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APPENDICES

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APPENDIX A : ANALYTICAL TECHNIQUES

1. DETERMINATION OF MOISTURE CONTENT

Porcelain crucibles were oven dried at 105° C for 24 h and weighed (W 0) after being cooled in a dessicator. Approximately 5.0 g of sample was weighed into crucibles and weighed again (W 1). The crucibles with the samples were oven dried at 105° C for 48 h, cooled in a dessicator before being weighed again (W2). The moisture content of the sample was computed with the following formula and expressed as the percentage per gram sample.

$$\% \text{ Moisture} = \frac{(W1 - W2)}{(W1 - W0)} \times 100$$

2. DETERMINATION OF TOTAL NITROGEN

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 colour of light pink at the end of the titration. The percentage of total nitrogen was computed using the formula as shown :

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

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

3.DETERMINATION OF TOTAL CARBON

Reagents

i. Dichromate mixture (0.0675 M)

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 1 L with distilled water and stored in a dark bottle.

ii. Ferrous Ammonium Sulfate (0.4 M)

Approximately 156.86 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 5.0g barium chloride and 0.3 g barium diphenylamine sulphonate were dissolved in 100 ml of distilled water.

Procedure

Total carbon was determined through 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 0.4M ferrous ammonium sulfate. The total carbon content was calculated with the formula and expressed as the percentage of 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

4. LACCASE ACTIVITY

Reagents

0.1 mM syringaldazine (4-Hydroxy-3,5-dimethoxybenzaldehyde azine) in 50% ethanol. The substrate was dissolved in 50% ethanol after 13hr of stirring. 50mM 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 = 525nm.

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 :

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

5. XYLANASE ACTIVITY

Reagents

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

Procedure

About 1.8 ml substrate solution was mixed with 0.2ml 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.

Calculation of Unit of Activity

The unit of enzyme activity was µmole 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:

$$\text{Xylanase (U/ml)} = \frac{\text{(Final absorbance)}}{0.5575} \times \frac{\text{dilution factor}}{0.2\text{ml}} \times \frac{1}{60\text{min}} \times \frac{1}{1\text{mg}} \times \frac{1000\mu\text{g}}{150.13\mu\text{g}} \times \mu\text{mole}$$

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

6. 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 amounts 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 400g 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 (2.0ml) was added to 3.0ml DNS reagent. The solution was mixed and incubated in a boiling water bath for 15 min. Upon removal from the water bath approximately 1.0ml 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 = 575nm. The reducing sugar concentrations of the test solution was calculated by extrapolation from a linear glucose standard graph.

Reducing Sugars Assay of test solution

Test solution (2.0ml) 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.

7. CARBOXYMETHYL CELLULASE ACTIVITY (CMCase)

Reagents

1% (w/v) solution of sodium carboxymethyl cellulose (medium viscosity) in 50mM 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 analysed 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 1µmole glucose released/min/g of substrate.

$$\text{CMCase (U/ml)} = \frac{\text{Final absorbance}}{0.2894} \times \frac{\text{dilution factor}}{0.2\ \text{ml}} \times \frac{1}{30\text{min}} \times \frac{1000\mu\text{g}}{180.16\mu\text{g}} \times \frac{1\mu\text{mole}}{1\text{mg}}$$

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

8. DETERMINATION OF SOLUBLE PROTEIN

Reagents

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50ml 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 100μg 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 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 grapgh with the following formula :

$$\text{Soluble protein (mg/ml)} = \frac{\text{(Final absorbance)}}{0.0112} \times \frac{1}{0.1 \text{ ml}} \times \frac{1 \text{ mg}}{1000 \ \mu g}$$

BSA stock solution = 1000 μg/

APPENDIX B : BUFFER AND MEDIA

1. BUFFER

Sodium citrate buffer (0.05M, pH 4.8)

To prepare 1 L of 1 M solution of sodium citrate, dissolve 210.0 g of citric acid monohydrate in 750ml of distilled water. Adjust the pH to 4.3 with sodium hydroxide pellets (approximately 50-60g). Dilute to 1L 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.

2. MEDIA

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

1. Enzyme Profile during SSF for 21 days for various agro-residues

Table 1: Xylanase activity (U/g substrate)

Day	sawdust	OPFPt	Hampas'
0	5.21	3.21	1.15
4	5.41	3.41	3.79
7	5.39	3.59	5.86
9	5.45	6.05	6.22
11	6.07	9.15	7.64
13	6.92	9.11	6.44
15	7.35	9.12	6.38
17	8.56	8.82	6.29
19	6.56	6.28	6.10
21	6.22	6.12	5.74

Table 2: Laccase activity (U/g substrate)

Day	sawdust	OPFPt	Hampas'
0	0.79	0.84	0.24
4	4.58	1.34	0.82
7	3.72	5.38	3.46
9	5.06	6.62	5.22
11	5.68	7.52	7.60
13	5.66	7.50	6.42
15	5.56	7.38	4.12
17	5.30	5.31	3.64
19	5.25	5.36	3.24
21	5.20	5.40	3.12

Table 3: CMCase activity (U/g substrate)

Day	sawdust	OPFPt	Hampas'
0	3.25	2.83	0.86
4	3.27	2.67	2.82
7	5.39	5.03	3.36
9	5.51	6.59	6.24
11	6.46	6.27	4.96
13	6.42	5.87	5.37
15	6.36	5.42	3.98
17	6.20	5.11	3.42
19	5.82	5.04	2.58
21	5.22	5.02	2.51

Table 4: Soluble protein content (mg/g substrate)

Day	sawdust	OPFPt	hampas'
0	-0.44	0.46	-0.04
4	0.04	0.91	0.06
7	0.45	2.16	0.10
9	0.42	2.24	0.64
11	0.46	4.30	0.89
13	0.49	4.28	1.02
15	0.74	3.96	0.93
17	0.80	3.95	0.92
19	0.78	3.76	0.89
21	0.77	3.25	0.90

2. Laccase and Xylanase Profile during Optimization of temperature, tsolvent medium and pH

Table 5: Laccase activity (U/g substrate)

Day	4-B	4-T	R-B	R-T
0	0.92	2.05	2.20	2.00
2	1.36	5.40	8.20	7.50
4	1.85	7.10	11.10	10.55
6	4.84	15.80	25.20	23.65
8	6.20	19.60	29.50	28.44
10	7.46	24.80	31.20	30.60

Table 6: Xylanase activity (U/g substrate)

Day	4-B	4-T	R-B	R-T
0	3.24	3.20	3.05	3.10
2	3.55	3.35	3.87	3.87
4	3.62	3.55	3.60	3.62
6	4.50	4.50	4.66	4.74
8	5.24	5.50	5.88	5.95
10	7.72	7.80	7.95	7.92

Note :

4-B : 4°C with sodium citrate buffer

4-T : 4°C with tap water

R-B : room temperature with sodium citrate buffer

R-B : room temperature tap water

Table 7: Laccase activity (U/g substrate)

Day	pH 4	pH 5	pH 6	PH 7	pH 8
0	3.85	4.05	1.98	1.85	2.05
2	10.5	11.26	5.46	5.6	7.66
4	21.5	24.65	9.64	9.5	10.64
6	30.5	32.8	23.4	23	24.05
8	38.9	40.5	25.5	25.42	28.45
10	45.6	46.5	29.54	29.64	30.58

Table 8: Xylanase activity

Day	pH 4	pH 5	pH 6	PH 7	pH 8
0	3.08	3.06	3.09	3.08	3.10
2	3.72	3.75	3.84	3.82	3.87
4	3.66	3.62	3.64	3.66	3.62
6	4.72	4.77	4.77	4.68	4.74
8	5.9	5.95	5.88	5.89	5.95
10	7.92	7.95	7.99	8.01	7.92

3. Laccase and xylanase activity during Optimization of inoculum age, inoculum density and nitrogen content of substrate

Table 9: Laccase activity (U/g substrate) (Optimization of inoculum age)

Day	2 wk	4 wk	6 wk
0	3.85	4.02	3.82
2	8.5	11.2	6.5
4	20.5	24.33	10.5
6	26.3	31.9	18.4
8	31.8	40.8	21.3
10	38.7	46.6	25.4

Table 10: Laccase activity (U/g substrate) (Optimization of inoculum density)

Days	10%	20%	30%
0	4	5.6	7.8
2	11.8	12.8	15.6
4	24.65	25.8	29.4
6	32.8	31.8	40.5
8	40.5	41.2	48.7
10	46.9	46.5	46.2

Table 11: Laccase activity (U/g substrate)(Optimization of nitrogen content)

Day	1.0 xurea	1.5 xurea	2.0 xurea	2.5 xurea
0	7.6	7.2	7.5	7.4
2	15.2	16.5	19.5	18.2
4	28.5	29.4	35.6	34.4
6	41.2	40.5	46.5	42
8	48.5	46.2	46	40.1
10	47.9	46	46.2	37.9

Table 12: Xylanase activity (U/g substrate) (Optimization of inoculum age)

Day	2 wk	4 wk	6 wk
0	3.04	3.06	3
2	3.22	3.94	3.02
4	3.34	4.12	3.12
6	3.86	4.84	3.55
8	4.02	6.15	3.72
10	4.45	8.42	3.86

Table 13: Xylanase activity (U/g substrate) (Optimization of inoculum density)

	10%	20%	30%
0	3.06	3.1	3.22
2	3.94	4.01	4.12
4	4.12	5.2	6.4
6	4.84	5.8	8.5
8	6.15	6.55	7.1
10	8.42	8.52	6.4

Table 14: Xylanase activity (U/g substrate)(Optimization of nitrogen content)

Day	1.0 xurea	1.5 xurea	2.0 xurea	2.5 xurea
0	3.24	3.2	3.18	3.2
2	4.15	4.6	6.12	6.08
4	6.2	7.5	8.22	7.9
6	8.14	8.5	9.12	7.5
8	7.22	8.45	8.5	6.8
10	6.2	8.1	7.8	5.5

Table 15:

ANOVA of effect of temperature/extraction medium on laccase activity

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Days	1604.8	5	320.97	15.88	2E-05 *
Temperature/medium	757.97	3	252.66	12.5	0.0002 *
Error	303.18	15	20.212		
Total	2666	23			

* P < 0.001

Table 16:

ANOVA of effect of pH on laccase activity

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Days	4569.3	5	913.86	96.075	3E-13 *
pH	671.12	4	167.78	17.639	2E-06 *
Error	190.24	20	9.512		
Total	5430.6	29			

* P< 0.001

Table 17:
ANOVA of effect of inoculum age on laccase activity

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Days	2486.2	5	497.25	28.234	1E-05
Age	449.1	2	224.55	12.75	0.0018 **
Error	176.12	10	17.612		
Total	3111.5	17			

** P< 0.01

Table 18:

ANOVA of effect of inoculum density on laccase activity

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Days	4588.2	5	917.64	441.05	2E-11
Density	132.68	2	66.338	31.884	5E-05 *
Error	20.806	10	2.0806		
Total	4741.7	17			

*P<0.01

Table 19:
ANOVA effect of nitrogen content on laccase activity

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Days	12423	5	2484.5	82.431	2E-10
Nitrogen	654.99	3	218.33	7.2436	0.0031 **
Error	452.11	15	30.141		
Total	13530	23			

** P < 0.01

Table 20:

ANOVA of effect of temperature/extraction medium on xylanase activity

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Days	61.992	5	12.398	486.4	5E-16
Temperature/medium	0.2512	3	0.0837	3.2843	0.0501 ns
Error	0.3823	15	0.0255		
Total	62.625	23			

ns : not significant
(P> 0.05)

Table 21:
ANOVA of effect of pH on xylanase activity

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Days	82.485	5	16.497	10877	1E-33
pH	0.0049	4	0.0012	0.8022	0.538 ns
Error	0.0303	20	0.0015		
Total	82.52	29			

ns : not significant
(P > 0.05)

Table 22:
ANOVA of effect of inoculum age on xylanase activity

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Days	13.219	5	2.6438	3.4648	0.0447
Age	10.11	2	5.0551	6.6248	0.0147 **
Error	7.6306	10	0.7631		

** 0.01 < P < 0.05

Table 23:
ANOVA of effect of inoculum density on xylanase activity

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Days	78.771	5	15.754	41.646	2E-06
Density	7.788	2	3.894	10.294	0.0037 *
Error	3.7829	10	0.3783		
Total	90.342	17			

* P< 0.01