African Journal of Biotechnology Vol. 8 (10), pp. 2283-2288, 18 May, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Mycelial growth interactions and mannan-degrading enzyme activities from fungal mixed cultures grown on palm kernel cake

F. B. Iluyemi[1] and M. M. Hanafi[2]*

[1]Department of Land Management, Faculty of Agriculture, Universiti Putra Malaysia 43400-UPM, Serdang, Selangor, Malaysia.
[2]Institute of Tropical Agriculture, Universiti Putra Malaysia 43400-UPM, Serdang, Selangor, Malaysia.

Accepted 23 April, 2009

Palm kernel cake (PKC), a by-product of the palm kernel oil extraction process contains mannan as its main polysaccharide. Mixed culture microbial degradation may enhance mannan-degrading enzymes production. Therefore, the objective of the study was to examine the nature of mycelial interactions and corresponding production of mannan-degrading enzymes of PKC. Fungal interactions was carried out using *Sclerotium rolfsii* and *Aspergillus niger* co-cultured with three *Trichoderma* strains (*Trichoderma harzianum, Trichoderma longiobrachiatum* and *Trichoderma koningii)* on potato dextrose agar (PDA) in disposable petri-dishes. Measurements of growth diameters were taken on days 2 and 13. For mannan-degrading enzyme production, single and co-cultures of these fungi were carried out under submerged cultivation for 13 days with PKC as the carbon source. About 57% of observed interactions on PDA were deadlock, 29% replacement and 14% intermingling. In *Trichoderma* sp./*A. niger* mixed cultures, there was an overall significant enhancement of enzyme: 2 to 200 fold (β-D-mannanase), 8 to 25 fold (β-mannosidase) and from no change to 15 fold increase (α-galactosidase). There was no obvious relationship between enzyme production and protein yield. However, co-culturing of *A. niger* with the *Trichoderma* strains showed an enhancement of mannan-degrading enzyme activities without reducing biomass yield.

Key words: α-Galactosidase, β-D-mannanase, β-mannosidase, fungi, co-cultures, palm kernel cake.

INTRODUCTION

Enzymes have been in commercial use in the food, feed, brewing, textile and other industries for their ability to speed up reaction rates by lowering the activation energy of chemical reactions. A variety of plants, animal and microbial sources are used for the production of industrial enzymes (Frost and Moss, 1987). However, microbial enzymes are more in use due to cheaper substrates and ease of process modification. In microbial enzyme and biomass production, defined mixed culture method in which more than one organism grows simultaneously can result in increased biomass and enzyme production. This is because under natural conditions, microbial degradation of various substrates involves the combined activity of different microorganisms. The main advantages of mixed culture methods are increase in substrate utilization due to the pooling together of the degradation abilities of a number of microorganisms and more resistance to contamination due to the increased competition that a contaminant experiences (Mitchell, 1992). Several authors have reported the enhancement of enzyme activity, for example cellulase in particular by the mixed culturing of different *Trichoderma* and *Aspergillus* strains grown on different substrates (Ghose et al., 1985; Duff et al., 1986; Gutierrez-Correa and Tengerdy, 1997). Apart from growth conditions and microbial interactions, the reported effect of nutritional supplement on enzyme production in mixed cultures is conflicting (Wan Yusoff and Thayan, 1991; Madamwar and Patel, 1992; Castillo et al., 1994), thus necessitating a case-by-case study.

*Corresponding author. E-mail: mmhanafi@agri.upm.edu.my. Tel: +603-89467842.

Studies on microbial production of mannanase have not received as much attention as xylanase, probably due to the fact that xylan is the predominant non-cellulosic polysaccharide in hardwood. However, some plant residues such as palm kernel cake (PKC), a by-product of the palm kernel oil extraction contain mannan as its main polysaccharide (Daud and Jarvis, 1992). Since PKC is mainly used as an animal feed ingredient, possible enzymatic hydrolysis of its polysaccharides in addition to increase in its protein content may improve its *in-vitro* utilization in simple stomached animals such as fish. Previous studies carried out in our laboratory indicated that *Sclerotium rolfsii* and *Aspergillus niger* produce relatively high mannan-degrading enzyme activities when grown on PKC (Iluyemi et al., 2001). We have also observed that the *Trichoderma* strains are able to enhance biomass protein (biomass) yield when grown on PKC (Iluyemi et al., 2005). In this present study, *S. rolfsii* and *A. niger* were co-cultured with three *Trichoderma* strains under submerged liquid cultivation (SLC) with PKC as the sole carbon source. The aim was to investigate the effect of mixed culturing on the mannan-degrading enzymes production of the *Trichoderma* strains while maintaining their ability to enhance protein yield. Mycelia interaction of these fungi on PDA was also investigated.

MATERIALS AND METHODS

Fungal species and combinations

The 5 fungal strains used were: *Trichoderma koningii* (TK), *Trichoderma harzianum* (TH), *Trichoderma longibrachiatum* (TL), *A. niger* (AN) and *Sclerotium rolfsii* (SR). Seven fungal combinations comprising of TK/AN, TK/SR, TH/AN, TH/SR, TL/AN, TL/SR and AN/SR were evaluated.

Culture medium and conditions

All fungal cultures were grown on PDA for 5 days before inoculating them into the culture medium. For growth interaction studies, dual direct opposition mycelial cultures were carried out. A 5 mm circular mycelia discs made with cork borer were inoculated into freshly prepared PDA in 9 cm disposable petri dishes in triplicates. Mycelia were inoculated 5 cm apart. For the individual growth studies, mycelia were inoculated onto the middle of the dish. The inoculated dishes were incubated at ambient temperature (27 ± 2°C) for 13 days. Daily observations were carried out, but measurements of growth diameters were taken on days 2 and 13. Photographs were also taken on these days.

Submerged liquid culture medium was prepared using 0.06% $MgSO_4.7H_2O$, 1.5% KH_2PO_4 and 0.03% (v/v) trace element solution. The trace elements composition is based on that of Sachslehner et al. (1997). The pH of the media was adjusted to 5.0 using 10% sulfuric acid. 50 ml of the medium was measured into 150 ml volumetric flasks and 2 g of ground and sieved (≤ 1.0 mm) PKC added to give a 4% PKC level. The flasks were then auto-claved at 121°C for 30 min, cooled and inoculated with 5 mm circular mycelia discs made with cork borer. Incubation was carried out in triplicate at ambient temperature with continuous shaking at 125 rpm for 13 days.

Enzyme activity assay

At the end of the incubation period, samples were centrifuged at 2500 rpm at 4°C and cell-free extracts (supernatant) were used as the crude enzyme preparations for enzyme activity and soluble protein assay. The residues were used for the determination of crude protein and amino acids. Extracts were assayed for β-D-mannanase, β-mannosidase and α-galactosidase activities. Enzyme activities were assayed based on the method of Ghose (1987). β-D-mannanase activity was assayed using a substrate of 0.5% solution of locust bean galactomannan in 0.05 M sodium citrate buffer, pH 4.50. Substrate solution and crude enzyme extract in the ratio of 3:1 were pipetted into test tubes and incubated for 15 min in a water bath at 50°C. The reducing sugar released was measured as mannose equivalent by the Somogyi-Nelson method (Somogyi, 1952; Nelson, 1944). The α-galactosidase and β-mannosidase activities were quantified in a similar manner using 10 mM of p-nitrophenyl-α-D-galactopyranoside and p-nitrophenyl-β-D mannopyranoside as substrates, respectively. The reaction was stopped, by adding 2.0 ml of 1 M Na_2CO_3. The absorbance of the liberated p-nitrophenol was measured at 405 nm. 1 unit (U) of enzyme activity is defined as the amount of enzyme that liberates 1 μmol product per min (mannose equivalent for β-D-mannanase; p-nitrophenol for α-galactosidase and β-mannosidase).

Biomass determination

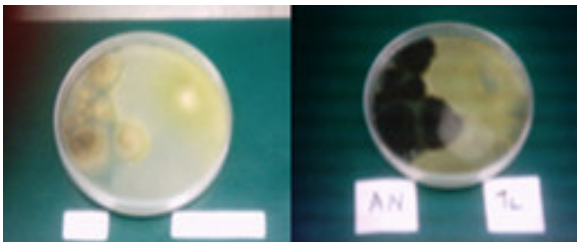
The residues obtained after incubation were oven dried at 40°C for 24 h. Indirect estimation of fungal biomass was carried out by determining the protein content of the residue. Analysis of total nitrogen content was determined by the Kjeldahl method. Crude protein level was obtained by multiplying total nitrogen by 6.25. Soluble protein was determined on the supernatant by the Lowry method (Lowry et al., 1951).

Statistical analysis

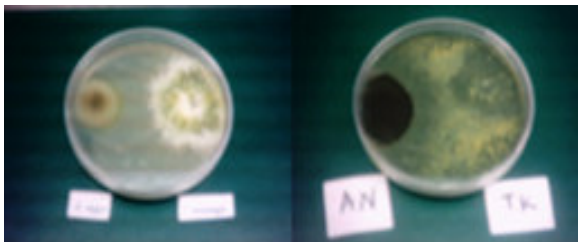
Analysis of variance (ANOVA) was performed on all data using the SAS (1985) statistical package. The mean values were compared by the least significant difference (LSD) test at 5% level of confidence.

RESULTS

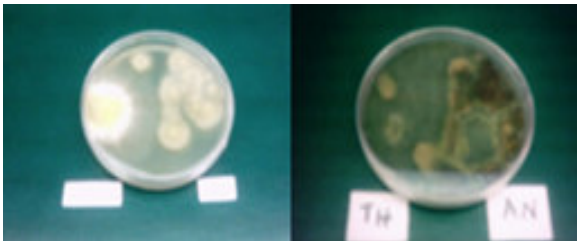
Visible assessment of interactions between opposition colonies were made based on the keys given by Stahl and Christensen (1992). Using these keys, the most common interaction between opposition colonies that was observed is deadlock (Figure 1, Table 1). Deadlock with inhibition accounted for 43% of the observed interactions, while 14% was deadlock on contact. Re-placement interaction in which one effectively grows over the other accounted for only 29% of all interactions in this study. These were replacements of *T. koningii* by *S. rolfsii* and *S. rolfsii* by *T. longibrachiatum*. The only form of intermingling was between *A. niger* and *T. harzianum*



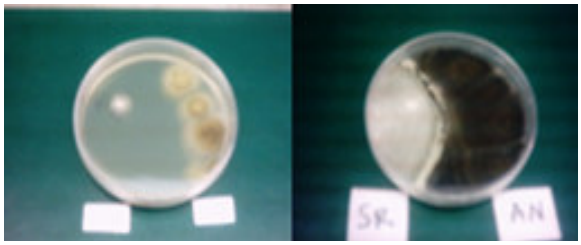
A. niger (L)/ *T. longibrachiatum* (R)



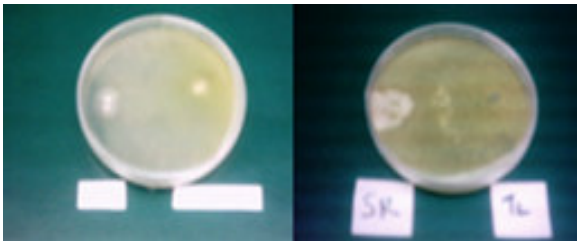
A. niger (L)/*T. koningii* (R)



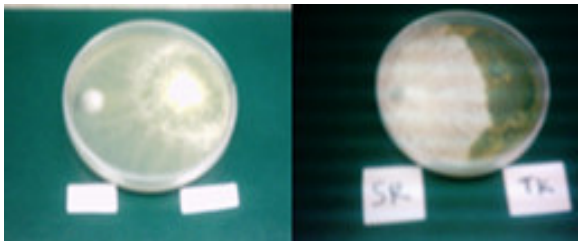
T. harzianum (L)/*A. niger* (R)



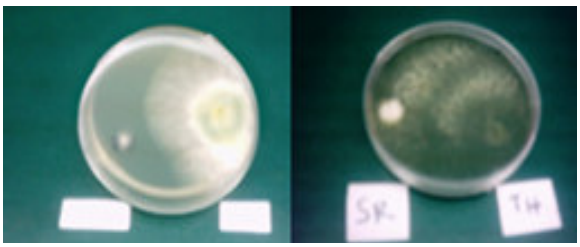
S. rolfsii (L)/*A. niger* (R)



S. rolfsii (L)/*T. longibrachiatum* (R)



S. rolfsii (L)/*T. koningii* (R



S. rolfsii (L)/*T. harzianum* (R)

Figure 1. Interactions of 5 fungal strains grown in pairs (5 cm apart) on PDA in 9 cm disposable petri dishes. (1st petri dish is day 2 and 2nd Petri dish is day 13 photograph. R = right side and L = left side of petri dish).

contributing about 14% of the observed interactions.

Biomass production was determined by the protein value. For *Trichoderma* sp./*A. niger* co-cultures, protein increase ranged from 13 to 35%, significantly higher than in *A. niger* single culture (Table 2). Among the mixed cultures, mycelial interaction appeared to explain biomass and enzyme yield. For *A. niger*/*T. koningii*, deadlock was the observed interaction. However, the growth diameter of *T. koningii* was more than double that of *A. niger* by day 13 and there was no significant increase in α-galactosidase activity. The activities of β-mannosidase and β-D-mannanase were significantly increased compared to *T. koningii* alone. Deadlock was also observed for *A. niger*/*T. longibrachiatum*, but in this case, the growth diameter of *T. longibrachiatum* was just 0.4 cm more than that of *A. niger*. There was significant enhancement of all the enzymes assayed. Between *A. niger* and *T. harzianum*, intermingling was the observed

Table 1. Interactions of 5 fungal strains grown in pairs (5 cm apart) on PDA in 9 cm disposable petri dishes.

Interacting organisms	Colony diameter (cm)		Type of interaction and observations on day 13
	Day 2	Day 13	
TL SR	6.0 1.0	6.4 2.0	Replacement. *S. rolfsii* has ceased to expand its growth and *T. longibrachiatum* mycelia are gradually growing over those of *S. rolfsii*.
TK SR	5.0 1.0	3.0 6.5	Replacement. Due to its fast growth, *T. koningii* has accumulated biomass before being overgrown by *S. rolfsii*.
TH SR	5.2 0.8	7.5 1.0	Deadlock with inhibition. *S. rolfsii* has ceased to expand its growth diameter, though it is accumulating mycelia. There is a demarcation line between it and *T. harzianum*.
TL AN	4.5 4.0	4.4 3.5	Deadlock with mutual inhibition. There is a demarcation line, over which neither mycelia grew.
TK AN	4.0 2.5	5.7 2.6	Deadlock. There is a demarcation line. However, the growth of *A. niger* appears to have been inhibited by *T. koningii*.
TH AN	3.0 4.0	4.2 3.7	Intermingling. *T. harzianum* mycelia had grown over those of *A. niger*, but *A. niger* mycelia are still visible on both upper and lower surfaces of petri dishes.
AN SR	2.5 1.0	4.7 3.6	There is contact of mycelia at the boundary. There appears to be no replacement, as the mycelia of both fungi are still visible.

TK = *Trichoderma koninggi*; TH = *Trichoderma harzianum*; TL = *Trichoderma longibrachiatum*; SR = *Sclerotium rolfsii*; AN = *Aspergillus niger*;

Table 2. Protein production and enzyme activities in single and submerged liquid cultivation with palm kernel cake as the sole carbon source.

Fungi	Crude protein (%)	Soluble protein (mg/ml)	β-D Mannanase (U/ml)	β-Mannosidase (U/ml)	α-Galactosidase (U/ml)
TK	31.27^{a}	0.61^{a}	3.97^{def}	0.06^{f}	8.08^{c}
TH	25.95^{de}	0.34^{cde}	0.03^{g}	0.04^{f}	0.71^{f}
TL	28.18^{bcd}	0.41^{bcd}	3.44^{ef}	0.03^{f}	1.04^{ef}
AN	22.66^{g}	0.43^{c}	5.61^{abcd}	0.85^{a}	8.34^{c}
SR	22.08^{g}	0.21^{f}	5.26^{bcd}	0.17^{e}	2.63^{e}
TK/AN	30.64^{ab}	0.37^{cde}	6.75^{abc}	0.46^{d}	7.90^{c}
TK/SR	30.33^{ab}	0.61^{a}	5.096^{cde}	0.06^{f}	6.15^{d}
TH/AN	26.12^{cde}	0.33^{de}	6.79^{abc}	0.60^{c}	10.48^{b}
TH/SR	28.48^{cd}	0.31^{ef}	0.13^{g}	0.04^{f}	0.70^{f}
TL/AN	25.69^{ef}	0.39^{bcde}	7.34^{a}	0.76^{b}	10.62^{b}
TL/SR	30.99^{a}	0.44^{b}	3.03^{f}	0.03^{f}	1.05^{ef}
AN/SR	23.41^{fg}	0.45^{b}	6.91^{ab}	0.88^{a}	12.29^{a}
Control	13.63^{h}	0.33^{de}	-	-	-
Stderr	±1.143	±0.044	± 0.808	±0.026	± 0.748

Stderr = standard error; TK = *Trichoderma koninggi*; TH = *Trichoderma harzianum*; TL = *Trichoderma longibrachiatum*; SR = *Sclerotium rolfsii*; AN = *Aspergillus niger*; Control = palm kernel cake.
Means in the same column with same superscript are not significantly different.

interaction. There was also significant enhancement of all the enzymes assayed in comparison to single culture of *T. harzianum.* In comparison to its single culture, crude protein yield was significantly increased in all the mixed cultures of *S. rolfsii* ranging from 29 to 40%. For *S. rolfsii*/*T. koningii*, the mycelia of *S. rolfsii* had replaced most of *T. koningii* by day 13, but *T. koningii* had accumulated a lot of biomass before the replacement (Figure 1). There was significant enhancement of β-D-mannanase and α-galactosidase activities but the activity of β-mannosidase was significantly reduced compared to *S. rolfsii* single culture. In the case of *S. rolfsii*/*T. longibrachiaum*, mycelial growth of *S. rolfsii* was really small (2 cm) before it was replaced by *T. longibrachiaum*. Enzyme activities were basically the same as in *T. longibrachiaum* single culture and this was significantly lower than in *S. rolfsii* single culture. Deadlock with inhibition was the observed interaction between *S.*

rolfsii/*T. harzianum*; however, growth diameter of *S. rolfsii* was only 1.0 cm. Enzyme activities were same as in *T. harzianum* single culture. Co-culturing *S. rolfsii* with *A. niger* resulted in deadlock with contact. There was no significant increase in biomass and β-D-mannanase activity, but the activities of the auxiliary enzymes namely α-galactosidase and β-mannosidase were significantly enhanced.

DISCUSSION

Many authors have also found deadlock to be the most common interactions among fungi, when they are grown in nutrient-rich medium (Stahl and Christensen, 1992; Shearer and Zare-Maivan, 1988; Webber and Hedger, 1986). Requirement for similar resources could have caused each fungus to claim its own territory and defend it by preventing the invasion of the other. The overall small growth diameter recorded for *S. rolfsii* may be due to slow growth as well as inhibition by the opposing colony. This is because its growth diameter on day 2 (grown on individual petri dish) was 1.0 cm compared to the others which were at least 5.0 cm. Based on the observed mycelial growth interactions on PDA, the increased protein content of *Trichoderma sp.*/*A. niger* and *Trichoderma sp.*/*S.rolfsii* co-cultures is probably due to *Trichoderma* mycelia yield and not to improve growth of *S. rolfsii* or *A. niger*. Similar result was reported by Gutierrez-Correa et al. (1999), using mixed cultures of *T. reesei* and *A. niger* on sugar cane bagasse with inorganic supplements. They reported a 33% increase in biomass content compared to *A. niger* alone. In this study, soluble protein concentrations were generally lower than in the control except for mixed culture of *T. koninggi/S. rolfsii* as well as the single culture of *T. koninggi*. This reduction in soluble protein may be due to protease production by these fungal strains. Other authors have reported the enhancement of enzyme activities in mixed cultures (Duenas et al., 1995; Duff et al., 1987). On the other hand, Carvalheiro et al. (1994) reported a reduction in cellulase activity by *Trichoderma reesei* and *Sporotrichum* sp. mixed culture on tomato pomace. It appears that enhancement or depression of enzyme activity depends on not just the microbes in use but on the substrate as well as the enzymes being assayed. There was no obvious correlation between protein production and enzyme activity, but it appears that mixed culturing of *A. niger* and the other fungi strains enhanced enzyme production more than growth. Gutierrez-Correa et al. (1999) also noted that there was a synergistic interaction in the co-culture of *T. reesei* and *A. niger* on sugar cane bagasse that helped enzyme production rather than growth.

Observed fungal interaction on PDA in this study may not be exactly how they interact on PKC where nutrients may not be as readily available. However, it does provide a good indication of compatibility among the fungi used in this study. Results obtained showed that:

i.) Co-culturing of the *Trichoderma* strains with *A. niger* irrespective of the observed form of mycelial interaction enhanced β-D-mannanase and α-galactosidase production but the highest increase was obtained from *T. harzianum*/*A. niger* mixed culture where the observed mycelial interaction was intermingling.
ii.) Co-culturing of the *Trichoderma* strains with *A. niger* and *S. rolfsii* did not reduce biomass yield.

The need to strike a balance in terms of biomass production and enhanced enzyme activities is very crucial as far as employing mixed fungal culture for protein increase and fibre reduction of agricultural residues. Further evaluation of *T. koninggi* and *A. niger* in terms of phasing may be worthwhile. This is because *A. niger* was able to boost enzyme production, while maintaining the biomass production of *T. koninggi*.

REFERENCES

Carvalheiro F, Roseiro JC, Collaco MTA (1994). Biological conversion of tomato pomance by pure and mixed fungal cultures. Process Biochem. 29: 601-605.

Castillo MR, Gutierrez-Correa M, Linden JC, Tengerdy RP (1994). Mixed culture solid substrate fermentation for cellulolytic enzyme production. Biotechnol. Lett. 16: 967-972.

Daud MJ, Jarvis MC (1992). Mannan of oil palm kernel. Phytochemistry, 31(2): 463-464.

Duenas R, Tengerdy RP, Gutierrez-Correa M (1995). Cellulase production by mixed fungi in solid-substrate fermentation of bagasse. World J. Microbiol. Biotechnol. 11: 333-337.

Duff SJB, Cooper DG, Fuller OM (1986). Evaluation of the hydrolytic potential of a crude cellulase from mixed cultivation of *Trichoderma reesei* and *Aspergillus phoenicis*. Enzyme Microb. Technol. 8(5): 305-308.

Duff SJB, Cooper DG, Fuller OM (1987). Effect of media composition and growth conditions on production of cellulase and □-glucosidase by a mixed fungal fermentation. Enzyme Microb. Technol. 9(1): 47-52.

Frost GM, Moss DA (1987). Production of enzymes by fermentation. In: Kennedy JF(ed) Biotechnology. Verlag chemie, Weinheim. 7a: 65-211.

Ghose TK (1987). Measurement of cellulase activities. Pure Appl. Chem. 59: 257-263.

Ghose TK, Panda T, Visaria VS (1985). Effect of culture phasing and mannanase on the production of cellulase and hemicellulase by mixed cultures of *Trichoderma reesei* D1-6 and *Aspergillus wentii* Pt 2804. Biotechnol. Bioeng. 27: 1353-1361.

Gutierrez-Correa M, Tengerdy RP (1997). Production of cellulase on sugar cane bagasse by fungal mixed culture solid substrate fermentation. Biotechnol. Lett. 19 (7): 665-667.

Gutierrez-Correa M, Portal L, Moreno P, Tengerdy RP (1999). Mixed culture solid state fermentation of *Trichoderma reesei* with *Aspergillus niger* on sugar cane bagasse. Bioresour. Technol. 68: 173-178.

Iluyemi FB, Hanafi MM, Radziah O, Kamarudin MS (2001). Production of mannan degrading enzymes by fungi grown on palm kernel cake. Pak. J. Appl. Sci. 2: 99-103.

Iluyemi FB, Hanafi MM, Radziah O, Kamarudin MS (2006). Fungal

solid state culture of palm kernel cake. Bioresour. Technol. 97(3): 447-482.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.
Madamwar D, Patel S (1992). Formation of cellulases by co-culturing of *Trichoderma reesei* and *Aspergillus niger* on cellulosic waste. World J. Microbiol. Biotechnol. 8: 183-186.
Mitchell DA (1992). Microbial basis of process. In: Doelle HW, Mitchell DA, Rolz CE Solid Substrate Cultivation. Elsevier Science Publishers, London, p. 24.
Nelson N (1994). A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.
Sachslehner A, Haltritch D, Nidetzky B, Kulbe KD (1997). Production of hemicellulose and cellulose degrading enzymes by various strains of *Sclerotium rolfsii*. Appl. Biochem. Biotechnol. 63-65: 189-201.
SAS Institute (1985). SAS user's guide: Statistics. NC: SAS Institute Inc.
Shearer CA, Zare-Maivan H (1988). *In vitro* hyphal interactions among wood- and leaf-inhabiting ascomycetes and fungi imperfecti from freshwater habitats. Mycologia 80: 31-37.
Somogyi M (1952). Notes on sugar determination. J. Biol. Chem. 195: 19-23.
Stahl PD, Christensen M (1992). *In-vitro* mycelial interactions among members of a soil microfungal community. Soil Biol. Biochem. 24(4): 309-316.
Wan Yusoff WM, Thayan R (1991). Synergism of □-glucosidase and cellulase in mixed culture fermentations. Malays. Appl. Biol. 20: 215-222.
Webber JF, Hedger JN (1986). Comparison of interactions between *Ceratocystis ulmi* and elm bark saprobes *in vitro* and *in vivo*. Trans. Br. Mycol. Soc. 86: 93-101.