APPENDICES

APPENDIX 1

CASE STUDY 1 - SR

(3 DRAFTS)

UNITED ASIAN BANK BERHAD

Fungi on Driftwood collected from ~~sandy~~ the Sandy Beaches of ~~Blue Lagoon~~, Port Dickson.

Introduction :
INTRODUCTION:

The sandy beaches harbor interesting organisms that are adapted to living in the water-filled spaces between grains of sand. ~~These spaces are~~ These spaces are small, mostly microscopic and accordingly, the organisms are minute and provided with adaptations to life in such a habitat. The mycota of such habitats is also not well known. and investigations of sand-inhabiting or arenicolous fungi have been restricted to mainly to taxonomic and morphological studies, whereas ecological observations are rare and have only started recently. (Kohlmeyer, 1966a; (1) Wagner-Mernar, 1972; (2)

Arenicolous fungi can be defined as fungi living among or on grains of sand without implying that they obtain any nutrients from the sand itself.

Pieces of Driftwood found on the sandy beaches along the shore and pieces of wood partially buried in the sand are rich sources of sand inhabiting fungi or arenicolous fungi.

These microscopic fungi plays an important role in degradation of organic material namely lignocellulose found in wood.

Although there are ~~extensive~~ many sandy beaches along the Malaysian coastline, they have not been

investigated for marine fungi. In order to characterise the fungi found on driftwood along sandy beaches, a wide range of localities have to be investigated and an estimate made as to their frequency of occurence. There is also lack of information as to the dominant fungi found.

Materials and methods.

Collection of driftwood was made at the sandy beaches of Pantai Port Dickson, Port Dickson. Blue Lagoon and Driftwood was collected randomly from the sandy beaches and placed in polythene bags for return to the laboratory. The wood was examined for the presence of fungi, and then incubated in the laboratory as described by Jones (1971)(4) Further fungi appeared on incubation and these were added to the list of species already recorded.

Results and Discussion

Table 1. Fungi from driftwood from Port Dickson, Malaysia.

Corollospora pulchella	8 7
Corollospora maritima	5
Arenariomyces trifurcatus	7
Humicola ~~atropatonella~~ sp.	8
Periconia prolifica.	2
Corollospora gracilis	

~~Perisporiales~~

Cirrenalia pygmaea. 2

Zalerion ~~maritimum~~ ~~varium~~ sp. 2

Carbosphaerella leptosphaericides 3

Total Number of collections 15

Empty Perithecia 3

collections with no fungi

Total ___

Total species ___

* ~~Tot~~ - To be confirmed.
Unidentified species.

A = Ascomycotina

D = Deuteromycotina.

Time of incubation

~~From that observations~~

Table 1 lists the fungi encountered. The most common species were Savoryella lignicola, Corollospora pulchella and periconia prolifica.

~~Discussion~~

Little is known of the arenicolous fungi from the tropics. ~~There has been not~~ no reports In fact little is known of the arenicolous fungi in Malaysia.

More ~~was~~ investigative work has to be done in this area in order to ~~make~~ classify

the 1st marine fungi found on drift wood and sand. There only ~~summary~~ has been work done on mangricolous fungi in Malaysia and Thus ~~Ist accounts~~ reported by Jones and Tan (1987)

Most of the arenicolous fungi so far observed have carbonaceous ascomata which may be an adaptive feature for resistry ~~desica~~ desiccation in the habitat of the sandy beaches (Kohlmeyer 1966)

References:

(1) Kohlmeyer 1966

(2) Wagner - Meene 1972

(3) Koch 1974

(4) Jones, E.B.G. 1971 Aquatic fungi In Methods in Microbiology, Vol. 4 (Eds Norris, J.R. Ribbon D.W and Both, C) Academic Press, New York.

(5) Jones, E B G and Tan, T.K. 1987 Observations on mangricolous fungi from Malaysia. Transactions of the British Mycological Society. 89. 590

(6) Kohlmeyer J. 1987 Taxonomic studies of marine Ascomycetes. In the Biology of Marine Fungi.

BL001	Periconia prolifica	3
BL002	Thamnidium sp soil	1
BL004	Stachybotrys (atra ?) (soil)	2
BL005	Curreniella pygmea	2
BL006	Diplo diplodia	
BL007	unidentified.	
BL008	Periconia prolifica.	
BL009	Carbosphaerella leptosphaerioides	
BL009	Savoryella lignicola.	20
	Savoryella appendiculata	3
PPD1	Stachybotrys (atra ?) (soil)	1
PPD2	Diplodiplodia sp.	2
PPD3		
PPD4	Biconiosporella like species	1
PPD5	Pestalotia like species. *	1
PPD6	Alternaria sp	2
PPD7	Trichocladium linderii like	1
PPD8	Savoryella elongata.	1
PPD9	Monodictys sp.	3
	Trichocladium opacum	2

Fungi on Driftwood collected from the Sandy Beaches of Port Dickson, Malaysia.

~~Smt~~ R. SUNDARI

Introduction.

The sandy beaches harbor interesting organisms that are adapted to living in the water-filled spaces between grains of sand. These spaces are small & and mostly microscopic. The organisms that live there are therefore minute and provided with adaptations to life in such a habitat. The mycota of such habitats is also not well known and investigations of sand-inhabiting or arenicolous fungi have been restricted mainly to taxonomic and morphological studies. Ecological observations are rare and have only started recently. (Kohlmeyer, 1966a; (1) Wagner-Merner (2), 1972; Koch, 1974 (3).)

Arenicolous fungi can be defined as fungi living among or on grains of sand without implying that they obtain any nutrients from the sand itself. Pieces of driftwood found on the sandy beaches, along the shore and pieces of wood partially buried

in the sand are rich sources of sand-inhabiting fungi. These microscopic fungi play an important role in degradation of organic material namely lignocellulose found in wood.

Although there are many sandy beaches along the Malaysian coastline, they have not been investigated for marine fungi. In order to characterise the fungi found on driftwood along sandy beaches, a wide range of localities have to be investigated and an estimate made as to their frequency of occurrence.

Materials and methods.

Collection of driftwood was made at the sandy beaches of Blue Lagoon and Pantai Port Dickson, Port Dickson. Driftwood was collected randomly from the sandy beaches and placed in polythene bags for return to the laboratory. The wood was examined for the presence of fungi, and then incubated in the laboratory as described by Jones 1971 (4). Further fungi appeared on incubation and these were added to the list of species already recorded

Results and Discussion

Table I lists the fungi encountered. The most common species were Savoryella lignicola, Corollospora pulchella and Periconia prolifica.

Table I. Fungi found on driftwood from Port Dickson, Malaysia.

	Number of collections
*A Savoryella lignicola	20
*D Periconia prolifica	8
*A Corollospora pulchella	8
*A Corollospora maritima	7
*D Humicola sp.	7
*A Arenariomyces trifurcatus	5
*A Carbosphaerella leptosphaerioides	4
*A Diplodiplodia sp.	4
*A Savoryella appendiculata	3
*D Monodictys sp.	3
*D Zalerion sp.	2
*D Trichocladium opacum	2
*D Alternaria sp.	2
*D Cirrenalia pygmea	2
*A Savoryella elongata	1
*D Trichocladium linderii like	1
*A Biconiosporella sp like	1
Total number of collections	80
Empty Perithecia	15
Collections with no fungi TOTAL	96

Total species 14

A = Ascomycotina

D = D.eutero mycotina

~~Its~~ little is known of the arenicolous fungi from the tropics. In fact little is known of the arenicolous fungi in Malaysia. More investigative work has to be done in this area in order to characterise the marine fungi found on drift wood and sand. There has been some work done on mangiicolous fungi in Malaysia ~~and~~ as reported by Jones and Tan (1987)15. Most of the arenicolous fungi so far observed have carbonaceous ascomata which may be an adaptive feature for resisting desiccation in the habitat of the sandy beaches. (Kohlmeyer 1986)(1).

References.

1) KOHLMEYER, ~~Hft~~ J. (1966a). Ecological observations on arenicolous marine fungi. Z. Allg. Mikrobiol. 6, 94-105.

2) WAGNER-HERNER, D. T. (1972). Arenicolous fungi from the south and central Gulf Coast of Florida. Nova Hedwigia 23, 915-922.

3. KOCH. J. (1974). Marine fungi on driftwood from the west coast of Jutland. Denmark. Frisia 10, 209-250.

4) JONES, E. B. G. (1971) Aquatic fungi. In Methods in Microbiology, Vol. 4 (Eds. Norris, J. R. Ribbon D. W. and Both, C.). Academic Press, New York.

5) JONES, E. B. G. and Tan, T. K. (1987). Observations on manglicolous fungi from Malaysia. Transacts of the British Mycological Society. 89, 390-39:

(6) Kohlmeyer J. (1984) Taxonomic studies of marine Ascomycotina. In the Biology of Marine Fungi. (Ed. Moss S. T.) Cambridge University Press, Cambridge, pp. 199-210.

FUNGI ON DRIFTWOOD COLLECTED FROM THE SANDY BEACHES OF PORT DICKSON, MALAYSIA.

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INTRODUCTION

The sandy beaches harbor interesting organisms that are adapted to living in the water-filled spaces between grains of sand. These spaces are small and mostly microscopic. The organisms that live there are therefore minute and provided with adaptations to life in such a habitat. The mycota of such habitats is not well known and investigations of sand-inhabiting or arenicolous fungi have been restricted mainly to taxonomic and morphological studies. Ecological observations are rare and have only started recently (1, 2, 3).

Arenicolous fungi can be defined as fungi living among or on grains of sand without implying that they obtain any nutrients from the sand itself. Pieces of driftwood found on the sandy beaches and pieces of wood partially buried in the sand are rich sources of sand - inhabiting fungi. These microscopic fungi play an important role in degradation of organic material namely lignocellulose found in wood.

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Results and Discussion

Table I lists the fungi encountered. The most common species were *Savoryella lignicola*, *Corollospora pulchella* and *Periconia prolifica*.

Table I: Fungi found on driftwood from Port Dickson, Malaysia

Fungi	Number collections
Savoryella lignicola[a]	20
Periconia prolifica[d]	8
Corollospora pulchella[a]	8
Corollospora maritima[a]	7
Humicola sp.[d]	7
Arenariomyces trifurcatus[a]	5
Carbosphaerella leptosphaerioides[a]	4
Diplodia sp.[a]	4
Savoryella appendiculata[a]	3
Monodictys sp.[d]	3
Zalerion sp.[d]	2
Trichocladium opacum[d]	2
Alternaria sp.[d]	2
Cirrenalia pygmea[d]	2
Savoryella elongata[a]	1
Trichocladium linderii like[d]	1
Siroconiosporella sp. like[a]	1
Total number collections	80
Empty perithecia	15
Collections with no fungi	3
Total	98

[a] - Ascomycotina
[d] - Deuteromycotina

Little is known of the arenicolous fungi from the tropics. In fact little is known of the arenicolous fungi in Malaysia. More investigative work has to be done in this area in order to characterise the marine fungi found on driftwood and sand. There has been some work done on manglicolous fungi in Malaysia as reported by (5). Most of the arenicolous fungi so far observed have carbonaceous ascomata which may be an adaptive feature for resisting desiccation in the habitat of the sandy beaches (6).

References

1. KOHLMEYER, J. (1966a). Ecological observations on arenicolous marine fungi. *Z. Allg. Mikrobiol.* 6, 94-105.

2. WAGNER-MERNER, D.T. (1972). Arenicolous fungi from the south and central Gulf Coast of Florida. *Nova Hedwigia*. 23, 915-922.

3. KOCH, J. (1974). Marine fungi on driftwood from the West Coast of Jutland. ~~Denmark~~ Denmark (not included) *Friesia* 10, 209-250.

4. JONES, E.B.G. (1971). Aquatic fungi. In Methods in Microbiology, Vol. 4 (East Norris, J.R. Ribbons, D.W. and Both, C.). *Academic Press, New York.*

5. JONES, E.B.G. and TAN, T.K. (1987). Observations on manglicolous fungi from Malaysia. *Transactions of the British Mycological Society*. 89, 390-392.

6. KOHLMEYER, J. (1986). Taxonomic studies of marine Ascomycotina. In the Biology of Marine Fungi (Ed. Moss, S.T.) *Cambridge University Press, Cambridge.* pp. 199-210.

APPENDIX 2

CASE STUDY 2 - SL

(5 DRAFTS)

Draft 1

Growth of *Pleurotus sajor-caju* on oil palm frond parenchyma tissue

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Department of Biochemistry, University of Malaya[a]

MATERIALS AND METHODS

Raw substrate

Oil palm frond parenchyma tissue (OPFPt) was obtained from the United Plantation, Telok Intan, Perak.

Inoculum

Pleurotus sajor-caju was obtained from MARDI, Serdang and used as the spawn culture for all experiments.

Fermentation

In this experiment, about 25 g ~~substrate~~ OPFPt was weighed into each 250 ml conical flask with 1% calcium carbonate. Two types of nitrogen supplementation were used, ie. 20% POME and one level of urea at 1.0 nitrogen unit (wrt 20% POME). The culture flasks were autoclaved at 121°C, 15 psi for 30 min. The moisture content was adjusted to 75% (v/w). Each flask was then inoculated with 10% (w/w) spawn culture. Incubation was done at room temperature for 30 days without shaking.

Enzyme harvesting

At suitable interval of time, triplicate culture flasks from each treatment (20% POME and urea) were sampled at random. About 75 ml of cold 50 mM sodium acetate, pH 4.8 was added to the content of each flask. The flasks were transferred ~~into~~ to an incubator cum shaker at 4°C, 150 rpm ~~and allowed to be shaken overnight~~ (approximately 18 hours).

~~On the following morning, the culture flasks were removed from chamber~~ and the solids removed through centrifugation at 5000 rpm at 4 °C for 20 min. ~~In all the procedures, the slurry and supernatant must be kept at 4 °C with ice chips.~~ The supernatant, designated as crude culture extract contained the fungal enzymes, and was ~~stored~~ kept in small vials at -20°C for enzyme assays.

Analytical determination

Enzyme assays.

(1) *Exocellulase.* A quantity, 0.5 ml of the crude culture extract was added to 1.0 ml of 20 mg Avicel/ml 50 mM sodium acetate buffer, pH 4.8 and incubated for 4 h. at 40°C (Wood and Bhat, 1988). The reducing sugars released after incubation was determined by the dinitrosalicylic (DNS) method (Miller, 1959). The enzyme activity is expressed as µmol glucose released/min/g of substrate.

(2) *Endocellulase.* The reaction mixture, consisted of 1.8 ml 1% solution of sodium carboxymethyl cellulose in 50 mM sodium acetate buffer, pH 4.8 and 0.2 ml of crude culture extract was incubated for 30 min at 40°C (Dong *et al.*, 1992). The reducing sugar released was measured by the dinitrosalicylic (DNS) method (Miller, 1959). One unit of enzyme activity was expressed as 1 µmol of glucose released/min/g substrate.

(3) *β-glucosidase.* Activity towards p-nitrophenyl-β-D-glucopyranoside (PNPG) was estimated by measuring spectrometrically the release of p-nitrophenol from PNPG (Dong *et al.*, 1992). One unit of enzyme activity was expressed as 1 µmol of p-nitrophenol produced/min/g substrate.

(4) *Xylanase.* Xylanase activity was assayed by mixing 0.2 ml of crude culture extract with 1.8 ml of 1% xylan in 50 mM sodium acetate buffer, pH 4.8 and incubated for 1 h. at 40°C (Bailey *et al.*, 1992) in a water bath with moderate shaking. Reducing sugar was measured with dinitrosalicylic acid reagent (DNS)(Miller, 1959). One unit of enzyme activity was defined as 1 µmol of xylose liberated/min/g substrate.

(5) *Laccase.* The assay mixture consisted of 0.1 ml crude culture extract, 3.0 ml distilled water and 0.1 ml of 1% syringaldazine in ethanol (Harkin and Obst, 1973). One unit of enzyme activity is defined as the amount of enzyme producing 1 OD unit/min/g substrate at 527 nm.

Protein. The extracellular soluble protein was measured by the method of Bradford (1976). Crystalline bovine serum albumin served as the standard.

RESULTS

A time course study was performed to compare solid state fermentation by *Pleurotus sajor-caju* on oil palm frond parenchyma tissue (OPFPt) supplemented with POME or urea.

Cellulolytic activity

Pleurotus sajor-caju is lignocellulolytic, producing extracellular cellulases and β-glucosidase in OPFPt supplemented with POME or urea (Fig. 1). In POME supplemented culture, the changes in exocellulase activity was

slight throughout the course of fermentation (0.26-0.32 U/g substrate). The endocellulase activity also increased gradually with an initial activity of 0.54 U/g substrate and reaching a maximum value of 2.91 U/g substrate at the end of fermentation period. The initial β-glucosidase activity was low which rose gradually and attained 0.23 U/g substrate towards the end.

The exocellulase activity of culture with urea supplementation showed a similar pattern with that of POME supplementation. However, endocellulase activity showed a more pronounced increase during the first 10 days with a maximum activity of 4.38 U/g substrate. Then the activity decreased which later stabilised at 3.03-3.5 U/g substrate. The initial β-glucosidase activity was also low at 0.0096 U/g substrate. This increased gradually to a maximum of 0.35 U/g substrate at the end of fermentation.

Xylanolytic activity

The xylanase activity of culture with POME supplementation showed a similar profile with that of endocellulase with a maximum activity of 2.85 U/g substrate at day 30. However, in urea supplemented culture, a very sharp increase of activity was observed during the first 5 days of which the maximum activity was 6.6 U/g substrate on day 5. After that, there was a decreasing trend in the activity, the lowest activity been 4.17 U/g substrate on day 30.

Ligninolytic activity

For both the POME and urea supplemented culture, there was a rapid increase in laccase activity, peaking on day 10 with activities of 10.53 and 15.93 U/g substrate respectively. From day 10 onwards, the activity decreased rapidly to values in the range of 6.6-7.6 U/g substrate on day 15. After this, there was a slight increase in activity ranging between 6.5-9.5 U/g substrate until the end of fermentation.

Soluble protein

The initial concentration of soluble protein was in the range of 0.14-0.21 mg/g substrate. For POME supplemented culture, the extractable protein reached 1.1 mg/g substrate with the first 15 days of incubation. An increase in the soluble protein concentration was also observed after day 20.

In urea supplemented culture, the increase in soluble protein was more rapid peaking on day 10 and 25 with values of 2.07 and 2.67 mg/g substrate respectively. In both cases, the soluble protein decreased sharply after day 25.

DISCUSSION

The present study indicated that the general composition of the oil palm frond parenchyma tissue (OPFPt) would support good fungal growth, though requiring supplementary nitrogen to maximise protein yield. This was confirmed by the results of growth experiments (Dinesh, 1994).

Appreciable amounts of enzyme activities were measured during the course of fermentation. Generally, culture with urea supplementation is more favourable in terms of higher enzyme activities compared to POME supplementation, which effected an increase degradation of the substrate. The detection of high level of laccase activity in the early stage showed that lignin was consumed preferably.

The soluble protein can be used to relate the growth of fungus in the substrate. First, the usual fungal growth and germination occurred, followed by exponential growth, deceleration and finally autolysis. Higher levels of soluble protein is correlated with increase in enzyme activities.

CONCLUSION

Oil palm frond parenchyma tissue can be a potential substrate for P. sajor-caju
As the growth conditions of solid state fermentation are closed to the natural ones, the results may be useful for investigating biodegradation in nature, improving the existing techniques, product quality and to increase our knowledge of fungal growth on solid substrate.

ACKNOWLEDGEMENTS

The authors would like to thank the University of Malaya for providing the grant for this study; United Plantation Bhd., Telok Intan, Perak; Forest Research Institute of Malaysia (FRIM); and the Canadian High Commission, Malaysia for travel support.

REFERENCES

1. Bailey, M. J., P. Biely and K. Poutanen. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology*. 23: 257-270.

2. Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry*. 72: 248-254.

3. Dinesh, N. (1994). Bioconversion of oil palm frond parenchyma tissue and palm oil sludge solid by selected fungi. M. Biotech. Thesis submitted to University Malaya as partial fulfillment for M. Biotech. Degree, 1994.

4. Dong, W. K., S. K. Tae, K. J. Young and K. L. Jae. 1992). Adsorption kinetics and behaviors of cellulase components on microcrystalline cellulose. *Journal of Fermentation and Bioengineering*. 73: 461-469.

5. Harkin, J. M. and J. R. Obst. (1973). Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experientia*. 29: 381-387.

6. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. 31: 426-428.

7. Wood, T. M. and K. M. Baht. (1988). Methods for measuring cellulase activity. *Methods in Enzymology*. 160A: 87-112.

Growth of *Pleurotus sajor-caju* on oil palm frond parenchyma tissue

[redacted] S. Vikineswary and S. Balabaskaran*
Institute of Advanced Studies, University of Malaya
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Fermentation

~~In this experiment~~, about 25 g OPFPt was weighed into each 250 ml conical flask ~~with~~ and 1% calcium carbonate. Two types of nitrogen supplementation were used, ie. 20% POME and one level of urea at 1.0 nitrogen unit (wrt 20% POME). The culture flasks were autoclaved at 121°C, 15 psi for 30 min. The moisture content was adjusted to 75% (v/w). Each flask was then inoculated with 10% (w/w) spawn culture. Incubation was done at room temperature for 30 days without shaking.
The urea solution was filter sterilized and added to the contents of cooled flasks.

Enzyme harvesting

At ~~suitable~~ regular intervals of time, triplicate culture flasks from each treatment (20% POME and urea) were sampled at random. About 75 ml of cold 50 mM sodium acetate, pH 4.8 was added to the contents of each flask. The flasks were transferred to an incubator shaker at 4°C at 150 rpm for approximately 18 hours.

The solids were removed through centrifugation at 5000 rpm at 4 °C for 20 min. The supernatant, designated as crude culture extract contained the fungal enzymes, and was kept in small vials at -20°C for enzyme assays.

Analytical determination techniques

Enzyme assays.

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A time course study was performed to compare solid state fermentation by *Pleurotus sajor-caju* on oil palm frond parenchyma tissue (OPFPt) supplemented with POME or urea.

Cellulolytic activity

Pleurotus sajor-caju is lignocellulolytic, producing extracellular cellulases and β-glucosidase in OPFPt supplemented with POME or urea (Fig. 1 & 2). In POME supplemented culture, the changes in exocellulase

activity was slight throughout the course of fermentation (0.26-0.32 U/g substrate). The endocellulase activity also increased gradually with an initial activity of 0.54 U/g substrate and reaching a maximum value of 2.91 U/g substrate at the end of fermentation period. The initial β-glucosidase activity was low which rose gradually and attained 0.23 U/g substrate towards the end.

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In urea supplemented culture, the increase in soluble protein was more rapid peaking on day 10 and 25 with values of 2.07 and 2.67 mg/g substrate respectively. In both cases, the soluble protein decreased sharply after day 25.

DISCUSSION

The present study indicated that the general composition of the oil palm frond parenchyma tissue (OPFPt) would support good fungal growth, though requiring supplementary nitrogen to maximise protein yield. This was confirmed by the results of growth experiments (Dinesh, 1994).

Appreciable amounts of enzyme activities were measured during the course of fermentation. Generally, culture with urea supplementation is more favourable in terms of higher enzyme activities compared to POME supplementation, which effected an increase degradation of the substrate. The detection of high level of laccase activity in the early stage showed that lignin was consumed ~~preferably~~. activity by the fungus

The soluble protein can be used to relate the growth of fungus in the substrate. First, ~~the usual~~ fungal growth ~~and germination~~ occurred, followed by exponential growth, deceleration and finally autolysis. Higher levels of soluble protein is correlated with increased enzyme activities.

CONCLUSION

Oil palm frond parenchyma tissue can be used as a potential substrate for *Pleurotus sajor-caju*. As the growth conditions of solid state fermentation are closed to the natural ones, the results may be useful for investigating biodegradation in nature, improving the existing techniques, product quality and to increase our knowledge of fungal growth on solid substrate.

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REFERENCES

1. Bailey, M. J., P. Biely and K. Poutanen. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology*. 23: 257-270.

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3. Dinesh, N. (1994). Bioconversion of oil palm frond parenchyma tissue and palm oil sludge solid by selected fungi. M. Biotech. Thesis submitted to University Malaya as partial fulfillment for M. Biotech. Degree, 1994.

4. Dong, W. K., S. K. Tae, K. J. Young and K. L. Jae. 1992). Adsorption kinetics and behaviors of cellulase components on microcrystalline cellulose. *Journal of Fermentation and Bioengineering*. 73: 461-469.

5. Harkin, J. M. and J. R. Obst. (1973). Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experientia*. 29: 381-387.

6. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. 31: 426-428.

7. Wood, T. M. and K. M. Baht. (1988). Methods for measuring cellulase activity. *Methods in Enzymology*. 160A: 87-112.

Draft 3

Growth of *Pleurotus sajor-caju* on oil palm frond parenchyma tissue

[redacted] S. Vikineswary and S. Balabaskaran+
Institute of Advanced Studies, University of Malaya
+ *Department of Biochemistry, University of Malaya*

MATERIALS AND METHODS

Raw substrate

Oil palm frond parenchyma tissue (OPFPt) was obtained from the United Plantation, Telok Intan, Perak, Malaysia.

Inoculum

Pleurotus sajor-caju was obtained from MARDI, Serdang, Malaysia and used as the spawn culture for all experiments.

Fermentation

About 25g OPFPt was weighed into each 250ml conical flask and 1% calcium carbonate was added. Two types of nitrogen supplementation were used, ie. 20% POME and one level of urea at 1.0 nitrogen unit (wrt 20% POME). The culture flasks were autoclaved at 121°C, 15psi for 30min. The urea solution was filter sterilised and added to the contents of autoclaved flasks. The moisture content was adjusted to 75% (v/w). Each flask was then inoculated with 10% (w/w) spawn culture. Incubation was done at room temperature for 30 days without shaking.

Enzyme harvesting

At regular intervals of time, triplicate culture flasks from each treatment (20% POME and urea) were sampled at random. About 75ml of cold 50mM sodium acetate, pH 4.8 was added to the contents of each flask. The flasks were transferred to an incubator shaker at 4°C and 150rpm for approximately 18h.

The solids were removed through centrifugation at 5000rpm at 4°C for 20 min. The supernatant, designated as crude culture extract contained the fungal enzymes, and was kept in small vials at -20°C for enzyme assays.

Analytical Techniques

Enzyme assays

(1) *Exocellulase.* A quantity, 0.5ml of the crude culture extract was added to 1.0ml of 20mg Avicel/ml 50mM sodium acetate buffer, pH 4.8 and incubated for 4h. at 40°C (Wood and Bhat, 1988). The reducing sugars released after incubation was determined by the dinitrosalicylic (DNS) method (Miller, 1959). The enzyme activity is expressed as µmol glucose released/min/g of substrate.

(2) *Endocellulase.* The reaction mixture, consisted of 1.8ml 1% solution of sodium carboxymethylcellulose in 50mM sodium acetate buffer, pH 4.8 and 0.2ml of crude culture extract was incubated for 30min at 40°C (Dong *et al.*, 1992). The reducing sugar released was measured by the dinitrosalicylic (DNS) method (Miller, 1959). One unit of enzyme activity was expressed as 1µmol of glucose released/min/g substrate.

(3) *β-glucosidase.* Activity towards p-nitrophenyl-β-D-glucopyranoside (PNPG) was estimated by measuring spectrometrically the release of p-nitrophenol from PNPG (Dong *et al.*, 1992). One unit of enzyme activity was expressed as 1µmol of p-nitrophenol produced/min/g substrate.

(4) *Xylanase.* Xylanase activity was assayed by mixing 0.2ml of crude culture extract with 1.8ml of 1% xylan in 50mM sodium acetate buffer, pH 4.8 and incubated for 1h. at 40°C (Bailey *et al.*, 1992) in a water bath with moderate shaking. Reducing sugar was measured with dinitrosalicylic acid reagent (DNS)(Miller, 1959). One unit of enzyme activity was defined as 1µmol of xylose liberated/min/g substrate.

(5) *Laccase.* The assay mixture consisted of 0.1ml crude culture extract, 3.0ml distilled water and 0.1ml of 1% syringaldazine in ethanol (Harkin and Obst, 1973). One unit of enzyme activity is defined as the amount of enzyme producing 1OD unit/min/g substrate at 527nm.

Protein. The extracellular soluble protein was measured by the method of Bradford (1976). Crystalline bovine serum albumin was used as the standard.

RESULTS

The enzyme activities during the growth of *Pleurotus sajor-caju* on OPFPT in SSF is given in Fig 1-4.

A time course study was performed to compare solid state fermentation by *Pleurotus sajor-caju* on oil palm frond parenchyma tissue (OPFPt) supplemented with POME or urea.

Cellulolytic activity

Pleurotus sajor-caju is lignocellulolytic, producing extracellular cellulases and β-glucosidase in OPFPt supplemented with POME or urea (Fig. 1 & 2). In POME supplemented culture, the changes in exocellulase activity was slight throughout the course of fermentation (0.26-0.32U/g

substrate). The endocellulase activity also increased gradually with an initial activity of 0.54U/g substrate and reaching a maximum value of 2.91U/g substrate at the end of fermentation period. The initial β-glucosidase activity was low which rose gradually and attained 0.23U/g substrate towards the end.

The exocellulase activity of culture with urea supplementation showed a similar pattern with that of POME supplementation. However, endocellulase activity showed a more pronounced increase during the first 10 days with a maximum activity of 4.38U/g substrate. Then the activity decreased which later stabilised at 3.03-3.5U/g substrate. The initial β-glucosidase activity was low at 0.0096U/g substrate. This increased gradually to a maximum of 0.35U/g substrate at the end of fermentation.

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Ligninolytic activity

For both the POME and urea supplemented culture, there was a rapid increase in laccase activity, peaking on day 10 with activities of 10.53 and 15.93U/g substrate respectively (Fig. 4). From day 10 onwards, the activity decreased rapidly to values in the range of 6.6-7.6U/g substrate on day 15. After this, there was a slight increase in activity ranging between 6.5-9.5U/g substrate until the end of fermentation.

Soluble protein

The initial concentration of soluble protein was in the range of 0.14-0.21mg/g substrate (Fig. 5). For POME supplemented culture, the extractable protein reached 1.1mg/g substrate with the first 15 days of incubation. An increase in the soluble protein concentration was also observed after day 20.

In urea supplemented culture, the increase in soluble protein was more rapid peaking on day 10 and 25 with values of 2.07 and 2.67mg/g substrate respectively. In both cases, the soluble protein decreased sharply after day 25.

DISCUSSION

The present study indicated that the general composition of the oil palm frond parenchyma tissue (OPFPt) would support good fungal growth, though requiring supplementary nitrogen to maximise protein yield. This was confirmed by the results of growth experiments (Dinesh, 1994).

Appreciable amounts of enzyme activities were measured during the course of fermentation. Generally, culture with urea supplementation is more favourable in terms of higher enzyme activities compared to POME supplementation, which effected an increase degradation of the substrate. Considering the huge amount of OPFPt available in the plantation, laccase activity as high as 15900U/kg substrate can be obtained in the upscaled processes. The detection of high level of laccase activity in the early stage showed that lignin was consumed actively by the fungus.

The soluble protein can be used to relate the growth of fungus in the substrate. First, fungal growth occurred, followed by exponential growth, deceleration and finally autolysis. Higher levels of soluble protein is correlated with increased enzyme activities.

CONCLUSION

Oil palm frond parenchyma tissue can be used as a potential substrate for *Pleurotus sajor-caju*. As the growth conditions of solid state fermentation are closer to the natural ones, the results may be useful for investigating biodegradation in nature, improving the existing techniques, product quality and to increase our knowledge of fungal growth on solid substrate.

ACKNOWLEDGEMENTS

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REFERENCES

1. Bailey, M. J., P. Biely and K. Poutanen. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology*. 23: 257-270.

2. Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry*. 72: 248-254.

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5. Harkin, J. M. and J. R. Obst. (1973). Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. *Experientia*. 29: 381-387.

6. Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. 31: 426-428.

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Growth of *Pleurotus sajor-caju* on oil palm frond parenchyma tissue

Draft 4

[redacted] S. Vikineswary, and S. Balabaskaran*

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Introduction

ATERIALS AND METHODS

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At regular intervals of time, triplicate culture flasks from each treatment (20% POME and urea) were sampled at random. About 75ml of cold 50mM sodium acetate pH4.8 was added to the contents of each flask. The flasks were transferred to an incubator shaker at 4°C and 150rpm for approximately 18h.

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Protein // italics

The extracellular soluble protein was measured by the method of Bradford (1976). Crystalline bovine serum albumin was used as the standard.

RESULTS

The results of the time course study was performed to compare solid state fermentation by *Pleurotus sajor-caju* of oil palm frond parenchyma tissue (OPFPt) supplemented with POME or urea.

Cellulolytic activity

Pleurotus sajor-caju is lignocellulolytic, producing extracellular cellulases and ß-glucosidase in OPFPt supplemented with POME or urea (Fig. 1 & 2). In POME supplemented culture, the changes in exocellulase activity was slight throughout the course of fermentation (0.26-0.32U/g substrate). The endocellulase activity also increased gradually with an initial activity of 0.54U/g substrate and reaching a maximum value of 2.91U/g substrate at the end of fermentation period. The initial ß-glucosidase activity was low which rose gradually and attained 0.23U/g substrate towards the end. at 30 days of incubation.

The exocellulase activity of culture with urea supplementation showed a similar pattern with that of POME supplementation. However, endocellulase activity showed a more significant increase during the first 10 days with a maximum activity of 4.38U/g substrate. Then the activity decreased which later stabilised at 3.03-3.5U/g substrate. The initial ß-glucosidase activity was low at 0.0096U/g substrate. This increased gradually to a maximum of 0.35U/g substrate at the end of fermentation.

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The xylanase activity of culture with POME supplementation showed a similar profile with that of endocellulase with a maximum activity of 2.85U/g substrate at day 30 (Fig. 3). However, in urea supplemented culture, a very sharp increase of activity was observed during the first 5 days of which the maximum activity was 6.6U/g substrate on day 5. After that, there was a decreasing trend in the activity and the lowest activity was 4.17U/g substrate on day 30.

Laccase
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For both the POME and urea supplemented culture, there was a rapid increase in laccase activity, peaking on day 10 with activities of 10.53 and 15.93U/g substrate respectively (Fig. 4). From day 10 onwards, the activity decreased rapidly to values in the range of 6.6 - 7.6U/g substrate on day 15. After this, there was a slight increase in activity ranging between 6.5-9.5U/g substrate until the end of fermentation.

Soluble protein

The initial concentration of soluble protein was in the range of 0.14-0.21mg/g substrate (Fig. 5). For POME supplemented culture, the extractable protein reached 1.1mg/g substrate with the first 15 days of incubation. An increase in the soluble protein concentration was also observed after day 20.

In urea supplemented culture, the increase in soluble protein was more rapid peaking on day 10 and 25 with values of 2.07 and 2.67mg/g substrate respectively. In both cases, the soluble protein decreased sharply after day 25.

DISCUSSION

The present study indicated that the general composition of the oil palm frond parenchyma tissue (OPFPt) would support good fungal growth, though requiring supplementary nitrogen to maximise protein yield. This was confirmed by the results of growth experiments (Dinesh, 1994).

Appreciable amounts of enzyme activities were measured during the course of fermentation. Generally, culture with urea supplementation is more favourable in terms of higher enzyme activities compared to POME supplementation, which affected an increase degradation of the substrate. Considering the huge amount of OPFPt available in the plantation, laccase activity as high as 15900U/kg substrate can be obtained in the upscaled processes. The detection of high level of laccase activity in the early stage showed that lignin was consumed actively by the fungus.

The soluble protein can be used to relate the growth of fungus in the substrate. First, fungal growth occurred, followed by exponential growth, deceleration and finally autolysis. Higher levels of soluble protein is correlated with increased enzyme activities.

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REFERENCES

BAILEY, M. J.; BIELY, P. and POUTANEN, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. **Journal of Biotechnology. 23:** 257-270.

Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. **Analytical Biochemistry. 72:** 248-254.

DINESH, N. 1994. Bioconversion of oil palm frond parenchyma tissue and palm oil sludge solid by selected fungi. M. Biotechnology. Thesis submitted to University Malaya as partial fulfillment for M. Biotechnology. Degree. 1994.

DONG, W. K.; TAE, S. K.; YOUNG, K. J. and JAE, K. L. 1992. **Adsorption kinetics and behaviors of cellulase components on microcrystalline cellulose. Journal of Fermentation and Bioengineering. 73:** 461-469.

HARKIN, J. M. and J. R. OBST. 1973. Syringaldazine, an effective reagent for detecting laccase and peroxidase in fungi. **Experientia. 29:** 381-387.

MILLER, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Chemistry. 31:** 426-428.

WOOD, T. M. and BHAT, K. M. 1988. Methods for measuring cellulase activity. **Methods in Enzymology. 160A:**

Growth of *Pleurotus sajor-caju* on oil palm frond parenchyma tissue

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**Department of Biochemistry, University of Malaya*

INTRODUCTION

Lignocellulosic material is continuosly produced in large quantities as residual wastes in agriculture and forestry and constitutes an abundant and underutilised source of renewable biomass. The oil palm industry produces a variety of by-products. In the field, a large quantity of oil palm fronds are produced during pruning and replanting programmes, which amount to 11 tonnes/hectare/year and 16 tonnes/hectare respectively (Mohamad *et al.*, 1981). This agricultural residue which contain lignocellulose in principle, can be utilised by white rot fungi in solid state fermentation. Preliminary studies by Dinesh (1994) have shown that *Pleurotus sajor-caju* can grow in oil palm frond parenchyma tissue (OPFPt) and the biomass produced has potential as animal feed supplement. This paper reports the biochemical changes related to the growth of the fungus in OPFPt.

References

MATERIALS AND METHODS

Substrate

Oil palm frond parenchyma tissue (OPFPt) was obtained from the United Plantation, Telok Intan, Perak, Malaysia.

Inoculum

Pleurotus sajor-caju was obtained from MARDI, Serdang, Malaysia and used as the spawn culture for all experiments.

(b) ß-glucosidase

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(d) Xylanase

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RESULTS

The enzyme activities during the growth of *Pleurotus sajor-caju* on OPFPt in SSF is given in Fig. 1-4.

Cellulolytic activity

Pleurotus sajor-caju is lignocellulolytic, producing extracellular cellulases and ß-glucosidase in OPFPt supplemented with POME or urea (Fig. 1 & 2). In POME supplemented culture, the changes in exocellulase activity was minimal throughout the course of fermentation (0.26-0.32U/g substrate). The endocellulase activity increased gradually with an initial activity of 0.54U/g substrate and reaching a maximum value of 2.91U/g substrate at the end of fermentation period. The initial ß-glucosidase activity increased gradually and attained 0.23U/g substrate at 30 days of incubation.

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CONCLUSION

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REFERENCES

BAILEY, M. J.; BIELY, P. and POUTANEN, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. **Journal of Biotechnology. 23:** 257-270.

BRADFORD, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. **Analytical Biochemistry. 72:** 248-254.

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MILLER, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Chemistry. 31:** 426-428.

MOHAMAD, H.; H. ABD. HALIM and M. AHMAD TARMIZI, 1986. Availability and potential utilisation of oil palm trunks and fronds up to the year 2000. **Porim Occasional Paper, No. 20.**

WOOD, T. M. and BHAT, K. M. 1988. Methods for measuring cellulase activity. **Methods in Enzymology. 160A:** 87-112.

APPENDIX 3

CASE STUDY 3 - CL

(2 DRAFTS)

Draft 1

Growth and product formation of *Ankistrodesmus convolutus* in an air-lift fermenter

[redacted] S.M. Phang and S.H. Goh
Institute of Advanced Studies, University of Malaya, 59100 Kuala Lumpur, MALAYSIA

Absract

Growth and production of lipids, fatty acids, proteins, carbohydrates and pigments in the green alga *Ankistrodesmus convolutus* were investigated. Four batches of cultures in an airlift fermenter were compared for growth and product formation. The four batches were grown under the following conditions: (1) unbuffered at low light intensity, (2) buffered at low light intensity, (3) buffered at high light intensity and (4) buffered at low light intensity and aerated with 5% CO_2. The CO_2-aerated culture attained the highest biomass (308 mg dry weight/L) compared to the other batches (122 - 150 mg dry weight/L). Cells grown at high light contained more lipids (26.0% dry weight) than under other culture conditions studied. No significant variations in fatty acid composition (predominantly 18:3n-3) and pigmentation were observed in the four batches of cultures. *Ankistrodesmus convolutus* produced significant amounts of carotenoids (especially lutein).

Introduction

Microalgae are potential sources of a wide range of useful natural products. The products which are commercially important include specialty lipids (eg. arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid) and pigments such as carotenoids and phycobiliproteins (1). Mass cultures of microalgae is vital in generating sufficient biomass for the extraction of various products. In parallel with the advancement of microalgal biotechnology, various mass-culture systems have been developed. Mass culture systems of microalgae include both open (raceway ponds) and closed systems (phototubular bioreactor and air-lift fermenter). Closed systems are more suitable than open systems for physiological and productivity studies which require strict sterile conditions.

Air-lift bioreactors are advantageous for the culturing of microalgae because they offer simple and effective mixing with no moving parts, high gas absorption efficiency and good heat transfer characteristics (2).

Ankistrodesmus convolutus has been selected for the present study after a preliminary screening programme of potential microalgal resources of Malaysia for useful natural products. This green alga appears to be a fast growing species with appreciable amounts of carotenoids and polyunsaturated fatty acids (3). Growth performance and product formation of *Ankistrodesmus convolutus* in four batches of cultures in an air-lift fermenter

were compared. The four batches were grown under the following conditions: (1) unbuffered at low light intensity, (2) buffered at low light intensity, (3) buffered at high light intensity and (4) buffered at low light intensity and aerated with 5% CO_2.

Materials and Methods

Culture conditions. *Ankistrodesmus convolutus* Corda (isolate No. 101) in this study was isolated from a freshwater pond and deposited at the Microalgal Culture Collection Centre at the Institute of Advanced Studies, University of Malaya. The alga was grown in an airlift fermenter (cap. 6.5 L) using Bold's Basal Medium, BBM (4). A 10% innoculum of an exponential phase culture with an optical density at 620 nm of 0.2 was used.

At low irradiance (31.9 $\mu Em^{-2}s^{-1}$) light was provided by four fluorescent lamps (True-Lite) attached to two parabolic reflectors. At high irradiance (63.8 $\mu Em^{-2}s^{-1}$) light was provided by eight fluorescent lamps. Illumination was set on a 12 h light and 12 h dark cycle.

Filter sterilised air was pumped through the sparger-loop at a rate of 1,500 mL/min. For the experiment to investigate the effect of CO_2, aeration of 5% CO_2 was used instead of air. Cooling water (20°C) was allowed to run down over the water jacket to maintain the culture temperature at 28°C.

Chemical analyses. Lipids were extracted in methanol-chloroform-water (2:1:0.8) and determined gravimetrically (5). Fatty acid methyl esters were analysed by gas chromatography after transesterification of the lipids in 1 N sodium methoxide at 60° C for 20 min (6). The gas chromatograph was equipped with a polar capillary column (DB 23). The following temperature program was used : 130°C (2 min) increased to 200°C (1 min) at a rate 3° C/min then further increased to 230°C at 2°C/min and held for 5 min.

Proteins were extracted in 0.5 N NaOH (80°C, 20 min) and assayed according to the dye-binding method (7). Carbohydrates were determined using the phenol-sulphuric method (8) after extracting the cells with 2 N HCl (80°C, 20 min).

Pigments were extracted by sonication (2 min) in 100% acetone (HPLC grade). After centrifugation, the supernatants were removed, evaporated and redissolved in 1 mL of acetonitrile-methanol-acetone (56:40:4) before analysis by HPLC. The HPLC system consists of a Rheodyne valve injection port (200 μL loop), a Shimadzu LC 7A pump and a Shimadzu M6A photodiode array detector. The pigments were resolved using a reversed phase column (C18) filled with 5 μ materials and a dimension of 300 X

39 mm. The pigments were separated isocratically using mobile phase consisting of acetonitrile:methanol:acetone (56:40:4) at a flow rate of 1 mL/min. Identification and quantification of the pigments were based on comparison and calibration with authentic standards supplied by Sigma and Fluka Chemicals.

Results and discussion

Growth of *Ankistrodesmus convolutus* based on cell counts and OD_{620} are shown in Figures 1(a) and 1(b) respectively. The culture which was bubbled with 5% CO_2 attained higher cell density and OD_{620} compared to other batches throughout the culture period (10 days) studied. No significant difference was observed in growth performance among the other batches of cultures studied.

The unbuffered culture showed a variation of pH ranging from 6.8 at the initial to 10.0 at the end of the study. The increase in pH could be attributed to assimilation of nitrate which release OH^{-1} (9). However, the increase in pH did not appear to have any adverse effect on the growth of *Ankistrodesmus convolutus*. This was because buffering of the medium (pH within 6.8 - 7.0 throughout the growth period) did not result in any improvement in growth.

Light intensity of 31.9 μEms^{-1} did not limit the growth of *Ankistrodesmus convolutus*. This was because a doubling of light intensity (63.8 μEms^{-1}) did not improve the growth performance of this alga.

The medium used seemed to be limited in carbon supply. This was shown by the improvement in growth after the supplementation of 5% CO_2. Biomass based on dry weight of the cultures aerated with 5% CO_2 was almost doubled that of the yields attained from other batches of cultures (Table 1). The addition of CO_2 was reported to improve biomass production in *Tetraselmis suecica* (10).

Table 1 shows the biochemical composition of the various batches of cultures. Carbohydrates constitute the lowest proportion (7.5 - 12.0% dry weight) of this alga. A slightly lower carbohydrate content (7.5% dry weight) was shown by the culture grown at low light in unbuffered medium compared to other culture conditions (10.0 - 12.0% dry weight). An increase in lipid content was observed in cultures grown in the buffered medium (23.4% dry weight) compared to the unbuffered medium (5.6% dry weight). The culture at high light seemed to afford higher lipid content (26.0% dry weight) compared to other cultures. The buffered culture had a higher protein content than the other cultures. Similar protein contents (around 18% dry weight) were produced in the other cultures. Owing to the higher biomass attained by the CO_2-aerated culture, the yields of the biochemicals (in mg/L) were higher than the other cultures.

Table 2 shows the fatty acid compositions of the different

batches of cultures grown in the airlift fermenter. The predominant fatty acid produced in all the batches was α-linolenic acid (18:3n-3) which ranged from 73.1 - 85.7% of the total fatty caids. Other fatty acids produced were mainly unsaturated fatty acids which included 16:4, 18:1, 18:2 and 18:4. The only saturated fatty acid produced were 16:0 and 18:0.

The unbuffered culture exhibited a slightly lower proportion of 16:0 but a slightly higher proportion of 18:3n-3 compared to the other batches. The CO_2-aerated culture produced less 16:0, 16:4, 18:1, 18:2 and 18:4 but more 18:3n-3 compared to other batches of cultures. No stearic acid (18:0) was produced by the CO_2-aerated culture. Total fatty acid content remains almost constant for all the batches of cultures (Table 2).

Pigment composition did not differ significantly among the various batches of cultures (Table 3). The pigments of this alga include lutein, violaxanthin, neoxanthin, α-carotene, ß-carotene, chlorophyll-a and chlorophyll-b. These pigments are commonly found in other green algae. However, antheraxanthin, zeaxanthin and loroxanthin which are known to present in other green algae (11) were not detected in the present study.

Pigment composition did not differ significantly among the various batches of cultures studied. Appreciable amounts of xanthophylls (10.7 - 12.4 mg/g dry weight) with the predominance of lutein were observed. Very low contents of carotenes were detected and more α-carotene was produced than ß-carotene. Total carotenoid content (12.2 -16.0 mg/g dry weighht) of *Ankistrodesmus convolutus* was much higher than the content for another species *Ankistrodesmus* (4.1 mg/g dry weight) reported by (12).

In conclusion, aeration with CO_2 medium improved growth of *Ankistrodesmus convolutus* in the airlift fermenter. A slight variation in biochemical composition but no significant variation in fatty acid composition and pigmentation were observed in the four batches of cultures. *Ankistrodesmus convolutus* produced appreciable amounts of carotenoids (especially lutein). Further manipulative studies to improve growth and carotenoid production are in progress.

Acknowledgement

The above study was sponsored by a grant (R & D 1/026/01) from the Malaysian government.

References

1. Cohen Z. (1986) Products from microalgae. In Richmond A. (ed), CRC Handbook of Microalgal Culture. CRC Press, Boca Raton, pp. 421-454.
2. Merchuk J.C. (1990) **Tibtech. 8**: 66-71.
3. Chu W.L., Phang S.M., Goh S.H. and Phang S.M. (1992) Promising microalgae for production of useful chemicals. In Shaari K.,

Kadir A.A., Ali A.R.M. (eds), Proc. Conf. Medicinal Products from Tropical Rain Forest Research Inst. Malaysia, Kuala Lumpur, pp. 338-345.
4. Nichols H.W. (1973) Growth media-freshwater. In Stein JR (ed), Handbook of Phycological Methods: Culture Methods and Growth Measurments. Cambridge U.P., Cambridge, 7-24.
5. Bligh E.G. and Dyer W.J. (1959) **Can. J. Biochem. Physiol. 37:** 911-917.
6. Christie W.W. (1989) Gas Chromatography and Lipids. The Oily Press of Scotland, pp. 69-70.
7.Bradford M.M. (1976) **Anal. Biochem. 72:** 248-254.
8.Kochert A.G. (1987) Carbohydrate determination by the phenol-sulphuric acid method. In Hellebust J.A. and Craigie J.S. (eds), Hnadbook of Phycological Methods - Physiological and Biochemical Methods. Cambridge U.P., Cambridge, pp. 95-97.
9. Goldman J.C., Bennert M.R. and Riley C.B. (1982) **Biotech. Bioeng. 24:** 619-631.
*10. Fabregas J. et al. (1984) **Aquaculture 42:** 207-215.
11. Goodwin T.W. (1980) The Biochemistry of the Carotenoids. Vol. 1. Plants. Chapman and Hall, London, pp. 207-256.
12. Paerl et al. (1984)

Table 1. Biomass and biochemical composition of batch cultures of *Ankistrodesmus convolutus* grown in air-lift fermenter.

Culture condition	Biomass (mg/L)	Lipids (% DW)	Carbohydrates (% DW)	Proteins (% DW)
Unbuffered[1]	122	15.6 (19.0*)	12.0 (14.6)	18.3 (22.3)
Buffered[2]	124	23.4 (29.0)	7.5 (9.3)	23.6 (35.4)
High Light[3]	150	26.0 (39.0)	10.0 (15.0)	18.4 (27.6)
+ 5% CO_2[4]	308	17.2 (53.0)	12.0 (37.0)	18.3 (56.4)

* Figures in parentheses indicate values expressed in mg/L
1 At 2,500 Lux
2 At 2,500 Lux, buffered with 10 mM HEPES
3 At 7,500 Lux, buffered with 10 mM HEPES
4 At 2,500 Lux, buffered with 10 mM HEPES and bubbled with 5% CO_2

10 Fabregas J, Abalde J, Herrero C, Cabezas BV
Veiga M (1984)
Growth of the marine microalga Isochrysis sp. in batch cultures with different salinities and nutrient concentrations
Aquaculture 42: 207–215

Table 2. Fatty acid composition (% total fatty acids) of batch cultures of *Ankistrodesmus convolutus* grown in an air-lift fermenter.

Culture condition	Fatty acid							TFA*	
	16:0	16:4	18:0	18:1	18:2	18:3	18:4	% Lipids	%DW
Unbuffered[1]	7.2	1.8	0.4	7.7	8.2	73.1	1.6	4.6	0.7
Buffered[2]	5.3	2.0	0.4	5.1	5.7	79.6	0.8	3.1	0.7
High Light[3]	5.6	2.3	1.0	5.4	6.5	78.6	0.8	2.5	0.7
+ 5% CO_2[4]	4.4	0.9	-	4.0	4.0	85.7	0.5	3.1	0.6

1 At 2,500 Lux
2 At 2,500 Lux, buffered with 10 mM HEPES
3 At 7,500 Lux, buffered with 10 mM HEPES
4 At 2,500 Lux, buffered with 10 mM HEPES and bubbled with 5% CO_2

Table 3. Pigment composition of batch cultures of *Ankistrodesmus convolutus* grown in air-lift fermenter.

Pigment (mg/g dry wt.)	Culture condition			
	Unbuffered[1]	Buffered[2]	High light	+ 5% CO_2[4]
Chlorophyll-a	32.6	30.9	26.7	32.4
Chlorophyll-b	12.0	15.7	13.0	13.2
Chl-a:chl-b	2.7	2.0	2.1	2.5
Total chlorophylls	44.6	46.6	39.7	45.6
Lutein	5.9	5.7	4.9	5.4
Neoxanthin	2.7	2.9	2.3	3.3
Violaxanthin	3.5	3.8	3.5	3.6
Total xanthophylls	12.1	12.4	10.7	12.3
α-carotene	1.6	1.0	0.9	1.5
ß-carotene	0.9	0.7	0.6	0.9
α-car:ß-car	1.8	1.4	1.5	1.7
Total carotenes	2.5	1.7	1.5	2.4
Total carotenoids	14.6	14.1	12.2	14.7
Σcarotenoids: Σchlorophylls	0.3	0.3	0.3	0.3

1 At 2,500 Lux
2 At 2,500 Lux, buffered with 10 mM HEPES
3 At 7,500 Lux, buffered with 10 mM HEPES
4 At 2,500 Lux, buffered with 10 mM HEPES and bubbled with 5% CO_2

Draft 2

PO11

Growth and Product Formation of *Ankistrodesmus convolutus* in an Air-lift Fermenter

[redacted] S.M. Phang and S.H. Goh
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Abstract- Four batches of *Ankistrodesmus convolutus* cultures in an airlift fermenter were investigated in terms of growth and biochemical composition. The contents of lipids, fatty acids, proteins, carbohydrates and pigments in the green alga were compared. The highest biomass (308 mg dry weight/L) was attained by the culture aerated with 5% CO_2. Cells grown at high light contained more lipids (26.0% dry weight) than at other culture conditions studied. No significant variations in fatty acid composition (predominantly 18:3n-3) and pigmentation were observed from the four batches of cultures studied. *A. convolutus* produced appreciable amounts of carotenoids (especially lutein).

Introduction

Microalgae are potential sources of a wide range of commercially valuable natural products which include specialty lipids (eg. arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid) and pigments such as carotenoids and phycobiliproteins (1). Generation of sufficient algal biomass for the extraction of the various products is vital. Air-lift bioreactors are advantageous for the culturing of microalgae because they offer simple and effective mixing with no moving parts, high gas absorption efficiency and good heat transfer characteristics (2).

A. convolutus is a fast growing alga with appreciable amounts of carotenoids and polyunsaturated fatty acids (3). Growth and product formation of four batches of *A. convolutus* cultured in an air-lift fermenter were examined in the present study.

erials and Methods

onvolutus was grown in an air-lift fermenter (cap. 6.5 L) using Bold's Basal Medium, BBM (4). innoculum of 10% in the exponential phase with an optical density of 0.2 at 620 nm was used.

r batches of cultures were grown in the air-lift fermenter under the following conditions: (1) unfered at low light intensity (31.9 $\mu Em^{-2}s^{-1}$), (2) buffered with 10 mM N-2-hydroxyethyl erazine-1-ethanesulphonic acid (HEPES) at low light intensity (3) buffered at high light intensity .8 $\mu Em^{-2}s^{-1}$) and (4) buffered at low light intensity and aerated with 5% CO_2. Batches 1, 2 and 3 e aerated with filter-sterilised air whilst batch 4 with 5% CO_2 at 1,500 mL/min. Illumination was on a 12 h light and 12 h dark cycle. Temperature was maintained at 28° C throughout the study.

)wth was monitored daily by cell counting and OD measurements at 620 nm. Lipids were extracted nethanol-chloroform-water (2:1:0.8) and determined gravimetrically (5). Fatty acid methyl esters re analysed by gas chromatography after transesterification of the lipids in 1 N sodium methoxide at ' C for 20 min (6). Proteins were extracted in 0.5 N NaOH (80°C, 20 min) and assayed according to : dye-binding method (7). Carbohydrates were determined using the phenol-sulphuric method (8) er extracting the cells with 2 N HCl (80°C, 20 min).

Pigments were extracted by sonication (2 min) in 100% acetone (HPLC grade). After centrifugation, the supernatants were removed, evaporated and redissolved in 1 mL of mobile phase consisting of acetonitrile-methanol-acetone (56:40:4) before analysis by HPLC.

Results and Discussion

The culture aerated with 5% CO_2 attained higher cell density and OD_{620} compared to other batches throughout the culture period studied (Figures 1a and 1b). No significant difference was observed in growth among the other batches of cultures studied.

The pH of the unbuffered culture increased from an initial value of 6.8 to 10.0 at the end of the study. The increase in pH could be attributed to assimilation of nitrate which release OH^{-1} (9). However, the increase in pH did not appear to have any adverse effect on the growth of *A. convolutus*. Buffering of the medium to maintain pH within 6.8 - 7.0 did not improve growth.

Light intensity at 31.9 μEms^{-1} was not limiting for the growth of *A. convolutus*. A doubling of the light intensity (63.8 μEms^{-1}) did not improve the growth of this alga.

An improvement in growth was achieved with the supplementation of 5% CO_2. Biomass based on dry weight of the culture aerated with 5% CO_2 was almost doubled that of other batches of cultures (Table 1). The addition of CO_2 was reported to improve biomass production in *Tetraselmis suecica* (10).

An increase in lipid content was observed in the buffered culture (23.4% dry weight) compared to the unbuffered culture (15.6% dry weight) at low light (Table 1). The culture subjected to high light afforded a higher lipid content (26.0% dry weight) compared to the other cultures. Cells grown in the buffered medium had a higher protein content than the cells of other culture conditions. Carbohydrates constitute the lowest proportion (7.5 - 12.0% dry weight) of the algal biomass. A slightly lower carbohydrate content (7.5% dry weight) was shown by the culture grown at low light in unbuffered medium compared to other culture conditions (10.0 - 12.0% dry weight). Yields of the biochemicals from the culture aerated with 5% CO_2 were higher than the other batches due to the higher biomass attained.

The predominant fatty acid produced in all the batches of cultures was α-linolenic acid (18:3n-3) which ranged from 73.1 - 85.7% of the total fatty acids (Table 2). Other fatty acids produced were mainly unsaturated fatty acids which included 16:4, 18:1, 18:2 and 18:4. The saturated fatty acids produced were 16:0 and 18:0.

The unbuffered culture exhibited a slightly lower proportion of 16:0 but a slightly higher proportion of 18:3n-3 compared to the other batches. The culture aerated with CO_2 produced less 16:0, 16:4, 18:1, 18:2 and 18:4 but more 18:3n-3 compared to other batches of cultures. No stearic acid (18:0) was produced by the CO_2-aerated culture. Total fatty acid content remained almost constant for all the batches of cultures (Table 2).

Pigment composition did not differ significantly among the various batches of cultures (Table 3). The pigments of this alga include lutein, violaxanthin, neoxanthin, α-carotene, ß-carotene, chlorophyll-a and chlorophyll-b. These pigments are commonly found in other green algae. However, antheraxanthin, zeaxanthin and loroxanthin which are known to present in other green algae (11) were not detected in the present study.

Appreciable amounts of xanthophylls (10.7 - 12.4 mg/g dry weight) with the predominance of lutein were observed. Very low content of carotenes was detected and more α-carotene was produced than ß-

carotene. Total carotenoid content (12.2 -16.0 mg/g dry weight) of *A. convolutus* was much higher than the content reported for another species of *Ankistrodesmus* (4.1 mg/g dry weight) (12).

Conclusion

Growth of *A. convolutus* in the air-lift fermenter improved with the aeration of 5% CO_2. A slight variation in biochemical composition but no significant variation in fatty acid composition and pigmentation were observed in the four batches of cultures. *A. convolutus* produced appreciable amounts of carotenoids (especially lutein). Further manipulative studies to improve growth and carotenoid production are in progress.

Acknowledgement

The above study was sponsored by a grant (R & D 1/026/01) from the Malaysian government.

References

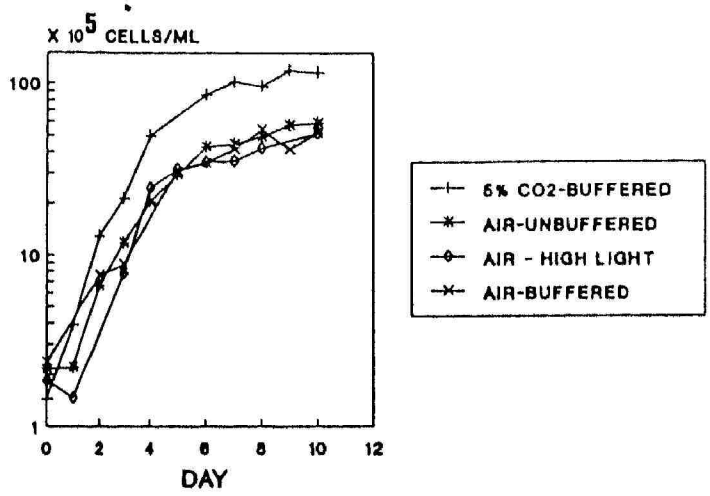
1. Cohen Z. (1986) Products from microalgae. In Richmond A. (ed), **CRC Handbook of Microalgal Culture.** CRC Press, Boca Raton, pp. 421-454.
2. Merchuk J.C. (1990) **Tibtech.**, 8: 66-71.
3. Chu W.L., Phang S.M., Goh S.H. and Phang S.M. (1992) Promising microalgae for production of useful chemicals. In Shaari K., Kadir A.A., Ali A.R.M. (eds), Proc. Conf. **Medicinal Products from Tropical Rain Forest**, Forest Research Inst. Malaysia, Kuala Lumpur, pp. 338-345.
4. Nichols H.W. (1973) Growth media-freshwater. In Stein JR (ed), **Handbook of Phycological Methods: Culture Methods and Growth Measurements.** Cambridge U.P., Cambridge, 7-24.
5. Bligh E.G. and Dyer W.J. (1959) **Can. J. Biochem. Physiol.**, 37: 911-917.
6. Christie W.W. (1989) **Gas Chromatography and Lipids.** The Oily Press of Scotland, pp. 69-70.
7. Bradford M.M. (1976) **Anal. Biochem.**, 72: 248-254.
8. Kochert A.G. (1987) Carbohydrate determination by the phenol-sulphuric acid method. In Hellebust J.A. and Craigie J.S. (eds), **Handbook of Phycological Methods - Physiological and Biochemical Methods.** Cambridge U.P., Cambridge, pp. 95-97.
9. Goldman J.C., Bennert M.R. and Riley C.B. (1982) Biotech. Bioeng., 24: 619-631.
10. Fabregas J., Abalde J., Herrero C., Cabezas B.V. and Veiga M. (1984) **Aquaculture**, 42: 207-215.
11. Goodwin T.W. (1980) The Biochemistry of the Carotenoids. Vol. 1. Plants. Chapman and Hall, London, pp. 207-256.
12. Paerl H.W., Lewin R.A. and Cheng L. (1984) **Bot. Mar.**, 27: 257-264

Table 3: Pigment composition of *Ankistrodesmus convolutus* grown in air-lift fermenter

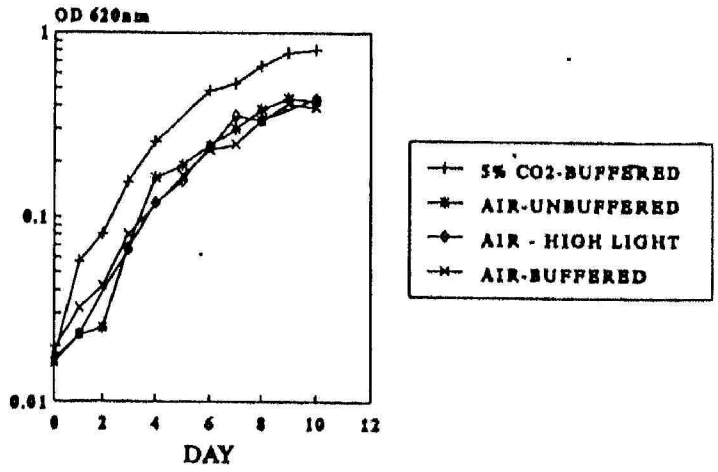
Pigment (mg/g dry wt.)	Culture condition*			
	Unbuffered[1]	Buffered[2]	High light	+ 5% CO_2[4]
Chlorophyll-a	32.6	30.9	26.7	32.4
Chlorophyll-b	12.0	15.7	13.0	13.2
Chl-a:chl-b	2.7	2.0	2.1	2.5
Total chlorophylls	44.6	46.6	39.7	45.6
Lutein	5.9	5.7	4.9	5.4
Neoxanthin	2.7	2.9	2.3	3.3
Violaxanthin	3.5	3.8	3.5	3.6
Total xanthophylls	12.1	12.4	10.7	12.3
α-carotene	1.6	1.0	0.9	1.5
β-carotene	0.9	0.7	0.6	0.9
α-car:β-car	1.8	1.4	1.5	1.7
Total carotenes	2.5	1.7	1.5	2.4
Total carotenoids	14.6	14.1	12.2	14.7
Σcarotenoids: Σchlorophylls	0.3	0.3	0.3	0.3

*Culture conditions 1 - 4 are described in Table 1.

a) ANKISTRODESMUS - AIRLIFT FERMENTER
CELL DENSITY



b) ANKISTRODESMUS - AIRLIFT FERMENTER
OD 620nm



APPENDIX 4

CASE STUDY 4 - DN

(3 DRAFTS)

Bioconversion of Dried Palm Oil Sludge Solids (POSS) into Animal Feed with *Myceliophthora thermophila* *

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bstract - Solid substrate fermentation (SSF) of dry POSS was carried out using the hermophilic microfungus *M. thermophila* under optimised conditions. The bioconverted substrate showed favourable improvements in its chemical characteristics and amino cid profile after 6 days of SSF. The crude protein content increased by 42.2% while crude fibre decreased by 25.3%. A solid inoculum was developed for *M. thermophila* sing POSS as the carrier substrate. The feed produced was free from aflatoxin contamination.

ntroduction

The Palm Oil industry in Malaysia is projected to generates approximately 15.3 million tonnes of Palm Oil Mill Effluent (POME) during oil extraction at the Palm Oil Mills by 1995 (Mohamad *et al.*, 1987). The POME is highly polluting and has caused severe water pollution problems in the past. One promising approach to address the problem is by drying the POSS and utilizing it as an ingredient in livestock feeds. This approach would also support the livestock industry in the country.

When used as animal feedstuff, POSS has been reported to have the limitation of having a high crude fibre content and poor availability of protein which permits only low inclusion levels in feeds especially for monogastic animals (Hutagalung *et al.*, 1982). Of late, SSF with fungi has shown promising results for improving the nutritional value of lignocellulosic agricultural by-products (Shim, 1992; Porim, 1992). In this respect the lignocellulolytic thermophilic microfungus *M. thermophila* shows great promise (Vikineswary, *et al.* 1988). This paper reports the improvement in the chemical characteristics of POSS after SSF with *M. thermophila*, for 6 days.

Materials and Methods

Substrate

Dried POSS was obtained from United Plantations, Sdn. Bhd., Teluk Intan, Perak. The substrate was used in its original granular form without any pretreatment.

Microorganism

The fungus used *M. thermophila* Apinis, was isolated from POME and maintained on Yeast Potato Starch agar (YpSs) slants at 4°C (Vikineswary, 1988).

Inoculum Preparation

A 10% spore suspension of 6 day old culture was aseptically inoculated into a POME: water (1:1) mixture and incubated in a rotary shaker (200 rpm) at 45±2°C for 24 hours. The resultant "porridgy" mycelial broth was blended and a 10% (v/v) inoculum was used to culture *M. thermophila* on POSS at 60% (v/w) moisture content. Incubation was carried out at 45±2°C in a humid chamber for 6 days without shaking. This resultant solid innoculant was used as the starter culture for the SSF of POSS.

* This work will form part of the M. Sc. thesis to be submitted by V. Direct to the Institute of Advanced Studies, University of Malaya.

id Substrate Fermentation of POSS

proximately 50g of autoclaved POSS (121°C, 20 min) with a 60% (v/w) moisture tent was inoculated with 3 teaspoons (3.6% w/w) *M. thermophila* solid inoculum. SSF was carried out at 45±2°C in a humid chamber for 6 days without shaking. e chemical characteristics of POSS before and after bioconversion was analysed in plicates.

alytical Techniques

e crude protein content was estimated by multiplying the kjeldahl nitrogen content by e factor 6.25 (AOAC, 1980). The crude fibre, crude fat, moisture and ash content is determined by the conventional methods (AOAC, 1980). The pH was measured shaking a 1:10 substrate to distilled water ratio and recording the pH with a digital meter. The gross energy of samples was determined using a calorimeter (PARR 61) with a water jacketed oxygen bomb (PARR 1108). The amino acid contents of mples with determined by HPLC. Samples were hydrolised with 4.3N LiOH.H_2O for yptophan determination, perfomic acid hydrolysis followed by 6N HCl hydrolysis for ethionine and cysteine and 6N HCl hydrolysis for the other amino acids. Hydrolysed mples were analysed using a Waters HPLC system with a 1.0x30 cm Pico.Tag amino id hydrolysate column, (1.5ml/ 30 minutes with CH_3CN:H_2O (6:4) solution, delivery ressure at 40 psi) and detected by UV at 254 and 280 nm).

oxicology

he substrate were screened for the mycotoxin producing fungi, *Aspergillus flavus* and *spergillus parasiticus* by randomly baiting 1.0g of substrate on Czapek Dox agar lates, incubating at room temperature, followed by visual observation. The samples ere also screened for the presence of aflatoxin B1, B2, G1, and G2 by extracted with $CHCl_3$:H_2O (10:1) solution followed by TLC [silica gel 60, mobile phase, $CHCl_3$:CH_3OH (95:5) for 25 minutes and viewed under UV light after spraying with 0% H_2SO_4].

Inoculum Storage and Viability

The solid *M. thermophila* inoculum on POSS was packed in test tubes and plastic bags and stored at 4°C, 10°C and at room temperature for up to 3 months. Containers were sampled for each treatment at month 0, 1, 2, 3 and the viability was assessed.

Results and Discussion

The results of the chemical characterization and amino acid profiles of POSS before and after SSF with *M. thermophila* is shown in Table 1 and 2 respectively. The crude protein content of POSS increased by 42.2%, the crude fibre content decreased by 25.3% while the changes in crude fat, ash and gross energy were negligible. The crude fat content of POSS was apparently not degraded by the fungus during growth and hence would serve as a dietary energy source when used as a feedstuff (Yeong and Mukherjee, 1983). The increase in crude protein and decrease in crude fibre is desirable as it would allow higher inclusion levels of the upgrade POSS in livestock feed ratios.

There was an improvement in all the amino acids contents except tryptophan. The glutamic acid content was the highest, at 2.04%, while cysteine and methionine were the lowest, at 0.19% and 0.18% respectively. Hence adequate supplementation with the latter two amino acids in envisaged when using upgraded POSS as a feedstaff.

The bioconverted feed produced was free from contamination by the mycotoxin producing fungi, *A. flavus* and *A. parasiticus* as well as from aflatoxin B1, B2, G1 and G2.

The solid POSS based inoculum of *M. thermophila* developed retained high viability (>95%) when stored in test tubes at 4°C for up to 4 months. The inoculum could also satisfactorily be stored in plastic bags at room temperature for up to 1 month. The use of solid inoculum for the SSF is advantageous as it is easier to prepare, handle and eradicates the need for the cumbersome liquid inoculum preparation. This would facilitate technology transfer to rural areas.

Conclusion

Palm Oil Sludge Solids can be upgraded bioconverted into a protein enriched feed with *M. thermophila* via simple low technology SSF methods.

Acknowledgements

The authors would like to sincerely thank United Plantations Sdn. Bhd., The Diagnostic Laboratory of the Veterinary Department, Petaling Jaya and The Department of Zoology, University of Malaya for their support, suggestions and technical assistance rendered during the course of this study.

References

AOAC (1980). Official Methods of Analysis (14th edn.). Association of official analytical chemists., Washington DC.

Hutagalung, R.I., Mahyuddin, M. and Jalaludin, S. (1982). The Oil Palm In Agriculture in the Eighties (Vol. II) Incorporated Society of Planters, Kuala Lumpur. pp. 609-622.

Mohamad, H., Zin Zawawi, Z. and Abdul Halim, H. (1987). Proceedings of the National Symposium on Oil Palm By-products for Agro-based Industries. 5-6th November 1985, Kuala Lumpur. PORIM, pp. 7-15.

Porim (1992). Porim Annual Report., Serdang pp.

Shim, Y.L. (1992). Utilization of Sago Hampas by Microfungi. M. Biotech. thesis, University of Malaya.

Vikineswary, S. (1988). Utilisation and Treatment of Palm Oil Mill Effluent by Selected Fungi. PhD. thesis, University of Malaya.

Yeong, S.W. and Mukherjee, T.K. (1982). MARDI Research Bulletin., 10: 399-408.

Table 1: Chemical ~~Principles~~ characteristics of upgraded POSS ~~bioconverted with~~ *M. thermophila*

Chemical ~~Principles~~ characteristics*	POSS	
	Before SSF	After SSF[a]
Crude Protein	14.2	20.2
Crude Fibre	25.3	18.9
Crude Fat	13.2	15.3
Moisture	3.2	3.4
Ash	17.3	17.7
Gross Energy (Cal/g)	5058	5257
pH	4.88	5.47

Table 2: Amino Acid Profile of upgraded POSS ~~bioconverted with~~ *M. thermophila*.

~~Acid~~ Amino Acid *	POSS ~~(% Dry Matter)~~	
	Before SSF	After SSF[a]
Alanine	0.93	1.49
Arginine@	0.51	0.65
Aspartic acid	0.74	0.90
Cysteine	0.07	0.19
Glutamic acid	1.52	2.04
Glycine	0.68	1.04
Histidine@	0.23	0.30
Isoleucine@	0.70	0.99
Leucine@	1.33	1.74
Lysine	0.42	0.46
Methionine@	0.09	0.18
Plenylalanine@	0.64	0.98
Proline	0.69	1.08
Serine	0.55	0.75
Threonine@	0.46	0.71
Tryptophan@	0.43	0.42
Tyrosine	0.36	0.65
Valine@	1.02	1.32

Note: * - All units are expressed as a precentage of dry matter unless otherwise stated.

a - SSF with *M. thermophila* [~~using~~ 60% moisture (v/w), incubated at 45±2°C for 6 days ~~period~~.]

@ - Essential Amino Acids

Draft 2

Bioconversion of Dried Palm Oil Sludge Solids (POSS) with *Myceliophthora thermophila*

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Abstract

Solid substrate fermentation of Dry Palm Oil Sludge Solids using the thermophilic microfungus, *Myceliophthora thermophila*, favourably improved the nutritional characteristics and amino acid profile of the substrate. The crude protein content increased by 47.3% while crude fibre decreased by 25.3%. A solid inoculum was developed for *M. thermophila* using POSS as the carrier substrate. The feed product was free from contamination with *A. flavus*, *A. parasiticus* and aflatoxins.

Introduction

The Palm Oil industry in Malaysia is projected to generate approximately 15.3 million tonnes of Palm Oil Mill Effluent (POME) in 1995 (Mohamad et al., 1987). The POME is highly polluting and has caused severe water pollution problems in the past. One promising approach to overcome the problem is by composting the POME and utilizing it as an ingredient in animal feeds. Besides helping to solve the pollution problem, this would also support the livestock industry in the country. However, when used as animal feed, POSS has been reported to have a high crude fibre content and poor availability of protein which limits its application levels in feeds especially for monogastic animals (Ong et al., 1982).

In recent years, solid substrate fermentation (SSF) with lignocellulosic agricultural by-products has shown promising results for improving the nutritional value of the substrate (Vikineswary, 1992; Shim, 1992). In this respect the lignocellulolytic thermophilic microfungus, *M. thermophila* shows great promise (Vikineswary, 1988). This paper reports the chemical characteristics of POSS after SSF with *M. thermophila*.

Materials and Methods

Substrate

Dried POSS was obtained from United Plantations, Sdn. Bhd., Teluk Intan, Perak.

Fungus

The fungus used, *M. thermophila* Apinis, was locally isolated from POME and maintained on Yeast Potato Starch agar (YpSs) slants at 4 C (Vikineswary, 1988).

Inoculum Preparation

A 10% spore suspension of 8 day old cultures was ~~aseptically inoculated~~ ~~...~~ incubated in 10% POME media at

~~water (1:1) mixture and incubated in a rotary shaker (200 rpm)~~ at 2 C for 24 hours, in a rotary shaker.

~~hours.~~ The ~~resultant "porridgy" mycelial~~ mycelial broth was blended and a 10% (v/v) inoculum was used to culture M. thermophila on POSS with a 60% (v/w) moisture content. Incubation was carried out at 45±2 C in a humid chamber for 6 days without shaking. This resultant solid innoculant was used as the starter culture for the SSF of POSS.

Solid Substrate Fermentation of POSS

Approximately 50g of POSS, in Erlenmeyer flasks ~~was~~ were autoclaved at 121 C for 20 min. The moisture content was ajusted to 60% (v/w). Each flask was then inoculated with 6 % (w/w) M. thermophila koji culture. Incubation was carried out at 45±2 C in a humid chamber for 6 days without shaking.

Analytical Techniques

The crude protein ~~content~~ (N x 6.25), crude fibre, crude fat, moisture and ash content was determined by the methods of AOAC (1980). The pH was measured by shaking the substrate in distilled water (1:10) after which ~~and~~ measurements were recorded with a digital pH meter. The gross energy was determined using a calorimeter with a water-jacketed oxygen bomb. The amino acid profile was determined using Waters High Performance Liquid Chromatography (HPLC) system with a 1.0x30 cm Pico Tag amino acid hydrolysate column and detected with UV at 254 and 280 nm.

Toxicology

The substrate was screened for the aflatoxin producing fungi, Aspergillus flavus and Aspergillus parasiticus by random plating the fermented substrate on Czapek Dox agar plates. Substrates were also screened for the aflatoxins B1, B2, G1, and G2 by extraction with $CHCl_3$:H_2O (10:1) solution followed by Thin Layer Chromatography (TLC) using silica gel 60 and detected with UV after spraying with 30% H_2SO_4.

Inoculum Storage and Viability

The solid M. thermophila inoculum on POSS was packed in test tubes and plastic bags and stored at 4 C, 10 C and at room temperature for 3 months. ~~and~~ The viability was assessed monthly.

Results and Discussion

The results of the chemical characterization and amino acid profiles of POSS before and after SSF with M. thermophila is shown in Table 1 and 2 respectively. The crude protein ~~content of POSS increased by 42.2% while~~ the crude fibre content decreased by 25.3% after SSF. The changes in crude fat, ash and gross energy were negligible. The crude fat content of POSS was apparently not utilised by the fungus during growth and ...

There was an overall improvement in the amino acid profile. The glutamic acid content was the highest, at 2.04%, while cystine and methionine were the lowest, at 0.19% and 0.16% respectively. Hence adequate supplementation with the latter two amino acids is envisaged when using upgraded POSS as a feedstaff.

The upgraded feed ~~product~~ was free from contamination with A. flavus , A. parasiticus and aflatoxin B1, B2, G1 and G2.

The solid POSS based inoculum of M. thermophila developed remained highly viabile (>95%) for up to 4 months when stored in test tubes at 4 °C. The inoculum could also satisfactorily be stored in plastic bags for up to 1 month in plastic bags. The use of solid inoculum for the SSF is advantageous as it is easier to prepare, handle and eradicates the need for the cumbersome liquid inoculum preparation, thus facilitating technology transfer to rural areas.

Conclusion

Palm Oil Sludge Solids can be upgraded into a protein enriched feedstuff with M. thermophila via solid state technology.

Acknowledgements

The authors would like sincerely thank United Plantations Sdn. Bhd. (the Diagnostic Laboratory of the Veterinary Department, Petaling Jaya) and the Department of Zoology, University of Malaya for their support, suggestions and technical assistance rendered during the course of this study. We also thank the University of Malaya for providing the grant for this study.

References

AOAC (1980). Official Methods of Analysis (15th. edn.), Association of official analytical chemists., Washington DC.

Hutagalung, R.I., Mahyuddin, M. and Jalaluddin, S. (1982). The Oil Palm In Agriculture in the Eighties (Vol. II). Incorpprated Society of Planters, Kuala Lumpur, pp. 609-622.

Mohamad, H., Zin Zawawi Z. and Abdul Halim, H. (1987). Proceedings of the National Symposium on Oil Palm By-products for Agro-based Industries. 5-6 November 1987, Kuala Lumpur. PORIM, pp. 7-15.

Porim (1992). Porim Annual Report., Serdang pp.

Yeo, Y.L. (1992). Utilization of Sago Hampas by Micro-fungi. M. Pintar thesis University of Ma

Yeoh, B.W. and Mukherjee, T.K. (1982). MARDI Research Bulletin, 10: 399-408.

Jorgensen, H. K. (1982) In Proc. of Regional Workshop on Palm Oil Mill Technology and Effluent Treatment. Universiti Pertanian Malaysia, Serdang, 19-21 August 1980. pp: 201-212.

Table 1: Chemical characteristics of upgraded POSS

	POSS	
	Before SSF	After SSF[a]
Crude Protein	14.2	20.2
Crude Fibre	25.3	18.9
Crude Fat	13.2	15.3
Moisture	3.2	3.[illegible]
Ash	17.3	17.7
Gross Energy (Cal/g)	5058	5257
pH	4.88	5.47

Note: - as below.

Table 2: Amino acid profile of upgraded POSS

Amino acid	POSS	
	Before SSF	After SSF
[illegible]	[illegible]	[illegible]
Arginine	[illegible]	[illegible]
Aspartic acid	[illegible]	[illegible]
Cysteine	[illegible]	[illegible]
Glutamic acid	[illegible]	[illegible]
Glycine	0.88	1.04
Histidine	0.23	0.30
[illegible]	0.70	0.99
Leucine	[illegible]	[illegible]
Lysine	0.42	0.46
Methionine	0.02	0.08
Phenylalanine	[illegible]	[illegible]
Proline	0.49	0.68
Serine	[illegible]	0.75
Threonine	0.46	0.71
Tryptophan	[illegible]	0.42
Tyrpsine	[illegible]	0.65
Valine	[illegible]	1.32

Note: - All units are expressed as a precentage of dry matter, unless otherwise stated.

SSF with M. thermophila moisture 60% (w/w), incubation at 45±2 C for 6 days.

- Essential amino acids

Draft 3

PO31

Bioconversion of Dried Palm Oil Sludge Solids (POSS) with *Myceliophthora thermophila*[*]

[redacted] S. Vikineswary and T. K. Mukherjee
Institute of Advanced Studies, University of Malaya, 59100 Kuala Lumpur, MALAYSIA

Abstract - Solid substrate fermentation (SSF) nof dry Palm Oil Sludge Solids (POSS) using the thermophilic microfungus, *Myceliophthora thermophila*, favourably improved the chemical characteristics and the amino acid profile of the substrate. The crude protein content increased by 42.2% while crude fibre decreased by 25.3%. A solid inoculum was developed for *M. thermophila* using POSS as the carrier substrate. The feed produced was free from contamination with *Aspergillus flavus*, *Aspergillus parasiticus* and aflatoxins.

Introduction

The Palm Oil industry in Malaysia is projected to generate approximately 15.3 million tonnes of Palm Oil Mill Effluent (POME) in 1995 (Mohamad *et al.*, 1987). Palm Oil Mill Effluent comprises mainly of the sterilizer condensate, clarifier sludge and hydrocyclone washing. Palm Oil Mill Effluent is highly polluting and has caused severe water pollution problems in the past. Among the various approaches to address this problem, drying the clarifier sludge to obtain POSS and utilising it as an animal feed has shown promise (Jorgensen, 1982). This approach would also support the livestock industry in the country. However, when used as animal feed, POSS has been reported to have a high crude fibre content and poor availability of protein which limits inclusion levels in feed rations especially for monogastric animals (Hutagalung *et al.*, 1982).

In recent years, SSF with fungi has shown promising results for improving the nutritive value of lignocellulosic agricultural by-products. In this respect the lignocellulolytic thermophilic microfungus, *M. thermophila* Apinis van Oorschot has shown great promise (Shim, 1992). This paper reports the chemical characteristics of POSS after SSF with *M. thermophila*.

Materials and Methods

Substrate

Dried POSS was obtained from United Plantation, Bhd., Teluk Intan, Perak.

Fungi

The fungus used, *M. thermophila*, was locally isolated from POME and maintained on Yeast Potato Starch agar (YpSs) slants at 4°C (Vikineswary, 1988).

Inoculum Preparation

A 10% spore suspension of 8 day old culture of *M. thermophila* was incubated in 50% POME media at 45±2°C for 24 hours in a rotary shaker. The resultant mycelial broth was blended and a 10% (v/v) inoculum was used to culture *M. thermophila* on POSS at 60% (v/w) moisture content. Incubation was

[*]This work will form part of the M. Biotech. thesis to be submitted by [redacted] to the Institute of Advanced Studies, University of Malaya.

carried out at 45 ± 2°C in a humid chamber for 6 days without shaking. This solid culture, koji was then used as the starter culture for the SSF of POSS.

Solid Substrate Fermentation

A preliminary experiment was carried out to optimize the growth of *M.thermophila* on POSS. In this study approximately 50g of POSS, in 2L Erlenmeyer flasks were autoclaved at 121°C for 20 min. The moisture content was adjusted to 60% (v/w). Each flask was then inoculated with 6 % (w/w) *M. thermophila* koji culture. Incubation was carried out at 45 ± 2°C in a humid chamber for 6 days without shaking.

Analytical Techniques

The substrates were analysed in triplicates for crude protein (N x 6.25), crude fibre, crude fat, moisture and ash content by the methods of AOAC (1990). The pH was determined by shaking the substrate in distilled water (1:10) after which measurements were recorded with a digital pH meter. The gross energy was determined using a calorimeter with a water jacketed oxygen bomb. The amino acid profile was determined using a Waters High Performance Liquid Chromatography (HPLC) system with a 1.0x30 cm Pico.Tag amino acid hydrolysate column and detected with UV light.

Toxicology

The substrates were screened for the aflatoxin producing fungi, *A. flavus* and *A. parasiticus* by randomly plating the fermented substrate on Czapek Dox agar plates. Substrates were also screened for the aflatoxins B1, B2, G1, and G2 by extraction of upgraded POSS with $CHCl_3$:H_2O (10:1) solution followed by Thin Layer Chromatography (TLC) using silica gel 60 and detected under UV light after spraying with 30% H_2SO_4.

Results and Discussion

The results of the chemical characterization and amino acid profiles of POSS before and after SSF with *M. thermophila* is shown in Tables 1 and 2 respectively. The favourable increase in crude protein by 42.2% and decrease in crude fibre by 25.3% after SSF would allow higher inclusion levels of upgraded POSS in livestock feed rations. The crude fat content of POSS was apparently not utilised by the fungus during growth and hence would serve as a dietary energy source when used as a feedstuff (Yeong and Mukherjee, 1983).

There was an overall improvement in the amino acid profile with glutamic acid being the highest, at 2.04%. However the low amounts of cystine and methionine of 0.19% and 0.18% respectively, would necessitate adequate supplementation when using upgraded POSS as a feed supplement.

The upgraded feed was free from contamination with *A. flavus* , *A. parasiticus* and the aflatoxins : B1, B2, G1 and G2. However as aseptic cultures are cost attractive in upscaled SSF operations, sound management practices are envisaged to minimise the risk of contamination.

Conclusion

Palm Oil Sludge Solids shows potential to be upgraded into a protein enriched feedstuff with *M. thermophila* via simple low technology SSF methods. Further upscaling experiments with more replicates is envisaged to verify these results.

Acknowledgements

The authors would like to sincerely thank United Plantation Bhd.; The Diagnostic Laboratory of the Veterinary Department, Petaling Jaya and The Department of Zoology, University of Malaya for their support and technical assistance rendered during the course of this study. We also thank the University of Malaya for providing the grant for this study.

References

1. AOAC (1990) **Official Methods of Analysis** (15th edn.) Vol.II. Association of Official Analytical Chemists., Washington DC.
2. Hutagalung, R.I., Mahyuddin, M. and Jalaludin, S. (1982) **The Oil Palm In Agriculture in the Eighties**, Vol. II. Incorporated Society of Planters, Kuala Lumpur (Eds. Pusparajah, E. and Chew, P. S.). pp. 609-622.
3. Jorgensen, H. K. (1982) Proceedings of **Regional Workshop on Palm Oil Mill Technology and Effluent Treatment**. 17-18 August 1982, Kuala Lumpur. PORIM. pp. 201-212.
4. Mohamad, H., Zin Zawawi, Z. and Abdul Halim, H. (1987) Proceedings of the **National Symposium on Oil Palm By-products for Agro-based Industries**. 5-6 November 1985, Kuala Lumpur. PORIM, pp. 7-15.
5. Shim, Y.L. (1992) **Utilisation of Sago Hampas by Microfungi.** M. Biotech. thesis, University of Malaya.
6. Vikineswary, S. (1988) **Utilisation and Treatment of Palm Oil Mill Effluent by Selected Fungi.** PhD. thesis, University of Malaya.
7. Yeong, S.W. and Mukherjee, T.K. (1982) **MARDI Research Bulletin., 10**: 399-408.

Table 1: Chemical characteristics[a] of upgraded POSS

	POSS	
	Before SSF[b]	**After SSF[c]**
Crude Protein	14.16 ± 0.29	20.20 ± 0.12
Crude Fibre	25.30 ± 0.38	18.39 ± 0.35
Crude Fat	13.23 ± 0.01	15.32 ± 0.04
Moisture	3.22 ± 0.02	3.42 ± 0.03
Ash	17.33 ± 0.04	17.66 ± 0.11
pH	4.88 ± 0.00	5.47 ± 0.01
Gross Energy (cal/g)	5058	5257

Note: See below.

APPENDIX 5

First Interview

I. Personal details

1. Name
2. Age
3. Current stage of education
4. Medium of Educational instruction

II. Writing Behaviour

1. Have you written a short paper before?
2. What sources did you consult to help you in preparing this paper and what kind of help did you receive from each source?
3. What kind of planning did you do before beginning to write the paper?
4. What were your goals in writing the paper? Was it necessary to consider the audience and the purpose of the paper? Why/Why not?
5. Describe your process of drafting from the point when you decided to write the paper right up to its completion.
6. Did you change your paper much while writing ? What aspects of the paper did you change?
7. Did you stop often while writing? If so, why did you stop?
8. What did you do if you were stuck for a word or phrase or unsure of how to proceed?

9. In what order did you write your paper? Is there any particular reason for this order?

10. Did you have problems organising your information? Were there particular sections of the paper where this was especially difficult?

11. How much time in total did you spend on the preparation of this paper?

12. What were you trying to achieve through the revisions that you made in the drafts?

13. Did you approach people to read and comment on your drafts? Who were these people?

14. How did you decide when to stop writing?

III General Variables

1. How do you feel about writing in general?

2. What kinds of writing do you do outside the academic environment?

3. Do you have any particular problems writing in English? What are they?

4. What are some of the different texts that you have to write in the course of your study at the Institute of Advanced Studies?

5. Do you generally rely on anyone to help you with your writing? Who?

6. What kind of writing do you generally prefer - academic or non-academic? Why?

Second Interview

1. What were you trying to achieve in each section of the paper?
2. Why is the section, _____, missing from your paper?
3. Elicit writer's specific reasons for each of the changes made in his/her drafts.
4. At what points in your writing did your revisions take place?
5. What were your general intentions in revising your paper?
6. In your opinion, what is revision?
7. How satisfied are you with the final paper?