The result of increased ADH I expression could be the higher yield of ethanol in mutant Fj, compared to its parent strain (Fig. 2). In Saccharomyces *cerevisiae,* two genes, *adh1* and *adh2,* code for two cytoplasmically expressed alcohol dehydrogenases: ADH I and ADH II. ADH I enzyme is involved primarily in ethanol production during fermentation. It is largely responsible for regeneration of NAD^+ in glycolysis[7]. Studies on yeast cells showed that furfural in the range of 1 to 2 g/l, inhibit function of alcohol dehydrogenase 1 enzyme and strongly affect the specific growth rate of the cells[17]. Enhancement of the expression of this enzyme in the mutant strain is in good correlation with its behavior, regarding production of more ethanol in the presence of furfural.

Another enzyme, expression of which has shown to be increased, was fructose-1, 6-bisphosphate aldolase (Table 1 and Fig. 4). Fructose-1, 6-bisphosphate aldolase is an essential glycolytic enzyme found in *Saccharomyces cerevisiae,* which catalyzes the cleavage of fructose 1, 6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate[3]. Furan derivatives like furfural in the range of 1 to 2 g/l, have reported to inhibit the aldolase enzymes in glycolytic pathway and affect the rate of growth of yeast cells[15].

After protein identification by mass spectrometry, we found an unnamed protein product (homologous to glyceraldehyde-3-phosphate dehydrogenase) with similar ranges of *M*r and p*I* (Table 1). The expression level of this protein was also increased after mutation by UV radiation and screening in the presence of furfural. The glyceraldehydes-3- phosphate dehydrogenase is sensitive to furfural[20], thus its over expression in mutant strain may be consider as a defense against environmental stress. This enzyme still is unnamed in *Saccharomyces cerevisiea* and is homologous to glyceraldehyde-3-phosphate dehydrogenase.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been considered a classical cytosolic glycolytic protein and in some microorganisms plays some role in cell flocculation[8]. This phenomenon has been considered very interesting in ethanol production, by facilitating the separation of the yeast cells from the fermenting medium[18].

Superoxide Mn dismutase (Mn-SOD), another protein which was found in higher level in the mutant, than the parent strain, catalyzes the conversion of superoxide anions to hydrogen peroxide and oxygen, thus provides the protection against toxic intracellular radicals that are produced under oxidative stresses[10]. The manganese-superoxide dismutase (Mn-SOD) is encoded by the gene sod 2 and is located in the mitochondrial matrix[11]. The presence of furfural causes known metabolic stresses, against which the over expression of Mn-SOD, may be of great significance. In previous reports it has been shown that furfural and phenolic compounds, such as vanillin, reduce and even inhibit the Mn-SOD activity and consequently cause yeast cells death[9].

Our results showed that after random mutation by UV, pattern of expression of proteins was altered meaningfully since identified proteins that had over expressed in mutant strain, played an important role in resistance against metabolic stress and metabolism of energy. Designing recombinant strains after exploration of related genes could be an interesting subject for further investigation.

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