Fermentation Performance of A Bakery Yeast Strain in Normal and Very High Gravity Media With Different Nitrogen Content

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ABSTRACT

Fermentation performance of the yeast *Saccharomyces cerevisiae* is influenced, among others, by growth media composition. Media with complex nitrogen source tend to give better fermentation performance. In the present study, we investigate fermentation performance of a bakery yeast strain in normal (20% w/v glucose) and very high (40% w/v glucose) gravity media with different nitrogen content. We used yeast extract – peptone (YEP) media with varying concentration of yeast extract, bacteriological peptone, ammonium sulphate, and potassium hydrogen phosphate in the media. For comparison, yeast nitrogen base (YNB) media was used. We found that increasing YEP media component in the media lead to better cell growth, cell health and fermentation performance. The cell appeared to overcome hyperosmotic stress due to high glucose concentration when higher content of YEP used in the media, as indicated by better cell viability. Surprisingly, cell grown in YNB media was observed has the highest viability throughout the fermentation, even though the fermentation performance was poorer. The best fermentation was observed when media with the highest YEP composition was applied. In this media, when normal and very high gravity media were used, ~97 and ~75% of the sugar were consumed, respectively. It was also found that ethanol yield was 0.327 and 0.277 $g.g^{-1}$ for normal and very high gravity media, respectively. The result of the present study showed that nitrogen content present in the media is really important for yeast growth and fermentation performance.

Key words: very high gravity media, ethanol fermentation, bakery yeast, nitrogen content

1. Introduction

Bioethanol is renewable and environmentally friendly fuel and therefore considered as one of major alternative fuel that can respond to the depletion of fossil fuel [1, 2]. Bioethanol is

produced from sugary, starchy or lignocellulosic materials and by the activity of microorganisms, especially *Saccharomyces cerevisiae*, they are converted to ethanol [2, 3]. One of the means to increase efficiency of the overall ethanol production process is by increasing ethanol productivity in the fermentation process. Increasing ethanol content at the final product may lead to lower energy cost for distillation process [4]. There are many efforts has been conducted to increase ethanol production, including modification of fermentation media, genetic engineering of microorganisms used in fermentation or combination of both [5-10].

One of the methods to increase ethanol production is by increasing available sugar in the fermentation media. According to Thomas *et al.* [11] sugar content in fuel ethanol fermentation process can be catagorized to normal (20-22%) and very high (>27%) gravity condition. Even though application of very high gravity condition can lead to higher ethanol production, it can also lead to lower sugar utilization due to hyperosmotic stress exposure to yeast cell that can lead to lower viability of the cell and reduce the ability of the cell to ferment sugar [12]. Supplementation by various compounds has been found to give positive effect on ethanol production, presumably by protecting yeast cell against hyperosmotic stress [5, 6, 11]

In VHG fermentation, the basal fermentation media need to be rich of nutrient, otherwise the fermentation process can not go into completion and will leave high amount of residual sugar in the fermentation media [13]. Increasing ethanol production in VHG fermentation can be achieved by supplementation of free amino nitrogen (e.g. by means of addition of yeast extract, peptone, dry spent yeast) [5, 14], osmoprotectant (e.g. proline, glycerol, glycine, and glycine betaine) [9, 15], and particular metal ions (e.g. Mg^{2+}, Zn^{2+}) [6, 10] in the fermentation media.

In the present study, we investigated the fermentation performance of a bakery yeast strain in normal and very high gravity media with varying nitrogen content. From this study, we expect to get information on the required nutrient in the media to achieve complete fermentation as indicated by high sugar consumption and high ethanol production.

2. Material and Methods

2.1. Yeast strain and maintenance

Yeast strain used in this study was a bakery strain (A12) [16] and maintained on slopes of a complete medium, yeast extract peptone (YEP), containing (w/v) 0.5% yeast extract, 0.5% bacteriological

peptone, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 1% glucose and 1.5% agar. Slopes were stored at 4°C and sub-cultured every 6 months.

2.2. Growth media and culture conditions

There were five different media used in the present study, four of which were YEP media as described on Table 1 and one media using YNB (referred as media E) representing poor nutrient media. The YNB was used as per manufacturer suggestion (0.67 g/L). For experimental culture, glucose was added to each media to reach final concentration of either 20 or 40% (w/v). The media was sterilized using autoclave at 121°C and 15 psi for 15 minutes.

Table 1 Media composition used in this study (g/100 mL solution).

Media Component	Media			
	A	B	C	D
Yeast extract	0.05	0.10	0.50	1.00
Bacteriological peptone	0.05	0.10	0.50	1.00
$(NH_4)_2SO_4$	0.03	0.06	0.30	0.60
KH_2PO_4	0.03	0.06	0.30	0.60

Cells for starter culture were grown in YEP broth medium containing (w/v) 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 2% glucose. Starter cultures were inoculated from slopes and grown overnight (~16 h) at 30°C and 180 opm in a water shaker bath.

Experimental cultures were prepared by sterilizing previously described media in sterile Erlenmeyer flasks, each sealed with an oxygen-permeable cotton wool bung, and then inoculating to give an initial viable cell number of ~10^6 cells/mL. The ratio of flask size to culture volume was maintained at 4:1 to ensure adequate oxygen mixing. Samples from the cultures were aseptically removed by drawing off with a micro pipette every 6 hours from 0 to 36 hours and followed by every 12 hours up to 144 hours. Examination of the samples included measuring growth rate by measuring optical density, viable cell numbers, and glucose and ethanol concentrations.

2.3. Growth rate

Yeast growth was monitored by measuring optical density of the culture at 600 nm (OD_{600nm}) using a Jenway 6305 UV-Vis spectrophotometer, making dilutions where necessary. Measurements were made using 1 mL (10 mm path length) PMMA cuvettes (Sarstedt).

2.4. Viability determination

Viable cell numbers were assessed using the methylene violet staining method and light microscopy (400× magnification) using a Neubauer-type haemocytometer. An equal volume of the sample was mixed with methylene violet solution (0.01% w/v in 2% sodium citrate solution) and observed after 2 minutes staining [17].

2.5. Ethanol and glucose determination

Glucose was determined using alkaline fericyanide assay as described by Walker & Harmond [18] while ethanol was determined using alcohol dehydrogenase assay as proposed by Ough & Amarine [19] and modified by Ishmayana *et al.* [20].

3. Results and Discussions

3.1. Cell growth in normal and very high gravity media

In the present study, five different media were used. Four of which were YEP media (A, B, C and D) with different concentrations of the media components and YNB media (E). Media C is normally used in fermentation experiments [16]. Even though YEP is considered as media with rich nutrition, limitation of the YEP component in the media may affect the growth and fermentation performance of the yeast cell.

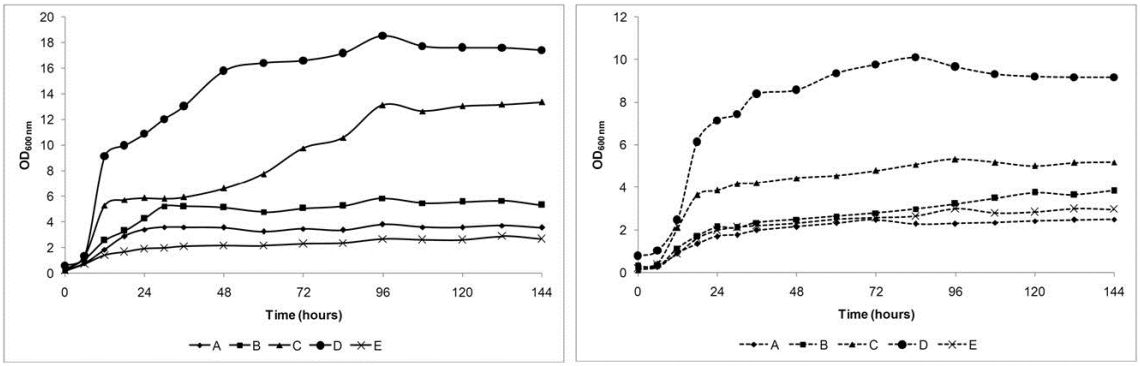

Figure 1. Growth of yeast cell grown in various media as indicated on the legend (refer to Table 1) with (a) 20% and (b) 40% w/v initial glucose.

A very clear difference in cell growth was observed when the cell grown in high and very high gravity media as can be observed on Figure 1(a) and 1(b). Yeast cell grown in normal gravity media has better cell growth compared to very high gravity media when the cell grown in media C and D. The OD value of cell grown in normal gravity media reached the highest OD of ~12 and ~18 when grown in media C and D, respectively. While in very high gravity media, the highest OD value reached ~5 and ~10 when grown in media C and D, respectively. However, when cell grown in media A, B and E with 40% (w/v) initial glucose, OD did not reach value more than ~3. From this result, it was apparent that very high gravity media repressed cell growth in poor or limited media, most likely due to exposure to hyperosmotic stress [12], and nutrition availability may enhance hyperosmotic stress tolerance of the cell [5, 14]). It also can be noted that cell growth was better when the media is rich with nutrition and readily available as observed when the cell grown in media C and D. When cell grown in media with poor or limited amount of nutrition, cell can not grow properly and this may also affect the performance of the cell to ferment sugar.

Cell growth in media A and B were similar to the growth of the cell in media E. Media A and B were actually used YEP which considered as rich nutrition media. However, the composition of the media component in media A and B were below what normally used in fermentation, and therefore limiting the amount of nutrition component in the media. Due to this limitation, the cell growth in these two media were retarded and become similar to cell grown in media E that used YNB, which considered as poor nutrition media. This result reveals that not only nutrition richness required by the cell to grow, but also the availability of the nutrition required that can support cell growth.

3.2. Cell viability

The cell viability data, as presented on Figure 2 (a) and 2(b), showed a tendency that the viability is decreased throughout the fermentation and cell grown in media C and D tend to have better cell viability especially in very high gravity media, even though this result need more confirmation for normal gravity media. Surprisingly, cell grown in media E has very high cell viability throughout the fermentation

(~90%). This phenomena was not observed on our previous study, when YNB media used for fermentation [13]. There are two main differences between the present study and the previous study. Firstly, the highest glucose concentration used in the previous study was 16% (w/v) whereas in the present study we used 20 and 40% (w/v) glucose. However, it is unlikely that 4% difference in sugar concentration may lead to such dramatic changes in viability. Secondly, in the previous study, we used filter sterilization when preparing fermentation media, whereas in the present study we used autoclave sterilization. There might be chemical changes during autoclave sterilization that produced protective agent that may assist the cell to maintain high viability or simply gave false viability result by interacting with methylene violet as staining agent. Further study to understand this observation is required.

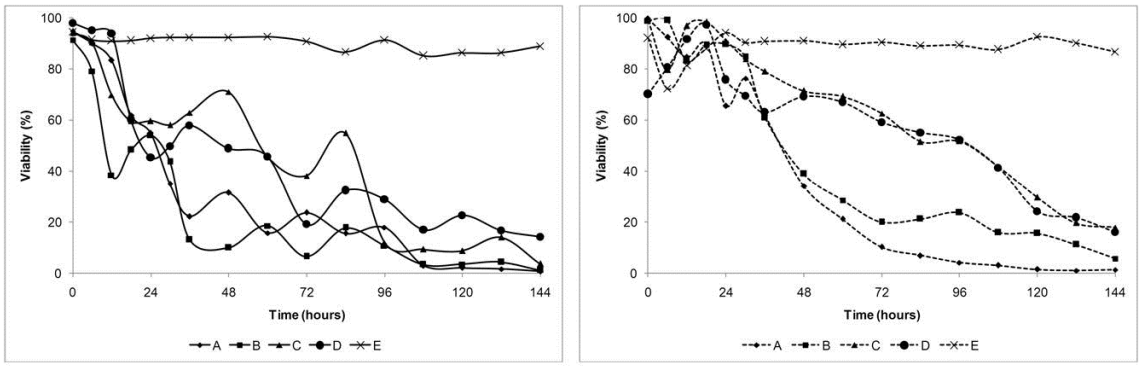

Figure 2. Viability of yeast cell grown in various media as indicated on the legend (refer to Table 1) with (a) 20% and (b) 40% w/v initial glucose

3.3. Fermentation performance

Figure 3(a) and 3(b) presents glucose consumption and ethanol production in different media. Glucose consumption is in agreement with increasing YEP composition in media, which indicate that higher YEP content lead to higher glucose consumption. Cell grown in YNB media showed lower glucose consumption and lower ethanol production compared cell grown in YEP media. In YEP media, fermentation performance may also relate to cell health as indicated by cell viability, in which healthier cell lead to better

glucose consumption and higher ethanol production. However, this observation was not accurate in case of cell grown in YNB media. Even though cell grown in YNB showed the highest viability throughout the fermentation, glucose consumption and ethanol production in this media was very poor. This result indicates that, assuming the cells were viable, even though the cell were viable they were loosing their ability to ferment sugar, creating viable but non-fermentable cell.

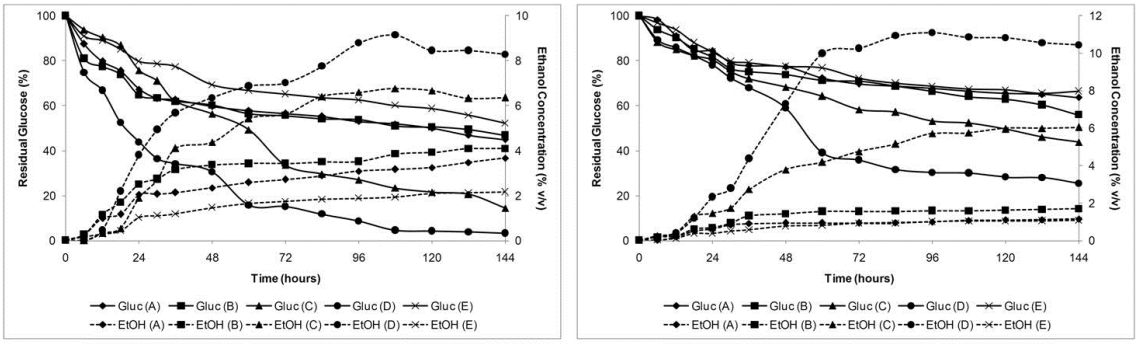

Figure 3. Glucose consumption and ethanol production of yeast cell grown in various media as indicated on the legend (refer to Table 1) with (a) 20% and (b) 40% w/v initial glucose

Fermentation performace parameter of cell grown in different media is presented on Table 2. Glucose consumption rate (*Qs)* for normal gravity fermentation is lower compared to very high gravity fermentation, but ethanol productivity (*Qp*) is actually higher, except for media D which have higher ethanol productivity. Even though glucose consumption rate is lower, the final glucose consumption for normal gravity condition is higher compared to very high gravity media. It seems that in very high gravity fermentation, even though the rate of glucose consumption was higher, due to osmotic stress, the cell can not consume all the available sugar (i.e. reduced fermenting ability), leading to lower ethanol productivity. Furthermore, in very high gravity fermentation, glucose is not only utilized to produce ethanol, but also to syntesize protecting agent (e.g. glycerol) to overcome hyperosmotic stress [12]. Whereas in media D, it is most likely that the nutrition component available in the media provide protection against hyperosmotic stress, and therefore synthesis of protecting agent is lower, lead

to higher ethanol productivity compared to other media with the same initial glucose concentration even though in the end the yield is still lower compared to the normal gravity media.

In term of ethanol yield ($Y_{p/s}$), cell grown in normal gravity media showed better performance as indicated by higher ethanol yield. In very high gravity media, even though glucose consumption rate were higher, the glucose was not entirely converted to ethanol, but also used to synthesize protecting agent so that the cell can survive against hyperosmotic stress. This was apparent especially in media E (YNB), which considered as poor media, and in media A and B which has lower concentration of YEP component in the media by very low ethanol yield.

Table 2. Fermentation performace of yeast cell grown in various media (refer to Table 1 for legend) with 20% and 40% w/v initial glucose

Glucose Conc. / Media	20% (w/v)				40% (w/v)			
	Q_s ($g.L^{-1}.h^{-1}$)	Q_p ($g.L^{-1}.h^{-1}$)	$Y_{p/s}$ ($g.g^{-1}$)	Gc (%)	Q_s ($g.L^{-1}.h^{-1}$)	Q_p ($g.L^{-1}.h^{-1}$)	$Y_{p/s}$ ($g.g^{-1}$)	Gc (%)
A	0.799	0.200	0.250	54.9	0.933	0.062	0.066	36.6
B	0.705	0.222	0.316	53.1	1.174	0.092	0.079	44.0
C	1.162	0.347	0.299	85.7	1.560	0.330	0.211	56.5
D	1.384	0.452	0.327	96.7	2.058	0.570	0.277	74.8
E	0.654	0.117	0.179	48.0	0.883	0.058	0.066	33.5

Note: *Q s*= glucose consumption rate, *Qp* = ethanol productivity, *Yp/s* = ethanol yield, Gc = Glucose consumption

In summary, fermentation in normal gravity media (20% w/v glucose) has better performance compared to very high gravity media (40% w/v glucose). Increasing the amount of YEP component in the media increased fermentation performance, but it still can not improve the fermentation performace at very high gravity media to reach the same performance as high gravity fermentation. More nitrogen source or supplementation with other compound might be required to reach better fermentation performance. Further investigation is required to confirm this.

4. Conclusions

The present study showed that nutrition availability, especially in the form of rich nutrition or complex nitrogen source, is very important to support yeast cell growth

and fermentation performance. Cell viability as an indication of cell health was also better when more nutrition available in the media. Surprisingly, cell grown in YNB, which considered as poor media, showed very high viability throughout the fermentation period but the fermentation performance was poor. Further study is required to investigate this observation.

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