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Solid-State Fermentation of Palm Kernel Cake with *Aspergillus flavus* in Laterally Aerated Moving Bed Bioreactor

Yoke Phooi Wong · Horng Yuan Saw · Jidon Janaun · Kamatam Krishnaiah · Auti Prabhakar

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Abstract Solid-state fermentation (SSF) was employed to enhance the nutritive values of palm kernel cake (PKC) for poultry feeding. *Aspergillus flavus* was isolated from local PKC and utilized to increase the mannose content of PKC via the degradation of β-mannan in PKC; evaluation was done for batch SSF in Erlenmeyer flasks and in a novel laterally aerated moving bed (LAMB) bioreactor. The optimum condition for batch SSF in flasks was 110% initial moisture content, initial pH 6.0, 30 °C, 855 μm particle size, and 120 h of fermentation, yielding 90.91 mg mannose g^{-1} dry PKC (5.9-fold increase). Batch SSF in the LAMB at the optimum condition yielded 79.61 mg mannose g^{-1} dry PKC (5.5-fold increase) within just 96 h due to better heat and mass transfer when humidified air flowed radially across the PKC bed. In spite of a compromise of 12% reduction in mannose content when compared with the flasks, the LAMB facilitated good heat and mass transfer, and improved the mannose content of PKC in a shorter fermentation period. These attributes are useful for batch production of fermented PKC feed in an industrial scale.

Keywords *Aspergillus flavus* · Bioreactor · Laterally aerated moving bed · Palm kernel cake · Poultry feed · Solid-state fermentation

Introduction

Palm kernel cake (PKC) is the fibrous material produced as by-product in the extraction of palm kernel oil from kernels of oil palm (*Elaeis guineensis*). PKC has been used as energy

Y. P. Wong · J. Janaun (✉) · K. Krishnaiah · A. Prabhakar
Chemical Engineering Program, School of Engineering and Information Technology,
Universiti Malaysia Sabah, Locked Bag No. 2073, 88999 Kota Kinabalu, Sabah, Malaysia
e-mail: jidon@ums.edu.my

H. Y. Saw (✉)
School of Engineering and Advanced Technology, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand
e-mail: sawhorngyuan@gmail.com

and compound feed for ruminants and mono-gastric animals because it is palatable, aflatoxin free, and contains considerably high protein level at 16–18% [1–4]. PKC also possesses chemical composition that is comparable to established feed like corn gluten and rice bran [5]. The inclusion of PKC in the diets of mono-gastric animals, particularly poultry, is however limited because PKC contains high content of indigestible fiber and is contaminated by shell of palm nut, resulting in grittiness. The indigestible portions are actually non-starch polysaccharides (NSP) comprising 78% β-mannan, 12% cellulose, 3% arabinoxylans, and 3% water-insoluble glucoxylans [4]. These characteristics are unfavorable to poultry, causing low palatability and indigestion. As a result, the inclusion of PKC in poultry diet has never been more than 40%, lest it will cause adverse effect to poultry [4, 6].

The strategy to increase the inclusion of PKC in poultry diet is to break down or degrade the NSP, particularly β-mannan, using enzymatic (β-mannanase) treatment, yielding simple carbohydrates or digestible sugars like mannose which could be easily absorbed and metabolized by poultry [4, 7]. Another alternative is through solid-state fermentation (SSF), a bioconversion technology in which microorganisms grow on solid substrates containing a certain level of water, degrading the substrates through production of enzymes and altering the chemical and physico-chemical properties of the substrates as the microbial growth and metabolism progress [8–10]. The choice of microorganisms generally concentrates on filamentous fungi due to their ability to grow completely on substrates and produce various extracellular enzymes [11]. Fungal strains that have been reported in the SSF of PKC include *Aspergillus niger* [9, 12], *Sclerotium rolfsii* and *Trichoderma* spp. [9], and *Aspergillus flavus* [2]. Most recently, a fungal strain had been isolated from PKC itself, and this strain was able to increase the reducing sugar content and decrease the hemicellulose (β-mannan) content of PKC when fermented in a novel laterally aerated moving bed (LAMB) bioreactor [13] and a fluidized bed bioreactor [14]. Upon identification with a Biolog (Biolog Inc., USA) procedure and database for filamentous fungi, this strain was designated *Aspergillus flavus* UMS01 [15].

This paper reports a study on the optimization of operating parameters on the SSF of PKC in Erlenmeyer flasks with *A. flavus* UMS01. The effects of initial moisture content, initial substrate pH, temperature, and substrate particle size on SSF were evaluated with the monitoring of microbial growth (biomass production). The quality of fermented PKC as poultry feed was appraised in terms of reducing sugar (mannose equivalent) production. Subsequently, for industrial application evaluation, the SSF of PKC was assessed in the LAMB bioreactor at the optimized condition with different superficial velocities of humidified air.

Materials and Methods

Substrate Preparation

PKC was obtained from a palm oil refinery (IOI Edible Oils Sdn. Bhd.) in Sandakan, Sabah, Malaysia. For SSF optimization study, PKC was sieved through 1.00-mm, 0.71-mm, 0.60-mm, and 0.425-mm standard mesh sieves (BS 410) to obtain mean particle sizes of 855 μm, 655 μm, and 513 μm. For SSF in LAMB, the particle size was <5 mm. Sieved PKC was oven-dried at 80 °C until constant weight (~24 h) and cooled to room temperature (30 °C) in desiccators filled with silica gels.

Cultivation of Microorganism

For SSF optimization study, *A. flavus* UMS01 was grown and maintained on potato dextrose agar (PDA) (Merck, USA) slants prepared in universal bottles at 30 °C and sub-cultured every month. Spores were harvested by adding 10 mL of sterilized distilled water containing 0.1% Tween 80 (Merck) to a sporulated (7-day-old) PDA. The bottle was shaken manually for 5 min to suspend the spores. The concentration of spores in the suspension was estimated with a series of dilution and plate count method. Then 0.1 mL of each dilution was pipetted and spread onto a PDA agar plate, and the plates were inverted and incubated at 30 °C for 16 h. Five replicates were done and the average spores count was 2×10^7 spores mL^{-1}. As for SSF in LAMB, the PDA slants were prepared in 500-mL Blue Cap bottles, and 300 mL of sterilized distilled water containing 0.1% Tween 80 was used to harvest a sporulated (7-day-old) PDA.

SSF of PKC in Erlenmeyer Flasks

Five grams of dried PKC in 200-mL Erlenmeyer flask was moistened with a desired amount of mineral medium (2 gL^{-1} KH_2PO_4, 5 gL^{-1} NH_4NO_3, 1 gL^{-1} NaCl, and 1 gL^{-1} $MgSO_4{\cdot}7H_2O$) and NaOH (1 M) to adjust to a required initial moisture content and initial pH. The flask opening was stuffed with cotton wool and covered with aluminum foil, and the flask was autoclaved at 121 °C for 15 min. Upon cooling down to room temperature, the sterilized flask content was inoculated aseptically with 1 mL (20% inoculum) of fungal spore suspension (2×10^7 spores mL^{-1}), mixed for 1 min with a vortex machine (IKS Works, USA), and incubated at a required temperature for 6 days in an incubator (Memmert, Germany). Three flasks (as triplicate) were removed from the incubator at each sampling time and were subjected to immediate analysis.

Optimization of SSF Parameters

The optimization followed a one-factor-at-a-time strategy (see for example Sabu et al. [16]), where evaluation on the effect of each parameter was done independently by keeping all other parameters constant. The optimized parameter was then incorporated into the next level of optimization. The order and range of parameters optimized were as follows: initial moisture content of substrate at 50–120% volume of water to sample weight, initial pH of substrate at 5–7, incubation temperature at 25–40 °C, and PKC particle size at 513–855 μm.

SSF of PKC in LAMB Bioreactor

The LAMB was developed in-house (Fig. 1). The bioreactor consisted of a perforated acrylic packed bed column with a free area of 15% (2 mm perforation diameter), 95 mm internal diameter and 855 mm in height. A perforated distributor pipe with a free area of 15% (1 mm perforation diameter) was located in the center of the column and was used for uniform distribution of humidified air introduced at the top of the bioreactor. The generation of humidified air was by passing dry air from a compressor (DIDATEC Technologie, France) through a humidifying column, and the relative humidity of air was monitored with a thermo-hygrometer (Radiance Industrial, China) and kept constant at 85±2%.

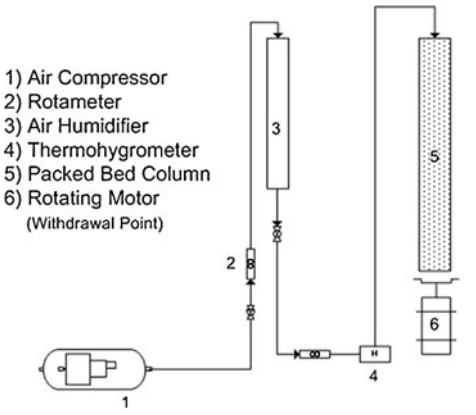

Fig. 1 Schematic diagram of laterally aerated moving bed (LAMB) bioreactor

The LAMB can perform batch and continuous SSF [13]. In this study, only batch SSF was performed. Dried PKC (1.5 kg) was moistened with a desired amount of mineral medium and NaOH (1 M) and autoclaved at 121 °C for 20 min. Upon cooling to room temperature in a laminar flow (ERLA, Malaysia), 20% of spore suspension was added little by little to the PKC and mixed thoroughly for about 10 min. The substrate was then filled into the LAMB column that was pre-sterilized with Dettol solution. Approximately 2 g of substrate was taken out from each of the five sampling points (height to diameter ratio=0.9, 2.8, 4.7, 6.6, and 8.5) of the column at an interval time of 24 h. The sample from each point was analyzed for reducing sugar, biomass, and moisture content, and the average value was used. Temperature of the substrate bed was recorded everyday by inserting a mercury thermometer into each sampling points, and the average value was used. The values for reducing sugar and biomass at different sampling locations were found to vary between 3% and 10%, moisture content between 10% and 20%, and temperature between 2 and 3 °C [15]. Three different superficial air velocities (4.4, 13.1, and 21.8 ms^{-1}), controlled by an air flow meter (Cole-Parmer Instrument Co., USA), were used to investigate the effect of aeration on heat transfer and bioconversion of PKC. The SSF was terminated after 120 h. SSF in LAMB was performed in duplicate.

Analysis on Fermented PKC

Reducing sugar (mannose equivalents) was estimated by dinitrosalicylic acid (Sigma, USA) method [17]. The color developed was read at 575 nm using a Novaspec II spectrophotometer. Mannose (Sigma) was used as standard.

Fungal biomass was estimated by determining *N*-acetyl glucosamine released by acid hydrolysis of chitin that was present in the cell wall of fungi [18]. Dried fermented PKC (0.5 g) was mixed with 2 mL of concentrated H_2SO_4 (Lab-Scan, Poland) at 30 °C for 24 h. The mixture was diluted to a 1-N solution with distilled water, autoclaved (121 °C; 15 min), neutralized with 10 N and 1 N NaOH (Mallinckrodt, USA), and made to 100 mL with distilled water. One milliliter of the solution was mixed with 1 mL acetyl acetone reagent (R&M Chemicals, Canada) and incubated in a boiling water bath (Memmert) for 20 min. After cooling, 6 mL of ethanol (95%) (KSS, Malaysia) was added, followed by the addition of 1 mL Erhlich's reagent (R&M Chemicals), and the mixture was incubated at 65 °C for 10 min. After cooling, the optical density of the reaction mixture was read at 530 nm with

the Novaspec II spectrophotometer against a reagent blank. The reagent blank was prepared from PKC without inoculum. Glucosamine (Sigma) was used as standard. The result was expressed as milligrams of glucosamine per gram of dry PKC.

For analysis on moisture content of fermented PKC in LAMB, oven-drying method at 100 °C for 24 h was used [14].

Results and Discussion

Effect of Initial Moisture Content

The first parameter optimized was initial moisture content (MC) at constant initial pH 7, particle size 855 μm, and 30 °C. MC is a fundamental parameter for mass transfer of water and solutes across substrate and microbial cells. Controlling or applying different levels of MC could modify the growth and biosynthesis of different metabolites [8, 11, 19]. Figure 2a and b shows the effect of initial MC on *A. flavus* UMS01 biomass and production of reducing sugar in Erlenmeyer flasks, respectively. Generally, both biomass and reducing sugar increased with increasing MC. The growth profiles during SSF were typical batch growth curves, which comprised lag phase, exponential phase, and stationary phase. The

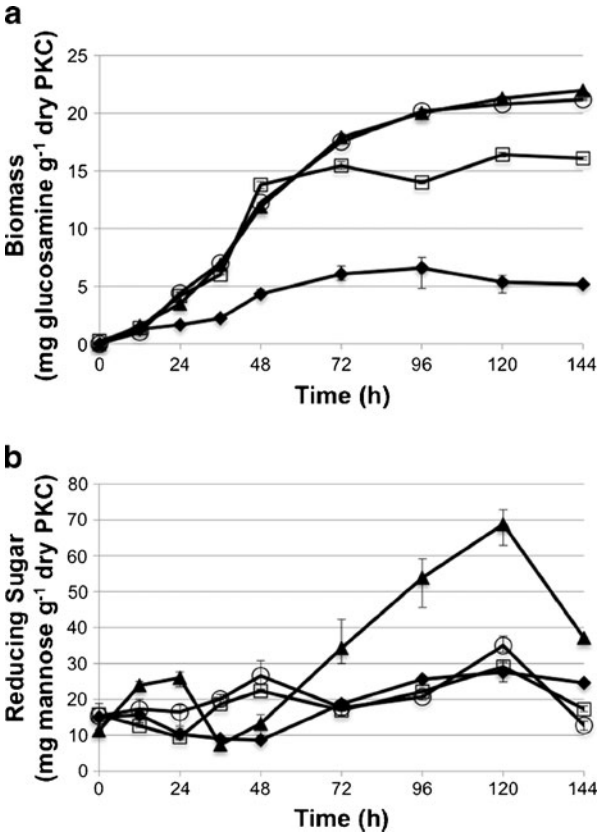

Fig. 2 Effect of initial moisture content on **a** fungal biomass and **b** reducing sugar in SSF of PKC in Erlenmeyer flasks at 20% inoculum, pH 7, 30 °C, and 855 μm PKC; 50% MC (*filled diamonds*); 100% MC (*open squares*); 110% MC (*filled triangles*); 120% MC (*open circles*). Results are mean of triplicate (n=3) with custom *error bars*

lag phase varied with different moisture contents. At 50% MC, the lag phase lasted for 48 h. At 110% and 120% MC, the lag phase was less than 12 h. The fungal growth then increased exponentially and generally entered the stationary phase after 120 h. However, it was observed that for 110% and 120% MC, fungal biomass continued to increase slightly after 120 h. The highest reducing sugar (68.78 mg mannose g^{-1} dry PKC) was produced at 110% MC and 120 h, corresponding to a biomass of 21.27 mg glucosamine g^{-1} dry PKC.

At optimum MC (110%), it was observed that *A. flavus* UMS01 germinated and grew all over the PKC particles. To support this growth, β-mannan (the main NSP of PKC) had to be degraded by β-mannanase produced by the fungus. Using water as mass transfer medium, the enzyme diffused into the PKC matrix and catalyzed the degradation of β-mannan into reducing sugars. The sugars were then consumed as nutrient by the fungus for growth and metabolites synthesis. At the optimum moisture content, PKC particles also swelled better, increasing the packing density of particles and possibly surface area to volume ratio, hence promoting better inter-particle oxygen diffusion for microbial growth and activity [20–22]. At 50% MC, low moisture level reduced the diffusion of enzyme and nutrients and resulted in poor β-mannan degradation and accessibility of mannose for fungal growth [8, 11]. The swelling of PKC was also lesser, reducing inter-particle oxygen diffusion [20–22]. On the contrary, flooding of inter-particle substrate space by water could have occurred at 120% MC, creating an adverse environment for oxygen transfer and proliferation of fungal mycelium [11, 19], leading to lower growth and mannose production.

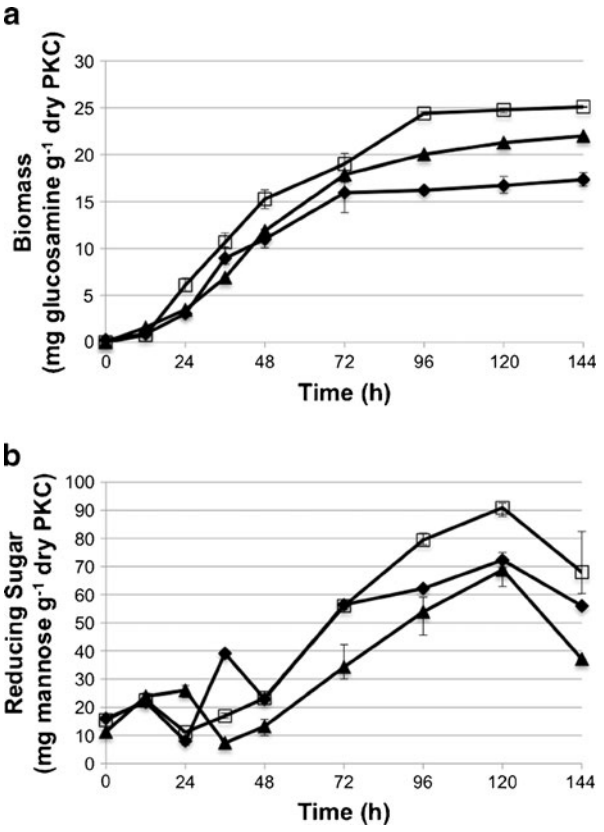

Fig. 3 Effect of initial pH on **a** fungal biomass and **b** reducing sugar in SSF of PKC in Erlenmeyer flasks at 20% inoculum, 110% MC, 30 °C, and 855 μm PKC; pH 5 (*filled diamonds*); pH 6 (*open squares*); pH 7 (*filled triangles*). Results are mean of triplicate (n=3) with custom *error bars*

Effect of Initial pH

Figure 3a and b shows the effect of initial pH on *A. flavus* UMS01 biomass and production of reducing sugar in Erlenmeyer flasks, respectively, at constant MC (110%), particle size (855 μm), and temperature (30 °C). The growth profiles were typical batch growth curves with short lag phase (12 h). The curve at pH 5 exhibited the shortest exponential phase (12–72 h), followed by the pH 6 curve (12–96 h). The fungal growth at pH 6 continued to increase slightly after 96 h. At the same pH, maximum reducing sugar of 90.91 mg mannose g^{-1} dry PKC was produced at 120 h.

Initial pH is an important environment parameter for SSF because extracellular enzymes produced could be stable at a certain pH and rapidly denatured at a lower or higher pH [23], affecting microbial growth and product formation. Initial pH 6 was adapted for subsequent optimization. This slightly acidic condition could also minimize bacterial contamination [11].

Effect of Temperature

Figure 4a and b shows the effect of temperature on *A. flavus* UMS01 biomass and production of reducing sugar in Erlenmeyer flasks, respectively, at constant 110% MC, initial pH 6, and particle size 855 μm. Batch growth curves were observed with a general lag phase of 12 h. The optimum temperature for fungal growth was between 30 °C and 35 °C, with a maximum

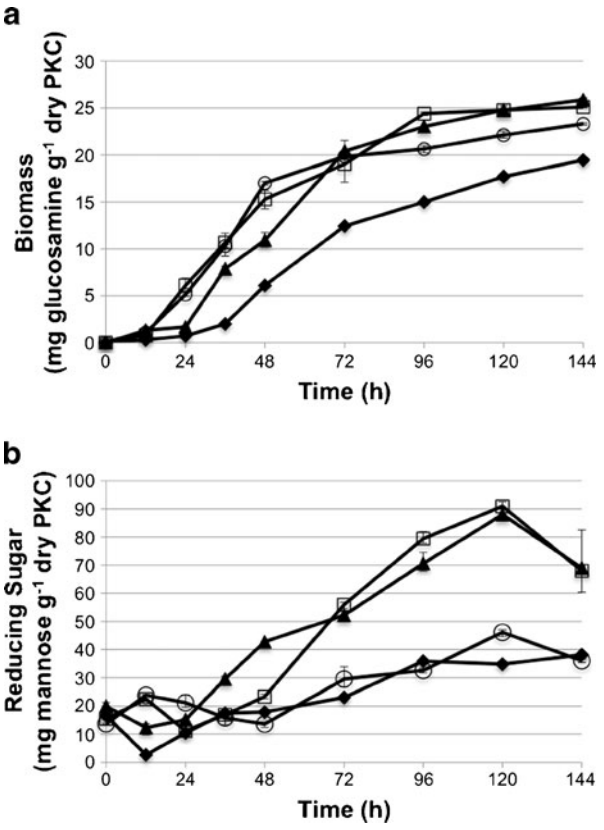

Fig. 4 Effect of temperature on **a** fungal biomass and **b** reducing sugar in SSF of PKC in Erlenmeyer flasks at 20% inoculum, 110% MC, pH 6, and 855 μm PKC; 25 °C (*filled diamonds*); 30 °C (*open squares*); 35 °C (*filled triangles*); 40 °C (*open circles*). Results are mean of triplicate (n=3) with custom *error bars*

growth of 25.09–25.86 mg glucosamine g^{-1} dry PKC at 144 h. However, the highest mannose yield was 90.91 mg mannose g^{-1} dry PKC achieved at 30 °C and 120 h. At 40 °C, heat could have denatured β-mannanase, resulting in lower conversion of β-mannan to mannose and directly affecting the fungal growth [8, 19].

Effect of Particle Size

Figure 5a and b shows the effect of particle size on *A. flavus* UMS01 biomass and production of reducing sugar in Erlenmeyer flasks, respectively, at constant 110% MC, initial pH 6, and 30 °C. The fungal growth curves were typical batch profiles. The highest biomass was achieved after 96 h, and the highest mannose yield was at 120 h, both with 855 μm PKC. This was because 855 μm PKC swelled more, resulting in higher surface area to volume ratio and packing density of particles, hence better gas exchange for fungal growth and activity. As particle size decreased, there was lower surface area to volume ratio and void space between particles, causing adverse effects to the SSF performance [20–22].

Optimum Parameters and Fermentation Period for SSF of PKC

The optimum condition for SSF of PKC with *A. flavus* UMS01 in Erlenmeyer flasks was 110% MC, initial pH 6, 30 °C, and with 855-μm particles. The lag phase for fungal growth

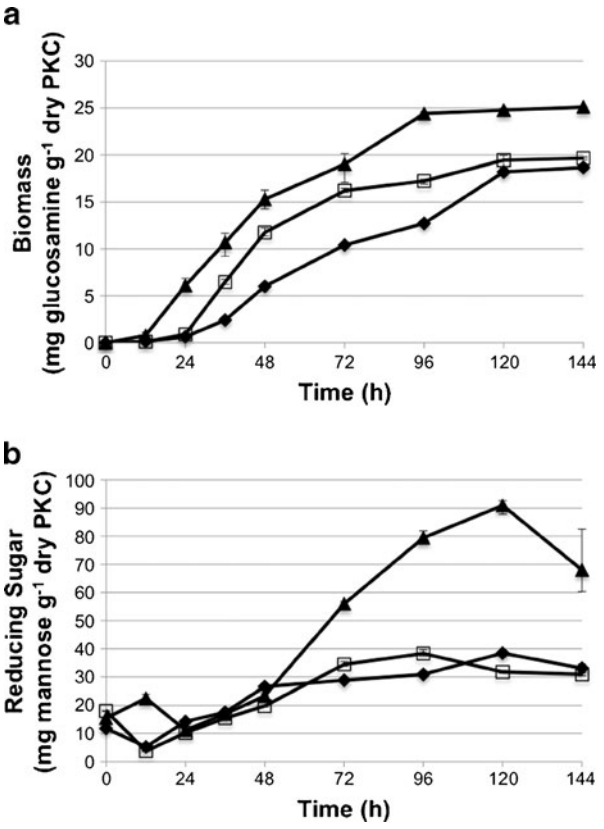

Fig. 5 Effect of particle size on **a** fungal biomass and **b** reducing sugar in SSF of PKC in Erlenmeyer flasks at 20% inoculum, 110% MC, pH 6, and 30 °C; 513 μm (*filled diamonds*); 655 μm (*open squares*); 855 μm (*filled triangles*). Results are mean of triplicate (n=3) with custom *error bars*

curve was 12 h, the exponential phase was from 12 h to 96 h, and the stationery phase from 96 h to 144 h. In the lag phase, there was only about 46% increase in reducing sugar (PKC originally contained 15.44 mg mannose g^{-1} dry PKC). This was strongly due to the availability of the initial reducing sugar that had caused carbon catabolite repression to the fungus, preventing the synthesis of enzymes for substrate utilization [24], which was β-mannanase for mannan degradation. Upon entering the exponential phase, the reducing sugar decreased sharply, indicating the utilization of the available sugar by the fungus to support its exponential growth. Once the reducing sugar content became limiting, the fungus began to produce β-mannanase slowly to break down mannan into reducing sugar, and the sugar content increased gradually. The reducing sugar production peaked at 120 h, giving a 5.9-fold increase from 15.44 mg mannose g^{-1} dry PKC to 90.91 mg mannose g^{-1} dry PKC. Beyond 120 h, the reducing sugar level dropped while fungal growth maintained, indicating that the metabolizable nutrient in the substrate was deteriorating, and the fungus was consuming the reducing sugar again.

SSF of PKC in LAMB Bioreactor

The SSF of PKC was performed in the LAMB bioreactor at the optimized parameters obtained from flask SSF for industrial application evaluation. However, particle size of <5 mm was used because the particle size distribution of PKC had shown that there was only about 11% of 855-

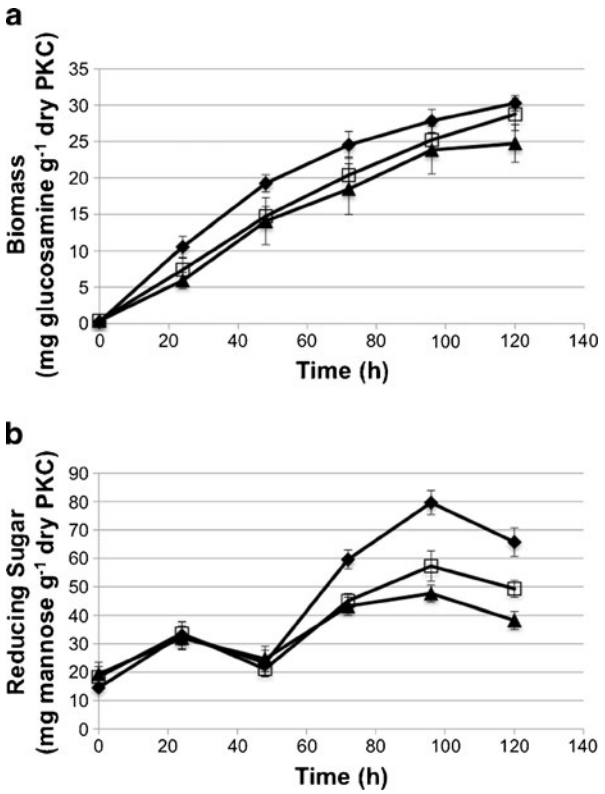

Fig. 6 Effect of superficial air velocity on **a** fungal biomass and **b** reducing sugar in SSF of PKC in LAMB bioreactor at 20% inoculum, 110% MC, pH 6, 30 °C, and <5 mm PKC; 4.4 m s^{-1} (*filled diamonds*); 13.1 ms^{-1} (*open squares*); 21.8 ms^{-1} (*filled triangles*). Results are mean of five determinations in duplicate (n=2) with custom *error bars*

μm particles [22]. In industrial practice, it would be more sensible to use a range of particle size instead of just one particle size with low fraction.

Figure 6 shows the effect of superficial air velocity on the SSF of PKC by *A. flavus* UMS01 in LAMB at 110% MC, initial pH 6, 30 °C, and with <5-mm particles. The fungal growth profiles in LAMB were different from the profiles in Erlenmeyer flasks. No lag phase was observed. The exponential phase started immediately and lasted until 96 h. From then on, there was sign of stationary phase. The biomass decreased with increasing

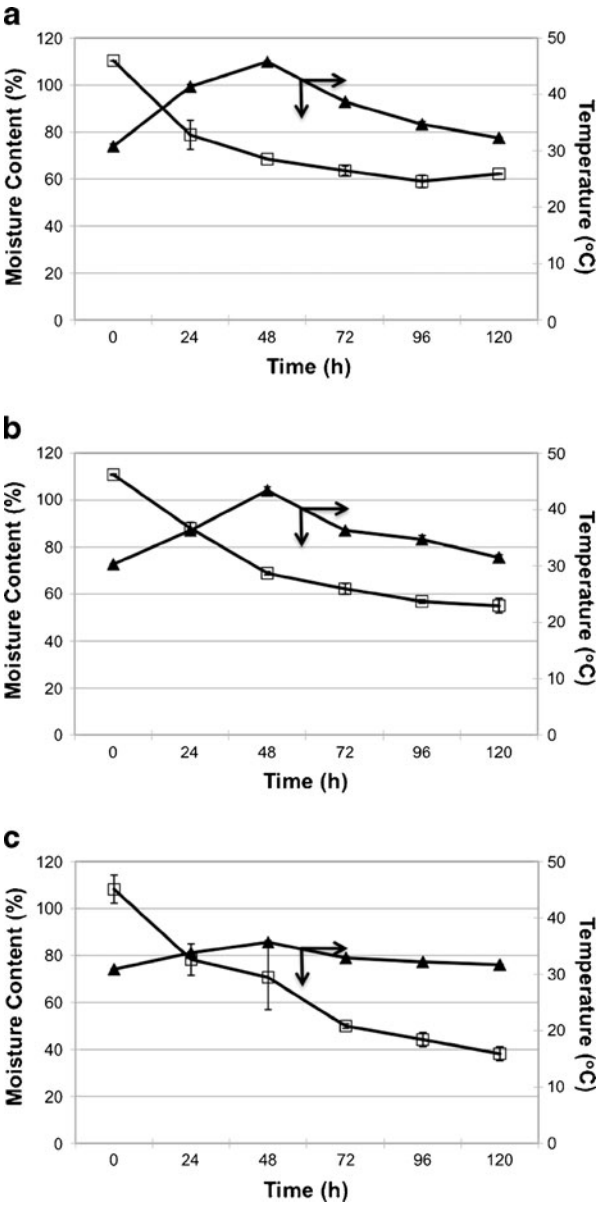

Fig. 7 Effect of **a** 4.4 ms^{-1}, **b** 13.1 ms^{-1}, and **c** 21.8 ms^{-1} superficial air velocity on bed moisture content and temperature in LAMB bioreactor at 20% inoculum, 110% MC, pH 6, 30 °C, and <5 mm PKC. Results are mean of five determinations in duplicate (n=2) with custom *error bars*

superficial air velocity, with the highest biomass of 30.28 mg glucosamine g^{-1} dry PKC achieved at 120 h with 4.4 ms^{-1} gas flow. Similarly, reducing sugar decreased with increasing superficial air velocity. There was an increase and drop of sugar yield within 48 h, indicating the consumption of sugar for exponential phase growth. Once the reducing sugar was limiting at 48 h, β-mannanase was produced to degrade mannan into mannose, and mannose increased again and peaked at 96 h, with a yield of 79.61 mg mannose g^{-1} dry PKC at 4.4 ms^{-1}.

Figure 7 shows the effect of superficial air velocity on the PKC bed moisture content and temperature in the LAMB. At 4.4 ms^{-1} gas flow, the bed temperature rose from ambient temperature to 41 °C after 24 h, and 46 °C after 48 h, and the moisture content dropped from 110% to 69% at 48 h. The rise in temperature was due to the generation of metabolic heat resulting from exponential phase growth. The drop in moisture was due to evaporation of water through metabolic heat evolution compounded by forced aeration, water consumption and liberation through fungal metabolism, and rapid uptake of water by growing spores [8, 14, 19]. After 48 h, the temperature and moisture content dropped and reached 32 °C and 62%, respectively, at 120 h.

At 21.8 ms^{-1} superficial air velocity, the bed temperature rose from 30 °C to 36 °C only (10 °C lower than that at 4.4 ms^{-1} air velocity) at 48 h, while the substrate moisture content dropped from 110% to about 71%. Within the next 48 h, the moisture content was further reduced to 44%. When SSF was terminated at 120 h, the bed temperature was 32 °C, similar to that at 4.4 ms^{-1}, and the bed contained 38% moisture, 24% lower than that at 4.4 ms^{-1}. The increase in superficial air velocity led to higher water evaporation, and this cooled the bed better due to very high latent heat of evaporation of water [25]. But the setback was drying up of bed moisture, and this was a major factor for lower conversion of β-mannan to mannose.

Comparison on SSF of PKC in Erlenmeyer Flasks and LAMB Bioreactor

In the industrial point of view, a short fermentation duration that can yield the optimum fungal biomass and reducing sugar in the SSF of PKC is desired. Figure 8 shows that in terms of fermentation time, the LAMB gave better fungal growth and reducing sugar. The optimum reducing sugar content in LAMB was 79.61 mg mannose g^{-1} dry PKC at 96 h, as compared to 90.91 mg mannose g^{-1} dry PKC at 120 h in the Erlenmeyer flask. There was a 12% discrepancy in the reducing sugar, most probably attributed to the difference in particle size used in the two bioreactors. In the LAMB, smaller particles were believed to have

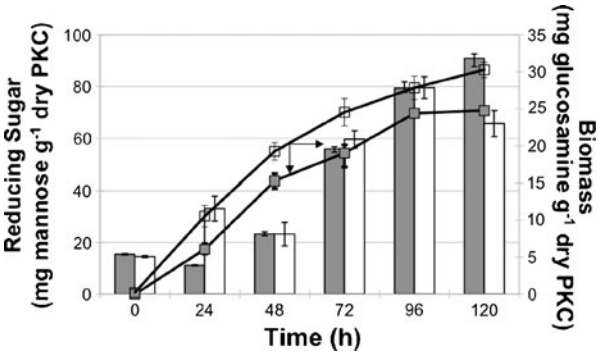

Fig. 8 Comparison of reducing sugar and fungal biomass production for SSF of PKC in Erlenmeyer flasks (*shaded gray*) and LAMB bioreactor (*white*) at optimum parameters; 20% inoculum, 110% MC, pH 6, and 30 °C. PKC sizes for flasks and LAMB were 855 μm and <5 mm, respectively

occupied the void spaces between larger particles, greatly decreasing the void fraction [20], and this could have affected the reducing sugar production. Nevertheless, the discrepancy was compensated by a fermentation period shorter by 24 h. The LAMB had provided better aeration to SSF when compared with the Erlenmeyer flask, facilitating better heat and mass transfer and speeding up the conversion of β-mannan to mannose through β-mannanase activity.

Conclusions

SSF with *A. flavus* isolated from PKC was applied on PKC to improve its nutritive values for animal feeding. SSF conditions were optimized in Erlenmeyer flasks, and batch SSF in flasks and LAMB were compared. The LAMB provided better heat and mass transfer and shortened the fermentation period; these attributes are useful in industrial-scaled production of nutritionally improved PKC, especially in poultry feeding where indigestion problem is evident. Focus of future research should involve the investigation on mycotoxin production during SSF with the strain and evaluation on the effects of fermented PKC on poultry feeding.

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