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Process Parameters Optimisation of Mannanase Production from *Aspergillus niger* FTCC 5003 Using Palm Kernel Cake as Carbon Source

[1]S. Abd-Aziz, [1]L.G.A. Ong, [1]M.A. Hassan and [2]M.I.A. Karim
[1]Department of Bioprocess Technology,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Biotechnology Engineering, Faculty of Engineering,
International Islamic University, Jalan Gombak, 53100 Kuala Lumpur, Malaysia

Abstract: The aims of this study are to optimised the process parameters involved for submerged fermentation of mannanase by *Aspergillus niger* FTC5003 using palm kernel cake (PKC) as carbon source. The parameters investigated include temperature, agitation speed, inoculum size and PKC concentration. The submerged fermentation was carried out for 10 days by using palm kernel cake as the sole carbon source with the addition of nitrogen source. Palm kernel cake can be considered as a suitable carbon source for the enzyme production due to it mainly consist of mannan and galactomannan, which is hemicellulose. The mannanase enzyme production using the optimised fermentation condition, which was conducted at agitation speed of 200 rpm, temperature 35°C, 1×10^4 spores mL^{-1} of inoculum size and 2% of palm kernel cake with productivity of 13.00 U/mL/day.

Key words: Mannanase, *Aspergillus niger*, palm kernel cake (PKC), optimised parameters, submerged culture

INTRODUCTION

Malaysia as an agricultural based country produces a lot of agricultural wastes per year. Example of agro-wastes abundantly produced in Malaysia includes cocoa pod husk, palm press fibre, palm kernel cake (PKC), palm oil mill effluent, oil palm trunk, empty fruit bunch, rice straw, rice husk, sago refuse and sugar cane bagasse. Large amounts of agro-wastes usually generate a lot of environmental problem and various efforts have been done to manage this problem. Large quantities of agricultural and agro-industrial residues are generated from diverse agricultural and industrial practices. These residues represent one of the most energy-rich resources on the planet (Francis *et al*., 2003; Nigam *et al*., 2001). They are in fact, one of the best reservoirs of fixed carbon in nature. Such resources are particularly attractive as they provide an inexpensive industrial substrate; furthermore it offers elimination of large-scale accumulation of biomass (Francis *et al*., 2003). PKC is the agro-industrial residue from the palm oil processing industries. In year 2001, Malaysia produces 11.8 million tones of crude palm oil, where it consist 3.3 million tones of palm kernel, 1.5 million tones of crude palm kernel oil and 1.7 million tones of PKC (Malaysia Palm Oil Board, 2002).

In most commercial enzymes production, the main carbon source used was sugar in which it will directly increase the cost of production. In this study the commercial carbon sources such as glucose, mannose etc. was replaced by PKC. PKC has become a draw back for monogastric livestock especially

Corresponding Author: Dr. Suraini Abd-Aziz, Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia Tel: +6-03-89467479 Fax: +6-03-89467510

poultry because the cell wall of PKC is composed of mainly linear and high crystalline mannan and a small quantity of galactomannan (Mohd-Jaafar and Jarvis, 1992). Utilisation of filamentous fungi in solid substrate fermentation (SSF) for fibre digestibility improvement has been widely applied (Raimbault, 1998; Pandey, 2003) *Aspergillus* sp. is among the most important microorganisms used in solid substrate fermentation process due to their physiological, enzymological and biochemical properties and also due to the wide application in (food) enzyme (Prasertsan *et al*., 1997; Ademark *et al*., 1998, 1999) and feed production. Besides that, *Aspergillus* has been reported by Ong *et al*. (2004), to produce abundant mannanase in the presence of PKC as sole carbon source in SSF. Enzymatic degradation of PKC now becomes an important and popular method in increasing PKC digestibility. The application of enzymes in hydrolysing mannan and galactomannan in PKC as fish meal has been reported by Ng and Chong (2002). This study focused on the optimisation of physical parameter that effect the mannanase production in submerged culture by *A. niger*.

MATERIALS AND METHODS

Microorganism

Aspergillus niger FTCC 5003 obtained from MARDI (Malaysia Agricultural Research and Development Institute) Culture Collection, Livestock Research Center, MARDI was used in this study. Potato dextrose agar slants were used for sporulation and storage. A suspension of 1×10^8 spores mL^{-1} was prepared in sterile 0.1% Tween 80 as an inoculum.

Culture Conditions

The fermentation medium used in this study was proposed according to Kusakabe and Takahashi (1988) which contained per liter: bacto-peptone 9.0 g, yeast extract 1 g, KH_2PO_4 10 g, $MgSO_4.7H_2O$ 0.5 g, corn steep liquor 0.5 g, PKC 20 g were used as the natural carbon source, media pH was 5.5.

Cultures were carried out in 500 mL Erlenmeyer flasks contained 150 mL of medium which was inoculated with $\times10^4$ spore mL^{-1} of *A. niger* (Abd-Aziz *et al*., 2003). Experiments were carried out in duplicate at 30°C and agitated at 150 rpm. For enzyme assays, sample was withdrawn each day and the mycelium was removed by centrifugation. The supernatant were collected and kept at -20°C for further analysis.

Fermentation Condition

For determination of optimum values of studied parameters, the following conditions were studied. For the effect of agitation speeds, the fermentation were carried out at the constant temperature of 30°C, 10% (v/v) of inoculum volume and 2% (w/v) of PKC as the main carbon source with varies agitation speeds of 100, 150, 200 and 250 rpm, respectively. For temperature effects, the fermentation were carried out at the constant agitation speed of 150 rpm, 10% (v/v) of inoculum volume and 2% (w/v) of PKC as the main carbon source with varies fermentation temperature of 25, 30, 35 and 40°C, respectively. For inoculum size effect, the fermentation were carried out at the constant agitation speed of 150 rpm, temperature 30°C and 10% (v/v) of inoculum size but with different percentage of PKC 1, 2, 4 and 6% (w/v), respectively.

Analysis

Mannanase activity was assayed according to the method proposed by Grobwindhager *et al*. (1999), using 0.5% solution of locust bean galactomannan as substrate. The reducing sugar was determined by dinitrosalicylic acid method using maltose as a standard according to Lu *et al*. (2003).

Protein concentration was determined according to Bradford method (1976), using bovine serum albumin as a standard. The yield of mannanase obtained was calculated using Eq. 1 below:

$$\text{Yield (U g}^{-1}\text{)} = \frac{\text{Maximum enzyme activity obtained (U mL}^{-1}\text{)}}{\text{Concentration of palm kernel cake (g mL}^{-1}\text{)}} \quad (1)$$

RESULTS AND DISCUSSION

Effect of Agitation Speed

Figure 1A-C shows the profile of mannanase activity, reducing sugar and soluble protein at different agitation speeds. The fermentations were carried out at the constant temperature of 30°C, 2% (w/v) of PKC with the 1×10^7 spores mL^{-1} but with different agitation speeds of 100, 150, 200 and 250 rpm. The mannanase activity increased almost at the same rate under those three agitation speeds that are 150, 200 and 250 rpm initially. However at 100 rpm the increment of the mannanase activity was slow as compared to the other three agitation speeds. After 6 days, the excretion rates were became different and higher maximum enzyme levels were reached at 200 rpm (168 U mL^{-1}), 150 rpm (150 U mL^{-1}) and 250 rpm (160 U mL^{-1}). Table 1 summarised the mannanase productivity with various agitation speeds.

Comparing Fig. 1B and C, it may suggested that the excretion of mannanase enzymes was parallel with the total soluble protein suggesting that the product was growth associated. Determination of soluble protein concentration is one of the indirect methods to estimate the biomass when insoluble substrate in used. The concentration of carbon source in PKC was indicated by reducing sugars. The consumption of sugar decreased as the agitation speed increased.

The concentration of carbon source i.e., palm kernel cake, was indicated by reducing sugars. The production and consumption of reducing sugars were nearly the same at all the agitation levels investigated. As the carbon source that present in this study are PKC which is in complex formed. The *A. niger* need to degrade the complex substrate into much simpler form before being consumed. Nevertheless, a slight difference could still be observed. The consumption of sugar declined from 100 to 200 rpm and was lowest at 250 rpm. A higher agitation speed increases the dispersion of macromolecules in the medium. Therefore, it might have contributed to the greater growth and better enzyme production that was observed in this study. However, the shearing effect induced by the higher agitation speed on the cells and enzyme inactivation may contribute negative effect towards cell growth and enzyme stability (Shioya *et al*., 1999; Feng *et al*., 2003). The study indicated that 200 rpm was best for *A. niger* FTCC 5003 to produce mannanase.

Effect of Temperature

The effects of different operating temperature are shown in Fig. 2A-C. The maximum enzyme activity was the highest at 35°C (263 U mL^{-1}) with the yield of 13180 U/g PKC and productivity of 26.40 U/mL/day (Table 2). As shown in Fig. 2B, the soluble protein concentration was typically temperature dependent. Figure 2A and B considered with preceding data again confirmed the observation that excretion of mannanase was largely growth associated. These phenomena also being observed by Feng *et al.* (2003).

As shown in Fig. 2C, the highest rate of reducing sugar consumption was observed at 25°C, while the corresponding consumption rate was the lowest at 35°C throughout the process. At 25°C, the residual reducing sugar concentration was less than 0.184 mg mL^{-1} after 5 days of fermentation. The association of soluble protein concentration and enzyme activity (Fig. 2A and B) suggested that reduced enzyme production at 25°C may be due to insufficient reducing sugar in the culture.

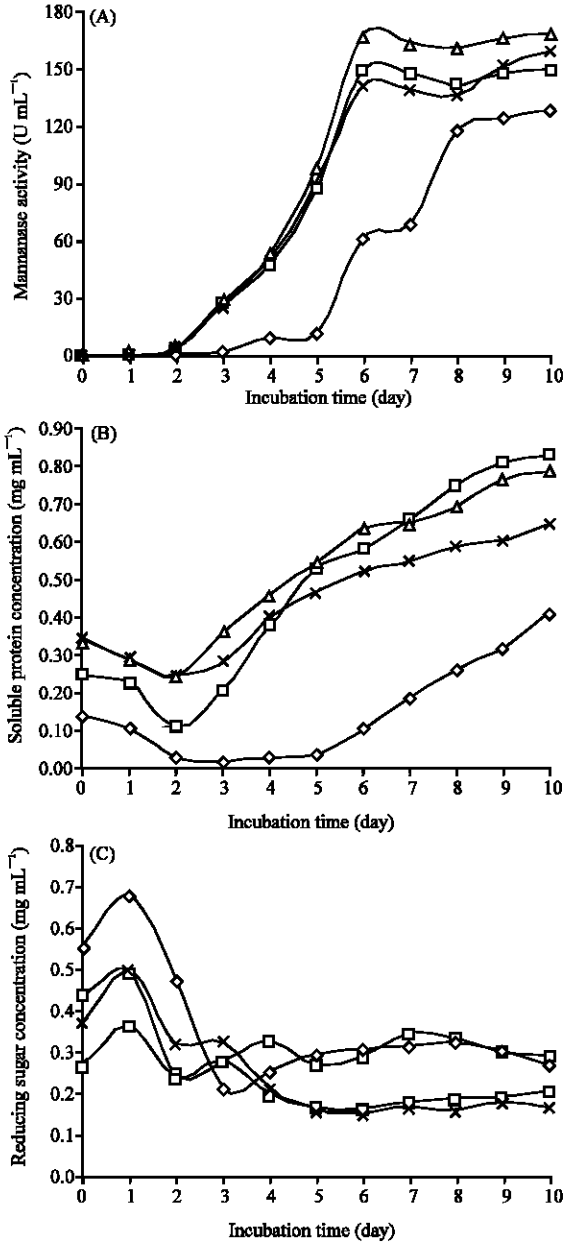

Fig. 1: Mannanase production by *A. niger* FTCC 5003 at different agitation speed of 100 rpm (◊), 150 rpm (□), 200 rpm (Δ) and 250 rpm (×). (A) enzyme activity, (B) soluble protein concentration and (C) reducing sugar concentration

Table 1: The mannanase production by *A. niger* FTCC 5003 from various agitation speeds in liquid fermentation

Agitation speed (rpm)	Maximum enzyme activity (U mL^{-1})	Yield (U/g PKC)	Productivity (U/mL/day)
100	128.90	6445	12.89
150	150.28	7514	15.03
200	168.47	8423	16.85
250	160.15	8007	16.01

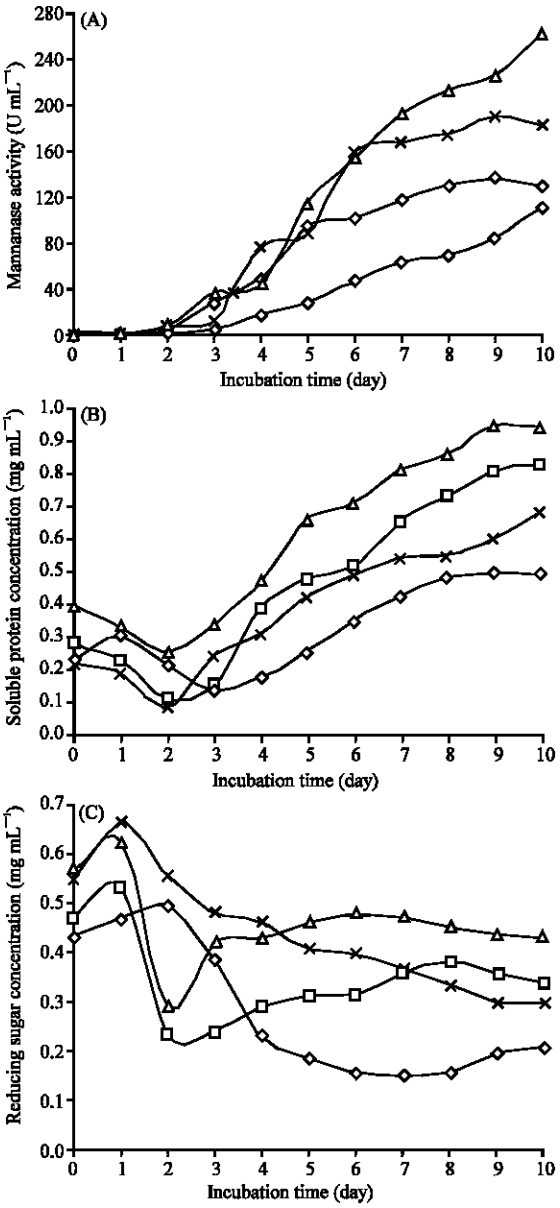

Fig. 2: Mannanase production by *A. niger* FTCC 5003 at different temperature of 25°C (◊), 30°C (□), 35°C (Δ) and 40°C (×). (A) enzyme activity, (B) soluble protein concentration and (C) reducing sugar concentration

Table 2: The mannanase production by *A. niger* FTCC 5003 from various temperatures in liquid fermentation

Temperature (°C)	Maximum enzyme activity (U mL^{-1})	Yield (U/g PKC)	Productivity (U/mL/day)
25	111.05	5552	11.10
30	136.81	6840	15.20
35	263.60	13180	26.40
40	189.83	9491	21.09

The influence of cultivation temperature on growth kinetics of *A. oryzea* has been investigated by Carlsen *et al*. (1996) in a series of batch experiments. The specific growth rate of the fungus, grown in the form of pellets with approximately the same size distribution, increased as the temperature raised from 27 to 35°C to reach its maximum value at 35°C. Pellet formation is affected by temperature. Braun and Vecht-Lifshitz (1991) suggested temperature reducing, among other possible approaches, for inducing mycelia pellet formation in fermentation culture.

Effect of Inoculum Size

If the inoculum concentration used was below 10^4, the inoculum size might be not sufficient for the production of enzyme and if the inoculum concentration used was above 10^7, the fungus might over grow and led to the phenomena where the fungus lacked of nutrient for the enzyme production (Table 3). In order to find the optimal inoculum volume, *A. niger* was cultivated under different inoculum sizes ranging from 1×10^4 to 1×10^7 spore mL^{-1} at a constant agitation speed of 150 rpm, temperature 30°C and 2% (w/v) of PKC as the main carbon source

Previous investigations shown that the inoculum size was one of the important factors that influenced mannanase production in liquid fermentation of *A. niger* FTCC 5003 (Abd-Aziz *et al*., 2003). It has also been reported that inoculum size influenced the mycelial growth and exo-biopolymer production (Park *et al*., 2001) and inoculum volume is another factor that influenced the mycelial growth and production of exopolysaccharides (Lee *et al*., 2004). Beside that, the inoculum concentration was related to the mycelia growth and mannanase production by *A. niger* FTCC 5003, which was in accordance with the results reported by other investigators (Park *et al*., 2001; Xiao *et al*., 2004).

In this study, the duration for the fermentation process was 10 days because the increment of enzyme production could be observed at day 7. Figure 3A and B shows the soluble protein concentration and reducing sugar during the 10 days of fermentation, respectively suggesting that the excretion of mannanase was largely growth associated.

Effect of Palm Kernel Cake Concentration

Fermentation media can be chemically defined (synthetic) or complex. When raw materials are used, they may require prior purification as some trace metals affect certain processes, such as the extensively studied citric acid fermentation by *A. niger* (Papagianni, 2004). In this study, palm kernel cake act as the major carbon source in the liquid culture.

Figure 4A-C shows the profile of mannanase activity, reducing sugar and soluble protein at different concentration of PKC. Table 4 shows the productivity of mannanase at different percentage

Table 3: The mannanase production by *A. niger* FTCC 5003 from various inoculum concentration in liquid fermentation

Inoculum concentration (spores mL^{-1})	Maximum enzyme activity (U mL^{-1})	Yield (U/g PKC)	Productivity (U/mL/day)
1×10^4	236.45	11822	23.65
1×10^5	196.63	9831	19.66
1×10^6	154.84	7742	17.20
1×10^7	114.43	5722	11.44

Table 4: The mannanase production by *A. niger* FTCC 5003 from various percentage of palm kernel cake in liquid fermentation

% of PKC	Maximum enzyme activity (U mL^{-1})	Yield (U/g PKC)	Productivity (U/mL/day)
1	74.89	7489	8.32
2	135.48	10163	15.05
4	186.03	6967	23.25
6	89.66	2241	12.81

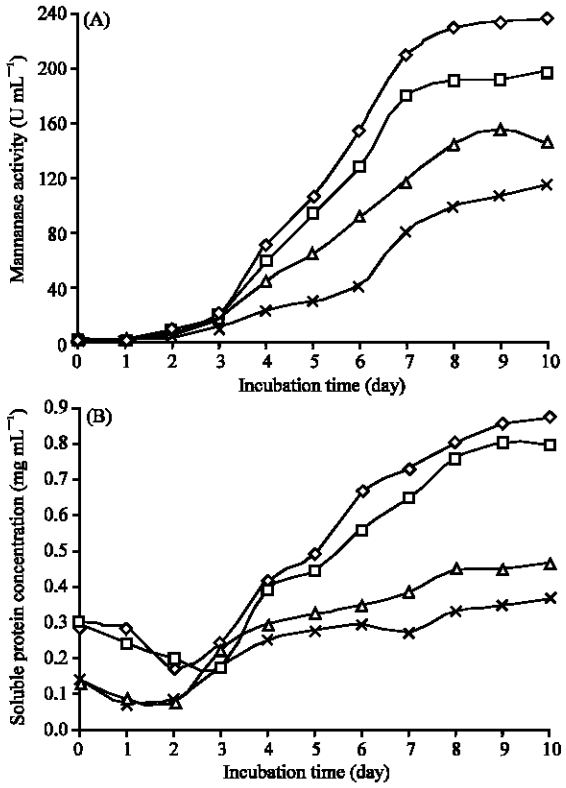

Fig. 3: Mannanase production by *A. niger* FTCC 5003 at different inoculum size of (◊) 1×10^4 spores mL^{-1}, (□) 1×10^5 spores mL^{-1}, (Δ) 1×10^6 spores mL^{-1} and (×) 1×10^7 spores mL^{-1}. Enzyme activity (◊) and soluble protein (□)

of PKC and the maximum enzyme activities at the percentage of PKC of 1, 2, 4 and 6% were 75, 135, 186 and 89 U mL^{-1}, respectively (Fig. 4). The type of growth was hardly be observed for the four different concentration of PKC studied. This might be due to the soluble protein concentration being an indirect method to measure the growth rate. Besides the mycelium can contribute to the changes of soluble protein concentration, enzymes also contribute to the changes of the protein concentration. The consumption of reducing sugar was decreased as the percentage of PKC decrease (Fig. 4C).

Pokhrel and Ohga (2007) found that carbon source was independently responsible in mycelial growth and polysaccharide production. Therefore, even if the same fungal species needs a different carbon source for specific metabolite production, it is possible that different carbon sources might have different effects on catabolic repression on the cellular secondary metabolism. Such a phenomenon was also claimed in submerged of several kinds of mushroom (Hwang *et al*., 2003; Kim *et al*., 2003).

Enzyme Production Using Optimised Parameters

A. niger FTCC 5003 was grown in liquid cultures for 10 days, using palm kernel cake as the major carbon source and optimized condition obtained (Fig. 5). The enzyme activity increased regularly starting at day 3 and reached the highest level after 8 days of culture (104 U mL^{-1}) and started to decrease after 9 days of fermentation with productivity of 13.00 U/mL/day. However, the soluble protein concentration, reached maximum after 9 days of fermentation.

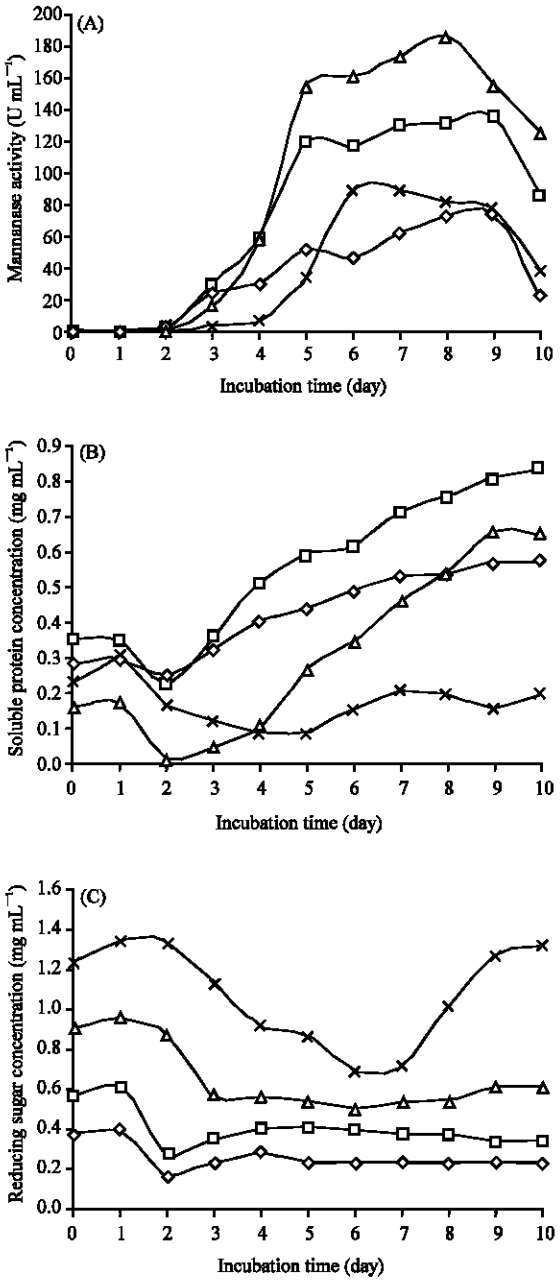

Fig. 4: Mannanase production by *A. niger* FTCC 5003 at different percentage of PKC 1% (◊), 2% (□), 4% (Δ) and 6% (×). (A) enzyme activity, (B) soluble protein concentration and (C) reducing sugar concentration

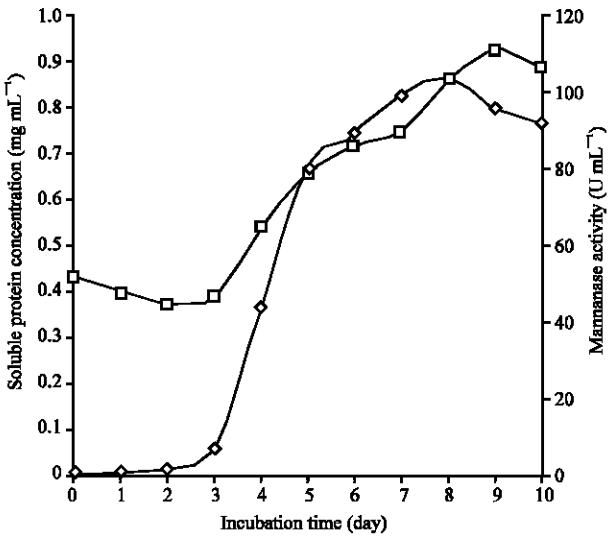

Fig. 5: Mannanase productions by *A. niger* FTCC 5003 using optimised parameter obtained. (◇) mannanase activity and (□) soluble protein concentration

CONCLUSION

Palm kernel cake was found suitable to be used as substrate for mannanase production by *A. niger* in submerged fermentation; with substantial enhancement in growth and enzyme production. The optimal culture condition for the production of mannanase using PKC was proposed at agitation speed of 200 rpm, temperature 35°C, 1×10^4 spores mL^{-1} of inoculum size and 2% of palm kernel cake. The final maximum activity obtained using the optimal fermentation condition was 104 U mL^{-1} and productivity of 13.00 U/mL/day.

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