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Research Article

Bioethanol Production from Sugarcane Bagasse Using *Neurospora intermedia* in an Airlift Bioreactor

Elvi Restiawaty[a,b*], Kindi Pyta Gani[b], Arinta Dewi[b], Linea Alfa Arina[b], Katarina Ika Kurniawati[b], Yogi Wibisono Budhi[a], Akhmaloka[c]

[a] *Research Group of Chemical Engineering Process Design and Development, Faculty of Industrial Technology, Institut Teknologi Bandung, Indonesia*

[b] *Biosciences and Biotechnology Research Center, Institut Teknologi Bandung, Indonesia*

[c] *Research Group of Biochemistry, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Indonesia*

ABSTRACT. Bagasse as solid waste in sugarcane industry can be utilized as one of the potential raw materials in the bioprocess industry. This research aims to investigate the conversion of bagasse to bioethanol using simultaneous saccharification and fermentation in an airlift bioreactor. Neurospora intermedia was used as a biological agent that carried out the saccharification and fermentation of sugarcane bagasse simultaneously for bioethanol production. Cell morphology of N. intermedia in the form of pellet was required to provide free movement in the axial flow of airlift bioreactor. The medium pH strongly affects the morphological shape of N. intermedia. Therefore, the formation of good pellets of inoculum was observed under acidic conditions, i.e. pH 3.0 – 3.5. The effect of the initial concentration of nutrient on the inoculum growth was also investigated. Inoculums cultured in potato dextrose broth (PDB) medium with a half the strength of the common nutrient concentration of PDB qualitatively indicated good growth in terms of the size and density of cells. The inoculums with good morphological form were fed into the airlift bioreactor, which already contained a liquid medium with initial pH of 3.5 and also contained pre-treated bagasse. In experiments using the airlift bioreactor, the pre-treated bagasse was added to various nutrient concentrations of the PDB infusion medium. The highest bioethanol production from bagasse was monitored in the medium culture of half strength PDB infusion. The yield of bioethanol obtained from total sugarcane bagasse and PDB in an air lift bioreactor achieved approximately 40%, which has an infusion medium with a half-strength PDB and initial pH of 3.0. ©2020. CBIORE-IJRED. All rights reserved

Keywords: Bioethanol, Airlift Bioreactor, Sugarcane Bagasse, *Neurospora intermedia*, Pellet

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1. Introduction

The potential biomass waste from agriculture in Indonesia has reached approximately 146.7 million tons per year or equivalent to 470 GJ per year (Dani & Wibawa, 2018). So far, the use of biomass as fuel is still largely in the form of solid fuels such as briquettes or charcoal (Arhamsyah, 2010). However, the use of solid fuels can produce combustion products that have a negative impact on the environment and health (Clancy *et al* 2006). Therefore, further studies on the use of biomass waste to produce liquid or gas fuels that support clean technology development are still needed.

The agricultural waste, such as rice husk, sugarcane bagasse, oil palm empty fruit bunches, vetiver leaves, etc. can be utilized as potential feedstock for liquid fuel production (Bukhari *et al.* 2014; Madu & Agboola 2018; Restiawaty *et al.* 2018; Restiawaty *et al* 2019). Indonesia has more than 450 thousand hectares of sugarcane field area, which produces more than 2.4 million tons of sugar (Directorate General of Estate Crops, Indonesian Ministry of Agriculture, 2016). As consequence, it results in sugarcane bagasse as solid waste. Currently, the main utilization of sugarcane bagasse is as a solid fuel of boiler in the sugar factory (about 97.4% of total waste), but it still remains huge amount of unutilized sugarcane bagasse (about 0.3 ton per year). The pile of unutilized sugarcane bagasse may induce the natural fermentation, generates unpleasant odors, and may increase the ambient temperature (Yuliani & Nugraheni 2010).

The sugarcane bagasse contains potential carbon sources, consisting of approximately 35% cellulose, 24% hemicellulose, and 22% lignin (Rezende *et al.* 2011). Cellulose and hemicellulose have long carbohydrate

* Corresponding author: erestiawaty@che.itb.ac.id

chains, which can be hydrolyzed into fermentable sugars as substrates for bioethanol production. However, the utilization of sugarcane bagasse as a feedstock needs a proper pretreatment to remove lignin, which is built from nonfermentable phenol containing polymer. In this case, the alkali treatment could be used as an effective method to remove the lignin (Restiawaty & Dewi, 2017). Nevertheless, the degradation products from lignin and sugar could give a negative impact to the hydrolysis and fermentation processes, in which some of degradation products could inhibit those processes (Zha *et al,* 2012; Karp, 2010).

The sugarcane bagasse has been widely utilized in bioethanol production. In the previous study, the production of bioethanol from sugarcane bagasse using *S. cerevisiae* (Wahono *et al.* 2015; Ramadoss & Muthukumar, 2016) and hydrolysate-resistant *E.* coli (Geddes *et al,* 2011) required hydrolysis enzymes to convert sugarcane bagasse into glucose. In addition to the use of enzymes, the utilization of hydrolyzing compounds, such as inorganic acids is also often found to convert cellulose into glucose. Nevertheless, the inorganic acid application can produce the side products, such as furan, phenolic, and acetic acid, which are possible to inhibit the fermentation process (Chandel *et al.* 2006). Therefore, the utilization of *Neurospora intermedia* or *N. crassa* in the bioethanol production from sugarcane bagasse can highly open a breakthrough because both have ability to carry out the simultaneous saccharification and fermentation (SSF) process. The SSF process has been examined previously using biomass, such as wheat bran (Nair *et al.* 2015), thin stillage (Ferreira *et al.* 2015), sorghum bagasse (Dogaris *et al.* 2009), and brewer's spent grain (Xiros *et al,* 2008). Sugarcane bagasse contains a high amount of cellulose and hemicellulose, which are complex carbohydrate chains, the sugarcane bagasse becomes very potential as substrate for bioethanol production using *Neurospora sp.*

This study focused on the production of bioethanol from the pretreated sugarcane bagasse using *N. intermedia* in an airlift bioreactor. The airlift bioreactor was chosen to provide perfect aeration and mixing that facilitates better mass transfer. The airlift bioreactor allows more uniform flow pattern along the axial direction (Figure 1), so that the cells may undergo the bioprocessing in the homogenous condition (Kaewpintong *et al.* 2007). *N. intermedia* is categorized as ascomycete filamentous fungi and will be hardly applied in the airlift bioreactor if the mycelia grows rapidly. For this reason, the pellet form of *N. intermedia* was needed to get a good mass transfer in the airlift bioreactor.

The airlift bioreactors are widely studied in the production of bioethanol using yeast. Modified airlift was used by Vicente *et al.* (1999) to produce bioethanol from glucose with a productivity of 16 g/L/h. Airlift bioreactor was also used by Ghanadzadeg and Ghorbanpour (2012) to investigate the best aeration rate for bioethanol production from cheese-whey using *Kluyveromyces fragilis* yeast. The best aeration was obtained at a rate of 0.4 vvm to produce 3 %-w/v of bioethanol. Then, bioethanol was produced from first generation biomass in the form of grains using yeast in the airlift bioreactor (Ferreira *et al,* 2015). Some studies used the airlift bioreactors for fermentation using fungi. Lennartsson *et al.* (2010) have examined the effect of pretreatment of lignocellulosic biomass using N-methylmorpholine-N-oxide (NMMO) in the production of bioethanol in the airlift bioreactor. This study produced 136 mg of ethanol per 1000 mg of birch wood as the highest ethanol production obtained. Nair *et al.* (2016) used the airlift bioreactor to scale up the palletization process of *N. intermedia*. The use of the airlift bioreactor to produce bioethanol from second generation biomass with pellet-shaped fungi has not been much studied. The objective of this study was to produce bioethanol in an airlift bioreactor using *N. intermedia* by considering the effect of pH and nutrient concentration. Both variations were investigated to determine the desired pellet shape and to obtain the desired size and density of the inoculum.

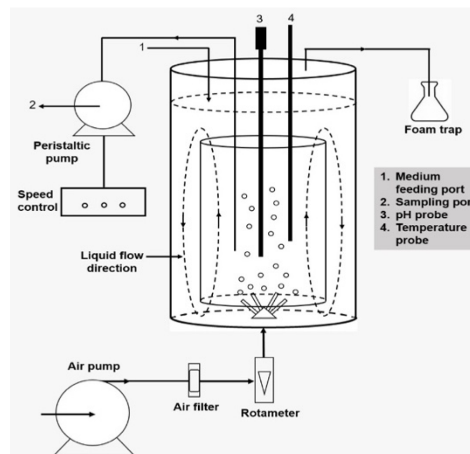

Fig. 1 Experimental setup of airlift bioreactor

2. Materials and Methods

2.1 Sugarcane bagasse pretreatment

The sugarcane (*Saccharum officinarum*) bagasse used as carbon source were obtained from sugarcane plantation, Bandung. The pretreatment of the sugarcane bagasse covered both physical and chemical treatments. The physical treatments included chopping and milling. The particle size of bagasse used in this study was in the range of 230 – 120 mesh or equivalent to 63 – 125 μm. Furthermore, modified alkali pretreatment (Mahamud and Gomes, 2012) was used as the chemical treatment of the biomass. The sugarcane bagasse was kept for 45 minutes at temperature of 60°C with 1% NaOH. After alkali treatment, the bagasse was neutralized using water and further dried at temperature of 60°C for 48 hours (Restiawaty *et al*., 2018).

2.2 Inoculum preparation

The ascomycete fungal strain of *N. intermedia* was obtained from Bioconversion Laboratory of Biosciences and Biotechnology Research Center, Institut Teknologi Bandung. The culture was maintained on potato dextrose agar (PDA) slants at 30°C incubator for 7 – 10 days. Inoculum preparation started from harvesting the spore solution of *N. intermedia.* The orange spore produced from the solid medium was flooded with physiological solution composed of NaCl 0.85% and Tween80. The spore solution of *N. intermedia* was checked using Spectrophotometer Genesys 10S UV-Vis at a wavelength of 660 nm. The spore

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solution was used as an inoculum if it has the optical density (OD_{660nm}) in the range of 1.0 – 1.2. The volume ratio of inoculum to the medium is 1:100 (v/v). The inoculum for airlift bioreactor was scaled up in two steps. The inoculum was cultured aerobically in 10 mL of potato dextrose broth (PDB) medium at room temperature for 24 h. The inoculum culture was then scaled-up in 100 mL of the potato infusion medium. Chloramphenicol was added for 1% of the total medium as the antibiotics. During incubation at room temperature, the culture was shaked at 155 rpm. The pellet of *N. intermedia* was obtained after the 48-hour scale-up.

2.3 Effect of initial pH and nutrient concentration

It was qualitatively observed the effect of nutrient concentration and *pH* medium to pellet formation of *N. intermedia*. The variations were made with PDB concentration and initial pH medium. The nutrient concentration was varied based on the PDB concentrations, i.e. 26.50 g/L (full strength PDB), 13.25 g/L (half strength PDB), and 6.63 g/L (quarter strength PDB) with the same initial pH 5.5. A full strength PDB contains 20 g/L glucose (dextrose) and 4 g/L infusion potatoes. The pellet formation (morphology of *N. intermedia*) was observed in different acid condition of medium, i.e. pH 2.5, 3.5, 4.5, and 5.5 in the same PDB concentration (half strength PDB).

2.4 Nutrient medium preparation for simultaneous saccharification and fermentation (SSF) in the airlift bioreactor

The nutrient medium used for initial fungal growth in bioreactor was potato infusion. Unpeeled potatoes were boiled in 2 L distilled water for 15 minutes. A certain concentration of glucose was subsequently added to the medium. The concentration of glucose depends on the strength of PDB concentration. The mixture of potato infusion and glucose was adjusted to pH 3.0 ± 0.1 by using 2 M of HCl. The compositions of potato infusion medium used in this research were (a) 200 g fresh potato/L and 20 g glucose/L (full strength PDB infusion); (b) 100 g fresh potato/L and 10 g glucose/L (half strength PDB infusion); (c) 50 g fresh potato/L and 5 g glucose/L (quarter strength PDB infusion).

2.5 SSF using airlift bioreactor

Internal loop airlift bioreactor was used in this research with working volume of 2.15 L from total 3 L volume and aeration rate of 1.6 vvm. This bioreactor was equipped with air pump, air filter, rotameter, sparger, pH probe, temperature probe, foam trap, port sampling, peristaltic pump, speed control, and draft tube that serves to help circulation or stirring static in bioreactor (see Fig. 1). Bioreactor was sterilized using an autoclave at 121°C for 25 minutes. The pretreated and sterilized sugarcane bagasse and the inoculum of *N. intermedia* were added to various nutrition concentration of the sterilized PDB infusion medium in the airlift bioreactor. Some drops of grapeseed oil were added as an antifoam agent to avoid foam formation during fermentation. This cultivation mixture was incubated at room temperature. The aeration was done in the first 24 h of cultivation to accelerate *N. intermedia* growth. The aeration was stop after 24 h cultivation and then the bioreactor was flowed with nitrogen gas for 15 min. The operation of bioreactor was then kept unaerated to alter the process from aerobic to semi-anaerobic growth condition.

2.6 Analysis

The sugar and bioethanol concentrations were analyzed using High Performance Liquid Chromatography (HPLC). A column of the resin ionic form of hydrogen (Phenomenex, Rezex ROA – Organic Acid) was used in series with a refractive index (RI) detector (Waters 2414). The process was conducted at 60°C with 0.005 M H_2SO_4 0.6 mL/min as the mobile phase (Xiros et al. 2008).

3. Results and Discussion

3.1 Pretreatment of sugarcane bagasse

The biomass pretreatment is necessary to increase the surface area, to separate cellulose from other lignocellulosic components, and to alter the structure of biomass to be easily hydrolyzed (Bezerra and Ragauskas, 2016). Previous research has been conducted for the effect of biomass pretreatment on the bioethanol yield. An appropriate pretreatment method for certain lignocellulosic biomass showed high bioethanol yield (Restiawaty and Dewi, 2017). Maryana *et al.* (2014) reported that pretreatment of sugarcane bagasse using alkali method resulted in high content of cellulose, i.e. 56-66%.

In this study, the addition of 1% NaOH caused an increase in cellulose content from 25.3% (before pretreatment) to 54.0% (after alkali pretreatment). Other compounds such as lignin and hot water soluble (HWS) were dissolved during the alkali pretreatment process (Gregg and Saddler, 1996), so that both compositions were reduced.

3.2 Effects of initial pH and PDB strength on inoculum morphology

Liao et al. (2007) and Ward (2012) reported that filamentous fungi in submerged culture might grow in different morphological forms, such as mycelial clumps or as pellets. The SSF carried out in an airlift bioreactor requires the morphological form of *N. intermedia* that can be circulated through the draft tube. This circulation process would give a good mass transfer condition. The suitable morphological form of *N. intermedia* for SSF process in an airlift bioreactor is pellets. The pellet formation of *N. intermedia* was strongly affected by pH. Nair *et al.* (2016) reported that the morphology of *N. intermedia* was perfectly pelletized in the PDB medium at pH 3.0 – 4.0 without the presence of lignocellulosic biomass.

This research investigated the appropriate pH medium to obtain a good inoculum pellets be applied to the SSF process in an airlift bioreactor that contains lignocellulosic biomass. The results in Fig. 2 indicated that the morphology of *N. intermedia* at pH 3.5 gave the good pellet shape and size. The formation of pellet also occurred in medium with pH 4.5 and 5.5, but the size of pellets was very small, and the cells were very dense.

The effects of initial nutrient concentration of the PDB infusion medium on inoculum growth is qualitatively

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presented in Fig. 3. Higher cell density and pellet diameter were obtained at higher carbon source concentrations. Full strength medium gave the biggest diameter of pellet, however it was slightly different than the diameter of pellets in half strength medium. Quarter strength medium have more clearly distinction than the other two variations. The pellets formed were less dense which indicates that the nutrients available in the media cannot produce more biomass cells.

The initial nutrient concentration also affected the yield of bioethanol. Fig. 4 depicts the bioethanol yield from pretreated bagasse using *N. intermedia* in 250 mL Erlenmeyer flask with various of PDB infusion strength. Bioethanol yield is defined as the mass percentage of bioethanol produced to the theoretical mass of bioethanol. The highest bioethanol yield was achieved from the cultivation using half strength PDB infusion. It gave about 44% of bioethanol yield.

3.3 SSF of bagasse using N. intermedia in the airlift bioreactor

The pellets of *N. intermedia* inoculum were introduced in SSF systems of air lift bioreactor. The filtered air was introduced from the bottom of bioreactor, which allowed the pellets and bagasse in medium to circulate through the draft tube inside the bioreactor. This circulation process would provide a good contact between the cells and bagasse and other nutrients in the medium. Bioethanol production was checked every 24 h in 96 h cultivation.

The maximum bioethanol concentration reached in different time of incubation for each PDB infusion strength (see Fig. 5). Full strength PDB infusion had the maximum bioethanol concentration after 96 h cultivation. The bioethanol concentration was 2.70 g/L or equivalent to about 16% of bioethanol yield. The SSF airlift system with a half strength PDB infusion medium produced bioethanol with concentration of 4.17 g/L (equivalent to 41% of bioethanol yield) in 72 h cultivation. Meanwhile, the use of PDB infusion medium with a quarter strength gave the maximum peak of bioethanol production from bagasse faster than the higher PDB strength. However, only about 0.98 g/L of bioethanol was detected as the maximum concentration that occurred in 24 h of cultivation.

The distinct pattern of this variation is shown from the trendline of bioethanol formation and depletion in Fig. 5. The quarter strength PDB infusion medium had a consistent trend result of decreasing bioethanol concentration for the entire incubation. The half strength PDB infusion medium showed increasing trend until 72 h of incubation and declining bioethanol production at the end of fermentation. Meanwhile, the full strength PDB infusion medium did not show any significant bioethanol production from the beginning to the end of incubation. The quarter strength PDB infusion medium had a lower bioethanol production (Fig. 5).

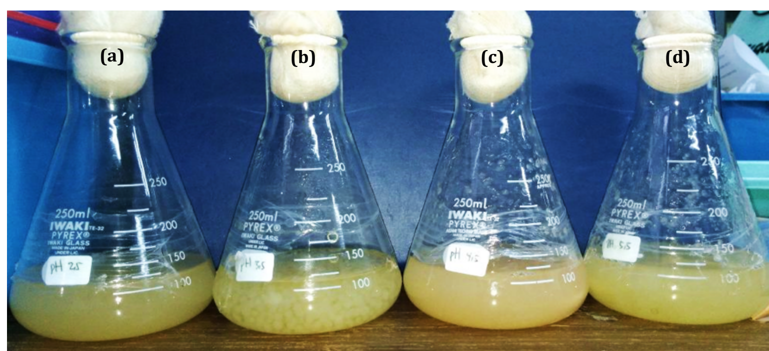

Fig. 2 The pellet formation of *N. intermedia* from various *pH*s of medium, i.e. (a) 2.5; (b) 3.5; (c) 4.5; and (d) 5.5.

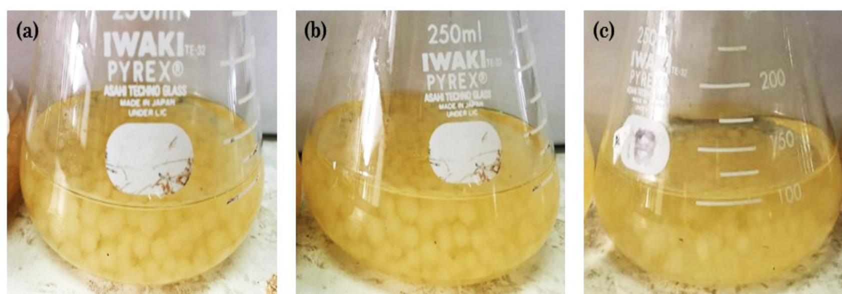

Fig. 3 The morphology of *N. intermedia* after 48 h cultivation in various nutrition concentration of PDB: (a) full strength, (b) half strength, and (c) quarter strength.

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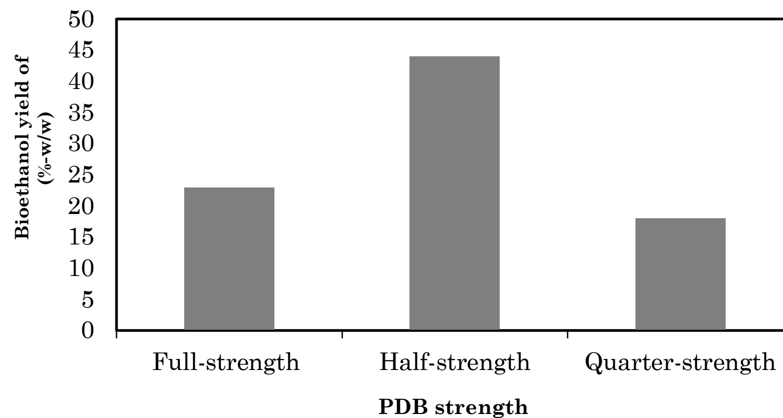

Fig. 4 Bioethanol yield from pretreated bagasse using *N. intermedia* in 250 mL Erlenmeyer flask with various of PDB strength. The incubations were done in room temperature for 24 h.

Bioethanol concentration in the half strength PDB infusion medium was higher than the quarter strength medium. It was induced that bioethanol was included in growth-associated product. With higher concentration of carbon source in the beginning of the half strength PDB infusion medium, more biomass of *N. intermedia* could be formed. Despande *et al.* (1986) described that the rate of bioethanol production could depend on the rate of glucose production from cellulose degradation by the cellulase enzyme activity. Higher *N. intermedia* biomass effects in higher enzyme was present to support bioethanol production.

In the quarter strength PDB infusion medium, decreasing of bioethanol concentration might be caused by limitation of *N. intermedia* biomass that resulted in low glucose availability at the beginning of fermentation as the effect of the low rate of cellulose and hemicellulose hydrolysis. In the half strength PDB infusion, after the 72 h of incubation, bioethanol concentration dropped in the presence of sufficient glucose concentration. Bioethanol could be toxic in high concentration, whereas the degradation of bioethanol into acetic acid is one of the possible defense mechanism of *N. intermedia* to maintain their ambient condition. *N. intermedia* has ADH1 (alcohol dehydrogenase 1) and ADH3 (alcohol dehydrogenase 3) genes to synthesize the alcohol dehydrogenase enzyme, the key enzyme in bioethanol production and utilization. The enzyme could also act as a biocatalyst in ester carboxylic production from alcohol and aldehyde, for example to produce acetic acid (Park *et al.* 2007). In the half strength PDB infusion medium, ethanol decrease occurred after 72 h of incubation from 4.17 g/L to 3.07 g/L (Fig. 5), while the acetic acid was observed to increase from 0.07 g/L to 0.10 g/L. The subsequent reaction of metabolic is the conversion of acetic acid into acetyl co-A before entering the TCA (tricarboxylic acid) cycle. As aforementioned, it can be concluded that the bioethanol could be consumed as carbon source for *N. intermedia* growth when concentration of the fermentable sugar was limited. In full strength PDB infusion medium, the presence of glucose was more exploited for cell growth than bioethanol production so that the concentration of bioethanol was lower than biomass growth.

In both type of reactors of flask and airlift, the half strength medium provided the highest bioethanol production. However, bioethanol production in Erlenmeyer flask was slightly higher than that of produced in airlift bioreactor. In order to achieve the same product concentration, a longer fermentation time was needed for large-scale bioreactor compared to small-scale one. Because of the longer fermentation time, the pH of the airlift bioreactor media changed, and the shape of the pellet was difficult to maintain. Some fungus stuck to the bioreactor wall when the incubation time was more than two days. This caused an increase in mass transfer barriers.

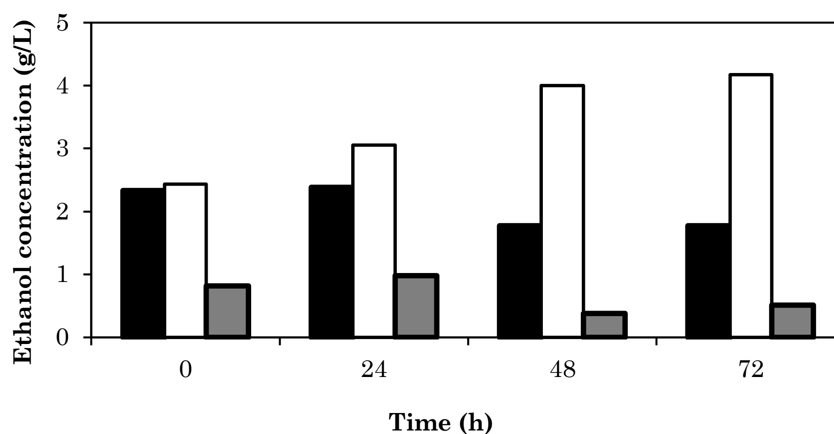

Fig. 5 Concentration of bioethanol production in different PDB infusion medium concentration: ■ full strength medium; □ half strength medium; ■ quarter strength

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4. Conclusion

The pellets of *N. intermedia* were well developed at a medium pH of 3.0 – 3.5. The half strength PDB infusion medium provided a good pellet density for pellet circulation in the airlift bioreactor. The use of PDB medium was needed to accelerate the growth of *N. intermedia*. However, the PDB concentration can affect the growth of *N. intermedia* cells. It has been proven that with a reduced PDB concentration can cause *N. intermedia* growth to be smaller. However, the half strength PDB infusion medium was a proper PDB concentration for bioethanol production in Erlenmeyer flask and airlift bioreactor. Approximately 41% of bioethanol yield was obtained from total carbon sources of sugarcane bagasse and PDB. For further research, the mass transfer study and scaling up the fungal cultivation for industrial applications using airlift bioreactor were recommended to be investigated.

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