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Growth and Batch Ethanol Fermentation of *Saccharomyces cerevisiae* on Sweet Sorghum Stem Juice under Normal and Very High Gravity Conditions

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Abstract: *Saccharomyces cerevisiae* NP 01 was grown on sweet sorghum stem juice under normal gravity (NG, total sugar of 240 g L^{-1}) and very high gravity (VHG, total sugar of 280 g L^{-1}) conditions. The effects of initial cell concentrations (1×10^6, 1×10^7 and 1×10^8 cells mL^{-1}) and nitrogen supplementation [0.5% $(NH_4)_2SO_4$] on growth and ethanol production were investigated. The experiments were carried out at 30°C in a 2-L fermenter under an agitation rate of 100 rpm. Under both NG and VHG fermentations, the initial cell concentration of 1×10^8 cells mL^{-1} and sweet sorghum stem juice without nitrogen supplementation gave the maximum ethanol production efficiencies. The concentration, productivity and yield of ethanol were 105.12±1.34 g L^{-1}, 2.92±0.04 g L^{-1} h^{-1} and 98.33±1.25% of theoretical yield, respectively at the fermentation time of 36 h under the NG condition. Under the VHG fermentation, the maximum ethanol concentration was not varied from that of the NG conditions, whereas the productivity and yield of ethanol were significantly lower than those of the NG conditions.

Key words: Ethanol fermentation, growth, $(NH_4)_2SO_4$, sweet sorghum stem juice, *Saccharomyces cerevisiae*

INTRODUCTION

Accumulation of carbon dioxide in the atmosphere due to large consumption of petroleum and coal has been recognized as a major factor of global warming and climate change. Bioethanol used as a replacement for gasoline can reduce vehicle carbon dioxide emissions by 90% (Ward and Singh, 2002). An increase use of ethanol as a fuel or a fuel additive, is therefore, one option to help the global warming.

Typically, ethanol fermentation is carried out under normal gravity (NG) conditions (fermentation of mashes containing 20-24% of dissolved solids) (Thomas *et al*., 1996). To increase the productivity and cost effectiveness of ethanol production, many process improvements have been studied including very high gravity (VHG) technology, which is defined as the preparation and fermentation to completion of mashes containing 270 g or more amount of dissolved solids per litre (Bayrock and Ingledew, 2001). This technology has several advantages for industrial applications such as the increase in both the ethanol concentration and the rate of fermentation, which reduce capital costs, energy costs per litre of alcohol and the risk of bacterial contamination (Thomas *et al*., 1996; Bvochora *et al*., 2000; Bayrock and Ingledew, 2001; Bai *et al*., 2004).

Ethanol can be produced from many different raw materials such as corn grain (in USA), sugarcane (in Brazil), tapioca and sugarcane molasses (in Thailand). Sweet sorghum, which is rich in fermentable carbohydrates, has also been an agricultural raw material of interest for biological transformation into ethanol for use as fuel or fuel additive (Schaffert, 1995; Göksungur and Zorlu, 2001; Laopaiboon *et al*., 2007). Sweet sorghum has been promised as a large scale energy crop because it can be cultivated in nearly all temperature and tropical climate areas. It is also often considered to be one of the most drought resistant agricultural crops as it has the capability of remaining dormant during the driest periods.

The ability of yeast to produce ethanol depends on many factors such as strains, growth factors and optimum environmental conditions. Moreover, it depends on the initial sugar concentration in the fermentation medium. To increase ethanol concentration, the higher initial sugar concentration above 200 g L^{-1} can be used. However, the substrate inhibition may be occurred under this condition

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(Thomas *et al*., 1996). In addition, the high ethanol concentration can cause an increased stress to yeast cells, resulting in stuck or sluggish fermentation. However, it was reported that under appropriate environment and nutritional conditions, *Saccharomyces cerevisiae* could produce and tolerate high ethanol concentrations (Thomas *et al*., 1996; Bafrncová *et al*., 1999). The VHG fermentation process exploits the observation that the growth of *S. cerevisiae* is promoted and prolonged when adequate but low levels of oxygen are present and when assimilable nitrogen levels are not limiting (Casey and Ingledew, 1986). Several investigators have observed that yeast extract (Jones *et al*., 1994; Bafrncová *et al*., 1999), ammonium (Jones *et al*., 1994), urea (Jones and Ingledew, 1994a), calcium and magnesium (Dombek and Ingram, 1986) have protective effects either on growth and fermentation or viability, which stimulate the fermentation rate and ethanol production. Thanonkeo *et al*. (2002) found that 0.5% $(NH_4)_2SO_4$ was an optimal concentration for ethanol production from sweet sorghum stem juice strain Keller by *S. cerevisiae* TISTR 5048. According to composition of sweet sorghum stem juice in Table 1, other nutrients may not be required. Laopaiboon *et al*. (2007) also showed that batch ethanol production from sweet sorghum stem juice strain Keller was dependent on the initial cell concentration of *S. cerevisiae* TISTR 5048. In addition, *S. cerevisiae* NP 01 isolated from Long-pang (Chinese yeast cake) for Sato (Thai rice wine) making was a high-ethanol-producing strain under VHG condition (Laopaiboon *et al*., 2008).

The aim of this study was to investigate effects of initial cell concentration of *S. cerevisiae* NP 01 and the absence of $(NH_4)_2SO_4$ as a nitrogen supplement on growth and ethanol production from sweet sorghum stem juice. The ethanol production efficiencies under NG and VHG fermentations were also compared.

Table 1: Characteristics of raw sweet sorghum stem juice cv. KKU40 (Laopaiboon *et al*., 2009)

Characteristics	Values
pH	4.9
Total soluble solid (°Bx)	18
Total sugar (g L^{-1})	173.02
Fermentable nitrogen (mg L^{-1})	626.38
NH_4^+-N (ppm)	21.4
NO_3^--N (ppm)	4.4
Total P (ppm)	20
Total K (ppm)	1790
Total Na (ppm)	170
Total S (ppm)	120
Total Ca (ppm)	166
Total Mg (ppm)	194
Total Fe (ppm)	2
Total Mn (ppm)	3
Total Cu (ppm)	0.3
Total Zn (ppm)	1.4

MATERIALS AND METHODS

Microorganism and inoculum preparation: The fermentative yeast *S. cerevisiae* NP 01 isolated from Long-pang (Chinese yeast cake) from Nakhon Phanom province, Thailand, was grown on yeast and malt extract (YM) medium on a rotating shaker at 100 rpm, 30°C for 18 h. The active cells were then harvested and used as inoculum for ethanol production.

Raw material: Sweet sorghum stem juice (strain KKU 40) was obtained from Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Thailand in Year 2007. After the extraction, the juice was kept at -18°C until use.

Ethanol production medium: Sweet sorghum stem juice containing total soluble solids of 18 °Bx was adjusted with sucrose to give total soluble solids of 24°Bx (NG conditions) and 28°Bx (VHG conditions). The juices were then supplemented with 0.5% $(NH_4)_2SO_4$ (Laopaiboon *et al*., 2007) and used as ethanol production (EP) medium. The EP medium was transferred into a 2 L fermenter (Biostat®B, B. Braun Biotech, Germany) with a final working volume of 1 L and autoclaved at 110°C for 40 min.

Fermentation conditions: To study the effect of initial cell concentration, *S. cerevisiae* NP 01 was inoculated into the sterile EP medium containing various sugar concentrations (NG and VHG conditions). The initial yeast cell concentrations in the medium were approximately 1×10^6, 1×10^7 and 1×10^8 cells mL^{-1}. The fermentation was operated in batch mode at 100 rpm and temperature was controlled at 30°C. Samples were withdrawn at appropriate time intervals for analysis. The optimum cell concentration (giving the highest ethanol production) under NG and VHG conditions was selected for the subsequent experiments.

To study the effect of $(NH_4)_2SO_4$ as a nitrogen supplement on ethanol production from sweet sorghum stem juice, 0.5% $(NH_4)_2SO_4$ was withdrawn from the EP medium under both NG and VHG conditions. The fermentation processes were carried out as previously described. The samples were withdrawn at appropriate time intervals for analysis.

Analytical methods: The viable yeast cell numbers and total soluble solids of the fermentation broth were determined by direct counting method with methylene blue staining using heamacytometer and hand-held refractometer, respectively (Zoecklien *et al*., 1995). The fermentation broth was centrifuged at 13,000 rpm for

10 min. The supernatant was then determined for residual total sugars by a phenol sulfuric acid method (Mecozzi, 2005). Ethanol concentration (P, g L^{-1}) was analyzed by gas chromatography (Shimadzu GC-14B, Japan, Solid phase: polyethylene glycol (PEG-20M), carrier gas: nitrogen, 150°C isothermal packed column, injection temperature 180°C, flame ionization detector temperature 250°C; C-R7 Ae plus Chromatopac Data Processor) and 2-propanol was used as an internal standard (Modified from Laopaiboon *et al*., 2007). The ethanol yield ($Y_{p/s}$) was calculated as the actual ethanol produced and expressed as g ethanol per g total sugar utilized (g g^{-1}). The ethanol productivity (Q_p, g L^{-1} h^{-1}) and the percentage of conversion efficiency or yield efficiency (E_y) were calculated by the following equations:

$$Q_p = \frac{P}{t}$$

and

$$E_y = \frac{Y_{p/s} \times 100}{0.54}$$

where, P is the actual ethanol concentration produced (g L^{-1}), t is the fermentation time (h) giving the highest ethanol concentration for batch and fed-batch fermentations and 0.54 is the maximum theoretical ethanol yield of sucrose consumption.

RESULTS AND DISCUSSION

Effects of initial cell concentrations on yeast growth under NG and VHG conditions: The time profiles of viable yeast cell number at various initial cell concentrations during batch fermentation on the EP medium under NG and VHG conditions are shown in Fig. 1a and b, respectively. No lag phase was observed after *S. cerevisiae* NP 01 cells were inoculated into the EP medium at all experimental conditions. This finding indicated that the sweet sorghum stem juice did not contain any substance at a level that was inhibitory to the yeast cells and the yeast cells had excellent adaptation to new environment. Under both NG and VHG conditions, stationary phase occurred at 12 to 24 h of the cultivation depending on the initial cell concentrations. The higher initial cell concentration was the earlier stationary phase reached. Yeast cell numbers at the initial cell concentration of 1×10^8 cells mL^{-1} did not increase significantly after 6 h of the experiments. Cell concentrations at all conditions were similar within 24 h of the experiment with the value of log 8.36±0.04 cells mL^{-1} and they remained constant throughout the experiments.

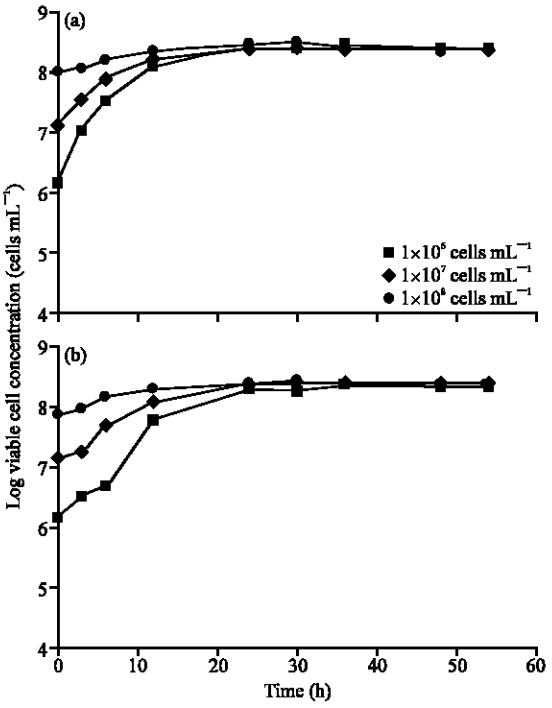

Fig. 1: Growth curve of *S. cerevisiae* NP 01 in the EP medium under (a) NG and (b) VHG conditions at various initial cell concentrations

Table 2: Maximum specific growth rate (μ_{max}) of *S. cerevisiae* NP 01 in the EP medium under NG and VHG conditions at various initial cell concentrations

NG/VHG	Initial cell concentrations (cells mL^{-1})	μ_{max} (h^{-1})*
NG	1.45×10^6	0.36±0.02[a]
	1.32×10^7	0.15±0.02[b]
	0.95×10^8	0.08±0.02[c]
VHG	1.50×10^6	0.31±0.04[a]
	1.45×10^7	0.19±0.02[b]
	1.45×10^8	0.08±0.03[c]

*The results were expressed as mean±the range of duplicate experiments. Means followed by the same letter within a same column are not significantly different using Duncan's multiple range test at the level of 0.05

The maximum specific growth rates (μ_{max}) at various initial cell concentrations are illustrated in Table 2. Under both NG and VHG conditions, μ_{max} were decreased with the increase in the initial cell concentrations and μ_{max} were similar at the same initial cell concentrations regardless of the initial sugar concentrations tested. The results indicated that the sugar level under NG and VHG conditions at concentrations up to 280 g L^{-1} did not affect on microbial growth and the tested initial cell numbers did not cause any adverse effect on final cell numbers.

Effects of initial cell concentrations on ethanol production under NG and VHG conditions: Figure 2 shows the time profiles of residual total sugar, total soluble solids, pH and ethanol concentration during batch

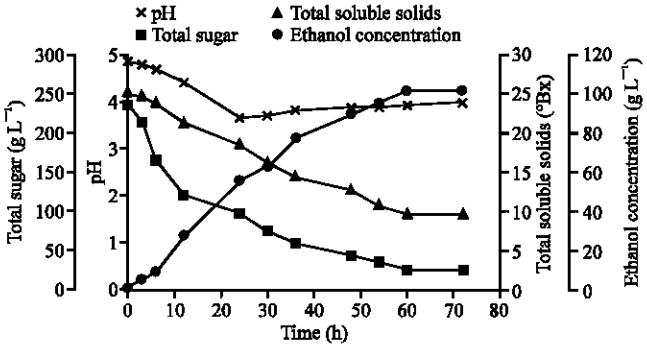

Fig. 2: Batch culture profile of ethanol production at initial cell concentrations of 1×10^6 cells mL^{-1} from the EP medium under NG condition

fermentation from the EP medium at the initial cell concentration of 1×10^6 cells mL^{-1} under NG fermentation. The total sugar and total soluble solids at the beginning of the fermentation were 235.37 g L^{-1} and 25.0°Bx, respectively. The values were decreased against time, while ethanol concentration in the broth was increased until 60 h of the fermentation. The pH value was decreased within 24 h and then relatively constant at about 4.0 throughout the experiment. This might be due to carbon dioxide production by *S. cerevisiae* NP 01 during fermentation. Carbon dioxide was able to dissolve in fermentation broth, became to carbonic acid and changed to ion carbonate and proton. Therefore, pH of the fermentation broth was constant (Shen *et al*., 2004). Changes of total sugar, total soluble solids, pH and ethanol concentration at the other initial cell concentrations under NG and VHG conditions were similar to those found in Fig. 2 (data not shown). Figure 3a and 3b compare sugar consumption and ethanol production at all tested conditions. Under NG and VHG conditions, the amount of total sugar remaining in the broth at the end of the fermentation was approximately 20 and 70 g L^{-1}, respectively at all initial cell concentrations, while ethanol was produced swiftly at the highest initial cell concentration. However, the final ethanol concentration at the highest initial cell concentration was closed to those of 1×10^6 and 1×10^7 cells mL^{-1}. The results also showed that the initial cell concentration affected fermentation time giving the highest ethanol concentration. The fermentation time at the lower initial cell concentration was longer than that of the higher initial cell concentration under both NG and VHG conditions.

The stuck fermentation was clearly observed especially under the VHG conditions. This might be due to thermal stress as described by Jones and Ingledew (1994b). They found that the amount of sugar that could be fermented decreasing when fermentation temperature

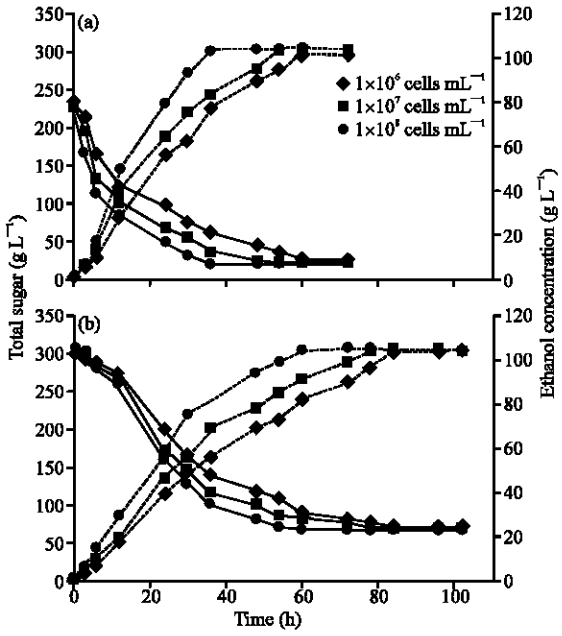

Fig. 3: Changes of total sugar (—) and ethanol concentration (----) during batch ethanol fermentation from in the EP medium under (a) NG and (b) VHG conditions at various initial cell concentrations

was above 25°C. However, lower temperature might cause lower ethanol productivity. This was supported by Bai *et al*. (2008) who reported that the negative impact of high temperature on ethanol fermentation performance was much worse under the VHG conditions than the regular fermentation.

Table 3 shows the important kinetic parameters (P, Q_p and $Y_{p/s}$) of ethanol fermentation under various conditions. The results showed that under NG conditions, final ethanol concentration and ethanol yield at the initial

Table 3: Kinetic parameters of batch ethanol production from the EP medium under NG and VHG conditions at various initial cell concentrations

		Parameters*				
NG/VHG	Initial cell concentrations (cells mL^{-1})	P (g L^{-1})	Q_p (g L^{-1} h^{-1})	$Y_{p/s}$ (g g^{-1})	E_y (%)	t (h)
NG	1.45×10^6	101.33±0.30[a]	1.69±0.01[a]	0.48±0.00[a]	89.33±0.34[a]	60
	1.32×10^7	103.62±0.40[b, c]	1.92±0.02[b]	0.50±0.00[b]	91.71±0.66[b]	54
	0.95×10^8	103.03±1.23[b, c]	2.86±0.03[c]	0.50±0.01[b]	92.59±1.09[b]	36
VHG	1.50×10^6	103.47±0.42[b]	1.23±0.01[d]	0.46±0.00[c]	85.18±1.22[c]	84
	1.45×10^7	104.43±0.52[c]	1.34±0.02[e]	0.45±0.00[d]	83.33±0.78[c]	78
	1.45×10^8	104.33±0.44[b, c]	1.74±0.01[f]	0.44±0.00[e]	81.48±0.63[d]	60

*P: Ethanol concentration; Q_p: Ethanol productivity; $Y_{p/s}$: Ethanol yield; E_y: Yield efficiency and t: Fermentation time. The results were expressed as mean±the range of duplicate experiments. Means followed by the same letter within a same column are not significantly different using Duncan's multiple range test at the level of 0.05

cell concentrations of 1×10^7 and 1×10^8 cells mL^{-1} were not significantly different and were slightly higher than those of 1×10^6 cells mL^{-1}. The initial cell at 1×10^8 cells mL^{-1} gave the maximum productivity with the value of 2.86±0.03 g L^{-1} h^{-1}. The value enhanced approximately 69 and 49% of those at the initial cell of 1×10^6 and 1×10^7 cells mL^{-1}, respectively. Higher ethanol productivity was mainly due to shorter fermentation time. According to the ethanol productivity (Table 3), the initial cell at 1×10^8 cells mL^{-1} was suitable for ethanol production from sweet sorghum stem juice under NG condition.

Under the VHG conditions, the initial cell at 1×10^8 cells mL^{-1} also gave the maximum ethanol productivity with the value of 1.74±0.01 g L^{-1} h^{-1}, which was approximately 42 and 30% higher than those of the initial cell at 1×10^6 and 1×10^7 cells mL^{-1}, respectively. However, the initial cell concentration did not affect on final ethanol concentration and ethanol yield under the VHG conditions. When compared to the NG conditions, the final ethanol concentrations under both conditions were similar but the ethanol yields under the VHG conditions were lower than those under the NG conditions, indicating that more byproducts might be produced under the VHG conditions. In addition, the ethanol productivities at the VHG levels were lower than those at the NG levels when compared at the same initial cell concentrations (Table 3), implying that substrate inhibition was occurred to some extent under the VHG conditions.

Effects of $(NH_4)_2SO_4$ supplementation on ethanol production from the EP medium under NG and VHG conditions: In our previous study (Thanonkeo *et al*., 2002), the EP medium (sweet sorghum stem juice) was supplemented with 0.5% $(NH_4)_2SO_4$. However, the sweet sorghum strain Keller (used in Thanonkeo *et al*., 2002) had been improved to be a new cultivar with higher sugar yield, namely strain KKU 40 (used in this research). It is possible that $(NH_4)_2SO_4$ supplementation as a nitrogen supplement in the sweet sorghum stem juice may not be necessary for ethanol production.

Comparison of the profiles of the total sugar, viable cells and ethanol during batch ethanol fermentation from the EP medium with and without $(NH_4)_2SO_4$ supplementation under the NG and VHG conditions at the initial cell concentrations of 1×10^8 cells mL^{-1} are shown in Fig. 4a and 4b, respectively. The rate of sugar consumption by *S. cerevisiae* NP 01 from the juice supplemented with $(NH_4)_2SO_4$ were slightly higher than that of the juice without $(NH_4)_2SO_4$ under the NG conditions (Fig. 4a). Sugar and ethanol concentrations were constant at 36 h in both media. However, the sugar remaining in the absence of $(NH_4)_2SO_4$ was approximately 15 g L^{-1} higher than that in the presence of $(NH_4)_2SO_4$. The profiles of viable cell and ethanol production in the two media were similar throughout the experiments.

Under the VHG conditions, total sugar and ethanol concentration remained constant at 60 h. Changes of total sugar, viable cell and ethanol concentrations during VHG fermentation under the presence and absence of $(NH_4)_2SO_4$ were similar.

Table 4 summarizes the important kinetic parameters of batch ethanol production from the juice with and without $(NH_4)_2SO_4$ supplementation under the NG and VHG conditions at the initial cell concentrations of 1×10^8 cells mL^{-1}. The results showed that ethanol concentration and ethanol productivity in the presence and absence of $(NH_4)_2SO_4$ were similar, while ethanol yield in the presence of extra nitrogen source was lower under both NG and VHG conditions. The results obtained were consistent with those of Laopaiboon *et al*. (2009). Negative effect of $(NH_4)_2SO_4$ on ethanol yield might be due to more byproducts occurred. Some studies also reported that excessive ammonium addition might cause the increase in higher alcohols (Beltran *et al*., 2005), acetic acid (Bely *et al*., 2003) or hydrogen sulphide (Wang *et al*., 2003). However, the byproducts were not monitored in our experiment. The results indicated that $(NH_4)_2SO_4$ seems not be a suitable nitrogen source for ethanol production from sweet sorghum stem juice by *S. cerevisiae* NP 01 and/or the juice may contain enough nitrogen source for ethanol production. Opposite results were observed by Nahvi *et al*. (2002), Ruanglek *et al*. (2006), Xu and Liu

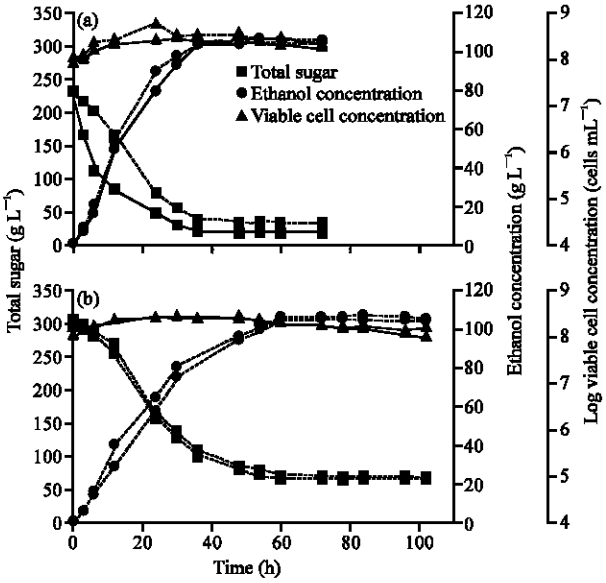

Fig. 4: Total sugar consumption and ethanol production during ethanol fermentation from the EP medium under (a) NG and (b) VHG conditions supplemented (—) and non-supplemented (----) with 0.5% $(NH_4)_2SO_4$

Table 4: Kinetic parameters of batch ethanol production from the EP medium with and without $(NH_4)_2SO_4$ supplementation under NG and VHG conditions

		Parameters*				
NG/VHG	EP medium	P (g L^{-1})	Q_p (g L^{-1} h^{-1})	$Y_{p/s}$ (g g^{-1})	E_y (%)	t (h)
NG	with $(NH_4)_2SO_4$	103.03±1.23^a	2.86±0.03^a	0.50±0.01^a	92.59±1.09^a	36
	without $(NH_4)_2SO_4$	105.12±1.34^b	2.92±0.04^b	0.53±0.01^b	98.33±1.25^b	36
VHG	with $(NH_4)_2SO_4$	104.33±0.44^{a,b}	1.74±0.01^c	0.44±0.00^c	81.48±0.63^c	60
	without $(NH_4)_2SO_4$	105.88±0.63^b	1.76±0.01^c	0.47±0.00^d	86.69±0.30^d	60

*P: Ethanol concentration; Q_p: Ethanol productivity; $Y_{p/s}$: Ethanol yield; E_y: Yield efficiency and t: Fermentation time. The results were expressed as mean±the range of duplicate experiments. Means followed by the same letter within a same column are not significantly different using Duncan's multiple range test at the level of 0.05

(2009) who found that $(NH_4)_2SO_4$ promoted growth and/or ethanol production. The different results might be due to the difference in raw materials, microorganisms and fermentation conditions.

The results obtained in this study implied that *S. cerevisiae* NP 01 was a suitable microorganism for ethanol production because the yeast could survive and retain its metabolism under very high ethanol concentration up to 105.88 g L^{-1} (13.40 % v/v) with the viable cells remaining approximately 2.04×10^8 cells mL^{-1} at the end of the fermentation.

CONCLUSIONS

The results obtained from this research demonstrated that the initial yeast cell concentration affected on ethanol production efficiency in terms of ethanol productivity. The VHG ethanol fermentation from sweet sorghum stem juice without nutrient supplementation could not improve ethanol production efficiency. In addition, $(NH_4)_2SO_4$ might not be suitable for use as a nitrogen supplement in sweet sorghum stem juice because it did not promote ethanol production under both NG and VHG levels. Supplementation with other nitrogen sources and/or other nutrients as well as other fermentation processes should be further investigated to improve ethanol productivity and complete sugar consumption under NG and VGH conditions.

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