British Biotechnology Journal
2(3): 133-145, 2012

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Characterization of Solid State Fermentation Culture Conditions for Growth and Mananase Production by *Aspergillus niger* USM F4 on Rice Husk in Tray System

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Authors' contributions

This work was carried out in collaboration between all authors. DI and RAR designed the study, performed the statistical analysis and wrote the protocol. HP wrote the first draft of the manuscript and managed the analyses of the study. Author LSH managed the literature searches. All authors had read and approved the final manuscript.

Research Article

Received 18th April 2012
Accepted 14th June 2012
Online Ready 27th June 2012

ABSTRACT

Aim: The study evaluated various fermentation conditions for the production of mannanase.
Place and Duration of Study: Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia between May 2009 and September 2010.
Methodology: Solid substrate fermentation was carried out in a shallow aluminum tray system (16 cm x 16 cm x 5 cm) for maximum mannanase production by *Aspergillus niger* USM F4 using rice husk as a substrate.
Results: The maximum mannanase activity of 119.91 U/g substrate was achieved on the 6 days of cultivation when the optimized physical parameters were used (substrate thickness of 1.6 cm or equivalent to 80 g of 0.75 mm rice husk, moisture content to substrate ratio of 1:1 (w/v), cultivation temperature at room temperature (28±2ºC), inoculum size of $6x10^6$ spores/ml and in static condition (no mixing during the

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fermentation process). The results showed an increment of about 30.79% of mannanase activity after the optimization (119.91 U/g substrate) compared to before optimization (91.68 U/g substrate).
Conclusion: The results obtained from this study revealed that rice husk can be used as a substrate for mannanase production in solid state fermentation process.

Keywords: Mannanase; Aspergillus niger; rice husk; solid substrate fermentation (SSF).

1. INTRODUCTION

Commercial interest in production of mannanase (1, 4-β-D-mannan mannohydrolase, E.C. 3.2.1.78) is increasing (Mudau et al., 2008) because of a wide variety of applications. Mannanase can be used to improve quality of animal feed (Hagglund et al., 2003; Aderolu et al., 2008), biobleaching of pulp in the paper industry (Wong and Saddler, 1993), bioconversion of biomass wastes to fermentable sugars (Chandrakant and Bisaria, 1998) and also in reducing the viscosity of coffee extracts (Dhawan and Kaur, 2007).

Malaysia is an agricultural-based country and produces large quantities of dgrowastes, of which the majority is desposed of due to low nutritional qualities including the presence of antinutritional factors. Some of these wastes include rice husks that usually are disposed or burned. However, based on solid substrate fermentation (SSF) processes, agricultural waste can readily be used as substrates for the cultivation of numerous microorganisms for the production of various secondary metabolites which are important for industrial applications. Syarifah et al. (2011) have reported using palm kernal cake as solid substrate in SSF by cultivated it with filamentous fungus able to produce mananase activity as 433.84 U/g.

Rice husk is an agrowaste which is abundantly available in Malaysia. It is a by product of rice milling and during the milling of paddy about 78% of weight is received as rice, broken rice and bran whereas the rest of about 22% of the paddy is received as husk. Currently, rice husk is only used to fuel boilers (Mansary and Ghayl, 1998) and the ash from the husk is used as fertilizers. However, in certain parts of the country rice husk has been used as ingredient in poultry and ruminant feeds but the problem of low nutrients digestibility, high silica and ash content besides abrasive characteristics limit its utilization. Rice husk is reported to be composed of 2.9 – 3.6% of crude protein, 8.0 – 12.0% of oil, 39.0 – 42.0% of crude fibre and ash of about 15.0 – 22.0% (How and Ibrahim, 2004). Based on its composition, it is suggested that rice husk can be used as a substrate in SSF (Mansary and Ghayl, 1998; Krishna, 2005) for enzyme production, such as mannanase.

SSF is defined as the cultivation of microorganisms on solid substrates that deficient in free flowing water. However, the substrates have to posses enough moisture to support growth and metabolism of microorganisms. SSF is considered more economical compared to submerged fermentation, mainly due to the substrates (agricultural wastes) are cheap and abundantly found (Pang and Ibrahim, 2005; Gassara et al., 2010). Moreover, SSF has other advantages over submerged fermentation including higher yield of products, simplicity of the machinery, easier in scaling up process, no foam build up during the fermentation process, lower of moisture content and lower of contamination risk (Pandey, 1992; Robinson et al., 2001).

This paper described the investigations of mannanase production by *Aspergillus niger* USM F4 using rice husk as a potential substrate in a tray system. Some of the governing parameters on the production of mannanase in the SSF system were studied.

2. MATERIALS AND METHODS

2.1 Microorganism and Inoculum Preparation

The fungus, *Aspergillus niger* USM F4 was obtained from the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia (USM), Penang, Malaysia. The fungal culture was grown on Potato Dextrose Agar (PDA) slants until sporulation (4-6 days) at 30ºC. The inoculum was prepared by adding 5 ml of sterile distilled water which contained 0.1% (v/v) Tween 80 to the agar slant and shook vigorously. The spore suspension was adjusted to the spore concentration of $6x10^6$ spores/ml (as the initial inoculum size).

2.2 Substrate Preparation

Rice husk which was obtained from a local rice mill that is rich with lignocellulosic material was used as a substrate. The rice husks were dried under sunlight until constant weight and ground to 0.75 mm particle size of substrate (as the initial particle size of substrate) prior used.

2.3 Cultivation System for Solid Substrate Fermentation

The cultivation of the fungus in the SSF system was carried-out in a shallow aluminum tray of 16 cm x 16 cm x 5 cm. Forty gram of rice husk was moistened with 32 ml of growth medium containing 1.6% (w/w) of locust bean gum and 1.2% (w/w) of ammonium nitrate, autoclaved at 121ºC for 15 minutes. After cooled, it was inoculated with 8 ml of $6x10^6$ spores/ml. The inoculums and the solid substrate were mixed well by using sterile spatula to ensure a uniform distribution. The initial substrate thickness and the ratio of moisture content for this study was 0.8 cm (40 g of rice husk) and 1:1 (w:v), respectively. Before incubating at room temperature (28±2ºC) for 10 days aerobically, the inoculated trays were covered with aluminum foil. The experiments were carried-out in triplicate and the results obtained were reported as mean of the triplicate experiments.

2.4 Sampling

Five gram of the fermented solid substrate was transferred into a 250 ml Erlenmeyer flask and added with 40 ml of 0.1% (v/v) of Tween 80 in distilled water. The sample was then mixed using a rotary shaker (150 rpm) at 25ºC for 1 hour. The suspension was filtered using Whatman No. 1 filter paper and the clear cell free filtrate was used as the enzyme source.

2.5 Optimization of SSF System for Mannanase Production

The optimization of SSF system for mannanase production was performed based on the modification of the physical parameters which included the substrate thickness in the range of 0.8 cm to 3.2 cm, while for the ratio of moisture content in the range of 1:0.5 – 1:3 (w:v), for cultivation temperatures in the range of 25-45ºC and for inoculum size in the range of $6x10^4$ to $6x10^8$. The effect of mixing frequency for mannanase production was also

examined including mixing at every 12 hours, 24 hours, 36 hours, 48 hours, 60 hours and no mixing incurred (static). All experiments were carried-out in triplicate and the results were presented as mean of the triplicate experiments.

2.6 Analysis

Mannanase activity was determined as previously described by Lin and Chen (2004). Enzyme solution (0.5 ml) was added in to 0.5 ml of 50 mM citrate buffer pH 4.0 which containing 0.5 % (w/v) locust bean gum. The mixture was incubated in a water bath at 60ºC for 30 minutes. The reaction was terminated by adding 1.5 ml of dinitrosalicyclic acid (DNS) followed by boiling for 5 minutes. The mixture was measured spectrophotometrically at 575 nm. One unit of mannanase activity (U) was defined as the amount of 1 µmol mannose released by the enzyme for every minute under assay conditions. Mannanase activity was expressed as unit per gram of dry weight of fermented rice husk (U/g substrate).

Fungal growth was determined by glucosamine method as described by Swift (1973) and expressed as mg glucosamine per g dried fermented substrate (mg glucosamine/g substrate). All the experiments were carried out in triplicate. All the data was presented as mean of the triplicate. The significant different of the mean data was analyzed using one way analysis of variance (ANOVA) and Duncan test (PASW Statistics 18 version) with 5% of confidence level.

3. RESULTS AND DISCUSSION

Solid substrate fermentation (SSF) is the preferred method for the enrichment of agricultural waste since it stimulates the natural environment of most microorganisms, especially filamentous fungi (Couto and Sanroman, 2005).

3.1 Effect of Substrate Thickness

The major challenge in the scale-up of SSF is the transfer of oxygen in to the substrate bed to obtained high cell densities where critical bed thickness (bed height) plays a prominent role. In SSF heat removal is also a major concern where it is more difficult to remove the waste metabolic heat from a bed of solids while preventing water being evaporated.

Fig. 1 shows the mannanase activity and fungal growth of *A. niger* USM F4 from various substrate thicknesses (0.8, 1.6, 2.4 and 3.2 cm). The mannanase production achieved its maximum activity (119.59 U/g substrate, $p<0.05$) at the substrate thickness of 1.6 cm or equalized to 80 g (w/w). The highest fungal growth (1.98 mg glucosamine/g substrate) was also achieved on the same substrate thickness. The mannanase production was decreased at the substrate thickness of 2.4-3.2 cm with the activity of 63.76 U/g substrate and 44.31 U/g substrate, respectively. The results indicated that the highest mannanase production was produced in a thinner substrate thickness.

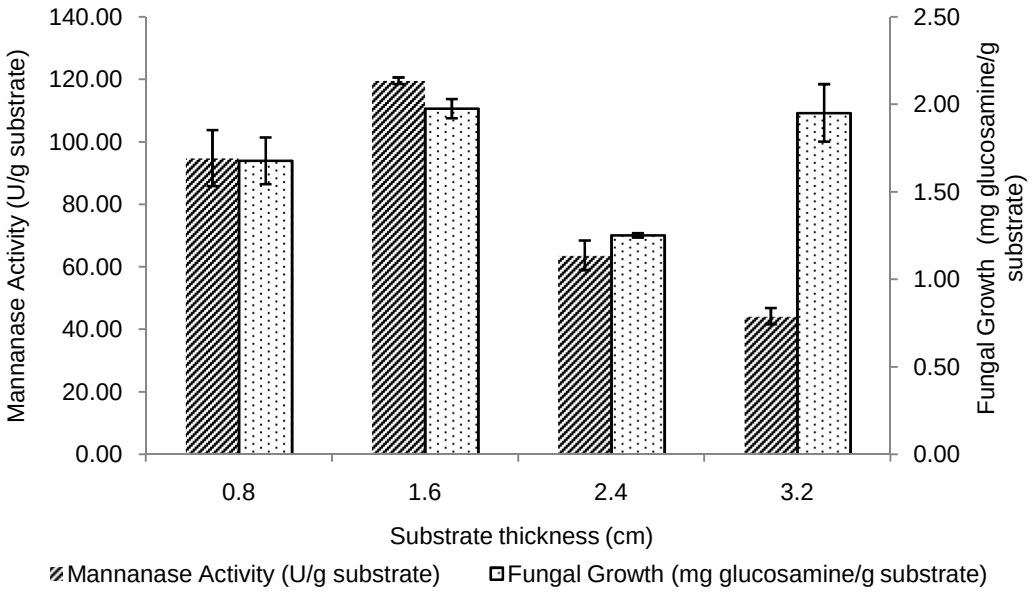

Fig. 1. Effect of substrate thickness on the production of mannanase and fungal growth of *Aspergillus niger* USM F4 in a tray system

Note: The experiment was carried out in triplicate (n=3).

Lower of enzyme production could be resulted due to the increasing of substrate thickness (Annuar et al., 2010). Moreover, it was also reported that the oxygen availability in the tray system has correlation with the substrate thickness (Raghavarao et al., 2003). It has been reported that in higher bed thickness, the oxygen availability is getting decrease at the middle and bottom area of substrate. This condition is due to the fast colonization of the fungus on the surface area which promotes the high density of the substrate. This condition has caused the exhausting of oxygen which affects the fungal growth as well as the enzyme activity. Therefore, thicker bed height than the optimum leads to undesirable situations like cell lysis and anaerobic conditions. On the other hand, a thinner bed height is usually chosen in a tray system since it easily to be fermented (Suryanaran, 2003) and permits better oxygen supply and heat removal (Gervais and Molin, 2003).

3.2 Effect of Moisture Content

Moisture content is one of the key factors which could affect the metabolite production in SSF (Archana and Satyanarayana, 1997). Water affects the physical properties of the solid substrate mainly by causing swelling of the substrate and facilitates effective absorption of the nutrients from the substrates for growth and metabolic activities (How and Ibrahim, 2004). The moisture content was adjusted by adding the moisturizing agent to give the moisture content ranging from the ratio of 1:0.0 to 1:3 (w:v). As shown in Fig. 2, the ratio of moisture content 1:1 resulted significant mannanase production with the highest activity of 114.52 U/g substrates and fungal growth of 1.47 mg glucosamine/g substrate. The activity of mannanase at the moisture content of 1:1 (w:v) was significant different ($p<0.05$) compared to other moisture contents (w:v) [1:0.5 (56.66 U/g substrate); 1:1.5 (26.10 U/g substrate); 1:2 (15.84 U/g substrate); 1:2.5 (1.44 U/g substrate)]. The result indicated that the fungus grew

well and produced highest mannanase activity at lower moisture content. This condition gives significant advantage in lowering the risk of contamination particularly from bacterial species (Pang and Ibrahim, 2005).

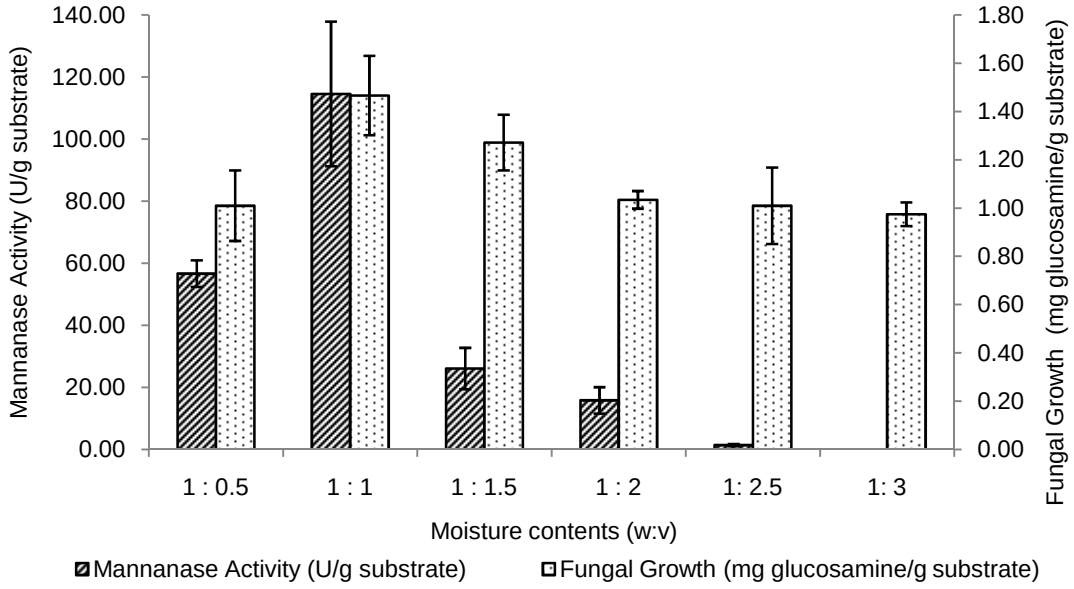

Fig. 2. Effect of moisture content on the production of mannanase and fungal growth of *Aspergillus niger* USM F4 in a tray system
Note: The experiment was carried out in triplicate (n=3)

However, moisture content below the optimum level (too low moisture content) might reduce the nutrient solubility in the substrate and affect the initial growth of fungal spores (which could disrupt the fungal growth and enzyme activity) (Murthy et al., 1993; Battan et al., 2006). Low water content is usually related to insufficient substrate swelling which prevented the nutrient absorption from the substrates. Low moisture content not only lessened substrate swelling but also reduced nutrient solubility and caused higher water tension which also result poor fungal growth (Syarifah et al., 2011). On the other hand, higher moisture contents resulted reduction in substrate porosity and caused oxygen limitation within the substrates which consequently affected the oxygen transfer within the substrate and thus resulting poor growth (Ramesh and Lonsane, 1990; Sandhya et al., 2005).

3.3 Effect of Cultivation Temperature

Temperature has a significant effect in the germination of spores which could determine the successful of the SSF system. Therefore, temperature become one of key parameters in SSF system (How and Ibrahim, 2004; Pang and Ibrahim, 2005). Fig. 3 shows the effect of cultivation temperature on mannanase production. The highest mannanase activity was achieved at the room temperature (28±2ºC) with the value of 97.00 U/g substrate and the fungal growth of 1.44 mg glucosamine/g substrate.

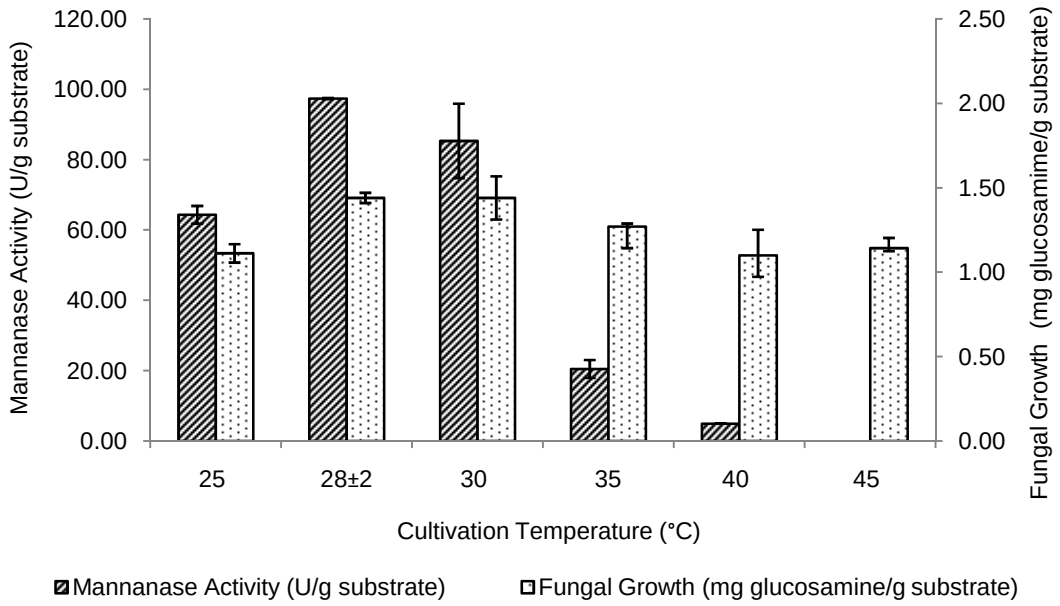

Fig. 3. Effect of temperature on the production of mannanase and fungal growth of *Aspergillus niger* USM F4 in a tray system

Note: The experiment was carried out in triplicate (n=3).

However there was no significant difference ($p>0.05$) on mannanase activity between cultivation temperature of 28±2ºC compared to 30ºC (85.37 U/g substrate). Lower or above both temperatures (28±2ºC and 30ºC) had resulted lower mannanase activity in the range of 4.92-64.27 U/g substrate, except for temperature at 45ºC (there was no activity detected). Nevertheless, room temperature (28±2ºC) was chosen as the optimum temperature for this study since it considered more economical compared to other cultivation temperatures. Furthermore, lower temperature can prevent the increasing of excessive temperature due to the gradient temperature which generating during fermentation (Syarifah et al. 2011). In addition, room temperature as the optimum cultivation temperature also similar to the natural habitat of *Aspergillus niger* USM F4 since the fungus is classified to the mesophilic group. The temperature in SSF system is influenced only by the environmental temperature, but also by the increase in temperature generated from the metabolic activities of the fungi growing on the solid substrates. Decline in enzyme production at higher temperature also can be caused by the denaturation of enzyme or its inactivation at higher temperatures. Therefore, the subsequent experiment was conducted at an incubation temperature of room temperature.

3.4 Effect of Inoculum Size

The effect of inoculum size on mannanase production was examined using the spore suspension ranging from $6x10^4$-$6x10^8$ spores/ml (Fig. 4). The results showed that the highest mannanase activity (119.91 U/g substrate, $p<0.05$) and the fungal growth (1.98 mg glucosamine/g substrate) were achieved at inoculum size of $6x10^6$ spores/ml. Lower and higher inoculum size gave significant effect to the mannanase activity with the value in the range of 84.67-89.57 U/g.

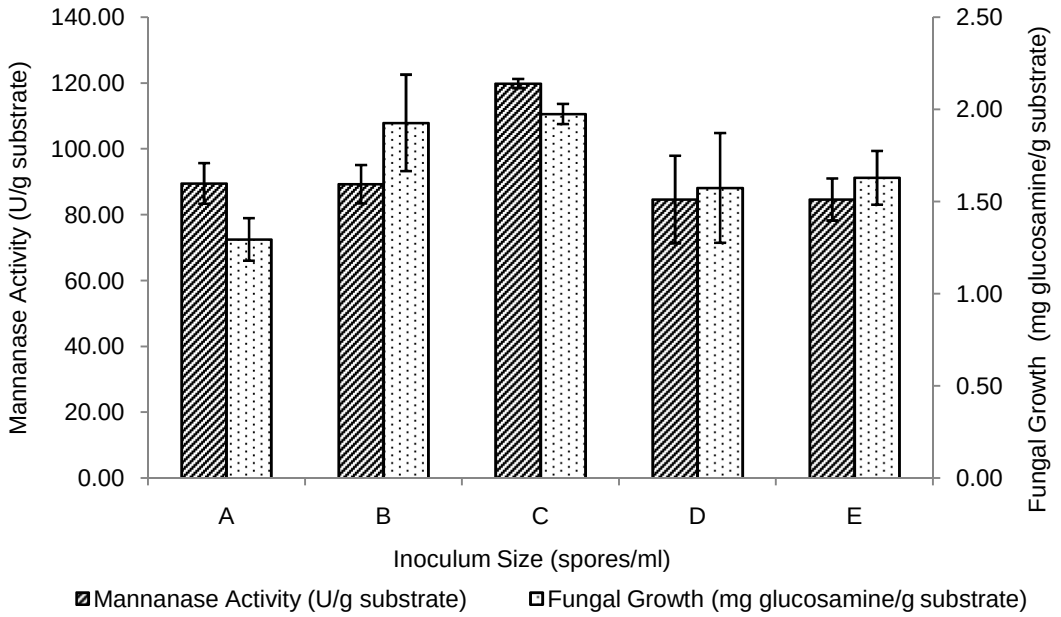

Fig. 4. Effect of inoculum size on the production of mannanase and fungal growth of *Aspergillus niger* USM F4 in a tray system.

A : 6x10^4 spores/ml; B : 6x10^5 spores/ml; C : 6x10^6 spores/ml; D : 6x10^7 spores/ml; E: 6x 10^8 spores/ml
Note: The experiment was carried out in triplicate (n=3).

In SSF, inoculum size must be distributed homogenously and must be in sufficient numbers for the microorganism to grow well. The fungal spores or fragments which initially attached on the outer surface of the substrate particle is slowly growing, multiplying and penetrating in to the substrate. Therefore, a suitable inoculum size is needed to have the highest mannanse production. The results also showed that below and above inoculum size of 6x10^6 spores/ml gave significant effect to the mannanase activity with the value only in the range of 84.67-89.57 U/g. Lower inoculums sizes resulted a lag phase on the first day of cultivation where no fungal growth and production was obtained. At lower inoculums sizes it was observed that the time taken to achieve maximum growth or enzyme production was much longer. Lower inoculum size was able to retard the proliferation of biomass. Thus, the degradation of the substrates by the microbes is slower and affects the metabolite production (Ramachandran et al., 2004). However, high inoculum sizes are inhibitory in nature where the overall trends shown that an increased in spore concentration adversely affect the enzyme production. This condition may be correlated to the amount of available oxygen and nutrients in the early stage of inoculation where rapid growth of the fungus resulted higher degradation of the substrates and increased availability of the nutrients (Kashyap et al. 2002). Kumar et al., (2010) and Baysal et al., (2008) also reported where higher inoculum than the optimum may produce too much biomass and may deplete the nutrient that necessary for microbial metabolite production.

3.5 Effect of Mixing Frequency

Fig. 5 shows that control or static condition (no mixing incurred the fermentation process) produced the highest mannanase activity (119.28 U/g substrate, $p<0.05$) and fungal growth (1.97 mg glucosamine/g substrate). Tray with mixing frequency of every 24 hours, 36 hours, 48 hours and 60 hours exhibited mannanase activity with the values of 3.01, 68.08, 73.39, and 47.98 U/g substrate, respectively. Meanwhile, there was no activity detected in the tray with every 12 hours of mixing frequency.

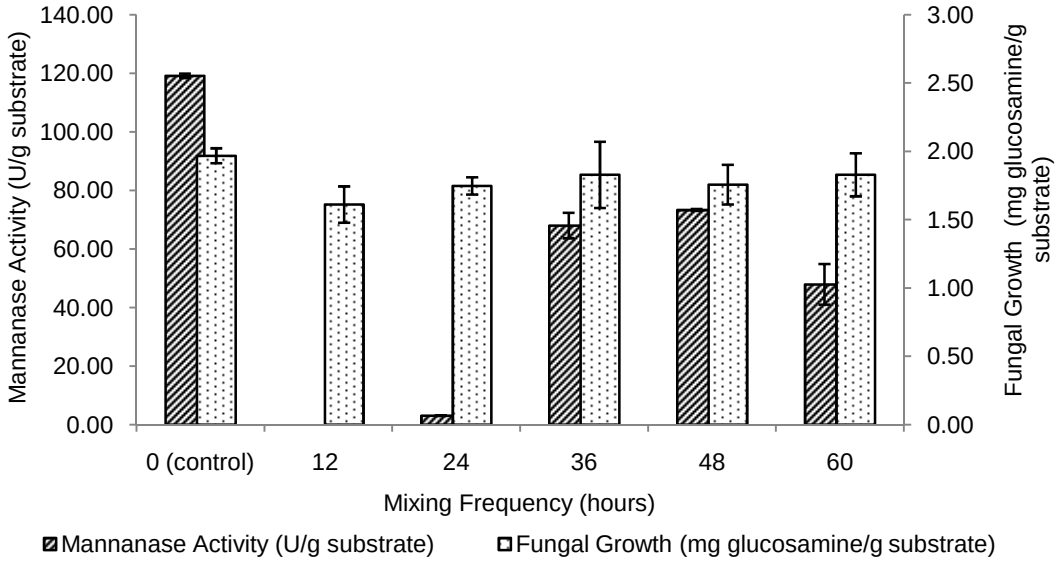

Fig. 5. Effect of mixing frequency on the production of mannanase and fungal growth of *Aspergillus niger* USM F4 in a tray system
Note: The experiment was carried out in triplicate (n=3).

Mixing in SSF might promote the mass and the heat transfer during fermentation as well as to uniform the growth of the fungus (Lonsane et al., 1992). However, in this case static condition found to be the best condition for the mannanase production. The porosity of the rice husk allowed the fungal hyphae to grow well and use-up oxygen that available in the substrate. Furthermore, in static condition, fungal hyphae were not broken in to fragments and mature hyphae always produced significant amount of mannanase (Syarifah et al., 2011). The only problem with static condition is it can cause substrate to become compress, inconsistent fungal distribution and growth, heat blockage and also the distribution of substrate moisture. Furthermore, a frequent mixing affects the fungal sporulation at the earlier growth stage which finally inhibited the enzyme production. Based on our observation we found that the substrate thickness of 1.6 cm was suitable for the fungus to growth efficiently and reached the bottom area of the substrate. Therefore, the mixing process was not necessary in this condition.

3.6 Profile of Mannanase Activity and Fungal Growth after the Optimization of Physical Parameters

Fig. 6 shows the profile of mannanase activity and fungal growth after the physical parameters optimization in a tray system for 10 days of cultivation time. The highest mannanase activity was achieved on day 6 of cultivation time with the activity of 116.57 U/g substrate. The fungal growth was also highest on that cultivation time with 1.78 mg glucosamine/g substrate. Mannanase activity increased up to 27.15% after the physical optimization. Increased in mannanase production in rice husk can be attributed to its content of more enriched and easily biodegradable ingredients.

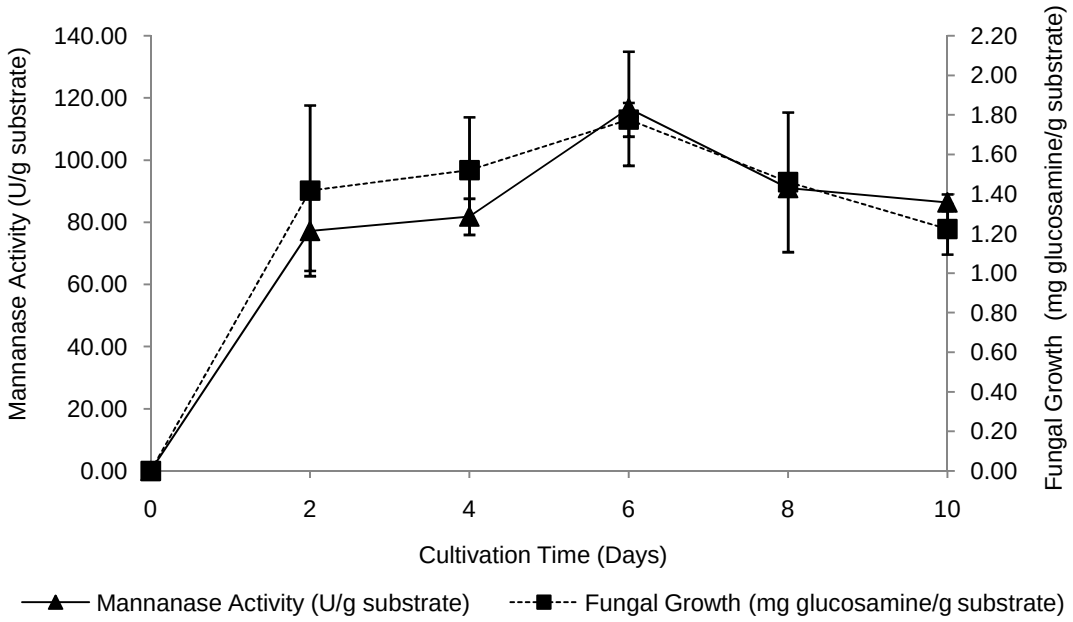

Fig. 6. Profile of mannanase production and fungal growth of *Aspergillus niger* USM F4 after the optimization of physical parameters in a tray system

Note:The experiment was carried out under SSF tray system with condition: 80 gram of substrate, substrate thickness of 1.6 cm, 0.75 mm particle size of substrate, moisture content of 1:1 (w:v), cultivation temperature at 28±2ºC (room temperature), inoculum size of $6x10^6$ spores/ml, at static condition, and with the supplementation of 1.6% (w/w) LBG and 1.2% (w/w) ammonium nitrate.

The result of optimization study proved that rice husk has potential to be a substrate for the mannanase production in SSF. Celluloses and hemicelluloses are the main component in rice husk (35–40% cellulose, 25–35% hemicelluloses and 12–18% lignin) and during the SSF process rice husk break-down in to compounds of smaller molecules with high availability components such as glucose and microbial proteins.

4. CONCLUSION

In the view of results obtained, we were able to establish that rice husk from paddy plantation which have not been exploited commercially for any industrial application and are poorly disposed could effectively be used as a substrate for mannanase production through SSF system. The optimization of physical parameters of SSF for mannanase activity which fermented by *Aspergillus niger* USM F4 was successfully achieved with the maximum activity of 119.91 U/ gram substrate. This study suggested that there is possibility to scale up the production of mannanase in a bigger SSF tray system and yet it can make the process more cost effective.

ACKNOWLEDGEMENTS

The authors would like to thanks Ministry of Science and Innovation, Malaysia for the financial support and Universiti Sains Malaysia for awarding USM Fellowship to Hapsari Puspitaloka.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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