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Modulation of Palm Wine Fermentation by the Control of Carbon and Nitrogen Source on Metabolism of *Saccharomyces cerevisiae*

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Abstract: The Indian palm trees (*Borassus flabellifer*) produce palm juice which has been traditionally used for palm wine production. The fermentation process was studied in the presence of different carbon sources such as glucose, glycerol and glucose-glycerol combination as substrates. The *Saccharomyces cerevisiae* (NCIM 3045) mutant strains had the ability to utilize the two types of carbon sources i.e., glucose and glycerol in three different types of fermentation media i.e., glucose, glycerol and glucose-glycerol combined. Although, glucose showed high ethanol production, (63.34 g L^{-1}) was seen after the 1st day, on the 3rd day, the yield (124.54 g L^{-1}) was the highest. The carbon utilization rates from different sources were found to be of different magnitudes. Further, the product as well as biomass formation also varied while correlating with the carbon utilization rate. Of the two nitrogen sources used viz. urea and di-ammonium hydrogen phosphate, urea induced the cell for high rate of sugar utilization and the highest yield of ethanol was obtained as 152.20 g L^{-1}. About 6 g L^{-1} of urea gave the optimum ethanol production.

Key words: Fermentation, glucose, glycerol, palm juice, palm wine, urea

INTRODUCTION

Wine fermentation is carried out by *Saccharomyces cerevisiae*. Yeast cells encounter variable concentration of different carbon sources. The changes in sugar content affect the fermentation process which can be established by the study of kinetic behavior of fermentation process (Wang *et al*., 2004).

The glucose concentration and level of glycolytic intermediates regulate the expression of several glucose transporters and some glycolytic gene (Carlson, 1999). Therefore, the main glucose repression pathway ensures that the preferred sugar are metabolized before the consumption of alternative carbohydrates i.e., maltose and galactose (Verstrepen *et al*., 2004).

Glycerol is another carbon source which has been demonstrated to serve as the major osmolyte of *S. cerevisiae*. Glycerol is an important cellular compound in yeast. It can serve as a carbon source as well as it has a metabolic function because its synthesis contributes to the maintenance of the cellular redox balance (Ansell *et al*., 1997). It is a precursor for the phospholipids which may in turn play a role in signaling (Siderius *et al*., 2000). Glycerol is used by the yeast as the sole source of carbon and energy. The glycerol kinase is a key enzyme for controlling the glycerol catabolic pathway in yeast. The structural genes glycerol kinase (GUT1) and FAD dependent glycerol-3-phosphate dehydrogenase (GUT2) have shown similarity to prokaryotic and eukaryotic homology (Pavlik *et al*., 1993; Ronnow, 1992). The gut1 and gut2 mutants are unable to use glycerol as a sole source of carbon and energy (Sprague and Cronan, 1977) which suggests that the phosphorylative pathway is the major route for glycerol assimilation in *S. cerevisiae* (Grauslund *et al*., 1999). Another important aspect is that glycerol maintains the signaling competent state of the cell (Siderius *et al*., 2000). It was proposed, based on the observation of increased glycerol intracellular levels at high temperature, of HOG pathway, mutants could be related with increase of glycerol.

Quantitatively, nitrogen is the second most abundant nutrient in wine fermentations. It is essential for yeast metabolism and growth. Consequently, lack of nitrogen triggers sluggish fermentations (Alexandre and Charpentier, 1998; Boulton *et al*., 1996; Fleet and Heard, 1993). The timing of the addition is key for ensuring a successful fermentation (Bely *et al*., 1990; Salmon, 1989). Early addition affects both the fermentation rate and the biomass yield. Late addition has a minimal effect on biomass formation but however increases the fermentation rate (Bely *et al*., 1990).

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Depending on the particular yeast race and fermentation conditions, urea may be used as a nitrogen source by *S. cerevisiae*. In this case, urea is degraded to ammonium ion and CO_2 by the multipurpose enzyme ATP-urea amidolyase (Whitney and Cooper, 1972). Ethyl carbamate is a well-known carcinogen found in fermented foods and drinks (Ough, 1976) which are produced during wine fermentation in the presence of ethanol and urea. Therefore, high concentration of urea is avoided during fermentation. The ammonium salt (diammonium hydrogen phosphate) is also used as a good nitrogen source for the fermentation. It not only prevents the sluggish fermentation but it is also used for improvement of fermentation kinetics by time optimization (Bell and Henschke, 2005).

The present research deals with individual as well as the combined effect of carbon source i.e., glucose and glycerol on palm wine fermentation carried out by *S. cerevisiae*. The effect of nitrogen supplementation on glucose and glycerol during fermentation is also studied.

MATERIALS AND METHODS

Chemicals: Dextrose, Glycerol (GR), KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, Urea, Di-ammonium hydrogen phosphate were purchased from Merck, India. Yeast extract and Peptone were procured from Himedia, India, 3,5-dinitrosalicylic acid used was from Loba Chemie, India.

Microorganism and culture preparation: Stock culture of *Saccharomyces cerivisiae* (NCIM 3045) was procured from National Chemical Laboratory (NCL) Pune, India. The culture media prepared consisted of malt extract 0.3, glucose 1.0, yeast extract 0.3, peptone 0.5, all in g 100 mL. The organisms were grown at a temperature of 30°C and pH 6.5. The incubation period was 48 h. After incubation, the culture was stored at 4°C in a refrigerator.

Preparation of fermentation media: The palm juice was collected from rural areas of West Bengal, India. It was put in a cooling bag and preserved at -20°C in a freezer (C340, New Brunswick Scientific) within 2-3 h from the time of collection. For fermentation, carbon, nitrogen and other trace elements were added to the palm juice in appropriate amounts. The three different flasks contained the fermentation media as glucose 1.0, glucose: glycerol (1:1) 1.0, glycerol 1.0, (g/100 mL) respectively and others components kept the same for the three flasks were KH_2PO_4 0.05, K_2HPO_4 0.05, $MgSO_4.7H_2O$ 0.05, $FeSO_4.7H_2O$ 0.001 (g/100 mL). For optimization of nitrogen sources, the fermentation media contained optimized carbon source along with other trace elements as per previous media.

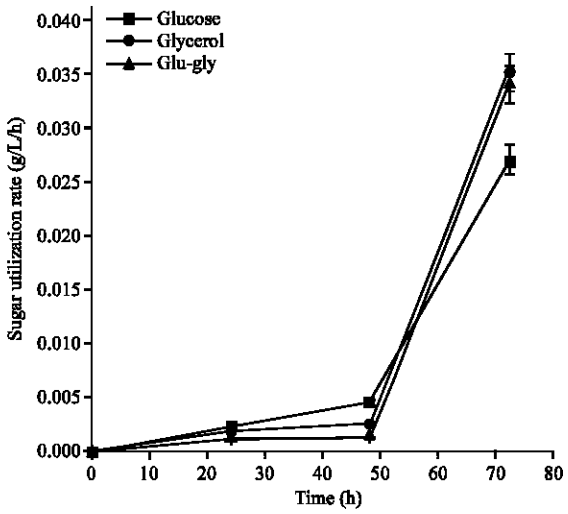

Fig. 1: Influence of different carbon source on sugar utilization in palm wine fermentation

Fermentation was performed in a 250 mL flask with 100 mL of fermentation media and inoculated with 1 mL of yeast culture solution. The culture solution was prepared by sterile distilled water mixed with yeast slant and concentration of yeast cells in OD was 0.5. The pH and temperature were adjusted to 5.5 and 32°C, respectively in anaerobic condition. The incubation time was 3 days. The samples were withdrawn at appropriate time intervals for analysis. All the experiments were performed thrice Fig. 1.

Analytical method

Estimation of ethanol, sugar and protein concentration: About 5 mL of fermented sample was centrifuged (Remi C-24, Mumbai, India) at 6500 g for 10 min. The supernatant solution was used to determine the ethanol concentration by Gas Chromatography (Perichrom SGE D11, column BP1-dimethyl polysiloxane). The absorbance of the sugar solution was determined spectrophotometrically (Model no. 2800 Hitachi, Japan) at 540 nm by DNS method (Plummer, 2007). Protein content of juice was estimated by Lowry method at 650 nm (Plummer, 2007).

Estimation of biomass concentration: The Biomass concentration was determined by the dry weight method. The cells were separated by centrifuging 5 mL of fermented broth (Remi C-24, Mumbai, India) at 1200 g for 20 min consecutively twice with saline water. The cells were dried at 65°C for 2 days in hot air oven (Concept International, Kolkata, India). Dilutions of the culture were made and the absorbance was measured. The calibration curve correlating absorbance and dry weight gave a straight line (Chowdhury *et al*., 2003).

RESULTS AND DISCUSSION

In the process of the palm wine fermentation, the effect of different sugars on *S. cerevisiae* and their performance in the fermentation process has been studied. The organism has shown varying sugar utilization rate, biomass formation rate, ethanol production rate, when the carbon source (i.e., glucose, glycerol, combination of glucose: glycerol) was varied. Table 1 shows the influence of different carbon sources on metabolic activity of the biomass i.e., sugar utilization rate, rate of ethanol production, rate of protein utilization and yield of product coefficient (Yp/s) that relates the amounts of products formed per unit mass of substrate consumed.

Role of different sugars on palm wine fermentatios: In the palm wine fermentation, glucose, glycerol and the combined effect of glucose: glycerol very much influenced the yeast activity. Generally, yeasts are glucophilic organism but under some specific conditions (e.g., in the presence of nitrogen), rapidly utilize sugars other than glucose (Berthels *et al*., 2004). From the beginning and upto 2nd day, the sugar utilization rate was higher in glucose containing flask it was 0.0047 g/L/h, rather than glycerol flask (0.0027 g/L/h) or glucose: glycerol flask (0.0014 g/L/h) (Fig. 1). But on the 3rd day sugar utilization rate (0.0363 g/L/h) was highest in glycerol flask and the rate decreased from glucose: glycerol to glucose these were 0.0351 and 0.0279 g/L/h, respectively.

Figure 2 shows that initially the production of ethanol was high in glycerol flask from 2nd day onwards it was 105.79 g L^{-1}. Other two flasks had near about the same ethanol concentration upto 2nd day these were 95.1 g L^{-1} in glucose flask and 97.67 g L^{-1} in glucose: glycerol. Finally after 3rd day, glucose containing flask produced highest ethanol which was 124.54 g L^{-1}.

Table 2 shows the statistical significance of ethanol production (one way ANOVA, Microsoft Excel, Windos 2007) with different sugars. The experimental results were F ratio value 0.000439, p value 0.999561. The F ratio value was lower than the value of F critical at the 5% level of significance. Therefore least significance test was not needed (Gacula and Singh, 1984).

Influence of urea in wine fermentation: Nitrogen helps to avoid sluggish fermentation (Alexandre and Charpentier, 1998; Boulton *et al*., 1996). In this study, it has been found that the sugar utilization was enhanced by using supplemented nitrogen in the fermentation broth. Figure 3 shows that sugar utilization in the control (in absence of urea) was slower than urea containing media (6 g L^{-1}).

The rates of sugar utilization in urea (6 g L^{-1}) flask were 0.033, 0.087 and 0.032 g/L/h from the 1st-3rd day, respectively. The highest ethanol production in 6 g L^{-1} urea flask was 152.20 g L^{-1} after 72 h. Another nitrogen source, di-ammonium hydrogen phosphate was also used in the fermentation broth to study its effect on the process of fermentation. It was found that di-ammonium hydrogen phosphate accelerated the

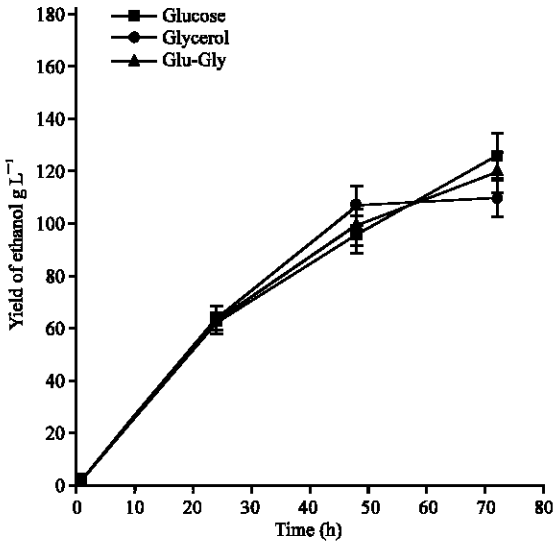

Fig. 2: Influence of different carbon source on ethanol production in palm wine fermentation

Table 1: The experimental values of different substrates utilization rate for the production of ethanol in palm wine fermentation

Substrates	Glucose	Glycerol	Glucose-Glycerol
Ethanol production (g/L/h)	2.51±0.06	2.67±0.04	2.50±0.07
Sugar utilization (g/L/h)	0.0025±0.002	0.0019±0.004	0.0013±0.001
α Nitrogen utilization (g/L/h)	0.004±0015	0.015±0.003	0.006±0.001
Biomass formation (g/L/h)	0.69±0.1	0.54±0.02	0.47±0.3
Yield coefficient of product (Yp/s)	12.45	10.86	11.83

Table 2: Analysis of Variance (ANOVA) for different substrate utilization rate for the production of ethanol (Table 1)

Source of variation	SS	df	MS	F	p-value	F critical
Between groups	1.503731	2	0.751865	0.000439	0.999561	5.143253
Within groups	10268.51	6	1711.418	-	-	-
Total	0270.01	8	-	-	-	-

SS = Sum of Squares, df = degree of freedom, MS = Mean of Squares, p = probability

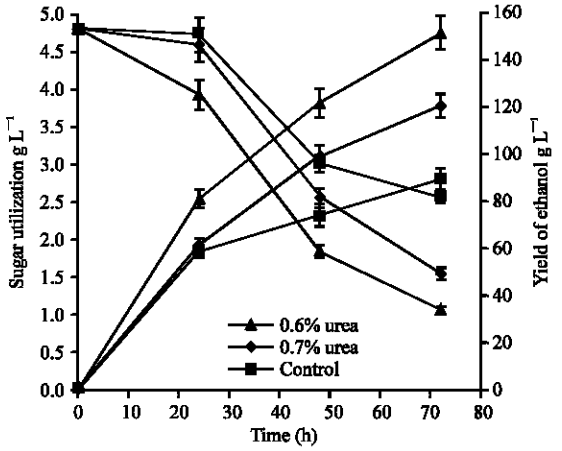

Fig. 3: Influence of urea on sugar utilization and ethanol production in palm wine fermentation. Composition of fermentation media-Glucose 1 g/100 mL, other trace element as per media composition. Control has no urea

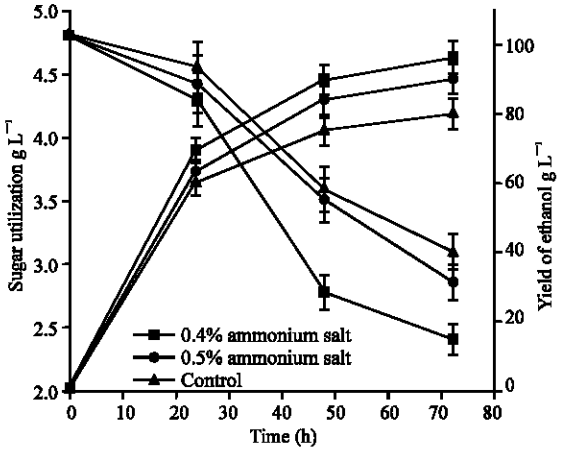

Fig. 4: Influence of di-ammonium hydrogen phosphate on sugar utilization and ethanol production in palm wine fermentation. Composition of fermentation media- Glucose 1 g/100 mL, other trace element as per media composition. Control has no diammonium hydrogen phosphate

fermentation process and increased the ethanol production. Optimum concentration of di-ammonium hydrogen phosphate was found to be 4 g L^{-1} and yield of ethanol was 96.38 g L^{-1} after 72 h. The rates of sugar utilization were 0.020, 0.063 and 0.0154 g/l/h from 1st-3rd day, respectively. The 7 g L^{-1} urea and 5 g L^{-1} di-ammonium hydrogen phosphate containing flask produced lower quantity of ethanol than the 6 g L^{-1} urea and 4 g L^{-1} di-ammonium hydrogen phosphate containing flask it may be due to the higher concentration of nitrogen inhibited the cellular activity of the yeast and ethanol production was affected (Fig. 3 and 4). It can also be concluded from the result that urea is a better source of nitrogen than di-ammonium hydrogen phosphate with respect to ethanol production.

Figure 5 shows α-nitrogen utilization and biomass formation with respect to time. The α-nitrogen utilization was overall high in glucose flask, the concentration of α-nitrogen come down from the 1st-2nd day these were 0.9 and 0.436 g L^{-1} and the utilized α-nitrogen were 0.3 and 0.464 g L^{-1} as can be calculated from Fig. 5.

After 2nd day utilization slowed down, α-nitrogen concentration was 0.363 g L^{-1}. In the 3rd day concentration of utilized α-nitrogen it was 0.073 g L^{-1} and total utilization upto 3rd day was 0.837 g L^{-1} (data was not shown in Fig. 5). Whereas other two flasks, there were α-nitrogen concentration came down from the 1-2 days were 1.04 g and 0.586 g L^{-1} for glycerol and 1.12 and 0.594 g L^{-1} for glucose: glycerol flask, respectively. The utilized α-nitrogen concentrations from 1-2 days were for

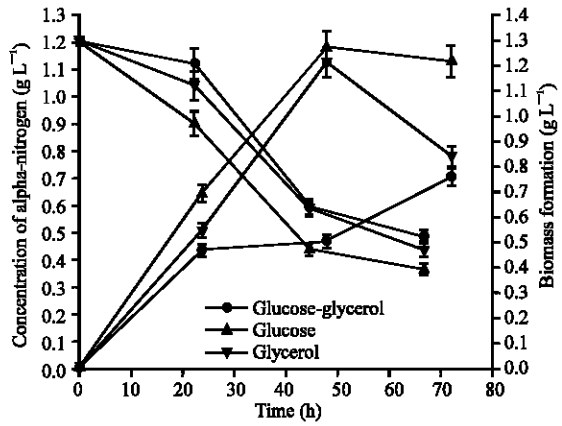

Fig. 5: α-nitrogen utilization and biomass formation in palm wine fermentation. Constituent of fermentation media- Glucose 1 g/100 mL, other trace element as per media composition and urea 0.6 g/100 mL

glycerol 0.164 and 0.45 g L^{-1} and for glucose: glycerol were 0.076 and 0.53 g L^{-1}, respectively (data were calculated from Fig. 5). In the 3rd day α-nitrogen concentration were 0.433 and 0.484 g L^{-1} for glycerol and glucose: glycerol flask, respectively. The total α-nitrogen utilization upto 3rd day were 0.716 and 0.767 g L^{-1} for glycerol and glucose: glycerol flask consecutively (data were not shown in graph). The highest biomass formation, observed in glucose containing flask upto the 2nd day was 1.273 g L^{-1} after that the rate of formation decreased and stationary phase set in. In case of glycerol upto 2nd

day biomass formation was 1.214 g L^{-1} and it rapidly decreased and the cells entered death phase after the 2nd day. But in case of glucose: glycerol, biomass formation increased up to 3rd day with a stationary phase found in the 2nd day. From zero to 1st day biomass increased to (0.471 g L^{-1}) but from 1st-2nd day growth was nearly stagnant it was 0.033 g L^{-1}. Again in the 3rd day growth became exponential and the biomass concentration increased by 0.26 g L^{-1}. This has happened probably because there is a mixed substrate utilization which led to a diauxic growth curve.

The predicted utilization of different carbon sources during the palm wine fermentation was observed. In general, yeast consumed glucose faster than the other sources of carbon, though some yeast strains prefer fructose (Schutz and Gafner, 1995). Results show that sugar utilization rate decreased in the order of glucose, glycerol and glucose: glycerol upto the 2nd day. The reason behind this was that the glucose was the first choice than other carbon sources. The ethanol production was high in glycerol flask and then ethanol concentration decreased from glucose: glycerol to glucose flask in that order upto the 2nd day. This is because in case of ethanol production, glycerol was converted to pyruvic acid faster than the glucose and this pyruvic acid was converted to ethanol by alcoholdehyrogenase. Therefore glycerol containing flask produced more ethanol than the glucose flask. On the 3rd day, sugar utilization rate increased from glucose, glucose: glycerol and glycerol containing flasks and ethanol production decreased from glucose, glucose: glycerol and glycerol flask respectively. In the glycerol containing flask, after 2nd day glycerol was depleted from the media, therefore ethanol production also decreased. But glycerol containing flask needed carbon source for ethanol production as well as to maintain cellular activity of yeast cells. But that much energy was not available from glycerol after the 2nd day. That is why glucose was utilized faster in glycerol flask to recover that energy. In case of glucose flask, on the 3rd day sugar utilization rate increased for first two days but that change normally occurred for ethanol production and routine cellular activity.

The glucose and glycerol penetrated into the cell by different pathways. When glycerol was the main carbon source for *S. cerevisiae*, it can permeate the plasma membrane of cells by three mechanisms-passive diffusion (Gancedo *et al*., 1968), facilitated diffusion through the Fps1p channel protein (Luyten *et al*., 1995) and glycerol/protein symport system (Lages and Lucas, 1997). In case of glucose, it is known that the glucose, glucose-carrier was constitutive (Eddy, 1982). Busturia and Lagunas (1986) showed that the glucose penetration by facilitated diffusion used different carriers, one of them had high affinity, others had low affinity (fructose crosses over the membrane using the same carriers as the glucose but their affinity was lower) (Busturia and Lagunas, 1986). From the kinetic studies of Bisson and Fraenkel (1984) and Bisson (1988) it has been established that the low affinity system was constitutive, it was very efficient during the growth phase and less efficient during the stationary phase (Bisson and Fraenkel, 1984; Bisson, 1988). The high affinity system was repressed by high glucose concentration. The rate of each of this carrier does not seem clear and these carriers are interconvertive depending on the metabolic conditions of the cell. From the above said evidences we can say that there were chances for the competitive inhibition to occur between glucose and glycerol. That's why sugar utilization rate was always lower compared to the other two flasks i.e., glycerol and glucose.

The glucose and glycerol degradation pathway was different. Glycerol degradation occurs via a phosphorylative pathway (Sprague and Cronan, 1977). Glycerol is converted to glycerol-3 phosphate by cytosolic glycerol kinase. Glycerol-3 phosphate then passes the outer mitochondrial membrane and oxidized to dihydroxy acetone phosphate (DHAP) by an inner mitochondrial membrane enzyme, FAD-dependent glycerol-3 phosphate dehydrogenase. DHPA returns to the cytosol where it was catabolized by glycolysis to produce pyruvic acid. Glucose catabolism occurs via EMP pathway (Voet *et al*., 2008). More steps are needed for degradation of glucose than the glycerol break down. The glycerol has highly reduced nature of carbon atoms and conversion of glycerol into the glycolytic intermediate, Phosphoenol Pyruvate (PEP) generate the twice amount of reducing equivalents than that produced by the metabolism of glucose (Syed and Ramon, 2007). This is also one of the reasons for higher rate of ethanol production in glycerol flask since the first two days.

On the 3rd day, ethanol production was high in glucose flask but sugar utilization rate was high in glycerol and glucose: glycerol containing flask rather than only glucose containing flask, the reason being that glucose is also needed for cellular activity which was not fulfilled by glycerol.

Nitrogen is an essential nutrient that is critical for fermentation efficiency and generally become limiting during wine fermentation (Salmon, 1989). The depletion of the nitrogen source, in combination with the rapid turnover of sugar transporters in the stationary phase was thought to be responsible for subsequent reduction in the fermentation rate observed towards the end of fermentation (Lagunas *et al*., 1982). It has been found

that nitrogen deficiency has an impact on the transporter turnover rate and on the expression of at least one transporter, HXT1 (Bisson, 1999). From the study, we find that 6 g L^{-1} of urea concentration was optimum for ethanol fermentation, it produced 152.20 g L^{-1} amount of ethanol compared to 4 g L^{-1} of diammonium hydrogen phosphate producing 96.38 g L^{-1}.

The diammonium hydrogen phosphate is a good nitrogen source whereas urea is a poor nitrogen source for wine fermentation carried out by *S. cerevisiae*. But for *S. cerevisiae* metabolism the better nitrogen source decreased the enzyme level and permeases required for the utilization and uptake of poor nitrogen this mechanism called nitrogen catabolite repression this is the reason urea produced more ethanol rather than diammonium hydrogenphosphate (Schutz and Gafner, 1995). The nitrogen source induced the yeast metabolism, therefore sugar utilization was higher in nitrogen containing media than the control (Alexandre and Charpentier, 1998; Boulton *et al*., 1996).

The amino nitrogen is a very much essential compound for fermentation. It is taken up during the first part of the *S. cerevisiae* growth phase. Biosynthetic pools of amino acids are filled and the remaining nitrogenous compounds are utilized as nitrogen source (Copper, 1982). Nitrogen catabolite repression is attributed to the action of three proteins, GLN3, URE2 and GAP1 (Magasanik, 1992). GAP1, the general amino acid permease that transports all biological amino acid across the plasma membrane (Jauniaux and Grenson, 1990) is regulated at the transcriptional level by GLN3 and URE2 and is inactivated by dephosphorylation in the presence of glutamate and glutamine (Stanbrough and Magasanik, 1995). Proline and arginine are the most abundant amino acid in fruit juice but *S. cerevisiae* is not able to completely utilize these two amino acids during alcoholic fermentation. Derepression for the assimilation of amino acid in URE2 mutant strains of *S. cerevisiae* lead to better amino acid assimilation during alcoholic fermentation. But the results showed that α amino nitrogen utilization rate was high in glucose containing flask and the rate decreased from glycerol to glucose: glycerol containing flask with time. Therefore, it may be concluded that nitrogen utilization was also controlled by main constituent of media i.e., carbon source.

CONCLUSION

In this study, it has been seen that glucose is the main carbon source for producing highest ethanol upto 3rd day of fermentation. In case glycerol, it helps in faster ethanol production than glucose till the 2nd day, therefore glycerol can be used as an inducer of short time fermentation for ethanol production in industry. It is also seen that organic nitrogen is better than inorganic nitrogen in the yeast mediated fermentation.

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