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Increase in Bioethanol Production by Random UV Mutagenesis of *S.cerevisiae* and by Addition of Zinc Ions in the Alcohol Production Media

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Abstract

Bioethanol is regarded as one of most demanding fuel as an alternative to fossil fuel as it is renewable and environment friendly. In industry, bioehanol is produced usually by using molasses as the carbon source with the help of *S.cerevisiae.* But due to steep hike in the prices of molasses, the production cost of biotehanol is on constant rise. Therefore, there is urgent need to evolve alternative strategy which can lower down the production cost of bioehanol.This study is directed towards finding out some rationale approaches which can increase the production rate of biotehanol, thereby decreasing the production cost.We have applied random mutagenesis approach by UV exposure for strain improvement of existing strain which is not alcohol tolerant. 25 minutes UV exposure to existing strain produced a mutant strain which produced 12% (v/v) alcohol in fermentor whereas WT *S. cerevisiae* produced only 8% (v/v).We have also attempted to register increase in alcohol production by adding of Zinc ion in alcohol producing media. It was standardized that 20mM Zinc sulphate addition to YEPD broth resulted in maximum increase in alcohol production in shake flask.When 20mM Zinc sulphate was added to fermentation broth in 2 litre laboratory based fermentor, it resulted in 13.2% (v/v) alcohol production as compared to 8% (v/v) alcohol production by WT strain. There was also 3.2 fold increases in alcohol dehydrogenase activity when it was measured in presence of 20 mM zinc sulphate as compared to control (ADH activity measured using same cell lysate in the absence of zinc sulphate).

Keywords: Bioethanol, UV mutagenesis, Zinc ion

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1. Introduction

In recent years, due to constantly increasing emission of greenhouse gasses, major thrust has laid upon production of biofuels instead of fossil fuels. Bioethanol has been regarded as a favorable alternative energy source, which is both renewable and environmental friendly (Ogawa et al., 2000). Nearly, 73% of the whole ethanol produced globally is used as biofuel (Cardona and Sanchez, 2007; Balat et al., 2008; Zhang et al., 2010). *Saccharomyces cerevisiae* is used universally for industrial ethanol production because of its ability to produce high concentrations of ethanol and high inherent ethanol tolerance. (Gunasekaran and Raj, 1999).*Saccharomyces cerevisiae* is utilized mostly in batch fermentations to convert sugars. Molasses has been used in laboratories to produce ethanol using *Saccharomyces cerevisiae* at bench scale as well as in continuous culture at industrial scale. But the cost of molasses is increasing rapidly and distillers are concerned by the price hike.Other attractive alternative is the readily available lignocellulosic biomass, which has a considerable promise as a raw material for liquid fuels. To promote bioethanol utilization, it is necessary to reduce its production cost.Wood and related lignocellulosic material are one of the most adequate and great resources of the lignocellulosic materials used for bioethanol production (Okuda et al., 2007).

Another important approach to reduce the cost of alcohol production is the strain improvement of existing *S. cerevisiae*.Therefore; strain improvement could result in increasing the ethanol production capacity of current fermentation plants and thereby decreasing the cost of production (Dombek and Ingram, 1987). Induced mutagenesis by application of physical and chemical mutagens is an uncomplicated process and straight forward method for yeast strain improvement (Sridhar et al., 2002).

Apart from strain improvement by mutagenesis, some alternative approach can also be attempted.It is reported that Zinc can induce the activity of alcohol dehydrogenase (ADH) protein in human system as this protein has binding sites for zinc (Leskovac et al.,1976). NADH dependant ADH protein catalyses the last step of bioethanol production e.g. the conversion of acetaldehyde to ethanol. If the rate of conversion of acetaldehyde to ethanol is increased, definitely it will augment the overall alcohol production. So, it is hypothesized that addition of Zinc ion (in the form of zinc salts) in alcohol producing media can increase the alcohol production.

Therefore, this present study is aiming at increasing the alcohol production by twin approach e.g.1strain improvement 2 increasing the activity of ADH by using metallic activators of enzyme Zinc ions respectively.

2. Materials & Methods

2.1. Material

***S.cerevisiae*:** Fermenting yeast (*S.cerevisiae*) procured from Institute of Microbial Technology (IMTECH), Chandigarh was used in the present study.

2.2. Maintenance, propagation and mutation of S.cerevisiae:

YEPD media (yeast extract peptone dextrose) was used for maintenance and propagation of *S.cerevisiae*. First, a UV survival curve for *S.cerevisiae* was developed by exposing 200 µl of liquid culture (in YEPD medium) for different time interval e.g.01, 2.5, 5, 10, 15, 20, 30 minutes.The time interval which produced 2-3% survival rate was taken for creation of mutant.*S.cerevisiae* was streaked on YEPD plate and incubated at 30^0C overnight to get single colony and it was exposed to UV light for the time interval 20, 25, 30 minutes.

2.3. Alcohol estimation in culture broth by titration method :

Four colonies of each time interval for UV exposure e.g.20, 25, 30 minutes were picked up from the respective YEPD-agar plates and incubated in 50 ml YEPD broth and incubated in rotary shaker at 200 rpm at 30^0C for 48 hours.WT *S.cerevisiae* colony was also inoculated in 50 ml YEPD broth as control.After 48 hrs incubation of microbial broth, 1ml of culture from each flask was centrifuged .50µl filtrate were taken out in 100ml flask .In the filtrate, 10ml of Potassium dichromate solution were added & kept for overnight incubation. One water control which contained 50 µl water (in place of 50 µl culture filtrate) was taken and 10 ml potassium dichromate solution was added and incubated overnight.Next day, 1ml of Potassium Iodide solution was added in each flask followed by addition of 90ml distilled water and titrated against sodium thiosulphate until straw yellow color developed. Once straw yellow color came out, 1ml of starch indicator was added and titration was continued until solution became colorless.

2.4. Induction of Alcohol production by addition of zinc sulphate in S.cerevisiae culture broth:

To determine the induction in alcohol production by Zinc sulphate, different concentrations of Zinc Sulphate (from 2 mM to 40 mM) were added to 50ml YEPD broth with *S.cerevisiae* and grown at 30^0C for 48 hours on rotary shaker. A control containing *S.cerevisiae* in YEPD media without Zinc sulphate was also grown at 30^0C for 48 hours on rotary shaker. Amount of alcohol was estimated in all the samples along with control by titration method as stated earlier.

2.5. Alcohol Dehydrogenase Assay:

S.cerevisiae cells were harvested from 1.5 ml culture broth and dissolved in Sodium citrate buffer, pH6.2 (sodium citrate contains 2mM Sodium citrate, 2mM beta mercaptoethanol) by vortexing**.** The cells were disrupted by six s periods of 30 s in a sonicator and the tubes kept in an ice bath during the resting periods. The supernatant separated by centrifugation (10 min at 12,000 rpm at 4^oC) was used for the alcohol dehydrogenase assay.NADH dependant ADH activity was assayed at 25^0C by measuring the decrease in absorption at 340 nm of NADH (Molar Absosptivity=6220 $M^{-1}cm^{-1}$).The reaction mixture consisted of 5.0 micro mole of acetaldehyde, 0.4 micromole of NADH, 75 micromole of pyrophosphate buffer (pH 7.8) and 100 microlitre of enzyme solution in a total volume of 2 ml.

2.6. Production of alcohol in Fermentor with control yeast strain (S.cerevisiae), mutated yeast stain (UV exposed S.cerevisiae) and control yeast & Mutated strain with 20mM Zinc Sulphate:

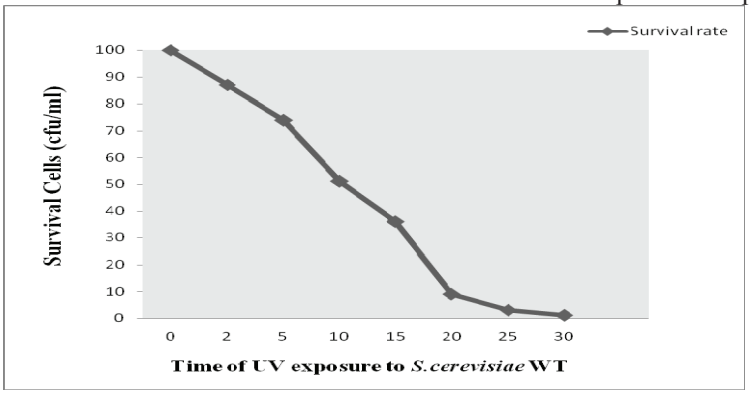
A seed culture in a one-liter Erlenmeyer flask containing 250 ml of YPS growth medium, was prepared using a 1.0 ml frozen suspension of *S.cerevisiae*. The culture was incubated at 28 °C for 12 hours at 200 rpm in an orbital shaker.Then inoculum was transferred to the Bioage Fermentor containing 1.50 liters of fermentation medium (the fermentor medium contains glucose: 70 gm/litre, sucrose: 80 gm/litre, hydrated magnesium sulphate: 10 gm/litre, potassium dihydrogen phosphate: 0.5 gm/litre; pH 5.5).The Fermentor was controlled at 30°C and pH 5.5. Aeration rate was set at 2.5 l min^{-1} (0.5 vessel volumes per minute), and agitation speed was 150 rpm. 29% NH_4OH base solution was used to control pH.Dissolved oxygen was controlled at 30% during the cell growth phase for 24 hours followed by an anaerobic production phase for another 48 hours.Amount of alcohol present in fermentation broth was measured by the method of Kaputi et al (1968).

3. Results and Discussion

3.1. UV mutation of S.cerevisiae (IMTECH, Chandigarh, strain no.3786):

Ethanol fermentation not only depends on the substrate used and conditions used for fermentation, but also depends upon the efficiency of *S.cerevisiae* strain to convert the sugar or other carbon sources to ethanol. In the present study; an attempt was made to carry out the strain improvement of existing strain by random mutation by UV exposure to *S.cerevisiae* for increased productivity of ethanol.

Survival Rate of *S.cerevisiae* when exposed to UV light: An UV survival curve for WT *S. cerevisiae* was developed to determine at which time interval of UV exposure, 2-3% survival rate of *S.cerevisiae* could be obtained. Usually 2-3% survival rate to UV exposure is suitable for getting good quality of mutants. A graph of no. of colonies of *S.cerevisiae* survived vs. different time interval of UV exposure was presented.



Graph 1: Effect of UV light on survival of *S.cerevisiae*

This graph showed that 25 minutes time interval of UV exposure (under the given condition) is efficient for creating 2-3% survival rate.

3.2. Measurement of Alcohol production by UV mutated yeast strain and comparison with exiting WT type S.cerevisiae:

20, 25, 30 minutes of incubation time were chosen for the UV exposure to create stable mutants. Mutated strains were cultured in YEPD media along in 50 ml shake flask with WT *S.cerevisiae* strains for 48 hours at 30^0C and culture supernatant was used for alcohol estimation by titration method and represented in table no.1.

Table 1. Amount of alcohol obtained from different UV mutated strains

Strain designation	% of alcohol produced (v/v)

S.cerevisiae	3.11 ±0.12
Mutant *S.cerevisiae* obtained after 20 Minutes UV exposure	3.86±0.145
Mutant *S.cerevisiae* obtained after 25 Minutes UV exposure	4.631±0.112
Mutant *S.cerevisiae* obtained after 30 Minutes UV exposure	4.632±0.134

From data mentioned in table no. 1, it is evident that mutant strain created by 25 minutes UV exposure achieved the highest increment in alcohol production. Mutant strain was able to produce 1.74 fold more alcohol as compared to WT strain.

3.3. Induction of alcohol production by addition of Zinc sulphate in YEPD broth:

Zymomonas mobilis is reported to have both iron and zinc dependant alcohol dehydrogenase genes whereas *S. cerevisiae* has only zinc dependant alcohol dehydrogenase gene (Mackenzie et al.,1989). The ADH enzyme of *S.cerevisiae* has Zinc binding site & binding of Zinc induces the activity of ADH enzyme.The dissociation of half (2 zinc atoms/monomer) of the total zinc content of the enzyme is associated with the full inhibition of its activity . Hence Zinc sulphate was used as additional component in the YEPD media as it might have assisted in the induction of alcohol production by augmenting the enzyme alcohol dehydrogenase activity of *S.cerevisiae*.

Table 2. Alcohol productivity in YEPD media in presence of different concentrations of Zinc sulphate

Concentration of Zinc sulphate added to alcohol producing broth using *S. cerevisiae* (in mM)	% of alcohol produced (v/v)
0	3.11±0.172
2mM	4.156±0.148
6mM	4.39±0.145
10mM	4.655±0.128
20mM	5.21±0.111
30 mM	3.92± .126
40mM	1.91±0.015

Different concentrations of Zinc sulphate starting from 2mM to 40mM were added in alcohol producing media to check the degree of increase in alcohol production on increasing the concentrations of Zinc ions. From the data mentioned in table no.2, it is evident that alcohol productivity increased gradually upto Zinc concentration, 20 mM. But above, 20mM, alcohol productivity decreased abruptly. Increase in alcohol productivity might be attributed due to the induction of ADH activity by increased concentrations of Zinc ions. But increased concentrations of Zinc ions above 20mM failed to increase alcohol productivity as increased concentrations of transition metal ions appeared to be toxic to yeast cells as it was evident from drastic decrease in cell grown when zinc concentrations was raised above 20mM (data not shown).

3.4. Production of alcohol in Fermentor by (a) WT Yeast strain with and without Zinc Sulphate (b) by mutated stain and estimation of alcohol by distillation method:

After optimizing the concentration of Zinc sulphate to be added to YEPD media for induction in biotehanol production and time period for UV exposure for creating suitable mutant, the alcohol production was scaled

up from shake flask to 2 litre laboratory based fermentor. After 24 hours of aerobic growth at 30°C, anaerobic growth was maintained for 48 hours at the same temperature. After fermentation, culture supernatant was subjected to fractional distillation and amount alcohol was measured according to the method of Kaputi et al (1968).

Table 3. Optical density of Fermented sample after distillation (with zinc sulphate & without zinc sulphate of UV exposed *S.cerevisiae* culture along with *S.cerevisiae* control).

Sample	Absorbance at 600 nm	% of alcohol (v/v)
Distilled filtrate from fermentor broth having WT *S.cerevisiae*	0.40	8%
Distilled filtrate from fermentor broth having mutated strain	0.57	11.5%
Distilled filtrate from fermentor broth having WT *S.cerevisiae* as inoculum with 20mM $ZnSO_4$, $7H_2O$	0.65	13.1%

Addition of 20mM Zinc sulphate in fermentation broth led to 1.63 fold increase in alcohol productivity with respect to WT strain. Mutant strain of *S. cerevisiae* led to an increase of 1.43 fold in biotehanol production as compared to WT strain. But, why is this increase in alcohol production in both situations? It has been speculated that activity of alcohol dehydrogenase may be increased due to the change in catalytic activity of ADH (in both the situations) which should be reflected in ADH assay. Therefore, we have attempted to assess the quantitative change in ADH activity by using cell lysate prepared from mutant strain.

3.5. ADH assay of cell lysate made from WT type and mutated strains:

An attempt has been made to check whether addition of Zinc sulphate can result in the increase in ADH activity in vitro by performing the ADH assay using cell lysate made from WT type and mutated strains of *S.cerevisiae*. The crux of this assay is the determination of utilization of NADH. More is the rate of utilization of NADH, more is the ADH activity. ADH needs NADH to convert acetaldehyde to ethanol and in the process NADH is oxidized to NAD^+.NADH absorbs at 340 nm, NAD^+ cannot absorb at 340 nm.

Table 4. ADH assay with cell lysate of WT and mutant strain of *S.cerevisiae*

Assay set up	Absorbance at 340nm
Blank without zinc(no cell lysate added)	0.612
Blank with Zinc (no cell lysate added)	0.690
Experimental set up without Zinc (cell lysate added)	0.498
Experimental set up with Zinc (cell lysate added)	0.328
Blank (all assay components added excluding cell lysate prepared from mutant strain)	0.624
Assay set up with cell lysate prepared from mutant strain) (all assay components including cell lysate)	0.318

Table 5. ADH activity of WT *S. cerevisiae* strain in presence and absence of Zinc sulphate and of mutant Strain

Sample	ADH Activity (Units/ml enzyme)

WT Cell lysate without Zinc sulphate	0.018 U/ml
WT Cell lysate with Zinc sulphate	0.058U/ml
Mutant Cell lysate without Zinc sulphate	0.049 U/ml

Therefore, the fold increases in ADH activity in presence of Zinc sulphate in WT *S.cerevisiae* =.058/.018 = 3.2 fold. As the ADH activity increases, it leads to more conversion of acetaldehyde to ethanol, therefore there is increase in alcohol productivity in presence of Zinc ions. The fold increase in ADH activity in mutant strain =.049/.018 = 2.7. In mutant strain, changes at the DNA level may result in catalytic domain which is reinforcing its activity.This strongly indicated that mutation had become successful in bringing about definite change/changes in ADH gene which resulted in increasing the catalytic activity. But what are the changes at DNA sequence level in ADH gene in mutant are yet to be known. It can be known by the sequencing of ADH gene fished out from mutant strain and process is underway.

In conclusion, it can be opined that UV mutation of *S. cerevisiae* and addition of Zinc ions in alcohol producing media has become successful in increasing the alcohol productivity and it will definitely assist in reducing the production cost of bioethanol at the commercial level.

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