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The use of dried spent yeast as a low-cost nitrogen supplement in ethanol fermentation from sweet sorghum juice under very high gravity conditions

Worawut Sridee[1] · Lakkana Laopaiboon[2,3] ✉ · Prasit Jaisil[4] · Pattana Laopaiboon[2]

1 Khon Kaen University, Graduate School, Khon Kaen, Thailand

2 Khon Kaen University, Faculty of Technology, Department of Biotechnology, Khon Kaen, Thailand

3 Khon Kaen University, Fermentation Research Center for Value Added Agricultural Products, Khon Kaen, Thailand

4 Khon Kaen University, Faculty of Agriculture, Department of Plant Science and Agricultural Resources, Khon Kaen, Thailand

✉ Corresponding author: lakcha@kku.ac.th
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Abstract Dried spent yeast (DSY) was used as a low-cost nitrogen supplement for ethanol fermentation from sweet sorghum juice under very high gravity (VHG) conditions by *Saccharomyces cerevisiae* NP 01. The fermentation was carried out at 30ºC in a 5-litre bioreactor. The results showed that DSY promoted ethanol production efficiencies. The ethanol concentration (P), productivity (Q_p) and yield ($Y_{p/s}$) of the sterile juice (total sugar of 280 g l^{-1}) supplemented with 8 g l^{-1} of DSY were not different from those supplemented with yeast extract and/or peptone at the same amount. The initial yeast cell concentration of 5 x 10^7 cells ml^{-1} was found to be optimal for scale-up ethanol production. In addition, an increase in sugar concentration in inoculum preparation medium (from 10 to 100 g l^{-1}) improved the ability of the inoculum to produce ethanol under the VHG conditions. When *S. cerevisiae* NP 01 grown in the juice containing 100 g l^{-1} of total sugar was used as the inoculum for ethanol fermentation, the P, Q_p and $Y_{p/s}$ obtained were 108.98 ± 1.16 g l^{-1}, 2.27 ± 0.06 g l^{-1} h^{-1} and 0.47 ± 0.01 g g^{-1}, respectively. Similar results were also observed when the ethanol fermentation was scaled up to a 50-litre bioreactor under the same conditions. The cost of the sweet sorghum for ethanol production was US$ 0.63 per litre of ethanol. These results clearly indicate the high potential of using sweet sorghum juice supplemented with DSY under VHG fermentation for ethanol production in industrial applications.

Keywords: bioethanol, dried spent yeast, scale-up, sweet sorghum juice, very high gravity fermentation

INTRODUCTION

Ethanol is an excellent transportation fuel which can be used as blend with gasoline. Ten and 20% blends are widely being used in Thailand and 85% blend is sold in some petrol stations in Bangkok, Thailand. Ethanol can be produced from biomass, a plentiful renewable resource. In Thailand, the main raw materials used for ethanol production are sugarcane molasses and cassava. Regarding the energy policy of Thai government, ethanol production will be increased to 3,000,000 litres day^{-1} in year 2012 and to 9,000,000 litres day^{-1} in year 2022 (Department of Energy Business, 2010). Therefore, it is possible that Thailand may face a shortage of sugarcane molasses and cassava.

Sweet sorghum (*Sorghum biocolor* (L.) Moench), a high biomass and sugar-yielding crops, has been of a particular interest as a substrate for ethanol production because its stalks contain high fermentable sugars, mainly sucrose and also fructose and glucose (Gnansounou et al. 2005; Sakellaviou-Makrantonaki et al. 2007; Laopaiboon et al. 2009; Wu et al. 2010). Moreover, it can be cultivated at

nearly all temperatures and tropical climate areas with the growing period of 120-150 days (Wu et al. 2010). Sorghum stalks are ideal for ethanol production, as the ethanol from sorghum is significantly cleaner than that from sugarcane (Prasad et al. 2007). In Thailand, the average yield of sweet sorghum cultivar KKU40, 90-100 days old, is 30-50 ton ha^{-1} corresponding to about 15-25 dry ton ha^{-1} (Jaisil et al. 2009). This yield is comparable to that (20-30 dry ton ha^{-1}) reported by Wu et al. (2010).

Improvement of ethanol production has been continuously investigated. A very high gravity (VHG) technology is one of the methods to enhance the ethanol production efficiency. It is defined as the preparation and fermentation to completion of medium containing sugar in excess of 250 g l^{-1} (Bai et al. 2008). 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 (Bvochorá et al. 2000; Bayrock and Ingledew, 2001; Bai et al. 2008). However, the substrate inhibition may occur under initial high sugar levels (Ingledew, 1999). In addition, the high ethanol concentration can cause an increase in the stress on yeast cells resulting in stuck or sluggish fermentation (Bai et al. 2004).

Table 1. Some nutrients and trace elements in yeast extract, peptone (HiMedia laboratory, India) and dried spent yeast (DSY) from Beerthip Brewery (1991) Co., Ltd., Bang Baan, Phra Nakhon Sri Ayutthaya, Thailand.

Constituents (%)	Yeast extract	Peptone	DSY
Total N[a]	10.65	11.49	6.44
Total P[b]	1.15	0.80	1.25
Total K[c]	4.96	1.41	1.59
Total Na[c]	0.82	2.99	0.34
Total Ca[d]	0.024	0.013	0.067
Total Mg[d]	0.054	0.014	0.174
Total Fe[d]	0.0052	0.0102	0.0081
Total Mn[d]	ND*	ND*	ND*
Total Cu[d]	ND*	ND*	ND*
Total Zn[d]	0.0087	0.0040	0.0044
Total Cl[e]	0.25	2.70	0.11
Total S[f]	0.35	1.00	0.35

[a] by Kjeldahl method; [b] Wet digestion (HNO_3:$HClO_4$) & Vanado molybdate method; [c] Wet digestion (HNO_3:HCl O_4) & Flame photometry method; [d] Wet digestion (HNO_3:HCl O_4) & Atomic absorption spectrophotometry method; [e] Dry ashing & $AgNO_3$ titration method; [f] Wet digestion (HNO_3:$HClO_4$) & Turbidimetric method. ND*: not detectable.

In general, *Saccharomyces cerevisiae* is used for commercial ethanol production. Our previous study showed that *S. cerevisiae* NP01 isolated from Loog-pang (Chinese yeast cake) for Sato (Thai rice wine) making was a high-ethanol-producing strain under VHG conditions (Laopaiboon et al. 2008). Under appropriate environmental and nutritional conditions, *S. cerevisiae* can produce and tolerate high ethanol concentrations (Bai et al. 2008). VHG technology exploits the observation that growth of *Saccharomyces* yeast is promoted and prolonged when adequate but very low levels of oxygen are present and when assimilable nitrogen levels are not limiting (Yue et al. 2010).

Our previous studies showed that sweet sorghum juice strain KKU40 was an optimal substrate for ethanol production. pH adjustment was not required, only nitrogen supplementation was essential under VHG fermentation to improve ethanol production efficiency (Laopaiboon et al. 2009; Nuanpeng et al. 2011). Nitrogen sources that are widely used to stimulate fuel alcohol, brewing, or winery fermentations are yeast extract and peptone (Bafrncová et al. 1999; Bvochorá et al. 2000; Reddy and Reddy, 2006; Laopaiboon et al. 2009), ammonium (Laopaiboon et al. 2007; Srichuwong et al. 2009) and urea (Yue et al. 2010). They are employed to increase yeast growth or viability and the rate of sugar utilization, and to reduce the fermentation time. However, several investigators reported negative effects of using ammonium and urea as nitrogen supplements in ethanol fermentation (Zoecklein et al. 1995; Bely et al. 2003; Wang et al. 2003; Beltran et al. 2005; Laopaiboon et al. 2009). Yeast extract and peptone are nitrogen supplements, which are the most widely used in a laboratory scale. Due to

high cost, they are not feasible for routine use in the industrial manufacture of a low profit margin, ethanol.

Low-cost nitrogen sources for replacement of yeast extract and peptone have been continuously investigated. These include finger millet (*Eleusine coracana L*) flour (Reddy and Reddy, 2006), corn steep liquor (Pereira et al. 2010) and fresh yeast autolysate (Jones and Ingledew, 1994), which are used for improvement of yeast growth and ethanol production under VHG fermentation. Brewer's yeast biomass is the second major by-product from brewery industry (after brewer spent grain); however, its use is still limited, being basically used as animal feed (Ferreira et al. 2010). Our preliminary study showed that dried spent yeast (DSY), a by-product from Beerthip Brewery, Thailand, contained high nitrogen and many essential mineral salts (Table 1). Therefore, it may be used as a low-cost nitrogen supplement for ethanol production from sweet sorghum juice instead of yeast extract and peptone.

The aim of this study was to compare the efficiency of ethanol production under VHG fermentation from sweet sorghum juice supplemented with DSY or yeast extract and peptone as nitrogen supplements. The optimum initial yeast cell concentration for scale-up ethanol production was determined, and the effects of the initial sugar concentration in inoculum preparation medium on yeast growth and ethanol production was investigated. The ethanol fermentation under non-sterilized condition and cost estimation of ethanol production by the sweet sorghum juice were also undertaken.

MATERIALS AND METHODS

Microorganism and inoculum preparation

S. cerevisiae NP01 isolated from Loog-pang (Chinese yeast cake) from Nakorn Panom province, Thailand, was inoculated into a 250-ml Erlenmeyer flask containing 150 ml of yeast extract and malt extract (YM) medium. The medium contained yeast extract, 3 g l^{-1}; peptone, 5 g l^{-1}; malt extract, 3 g l^{-1} and glucose, 10 g l^{-1} (Melzoch et al. 1994). The flask was incubated on a rotating shaker at 150 rev min^{-1}, 30ºC for 24 hrs. The yeast suspension (approximately 3%) was transferred into a 500-ml Erlenmeyer flask with 350 ml of the YM medium to give the initial cell concentration of approximately 5 x 10^6 cells ml^{-1}. The flasks were further incubated under the same conditions. After 15 hrs, the cells were harvested and used as an inoculum for ethanol production.

Raw materials

Mature sweet sorghum (cv. KKU40), approximately 100 days old, was harvested and the sweet sorghum stalks were squeezed to obtain the juice. The juice containing the total soluble solids of 18ºBx was concentrated to 75ºBx by direct heating. The concentrated juice was stored at 4ºC until use.

Dried spent yeast (DSY) obtained from Beerthip Brewery (1991) Co., Ltd., Bang Baan, Phra Nakhon Sri Ayutthaya, Thailand was kept at room temperature until use.

Ethanol production medium

The concentrated sweet sorghum juice was diluted with distilled water to a desired total sugar concentration (280 g l^{-1}) and used as an ethanol production (EP) medium.

Experiments

1. Composition of nutrient supplement. The main composition of yeast extract, peptone and DSY was analysed by Central Laboratory (Thailand) Co., Ltd., Khon Kaen, Thailand.

2. Effects of nitrogen sources. The EP medium was supplemented with YEP (3 g l^{-1} of yeast extract and 5 g l^{-1} of peptone) (Melzoch et al. 1994), YE (8 g l^{-1} of yeast extract), DSY8 (8 g l^{-1} of DSY) or DSY16 (16 g l^{-1} of DSY). The medium was transferred into a 5-litre stirred tank bioreactor (Biostat®B, B. Braun Biotech, Germany) with a final working volume of 3.5 litres and autoclaved at 110ºC for 60 min. *S. cerevisiae* was inoculated into the sterile five EP media (no supplement, YEP, YE, DSY8 and

DSY16) to give the initial cell concentration of approximately 1 x 10^8 cells ml^{-1}. The fermentation was carried out in batch mode at 30ºC and the agitation rate of 100 rev min^{-1}. The samples were collected at time intervals for further analysis.

3. Effects of initial cell concentrations. The optimum EP medium for ethanol production from Experiment 2 was prepared and transferred into the 5-litre bioreactor and autoclaved. The yeasts were inoculated into the sterile EP medium to give the initial cell concentration of approximately 1.0 x 10^7, 5.0 x 10^7 and 1.0 x 10^8 cells ml^{-1}. The fermentation was carried out as described above.

4. Effects of initial sugar concentrations in inoculum preparation medium. The YM medium, which was used as the inoculum preparation (IP) medium, was modified by increasing glucose concentration from 10 g l^{-1} to 50 and 100 g l^{-1}. The sweet sorghum juice containing 100 g l^{-1} of total sugar was also prepared and used as one of the IP media. The yeast suspension (approximately 3%) was inoculated into different IP media. After 15 hrs, the cells were harvested and inoculated into the sterile EP medium supplemented with DSY at the optimum concentration (from Experiment 2). The ethanol fermentation was carried out as described above.

5. Effects of EP medium sterilization. The optimum EP medium for ethanol production from Experiment 2 was prepared in the bioreactor without sterilization. *S. cerevisiae* NP01 grown in the optimum IP medium (from Experiment 4) were inoculated into the non-sterile EP medium. The initial cell concentration in the EP medium was selected from the results of Experiment 3. The fermentation was carried out as described above. Ethanol production from the sterile EP medium under the same conditions was also performed as the control treatment. The total plate counts of bacteria and acid bacteria in the non-sterile EP medium on nutrient agar with 0.1% (w/v) of $NaHCO_3$ and on de Man, Rogosa and Sharpe (MRS) agar, respectively were also determined during the ethanol fermentation (Zoecklein et al. 1995).

6. Scale-up ethanol production. The optimum EP medium for ethanol production was prepared in a 50-litre stirred tank bioreactor with a final working volume of 35 litres. The EP medium was sterilized at 90ºC for 30 min. After it was cooled down to 30ºC, *S. cerevisiae* NP01 (from Experiment 4) were inoculated. The fermentation conditions were the same as those in the 5-litre bioreactor.

Analytical methods

The viable yeast cell numbers and total soluble solids of the fermentation broth were determined by direct counting method using haemacytometer and hand-held refractometer, respectively (Zoecklein et al. 1995). The fermentation broth was centrifuged at 13,000 rev min^{-1} for 10 min. The supernatant was then determined for residual total sugar in terms of total carbohydrate by 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, 150ºC isothermal packed column, injection temperature 180ºC, flame ionization detector temperature 250ºC; C-R7 Ae plus Chromatopac Data Processor) and 2-propanol was used as an internal standard. The ethanol yield ($Y_{p/s}$, g g^{-1}) and volumetric ethanol productivity (Q_p, g l^{-1} h^{-1}) were calculated as previously described (Laopaiboon et al. 2009).

RESULTS AND DISCUSSION

Composition and cost of nitrogen sources

The appearance of yeast extract, peptone and DSY was similar (Figure not shown). Their main compositions are shown in Table 1. Total nitrogen content in DSY was about 60% of that in yeast extract and peptone. Sodium and chloride contents were much lower than those of yeast extract and peptone, whereas calcium and magnesium contents were higher. Other components detected in DSY were comparable with those of either yeast extract or peptone. In Thailand, the cost of yeast extract (HiMedia laboratory, India), peptone (HiMedia laboratory, India) and DSY (Beerthip Brewery (1991), Co., Ltd.) was approximately US$ 67, 83 and 0.7 /kg, respectively.

Effects of nitrogen sources on ethanol production

The time profiles of residual total sugar, viable yeast cell number, ethanol concentration and pH during the batch fermentation of *S. cerevisiae* NP01 from the sweet sorghum juice (total sugar concentration of 280 g l^{-1}) and the EP medium supplemented with YEP, YE, DSY8 and DSY16 are shown in Figure 1. Ethanol production from the EP medium without nitrogen supplement was also carried out as a control treatment. The changes of all parameters measured were similar among the different nitrogen sources. The sugar consumption in the control treatment was the lowest while those of other four treatments were similar (Figure 1a). The total sugar remaining in the control was 37.36 g l^{-1}, while it ranged from 16.00 to 21.80 g l^{-1} in other treatments. It was found that YE and DSY supplementation did not promote cell growth in the EP medium (Figure 1b). The yeast cell concentrations in the EP media supplemented with YE and DSY were similar to that of the control EP medium. The yeast cell concentrations in all EP media increased in the first 24 hrs and were relatively constant throughout the experiments. Slightly higher cell growth was observed in the EP medium containing YEP, indicating that the addition of peptone stimulated cell growth. Ethanol concentrations produced in the five EP media were also similar in the first 24 hrs (Figure 1c). After that, ethanol concentration in the control treatment was the lowest, while those of other EP media were similar. pHs of the EP media during fermentation were slightly decreased and were not different among the treatments (Figure 1d).

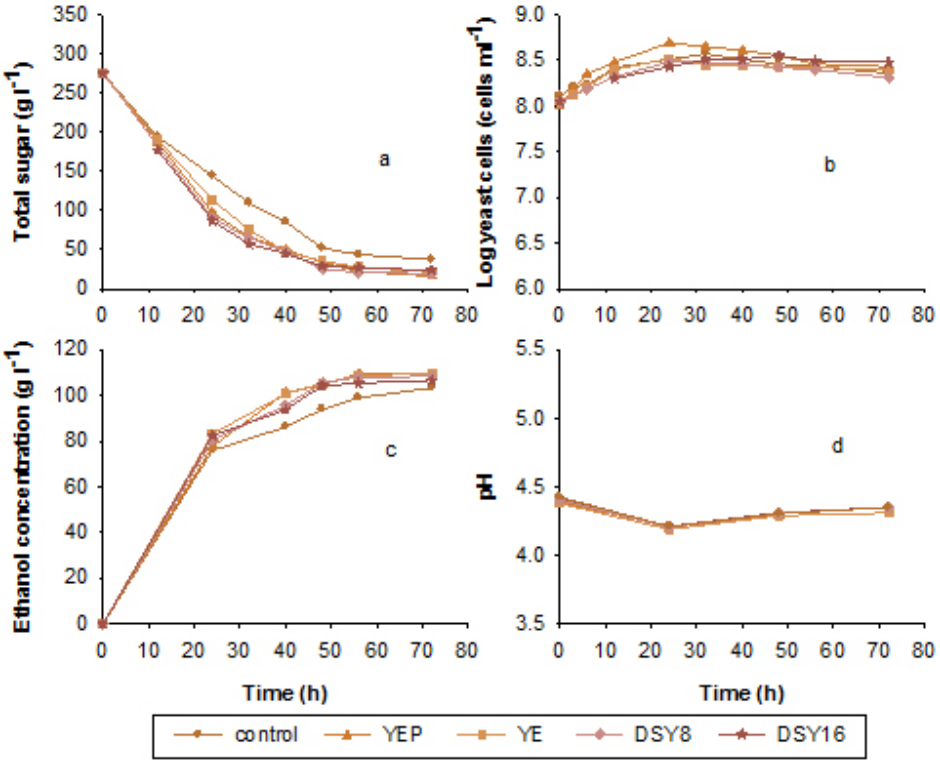

Fig. 1 Fermentation kinetics during batch ethanol production from the sweet sorghum juice containing 280 g l^{-1} of total sugar under various nitrogen supplements by *S. cerevisiae* NP 01. (a) total sugar, (b) log yeast cells, (c) ethanol concentration and (d) pH.

Bafrncová et al. (1999) found that yeast extract had a protective effect either on growth and fermentation or on viability during VGH ethanol fermentation from a synthetic medium containing 300 g l^{-1} of glucose, leading to an increase in the fermentation rate and ethanol production. In addition, Stewart et al. (1988) reported that when the concentrations of peptone and yeast extract in the VHG medium were increased, yeast could better tolerate to osmotic pressure and high temperature. However, in our study, yeast extract, peptone and DSY could stimulate ethanol fermentation but only peptone promoted growth in the sweet sorghum juice. The dissimilar results might be due to the

differences in the raw materials as well as the initial sugar concentration in the ethanol production medium.

It was found that nutrient supplementation was essential for ethanol production from sweet sorghum juice under the VHG conditions (Table 2). At 48 hrs of the fermentation, the ethanol concentration or *P* of the control was the lowest. The *P* values were increased in the EP media with nutrient supplements and ranged from 104.24 to 105.46 g l^{-1}, resulting in similar ethanol productivity or Q_p in the four EP media. Ethanol yields or $Y_{p/s}$ of the EP media supplemented with YEP, YE and DSY were also similar in the range of 0.44 to 0.45, while $Y_{p/s}$ of the control was only 0.42 indicating that more by-products were produced in the control EP medium.

Table 2. Kinetic parameters of batch ethanol production from the sweet sorghum juice containing total sugar of 280 g l^{-1} under various nitrogen supplements.

Nitrogen sources [a]	Parameters (mean ± SD)[b]			
	P (g l^{-1})	Q_p (g $l^{-1}h^{-1}$)	$Y_{p/s}$ (g g^{-1})	*t* (hr)
None	93.90 ± 1.21	1.96 ± 0.03	0.42 ± 0.01	48
YEP	104.60 ± 0.05	2.18 ± 0.00	0.44 ± 0.01	48
YE	105.22 ± 1.90	2.19 ± 0.03	0.45 ± 0.02	48
DSY8	105.46 ± 1.44	2.20 ± 0.02	0.44 ± 0.01	48
DSY16	104.24 ± 0.96	2.17 ± 0.03	0.44 ± 0.01	48

[a] YEP = 3 g l^{-1} of yeast extract and 5 g l^{-1} of peptone; YE = 8 g l^{-1} of yeast extract; DSY8 = 8 g l^{-1} of dried spent yeast; and DSY16 = 16 g l^{-1} of dried spent yeast. [b] *P* = ethanol concentration; Q_p = ethanol productivity; $Y_{p/s}$ = ethanol yield; *t* = fermentation time. * The experiments were performed in duplicate.

After 48 hrs, the *P* values were gradually increased in the control EP medium and the highest *P* value of 103.40 g l^{-1} was obtained at the end of the fermentation (72 hrs) (Figure 1c). This value was close to those of other EP media but the fermentation time giving the highest *P* value in the control EP medium was about 24 hrs longer than those of other EP media. These findings indicate that the nitrogen supplements stimulate the rate of ethanol production under the VHG fermentations.

YEP, YE and DSY at the same amount (8 g l^{-1}) promoted sugar consumption and ethanol production from the EP medium or the sweet sorghum juice and the ethanol production efficiencies were not different. The increase in DSY concentration might improve the ethanol production efficiencies. However, when DSY concentration in the EP medium was increased from 8 to 16 g l^{-1}, *P*, Q_p and $Y_{p/s}$ values of the two EP media were not different (Table 2). Similar results were observed by Jones and Ingledew (1994) who used fresh yeast autolysate as a source of extra free amino nitrogen (FAN). They found that the fresh yeast autolysate significantly accelerated the rates of sugar utilization and ethanol production from wheat mash under VHG fermentation conditions. Therefore, DSY at 8 g l^{-1} would be used as a low-cost nitrogen supplement in the subsequent experiments.

Effects of initial cell concentration on ethanol production

In the present study, *S. cerevisiae* NP01 at the initial concentration of 1 x 10^8 cells ml^{-1} in the EP medium could grow well in the first 24 hrs of the fermentation (Figure 1). However, in a large scale bioreactor, it was difficult to prepare high initial cell concentrations up to 1 x 10^8 cell ml^{-1} (except using in the form of active dried yeast or pressed yeast) (Siqueira et al. 2008). Therefore, lower initial cell concentration (lower than 1 x 10^8 cells ml^{-1}) might be used to reduce the cost of inoculum preparation. In this study, the initial cell concentrations for ethanol production were approximately 1 x 10^7, 5 x 10^7 and 1 x 10^8 cells ml^{-1}. The profiles of total sugar, viable yeast cell numbers, ethanol concentration and pH during the batch fermentation from the juice supplemented with 8 g l^{-1} of DSY at the different initial cell concentrations were similar (Figure 2). The sugar consumption rate at the initial cell at 1 x 10^8 cells ml^{-1} was higher than those at 5 x 10^7 and 1 x 10^7 cells ml^{-1}, respectively (Figure 2a). This indicated that the sugar consumption rate depended on the initial cell concentration. However, at the end of the fermentations, the total sugar remaining in all treatments was similar ranging from 19.74 to 29.84 g l^{-1}.

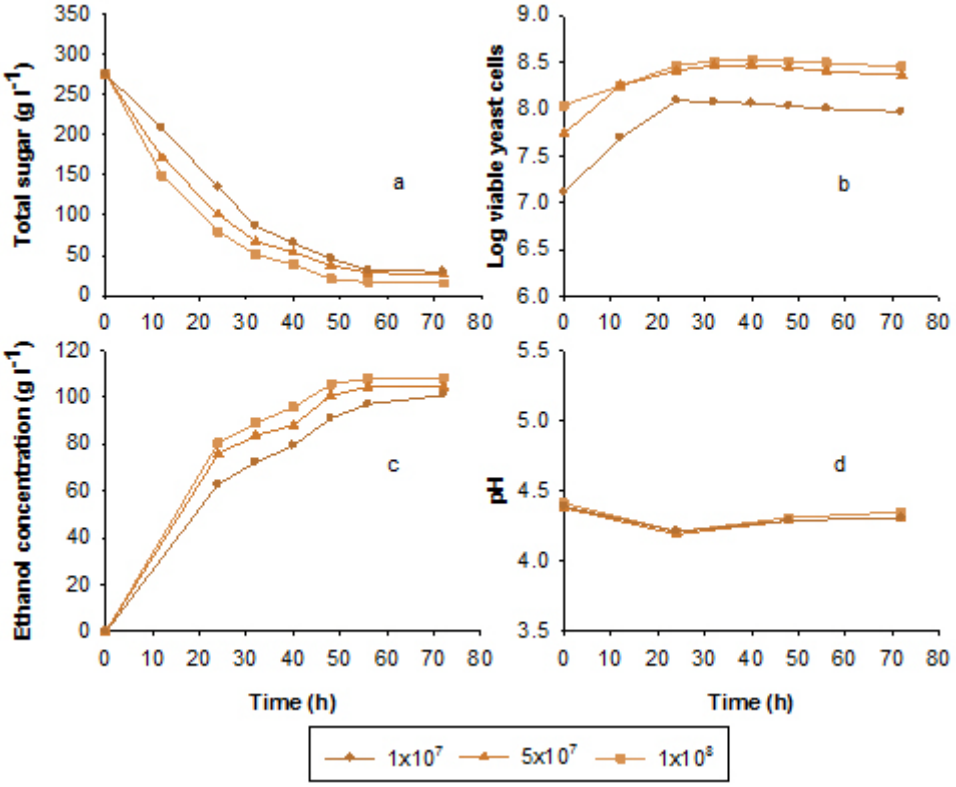

Fig. 2 Fermentation kinetics during batch ethanol production from the sweet sorghum juice supplemented with 8 g l^{-1} of DSY under different initial yeast cell concentrations of *S. cerevisiae* NP 01. (a) total sugar, (b) log viable yeast cells, (c) ethanol concentration and (d) pH.

Regarding yeast growth, at the initial cell concentration of 1 x 10^8 cells ml^{-1}, the cell numbers were increased about 3 times (3.22 x 10^8 cells ml^{-1}) within 24 hrs of the fermentation (Figure 2b). At the initial cell concentration of 5 x 10^7 cells ml^{-1}, the cell number increased to 1.78 x 10^8 cells ml^{-1} at 12 hrs, which was comparable to that of the highest initial cell concentration. At the lowest initial cell concentration, the yeast cell numbers increased and reached to the maximum value at 24 hrs. However, the value was approximately one log scale lower than the maximum values of the higher initial cell concentrations. The initial cell concentration also markedly affected the rate of ethanol production especially in the first 48 hrs (Figure 2c). After that, the ethanol concentrations or *P* values were slightly increased. The fermentation times obtaining the highest *P* values of the initial cell concentrations at 1 x 10^7, 5 x 10^7 and 1 x 10^8 cells ml^{-1} were 72, 56 and 48 hrs, respectively (Figure 2c). pHs of all treatments were changed in the same manner (Figure 2d).

Table 3 summarizes the important kinetic parameters of the ethanol fermentation at the fermentation time giving the highest ethanol concentration under the different initial cell concentrations. Ethanol production efficiency in terms of Q_p of the initial cell concentration of 5 x 10^7 cells ml^{-1} (1.86 g l^{-1} h^{-1}) was slightly lower than that of 1 x 10^8 cells ml^{-1} (2.20 g l^{-1} h^{-1}), while *P* and $Y_{p/s}$ were not different. Therefore, the initial cell concentration at 5 x 10^7 cells ml^{-1} was selected to be the optimum initial cell concentration for the subsequent experiments. The lower initial yeast cell concentration for ethanol production was reported by Wu et al. (2010). They used dry alcohol yeast (Ethanol Red) at the initial cell concentration of 1 x 10^7 cells ml^{-1} for batch ethanol fermentation from sweet sorghum juice with 25-30% (w/v) sugar contents and yeast extract supplementation, obtaining ethanol yields at 0.39-0.46. g g^{-1}.

Table 3. Kinetic parameters of batch ethanol production from the sweet sorghum juice supplemented with 8 g l^{-1} of DSY under different initial yeast cell concentrations at the time giving the highest ethanol concentration.

Initial yeast cell concentration (cells ml^{-1})	Parameters (mean ± SD)[a]			
	P (g l^{-1})	Q_p (g $l^{-1}h^{-1}$)	$Y_{p/s}$ (g g^{-1})	t (hr)
1.30 x 10^7	100.84 ±1.66	1.40 ± 0.02	0.42 ± 0.01	72
5.01 x 10^7	104.36 ± 1.90	1.86 ± 0.04	0.43 ± 0.01	56
1.10 x 10^8	105.46 ± 1.44	2.20 ± 0.02	0.44 ± 0.01	48

[a] P = ethanol concentration; Q_p = ethanol productivity; $Y_{p/s}$ = ethanol yield; t = fermentation time.* The experiments were performed in duplicate.

Effects of initial sugar concentrations in inoculum preparation (IP) medium for ethanol production

At high sugar concentrations under VHG fermentation, biomass concentration might decrease due to cell disruption by high osmotic pressure yielding low biomass and ethanol yields. In addition, high substrate concentrations also caused prolongation of complete sugar utilization and lower final ethanol concentration (Ozmihci and Kargi, 2007; Bai et al. 2008). Zhao and Bai (2009) also reported that VHG fermentation imposed severe stresses on yeast cells, namely the high osmotic pressure from the substrate sugar at the beginning and the strong ethanol inhibition during the production stage. Among various stresses that yeast cells encountered during ethanol fermentation, ethanol inhibition and osmotic pressure were the most important adverse conditions (Birch and Walker, 2000; Querol et al. 2003). One method to reduce those adverse effects is to increase sugar concentration in the IP medium. This process may facilitate the inoculum to become acclimatized under the higher sugar concentration, which is useful for ethanol fermentation under VHG condition.

In the present study, *S. cerevisiae* NP01 grown in YM medium containing different glucose concentrations (10, 50 and 100 g l^{-1}) and in the sweet sorghum juice containing 100 g l^{-1} of total sugar was used as the inoculum for ethanol production from the EP medium (the sweet sorghum juice supplemented with 8 g l^{-1} of DSY). The initial cell concentration in the EP medium was approximately 5 x 10^7 cells ml^{-1}. The pH changes under the different conditions were similar (data not shown). The results showed that the level of glucose concentration in YM medium did not affect the ability of yeast to utilize sugar and yeast growth in the EP medium (Figure 3a and 3b). Total sugar remaining and viable yeast cell number in the EP medium under the different initial glucose concentration in YM broth were not different. However, the inoculum prepared under higher glucose concentration could produce ethanol at higher levels (Figure 3c). The results indicated that the yeasts were acclimatized to some extent to high sugar concentrations, and might have a protective effect on ethanol inhibition during the ethanol production process.

Table 4 summarizes the important kinetic parameters of the ethanol fermentation by the inoculum grown under the different initial sugar concentrations in the IP medium. At 48 hrs of the fermentation time, the P values increased with increasing the sugar concentrations in the IP medium. The $Y_{p/s}$ values (ranging from 0.46 to 0.47) of the yeasts grown in 50 and 100 g l^{-1} of sugar were higher than those grown in 10 g l^{-1} of glucose. In addition, ethanol production by the inoculum grown in the sweet sorghum juice containing 100 g l^{-1} of total sugar corresponded to that grown in the YM medium containing 100 g l^{-1} of glucose. These findings indicated that the sweet sorghum juice consisted of essential nutrients in adequate amount for yeast growth as found in the YM medium. Therefore, the sweet sorghum juice containing 100 g l^{-1} of total sugar was selected to be used for inoculum preparation in the next experiment.

Ethanol production under non-sterilized condition

Contamination sources in large-scale yeast ethanol fermentations were categorized as either direct or indirect (Schell et al. 2007). Potential direct sources could originate from materials added to the fermentor, while potential indirect sources were dirty transfer lines or water used for pump and agitator seals. Many researchers had studied ethanol fermentation under non-sterilized conditions (Roukas,

1996; Echegaray et al. 2000; Tao et al. 2005) in order to save energy consumption and to meet the demands of ethanol industrialization. In addition, one of several advantages of VHG fermentation for industrial applications is that it can reduce the risk of bacterial contamination (Bai et al. 2008). Therefore, sterilization of EP medium at VHG levels is probably not necessary. Moreover, if sterilization is omitted, operation cost of raw material preparation and energy consumption will be reduced. To achieve these objectives, ethanol production from the EP medium with and without sterilization was compared.

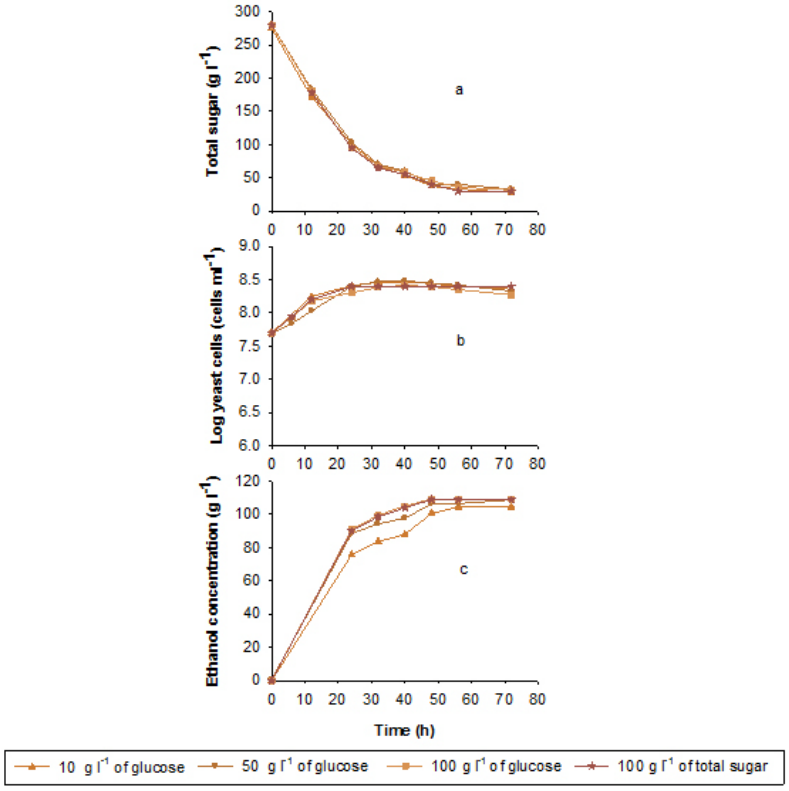

Fig. 3 Fermentation kinetics during batch ethanol production from the sweet sorghum juice supplemented with 8 g l^{-1} of DSY by *S. cerevisiae* NP01 grown in different initial sugar concentrations in IP medium. (a) total sugar, (b) log viable yeast cells and (c) ethanol concentration.

In Figure 4a, the initial total sugar concentration of the EP media with and without sterilization was similar at approximately 280 g l^{-1}, indicating that the sterilization condition of the media in the 5-litre stirred tank bioreactor at 110ºC, 60 min did not cause adverse effect or maillard reaction between sugar and nitrogen sources did not occur during sterilization process. A decrease in sugar concentration after autoclaving was observed in the study of Tao et al. (2005) who found that about 5% of glucose was lost during autoclaving process of fermentation medium in a 250-ml Erlenmeyer flask at 121ºC, 20 min.

The rate of sugar utilization and pH changes of the EP medium with and without sterilization during ethanol fermentation were not different (Figure 4a and b). The yeast cell numbers increased until 24 to 32 hrs. However, *S. cerevisiae* NP01 could grow in the sterile EP medium better than in the non-sterile medium at approximately 0.5 log scale (Figure 4b). Medium sterilization significantly affected ethanol production by the yeasts. The maximum ethanol concentration from the sterile EP medium was about 30 g l^{-1} higher than that of the non-sterile medium. The total bacterial plate count in the non-sterile EP medium during the fermentation was detected (Figure 5). The cell number of total bacteria increased about one log scale in 24 hrs, and then slightly decreased until the end of the experiment. The number of acid bacteria in the non-sterile EP medium at the beginning was about log 3.06 cfu ml^{-1}, indicating

that most bacteria contaminated in the medium were acid bacteria. Similar results were observed by Wu et al. (2010) who reported that bacteria in sweet sorghum juice were mostly lactic acid bacteria. In our study, the number of acid bacteria decreased after 24 hrs (data not shown) that might be due to high ethanol concentration in the broth. At the end of the fermentation, it was rarely detected (log 1.50 cfu ml^{-1} only).

Table 4. Kinetic parameters of batch ethanol production from the sweet sorghum juice supplemented with 8 g l^{-1} of DSY by *S. cerevisiae* NP01 grown under different initial sugar concentrations in inoculum preparation (IP) medium.

Initial sugar in IP medium (g l^{-1})	Parameters (mean ± SD)[a]			
	P **(g l^{-1})**	Q_p **(g $l^{-1}h^{-1}$)**	$Y_{p/s}$ **(g g^{-1})**	***t*** **(hr)**
10 g l^{-1} of glucose	100.67 ± 1.90	2.10 ± 0.04	0.43 ± 0.01	48
50 g l^{-1} of glucose	106.41 ± 1.40	2.22 ± 0.03	0.46 ± 0.00	48
100 g l^{-1} of glucose	109.30 ± 0.86	2.28 ± 0.02	0.46 ± 0.01	48
100 g l^{-1} of total sugar in SSJ[b]	108.98 ± 1.16	2.27 ± 0.04	0.47 ± 0.01	48

[a] See Table 3; [b] Sweet sorghum juice. * The experiments were performed in duplicate.

At 48 hrs, the *P*, Q_p and $Y_{p/s}$ values of batch ethanol production from the non-sterile EP medium supplemented with 8 g l^{-1} of DSY were 80.91 ± 3.13 g l^{-1}, 1.69 ± 0.07 g $l^{-1}h^{-1}$ and 0.35 ± 0.01 g g^{-1}, respectively. These values were significantly lower than those of the sterile medium at approximately 25 to 28% at the same fermentation time (Table 4). In the two EP media, the amount of sugar utilized were similar but the *P* values produced were different, indicating that the contaminants or bacterial cells in the non-sterile medium utilized the sugar and produced by-products which were not determined in this experiment. In addition, lower *P* value in the non-sterile EP medium might be due to lower yeast cell concentration during the fermentation. In some studies, low *P* values in non-sterile media were caused by contaminants (mainly acetic acid bacteria), which could utilize ethanol produced by fermentative yeasts as an energy source (González et al. 2005). Narendranath et al. (1997) found that only 10^5 cfu ml^{-1} of *Lactobacillus paracasei* was sufficient to reduce more than 2% (v/v) or 15.8 g l^{-1} of the final ethanol concentrations.

Medium sterilization was not necessary as reported in some studies. Roukas (1996) found that non-sterilized beet molasses was used successfully as a raw material for ethanol production by fed-batch fermentation. However, very high initial yeast cell concentration (2.8 x 10^8 cells ml^{-1}) and sterilized fermentor were required. In addition, contamination problem in ethanol production from non-sterilized soybean molasses could be avoided by the addition of appropriate antibiotic (Siqueira et al. 2008). Tao et al. (2005) showed that at pH 4.5-5.0, medium sterilization was not necessary for ethanol production from glucose medium by *Zymomonas mobilis*. In our study, the pH throughout the fermentation ranged from 4.1-4.5; however, 25% reduction of ethanol production was observed. The reduction was probably due to the contamination in the fermentation broth.

Scaling up of ethanol production

All previous experiments of ethanol fermentation were carried out in the 5-litre bioreactor. Therefore, scale-up ethanol production should be investigated to confirm the results before launching to an industrial production. The time profiles of total sugar concentration, cell viability, ethanol concentration and pH during batch fermentation from the juice supplemented with 8 g l^{-1} of DSY in the 5 and 50-litre stirred tank bioreactors by the inoculum grown in the juice containing 100 g l^{-1} of total sugar were not different (Figure 6). The total sugar markedly decreased in the first 48 hrs of the fermentation and remained approximately 27 g l^{-1} at the end of the fermentation (Figure 6a). The yeast cells rapidly grew in the first 12 hrs (Figure 6b). The number of viable yeast cells in the 50-litre bioreactor seemed to be slightly higher than that in the 5-litre bioreactor after 6 hrs with the difference of only 0.05-0.12 log scale.

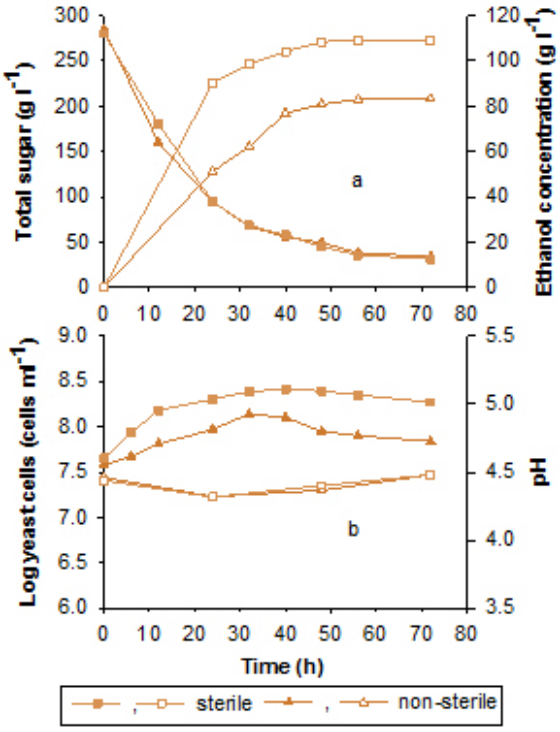

Fig. 4 Fermentation kinetics during batch ethanol production from sterile and non-sterile sweet sorghum juice supplemented with 8 g l^{-1} of DSY by *S. cerevisiae* NP01. (a) total sugar (closed symbol) and ethanol concentration (open symbol), (b) log viable yeast cells (closed symbol) and pH (open symbol).

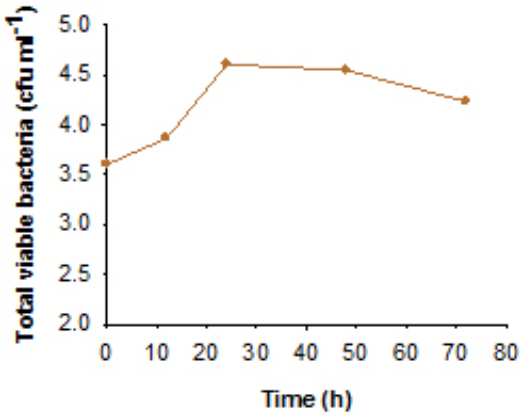

Fig. 5 Total viable bacteria during batch ethanol production from non-sterile sweet sorghum juice supplemented with 8 g l^{-1} of DSY.

In the first 40 hrs, the ethanol concentration of the 5-litre bioreactor was higher than that of the 50-litre bioreactor. However after 40 hrs, the values in the two bioreactors were comparable. At 48 hrs, the *P* value in the smaller bioreactor was slightly higher than that of the larger one at approximately 3%, whereas the Q_p *and* $Y_{p/s}$ values were similar (Table 5). The results indicated that the size of the bioreactor in this study did not markedly affect *P*, Q_p and $Y_{p/s}$ values. It was also implied that the agitation, mixing and temperature control system in the bigger reactor were mimiced those of the smaller reactor.

In the 50-litre bioreactor, the juice was sterilized at 90ºC because of the limitation of sterilization controller. However, this temperature was sufficient to protect contamination during the fermentation. Contaminant was rarely detected at the beginning and the end of the fermentation (data not shown). The number of the contaminant did not increase during the fermentation because most bacterial cells could not withstand high ethanol concentration and the acidic pH (pH 4) of the juice (Zoecklein et al. 1995).

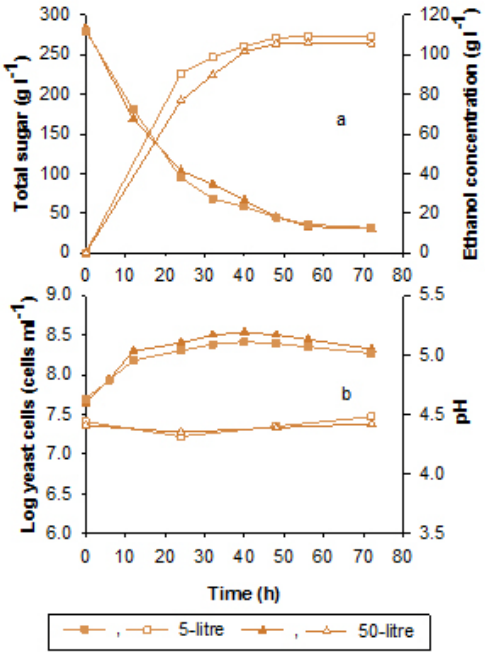

Fig. 6 Fermentation kinetics during batch ethanol production from the sweet sorghum juice supplemented with 8 g l^{-1} of DSY in 5-litre and 50-litre bioreactors. (a) total sugar (closed symbol) and ethanol concentration (open symbol), (b) log viable yeast cells (closed symbol) and pH (open symbol).

Cost of raw materials used for ethanol production in Thailand including sweet sorghum was compared in Table 6. The cost from sweet sorghum juice was lower than that from cassava and comparable to that from sugarcane molasses. Due to high fluctuation of molasses cost, the cost from sweet sorghum was occasionally lower than that from sugarcane molasses in some seasons. In addition, sweet sorghum can be planted 3 rounds/year with an average yield of 12.5 tons/acre/round or 37.5 tons/acre/year, while sugarcane can be cultivated only one round/year or 25 tons/acre/year. This clearly indicates the high potential use of sweet sorghum as one of raw materials for ethanol production in Thailand and other countries.

Table 5. Comparison of kinetic parameters of batch ethanol production from the sweet sorghum juice and supplemented with 8 g l^{-1} of DSY in 5 and 50-litre bioreactors.

Bioreactor	Parameters (mean ± SD)[a]			
	P (g l^{-1})	Q_p (g $l^{-1}h^{-1}$)	$Y_{p/s}$ (g g^{-1})	t (hr)
5-litre	104.09 ± 0.76	2.60 ± 0.03	0.47 ± 0.01	40
	108.98 ± 1.16	2.27 ± 0.06	0.47 ± 0.01	48
50-litre	101.34 ± 0.83	2.53 ± 0.02	0.47 ± 0.01	40
	105.49 ± 1.21	2.20 ± 0.03	0.46 ± 0.01	48

[a] See Table 3; * The experiments were performed in duplicate.

Table 6. Cost of ethanol productionfrom different raw materials in Thailand.

Raw material	Yield of raw material (tons/acre/year)	Cost of raw material (US$/ton)	Ethanol produced (litres/ton)	Cost of ethanol production (US$/litre)	
				From raw material only	Including production cost[f]
Sugarcane molasses	1.13[a]	100-150[d]	250[d]	0.40-0.6	0.57-0.77
Cassava	8.75[b]	91[b]	170[d]	0.54	0.75
Sweet sorghum	37.5[c]	25[c]	54[e]	0.46	0.63

[a] The value is calculated from the production yield of sugarcane (25 tons/acre/year) and one ton of sugarcane stalk produces 45 kg of molasses; [b] data from Bank of Thailand , Office of Agricultural Economics (Domestic and World Prices of Certain Commodities, 2010); [c] data from Jaisil et al. (2009); [d] data from Jaisil and Putto (2010); [e] data from this study according to sugar content and ethanol yield; [f] Production cost of sugar-based and starchy-based raw materials were US$ 0.17 and 0.21, respectively (Jaisil and Putto, 2010).

CONCLUDING REMARKS

The by-product from brewery industry or DSY could be used successfully as a low-cost nutrient supplement instead of yeast extract and peptone for batch ethanol fermentation from sweet sorghum juice under the VHG conditions. The initial yeast cell concentration of 5 x 10^7 cells ml^{-1} was optimal for the ethanol production. The sweet sorghum juice consisted of essential nutrients in adequate amount for yeast growth. The juice containing 100 g l^{-1} of total sugar without nutrient supplement could replace the typical inoculum preparation medium (YM medium). The scale-up ethanol production (up to 50-litre bioreactor) under VHG fermentation provided satisfactory results. To improve ethanol production efficiencies under non-sterilized conditions in industrial applications, the addition of an antibiotic into the sweet sorghum juice and keeping the sanitation of the working environment may be necessary.

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