ORIGINAL ARTICLE

Xylanase production by a local fungal isolate, *Aspergillus niger* USM AI 1 via solid state fermentation using palm kernel cake (PKC) as substrate

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Abstract

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Isolate USM A1 I which was identified to be *Aspergillus niger* was selected as a potential producer of xylanase via a solid state fermentation system (SSF) using palm kernel cake (PKC) as substrate. The modification of the physical conditions of the SSF system indicated that the xylanase activity was 23.97 U/g PKC at the moisture ratio of 1:0.75 of PKC: moistening agent with the inoculum size of 1¥10^4 spores/ml and cultivated at the ambient temperature (28±3ºC). The supplementation of additional carbon and nitrogen sources in the PKC medium could enhance enzyme productivity. The maximum production of xylanase and growth obtained with the supplementation of xylose at 0.75% (w/w) were 25.40 U/g and 1.69 mg glucosamine/g PKC. Moreover, the presence of $NaNO_3$ at 0.075% (w/w) as additional nitrogen source further enhanced xylanase production to 33.99 U/g PKC although the growth remained unchanged at about 1.67 mg glucosa-

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mine/g PKC. The optimized conditions showed an increased xylanase production by 157% compared to before the optimization of the SSF system. The xylanase productivity was 23.12 U/mg glucosamine after optimization and 11.72 U/mg glucosamine before optimization.

Key words : xylanase production, *Aspergillus niger*, solid state fermentation, palm kernel cake

Xylanases show great potential for industrial applications mainly for the bioconversion of lignocelluloses to sugar, ethanol, and other useful substances, clarification of juices and wines, improving the nutritional quality of silage and green feed and the de-inking processes of waste papers (Viikari *et al*., 2001). Haltrich *et al*., (1996) gave an overview of fungal xylanases and showed that the enzyme can be produced by a number of microorganisms including bacteria, yeasts and filamentous fungi such as *Trichoderma, Bacillus, Cryptococcus, Aspergillus, Penicillium, Aureobasidium, Fusarium, Chaetomium, Phanerochaete, Rhizomucor, Humicola, Talaromyces* and many more. These fungi produced xylanase enzymes extracellularly with a wide range of activities from 4-400 IU/ml using various substrates both in submerged and solid state fermentation (SSF) processes. Extracellular enzymes are considered important from the industrial viewpoint as they ease the extraction procedure. Although a number of xylanase productions were performed using submerged systems, solid state fermentation was found to be more economical mainly due to the cheap and abundant availability of agricultural wastes which can be used as substrates. Besides that SSF offers distinct advantages over submerged fermentation including economy of space needed for fermentation; simplicity of the fermentation media; no requirement for complex machinery, equipment and control systems; greater compactness of the fermentation vessel owing to a lower water volume; greater product yield; reduced energy demand; lower capital and low recurring expenditures in industrial operation; easier scale up processes; lesser volume of solvent needed for product recovery; superior yields; absence of foam build up; and easier control of contaminants due to the low moisture level in the system (Pandey, 1992).

Malaysia with abundant agricultural wastes mainly from the oil palm industries will be of great advantage, not only for the utilization of wastes for the production of value added chemicals but will also lessen the undesirable impact of the agricultural wastes on the environment. Agricultural wastes such as palm kernel cake, sugar cane baggase, paddy straw, rice husks and sago wastes contribute to more than 5 million tones of wastes per year. Based on the enormous amount of wastes, there is an urgent need to manage the bulk wastes effectively and economically. At the same time, it is also necessary to generate value added products from these wastes. SSF has been used for the production of fine chemicals of commercial value from microbial sources such as enzymes, antibiotics, flavouring compounds and also microbial biomass which was used as animal feeds. Our laboratory is particularly interested in xylanase enzymes which will be used in the de-inking processes of laser printed waste papers. With the objective of mass production of xylanase using local raw materials from indigenous isolates, this paper describes the selection of *Aspergillus niger* USM A1 I as a potential xylanase producer via SSF system and the optimization of the cultivation system and medium composition in enhancing the production of the enzyme.

Materials and Methods

Isolation of microorganisms and screening of the xylanolytic activities

The isolation of fungi was carried out on potato dextrose agar (PDA) using the samples obtained from agricultural soils, decaying woods, and agricultural waste materials. One g of each of

the samples was suspended in 10 ml of sterile distilled water and shake vigorously for 10 min. Later, 1.0 ml of the resulting liquid was spread on the surface of PDA using an L-shaped glass rod and incubated at 37ºC for 5-7 days. The fungal isolates formed were subcultured to purity and examined for xylanolytic activities. Screening for xylanolytic activities was performed on malt extract agar (MEA) containing 0.1% (w/v) of xylan from oat spelt. Positive xylanolytic isolates were detected based on the clear zones of hydrolysis on the xylan. The potential isolates were subcultured and maintained on MEA slants. The slants were stored at 4ºC prior to use.

Solid-state fermentation (SSF) cultivation systems

Cultivation of fungus was performed in 250 ml Erlenmeyer flask containing 10 g of solid substrate with the addition of 15 ml of Mandel's medium. The Mandel's medium was prepared with the following composition (g/l) 10.0g; urea, 0.3; peptone, 0.75; yeast extract, 0.25; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3 and trace elements (mg/l): $FeSO_4.7H_2O$, 5; $MnSO_4.4H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4 and $CoCl_2.6H_2O$, 20.0 (Mandels *et al*., 1976). The medium and the trace elements were autoclaved separately. The flask was cooled down at room temperature and a known amount of sterilized trace elements was added. The flasks were then inoculated with 1×10^6 spores/ml of the moistening agent and incubated for 5 or 7 days (see below) at the ambient temperature (28±3ºC). The inoculum was prepared by growing the isolate on malt extract agar at 37ºC until sporulation. The spores were harvested using 0.1% Tween 80 (Smith *et al*., 1996) and the spore number was estimated by direct microscopic counting using haemocytometer.

Enzyme extraction

Seventy millilitres of cold distilled water (4ºC) was added to the SSF medium (10 g substrate) after cultivation. The mixture was vigorously homogenized for 30 minutes at 200 rpm. The solid biomass residues were separated from the suspension by filtration through Whatmann filter paper No.1. The cell free supernatant was used as the source of crude enzyme preparation.

Identification of isolate USM A1 I, the potential producer of xylanase

Fungal identification was carried out based on the colony morphologies and structural characteristics as observed under the light and scanning electron microscopy (Leica Cambridge S-360). The fungal characteristics were described and identified based on the description given by Pitt and Samson (2000).

Optimization of medium composition and cultural conditions

The optimization of medium composition and cultural conditions were carried out based on the stepwise modifications of the governing parameters for xylanase production. The effect of various substrates for SSF, consisting of PKC, sugar cane baggase, paddy straw and rubber wood saw dust, was examined by adding 10 g of each substrate in a 250-ml Erlenmeyer flask with 10 ml of distilled water which was added to moisture the substrates. Cultivations were carried out at ambient temperature (28±3ºC) for 5 days.

The effect of moisture level on the enzyme production was determined by varying the ratio (w/v) of PKC to moistening agent at the ratio of 1:0.5, 1:0.75, 1:1.0 and 1:1.25. The moistening agent used was sterile distilled water. The effect of mineral salts solution and tap water as moistening agents on xylanase production was also determined as comparison to that of distilled water. The mineral salts solution used consist of (g/l): $FeSO_4.7H_2O$, 5; $MnSO_4.4H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4 and $CoCl_2.6H_2O$, 20.0

The effect of cultivation temperatures on the enzyme production was examined at ambient temperature (28±3ºC), 25, 30, 35 and 37ºC. Cultivations were carried out for 7 days.

The effect of inoculum size was determined by adding the spore suspension of concentration of 1×10^3, 1×10^4 and 1×10^5 spores/ml prepared using the moistening agent for moisture control in the

SSF system. Cultivations were carried out at ambient temperature (28±3ºC) for 7 days.

The effect of supplementation of additional carbon and nitrogen sources to PKC was examined using carbon sources consisting of glucose, xylose, malt extract, lactose, maltose or xylan at 5% (w/w). Similarly, the nitrogen sources examined were peptone, urea, $NaNO_3$, yeast extract, $(NH_4)_2SO_4$ and NH_4NO_3 at 0.075% (w/w). The optimum concentrations of the carbon and nitrogen sources were also determined. Cultivations were carried out at ambient temperature (28±3ºC) for 7 days.

Analyses

Xylanase activity was assayed using the method of Gessesse and Gashe, (1997) at 50ºC using 1% (w/v) oat spelt xylan as the substrate, which was dissolved in 50 mM citrate buffer, pH 4.5. One unit of xylanase activity is defined as the amount of enzyme that releases 1 µmol of reducing sugar equivalent to xylose per minute. Xylanase production was expressed as units (U) per gram of dry weight of substrate. Growth of the fungus was determined by the method of glucosamine as described by Swift (1972). Growth of the fungus was expressed as mg glucosamine per g of substrate.

Results and Discussion

Selection and identification of isolate USM A1 I

Based on the screening programme, a total of 55 isolates were capable of exhibiting xylanolytic activities on MEA-xylan agar with the diameter of the clear zones ranging from 3.5-4.5 cm. However, 5 isolates consisting of USM A1 I, USM E2, USM JI 3, USM D1 I and USM EI 7 were selected for further confirmation using the MEA-xylan agar plates. The isolates USM A1 I demonstrated reproducible zones of hydrolysis of 4.5 cm diameter and this isolate was selected as the potential producer of xylanase. Isolate USM A1 I was identified based on the structural morphologies as observed under the light and scanning electron microscopes. It was observed that the isolate possessed distinct conidiophores terminated by a swollen vesicle bearing flask-shaped phialides. The spores showed black colouration and were produced in long chains from the ends of the phialides. Growth on malt extract agar (MEA) showed that the initial white mycelia turned yellow and finally black upon maturation. The conidial heads were seen as black colouration with the diameter of more than 70 mm (Figure 1). Based on these characteristics, isolate USM A1 I was identified to be *Aspergillus niger* (Pitt and Samson, 2000).

Optimization of cultural conditions and medium composition for the production of xylanase by *Aspergillus niger* USM A1 I.

Effect of substrate types

Several agricultural wastes consisting of

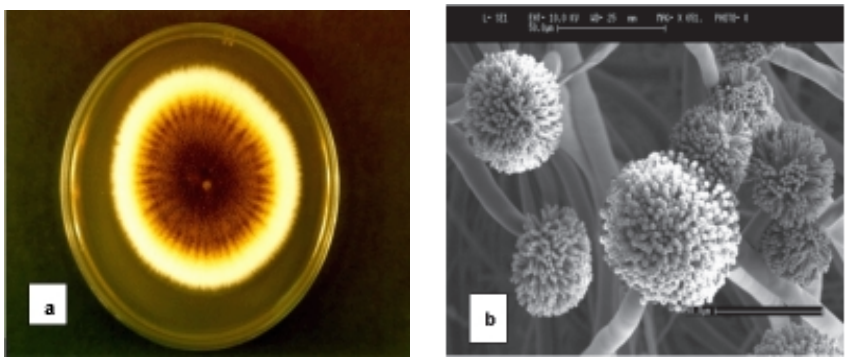

Figure 1. A locally isolated *Aspergillus niger* USM A1 I. under scanning electron microscopy showing structural morphologies with the conidiophores bearing conidia.

sugar cane baggase, paddy straws or rubber wood saw dusts were examined as subtrates in comparison to that of PKC for the growth and xylanase production by *A. niger* USM A1 I. The results after 5 days incubation at ambient temperature (28± 3ºC) shown in Figure 2 revealed that PKC remained the best substrate for xylanase production by *A. niger* AI 1 in SSF with the growth of 0.9 mg glucosamine/g substrate and xylanase production of 9.5 U/g subtrate. Other substrates of sugar cane baggase, paddy straws and rubber wood saw dusts, although supporting the growth in the range of less than 0.5 mg glucosamine/g substrate, did not allow any synthesis of xylanase by the fungus. The xylanase activity observed was only in the range of less than 0.2 U/g substrate. The high growth of the fungus on PKC corresponded closely to the production of xylanase. The suitability of PKC as substrate is related to the nutritional content of the substrate. PKC has higher protein content of 16% compared to other substrates which are in the range of 1-3% as determined by the method of Macro-Kjeldahl (AOAC, 1997). PKC was also found to contain a higher moisture content of about 6-7 % compared to the other substrates used in the study. At the same time, the particle size of PKC was relatively smaller than other substrates giving a higher surface area which would ease oxygen diffusion and nutrient absorption and assimilation by the mycelia.

Effect of cultivation temperature

Temperature is one of the important parameters that determines the success of SSF system. Therefore, the effect of temperature on xylanase production by *A. niger* USM A1 I was examined and the results obtained are shown in Figure 3. The production of xylanase was maximum at the ambient temperature (28±3ºC) with an activity of 14.4 U/g and the growth about 1.8 mg glucosamine/g substrate. A lower activity in the range of 2-6 U/g and growth of about 1.2 mg glucosamine/g substrate were obtained with cultivation temperatures lower or above the ambient temperature. At 25ºC, the activity of xylanase was 2.2 U/g. The results obtained indicated that the enzyme production corresponded closely to the growth of the fungus. The optimum temperature for xylanase production is similar to the optimum temperature for the growth of the fungus. This observation was in agreement with those reported by Sudgen *et al*., (1994) and Biswas *et al*., (1990), who showed that the highest xylanase activities were obtained at temperatures that were optimum for the growth of the fungi in solid-state fermentation. Higher xylanase production was also reported from alkalophilic *Bacillus* sp. using SSF at the optimum growth conditions (Gessesse and Mamo, 1999). For *A. niger* USM A1 I, the ambient temperature which was the optimum temperature for xylanase production was similar to the temperature of the

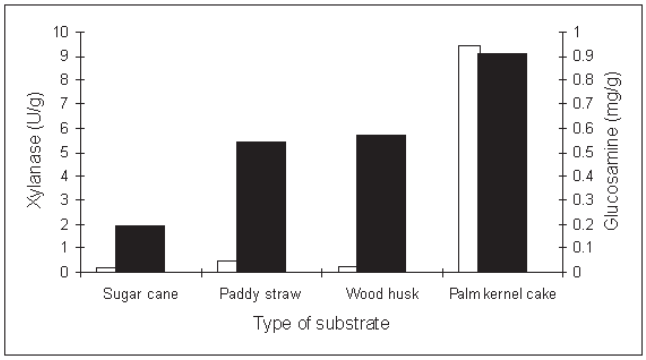

Figure 2. SSF cultivation systems by *Aspergillus niger* USM A1 I on various agricultural wastes as substrates grown at ambient temperature (28±3ºC) for 5 days. Symbols: xylanase activity (□) and growth (■)

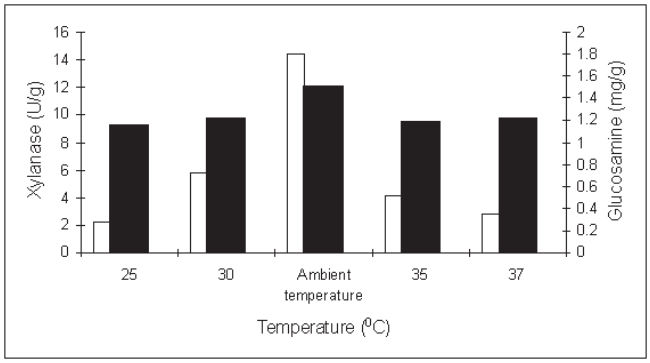

Figure 3. Effect of cultivation temperature on SSF system of *Aspergillus niger* USM A1 I using PKC as substrate for 7 days.
Symbols: xylanase activity (□) and growth (■)

natural habitat of the fungus where it was initially isolated.

Effect of moisture content

Solid substrates used in SSF are insoluble in water, therefore water will have to be absorbed onto the substrate particles, which can be used by the microorganisms for growth and metabolic activity (Pandey, 1992). It is also expected that the rate of water absorbed by different substrates varies from one substrate to another. This is another possible explanation for the variation in the xylanase production using different substrates. Thus, it is concluded that the degree of hydration of the substrate plays an important role on the growth of the fungi and subsequently the enzyme production. Water causes the swelling of the substrate and facilitates good utilization of substrates by the microorganisms. Increasing moisture level is believed to have reduced the porosity of substrate, thus limiting the oxygen transfer into the substrate (Raimbault and Alazard, 1980 and Feniksova *et al*., 1960). Likewise, a lower moisture ratio leads to reduced solubility of the nutrients of the solid substrate, lower degree of swelling and a higher water tension (Ikasari and Mitchell, 1994).

The moisture content of the substrate was examined by adding external water in the cultivation system using distilled water at different ratio between the substrate, PKC and the amount of water added. As indicated in Figure 4a, the xylanase production was optimum using the PKC which was moistened with moistening agent in the ratio of 1:0.75 with the production of 19.5 U/g substrate and growth of 1.6 mg glucosamine/g substrate. The fact that the fungus grows and produces maximum xylanase activity at lower water ratio offers significant advantage in reducing the risk of contamination, since most bacterial species are unable to grow at reduced moisture level.

Besides distilled water, other moistening agents consisting of mineral salt solution and tap water were also examined. The results obtained were shown in Figure 4b. As shown in the figure, mineral salts solution and tap water resulted higher xylanase production compared to distilled water. However, the enzyme production using tap water of 24.23 U/g showed no significant difference from that of using the mineral salts solution of 25.12 U/g. Thus, tap water was chosen as the moistening agent for economical reasons.

Effect of inoculum size

The inoculum size based on the number of spores was examined using the spore concentration of 1×10^3, 10^4 and 10^5 spores/ml of the moistening agent (Figure 5a, 5b and 5c).

It was found that the increase in inoculum size resulted in a rapid increase in xylanase production although the growth did not show rapid

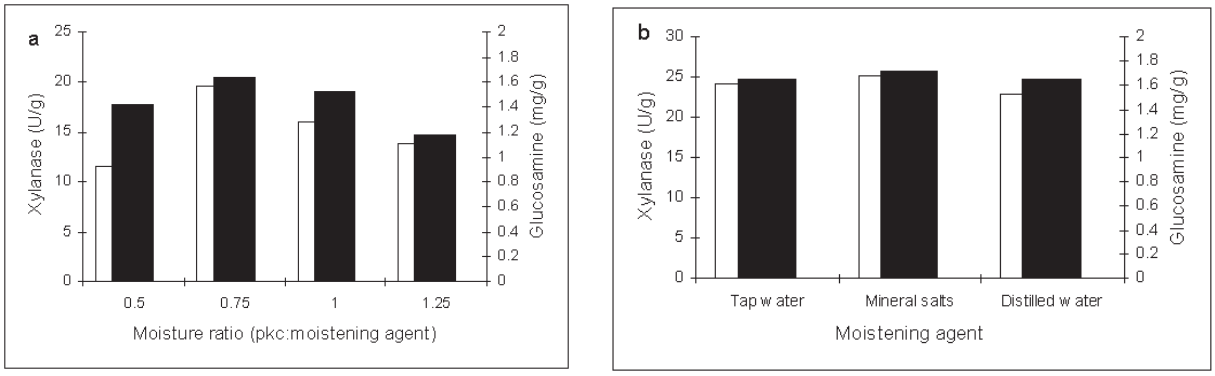

Figure 4. Effect of moisture content on SSF system of *Aspergillus niger* USM A1 I at ambient temperature (28±3ºC) for 7 days
a) Effect of the ratio between the substrate PKC to added distilled water as moistening agent.
b) Effect of other moistening agents used at the moisture ratio of 1:0.75 (pkc: moistening agent) compared to distilled water
Symbols: xylanase activity (□) and growth (■)

increment. The time taken to achieve maximum xylanase production using the inoculum size of 10^3, 10^4 and 10^5 spores/ml were 12, 8 and 4 days, respectively, giving the production rates of 3.3, 4.4 and 3.6 U/g/day, respectively. In the system using the inoculum size of 1×10^5 spores/ml, it was observed that xylanase production increased significantly on the 16th day of the fermentation. Similar observation was reported by Raimbault and Alazard (1980) who showed that maximum enzyme production and declination was achieved much faster due to the rapid degradation of substrate as a consequence of rapid growth. Nevertheless, the degradable products, which were gradually formation, were used up by biomass to enhance xylanase production later in the fermentation process.

Based on the production rate, the inoculum size of 1×10^4 was chosen to be used in the subsequent experiments.

Effect of supplementation of additional carbon and nitrogen sources

The production of primary metabolites by microorganisms are highly influenced by their growth, which is determined by the availability of the nutrients in the substrates. Therefore, it is expected that the improvement of the nutritional value of PKC by the supplementation of carbon or nitrogen sources will also improve the growth of *A. niger* USM A1 I and subsequently the enzyme production. Figure 6 shows the supplementation of sugars, which may act either as carbon sources or inducers. As shown in the figure, the addition of xylose resulted in an increment of 32% in xylanase production compared to the cultivation in the absence of xylose (Figure 6a). Xylose has been described as an effective inducer and carbon source for xylanase production in several microorganisms for xylanase production including *A. pullulans* (Priem *et al*., 1991), Fusarium oxysporum (Singh *et al*., 1992) and *T. lanuginosus* (Purkarthofer *et al*., 1993). The effect of xylose concentration on the xylanase production was examined within the range of 1-5% (Figure 6b) and subsequently in the range of 0.25-1.0% (Figure 6c). As shown in Figure 6b, it was observed that the optimum xylose concentration was 1.0%, suggesting that the sugar acted as inducer rather than as carbon source. There is also evidence from the observation that the fungal growth did not show any significant difference with increasing

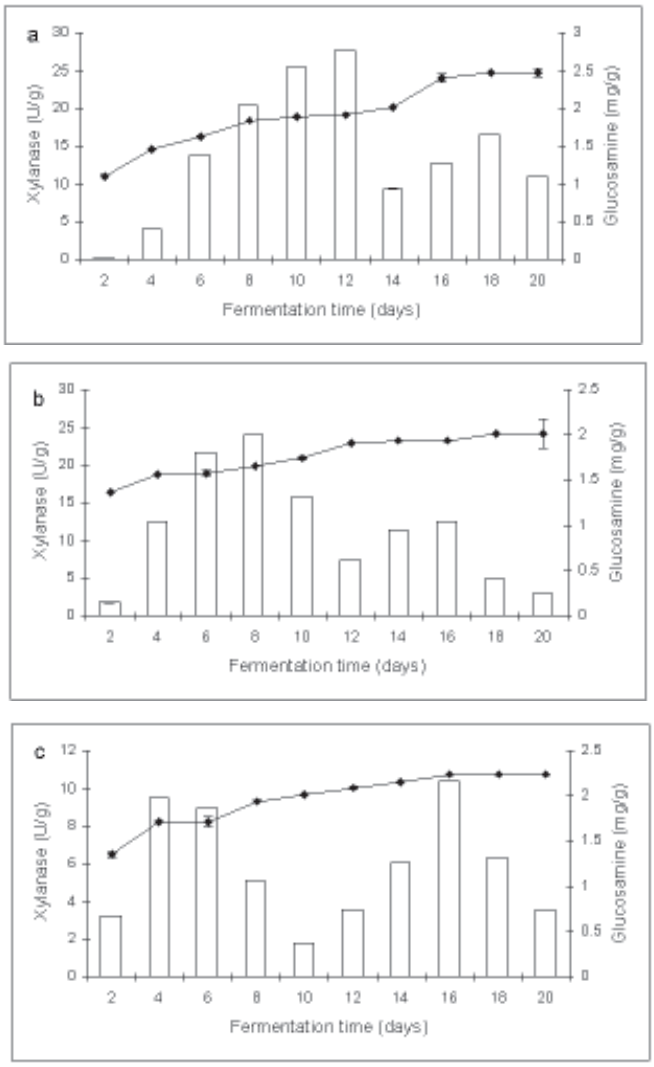

Figure 5. Effect of inoculum size on the SSF system of *Aspergillus niger* USM A1 I at ambient temperature (28±3ºC) for 7 days
Inoculum sizes of a) 1×10^3, b) 1×10^4, c) 1×10^5 spores/ml in sterile tap water as moistening agent.
Symbols: xylanase activity (□) and growth (■)

xylose concentration. Figure 6c shows that the optimum concentration of xylose was 0.75% giving the maximum enzyme production; however, a drop in the xylanase production was observed with lower xylose concentration, which indicates a state of xylose inadequacy for growth (Figure 6c). The results obtained so far suggested that the existence of the repression phenomenon on xylanase production at the xylose concentration exceeding 1%. The phenomenon of repression was also reported by Gessesse and Mamo (1999), who showed that xylose strongly repressed xylanase production by *Bacillus* sp. at the concentration of higher than 1% (w/w).

The effect of nitrogen source supplementation on the production of xylanase by *A. niger*

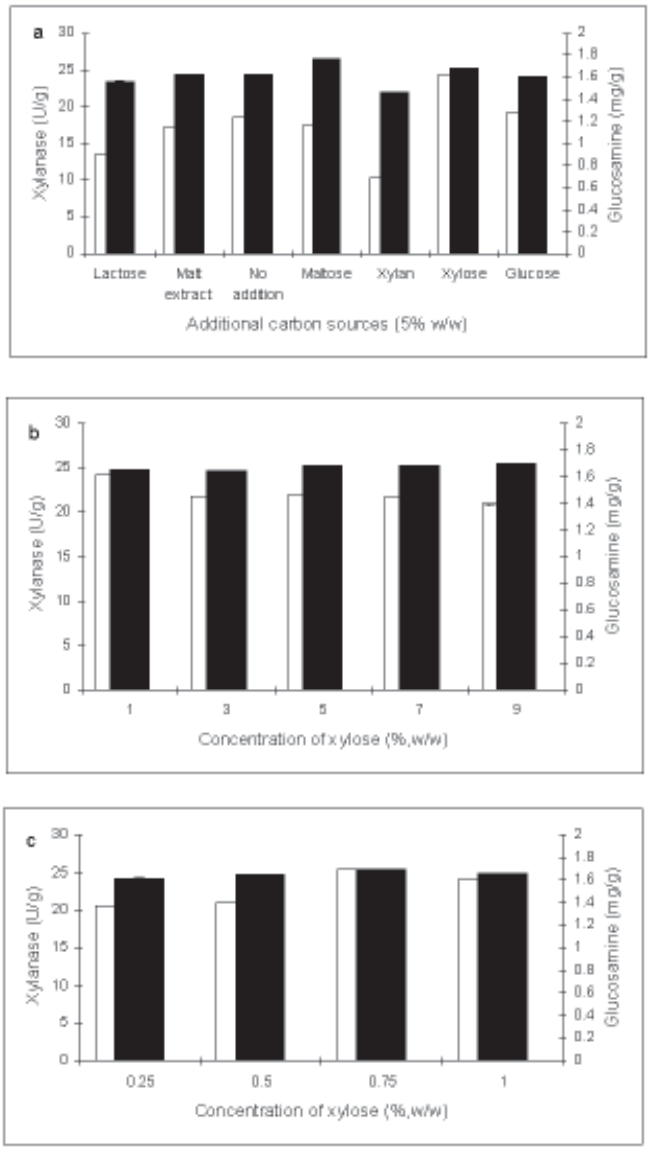

Figure 6. Effect of carbon sources supplemented to the SSF system.
a) Effect of various carbon sources on growth and xylanase production at 5% (w/w).
b) Effect of xylose concentration in the range of 1-9% (w/w)
c) Effect of xylose concentration in the range of 0.25-1.0% (w/w)
Cultivations were carried out at ambient temperature (28±3ºC) for 7 days
Symbols: xylanase activity (□) and growth (■)

USM A1 I was also examined. The results obtained using various nitrogen sources are shown in Figure 7. As shown in Figure 7a, when compared to the cultivation in the absence of any nitrogen sources, the presence of nitrogen sources have improved xylanase production by about 12-46%. Among the nitrogen sources tested, $NaNO_3$ was found to enhance the production of xylanase at 0.075% (w/

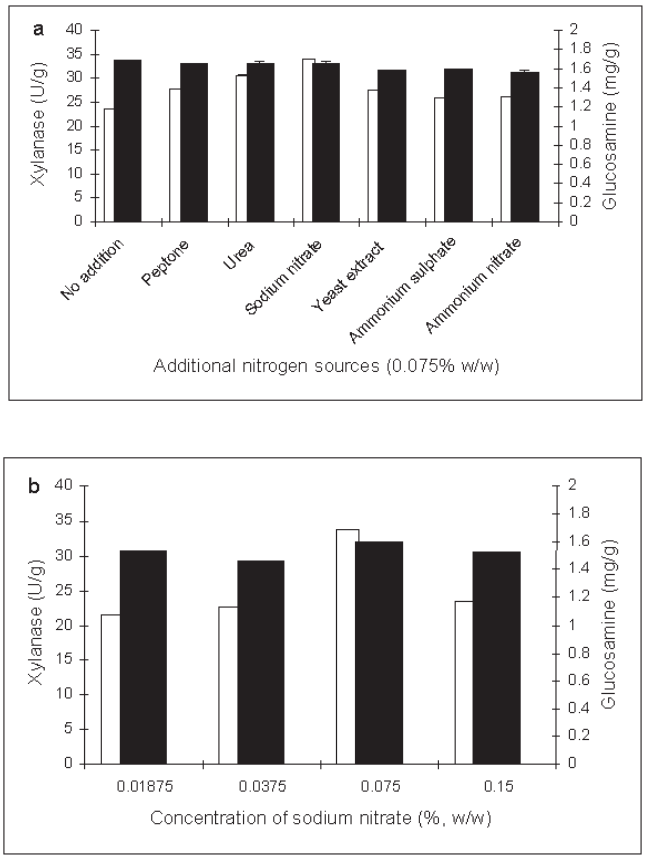

Figure 7. Effect of nitrogen sources supplemented to the SSF system
a) Effect of various nitrogen sources on growth and xylanase production at 0.075% (w/w)
b) Effect of $NaNO_3$ concentration in the range of 0.019-0.15% (w/w)
Cultivations were carried out at ambient temperature (28±3ºC) for 7 days
Symbols: xylanase activity (□) and growth (■)

w) by about 46% compared to the cultivation without the addition of any nitrogen sources (Figure 7a). The results obtained also did not exhibit any significant difference between the organic or inorganic nitrogen sources on the production of xylanase by *A. niger* USM A1 I.

The effect of the concentration of $NaNO_3$ between 0.018 and 0.15% on the enzyme production was examined. The results obtained indicated that the optimum concentration was 0.075% (w/w) with the xylanase production of about 34 U/g substrate (Figure 7b). Although the growth was unaffected, increased concentration of $NaNO_3$ did not enhance xylanase production.

Xylanase production by *Aspergillus niger* USM A1 I before and after optimization

Figure 8 shows the profiles of the growth and xylanase production by *A. niger* USM A1 I via SSF before and after optimization of the cultural conditions and medium composition. As shown in the figure, xylanase production increased by 157% while the growth increased by 40% after optimization. The maximum xylanase activity was about 35 U/g PKC obtained at the growth of about 1.5 mg glucosamine/g PKC. The production rate of xylanase using the optimized conditions was 6.2 U/g/day, while specific enzyme productivity was 23.12 U/mg glucosamine. On the other hand,

the production rate and productivity before optimization were 1.75 U/g/day and 12.0 U/mg glucosamine, respectively. Reports on the use pf PKC for enzyme production including xylanases were not available; however, the production of xylanases using other substrates such sugar cane baggase, wheat bran, wheat straws, corn cobs, soy bean hull and rice straw has been reported. One of those reports was from Gawande and Kamat (1999), using *Aspergillus terreus* and *Aspergillus niger* for xylanase production on wheat bran with the production of 61.9 U/ml and 74.5 U/ml, respectively. Another strain of *Aspergillus niger* 3T5B8 showed a varying activity in the range of 14-40 U/ml in SSF system, also using wheat bran as substrate. Higher xylanase production of about 600 U/g of wheat bran as substrate was obtained using *Bacillus* sp. AR-009 (Gessesse and Mano, 199). However, *Bacillus licheniformis* show lower xylanase production of 15-17 U/g wheat bran (Archana & Satyanarayana, 1997). Zychlinski *et al*. (1994) studied various solid substrates consisting of wheat bran, beet pulp, wheat straw, apple pomace, coarse rye meal, and wheat meal and showed that the xylanase production by either *Chaetomium globosum* and *Aspergillus niger* were in the range of 2.5-17.5 U/g substrates. Our findings of 35 U/g PKC xylanase production suggested that the xylanase production by *Aspergillus niger* USM A1 I is comparable to several earlier reports. Although there are strains which showed higher productivity, the comparison remains inconclusive since in the studies of those strains, the substrates and cultural conditions used were different.

In conclusion, the results obtained from this work strongly indicate that the SSF system using PKC as substrate is an economical method for the production of xylanase at extremely low operational cost based on the fact that PKC is one of the cheap and abundant agrowaste by-products of palm oil industry. The cultivation systems can easily be modified to enhance the productivity of the enzyme formation by the fungus, which will facilitate the scale up processes for mass production. The xylanase from *A. niger* USM A1 I has been produced in tray system, characterized and is currently being used for the de-inking processes of laser printed waste papers. Some of the findings from these work will be published subsequently.

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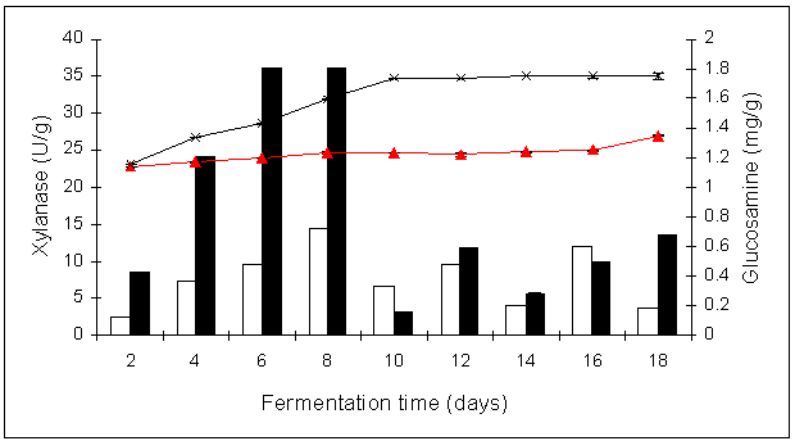

Figure 8. Profiles of xylanase production and growth of *Aspergillus niger* USM A1 I in SSF system before and after optimization of cultural conditions and medium composition.
Symbols: Before optimization: xylanase activity (□) and growth (▲)
After optimization: xylanase activity (■) and growth (×)

Ministry of Sciences, Technology and Innovation, Malaysia. The work forms a part of the Ph.D thesis of Pang Pei Kheng.

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