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Growth kinetic and modeling of ethanol production by wilds and mutant *Saccharomyces cerevisae* MTCC 170

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ABSTRACT

Saccharomyces crevisiae was treated with physical (ultraviolet and microwave irradiation), and chemical mutagen (5- bromouracil, allylthiourea, dithiotritol, acriflavin and acridine) to develop mutant with high ethanol producing efficiency. Two mutant, S. crevisiae SUVaM1, SCD10M3 produced high ethanol yield exceeding that of the wild type. Both wild and mutants show ethanol tolerance up to 10%. Maximum substrate tolerance for wild and SUVaM1 was10% but mutant SCD10M3 has 20% glucose. The best pH and temperature was 6 and 30^{0}C respectively for ethanol production for wild & mutants. This study also investigated the growth kinetics and ethanol productivity with Monod model. The resulting ethanol yield was 2.9-fold more than that of the wild type strain.

Keywords: S*accharomyces crevisiae*, ethanol, Mutation, growth and product kinetics

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INTRODUCTION

Bioethanol has significant environmental advantages over petroleum as a liquid fuel [1, 2]. During the last years substantial progress has been made in the development of genetically-modified organisms. To improve the microbial strains, random mutagenesis, classical breeding and genetic crossing are used for enhanced properties of interest. Genetic recombination methods are very tedious and lengthy process. Microorganisms are genetically capable with a mechanism that adjunct the production of metabolic cells to a level that should meet their own needs. Exposing a culture of a microorganism to UV light or chemicals enhances the mutations occurrence rate. *S. cerevisiae* is a very striking life form due to its application in the ethanol production and nonpathogenic, character. It is used extensively in batch fermentations for ethanol production of beverages [3]. Although the improved fermentative engineering and optimal cultural conditions can quantitatively enhance the microbial products, but this will only be up to a limit. Genetic improvement of the organism is fundamental to the success of fermentation technology. This study was made in order to obtain mutants capable to produce high yield of ethanol with respect to the wild *S. cerevisiae*. Physical (ultraviolet and microwave irradiation), and chemical mutagen (5- bromouracil, allyl thiourea, dithiotritol, acriflavin and acridine) were tested for high ethanol yield. This study also investigated the growth kinetics and ethanol productivity by Monod model [4].

MATERIALS AND METHODS

Microorganism:
The pure yeast *S. cerevisiae* (MTCC No. 170) purchased from Institute of Microbial Technology Chandigarh-India, was revived by growing on medium having components (g l^{-1}) Glucose, 200; Yeast Extract,30; Peptone,20. Agar slant was prepared for the preservation of culture and stored at 4^0C

Strain Improvement by UV radiations:
S. cerevisiae culture was diluted by serial dilution (10^{-1} to 10^{-10}) method and 100 µl of culture was spreaded on petriplates having solidified agar medium. The plates were exposed to UV light at a distance of 55 cm for various time intervals (5, 10, 15, 20 and 25 min.). The treated petri-plates were covered by dark paper & incubated at 30°C for 3 days. Different colonies on agar plates were picked up with inoculating needle and placed in liquid media & incubated for 3 days. Liquid samples were collected and solid biomass was separated with centrifugation (8000 rpm) for 20 minutes. Ethanol content was analyzed in the supernatant.

Strain Improvement by microwave radiations:
S. cerevisiae culture was diluted by serial dilution (10^{-1} to 10^{-10}) method exposed to microwave radiation of variable frequency (350, 500 and 650 MHz) for 30 s. 100 µl of exposed culture sample was spreaded on petriplates was incubated at 30°C for 3 days. Different microorganism was isolated as per previous experiment and ethanol production efficiency was analyzed.

Strain Improvement by *chemical method:*
Liquid medium was prepared and sterilized for 15 min. at 121^0C in test tubes. Different mutagenic chemicals (5 bromouracil, allylthiourea, dithiotritol, acriflavin and acridine) with different concentration (10 to 40µM) were added in the medium sterilized test tubes. The pure yeast culture of *S.cerevisiae* was inoculated in different test tubes and incubated at 30°C for 3 days. After 72 h samples were withdrawn and ethanol content was determined by standard procedure.

Ethanol tolerance of wild and mutant microorganism:
Ethanol tolerance of wild and mutant *S. cerevisiae* was analyzed. The broth medium was prepared and sterilized for 15 min. at 121^0C Different concentration of absolute ethanol 5-25% (v/v) was added in different flasks and inoculated with the wild and mutant strains. Cultures was incubated at 30°C for 3 days. Cell growth was determined by spectrophotometer by taking O.D at 600 nm against the medium as blank.

Effect of operational parameters on ethanol production of wild and mutant microorganism:
Effect of glucose, 5-25% (w/v), temperature and pH on ethanol fermentation was carried out by varying the glucose, incubation temperature and pH for wild and mutant microorganisms.

Growth and product kinetics of wild and mutant microorganism
Monod Model [4] was used to determine the Growth and product kinetics of wild and mutant microorganisms. The 100 ml liquid medium with different glucose composition (5, 10, 15 and 20%), pH 6 was prepared and sterilized at 121^0C for 15 min. One wild and two mutants SUVaM1, SCD10M3 were inoculated in separate flask of different substrate concentration and incubated at optimum temperature 30^0C. Samples were withdrawn after different time intervals and centrifuged (8000 rpm for 20 min.). Pellets were dried for determination of biomass. Supernatant was used for determination of ethanol and glucose concentration [5]. The Monod [4] equation is:

$$\mu = \mu_{\max}\frac{S}{K_s + S}$$

Where μ is the specific growth rate (h^{-1}) of the microorganisms, μ_{max} is the maximum specific growth rate of the microorganisms, S is the concentration of the limiting substrate for growth, K_s is Monod Constant.

Estimation of ethanol by potassium dichromate method:
Ethanol content was estimated by acidified potassium dichromate [6]. One ml of aliquot was taken in distillation flask and distilled at 74^0C. Distillate was collected in 25 ml of acidified potassium dichromate [36 g of potassium dichromate was dissolved in 500 ml solution (325 ml conc. sulphuric acid and 175 ml water) and made final vol.

1000ml]. The test tubes were incubated at 75°C for 15 min. Optical density was taken at 600 nm. Ethanol concentration was determined from the standard plot.

RESULTS AND DISCUSSION

Improvement of S. cerevisiae by UV and microwave radiations:

UV mutagenesis was performed by exposing the wild strain of *S. cerevisiae* under UV radiation. Seventy five cultures was isolated and screened for the ethanol production. Only one mutant SUVaM1 produced ethanol 10.12g l^{-1} as compare to wild, 4.3 g l^{-1}. All other isolated culture exhibited decrease in ethanol production. Petriplates with exposure time of 25 min shows no growth. Similar results were obtained from UV radiations to cause mutations in *S.cerevisiae* (Sridhar *et al*., 2002). In second phase microorganism was exposed to microwave radiation of different frequency (350, 500 and 650 MHz) for 30 s to improve ethanol production. Radiation of high frequency was more effective and decreased ethanol production in mutant as compare to the low frequency. All isolated mutant show decline in ethanol production as compared to wild (Table. 1*)*. This can be due to fact of reduced growth rate by forming DNA lesions in *S.cerevisiae* [7].

Table 1. Effect of microwave radiations on ethanol production

Frequency	Microorganism	Ethanol Production (g l^{-1})
----	Wild	4.3
350	SM 1	3.9
500	SM 2	2.8
650	SM 3	1.5

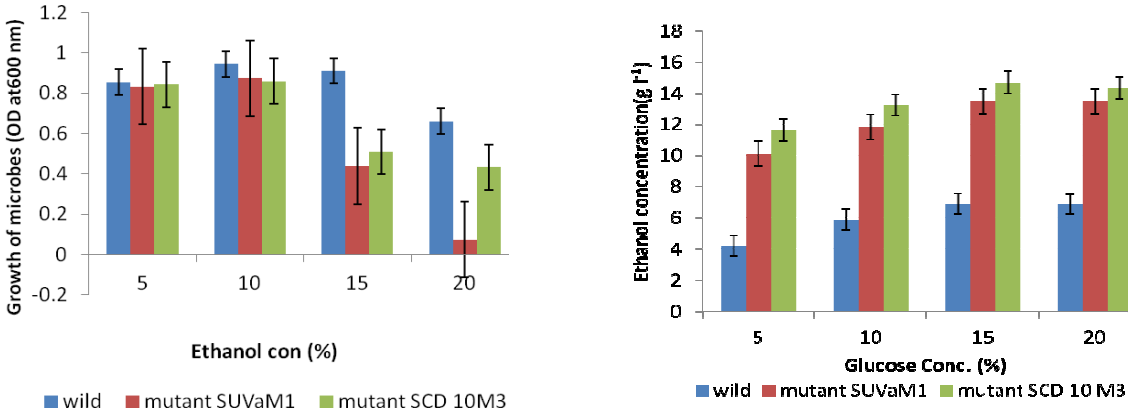
Table 2. Effect of chemical mutagens on ethanol production

Mutagen	Conc. of mutagen(µM)	Microorganism	Ethanol production (gl^{-1})
---------	---	Wild	4.3
5-bromouracil	10	SCB10	2.15
	20	SCB20	3.7
	30	SCB30	2.6
	40	SCB40	1.5
Acriflavin	10	SCAc10M2	1.25
	20	SCAc20	1.1
	30	SCAc30	0.65
	40	SCAc40	0.55
Dithiotrteol	10	SCD10M3	11.4
	20	SCD20	1.2
	30	SCD30	1.1
	40	SCD40	1.0
Allylthiourea	10	SCAl10	0.9
	20	SCAl20	0.86
	30	SCAl30	0.7
	40	SCAl40	0.5
Acridine	10	SCAcr10	0.8
	20	SCAcr20	0.9
	30	SCAcr30	0.5
	40	SCAcr40	0.4

Mutation of S. cerevisiae by chemical method:

Chemical mutation was performed with various chemical mutagen (5-bromouracil, acriflavin, dithiotritol, allylthiourea and acridine) in different ranges of concentration (10 to 40 µM). All chemicals have different mode of action to cause mutations. Microorganisms were treated with chemicals followed by incubation at 30°C for 3 days. Mutant isolated by bromouracil, allylthiourea and acridine treatment exhibit decrease in ethanol potential as comparison to wild. Dithiotrteol was effective mutagen to enhance ethanol production with respect to other mutagens. The mutant strain SCD10M3 produced, 11.03 g l^{-1} ethanol by dithiotrteol mutant at 1010 µM concentration. But higher concentration of mutagen (>10 µM) was less effective to enhance ethanol production. It means higher conc. of chemicals results in decreased ethanol production in mutants. This can be due to structural effect at molecular level by mutagens which cause frame shift mutations. These changes directly effects growth & product formation of microorganisms (Nasim and Brychcy, 1979). After the treatment with physical & chemical

mutagen two mutant strain SUVaM1, SCD10M3 were selected which has higher ethanol production potential as comparison to wild. These isolated mutants were used for further study.



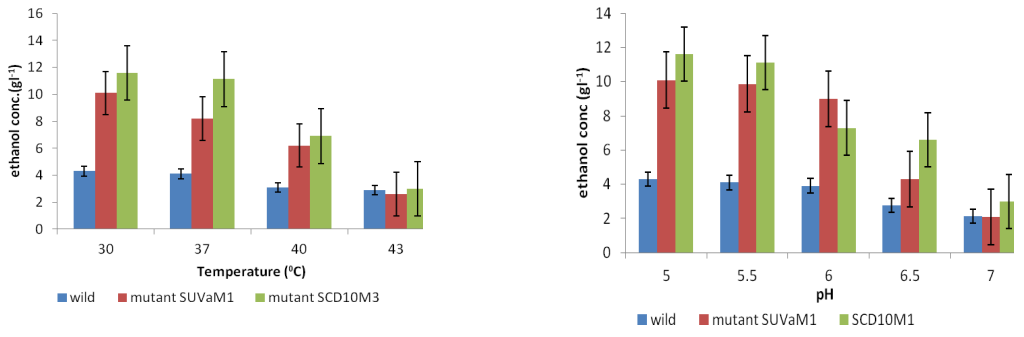
(a) **(b)**

Fig. 1 Effect of (a) ethanol concentration on growth and (b)glucose concentration on ethanol production in wild & mutants microorganism

Ethanol tolerance of wild and mutant microorganisms:

After selection of mutants ethanol tolerance was observed for the growth of microorganisms. Wild and mutant strains were grown on media with different conc. of absolute ethanol. Mutant microorganisms have high ethanol tolerance (Fig.1.a). With increase in ethanol concentration above 10%, the growth of microorganisms declined because ethanol is a toxic metabolite for the fermentation rate. The yeast *S. cerevisiae* which has monounsaturated fatty acids in their cell membrane showed high degree of tolerance towards the ethanol.

To evaluate the substrate (glucose) tolerance, microorganisms were grown at different substrate conc. (5, 10, 15 and 20%). Maximum ethanol production by the mutant SCD10M3was observed at 20% (w/v) glucose concentration, while SUVaM1 and wild has maximum substrate tolerance at 10%.



(a) **(b)**

Fig.2.Effect of (a) temperature and (b) pH on ethanol production in wild & mutants microorganisms for ethanol production

Effect of Temperature and pH on the ethanol production:

The optimum temperature for ethanol production for wild and mutant SUVaM1 and SCD10M3 was determined. The wild and mutant strain showed maximum ethanol production at 30 ^{0}C (Fig.2,a). Temperature plays important role in the ethanol production and ethanol tolerance. The growth of yeast was affected above a temperature range of 34°C and ethanol production was decreased above 37°C temperature range. The ethanol production rate and ethanol tolerance was affected above the temperature range of 37°C showed temperature inhibition on *Saccharomyces spp.* [8]. The Optimum pH for screened strains was detected by ethanol production at various pH ranges (5, 5.5, 6.0, 6.5 and 7.0). All strains (wild and mutants), showed maximum ethanol production at 6.0 (Fig. 2,b). Thus by increasing or decreasing the pH, the ethanol production decreases. The various data suggest that Yeast strains are able to grow

at optimum pH and by increasing or decreasing the pH the growth and ethanol productivity is affected. Yeast *S. cerevisiae*, grow at 25-30°C at a pH range of 3.0-6.0 in ethanol concentration of 2.5-15%. As the pH decreased or increased from 6.0-3.0 the ethanol production decreases [9].

Growth and product kinetics of wild and mutants' microorganism

Growth and product kinetics was calculated by Monod [4] (Table.3). Maximum specific growth rate (µmax) for the wild was 0.133, which were increased for mutants SUVaM1 and SCD10M3 . 0.145 and 0.116 respectively, they shows higher growth rate than wild. The Substrate utilization constant (K_s) is the measure of substrate affinity for the microorganism. Low value of ks indicates high affinity for substrate; it means consumption rate of glucose is higher. Substrate utilization constant (K_s) for the wild and mutant SUVaM1was 3.7, but which was lower (3.0) for mutant for SCD10M3. It means that the consumption of glucose increased for mutant SCD10M3 &decreased for mutant SUVaM1 with respect to wild strain. The biomass yield ($Y_{x/s}$) is based on utilization of substrate which is defined in terms of mass of new cells per mass of substrate utilized.i.e.$Y_{x/s}$ =dX/dS. The biomass yield ($Y_{x/s}$) decreased for mutant SCD10M3(0.11) but increased for mutant SUVaM1 (0.21) as compared to wild which is 0.185; lower the value of Yx/s means higher the consumption of substrate used by the mutant SCD10M3 for growth. Product formation was described by Ledderking Pirt kinetics model. The product formation rate depends upon biomass convnetarion as well as growth rate (dx/dt). The specific rate of utilization (q_s) is defined as the gram of substrate utilized per gram of cell produced per hrs.

$$q_s = -ds/Xdt \quad (1)$$

Here minus sign indicates consumption of glucose. The specific rate of substrate utilization (q_s) was increased for SCD10M3 (0.38) while that for wild and SUVaM1 were 0.22 and 0.199 respectively. The relationship between q_s and $Y_{x/s}$; they are inversely proportional to each other, lower the value of qs while higher the $Y_{x/s}$ and vice versa. Ethanol yield ($Y_{p/s}$) is based on ethanol production which is defined as; mass of new cells per mass of ethanol produced.

$$Y_{p/s} = dX/d_p \quad (2)$$

Ethanol yield was maximum for mutant SUV20aM1 (0.95) as comparison to wild (0.76) and mutant SCD10M3 (0.70). Less value of $Y_{p/s}$, means more production of ethanol. Thus mutant strain SCD10M3 produces more ethanol as comparison to wild & SUV20aM1. The specific rate of ethanol production (q_p) is defined as the gram of ethanol produced per gram of cell produced per hr i.e. q_p= dp/Xdt. The specific rate of ethanol production (q_p) gm was maximum for wild i.e. 0.22 and for mutants SUVaM1 and SCD10M3 were decreased to 0.04 and 0.06 respectively.

Table 3. Growth & product kinetics of wild & mutant microorganisms

*Kinetics Parameters	Microorganisms		
	Wild	SUVaM1	SCD10M3
μ_{max}	0.133	0.145	0.116
K_s	3.7	3.7	3
q_s	0.22	0.19	0.38
q_p	0.05	0.04	0.06
$Y_{x/s}$	0.185	0.21	0.11
$Y_{p/s}$	0.76	0.95	0.70

**µ (maximum growth rate per hour), K_s (Substrate saturation constant mg liter),q_s (gm of substrate utilized /gm of cell produced/hour), q_p (gm of Ethanol produced /gm of cell produced/hour), $Y_{x/s}$ (biomass produced in gm/gm of substrate utilized), $Y_{p/s}$ (biomass produced in gm /Ethanol produced in gm).*

CONCLUSION

In this investigation, UV radiation and chemical mutagen dithiothretol increased ethanol production to 2.9 fold from a wild strain. In addition, optimization process caused more ethanol production. The outcome strongly supports that mutation and optimization of the variables in combination not only reinforced ethanol overproduction up to 2.0 fold, but also diminished the cost of the production process. This study also concluded that using hybrid techniques such as mutation and culture optimization together can result better and faster for having overproducer strains in industrial microbiology and biotechnology.

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