Bioethanol Production: Optimization of parameters for ethanol production by using *Saccharomyces cerevisiae*

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ABSTRACT

In present study, ethanol fermentation from sugarcane molasses was carried out using *Saccharomyces cerevisiae*. For this, the native yeast species were screened from local molasses samples collected and identified. The most efficient species obtained from primary screening was used for further optimization studies. Optimization of different fermentation parameters such as of stationary and shake flask fermentation conditions, inoculum size, pH, different sugars and metal ion concentration were studied. The results revealed that stationary fermentation condition, pH-6, inoculum size of 2g % w/v , 25% sucrose, 20% glucose concentration, 1.75mM $CaCl_2$ concentration were found to be optimum for ethanol fermentation.

KEYWORDS: *Saccharomyces cerevisiae, fermentation, sugarcane molasses*.

INTRODUCTION

Fermentation is one of the oldest biochemical processes known to Mankind. It has been used to produce a variety of valuable products, including foods, flavorings, beverages, pharmaceuticals, and value-added chemicals like ethanol. Bioethanol has the advantages of being renewable, cleaner burning and produces no greenhouse gases. Biofuels are not only promising sources of environment-friendly energy, but also provide an economic opportunity for the agriculture industry worldwide. An efficient method for conversion of biomass into fuel is by ethanol production because ethanol is an economical as well as environmentally friendly fuel (Martin *et al*., 2002). With industrial development taking place rapidly, there is a need for environmentally sustainable energy sources. 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 (Zaldivar *et al*., 2001).

Saccharomyces cerevisiae is the most commonly used microorganism for ethanol production by fermentation. It is facultative anaerobe and under anaerobic conditions can ferment glucose to ethanol. *S. cerevisiae* is ideal for ethanol production due to several properties including fast growth rates, efficient ethanol production and a tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels (Elena *et al*., 2009). The future of the fermentation industry with respect to Bioethanol

production depends on three major strategies. First, its ability to exploit a variety of microorganisms that are capable of efficient ethanol production by fermentation; second, to utilize various substrates such as sugars, starches or celluloses derived from a variety of different sources; and third, since utilizing starches and celluloses requires enzymes, to locate, develop and investigate relatively inexpensive sources of enzymes. 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. The second category is much inexpensive as being a waste material, it is more ethical to use it for bioethanol production as compared to the first category (Elena *et al.*, 2009). A significant aspect in the fermentation of biomass to ethanol is the cost of the medium used; efficient ethanol production processes and cheap substrates being used and efficient strains of yeasts showing great tolerance to adverse conditions and giving relatively higher yields. Thus, the present study aims at optimizing different environmental parameters for ethanol production using yeast strain (RGK1) which is a local native strain isolated from molasses samples.

MATERIALS AND METHODS

Feedstock collection and characterization

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

Reagents and chemicals

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

Yeast strain

Saccharomyces cerevisiae strains were maintained on Yeast extract Peptone Dextrose agar and Sub cultured once in every month.

Composition of media:

Inoculum media: Malt extract (3.0gm), Glucose (1gm), Yeast extract (0.3gm), Peptone (0.5gm) per 100ml distilled water and pH 6.5

Fermentation Media: Magnesium sulphate (0.2gm), Urea (0.2gm), Sucrose (15gm) per 100ml distilled water and pH 6.7

Factor optimization for ethanol production: The following parameters were selected for optimization - Stationary and Shake Flask Method, Inoculum size, pH, Sugar concentration-Glucose and Sucrose, Metal ion – $CaCl_2$. For all the optimization experiments Standard ethanol fermentation protocol was followed.

Stationary and Shake Flask Method: Fermentation flasks were incubated at 28°C, at 150 rpm in incubator shaker for aeration and agitation with 15 gm% sucrose. Static conditions were maintained by keeping flask at 28°C incubator (Kong kiattikajorn Jirasak *et al.*, 2007). 24 hours old culture was used for

inoculum preparation, O.D. of which was adjusted to 0.1 and 0.5 ml was added in fermentation medium. Samples were removed for four successive days at an interval of 24 hr for estimation of ethanol.

Inoculum size: Fermented broth was centrifuged in pre-weighed sterile centrifuge tube (15 ml) at 9800 rpm at 20^0C for 20 min. Supernatant was discarded and pellet was used as an inoculum. Amount of culture added in the fermentation medium was optimized (0.5, 1, 2 g % w/v) (Laluce *et al.*, 2009). Sucrose concentration used 15%. Samples were removed for four successive days at an interval of 24 hours for estimation of ethanol.

pH: To observe the effect of pH on ethanol production, fermentation medium was adjusted to different pH ranges as (pH 5, 6, 7 and 8). Acetate buffer (pH 5) and phosphate buffer (pH 6, 7 and 8) were used to adjust pH (Hoek Pim Van *et al*., 1998, Teun Van De Laar *et al*., 2006). Samples were removed for four successive days at an interval of 24 hrs for estimation of ethanol.

Sugar concentration

Glucose: To observe optimum glucose concentration for ethanol production, different glucose concentrations (10%, 15%, 20%, 25%, 30 and 35%) were supplemented separately in fermentation medium. Samples were removed for four successive days at an interval of 24 hours for estimation of ethanol (Nghiem *et al*., 2000, Schroeder *et al*., 2001)

Sucrose: To observe optimum sucrose concentration for ethanol production, different sucrose concentrations (10%, 15%, 20%, 25%, 30 and 35%) were supplemented separately in fermentation medium. Samples were removed for four successive days at an interval of 24 hours for estimation of ethanol (Laluce *et al.*, 2009).

Metal ion concentration: $CaCl_2$: To study the influence of metal ions on ethanol production $CaCl_2$ was supplemented separately in fermentation medium. Different $CaCl_2$ concentrations (0.25, 0.75, 1.25, 1.75 mM) were used for optimization of CaCl2 for ethanol production (Regina *et al*., 1988, Schroeder *et al*., 2001). Samples were removed for four successive days at interval of 24 hrs for estimation of ethanol.

RESULTS:

Selection of strain: The experiment was performed to estimate the ethanol production by *Saccharomyces cerevisiae*. Out of screened strains, the strain with highest ethanol production (RGK1) was selected. The other strains were found to produce less ethanol during fermentation. The further studies on optimization were carried out using *S. cerevisiae* (RGK1).

Effect of stationary and shaker method: Approximately 4.6% of ethanol was produced at stationary conditions which were much more than fermentation process carried out in shaker incubator having 3.4% ethanol production.

Optimization of fermentation parameters

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 hrs. 0.5 g % (w/v) and 1 g % (w/v) gave effective ethanol production only at 48 hr of incubation.

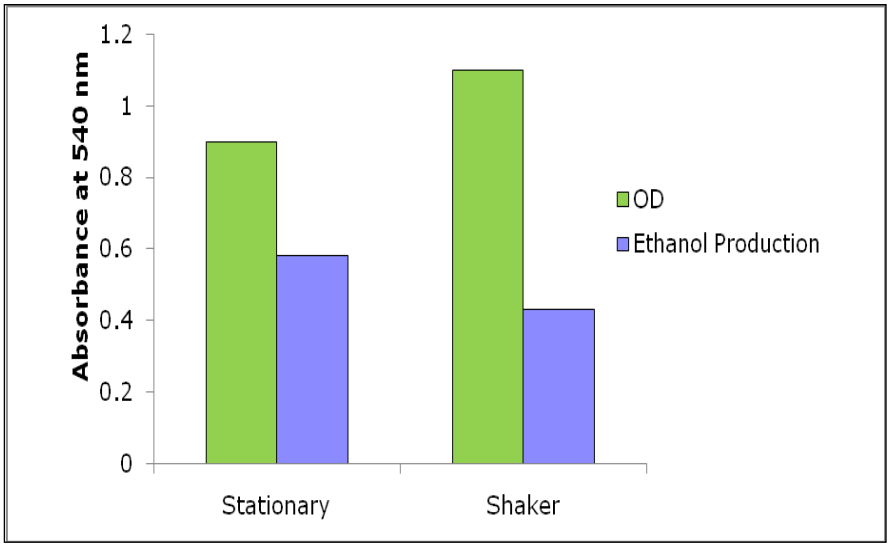


Fig 1: Effect of stationary and shaker on ethanol production

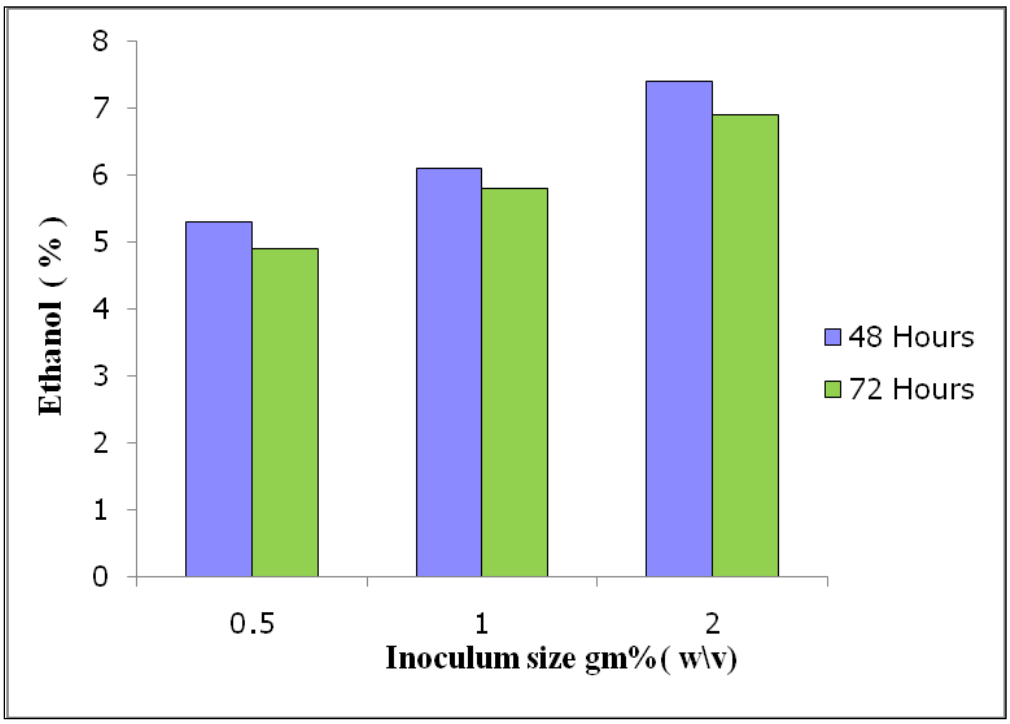


Fig 2: Effect of inoculum size on ethanol production

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.

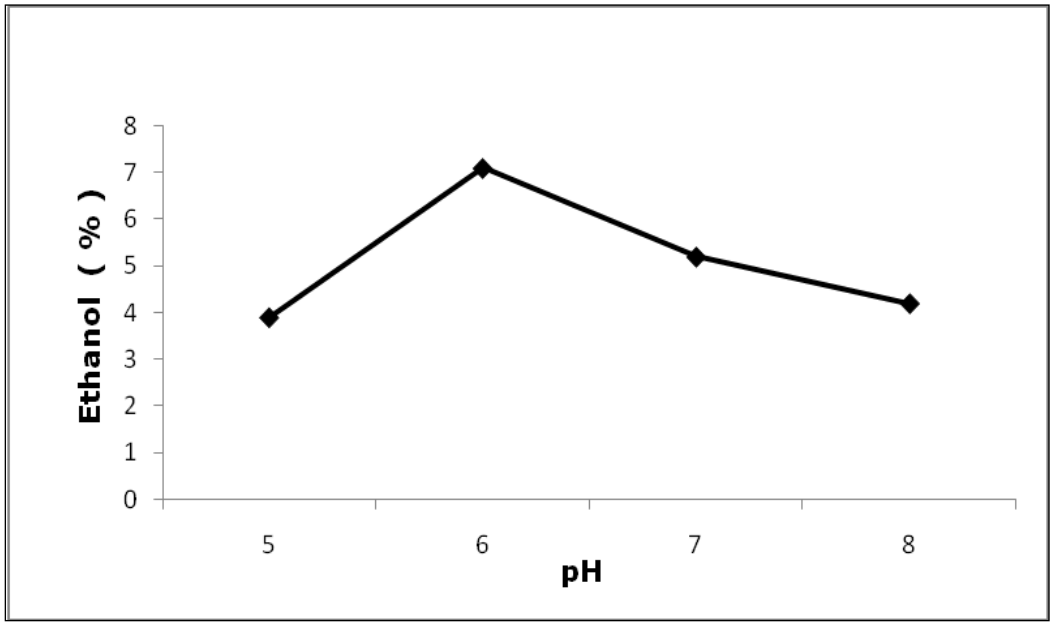


Fig 3: Effect of pH on ethanol production

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.

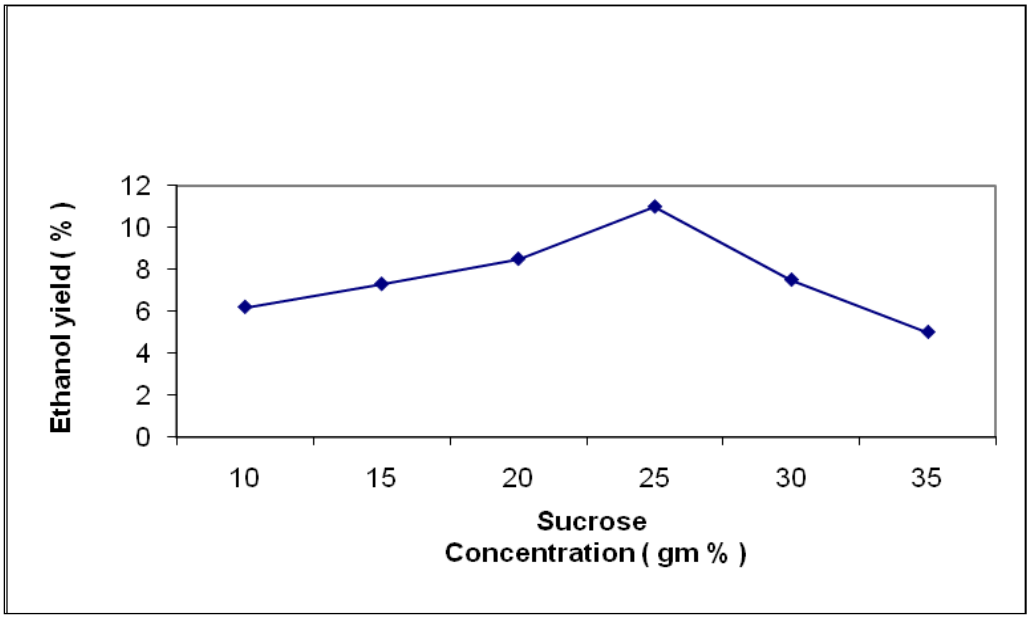


Fig 4: Effect of Sucrose concentration on ethanol production

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.

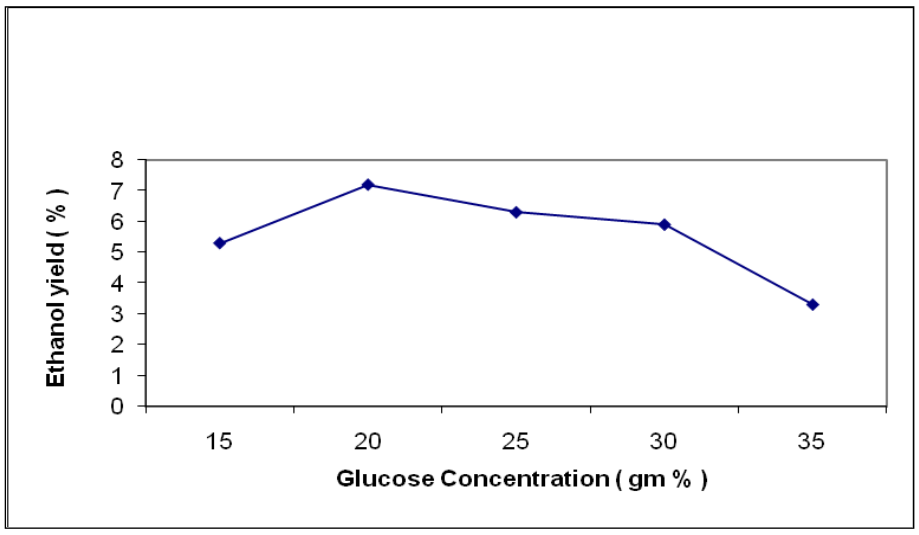
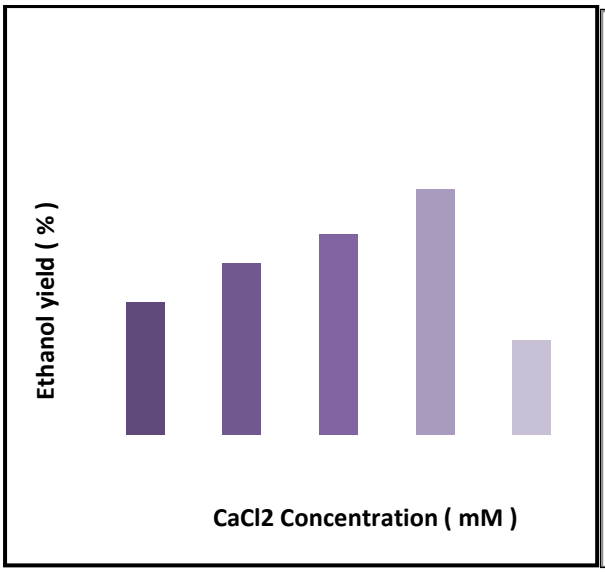


Fig 5: Effect of glucose concentration on ethanol production

Effect of Metal ions on ethanol production:

The study on the influence of metal ion on ethanol production indicated that ethanol production was increased by $CaCl_2$ addition even after 48hrs incubation.1.25 mM concentration was found to be optimum for ethanol production.



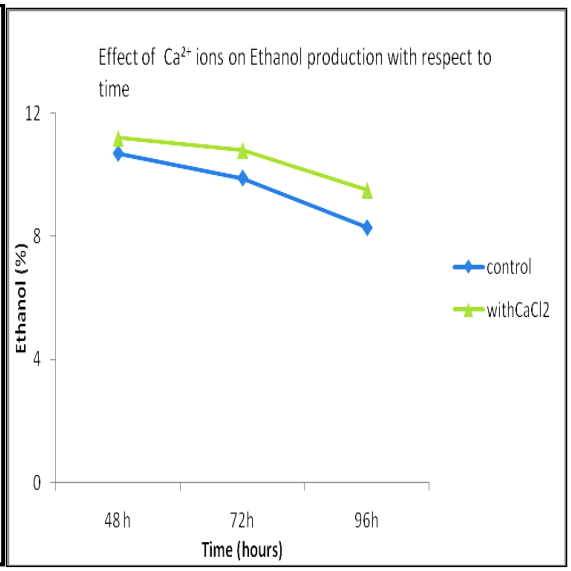


Fig 6: Effect of $CaCl_2$ concentration

Fig 7: Effect of $CaCl_2$ concentration

Effect of ethanol on yeast cell growth: By taking into consideration total viable count, dry weight, Optical density it was found that after 48hrs of incubation when ethanol is produced the yeast cell viability was decreased. Thus yeast cell viability is inversely proportional to ethanol production.

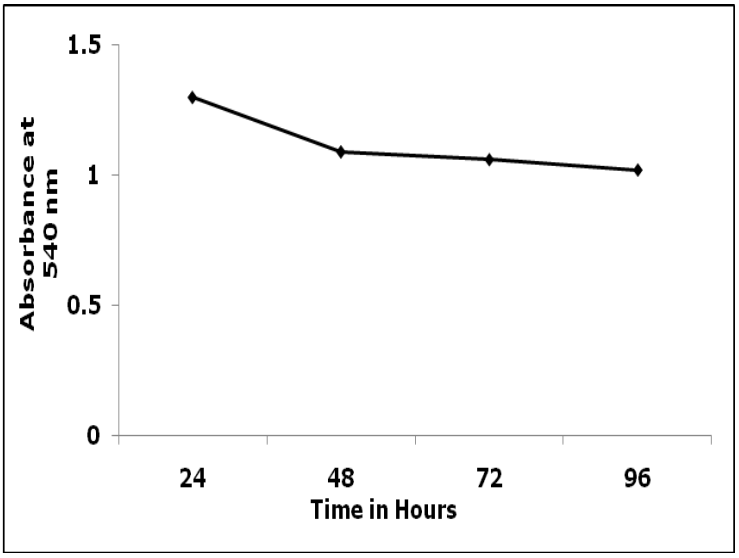


Fig 8: Effect of ethanol on growth of yeast

DISCUSSION

Selection of strain of *Saccharomyces cerevisiae* RGK1 was done on the basis of their efficiency of ethanol production (Arisra *et al*., 2007). This screening was done by using ethanol fermentation protocol (Teun Van De Laar *et al*., 2006) Estimation of % ethanol yield was determined by using Potassium Dichromate method. It was found that among fifty strains RGK1 was showing maximum10 % ethanol yield, so this strain was selected for further experiments. Reliability of selected strain RGK1 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 (Kilonzo *et al*., 2008, Arisra *et al*., 2007) and by monochrome staining. Selected strains were subjected for optimization of Stationary and shaker condition, inoculum size, pH, Sugar concentrations (glucose and sucrose) and $CaCl_2$ ions.

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 30^0C for 48 hrs. 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 (Kong kiattikajorn Jirasak *et al*., 2007).

To study the effect of inoculum size on ethanol production, amount of culture added in the fermentation medium was optimized (0.5, 1, 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 % and1gm% w/v, inhibitory effect of ethanol, after 48 hours was also less (Laluce *et al*., 2009). 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* RGK1. 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 (15, 20, 25, 30 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 ceases (Nghiem *et al*., 2000, Schroeder *et al*., 2001).

Optimum Sucrose concentration was 25 g % for maximum ethanol production (11 %). Different concentration of sucrose (10, 15, 20,25,30,35 g %) 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 (Hoek *et al*., 1998). Optimum Sucrose concentration 25 g % at 28^0C was used for pH optimization (pH 5, 6, 7, 8). pH 6 was found to be optimum for ethanol production. Based on fermentation efficiency pH 6 was used for further experiments (Hoek *et al*., 1998).

Supplementation of Ca^{2+} ($CaCl_2$) in optimum 1.75 mM/ 100 ml in the fermentation medium lead to rapid production of ethanol by *Saccharomyces cerevisiae* RGK1 which was found to be 10.6 % of ethanol at 48 hr. With Supplementation of $CaCl_2$ *Saccharomyces cerevisiae* RGK1 was able to produce ethanol in significant amount even up to 96 hr which otherwise produce ethanol only up to 72 hr (Regina *et al*., 1988).

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