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Full Length Research Paper

Characterization of alcohol resistant yeast *Saccharomyces cerevisiae* isolated from Toddy

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In the present investigation *Saccharomyces cerevisiae* was isolated from Toddy and identified by based on morphological and sugar fermentation tests. In alcohol tolerance test, *Saccharomyces cerevisiae* tolerate up to 15% of ethanol in the medium. Optimize the culture conditions of yeast strain such as initial sugar concentration, pH, temperature and incubation period on ethanol production was also investigated. The fermentation process was carried out in Yeast extract peptone glucose medium (YPG) under optimum conditions such as initial sugar concentration-4%, pH-3, temperature-30°C and incubated for 72 hrs. The maximum amount ethanol production (19.83 g/l) was observed by Gas chromatography analysis.

Keywords: *Saccharomyces cerevisiae*, Toddy, Ethanol production, Alcohol tolerance, Fractional distillation, Gas chromatography

INTRODUCTION

On account of limited global supply of oil, ethanol has emerged as an alternative for petroleum based liquid fuels. It is used in automobiles as an alternative fuel has attracted worldwide attention for its production on a large scale while maintaining the economic status of a country. Biofuel production process; whether biogas, bioethanol or biodiesel are entirely different and unique. Through the processes of transesterification, fermentation or anaerobic digestion, biofuels are produced from organic material and refined, for use in vehicles or in other applications (Bertolini et al., 1991).

Ethanol production is quite unique on a global perspective. Using yeast, through fermentation process the sugars are converted into alcohol, carbon dioxide and some byproducts were produced. Outputs from the ethanol plant include, naturally ethanol, water, stillage, syrup, molasses, carbon dioxide, biomass and other alcohol. Brazil and the USA are the world's largest producers of bioethanol, counting with approximately 62% of world production (Maris et al., 2006).

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The yeast *Saccharomyces cerevisiae* is the primary organism used for ethanol production and efficiency of ethanol production is mainly dependent on the choice of yeast strain (Gera and Sharma, 1991). The most commonly used microorganism for ethanol production is ordinary baker's yeast *S. cerevisiae*. In the pretreatment process some inhibitors are formed and *S. cerevisiae* one of the most inhibitor tolerant microorganisms used for the conversion of hexoses such as glucose and mannose not pentoses such as xylose, arabinose that are found in the hemi cellulose portion (Olsson and Hahn, 1993).

The increasing demand for ethanol for various industrial purposes such as alternative source of energy, industrial solvents, cleansing agents and preservatives has necessitated increased production of this alcohol. Ethanol production is usually accomplished by chemical synthesis of petrochemical substrates and microbial conversion of carbohydrates present in agricultural products. Owing to depleting reserves and competing industrial needs of petrochemical feed stocks, there is global emphasis in ethanol production by microbial fermentation process. Increased yield of ethanol production by microbial fermentation depends on the use of ideal microbial strain, appropriate fermentation substrate and suitable process technology (Brooks, 2008).

MATERIALS AND METHODS

Isolation of Yeast strain

Toddy sample was collected in a sterile container from a local area of Sivakasi in Virdhunagar District and transferred to laboratory immediately. The sample was serially diluted and plated on Yeast extract peptone glucose agar medium (YPG). The plates were incubated at 30°C for 48 hrs. The isolates were maintained on YPG agar slants at 4°C for further studies (Martini, 1996).

Identification of yeast isolate by fermentation studies

Identification of yeast isolate was carried out by the fermentation patterns of different sugars like glucose, galactose, maltose, sucrose, xylose, arabinose, raffinose, cellulose and lactose. All these sugars were taken in a separate test tube with 5ml of Phenol red broth medium. Yeast isolates were inoculated and incubated at 30°C for 48 hrs. After incubation the tubes were observed. The sugar fermentation was indicated by color change from red to yellow due to acid production (Barnett et al., 1990).

Ethanol tolerance test

The yeast isolate was tested for ethanol tolerance. The yeast strain was inoculated in 10ml of YPG broth containing different concentration of ethanol (0, 2.5, 5, 7.5, 10, 12.5 and 15%). The tubes were incubated at 30°C for 48 hrs. After incubation the viability of yeast cells were checked by serially diluted with sterile distilled water and plated on YPG agar medium. After incubation the results were tabulated in the form of CFU/ml (Khaing et al., 2008).

Estimation of ethanol by potassium dichromate and sulphuric acid method

Ethanol assay from sample was tested by the method of Caputie et al. (1986). One ml of culture supernatant was taken and make up the volume to 5ml with distilled water then followed by 1ml of $K_2Cr_2O_7$ solution and 4ml of Conc. H_2SO_4 solution was added. The intensity of colour was read at 660nm in UV/VIS spectrophotometer (Systronics, 119). Blank is prepared in the same manner without ethanol. Ethanol production was assayed by comparing with standard graph.

Optimization of cultural conditions of yeast for ethanol production

Effect of initial sugar concentration on ethanol production

The effect of initial sugar concentration on ethanol production was tested by varying the concentration of glucose (1, 2, 3, 4, 5 and 6%) in Yeast extract Peptone Glucose medium (YPG). The overnight culture of yeast cells were inoculated and incubated at 30°C for 48 hrs. After incubation samples were drawn and tested for concentration of ethanol (Roukas, 1996).

Effect of incubation period on ethanol production

Effect of incubation period on ethanol production was tested by the yeast isolate grown in the YPG medium with 4% of glucose and incubated at 30°C for different time periods (24, 48, 72, 96 and 120 hrs). The samples were drawn for every 24 hrs interval and the ethanol concentration was estimated (Tahir et al., 2010).

Effect of temperature on ethanol production

Effect of temperature on ethanol production by the yeast isolate grown in the YPG medium with 4% of glucose and incubated at various temperature (25, 30, 35 and 40°C). After 72 hrs of incubation the ethanol concentration was estimated (Neelakandan and Usharani, 2009).

Effect of initial pH on ethanol fermentation

The effect of pH on ethanol production was tested by the yeast isolate grown in the YPG medium. The pH of the medium was maintained in the range of pH 2.5 to pH 5.5 and incubated at 30°C for 72 hrs. After incubation the ethanol concentration was estimated (Tahir et al., 2010).

Fermentation process

A 24 hrs cell suspension of *Saccharomyces cerevisiae* at OD_{660} = 0.6 was inoculated into 500ml of fermentation medium (in 1000ml Erlenmeyer flask) containing (g/l): glucose-40g; yeast extract-10g; peptone-10g. The pH of the fermentation medium was adjusted to pH-3. The fermentation was carried out at 30°C for 72 hrs

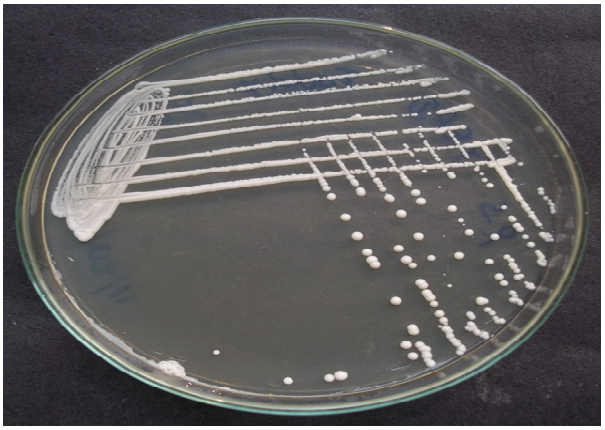

Figure1. *Saccharomyces cerevisiae* on Yeast extract peptone glucose agar plate

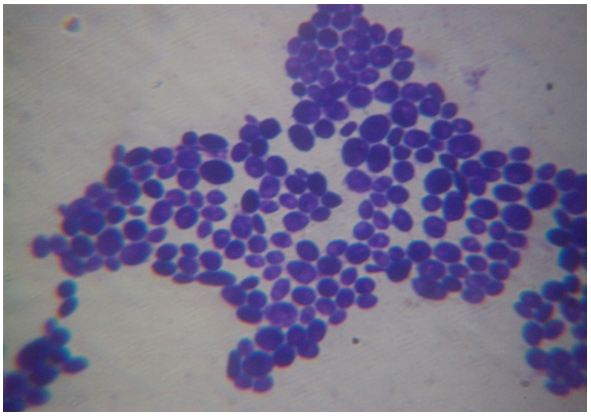

Figure 2. Microscopic Morphology of S*accharomyces cerevisiae*

(Manikandan et al., 2010).

Distillation of ethanol by fractional distillation method

The fermented broth was collected and the filtrate was obtained by centrifugation at 10000 rpm for 5min. Ethanol was distillated by fractional distillation method. The filtrate was boiled at 78.4℃ and condensed. The distillate (ethanol) was collected in the collection chamber (Khaing et al., 2008).

Ethanol analysis by Gas chromatography

The concentration of ethanol was estimated by using VARIAN-CP-3800 Gas chromatography (GC) with flame ionization detector (FID) and BPX-5 (SGE) column (30m length and 0.32 mm diameter) using Helium as the carrier gas at the rate of 1.3 ml/min. Hydrogen and compressed air are used as fuel gas and are regulated at a rate of 30µl/min and 300µl/min respectively. The injector, detector and oven temperature were programmed as 150℃, 180℃ and 60℃ respectively. Sample was injected into injector port by using a micro syringe (1-10 µl). The peak eluted was noted (using STAR WORKSTATION-system control version 5.51) and by knowing the area of peak and the concentration of ethanol was calculated using calibration chart (Manikandan et al., 2010). Gas chromatography analysis was carried out in Sargam Laboratory at Chennai.

RESULTS AND DISCUSSION

The indigenous yeast was isolated from Toddy. The sample was collected in sterile container, serially diluted and plated on Yeast extract peptone glucose agar medium (Figure 1). The morphology of the yeast isolate was tested by mounting with crystal violet, ascospore were seen at the time of microscopic observation of the slide (Figure 2). Biochemical identification of yeast isolate

Table. 1. Sugar fermentation test for indigenous yeast isolate

Type of sugar	Fermentation of sugar by yeast isolate
Glucose	+
Galactose	+
Sucrose	+
Maltose	+
Xylose	-
Raffinose	+
Arabinose	-
Cellulose	+
Lactose	-

(+) Positive, (-) Negative

Table 2. Ethanol tolerance test for *Saccharomyces cerevisiae*

Ethanol concentration (%)	*Saccharomyces cerevisiae* (CFU/ml)
0	4.5×10^{8}
2.5	2.6×10^{8}
5	1.3×10^{8}
7.5	8×10^{7}
10	3×10^{7}
12.5	2.5×10^{7}
15	6×10^{6}

was tested by the fermentation patterns of carbon sugars, some of the sugars like glucose, galactose, maltose, sucrose, raffinose, cellulose are positive in fermentation process was indicated by color change from red to yellow due to acid production (Table. 1). The yeast isolate was identified as *Sccharomyces cerevisiae* by based on the morphology and sugar fermentation tests.

Similar results were reported by Walker et al. (2006) reported that all the isolate ferment at least one type of sugar. However a majority of these isolates which ferment glucose, galactose, maltose, sucrose and raffinose, this type of strain belonged to the genus *S. cerevisiae*. Martini, (1996) reported that indigenous yeast *S. cerevisiae* with very high ethanol producing capabilities in the natural environment are though to be very rare. The most commonly used microorganism for ethanol production is ordinary baker's yeast *S. cerevisiae*. In the pretreatment process some inhibitors are formed and *S. cerevisiae* one of the most inhibitor tolerant microorganisms used for the conversion of hexoses such as glucose and mannose not pentoses such as xylose, arabinose that are found in the hemi cellulose portion (Olsson and Hahn, 1993).

The effect of ethanol concentration on the cell growth and viability of *Saccharomyces cerevisiae* was tested. *Saccharomyces cerevisiae* tolerate up to 15% of ethanol in the medium (Table. 2). Similar results were reported by Khaing et al. (2008). They reported that the *S. cerevisiae* (KY1&KY3) strains has tolerate up to 15% of ethanol in the medium and *S. cerevisiae* (KY2) tolerate up to 20% of ethanol has leads to maximum ethanol production over a long incubation period. Cassey, (1996) reported that the yeast strains survive to any extent in palm wines must have some degree of ethanol tolerance, which have some importance in choosing a yeast strain for industrial ethanol fermentation process. Use of efficient yeast strains with higher ethanol tolerance to improve ethanol yields in the fermented wash would reduce distillation costs and hence the profitability of the overall process (Chandrasena et al., 2006).

In the present study, the effect of initial sugar concentration on ethanol production was tested. At the 4% of initial sugar concentration *Saccharomyces cerevisiae* produced maximum amount of ethanol observed as 16.03 ± 0.17 g/l (Figure 3). Similar results were reported by Govindaswamy and Vane, (2010). They reported that 5% of initial glucose concentration resulted in yield of 0.49 g/g by *S. cerevisiae* for duration of 72 hrs of incubation period yield maximum amount ethanol (85 g/l). Sugar concentration is also critical in fermentation process and influencing the rate of production and the yield in addition to physiological growth of yeast. Initial

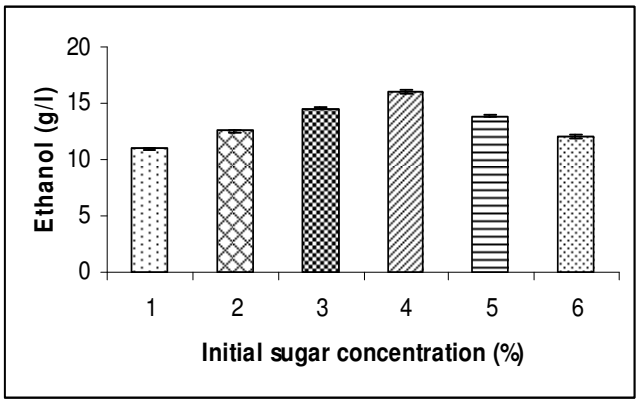

Figure 3. Effect of Initial Sugar Concentration on Ethanol production

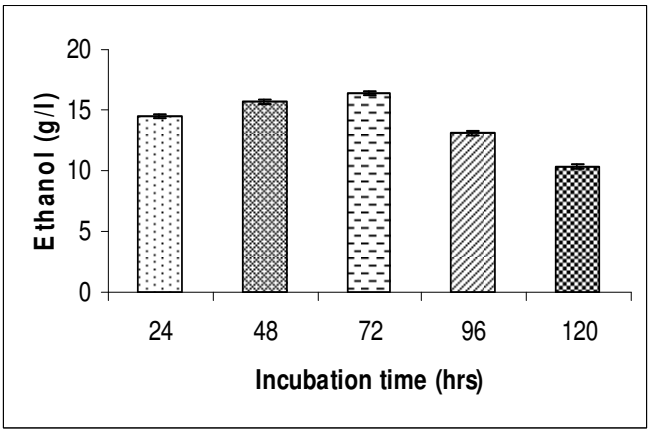

Figure 4. Effect of Incubation Period on Ethanol Production

sugar concentration has also been found to determine the amount of alcohol (Mariam et al., 2009). Mishima et al. (2008) reported that 14.4 g/l of ethanol from water hyacinth used as substrate at the initial sugar concentration 30 g/l and water lettuce as substrate produce maximum ethanol 14.9 g/l at the sugar concentration 33 g/l.

The effect of incubation period on ethanol production was tested in this paper. *Saccharomyces cerevisiae* produced maximum ethanol (16.42 ± 0.14 g/l) at 72 hrs of incubation (Figure 4). Similar results were reported by Walkins et al. (2006). They reported that the work done with two ethanologenic yeast strain *S. cerevisiae* and *Kluyveromyces marxianus,* were used to ferment hydrolyzed sugars extracted from orange peel waste. In these conditions *S. cerevisiae* produced more ethanol than *Kluyveromyces marxianus* at 72 hrs of incubation period. Ferrai et a*l*. (1992) reported that production of maximum ethanol concentration 12.6 g/l in fermentation time 72 hrs from eucalyptus wood hemicellulose by *Pichia stipitis.* Ballesteros et al. (2004) reported after 72 hrs of incubation 16-19 g/l ethanol production from different lignocellulosic biomass in simultaneous saccharification and fermentation process by *Kluyveromyces marxianus* CECT 10875 at 42°C.

The effect of temperature on ethanol production was also analyzed. *Saccharomyces cerevisiae* produced maximum yield of ethanol (18.13 ± 0.16 g/l) at 30°C (Figure 5). Similar results were reported by Pertrova and Ivanova, (2010). The fermentation of olive tree pruning hydrolysate containing xylose by *Pachysolan tannpphilus* was produced maximum ethanol 0.38 g/g at 30°C and pH 3.5. Mariam et al. (2009) reported that the maximum amount of ethanol (7.5%) was obtained from fermentation by *S. cerevisiae* at the optimum pH 3.5 and incubation temperature 30°C.

The effect of pH on ethanol production was tested. The maximum amount of ethanol (18.60 ± 0.14 g/l) was produced by Saccharomyces *cerevisiae* at pH 3 (Figure 6). Similar results were reported by Narendran et al. (2004). They reported that *S. cerevisiae* is an acidophilic organism and grow better under acidic conditions. The

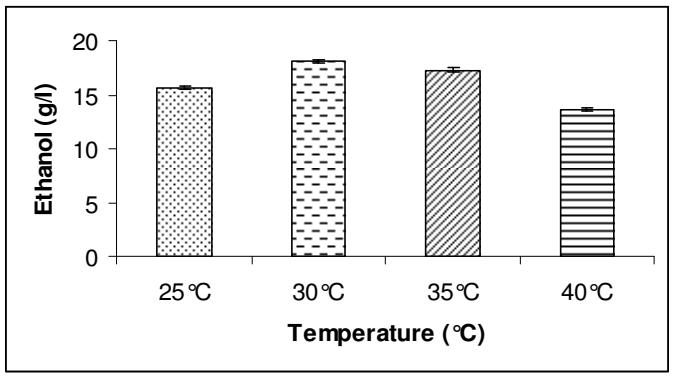

Figure 5. Effect of Temperature on Ethanol Production

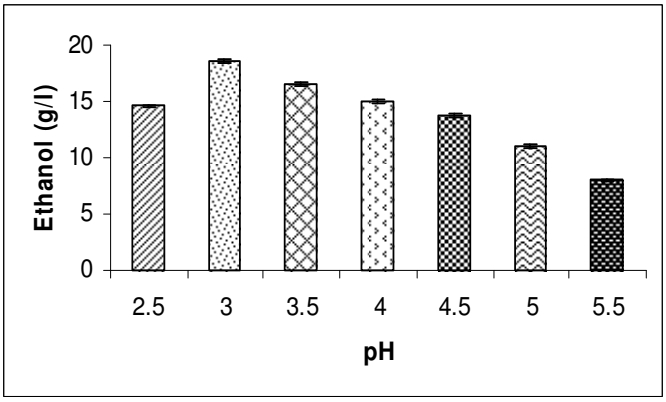

Figure 6. Effect of pH on Ethanol Production

optimal pH range for yeast growth can be varied from pH 3-6. The intracellular enzymes of yeast work best at its optimal pH it leads to maximal conversion of sugar into ethanol. Goksungur and Zorlu, (2001) reported that continuous production of ethanol from beet molasses by Ca-immobilized *S. cerevisiae* at 30℃ and pH 3 are optimum for maximum ethanol production. The maximum amount of ethanol (7.5%) was obtained from fermentation by *S. cerevisiae* at the optimum pH 3.5 and incubation temperature 30℃. Manikandan et al. (2010) reported that *S. cerevisiae* yeast isolated from toddy and maximum yield of ethanol (40 g/l) compared with baker's yeast *S. cerevisiae* in the optimum pH 3.0, temperature 30℃ and initial sugar concentration 20%. Toddy is produced abundantly as a cheaper source for the development of suitable yeast strains for ethanol production.

The fermentation process was carried out in Yeast extract peptone glucose medium (YPG) under optimum conditions such as initial sugar concentration-4%, pH-3, temperature-30℃ and incubated for 72 hrs. After incubation, ethanol was distillated from the fermented broth by fractional distillation method. *Saccharomyces cerevisiae* yield 19.83 g/l of ethanol was analyzed by Gas chromatography. Amore *et al.* (1998) reported that the maximum of 12.3% of alcohol was produced from a concentration of 20% glucose in the medium after 72 hours of incubation. Similar results were reported by Uma and Polasa (1990) isolated *S. cerevisiae* from palm wine, which produced increased amount of ethanol in Yeast extract peptone glucose medium. Bertolini et al. (1991) reported that isolated new strains of *S. cerevisiae* on basal medium containing 48% sucrose from fermenting sample collected from Brazilian alcohol factories. Isolated strains fermented concentrated sugarcane syrups as well as high sucrose solution in synthetic medium with conversion efficiency of 89-92%.

CONCLUSION

S. cerevisiae one of the most commonly used microorganism for industrial fermentation process. Use of efficient yeast strains with higher ethanol tolerance to improve ethanol yields in the fermented wash would reduce distillation costs and hence the profitability of the overall process while save the environment for pollutants.

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