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# Improvement of Local *Saccharomyces Cerevisiae* Strain by Mutagenesis for Enhanced Production of Bioethanol

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## Abstract
Microbial production of ethanol is a very popular concept in respect of alleviating energy demand nowadays. In industry, bioethanol is produced usually from molasses as the carbon source by using *S.cerevisiae.* But, due to sharp increase in the prices of molasses, the production cost of bioethanol is on constant rise. Thus, there is a demand to find out an alternate strategy that can help to lower down the production cost by increasing the yield of bioethanol. Therefore, the present study aims at applying strain improvement strategy to the wild type *S. cerevisiae* thereby increasing the production and tolerance to ethanol. For this, wild strain of *S. cerevisiae* was adapted over an extended period to different concentration of alcohol ranging from 7% to 21%. The wild strain was found to tolerate 15% alcohol concentration. The adapted wild strain of *S.cerevisiae* was exposed to U.V. radiation for ten minutes and a mutant strain was obtained that gave 12.5% (+/- 0.02%) of alcohol whereas the wild *S. cerevisiae* was giving only 10.0% (+/-0.4%) alcohol yield. Thus, after mutagenesis programme, a superior improved strain was obtained that may have potential for mass scale production of Bioethanol.

**Keywords**
Bioethanol; Molasses; *S.cerevisiae*; Adaptation; UV mutagenesis.

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**INTRODUCTION:**
With industrial development taking place rapidly, there is a need for environmentally sustainable energy sources. In recent years, due to constantly increasing emission of greenhouse gases, major thrust has led upon production of biofuels instead of fossil fuels. Biofuels are not only promising sources of environment-friendly energy, but also provide an economic opportunity for the agriculture industry worldwide. Bioethanol is an attractive, sustainable energy source to fuel transportation. Based on the premise that fuel bioethanol can contribute to a

cleaner environment and with the implementation of environment protection laws in many countries demand for this fuel is increasing [2].

*Saccharomyces cerevisiae* is used universally for industrial ethanol production due to its ability to produce high concentration of ethanol and high inherent ethanol tolerance [3]. There are two major categories of biomass that are used for Biofuel production. The first category is crops and grains like corn, wheat, sugarcane, soybeans, etc. and the second category contains waste biomass such as straw, corn stover and waste wood. As second category is much inexpensive as being a waste material, it is more ethical to use for bioethanol production as compared to the first category [1]. Molasses has been used in laboratories to produce ethanol using *S.cerevisiae* at bench scale as well as in continuous culture at industrial scale. But the cost of molasses is on constant rise and distilleries are concerned by the price hike. To promote bioethanol utilization, it is necessary to reduce its production cost thereby increasing its production rate [4]. An important approach in reducing the cost of alcohol production is Strain improvement of the existing *S. cerevisiae*. The strain improvement could result in increasing the ethanol production capacity of current fermentation plants, thereby decreasing the production cost. Induced mutagenesis by application of physical and chemical mutagens is an uncomplicated process and straight forward method for yeast strain improvement [12]. Therefore, the present study was undertaken to adapt the wild type *S. cerevisiae* to gradually increased levels of alcohol concentration and also to mutate the same by random UV mutagenesis to develop mutant *S. cerevisiae* with possible ability of increased alcohol yield.

## MATERIALS AND METHODS

### Materials:

### Molasses:

Molasses samples were collected from Shiddeshwar Sugar Industry, pvt ltd. Solapur, Maharashtra, India and Lokmangal Sugar Industry, pvt ltd. Solapur, Maharashtra, India.

### Reagents and chemicals

Potassium Dichromate reagent was prepared by using Standard reference. All the other chemicals were of high purity and analytical grade purchased from Hi Media.

### Yeast strain

*Saccharomyces cerevisiae* was isolated from molasses sample and identified on the basis of morphological and biochemical characterization and was maintained on Yeast extract Peptone Dextrose agar (YEPD) and Subculture once in every month.

### Methods:

### Isolation and identification of yeast:

Molasses samples were used for isolation of yeast. The samples were serially diluted and plated onto YEPD agar medium. Monochrome staining of yeast suspension was carried out using methylene blue stain and the morphology structure of yeast was observed [16, 17]. The biochemical tests were carried out involving fermentation patterns of different sugars like sucrose, glucose, maltose, raffinose, xylose, lactose and fructose. The ability to ferment carbohydrate was examined anaerobically by looking for formation of gas ($CO_2$) in Durham tube and color change in fermentation media after incubation [18].

### Inoculum development:

For this, the inoculum media (Malt extract (3.0g), Glucose (1g), Yeast extract (0.3g), Peptone (0.5g) per 100ml distilled water and pH 6.5) was prepared and autoclaved at 121$^{o}$C for 15 psi and then inoculated with 24h old yeast culture and incubated at 28$^{o}$ C for 48hr in vigorous shaking condition (180 rpm).

### Factor optimization for ethanol production (O-FAT Method):

The following parameters were selected for optimization by one factor at a time method viz Stationary and Shake Flask Method, Inoculum size, pH and Sugar concentration (Glucose and Sucrose). For all the optimization experiments Standard ethanol fermentation protocol was followed.

### Stationary Condition:

Fermentation flasks containing fermentation media (Magnesium sulphate (0.2g), Urea (0.2g), Sucrose (15g) per 100ml distilled water and pH 6.7) supplemented with 2g% of inoculum, were incubated at 28°C, at 150 rpm in incubator shaker for aeration and agitation with 15 g% sucrose. Static conditions were maintained by keeping flask at 28°C incubator [10]. Samples were removed for four successive days at an interval of 24 hr for estimation of ethanol.

**Inoculum size:**

Inoculum media inoculated with yeast culture and incubated for 48hr was centrifuged in pre-weighed sterile centrifuge tube (15 ml) at 9800 rpm at 20$^0$C for 20 min. Supernatant was discarded and pellet was used as an inoculum. Amount of culture added in the fermentation medium was optimized (0.5g%, 1g% and 2 g % w/v respectively) [7]. Samples were removed for four successive days at an interval of 24 hr for estimation of ethanol.

**pH:**

To observe the effect of pH on ethanol production, fermentation medium was inoculated with 2g% inoculum was adjusted to different pH ranges as (pH 5, 6, 7 and 8) with acetate buffer (pH 5) and phosphate buffer (pH 6, 7 and 8) [6]. Samples were removed for four successive days at an interval of 24 hr for estimation of ethanol.

**Sugar concentration**

**Glucose:**

Different glucose concentrations (10g%, 15g%, 20g%, 25g%, 30g% and 35g%) were supplemented separately in fermentation medium inoculated with 2g% of inoculum. Samples were removed for four successive days at an interval of 24 hr for estimation of ethanol [8].

**Sucrose:**

Different sucrose concentrations (10g%, 15g%, 20g%, 25g%, 30g% and 35g%) were supplemented separately in fermentation medium inoculated with 2g% of inoculum. Samples were removed for four successive days at an interval of 24 hr for estimation of ethanol [7].

**Adaptation to different ethanol concentrations:**

Adaptation of *Saccharomyces cerevisiae* strain was carried out by transferring loopful of 24 hr old culture in fermentation broth containing 7% ethanol and incubated for 7 days. Growth from this tube was transferred serially to fermentation medium containing ethanol concentration from 9%, 11%, 13%, 15%, 17%, 19% and 21% respectively and incubated over an extended period [13]. The viability of inoculum was checked at every transfer.

**Mutation Experiment:**

**UV treatment and selection of mutants:**

The adapted *S.cerevisiae* culture was inoculated in sterile YEPD medium and incubated overnight at 28$^o$C until cells reached a density of 1× 10$^8$ cells/ml. The cells were further diluted to reach a final density 10-100 cells/ml. Then 0.1ml of this suspension was plated onto sterile YEPD agar plate. The plates were exposed to short wavelength UV light (280 nm) from a distance of 15 cm for 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 min respectively [14]. To stop photo reactivation, plates were incubated in dark for 48h at 28$^o$C. The survival rate for the *S. cerevisiae* was developed to determine at which time interval of UV exposure, 2-3% survival rate could be obtained. 2-3% survival rate after UV exposure is suitable for getting good quality mutants. The selection of mutants was based on colony morphology, fast growth and pigment production.

**Alcohol production by mutated *S. cerevisiae*:**

Three variants after UV mutagenesis were selected and labeled as RGK*1, RGK*2, RGK*3 respectively and were subsequently cultured separately in Inoculum medium for ethanol production. The inoculum was transferred to the fermentation medium and was subjected to fermentation. Aliquots of fermentation medium were removed after every 24hr and were further subjected to ethanol production by distillation and the distillate was estimated for ethanol production using potassium dichromate method.

**RESULTS:**

**Isolation and Identification of yeast:**

The yeast isolate formed butyrous, smooth white raised colonies onto YEPD plate (Figure 1). The microscopic observation of the isolated suspension revealed the budding stage of yeast (Figure 1) [16, 17]. *S. cerevisiae* showed variation in utilization of sugars (Table 1).

**Table 1: Carbohydrate Fermentation test for yeast isolate**

| Type of carbohydrate | Fermentation result by yeast isolate | |
|---|---|---|
| | Acid production | Gas production |
| **Sucrose** | + | + |
| **Glucose** | + | - |
| **Lactose** | - | - |
| **Xylose** | - | - |
| **Maltose** | + | + |
| **Raffinose** | + | - |
| **Fructose** | + | + |

**(+) Positive, (-) Negative**

**Table 2: Adaptation of yeast to different Ethanol concentrations:**

| Ethanol (Gm %) | Yeast (CFU/ml) |
|---|---|
| **7** | $9\times10^7$ |
| **11** | $4\times 10^7$ |
| **13** | $1.8\times10^7$ |
| **15** | $5\times10^6$ |

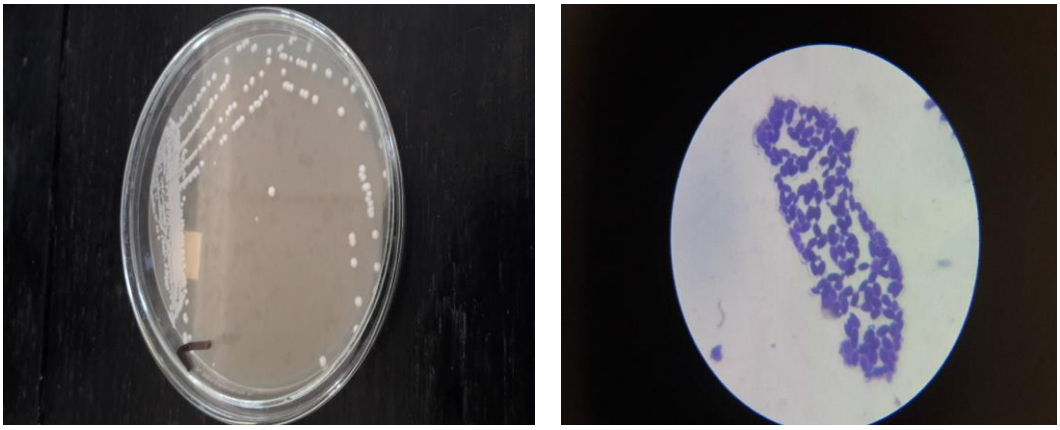

**Figure 1: Growth of *Saccharomyces* onto YEPD plate and microscopic observation of the same.**

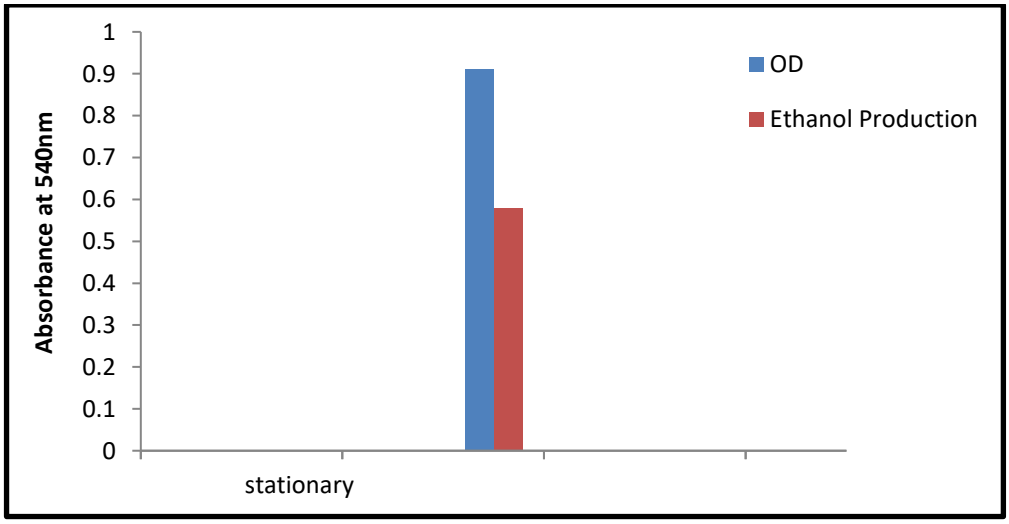


**Figure 2: Effect of stationary condition on ethanol production.**

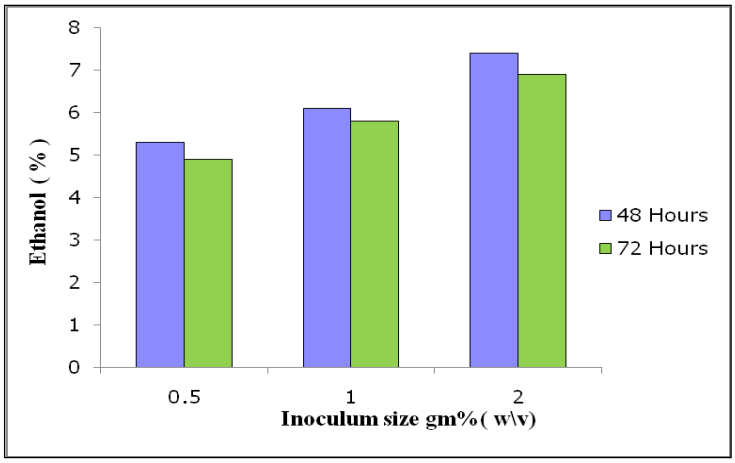


**Figure 3: Effect of inoculum size on ethanol production.**

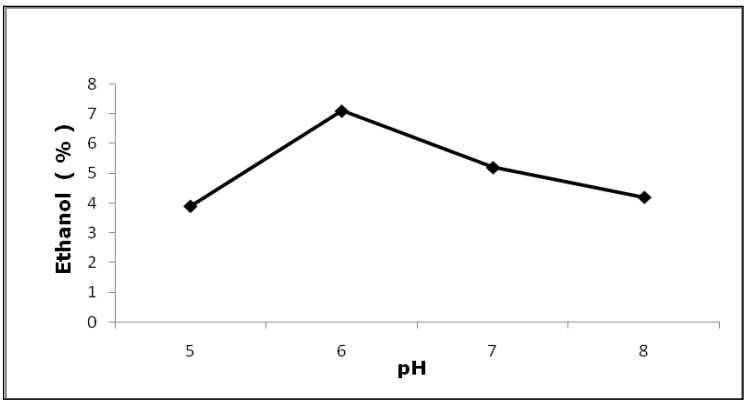


**Figure 4: Effect of pH on ethanol production.**

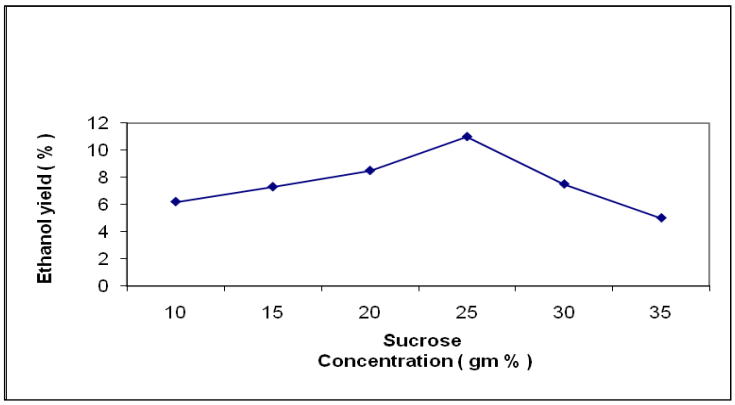


**Figure 5: Effect of Sucrose concentration on ethanol production.**

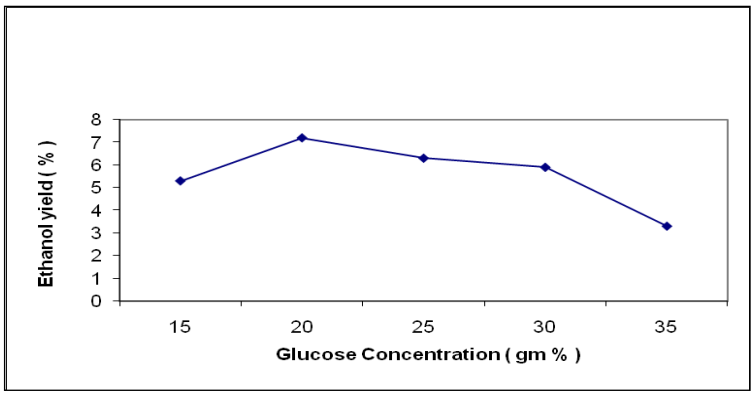


**Figure 6: Effect of glucose concentration on ethanol production.**

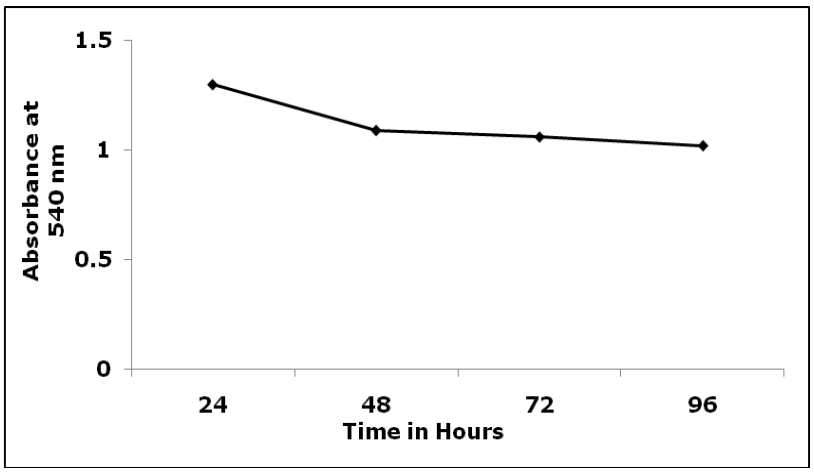


**Figure 7: Effect of ethanol on growth of yeast.**


**Figure 8: UV irradiated plate.**

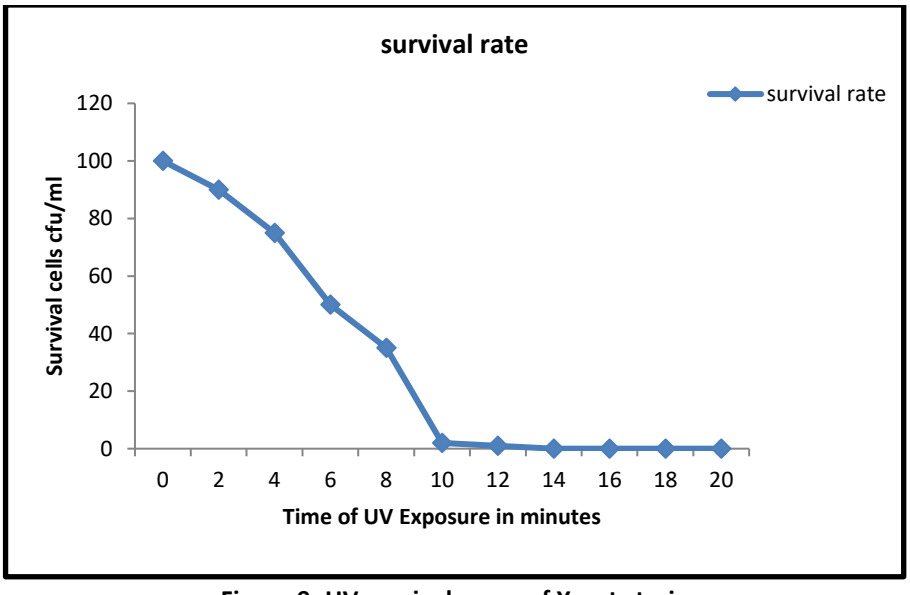


**Figure 9: UV survival curve of Yeast strain**

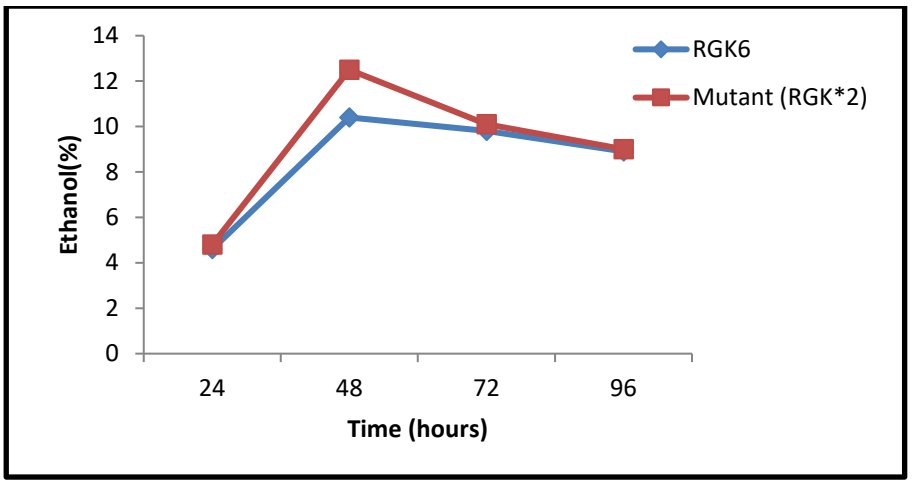


**Figure 10: Efficiency of mutant (RGK*2) for ethanol production compared with wild type (RGK6) *S. cerevisiae*.**

**Selection of strain:**

The selection of strain was based on ethanol production. Experiments were performed to estimate the ethanol production by the isolated strains. Out of screened strains, the strain labeled RGK6 with highest ethanol production that is of 10.0% (+/- 0.4%) was selected. The other strains were found to produce less ethanol during fermentation. The further studies on optimization were carried out using *S. cerevisiae* (RGK6).

**Optimization of fermentation parameters**

**Effect of stationary condition on growth and ethanol production:**

Approximately 4.6% of ethanol was produced at stationary conditions (Figure 2).

**Effect of inoculum size:**

Inoculum size was optimized to give effective ethanol production. 2 g % (w/v) culture gave maximum ethanol production during ethanol fermentation process even after 48 hr. 0.5 g % (w/v) and 1 g % (w/v) gave effective ethanol production only at 48 hr of incubation (Figure 3).

**Effect of pH:**

Maximum ethanol production was estimated at pH 6 and further increase or decrease in pH resulted in decrease in ethanol production. The ethanol production was found to be more also at pH 7 but it was less as compared to production at pH 6 throughout the fermentation process and therefore pH 6 was considered to be optimum pH for ethanol production (Figure 4).

**Effect of sugar concentration:**

**Effect of different Sucrose concentration**

Maximum 11 % ethanol production was found at 25 g % of sucrose throughout the fermentation process. Further increase or decrease in sucrose concentration resulted in decrease in ethanol production. Thus 25 g % sucrose concentration was considered to be optimum for ethanol production (Figure 5).

**Effect of different Glucose concentration:**

Maximum 7.2% ethanol production was found at 20 g % of glucose throughout the fermentation process. Further increase or decrease in glucose concentration resulted in decrease in ethanol production. Thus 20 g % glucose concentration was considered to be optimum for ethanol production (Figure 6).

**Effect of ethanol on yeast cell growth:**

By taking into consideration total viable count, dry weight, Optical density it was found that after 48hr of incubation when ethanol is produced the yeast cell viability was decreased. Thus, yeast cell viability is inversely proportional to ethanol production (Figure 7).

**Adaptation of *S. cerevisiae* to different ethanol concentrations:**

When *Saccharomyces cerevisiae* was transferred serially from medium containing 7% to 21% ethanol concentration, it was observed that up to 15% yeast cells were in vegetative state (Table 2). Above 15% that is from 17% ascospore production was observed. Similar results were reported by Khaing et al [13].

**Effect of UV mutagenesis:**

The UV exposure yielded number of variants. The selection of mutants was carried based on slight

changes in morphology, growth rate and pigmentation by colony (Figure 8).

**Effect of UV light on survival rate:**

The UV survival curve revealed that 10 minutes of UV exposure (under given condition) is efficient for creating 2-3% survival rate (Figure 9). 2-3% survival rate is efficient to obtain good quality mutants.

**Ethanol production by UV mutated variant (RGK*2):**

Out of selected mutated variant RGK*2 was found to give highest yield of ethanol. The mutated variant RGK*2 yielded 12.5% (+/- 0.02%) as compared to the wild type (RGK6) which yielded 10.4% of ethanol (Figure 10).

## DISCUSSION

Selection of strain of *Saccharomyces cerevisiae* RGK6 was done on the basis of their efficiency of ethanol production [10]. This screening was done by using ethanol fermentation protocol [11]. Estimation of % ethanol yield was determined by using Potassium Dichromate method. It was found that among fifty strains RGK6 was showing maximum that is 10.0 % (+/- 0.4%) ethanol yield, so this strain was selected for further experiments. Reliability of selected strain RGK6 was confirmed by carrying out experiments in triplicate. The purity of strains was checked by isolating the strain on YEPD (Yeast Extract –Peptone Dextrose Agar) medium [9] and by monochrome staining. Selected strains were subjected for optimization of Stationary and shaker condition, inoculum size, pH and Sugar concentrations (glucose and sucrose).

To study the effect of stationary and shake flask condition on ethanol production, fermentations were carried out both in stationary and shake flask condition (rotary shaker set at 150 rpm) at $30^0$C for 48 hours. It was observed that though turbidity was higher in shake flask condition, ethanol percent was higher in stationary condition which indicates that aeration and agitation is required for growth of the yeast, but stationary condition is favorable for ethanol production (Pasteur Effect). Hence further studies were carried out at stationary condition [10].

To study the effect of inoculum size on ethanol production, amount of culture added in the fermentation medium was optimized (0.5g%, 1.0g%, 2 g %, w/v). At 2 g %, w/v maximum ethanol production was observed. It indicated that due to increase in yeast cell number, maximum substrate was utilized and thus there was increased in ethanol production. As cell number was high as compared to 0.5 g % and1.0 g% w/v, inhibitory effect of ethanol, after 48 hr was also less [7]. Maximum ethanol production was estimated at pH6 and further increase or decrease in pH resulted in decrease in ethanol production. It indicated that slightly acidic conditions were favorable for ethanol fermentation by *Saccharomyces cerevisiae* RGK6. Optimum Glucose concentration was found to be 20 g% for wild type strain while optimum Sucrose concentration was 25 g % for maximum ethanol production (7.2 % and 11 % respectively). Different concentration of glucose (15g%, 20g%, 25g%, 30g% and 35 g %) were used. When glucose concentration was increased above 20 g % decrease in ethanol yield was observed. A high sugar concentration leads to create osmotic stress in the system as a result ethanol production cease [8].

Optimum Sucrose concentration was 25 g % for maximum ethanol production (11 %). Different concentration of sucrose (10g%, 15g%, 20g%, 25g%, 30g%, 35g%) were used. Sucrose utilizes invertase activity of *Saccharomyces cerevisiae. Sucrose* gives high ethanol production and is cheap source as compared to glucose. Thus, sucrose was used for further experiments [6]. Optimum Sucrose concentration 25 g % at $28^0$C was used for pH optimization (pH 5, 6, 7, 8). The pH 6 was found to be optimum for ethanol production. Based on fermentation efficiency pH 6 was used for further experiments [6]. The adaptation of *S. cerevisiae* to different concentrations was carried out, of which yeast showed adaptation up to 15% of ethanol concentration in the medium. Adaptation helps in use of efficient yeast strain with higher ethanol tolerance to improve ethanol yield which will ultimately reduce the distillation cost and hence profitability of overall process [15]. After UV treatment, 100% killing was obtained at 12 minutes of UV exposure. It was found that UV exposed variant (RGK*2) gave ethanol yield of 12.5% (+/- 0.02%) which was more as compared to wild type that yield 10.0% (+/-0.4%) ethanol. Thus, the increase in alcohol production by mutant variant was 20.1% more as compared to wild type *S. cerevisiae*.

**CONCLUSION:**

In present study, amongst the fifty strains checked, RSG6 was found to give highest ethanol production that is of 10.0% (+/-0.4%). Different fermentation parameters like Stationary condition, 2g% of inoculum size, pH 6, 25% sucrose, were found to be optimum for highest ethanol production. After mutagenesis programme, a superior improved strain (RGK*2) was obtained that may have potential for mass scale production of bioethanol. UV mutation of *Saccharomyces cerevisiae* has thus proved to be successful in increasing the ethanol yield upto 12.5% (+/-0.02%), so also use of efficient strain with higher ethanol tolerance to improve ethanol yield may reduce the production cost of bioethanol thereby increasing the profitability of the overall process.

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