Enhancement of very high gravity bioethanol production via fed-batch fermentation using sago hampas as a substrate

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Abstract. Very high gravity (VHG) ethanolic fermentation is a promising technology used for producing bioethanol. However, the technology is often associated with the excessive amount of glucose that is entirely supplied in the beginning of the culture causing the fermentation process to be sluggish and therefore inhibits complete utilisation of glucose. The high concentration of glucose in the fermentation medium also elevates the osmotic pressure, which has a destructive effect on yeast cells. This study aims to enhance the production of VHG bioethanol from sago hampas hydrolysate (SHH) via fed-batch fermentation. The fermentations were performed in a 2-L stirred tank bioreactor. Batch fermentation was conducted as a control. Our results showed that the maximum yeast cell concentration achieved was significantly improved by 1.5-fold when the fermentation was carried out in fed-batch mode. The ethanol yield attained in the fed-batch culture represents an enhancement of 22% over that achieved in the batch culture. Moreover, the ethanol productivity achieved in the fed-batch culture was found to be increased by 1.8 times in comparison to the productivity attained in the batch culture. In general, this work provides useful insights into promising techniques for enhancing VHG fermentations in the stirred tank bioreactor employing agricultural residues as feedstocks.

Keywords: Ethanol, fed-batch system, osmotic pressure, substrate inhibition, very high gravity (VHG) fermentation, yeast

INTRODUCTION

Bioethanol has emerged as a sustainable and eco-friendly alternative to the conventional fossil fuel that poses major environmental problems. Currently, there is an increasing interest to exploit lignocellulosic waste as feedstocks for producing bioethanol. Apart from being cheap feedstocks, the use of lignocellulosic waste for bioproduction may also overcome the disposal problem that may otherwise be caused by the bulk dumping of the waste to the environment (Gutierrez-Rivera *et al.*, 2012; Ishola & Taherzadeh, 2014; Vincent *et al.*, 2014). Lignocellulosic waste generally has high

content of starch and cellulosic components, which can be utilised to produce fermentable sugars and bioethanol (Janggu & Bujang, 2009; Linggang *et al.*, 2012; Awg-Adeni *et al.*, 2013; Jenol *et al.*, 2014).

One of the potential lignocellulosic waste is sago hampas, which is a side product of the sago extraction process. Every year, Sarawak exports about 44,000 tonnes of sago starch mainly to Peninsular Malaysia, Japan and other countries (Awang-Adeni *et al.*, 2010). For every tonne of starch produced, one tonne of sago hampas are

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released into the river (Bujang *et al.*, 1996). It is estimated that at least 40 tonnes of sago hampas are produced daily in Sarawak. Currently, there is no proper way to treat sago hampas, hence most of them are disposed to the river causing severe environmental pollution. Sago hampas on a dry basis has 58% starch apart from other lignocellulosic components (Linggang *et al.*, 2012). Our previous works have highlighted the feasibility of using sago hampas as a substrate for bioethanol production (Awang-Adeni *et al.*, 2013; Mohammad *et al.*, 2020). The remaining challenge in using sago hampas as a feedstock for bioethanol production, however, lies in the technique used to enhance the productivity of bioethanol fermentation.

Very high gravity (VHG) fermentation is a promising technology for producing bioethanol using high concentration of sugar (>250 g/L) at the low expense of water and energy (Ingledew & Lin, 2011). However, in batch fermentations under VHG conditions, the yeasts undergo various stresses and toxic effects induced by the high concentrations of sugars and ethanol. This has resulted in incomplete fermentation of ethanol (Dodic *et al.*, 2009; Puligundla *et al.*, 2011). In order to lower the drawback of batch VHG fermentation, adoption of a fed-batch system offers an advantage whereby the system can control the feeding rate of the fermentation medium. Thus, it has the potential to minimise the inhibition effects of high concentrations of substrate and ethanol, which exist at the initial and at the end of the ethanol fermentation (Joannis-Cassan *et al.*, 2014). This work aims to investigate the feasibility of fed-batch fermentation system for enhancing the production of bioethanol under VHG condition using sago hampas hydrolysate (SHH) as a main substrate.

MATERIALS AND METHODS

Raw material preparation

Sago hampas was collected from Ubom Sago Sdn. Bhd. in Mukah and Herdsen Sago Industry Sdn. Bhd. in Pusa, Sarawak, Malaysia. The hampas was sun-dried for 3-5 days and oven-dried for 1-2 days to completely remove its moisture content. Sago

hampas was ground into fine powder with a particle size of approximately 1 mm. The ground sago hampas was then used as a substrate for glucose production via an enzymatic hydrolysis process.

Enzymatic hydrolysis of sago hampas

A suspension of sago hampas, 7% (w/v) was mixed with distilled water. The suspension was then boiled and gelatinised in a steel vessel at 85-90°C on a hot plate for 30 minutes. After the gelatinisation process, Liquozymes® SC DS (Novozyme, Denmark) was added into the suspension at a concentration of 2 µl/g and left at the same temperature for another 30 minutes for the liquefaction process to be completed. Throughout the process, the suspension was constantly stirred. Then, the suspension was cooled down to 50-60°C. Spirizyme® Fuel HS (Novozyme, Denmark), at a concentration of 1 uL/g was then added into the suspension for the saccharification stage and left inside the incubator shaker at a temperature of 50-60°C for another 5-6 hours. Three-cycle hydrolysis method as suggested by Awg-Adeni (2015) was performed in this study in order to increase the concentration of glucose in SHH.

Inoculum preparation

A commonly used instant dry baker's yeast, *Saccharomyces cerevisiae* was used in this study. Approximately, 1 g of yeast grains was activated in a sterile Yeast Malt Broth medium and the culture was incubated at 30°C overnight. Then, a single loop of the yeast culture was streaked onto Potato Dextrose Agar (PDA). For the batch system, after 24 h, a single colony from the incubated plates was inoculated into sterile 100 mL inoculum medium containing 100 g/L glucose and 5 g/L yeast extract. The culture was incubated at 30°C for 10-16 h. For the fed-batch system, the inoculum was prepared in two sets with each consisted of 50 mL inoculum media. The first inoculum was used as a starting culture for 500 mL fermentation media whereas the second inoculum was added after 24 h of incubation.

Bioethanol fermentation

Bioethanol fermentations were carried out in a 2 L stirred tank bioreactor (B Braun, Germany).

The fermentation medium comprised of 250 g/L glucose and 5 g/L yeast extract. The pH of the fermentation medium was adjusted to 5.5-5.6 using 1 M hydrochloric acid and 1 M sodium hydroxide before sterilisation and it was not controlled during the fermentation process. In the case of batch fermentation, the vessel was initially filled with 1 L medium and was then sterilised by autoclaving at 121°C for 20 minutes. In the case of fed-batch fermentation, the vessel was initially filled with 450 mL medium before it was autoclaved. Upon cooling to room temperature, an appropriate amount of yeast cells with the final concentration of 7×10^4 cells/mL was added to the fermentation medium.

For fed-batch experiment, 50 mL yeast culture was initially added into the 450 mL fermentation medium in the bioreactor and the mixture was agitated at 200 rpm. After 24 h, another 50 mL of yeast culture was inoculated into the culture. The two-stage addition of yeasts with the interval of 24 h was to avoid the decline of yeast count, which normally decreases after 30 h of incubation. At 30 h, another batch of fresh 450 mL medium was fed into the fermenter and the agitation speed was increased to 280 rpm. The addition of fresh medium was carried out after 30 h in order to allow the second batch of yeast culture, which was added at 24 h, to grow faster by consuming the residual glucose in the old medium, before consuming the newly added fresh medium. The temperature for both batch and fed-batch cultures was controlled at 30°C. For batch culture, the agitation speed was maintained at 280 rpm throughout the incubation. All fermentations were carried out for 72 h and the sampling was done every 6 h.

Analytical methods

Total viable cell count

The viable yeast cell numbers were determined by methylene blue staining and direct counting using a haemocytometer (Phukoetphim *et al.*, 2017). The formula to calculate the numbers of cells is as follows:

$$\text{Total no. of cells/mL} = \frac{\text{average no. of cells} \times \text{dilution factor}}{\text{factor} \times 10^4}$$

Determination of glucose and ethanol content

A total of 10 mL fermentation broth was withdrawn from the fermenter at every sampling hour. The sampled broth was centrifuged at 10,000 rpm for 10 minutes. The resulting supernatant was used to determine the concentration of glucose and ethanol by high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). The HPLC system is equipped with Shimadzu LC-20AT solvent delivery pump and Shimadzu RID-10A detector. The ethanol yield ($Y_{E/S}$) was calculated as the actual concentration of ethanol (g) produced for every gram of glucose utilised. The volumetric ethanol productivity (Q_p , g/L/h) was calculated by dividing the concentration of ethanol produced by the time taken to reach the highest concentration of ethanol. In addition, the ethanol efficiency (E_t) was calculated by finding the percentage of experimental ethanol yield divided by theoretical ethanol yield.

Statistical method

The data in this study were statistically analysed using SPSS software (SPSS Statistics Data Editor V.21, USA). T-test was used and the results were considered statistically significant if the p value < 0.05 .

RESULTS AND DISCUSSION

Cell growth

Figure 1 shows the growth profile of *S. cerevisiae* cultivated in batch and fed-batch mode. The total cell count in the batch culture increased sharply from 0 to 6 h of fermentation and reached its maximum peak of 2.48×10^6 cells/mL at 30 h before it decreased steadily towards the end of the fermentation. The final total cell number in the culture was 1.55×10^6 cells/mL. As depicted by Figure 1, the cell death in the batch culture was detected after 30 h of incubation. The relatively short survival period of the yeast cells can be associated with the high content of glucose in the batch medium that escalated the osmotic stress in yeasts, which consequently caused harmful effects on the yeast cells (Bafrcova, 1999).

In the fed-batch culture, the yeast growth increased steadily from 0 to 24 h of fermentation.

There was a slight decline of yeast count at 30 h, which was due to the feeding of fresh medium that caused the dilution of the culture. After 36 h of fermentation, the yeast count started to increase again and reached its maximum peak of 3.62×10^6 cells/mL at 42 h. The maximum yeast count achieved in fed-batch culture represents a 1.5-fold increment over that attained in the batch culture. Due to the feeding of the yeast cells at 24 h, the log phase of the cell growth in the fed-batch culture extended until 42 h in contrast to the batch culture where the growth declined at 30 h. In this work, the incubation was stopped at 72 h due to the plateau production of ethanol despite the presence of viable cells in the culture. Prolonging the incubation time may not increase the ethanol production nor glucose consumption by the yeast cells.

Furthermore, it can be inferred that the toxic effect of the medium in the fed-batch culture was reduced since it was observed that the cell death occurred when the ethanol concentration reached

80 g/L at 42 h. In contrast to the batch culture, the cell death took place at 30 h when the ethanol produced was 19 g/L. The cell death in the fed-batch fermentation might be due to the inhibitory effect of the high concentration of ethanol produced. Zhang *et al.* (2015) reported that the yeast growth completely stopped when the ethanol concentration exceeded 70 g/L. In contrast to the batch fermentation, the cell death may be contributed by the high concentration of glucose (169 g/L) at 30 h. The high concentration of glucose may introduce high osmotic pressure on the yeast cells and hence may lyse them. According to Stanbury *et al.* (1995), fed-batch fermentation can minimise substrate inhibition and toxic effects from the high concentration of the medium. In summary, the results presented in this work suggests that the fed-batch culture was more efficient in enhancing the yeast growth and in maintaining viable cells longer than in the batch culture.

Figure 1. Total cell count of *S. cerevisiae* in batch and fed-batch system within 72 h of fermentation.

Glucose consumption

The profile of glucose consumption in the batch and fed-batch cultures during the ethanol fermentation is illustrated in Figure 2. In the

batch culture, there was a steady decrease of glucose concentration throughout the fermentation until the final concentration of 104 ± 6.0 g/L. On the other hand, in the fed-batch

culture, the decrease of glucose concentration was observed in two phases namely from 0 to 30 h and from 36 to 72 h. The spike of glucose concentration between 30 and 36 h was due to the feeding of the fresh medium. Despite the approximately similar concentration of glucose in the beginning of fermentation for both batch and fed-batch cultures, there was a difference in terms of the amount of glucose consumed. In the batch culture, the amount of glucose consumed by *S. cerevisiae* during the ethanol fermentation was 155 ± 6.00 g/L. Meanwhile, in the fed-batch culture, about 224.71 ± 0.86 g/L of glucose was consumed making the consumption efficiency as 28% higher than that in the batch culture.

The low efficiency of glucose consumption by the yeast cells in the batch culture can be associated with the high osmotic pressure exerted on the whole yeast cells at the same time resulting in the decline of the cell growth at 30 h.

In contrast, in the fed-batch culture, the two-stage feeding of inoculum and medium was found to alleviate the impact of high osmotic pressure as what occurred in the batch culture. The remaining challenge in the fed-batch culture is to ensure complete glucose consumption by the end of the ethanol fermentation.

According to Bafrncova *et al.* (1999), supplementation of growth nutrients is crucial in order to ensure the optimal growth of *S. cerevisiae* and efficient consumption of the substrate supplied. This is parallel with some published works that reported the importance of growth supplements that help to improve sugar utilisation by *S. cerevisiae* (Ortiz-Muniz *et al.*, 2010; Li *et al.*, 2011; Sankh *et al.*, 2011; Harde *et al.*, 2014). The influence of growth supplements on the growth of *S. cerevisiae* during ethanol fermentation should be taken into consideration in the future work.

Figure 2. Residual glucose concentration in batch and fed-batch system during bioethanol fermentation.

Ethanol production

Figure 3 depicts the ethanol production by *S. cerevisiae* in batch and fed-batch fermentations. The highest ethanol produced in the fed-batch fermentation was 111.88 ± 2.28 g/L yielding an enhancement of 1.8 times higher than the

maximum ethanol concentration achieved in the batch fermentation. The same increment was also seen in the ethanol productivity where 1.55 g/L ethanol was produced per hour in the fed-batch culture in comparison to only 0.87 g/L ethanol produced per hour in the batch culture.

Meanwhile, the ethanol yield attained in the fed-batch culture represents an improvement of 22% over that achieved in the batch culture. The kinetic parameters of batch and fed-batch cultures are summarised in Table 1.

Our results for the ethanol yield in the fed-batch culture is parallel with what was reported by Khongsay *et al.* (2012) in which aeration was provided to the culture. In contrast, we managed to achieve the same yield without the use of aeration, which contributes an additional cost to the ethanol production. On the other hand, the ethanol yield achieved in the fed-batch culture in this study was found to be higher than that reported by Chen-Guang *et al.* (2012) in which VHG and redox potential-controlled conditions were applied. Similarly, the ethanol yield of the fed-batch culture in this study was higher than the value attained by Joannis-Cassan and co-workers (2014) where 3-stage fed-batch system was applied in the VHG ethanol fermentation. All the aforementioned works reported shorter fermentation time (53-56 h), which resulted in higher volumetric productivity (2.07-2.55 g/L/h). Despite longer fermentation time (72 h) employed and lower Q_p value attained in our study, the overall production cost was considered lower as

there was no major modification of the fermenter involved. The fed-batch technique employed in this work involved manual addition of inoculum and fresh medium into the unaerated culture. Moreover, the strain used in this work was not genetically modified unlike those that were used in some works (Almeida *et al.*, 2007; Liu, 2011; Ask *et al.*, 2013; Wei *et al.*, 2013). This clearly implies that the high ethanol productivity achieved in this work was contributed by the efficiency of the fed-batch technique employed. Our study intended to provide a solution for sago millers to manage sago hampas, which is otherwise disposed as waste. On site valorisation of sago hampas for production of value-added products such as bioethanol may help the millers to generate more economic opportunities. The simple bioethanol fermentation technique as proposed in this work ranging from the yeast strain used, Baker's yeast that is available at the local market, to the simple fed-batch procedure, is seen as economically viable, which can be easily applied by sago millers. Future studies should focus on the effects of other essential parameters on the yeast growth and ethanol production such as pH, oxygen, temperature, dissolved solids, organic acids and immobilization of yeast cells.

Figure 3. Profile of ethanol production by *S. cerevisiae* in batch and fed-batch fermentations.

Table 1. Comparison of kinetic parameters between batch and fed-batch fermentations.

Kinetic Parameters	Batch	Fed-batch
Initial glucose (g/L)	255.00 ± 0.31	256.91 ± 4.59
Residual glucose (g/L)	104.00 ± 6.00	32.19 ± 3.73
Glucose consumption efficiency (%)	59.22 ± 2.35	87.47 ± 0.87
Ethanol produced, P _E (g/L)	62.65 ± 3.06	111.88 ± 2.28
Q _p (g/L/h)	0.87 ± 0.06	1.55 ± 0.005
Y _{E/s} (g/g)	0.41 ± 0.01	0.5 ± 0.004
E _f (%)	80.23 ± 2.35	97.26 ± 1.09

Q_p = Volumetric ethanol productivity, Y_{E/s} = Ethanol yield, E_f = Ethanol efficiency

CONCLUSION

This work has demonstrated the feasibility of fed-batch fermentation to enhance the VHGH bioethanol production using SHH as a main substrate. Our results showed that the *S. cerevisiae* growth was significantly higher in the fed-batch culture than in the batch culture. The maximum yeast cell concentration achieved in the fed-batch fermentation was 1.5-fold higher than that achieved in the batch fermentation. The glucose consumption efficiency was also significantly improved in the fed-batch fermentation. Moreover, the ethanol yield was significantly enhanced by 22% when the fermentation was carried out in the fed-batch mode. The ethanol productivity achieved in the fed-batch culture was 1.8 times higher than that achieved in the batch culture. It can be concluded that the fed-batch fermentation technique as proposed in this work can serve as a promising approach for enhancing the production of VHGH bioethanol from sago hampas, which is known as a low cost and sustainable fermentation feedstock.

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