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Production of Cellullolytic Enzymes by a Newly Isolated, *Trichoderma* sp. FETL c3-2 via Solid State Fermentation Grown on Sugar Cane Baggase: Palm Kernel Cake as Substrates

[1]Pang Pei Kheng, [1]Darah Ibrahim, [2]Laszlo Poppe, [3]George Szackacs and [1]Ibrahim Che Omar
[1]Fermentation and Enzyme Technology Laboratory, School of Biological Sciences,
Universiti Sains Malaysia, 11800 Penang, Malaysia
[2]Institute for Organic Chemistry, [3]Department of Agricultural Chemical Technology,
Budapest University of Technical and Economics, Gellert ter 4, Budapest H-1111, Hungary

Abstract: The production of cellulase by a local isolate *Trichoderma* sp. FETL c3-2 via solid state fermentation system using sugar cane baggase:palm kernel cake as substrate mixture was investigated. The optimized Solid State Fermentation (SSF) system consists of 5 g of sugar cane baggase: palm kernel cake, moisture content of 75% (w/w), pH of moistening agent of pH 7.0, at 30°C and inoculum size of 1×10^8 spores mL^{-1}. The SSF system was also supplemented with 4% (w/w) dextrin and 6% (w/w) of yeast extract as additional carbon and nitrogen sources, respectively. Cellulose at the concentration of 0.2% (w/w) was found to be a significant inducer for cellulase production. Using the optimized SSF system, maximum FPase and CMCase production of 3.3 U g^{-1} substrate and 18.05 U g^{-1} substrate, respectively, after 4 days of fermentation time at 30°C were obtained. The modification of the SSF system resulted a 105% increment of FPase and 63% of CMCase production. Although no significant increase in growth after modification was observed, the results obtained indicated that the cellulase production by *Trichoderma* sp. FETL c3-2 is growth dependent.

Key words: Cellulolytic enzymes, *Trichoderma* sp. FETL c3-2, solid state fermentation, sugar cane baggase, palm kernel cake

INTRODUCTION

Malaysia generates about five million tonnes of agrowastes annually from its plantation crops mainly oil palm, rubber, rice, cocoa, coconuts and pineapples which are expected to increase at the rate of 10% a year. However, systematic management of these wastes has never been given serious attention, although everybody is aware of its consequences to the environment and other economic implications. Although a small amount of these wastes are use for composting under domestic scale, most of the wastes were either burned or allowed to decompose naturally with the exception of palm kernel cake which is exported to the European countries as animal feed. Thus, it is timely that Malaysia utilizes effectively the agrowastes as substrates for the production of added value products of commercial potentials. Enzymes constitute one of the products which have a wide market potential and highly demanded for industrial applications in Malaysia. Nevertheless, all the enzymes used in Malaysia are imported, mainly from Denmark, Netherlands, Belgium and others, mounting to about USD15.0 millions in year 2003, consumed by less than 5% of the total potential markets in Malaysia (Department of Statistics, Malaysia, 2003). The percentage is small mainly due to the high enzyme cost. Therefore, the production of enzymes in Malaysia, with the target of meeting local needs, should be an important and immediate agenda in manufacturing industries. Thus, if Malaysia is to embark on the enzyme production industries, the use of Solid State Fermentation (SSF) would be an excellent alternative to that of the submerged systems as a cheap and economical process which has been shown to be successful in several developed countries (Pandey, 1992).

Our laboratory has extensively carried out research activities on the utilization of agrowastes such as palm kernel cake, sugar cane baggase, oil palm fronds and trunks, rice husks and straws, rubber wood dusts, cocoa pods and coconut fibres and meals, for various applications including enzyme production via SSF system. Cellulase, xylanase, lipase, protease, chitinase, tannase and glucosidase are among the enzymes studied via SSF on agrowastes as substrates using local microbial

Corresponding Author: Ibrahim Che Omar, Fermentation and Enzyme Technology Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia Tel: 604-6534052 Fax: 604-6565125

isolates. In order to ensure viable commercialization of enzyme production via SSF system, a cheap system with hyper enzyme producers must be established. The current work focuses on cellulase production, one of the important enzymes for industrial applications in Malaysia. Most of the cellulases in Malaysia are used in the preparation of pre-digested animal feed, food industries and detergent industries. However, we are proposing the use of cellulase and xylanase in pulp and paper industries mainly for the enzymatic deinking of waste papers for paper recycling, of which the success depends on the availability of cheap and economical sources of enzymes. We have reported the production of xylanase by *Aspergillus niger* USM A1 I (Kheng and Omar, 2005) and lipase by *Aspergillus flavus* USM A10 (How and Ibrahim, 2004) and *A. terreus* E5 (How *et al*., 2004) and the current work complements the enzymes required in the enzymatic deinking process. This study describes the cellulase production by one of the potential Malaysian isolates, *Trichoderma* sp. FETL c3-2 using sugar cane baggase: palm kernel cake as substrates and the parameters governing the productivity of the enzymes.

MATERIALS AND METHODS

Source of microorganism: *Trichoderma* sp. FETL c3-2 was isolated from the soils obtained from the Northern Region of Peninsular Malaysia and kept at the Culture Collection of Fermentation and Enzyme Technology Laboratory (FETL), Universiti Sains Malaysia. The fungus was maintained on potato dextrose agar and kept at 4°C prior to use.

Substrates for Solid State Fermentation (SSF): The substrates used in the SSF system consist of sugar cane baggase, palm kernel cake and rice husks which were obtained locally in Malaysia. All the substrates were dried under sunlight until constant weight before use to avoid the interference in the moisture content of the substrates. Proximate analysis was carried out for all the substrates based on the methods described by AOAC (1997).

Cultivation system of SSF cultivation system of SSF: The cultivation of the fungus in the SSF system was performed in a 500 mL Erlenmeyer flask containing 5 g substrate with the addition of known volume of the moistening agent to give the desired moisture content in the SSF system. The moistening agent solution consists of (g L^{-1}) NH_4NO_3; KH_2PO_4; 5corn steep liquor; 2, NaCl; 1 and $MgSO_4.7H_2O$; 1. The moistening agent solution was also supplemented with trace elements consisting of (mg L^{-1}): $FeSO_4.7H_2O$; 5, $MnSO_4.4H_2O$; 1.6, $ZnSO_4.7H_2O$; 3.45 and $CoCl_2.6H_2O$ 2.0 of pH 7.0. Cultivation was carried out for known incubation period at 30°C with the inoculum concentration of 1×10^6 spores mL^{-1}. The inoculum was prepared by growing the isolate on malt extract agar at 37°C until sporulation. The spores were harvested using 0.1% (v/v) Tween 80 (Smith *et al*., 1996). The number of spores was estimated by direct microscopic counting using haemocytometer

Optimization of SSF system for cellulase production: The optimization of SSF system for cellulase production was performed based on the modification of the physical parameters and the supplementation of additional nutrients. The effect of physical parameters was determined based on the modification of moisture content in the range of 65-85% (w/w), pH of the moistening agent in the range of pH 6 -10, cultivation temperature in the range of 25-40°C and inoculum sizes in the range of 1×10^6 - 1×10^8 spores mL^{-1}.

The effect of supplementation of additional carbon and nitrogen sources and inducer on cellulase production was examined. The carbon sources examined consist of maltose, starch, sorbitol, lactose, dextrin and sucrose, while the nitrogen sources consist of peptone, urea, tryptone, yeast extract and sodium nitrate. The concentration at 4% (w/w) was used for both the carbon and nitrogen sources. Xylan, cellulose, carboxyl methyl cellulose and xylose were examined as inducer at 0.2% (w/w).

Cultivation was carried out for 4 days unless otherwise stated. All experiments were carried out in triplicates and data were presented as mean of the triplicates experiments.

Extraction of the enzyme: After the cultivation period, the biomass from each flask was extracted using 150 mL of distilled water containing 0.1% (w/v) of Tween 80 and allowed to stand for 2 h to further complete the extraction of the enzymes from the substrates. The extracted biomass was centrifuged at 6000 rpm for 10 min at 4°C and the clear supernatant obtained was used as the enzyme source.

Analysis: Growth of the fungus was determined based on the glucosamine method as described by Swift (1972). The cellulase activity was determined based on the Filter Paper (FPase) and the carboxylmethyl cellulose (CMCase) activities. FPase activity was determined by the method of Ghose (1987). A 1×6 cm Whatman No.1 filter paper strip was added to 1.5 mL of culture filtrate containing 0.05 M citrate buffer (pH 4.8). The samples were incubated for 1 h at 50°C. The hydrolysis was terminated by the

addition 3 mL of dinitrosalicylic acid solution, followed by boiling for 5 min. After cooling, 20 mL of distilled water were added and the absorbance at 540 nm was determined. One unit of filter paper activity is defined as the amount of enzyme that releases 1 μmol of glucose per min under the assay conditions. The carboxylmethyl cellulase activity (CMCase) was determined by the method of Gessesse and Mamo (1999). One unit of CMCase activity was defined as the amount of enzyme that releases 1 μmol of glucose per min under the above assay conditions. The mean data were presented as U per g substrate calculated from triplicate experiments.

RESULTS AND DISCUSSION

Production of cellulolytic enzymes on various agrowastes as substrates: Figure 1a shows the *Trichoderma* sp. FETL c3-2 under the scanning electron microscope showing the structures of conidiophores, phialides and conidia. Figure 1b also shows the fungal growth on the sugar cane baggase as substrate, while Fig. 1c shows the ramification of mycelia and conidiophores intertwining on the surfaces of the substrate. The growth rate and the rate of FPase and CMCase production by *Trichoderma* sp. FETL c3-2 on various substrates with different particle size and moisture content are shown in Table 1. The use of sugar cane baggase, palm kernel cake and rice husks revealed that the growth rate increased with the increase in moisture content, however, the enzyme production exhibited variations depending not only on the moisture content but also the particle size. Smaller particle size (1 mm) at higher moisture content did not improve the production. The maximum cellulase production rate obtained with sugar cane baggase was 0.44 U FPase/g substrate/day and 2.18 U CMCase/g substrate/day. Palm kernel cake (PKC) gave lower FPase and CMCase production rate although a higher maximum growth of 0.91 mg glucosamine g^{-1} substrate/day was obtained with PKC. The results suggested that PKC may be added in the SSF system to enhance the growth. With the moisture content set at 75%, the maximum enzyme production rates were 0.41 U FPase/g substrate/day and 2.76 U CMCase/g substrate/day with the fungal growth rate of 0.57 mg glucosamine per g substrate/day. In the subsequent experiments, the SSF system using the substrate mixture of sugar cane baggase and PKC was studied.

Proximate analysis of sugar cane baggase revealed that the cellulose and hemicellulose content of 47.34 and 29.30%, respectively are higher than in rice husk and palm kernel cake which is in the range of 20-30%. At the same time, palm kernel cake which contains 16% protein and more than 8% lipid content justify the good fungal growth on PKC. However, the lipid content did not support the synthesis of cellulolytic enzymes by the fungus. Rice husk with about 57% lignin contributes to poor fungal growth and low enzyme production. The use of baggases has also been reported previously, using different microorganisms with large variation in the cellulase productivities depending on the types of substrates and cultural conditions (Jecu, 2000; Ogel *et al*., 2001; Kang *et al*., 2004). Thus, it is important to determine the optimal cultural conditions in SSF for improving enzyme productivities by a particular isolate.

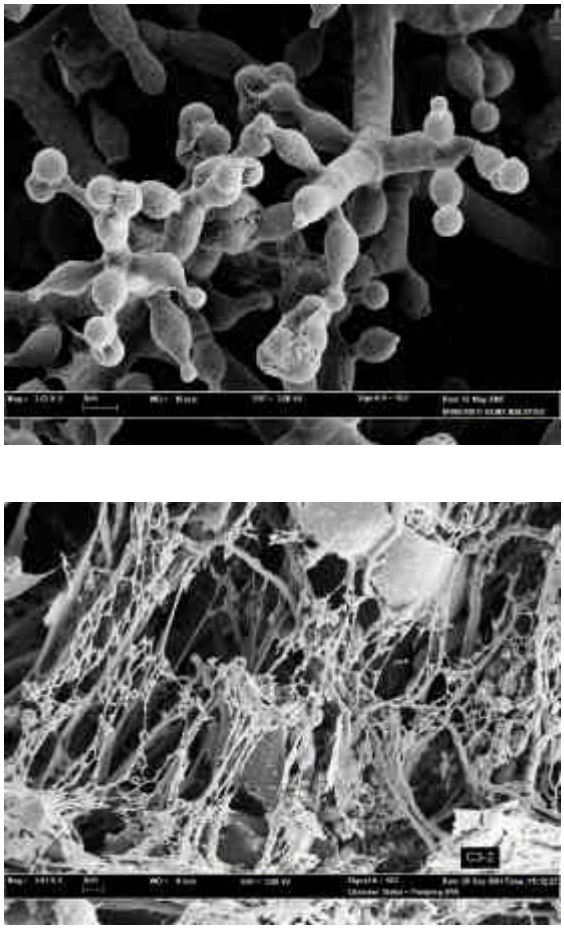

Fig. 1: *Trichoderma* sp. FETL c3-2 a) Fungal structural under scanning electron microscope showing the conidiophores (1), phialides (2) and the conidia (3) (3520×magnification) b) Growth on sugar cane baggase

Table 1: The production of cellulolytic enzymes by *Trichoderma* sp. FETL c3-2 on various substrates in SSF cultivation system

Substrate (particle size)	Moisture content (%)	Growth rate (mg glucosamine g^{-1} substrate/day)	FPase production rate (U g^{-1} substrate/day)	CMCase production rate (U g^{-1} substrate/day)
Single substrate				
Sugar cane baggase (SC) (2 mm)	80	0.55	0.44	2.18
Palm Kernel Cake (PKC)	50	0.91	0.17	1.43
Rice husk (RH) (2 mm)	50	0.27	0.19	1.43
RH (1 mm)	50	0.23	0.22	1.40
Single substrate				
Sugar cane baggase (SC) (2 mm)	83	0.69	0.46	2.15
Palm Kernel Cake (PKC)	60	0.91	0.16	1.30
Rice husk (RH) (2mm)	60	0.29	0.21	1.50
RH (1mm)	60	0.27	0.17	1.22
Mixed substrate*				
SC (2 mm) PKC	65	0.43	0.38	2.71
RH (2 mm) PKC	50	0.66	0.32	2.49
RH (2 mm) PKC (1 mm)	65	0.28	0.21	1.75
RH (1 mm) SC (2 mm)	65	0.27	0.19	1.54
Mixed substrate*				
SC (2 mm) PKC	75	0.57	0.41	2.76
RH (2 mm) PKC	75	0.63	0.24	2.37
RH (2 mm) PKC (1 mm)	75	0.29	0.20	1.61
RH (1 mm) SC (2 mm)	75	0.26	0.24	1.61

*The substrates used at the ratio of 50:50 (w/w).

Table 2: Effect of ratio of the SC:PKC on cellulase production by *Trichoderma* sp. FETL c3-2

Substrate ratio (%,w/w)	Moisture content (%)	Growth rate (mg glucosamine g^{-1} substrate/day)	FPase production rate (U g^{-1} substrate/day)	CMCase production rate (U g^{-1} substrate/day)
Control (50:50)	65	0.55	0.38	2.71
70:30	65	0.54	0.42	2.84
80:20	75	0.54	0.44	2.95
90:10	75	0.52	0.49	3.43
100:0	80	0.41	0.48	3.13
Control (50:50)	75	0.58	0.41	2.76
70:30	75	0.55	0.43	2.87
80:20	80	0.53	0.44	2.99
90:10	80	0.51	0.47	3.36
100:0	83	0.45	0.42	3.12

Effect of substrate ratio on cellulase production: The effect of the substrate ratio of sugar cane baggase and palm kernel cake was examined. PKC contains about 16% protein which is expected to provide the nitrogen content needed for growth. As indicated in Table 2, it was observed that increasing the amount of sugar cane baggase improved the cellulase activity significantly. A maximum production rates of 0.49 and 3.43 U g^{-1} substrate/day were obtained with FPase and CMCase, respectively with the substrate mixture of 90:10 (%,w/w) of sugar cane baggase and PKC. Increasing the amount of PKC, improved the growth, however the enzyme production rate decreased gradually. The high lipid content of PKC might have cause poor water absorption capacity and prevent effective oxygen diffusion within the substrate and biomass resulted in poor growth and enzyme production. On the other hand, high lipid content in PKC has been shown to be a good inducer for the production of lipase via SSF system (How and Ibrahim, 2004). Kang *et al.* (2004) also showed that the production of cellulases and hemicellulases by *Aspergillus niger* KKS was higher in rice straw: wheat bran mixture using submerged system. Nevertheless, higher activities were obtained using rice straw alone in the SSF system by *A. niger* KK2. It is suspected that mixing of substrates with different properties may interfere the physiology of the fungi and thus subsequently affected its metabolic activity and degradation process. In the case of sugar cane baggase and PKC, the small proportion of PKC helps to improve the growth of the fungus, especially during the early part of the SSF.

Effect of moisture content: Moisture content is an important parameter in SSF processes. Water causes the swelling of the substrate and facilitates effective absorption of the nutrients from the substrate. The moisture content was adjusted by adding the moistening agent to the substrate to give the moisture content ranging from 65 - 85%. As shown in Table 3, the moisture content of 75% was found to be optimum with the FPase production of 2.25 U g^{-1} substrate, while the CMCase production of 14.3 U g^{-1} substrate of moisture content. Lower moisture content of less than 75% or higher did not improve the enzyme production and the growth of the fungus. Low water content is related to insufficient substrate swelling which prevented the nutrient absorption from the substrate. Virupakshi *et al.* (2005) have shown that the moisture content also contributed to

Table 3: Effect of moisture content on the production of cellulases by *Trichoderma* sp. FETL c3-2

Moisture content (%, w/w)	FPase production (FPU g^{-1} substrate)	CMCase production (U g^{-1} substrate)	Growth (mg glucosamine g^{-1} substrate)
65	1.12±0.02	11.08±0.04	1.85±0.02
70	1.49±0.04	12.11±0.11	1.96±0.03
75	2.25±0.02	14.28±0.09	2.42±0.02
80	1.87±0.04	13.68±0.11	1.85±0.02
85	1.42±0.04	11.06±0.03	1.77±0.02

Table 4: Effect of cultivation temperature on the production of cellulases by *Trichoderma* sp. FETL c3-2

Temperature (°C)	FPase production (FPU g^{-1} substrate)	CMCase production (U g^{-1} substrate)	Growth (mg glucosamine g^{-1} substrate)
25	1.79±0.05	12.61±0.05	2.01±0.02
30	2.35±0.01	14.30±0.04	2.43±0.04
32	1.98±0.04	12.23±0.08	1.68±0.01
35	1.65±0.07	10.90±0.03	1.59±0.02
40	0	0	0

Table 5: Effect of pH of the moistening agent on the production of cellulases by *Trichoderma* sp. FETL c3-2

pH	FPase production (FPU g^{-1} substrate)	CMCase production (U g^{-1} substrate)	Growth (mg glucosamine g^{-1} substrate)
6	1.63±0.01	12.53±0.10	1.96±0.01
7	2.45±0.04	14.48±0.04	2.40±0.01
8	1.79±0.01	13.13±0.05	2.00±0.01
9	1.60±0.01	12.43±0.03	1.97±0.02
10	1.21±0.01	10.67±0.07	1.77±0.03

the gummy texture of the rice bran which affected oxygen transfer and diffusion. At the same time, higher water content resulted in the reduction of substrate porosity and caused oxygen limitation within the substrates which subsequently affected the growth and enzyme production.

Effect of temperature: Like the moisture content, temperature is also one of the determinants in the success of SSF. The temperature in a SSF system is a net resultant of the environmental temperature and temperature increment as a result of heat generated from the metabolic activity of the fungus growing on the solid substrate. The production of cellulase by *Trichoderma* sp. in the SSF system, shows maximum production at 30°C with the FPase production of 2.35 U g^{-1} substrate and CMCase of 14.30 U g^{-1} substrate (Table 4). The fungal growth dropped drastically at the temperature of 40°C and no growth and enzyme production were observed. Similar observation has been reported using other fungi and thus confirming the views that most SSF systems and the stability of the enzymes produced by these fungi were strongly related to the temperatures in its natural habitat (Szewczyk and Myszka, 1994; Jecu, 2000). For many fungi, the optimum temperature is in the range of 28-35°C.

Effect of pH of the moistening agent on cellulase production: pH is an important physiological parameter studied in numerous submerged fermentation processes. However, limited reports are available on the importance of pH in SSF processes. Furthermore, the mechanism of pH effect on the growth and metabolite production by microorganisms on solid substrates remains unclear. This is because the natural habitat of microorganisms on solid substrates can be highly influenced by rapid changes in several governing physical parameters in its environment, including changes in environmental pH. However, microorganisms are highly acclimatized to these changes and rarely show a drastic change in the growth and metabolic activities. The initial pH of the substrate was adjusted by adding the moistening agent of different pH. The results obtained as shown in Table 5 suggested that pH affected the growth and cellulase production by *Trichoderma* sp. FETL c3-2 in the SSF system. The optimum pH was pH 7 which gave the enzyme productivity of 2.45 U g^{-1} substrate of Fpase and 14.48 U g^{-1} substrate of CMCase (Table 5). Gradual drop in growth and enzyme production was observed after pH 7 which also suggested that the possibility of enzyme instability at higher pH might have occurred. Panagiotou *et al*. (2003) have shown that the pH effect is closely related to the moisture content; larger effect was observed with SSF system requiring higher moisture content.

Effect of inoculum size: The effect of inoculum size was examined using the spore suspension of concentration from 1×10^{6} -1×10^{8} spores mL^{-1} for a fermentation period of up to 7 days. The results of the cellulase activity and growth profiles using different inoculum sizes, shown in Fig. 2a-c are similarly observed by other fungi (Ikasari and Mitchell, 1994; Kheng and Omar, 2005). As shown in the figure, higher inoculum size of 1×10^{8} spores mL^{-1} enabled the maximum growth to be achieved in a shorter time giving a maximum FPase production of 2.55 U g^{-1} substrate and growth of 2.30 mg glucosamine g^{-1} substrate after 4 days of fermentation. With the inoculum size of 1×10^{7} spores mL^{-1}, a lower maximum production of 2.45 U g^{-1} substrate after 4 days of fermentation. However, a lag phase of 3 days was also observed before the exponential increase in the activity. On the other hand, with the inoculum size of 1×10^{6} spores mL^{-1}, after the 3-day lag phase, a gradual increase was observed with a maximum FPase of 2.45 U g^{-1} substrate after 4 days of fermentation. Generally, lower growth profiles were observed with lower inoculum sizes. Similar trend of CMCase activity profiles was also observed with the maximum production of about 15.0 U g^{-1} substrate obtained after 3-4 days of fermentation with the inoculum size of 1×10^{8} spores mL^{-1}. With higher inoculum size, the time taken for the inoculum to colonize the substrate will be shorten significantly with higher enzyme productivity.

At the same time, the enzyme production profiles correspond with the growth profiles, suggesting a growth dependent phenomenon in enzyme production. Similar observation has been reported by Raimbault and Alazard, (1980) who deduced that higher enzyme production at higher inoculum is related to the rapid growth of the fungus as a result of higher degradation of the substrates and the increase availability of the nutrients.

Effect of supplementation of carbon sources: The effect of additional carbon sources was examined at 4% (w/w) and the results obtained is shown in Table 6. When compared to the control system without the addition of any carbon sources, it was observed that dextrin was found to enhance the production of FPase of about 2.56 U g^{-1} substrate and the production of CMCase was about 14.74 U g^{-1} substrate. The growth of the fungus varied accordingly with the types of carbon sources supplemented, although the presence of exogenous carbon sources resulted in slightly lower growth compared to the cultivation without the supplementation of any additional carbon sources. Increase in enzyme production with additional carbon sources has been demonstrated by both the submerged and SSF systems as a result of good growth (Solis-Pereira *et al*., 1993) The effect of dextrin concentration was also determined in the range of 2-10% (w/w). The enzyme production and growth were maximum at 4% (w/w) and the production dropped gradually thereafter. Sternberg (1976) have shown that the drop in enzyme production and fungal growth in the presence of sugars was an indication on the impact of catabolite repression on enzyme synthesis. Based on the larger drop in enzyme production, catabolite repression is more obvious in the case of CMCase compared to that of FPase, suggesting that each enzyme in a complex enzyme system can be enhanced under different cultural conditions. Wen *et al.* (2005) have shown that production of enzyme mixture must be carried out under sub-optimal conditions in order to provide conducive conditions for the production of one enzyme, while preventing catabolite repression for another enzyme under high concentration of the carbon sources.

Effect of supplementation of nitrogen sources: The effect of supplementation of nitrogen sources in SSF system on the growth and enzyme production is shown in Table 7. As shown in the Table 7, the supplementation of peptone or yeast extract favours the production of FPase with the production of 2.7-2.8 U g^{-1} substrate, while the production of CMCase of 15.5-16.0 U g^{-1} substrate. Similar observation has been reported by Panagiotou *et al.* (2003) who showed that different

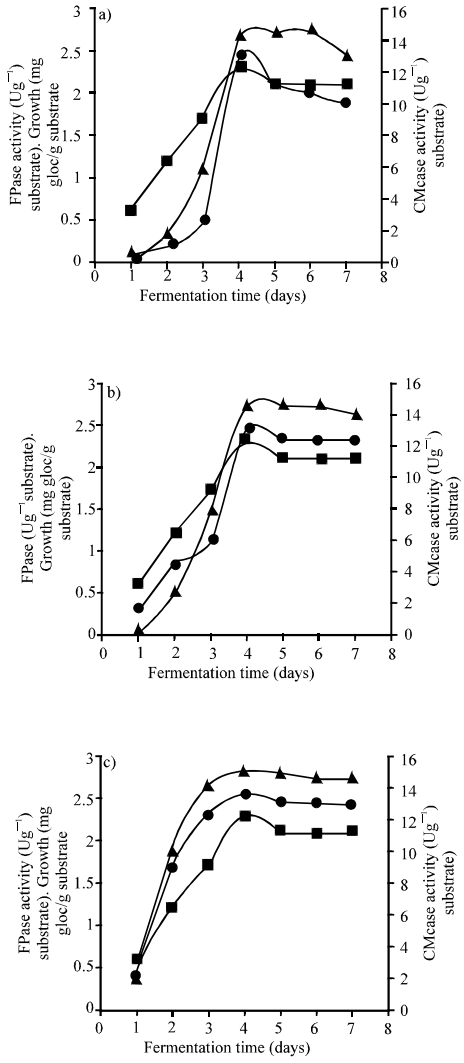

Fig. 2: Effect of inoculum sizes on the production of cellulases by *Trichoderma* sp. FETL c3-2. Inoculum sizes: a) 1×10^{6}, b) 1 x 10^{7} c) 1 x10^{8} spores mL^{-1}, FPase (●), CMCase (▲) and growth (■)

nitrogen sources exhibited different effect on each enzyme activity in the enzyme complex system. Other nitrogen sources resulted the FPase production in the range of 2.3-2.7 U g^{-1} substrate, while the CMCase production in the range of 12.0-15.0 U g^{-1} substrate. The effect of concentration of yeast extract of more than 6% (w/w) did not improve the enzyme production and growth of the fungus. The increase in the production of the cellulase in the presence of additional carbon and nitrogen sources suggested that the available sources in sugar cane baggase were inadequate or not accessible to the microorganisms for growth and enzyme synthesis.

Table 6: Effect of additional carbon sources in the SSF system on the production rate of cellulases by *Trichoderma* sp. FETL c3-2

Carbon sources (%, w/w)	FPase production (FPU g^{-1} substrate)	CMCase production (U g^{-1} substrate)	Growth (mg glucosamine g^{-1} substrate)
Maltose	2.11±0.01	13.74±0.01	1.97±0.01
Starch	1.91±0.01	13.66±0.01	1.95±0.01
Sorbitol	1.74±0.01	12.25±0.03	2.17±0.01
Lactose	1.84±0.01	13.11±0.01	2.21±0.01
Dextrin	2.56±0.06	14.74±0.07	2.23±0.01
Sucrose	1.81±0.02	12.15±0.01	1.91±0.02
Glucose	1.48±0.03	11.61±0.01	1.97±0.02

Table 7: Effect of additional nitrogen sources in the SSF system on the production rate of cellulases by *Trichoderma* sp. FETL c3-2

Nitrogen sources (%, w/w)	FPase production (FPU g^{-1} substrate)	CMCase production (U g^{-1} substrate)	Growth (mg glucosamine g^{-1} substrate)
Peptone	2.71±0.03	15.48±0.01	1.96±0.06
Urea	2.29±0.01	11.59±0.11	1.93±0.05
Tryptone	2.48±0.03	15.11±0.02	2.17±0.06
Sodium nitrate	2.65±0.04	15.39±0.10	1.69±0.01
Yeast extract	2.81±0.04	16.23±0.08	2.26±0.01

Table 8: Effect of inducers in the SSF system on the production rate of cellulases by *Trichoderma* sp. FETL c3-2

Inducers (%, w/w)	Fpase production (FPU g^{-1} substrate)	CMCase production (U g^{-1} substrate)	Growth (mg glucosamine g^{-1} substrate)
Xylan	2.52±0.02	16.50±0.10	2.44±0.01
Cellulose	3.33±0.03	18.10±0.05	2.30±0.01
Carboxylmethyl cellulose	2.65±0.01	16.59±0.01	2.70±0.01
Xylose	2.88±0.00	16.61±0.01	2.72±0.02

Effect of the supplementation of inducers: Most industrial enzymes are highly inducible and therefore the presence of inducers in the culture medium enhanced the enzyme production significantly. In the case of cellulase, the inducible compounds mainly cellulose are bound in the substrates. Therefore, growth and enzyme secretion become a pre-requisite which will subsequently releases the compounds which can now act as carbon source or inducer for enzyme production. However, growth and enzyme production will not take place simultaneously during the initial stage of the fermentation. Thus, in order to accelerate enzyme production as early as possible during the fermentation, inducible compounds must be supplemented. Several related compounds which act as inducers were examined. As shown in Table 8, the presence of cellulose enhanced the production of FPase to about 3.3 U g^{-1} substrate, while other inducers such as xylan, carboxymethyl cellulose and xylose resulted the production level in the range of 2.5-2.9 U g^{-1} substrate. As for the CMCase, the production was also affected by the presence of cellulose with production of about 18.05 U g^{-1} substrate. The concentration of cellulose of more than 0.2% (w/w) did not show any significant effect on the enzyme production or the growth of the fungus. Adsul *et al*. (2004) and Sudgen and Bhat (1994) have clearly shown that medium containing high cellulose concentration did not show superior productivities of cellulases compared to only the agrowastes, again suggesting the phenomenon of catabolite repression must have occurred.

The results obtained in the work indicated that with the modification of the SSF system, the production of FPase has improved from 1.61 U g^{-1} substrate to 3.30 U g^{-1} substrate. While, CMCase production increased from 11.05 U g^{-1} substrate to 18.00 U g^{-1} substrate. However, the growth was not significantly affected after the modification of the cultural conditions with an average of about 2.3 mg glucosamine g^{-1} substrate. Nevertheless, under many circumstances, it is demonstrated that higher growth resulted a higher enzyme production, suggesting that the enzyme production is growth dependent. Enzymes are primary metabolites and therefore the growth dependent of metabolite synthesis is most likely expected which has also been reported both in the SSF and submerged systems. Comparison of the enzyme productivities with other reported data poses several problems. This is because apart from the different microorganisms used, the substrates are water insoluble with large variation in physical properties and a wide range of structural composition. The structural and chemical composition of the substrates may also vary depending on the types of plant species and origin. Furthermore, the degradation of the lignocellulolytic biomass requires a multi component enzyme system. Therefore, quantitative comparison remains inaccurate and subjective. In the case of *Trichoderma* sp FETL c3-2, regardless of the enzyme production level, most importantly, it has been shown that the cellulases it produces is effective in enzymatic deinking of laser printed waste papers. Some of the findings on the deinking process will be reported subsequently.

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