APPENDIX A : ANALYTICAL TECHNIQUES

1. DETERMINATION OF CRUDE FIBER

The crude fiber content was determined by the Weende method (AOAC, 1990) using a Tecator Fibertec System 1. Approximately 1.0 g sample (W∅) was weighed into fritted glass crucibles and hydrolyzed with boiling 0.128M sulfuric acid, followed by boiling in 0.223M potassium hydroxide solution in a hot extractor. The residue was washed with preheated distilled water before being transferred to a cold extractor and washed with acetone. The residue and crucibles were oven dried at 105°C overnight and weighed (W1) before being ignited in a muffle furnace at 450°C for 8 h. The residual ash was first cooled in an oven at 105°C overnight, then cooled to room temperature in a dessicator and finally weighted (W2). The percentage of crude fiber in the sample was calculated with the formula:

$$\% \text{ Crude fiber} = \frac{W1 - W2}{W\varnothing} \times 100$$

2. DETERMINATION OF MOISTURE CONTENT

Clean porcelain crucibles were oven dried at 105°C for 24 h, cooled in a dessicator and weighed (W∅). Approximately 5.0 g sample was weighed into the crucibles and the whole crucible with the sample weighed again (W1). The crucibles with the samples were oven dried at 105°C for 48 h, and cooled in a dessicator before being re-weighed

(W2). The moisture content was computed with the following formula and expressed as the percentage per gram sample.

$$\% \text{ Moisture} = \frac{(W1 - W2)}{(W1 - W\varnothing)} \times 100$$

3. DETERMINATION OF CRUDE FAT

The crude fat content was determined gravimetrically using a Tecator Soxtec System H. Approximately 1.0 g sample was used (W∅). The samples were not hydrolyzed prior to extraction with ether. The fat was extracted into pre-weighed thimbles (W1), dried overnight at 105°C, cooled and weighed (W2).

The crude fat content was calculated with the formula:

$$\% \text{ Crude Fat} = \frac{(W2 - W1)}{W\varnothing} \times 100$$

4. DETERMINATION OF TOTAL NITROGEN AND CRUDE PROTEIN

Total nitrogen content was determined by the semi-micro Kjeldahl method (AOAC, 1990) using a Tecator Kjeldahl Auto 1030 Analyzer system. Approximately 1.0 g of sample with a 10.0 g mixture of potassium sulfate and copper sulfate (100:7) as the catalyst was digested in 25 ml concentrated sulfuric acid at 420°C. After digestion,

cooled samples were diluted with 75 ml of distilled water, distilled into 1% boric acid and titrated against 0.5 M hydrochloric acid. The bromocresol green and methyl red indicators used, changed to a final color of light pink at the end point of the titration. The percentage of total nitrogen and of crude protein were computed using the formulas:

$$\% \text{ Total Nitrogen} = \frac{(14.01 \times M \times 100)}{\text{mg sample}} \times (\text{ml titrant} - \text{ml blank}) \times 100$$

14.01 = the atomic weight of nitrogen, M = the molarity of the acid (mol/L)

$$\% \text{ Crude Protein} = \text{Total N} \times 6.25$$

Since, on the average, protein contains about 16% nitrogen, one can either divide the percentage of nitrogen by 0.16 or multiply it by a factor of 6.25 to obtain the crude protein content.

5. DETERMINATION OF TOTAL ASH

The ash content was determined using the same samples used in the dry matter determination. After the moisture content of the samples was determined, the samples were ignited in a muffle furnace at 550°C overnight, followed by cooling to room temperature in a dessicator and weighed (W3). The ash content was computed with the formula:

$$\% \text{ Total Ash} = \frac{(W2 - W3)}{W1 - W\varnothing} \times 100$$

6. DETERMINATION OF ACID DETERGENT FIBER

Reagents

Acid detergent solution

Approximately 29.0 g cetyl trimethylammonium bromide (CTAB) was dissolved in 1L of 1N H_2SO_4.

Procedure

Approximately 1.0 g sample (W1) was re-fluxed for 60 min in 50 ml acid detergent solution using a Tecator Fibretech System 1. This treatment with acid detergent solution solubilized all sugars, starch, fat, protein and hemicellulose in the sample, leaving cellulose, lignin and substantial amount of silica in the pre-weighed sintered glass crucible used. After re-fluxing, the reagent was filtered off with the aid of vacuum suction and rinsed twice with hot water, followed by twice with acetone. The crucible were oven dried at 105°C overnight and weighed (W2). The acid detergent fiber content was calculated with the formula:

$$\% \text{ Acid Detergent Fiber} = \frac{\text{Weight of residue (W2-W1)}}{\text{sample weight (W1)}} \times 100$$

Note: The same sample can be used for the determination of cellulose and lignin.

7. DETERMINATION OF LIGNIN

Reagents

i. Saturated Potassium Permanganate

Approximately 50.0 g reagent grade potassium permanganate was dissolved in 1L distilled water. The solution was stored in a dark bottle at 4°C.

ii. Buffer solution

Approximately 6.0 g ferric nitrate and 0.15 g argentum nitrate were dissolved in 100 ml distilled water. Approximately 500 ml glacial acetic acid, 5.0 g potassium acetate followed by 400 ml tertiary butyl alcohol were added and mixed thoroughly.

iii. Combined Permanganate Solution

Two parts of saturated potassium permanganate solution was mixed with one part buffer solution (v/v) just prior to use and stored in a dark bottle at 4°C.

iv. Demineralizing Solution

Approximately 50.0 g oxalic acid dihydrate was dissolved in 700 ml ethanol (95%) before being used with 50 ml of 12 N HCl and 250 ml distilled water.

Procedure

The lignin content was determined using the residue after acid detergent fiber determination (Van Soest and Wine, 1968). Approximately 25 ml of combined permanganate solution was added to the samples and the crucibles were immediately immersed in an enamel pan containing 2-3 cm depth of water. Individual glass rods were used to break up particle clumps to ensure maximal surface area was acted upon by the combined permanganate solution. The samples were allowed to stand for 90 ±

10 min at 20 - 25°C with purple color maintained through the period. Exhausted permanganate solution, which turned brown, was removed using vacuum suction and replaced with fresh solution. After the treatment period, the combined permanganate solution was filtered off without washing the remaining residues. The crucibles were then placed in a clean dry pan, filled half with demineralizing solution and allowed to stand for 5 min before being filtered off. Residues on the sides of the crucibles were carefully washed down with demineralizing solution. Yellow specks in the residues indicates that lignin is still present and the permanganate treatment should be repeated. After demineralization, the residues were washed twice with 80% alcohol solution, followed by twice with acetone. The samples were oven dried overnight at 105°C and weighed. The lignin content in the sample was calculated with the formula:

$$\% \text{ Lignin} = \frac{\text{Weight of residue - Weight of ADF}}{\text{sample weight}} \times 100$$

8. DETERMINATION OF CELLULOSE AND INSOLUBLE ASH

The cellulose content in the samples were determined gravimetrically, using the same sample used for the acid detergent fiber and lignin determination (Van Soest and Wine, 1968). After lignin determination, the residues were ashed at 450°C for 8 h, cooled and weighed. The cellulose content was computed with the formula:

$$\% \text{ Cellulose} = \frac{\text{Weight of residue after lignin determination} - \text{Weight of ADF}}{\text{sample weight}} \times 100$$

$$\% \text{ Insoluble Ash} = \frac{\text{Weight of ash}}{\text{sample weight}} \times 100$$

9. DETERMINATION OF TOTAL CARBON

Reagents

i. Dichromate mixture (0.0675M)

Approximately 19.86 g potassium dichromate and 200 ml ortho-phosphoric acid (SG 1.79) were slowly added to 400 ml of concentrated sulfuric acid. The mixture was made up to 1L with distilled water and stored in a dark bottle.

ii. Ferrous Ammonium Sulfate (0.4M)

Approximately 156.86 g ammonium ferrous sulfate was added to 20 ml of concentrated sulfuric acid and made up to 1L with distilled water. Required amount of this reagent was prepared fresh just prior to use and stored at 4°C.

iii. Indicator Solution

Approximately 5 g barium chloride and 0.3 g barium diphenylamine sulphonate were dissolved in 100 ml of distilled water.

Procedure

Total carbon was determined by the Rapid Titration Wet Oxidation method of Allen (1987). Approximately 0.5 g of sample was oxidized by boiling in 25 ml of dichromate mixture for 1 h. The resultant clear mixture was cooled and made up to 100 ml with distilled water. Exactly 5 ml of indicator solution was added and the remaining unused dichromate was titrated against 0.4 M ferrous ammonium sulfate. The total carbon content was calculated with the following formula and expressed as the percentage of the dry sample weight:

$$\% \text{ Carbon} = \frac{(25.0 - T) \text{ ml} \times 0.12}{\text{sample weight (g)}} \times 100$$

T = the titre of ammonium ferrous sulfate used

10. DETERMINATION OF pH

The pH was measured using a suitably calibrated pH meter. The pH of solids was measured by making a 10%(w/v) suspension by mixing vigorously (1000 rpm for 10 min) the solids in distilled water and taking the pH of the supernatant after the solids have settled. The measurements were determined at room temperature of 25 ± 2°C.

11. DETERMINATION OF REDUCING SUGAR (DNS METHOD)

Reagents

i. Dinitrosalicylic acid (DNS)

The DNS reagent used contained 1% (w/v) dinitrosalicylic acid, 0.2% (w/v) phenol, 0.05% sodium sulfite and 1% NaOH (Miller, 1959). Large batches of reagent was prepared without sulfite and stored in a dark bottle at 4°C. Appropriate amount of sodium sulfite was added to aliquots just prior to the time when the reagent was to be used.

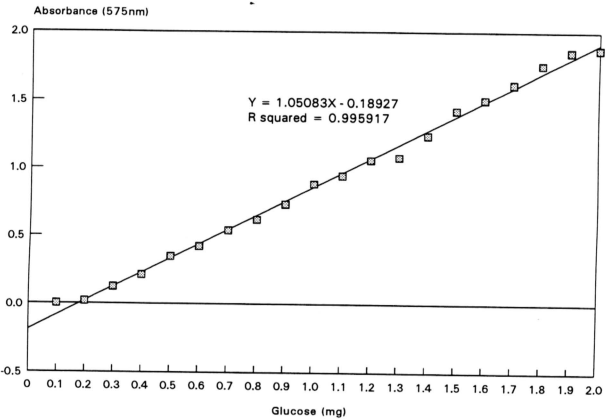
ii. Rochelle salt solution (40%)

Dissolve about 400 g of AR grade Rochelle salt (potassium sodium tartarate tetrahydrate crystals $C_4H_4KNaO_6.4H_2$) in 1L of distilled water.

Procedure for preparation of glucose calibration plot

Diluted sugar solution (2.0 ml) was added to 3.0 ml DNS reagent. The solution was mixed and incubated in a boiling water bath for 15 min. Upon removal from the water bath approximately 1.0 ml of 40% Rochelle salt solution was added to the reaction to stabilize the orange red color formed before it was cooled under running tap water. The intensity of the color was determined using a Shimadzu UV 160A Spectrophotometer (Japan) at the wavelength λ = 575 nm. The reducing sugar concentrations of the test solution was calculated by extrapolation from a linear glucose standard graph.

Glucose Calibration Plot



Reducing Sugars Assay of test solution

Test solution (2.0 ml) was assayed following the method described above. The absorbance values after subtraction of the substrate blank and enzyme blank were then translated into glucose equivalent using the glucose standard graph.

12. DETERMINATION OF SOLUBLE PROTEIN

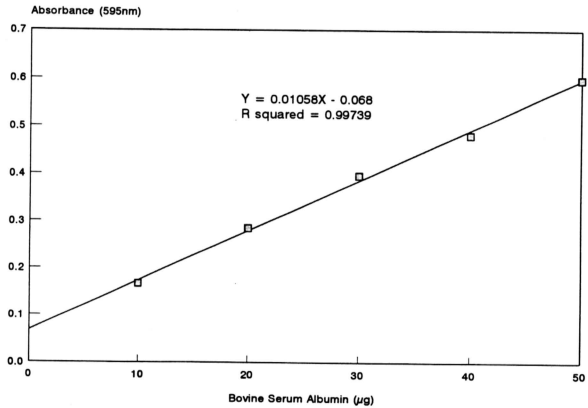
Reagents

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution, 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was made up to a final volume of 1 L.

Procedure for preparation of protein calibration plot

Bovine Serum Albumin (BSA) solution containing 10 to 100 μg protein in a volume up to 0.1 ml was pipetted into 12 x 100 mm test tubes. The volume in the test tube was adjusted to 0.1 ml with appropriate buffer. Then 5.0 ml of protein reagent was added into the test tubes and the contents mixed either by inversion or vortexing. The absorbance at λ = 595 nm was measured after 2 min and before 1 h in a 3.0 ml cuvette against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5.0 ml of protein reagent. The weight of the protein was plotted against the corresponding absorbance resulting in a standard curve.

Protein Calibration Plot



Protein assay of test solution

Solution containing protein in a volume of up to 0.1 ml was pipetted into 12 x 100 mm test tubes. The volume in the test tubes was adjusted to 0.1 ml with appropriate buffer and the protein content was measured following the procedure described above for protein standard curve. The amount of protein in the test solution was calculated by using the protein standard graph with the following formula:

$$\text{Soluble protein (mg/ml)} = \frac{\text{(Final absorbance - 0.068)}}{0.01058} \times \frac{1}{0.1\text{ml}} \times \frac{1\text{ mg}}{1000\mu\text{g}}$$

BSA stock solution = 1000μg/ml

13. CARBOXYMETHYL CELLULASE ACTIVITY (CMCase)

Reagents

1% (w/v) solution of sodium carboxymethyl cellulose (medium viscosity) in 50 mM sodium citrate buffer, pH 4.8. Dinitrosalicylic (DNS) acid reagent, Rochelle salt (40%), 50 mM sodium citrate buffer (pH 4.8).

Procedure

The reaction mixture was composed of 1.8 ml substrate solution and 0.2 ml of enzyme sample. The solution was mixed well and incubated at 40°C for 30 min in a water bath with moderate shaking. The reducing sugars released was analyzed according to the DNS method (Miller, 1959).

Calculation of unit of activity

A glucose standard graph was used and the reducing sugar expressed as glucose equivalent. Correction was made for absorbance due to the background color in the enzyme blank. The CMCase activity (Dong *et al.*, 1992) was calculated with the following formula and translated to an enzyme activity expressed as 1μmol glucose released/min/g of substrate.

$$\text{CMCase (U/ml)} = \frac{\text{(Final absorbance - 0.18927)}}{1.05083} \times \frac{\text{dilution factor}}{0.2\text{ml}} \times \frac{1}{30\text{min}} \times \frac{1000\,\mu\text{g}}{1\text{mg}} \times \frac{1\mu\text{mole}}{180.16\mu\text{g}}$$

Glucose stock solution = 1mg/ml Glucose MW = 180.16

14. FILTER PAPER HYDROLYSIS ACTIVITY (FPase)

Reagents

50 mM sodium citrate buffer (pH 4.8), Dinitrosalicylic (DNS) acid reagent, Rochelle salt (40%) and Whatman No. 1 filter paper strips of 1.0 cm x 3.0 cm (≈ 25 mg).

Procedure

Approximately 0.2ml of supernatant and 1.8 ml of sodium citrate buffer were added into test tubes containing 25 mg Whatman No. 1 filter paper strips (rolled). The mixture was incubated at 40°C for 1 h in a shaking waterbath (Elshafei *et al.*, 1990). The reducing sugars released was analyzed according to the DNS method (Miller, 1959).

Calculation of unit of activity

A glucose standard graph was used and the reducing sugar expressed as glucose equivalent. The absorbance values obtained (less substrate and enzyme blanks) were translated to an enzyme activity expressed as 1μmol glucose released/min/g of substrate.

$$\text{FPase (U/ml)} = \frac{\text{Final absorbance} - 0.18927}{1.05083} \times \frac{\text{dilution factor}}{0.2\text{ml}} \times \frac{1}{60\text{min}} \times \frac{1000\,\mu\text{g}}{1\text{mg}} \times \frac{1\mu\text{mole}}{180.16\mu\text{g}}$$

Glucose stock solution = 1mg/ml Glucose MW = 180.16

15. β-D-GLUCOSIDASE ACTIVITY

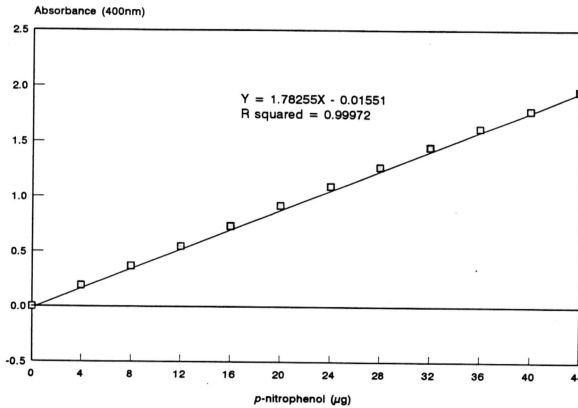
Reagents

0.5 mM *p*-nitrophenyl-β-D-glucopyranoside in 50 mM sodium citrate buffer, pH 4.8 and 1M sodium carbonate solution.

Procedure

The assay mixture consisted of 1.0 ml substrate and 0.1 ml of enzyme solution. All the test tubes were covered with aluminum foil as the liberated *p*-nitrophenol is light sensitive. The contents of the test tubes were mixed, and then incubated at 40°C for 30 min in a water bath with moderate shaking. The incubation was terminated by adding 2.0 ml of 1 M Na_2CO_3 and the liberated *p*-nitrophenol was measured at λ = 400 nm. The usual enzyme and reagent blanks were included. The absorbance values obtained (less the enzyme and substrate blanks) were translated to 1μmol nitrophenol released/min/g substrate using a standard graph relating μg of *p*-nitrophenol to absorbance (Dong *et al.*, 1992).

p-Nitrophenol Calibration Plot



Calculation of Unit of Activity

The unit of activity was the amount of enzyme required to release 1 μmol *p*-nitrophenol under the assay conditions and calculated as follows:

$$\beta\text{-glucosidase (U/ml)} = \frac{(\text{Final abs.} + 0.0155)}{1.7826} \times \frac{\text{diltn. factor}}{0.1\text{ml}} \times 0.04 \times \frac{1}{30\text{min}} \times \frac{1000\,\mu\text{g}}{1\text{mg}} \times \frac{1\mu\text{mole}}{139.1\mu\text{g}}$$

p-nitrophenol stock solution = 0.04 mg/ml *p*-nitrophenol MW = 139.1

16. XYLANASE ACTIVITY

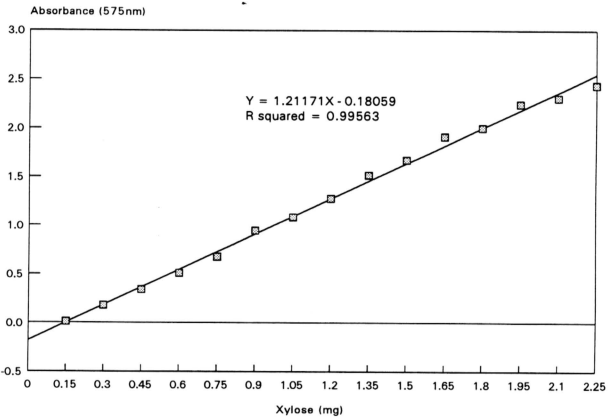
Reagents

1% (w/v) suspension of xylan in 50 mM sodium citrate buffer (pH 4.8). To prepare the substrate, heat 1%(w/w) suspension of xylan in the buffer to boiling point on a heating magnetic stirrer. Cool the suspension with continuous stirring overnight. Dinitrosalicylic (DNS) acid reagent, Rochelle salt (40%), 50 mM sodium citrate buffer, pH 4.8.

Procedure

About 1.8 ml substrate solution was mixed with 0.2 ml of enzyme solution. The mixture was mixed and incubated for 1 h at 40°C in a water bath with moderate shaking. The reducing sugars released in the reaction was determined using the DNS method. The standard used was pure xylose.

Xylose Calibration Plot



Calculation of Unit of Activity

The unit of enzyme activity was µmol reducing sugar released per minute. Correction was made for absorbance due to background color in the enzyme blank. By using the standard line for xylose, the corrected absorbance was converted to enzyme activity units. Finally, the activity in the original sample was calculated by multiplying activity units by the dilution factor using the following formula:

$$\text{Xylanase (U/ml)} = \frac{(\text{Final absorbance} - 0.18059)}{1.21171} \times \frac{\text{dilution factor}}{0.2\text{ml}} \times \frac{1}{60\text{min}} \times \frac{1000\,\mu\text{g}}{1\text{mg}} \times \frac{1\mu\text{mole}}{150.13\mu\text{g}}$$

Xylose stock solution = 1.5 mg/ml Xylose MW = 150.13

17. LACCASE ACTIVITY

Reagents

0.1 mM syringaldazine (4-Hydroxy-3,5-dimethoxybenzaldehyde azine) in 50% ethanol. The substrate was dissolved in 50% ethanol after 13 h of stirring. 50 mM sodium citrate buffer, pH 4.8.

Procedure

0.1 ml enzyme solution was mixed with 3.0 ml buffer at room temperature. Then 0.1 ml portion of 0.1 mM syringaldazine was added and mixed with a vortex mixer. The initial rate of color change was measured on a spectrophotometer at wavelength λ = 525 nm.

Calculation of Unit of Activity

One unit was defined as the enzyme producing one unit of absorbance change/min./g substrate. Laccase activity in the culture filtrate was calculated as follows:

$$\text{Laccase activity (U/ml)} = \text{Final absorbance} \times \frac{\text{dilution factor}}{0.1\text{ml}} \times 1\text{ min}$$

18. α-AMYLASE AND GLUCOAMYLASE ACTIVITY

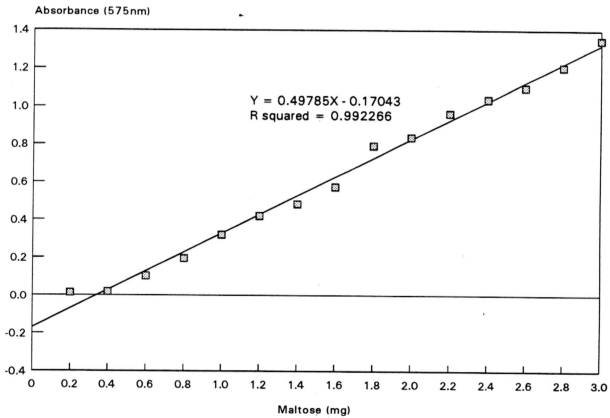
Reagents

Substrate for α-amylase was raw sago starch and for glucoamylase was cooked starch each at ($\leq 300\ \mu$) 100mg/ml suspended in distilled water. 50 mM sodium citrate buffer, pH 4.8, 0.12 N NaOH, Dinitrosalicylic (DNS) acid reagent, Rochelle salt (40%).

Procedure for α-amylase assay

The assay mixture contained 1.0 ml of enzyme filtrate, 1.0 ml of 50 mM sodium citrate buffer, pH 4.8 and 1.0 ml substrate. Addition of the raw sago starch is done while stirring on a magnetic stirrer. After incubation at 35°C for 1 h in a water bath with moderate shaking, the reaction was stopped with 1.0 ml of 0.12 N NaOH and then centrifuged at 2500 rpm for 10 min to sediment the excess starch. From the mixture, 2.0 ml of supernatant was pipetted out and 3.0 ml of DNS solution was added to it. The amount of reducing sugars was determined using the DNS method and a standard of pure maltose (SIGMA) was used.

Maltose Calibration Plot



The substrate blank in triplicates were included in the enzyme assay, which consisted of 1.0 ml of enzyme filtrate incubated with 2.0 ml of buffer without the raw starch substrate and assayed similar to the above. This was to determine the amount of sugar present in the culture filtrate as it had not been purified before being assayed for enzymes.

The enzyme blank was also set up consisting of 2.0 ml of buffer and 1.0 ml of raw starch substrate, to determine the sugar hydrolyzed when starch was shaken vigorously in acidic condition. The same was carried out for glucoamylase assay using cooked sago starch without any centrifugation.

Calculation of Unit of Activity

The unit of enzyme activity was µmol reducing sugar released per minute. Correction was made for absorbance due to background color in the enzyme blank. By using the standard line of maltose and glucose, for amylase and glucoamylase, respectively the corrected absorbance was converted to enzyme activity units. Finally, the activity in the original sample was calculated with the following formula:

$$\alpha\text{-amylase (U/ml)} = \frac{(\text{Final absorbance} - 0.17043)}{0.497857} \times \frac{\text{dilution factor}}{1.0\ \text{ml}} \times \frac{1}{60\text{min}} \times \frac{1000\ \mu\text{g}}{1.0\text{mg}} \times \frac{1\mu\text{mole}}{360.3\mu\text{g}}$$

Maltose stock solution = 1mg/ml Maltose MW = 360.3

$$\text{Glucoamylase (U/ml)} = \frac{(\text{Final abs.} - 0.18927)}{1.05083} \times \frac{\text{dilution factor}}{0.2\text{ml}} \times \frac{1}{30\text{min}} \times \frac{1000\ \mu\text{g}}{1\text{mg}} \times \frac{1\mu\text{mole}}{180.16\mu\text{g}}$$

Glucose stock solution = 1mg/ml Glucose MW = 180.16

APPENDIX B : BUFFERS AND MEDIA

1. BUFFERS

a. Sodium Citrate Buffer (0.05 M , pH 4.8)

To prepare 1 L of 1 M solution of sodium citrate, dissolve 210.0 g of citric acid monohydrate in 750 ml of distilled water. Adjust the pH to 4.3 with sodium hydroxide pellets (approximately 50-60 g). Dilute to 1 L and adjust the pH to 4.5 with sodium hydroxide if necessary. This is stock solution of 1 M sodium citrate buffer, pH 4.5. Dilute twenty times to get 0.05 M sodium citrate buffer solution with a final pH of 4.8.

b. Phosphate Buffer (0.1 M, pH 6.0)

Prepare stock solutions

(1) 0.2 M solution of monobasic sodium phosphate ($NaH_2PO_4.H_2O$). Dissolve 27.8 g in 1 L of distilled water.

(2) 0.2 M solution of dibasic sodium phosphate. Dissolve 53.65g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1000 ml of distilled water.

Mix 87.7 ml of solution (1) with 12.3 ml of solution (2) and dilute to a total volume of 200 ml with distilled water. This is a stock solution of 0.1 M phosphate buffer, pH 6.0.

c. Phosphate Buffer (0.01 M, pH 6.0)

Dilute 100 ml of 0.1 M phosphate buffer, pH 6.0 to 1000 ml with distilled water.

d. Citrate-phosphate Buffer (0.1 M)

Prepare stock solutions

A: 0.1 M solution of citric acid (19.21 g in 1 L)

B : 0.2 M solution of dibasic sodium phosphate (53.65g of $Na_2HPO_4.7H_2O$ or 71.7 g of $Na_2HPO_4.12H_2O$ in 1 L)

Mix x of A + y of B and dilute to 100 ml.

x (ml)	y (ml)	pH
39.8	10.2	3.0
30.7	19.3	4.0
24.3	25.7	5.0
17.9	32.1	6.0
6.5	43.5	7.0

e. Glycine-NaOH buffer (50 mM)

Prepare stock solutions

A : 0.2 M solution of glycine (15.01 g in 1 L)

B : 0.2 M NaOH

Mix 50 ml of A + x ml of B, and dilute to 200 ml.

x (ml)	pH
8.8	9.0
32.0	10.0

2. MEDIA

a. Potato Dextrose Agar (PDA)

Suspend 39 g of PDA and 2 g of Agar No. 3 (Oxoid) in 1 L distilled water and heat to boil to dissolve completely. Autoclave for 20 min at 15 psi, 115°C. Cool medium to 45-50°C and dispense into petri dishes. Final pH should be 5.6 ± 0.2 at 25°C.

APPENDIX C: DATA AND STATISTICAL TABLES

The results of the statistical analysis carried out in this study are tabulated as follows:

1. ANOVA of the effect of Inoculum Age and Density on Soluble Protein content of the culture filtrate

Source of variation	df	Sum of squares	F
Main effects			
Age	1	54.2293	24.907***
Density	2	5.9469	1.366 ns
Interactions			
Age x Density	2	0.0077	0.002 ns
Residual	97	211.1925	
Total	102	271.3764	

Residual Mean Square Error = 2.1772
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

2. ANOVA of the effect of Sampling Day and Inoculum Density on Soluble Protein content of the culture filtrate with a 2-week old inoculum

Source of variation	df	Sum of squares	F
Main effects			
Day	5	6.9712	54.689***
Density	2	2.0799	40.791***
Interactions			
Day x Density	10	0.0632	2.48 *
Residual	22	0.5608	
Total	39	10.2442	

Residual Mean Square Error = 0.0254
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

3. ANOVA of the effect of Sampling Day and Inoculum Density on Soluble Protein content of the culture filtrate with a 4-week old inoculum

Source of variation	df	Sum of squares	F
Main effects			
Day	6	198.9569	528.599***
Density	2	3.46579	27.624***
Interactions			
Day x Density	12	1.8403	2.445*
Residual	42	2.6346	
Total	62	206.8978	

Residual Mean Square Error = 0.0627
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

4. Grouping of ranked means of Soluble Protein content using a 4-week old inoculum with Student Newman-Keuls' test

Sampling Days	0	2	4	6	12	10	8
Count	9	9	9	9	9	9	9
LS Mean	0.0	0.0	1.329	2.583	4.152	4.153	4.212*

*Means underlined by same line are not significantly different at the 5% level of significance

5. ANOVA of the effect of Inoculum Age and Density on Reducing Sugars content of the culture filtrate

Source of variation	df	Sum of squares	F
Main effects			
Age	1	59.5239	0.173 ns
Density	2	1292.5379	1.882 ns
Interactions			
Age x Density	2	145.8706	0.212 ns
Residual	81	27809.788	
Total	86	29307.72	

Residual Mean Square Error = 343.3307
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

6. ANOVA of the effect of Inoculum Age and Density on Xylanase activity of the culture filtrate

Source of variation	df	Sum of squares	F
Main effects			
Age	1	224.2276	23.174 ***
Density	2	38.6823	1.999 ns
Interactions			
Age x Density	2	21.5045	1.111 ns
Residual	105	1015.9459	
Total	110	1300.3603	

Residual Mean Square Error = 9.6757
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

7. ANOVA of the effect of Sampling Day and Density on Xylanase activity of the culture filtrate with a 2-week old inoculum

Source of variation	df	Sum of squares	F
Main effects			
Day	5	130.3495	20.339 ***
Density	2	2.2179	0.865 ns
Interactions			
Day x Density	10	50.6499	3.952 **
Residual	29	37.1708	
Total	46	220.3883	

Residual Mean Square Error = 1.2817
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

8. Grouping of ranked means of Xylanase activity of culture filtrate using a 2-week old inoculum with Student Newman-Keuls' test

Sampling Days	0	12	6	10	8	4
Count	9	6	9	6	9	8
LS Mean	0.6909	4.5918	4.8163	4.8965	4.9864	5.0976*

*Means underlined by same line are not significantly different at the 5% level of significance

9. ANOVA of the effect of Inoculum Age and Density on Laccase activity of the culture filtrate

Source of variation	df	Sum of squares	F
Main effects			
Age	1	2256.9748	71.414 ***
Density	2	115.9302	1.834 ns
Interactions			
Age x Density	2	11.7775	0.186 ns
Residual	102	3223.6350	
Total	107	220.3883	

Residual Mean Square Error = 31.6043
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

10. Grouping of ranked means of Laccase activity of culture filtrate using a 2-week old inoculum with Student Newman-Keuls' test

Sampling Days	0	4	6	10	12	8
Count	9	6	6	6	5	4
LS Mean	0.0167	1.8500	3.3750	3.4750	3.5667	3.8250*

*Means underlined by same line are not significantly different at the 5% level of significance

11. ANOVA of the effect of Sampling Day and Density on Laccase activity of the culture filtrate with a 2-week old inoculum

Source of variation	df	Sum of squares	F
Main effects			
Day	5	80.2962	133.441 ***
Density	2	32.5998	135.441 ***
Interactions			
Day x Density	10	13.8983	11.549 ***
Residual	18	2.1663	
Total	35	128.9606	

Residual Mean Square Error = 0.1203
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

12. ANOVA of the effect of Sampling Day and Inoculum Density on Laccase activity of the culture filtrate with a 4-week old inoculum

Source of variation	df	Sum of squares	F
Main effects			
Day	6	2709.5254	489.731 ***
Density	2	102.6968	55.686 ***
Interactions			
Day x Density	12	331.8469	29.990 ***
Residual	42	38.7287	
Total	62	3182.7979	

Residual Mean Square Error = 0.9221
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

13. Grouping of ranked means of Laccase activity of culture filtrate using a 4-week old inoculum with Student Newman-Keuls' test

Sampling Days	0	2	4	12	10	6	8
Count	9	9	9	9	9	9	9
LS Mean	0.1778	1.9703	12.073	15.038	15.512	16.654	16.728*

*Means underlined by same line are not significantly different at the 5% level of significance

14. ANOVA of the effect of Inducers on Laccase activity of the culture filtrate on day 6 of fermentation

Source of variation	df	Sum of squares	F
Treatments	2	394.2272	43.476 ***
Residual	6	27.2033	
Total	8	421.4306	

Residual Mean Square Error = 4.5339
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

15. ANOVA of the effect of Inducers on Laccase activity of the culture filtrate on day 8 of fermentation

Source of variation	df	Sum of squares	F
Treatments	2	697.145	99.973 ***
Residual	6	20.920	
Total	8	718.065	

Residual Mean Square Error = 3.4867
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

16. ANOVA of the effect of Inducers on Laccase activity of the culture filtrate on day 6 of fermentation

Source of variation/Contrast	df	Sum of squares/ Contrast sum of squares	F
Treatments	2	394.23	43.476 ***
Vanillin vs Ferulic acid	1	1.76	0.38 ns
Inducers vs Control	1	392.47	86.63 ***
Residual	6	27.20	
Total	8	421.43	

Residual Mean Square Error = 4.53
*, **, *** Significant at 0.05, 0.01, 0.001 probability levels, respectively
ns = Not significant

APPENDIX D : STUDIES ON RECYCLING OF SPENT SAGO 'HAMPAS' AFTER SOLID SUBSTRATE FERMENTATION FOR MUSHROOM CULTIVATION

From the earlier trials, 'hampas', the fibrous pith residue after sago starch extraction was found to show potential for lignocellulolytic enzyme production with *Pleurotus sajor-caju* through solid substrate fermentation (SSF). Apart from that, the initiation of fruit body noted in the SSF cultures led to the belief that 'hampas' may also serve as a substrate for mushroom cultivation. Several workers have reported that cultivation of edible fungi is the most economical means of utilizing lignocellulosic wastes (Bisaria *et al.*, 1987; Zadrazil and Reiniger, 1988). The oyster mushroom, *P. sajor-caju*, a highly nutritious protein source is found to be cultivated with minimal input employing simple methods.

Successful cultivation of mushroom on the remaining solid residue after enzyme extraction by SSF (Rajarathnam *et al.*, 1992) and vice versa, i.e. successful cultivation of mushroom on agro-industrial waste and utilization of the residues later for enzyme production (Azizi *et al.*, 1990) has been reported. It has been reported that by adopting successive cultivation of mushrooms after enzyme extraction of a given substrate, bioconversion of the spent substrate could be accomplished. Some workers have even reported two mushroom crops in succession (Royse, 1992; Quimio *et al.*, 1990). The left over substrate after *Pleurotus* cultivation has been known to increase rumen digestibility (Zadrazil, 1977). The effect of supplementing wheat and rice straw with organic and inorganic nutrients on yield enhancement, decomposition of substrates,

protein and nitrogen content of the fruiting bodies of *P. sajor-caju* has been reported by Zadrazil (1980) and Zakia Bano *et al.* (1993).

Several types of containers are used for the cultivation of *Pleurotus* spp. Trays, polyethylene bags, jars, baskets, beds and blocks have been used in different parts of the world for raising *Pleurotus* spp. (Quimio *et al.*, 1990). Essentially, the success of mushroom cultivation would depend on a practical yet efficient method, using the resources available *in situ*. In the present study, attempts were made to evaluate the concept of reusing the spent sago 'hampas' after enzyme extraction from SSF and spent substrate from one cycle of mushroom growth, together with shredded paper as a co-substrate/filler using two different container systems. Palm Oil Sludge Solids (POSS) and calcium carbonate were added as supplements for mushroom growth.

Two systems of cultivation (tray and polyethylene bags) were used with modified Graham and Clyde method (1985). The substrates used were spent sago 'hampas' and shredded paper, because both were available in abundant supply. Spent sago 'hampas' was obtained as solid residue after SSF, and shredded paper, consisting of white waste paper, was obtained from office waste. An organic nitrogenous supplement, Palm Oil Sludge Solids (POSS) in granular form was added to the culture. POSS is available locally in the commercial form as an organic fertilizer (Trade name 'Supergro').

For the tray culture, about 700g moist spent sago 'hampas' from previous SSF with an initial moisture content of 75% (w/w) was used. Dry shredded paper (1 kg) was soaked in water for 3 days. The paper was then torn to smaller pieces and the excess

water was squeezed out. The torn pieces of paper were mixed with the moist spent sago 'hampas' and were supplemented with about 100 g of POSS and 20 g of $CaCO_3$. It was not necessary to sterilize the substrate and supplements. About 25% (w/w) of inoculum was added to the substrate. The *P. sajor-caju* inoculum was prepared using wheat grains as outlined by Quimio *et al.* (1990). The substrates, supplements and inoculum were mixed thoroughly and compacted into a plastic container measuring 36cm x 25cm x 10cm covered with a black plastic sheet. The tray was kept in a fairly cool and shaded area. The temperature was found to be in the range of 23 ± 2°C during the spawn run and fructification periods. Distilled water was sprayed daily onto the contents of the tray to keep it moist.

The polyethylene bag culture method is preferred by mushroom farmers and many research workers. The polyethylene bag culture method was chosen to compare the ability of spent sago 'hampas' after SSF and spent substrate after one cycle of mushroom cultivation to re-support the growth of *P. sajor-caju* when combined with shredded paper. The two treatments were: Polybag 1 - spent sago 'hampas' from SSF and shredded paper (1:1 w/w), and Polybag 2 - spent substrate reused after the last flush of *P. sajor-caju* from tray culture, containing significant amounts of the original nutrients. The spent substrate was removed, broken up and mixed with shredded paper (1:1 w/w). In Polybag 1, about 800 g of moist 'hampas' was used. Dry shredded paper was first soaked in water for 3 days and then torn to smaller pieces. The excess water was squeezed out and the approximate weight of the moist paper was 830g. About 100g of POSS and 10g of $CaCO_3$ were mixed thoroughly with the substrate as

supplements. The mixed substrate was left overnight in a covered plastic pail and then was compacted into autoclavable polyethylene bags (15 cm x 20 cm lay flat dimension) until half full, making sure that each bag had about the same amount of substrate. The polyethylene bags containing the mixed substrate were sterilized at 10 psi, 115°C for 20 min. Bags with the spent sago 'hampas' and shredded paper together with their supplements were closed with cotton plugs following spawning at a level of 2% (w/w) based on the dry substrate and were then incubated at room temperature (23 ± 2°C). In Polybag 2, about 830 g of moist spent substrate from tray culture was used. The spent substrate was first broken up and mixed with about 800g of moist shredded paper prepared as in treatment Polybag 1. The POSS supplementation, sterilization, inoculation and incubation temperature were similar to those in Polybag 1.

The mycelial colonization period lasted 14 days for the tray culture, after which the black plastic sheet cover was removed to expose the substrate surface to light and aeration. Only three flushes of mushrooms were collected from the tray culture. In the polyethylene bag culture, mycelial colonization lasted 35 days and five flushes of mushrooms were harvested at intervals of 5 to 7 days. Regular watering was done with distilled water on all polyethylene bags that were opened after sporophore formation. All data given are the values of triplicate cultures and the mushroom yields in all the cultures were calculated with the biological efficiency (BE) relation (Chang *et al.*, 1981):

$$\text{Biological efficiency } \% = \frac{\text{Weight of fresh mushroom harvested}}{\text{Weight of dry substrate}} \times 100$$

Statistical significance was tested by one-way Analysis of Variance with the significance level of $P<0.05$. The Least Significant Difference test was conducted to detect any significant difference among the biological efficiencies of the treatments.

From the first experiment, it was observed that the *P. sajor-caju* used in the present study colonized the spent sago 'hampas' rapidly in the tray culture within two weeks. After completion of the mycelial colonization, the substrate surface in the tray was exposed to light by removing the black plastic covering. The ambient temperature of $23 \pm 2°C$ was sufficient to stimulate fruiting bodies. Young fruiting bodies appeared within 24 to 36 hr after initial exposure to light. Mature fruiting bodies were harvested within 2 to 3 days after substrate exposure to light (Plate 1). The total yield of mushrooms picked was 375 g with 62% biological efficiency as seen in Table 1. The BE obtained in the present study is within the wide range of efficiencies reported in the literature for *P. sajor-caju* cultivation, ranging from 10 to 100% on various substrates with different nutrient supplementation (Bisaria *et al.*, 1987; El-Kattan *et al.*, 1991; Mahmoud and El-Kattan, 1989).

Plate 1. ***Pleurotus sajor-caju*** **fruit bodies on sago 'hampas' and shredded paper in tray culture (17 days from spawning)**

Table 1. Cultivation of ***Pleurotus sajor-caju*** **on spent sago 'hampas'**

Type of container	Approximate substrate composition	Total substrate (g) (dry weight)	Inoculum rate (%)	Appearance of primordia (days)#	Cumulative yield (g) (fresh weight)	BE (%)
Plastic tray	Spent sago 'hampas', paper, POSS, $CaCO_3$	605	25	14	375	61.9 a
Polybag 1	-"-	503	2	35	240	47.7 b
Polybag 2	Spent substrate,* paper, POSS, $CaCO_3$	490	2	35	189	38.5 c

Biological efficiency (BE) values with different alphabet within the same column indicated significant difference ($P<0.05$).

*Spent substrate taken after one cycle of mushroom harvest using tray culture

#Days following spawning

Initial moisture level and pH of substrate = 75% (w/w) and 7.0

Pleurotus sajor-caju produced significantly ($P<0.05$) higher yields within a shorter period of spawn run when mycelia grew in plastic trays. This could be mainly attributed to a combination of a higher spawning rate and greater amount of substrate which led to rapid colonization. However, Garcha *et al.* (1985) studying the efficacy of containers like trays, baskets and polyethylene bags for the production of *Pleurotus florida* and *Pleurotus sajor-caju*, noted that trays did not support much growth of the mycelium and no fruiting body was harvested. They attributed the poor response of tray system to a lesser degree of compactness of the substrate, thereby, enhancing the evaporation rate and carbon dioxide loss; two most important factors needed in the early stages of mycelial colonization. Stimulation of mycelial growth by high internal CO_2 levels in *Pleurotus* culture has been reported by Zadrazil (1975, 1978) which acted as a shield for *Pleurotus* against other undesirable micro-organisms.

Another factor which may affect the yield of *Pleurotus* is the moisture content of the substrate. The substrate in the tray culture required daily watering so that drying up of the substrate is prevented. Somewhat similar findings were reported by El Kattan *et al.* (1991) studying oyster mushroom cultivation in perforated and unperforated polyethylene bags.

From the second experiment, the polyethylene bag cultures took a longer period for spawn run of more than 30 days. Visual assessment done after two weeks of spawn run of the polyethylene bag cultures revealed that mycelial growth in treatment Polybag 2 was more rapid than in Polybag 1. This could possibly be attributed to the presence of residual nutrients left over from the previous mushroom cycle assisting in the rapid

colonization. However, significant increase in the mushroom yield, that could be attributed to the rapid colonization, was not observed in Polybag 2. Similar findings were reported by Mueller *et al.* (1984).

In the present study, BE obtained with polyethylene bag culture was about 35 to 50%, which was much lower than the BE obtained in tray culture. In general, the first flush of fruit bodies gave much more yields than the second, third, fourth or fifth flushes. The yields obtained in the first and second flushes were about 40 to 50% of the total yield (data not shown). Similar observation was found in studies of *P. sajor-caju* on different agro-wastes by Bisaria *et al.* (1987). The accumulation of toxic substances in the substrate together with loss of culture vigor and depletion of nutrients could have also caused the decline in mushroom yields of subsequent flushes. However, Chang *et al* (1981) observed uniform and consistent distribution of fruiting bodies in all four flushes of *P. sajor-caju* on cotton wastes.

In studies to recycle *Pleurotus* waste, Sharma and Jandaik (1985) found larger *P. sajor-caju* sporophore formation when the substrate was supplemented with either starch, peptone or 5% wheat bran. The 66% starch content in 'hampas' (Shim, 1992) could have also assisted fruit body formation in the present study.

In general, both the culture systems had advantages as well as disadvantages for fruit body formation. Table 2 summarizes some of the differences between tray and polyethylene culture for mushroom cultivation.

Table 2. Comparison of characteristics of tray and polyethylene culture for mushroom cultivation

Characteristics	Plastic tray	Polyethylene bag
Sterilization	Not required	Necessary
Inoculum rate	High	Low
Spawn run	Rapid and shorter	Slow and longer
Watering of substrate	Regular	Intermittent
Reuse of containers	Yes	No
Handling ease	Cumbersome	Convenient
Large scale cultivation	Not suitable	Practical
Biological efficiency*	High	Low

*Based on this study only.

In the present study, it is evident that the chances of obtaining higher yields in a shorter period using the simple tray culture are high, if the conditions were made favorable for *Pleurotus* growth. This study showed that the range of biological efficiencies obtained for the supplemented sago 'hampas' substrate were proximately in agreement as reported by other workers (Bisaria *et al.*, 1987; El-Kattan *et al.*, 1991). The differences in efficiencies found in this study can be attributed in part to the different treatment methods used and to the nutrients present in the substrate. It is also obvious that due to the very high saprophytic colonizing ability (Madan *et al.*, 1987; Zadrazil, 1980), this fungi could colonize *Pleurotus* waste and thus resulting in good yields of fruit bodies (Sharma and Jandaik, 1985).

In conclusion, these simple laboratory trials suggest that spent sago 'hampas' from SSF and the spent substrate from one cycle of mushroom cultivation can be reused as a substrate together with shredded paper for cultivation of *P. sajor-caju* with appreciable biological efficiency.

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APPENDIX E : PAPERS PRESENTED AND COMMUNICATED FOR PUBLICATION

1. Kumaran, S., Sastry, C. A. and Vikineswary, S. (1995) **Lignocellulolytic activities of *Pleurotus sajor-caju* grown on sago *hampas*.** Presented at the "International Conference on Biotechnology Research and Applications for Sustainable Development" (7-10 Aug., 1995) Bangkok, Thailand. (Abstract only).

2. Kumaran, S., Sastry, C. A. and Vikineswary, S. (1997) **Laccase, cellulase and xylanase activities during growth of *Pleurotus sajor-caju* on sago *hampas*.** *World Journal of Microbiology and Biotechnology* 13: 43 - 49.

3. Kumaran, S., Sastry, C. A. and Vikineswary, S. (1996) **Reusing spent sago 'hampas' from solid substrate fermentation for mushroom cultivation.** *Malaysian Applied Biology* (in press).