

APPENDIX

I. Culture media for isolation, identification and growth characterization of *Myrothecium verrucaria*

(1.) Potato dextrose agar (PDA)

- Suspended 39 g of PDA in distilled water and diluted to make a 1,000 mL solution.
- Boiled agar suspension until dissolved completely and sterilized at 121-124 °C for 15 minutes.
- Allowed the agar suspension to warm in water bath at 56 °C before pouring 2 mL in a plate.
- Warmed the agar plate at 37 °C until the surface was dried.

(2.) Potato dextrose broth (PDB)

- Suspended 24 g of PDB in distilled water and diluted to make a 1,000 mL solution.
- Warmed PDB suspension gently until dissolved completely.
- Filled in a 125 mL Erlenmeyer flasks with 25 mL of PDB suspension.
- Sterilized at 121-124 °C for 15 minutes.

II. Reagents for measurement of total and conjugated bilirubin by the bilirubin oxidase method

(1.) 0.1 mol/L Tris-SDS buffer, pH 8.0 for total bilirubin determination

- Dissolved 1.44 g of Sodium dodecyl sulfate and 12.10 g Trizma base in 900 mL of distilled water and mixed until well dissolved.
- Adjusted pH to 8.0 with 1 N NaOH or 1 N HCl.
- Filled up with distilled water to 1,000 mL.

(2.) 0.1 mol/L Lactic acid buffer, pH 3.7 for conjugated bilirubin determination

(2.1) 0.1 Lactic acid

- Transfer 7.52 mL of 13.3 mol/L Lactic acid to a 1 liter volumetric flask.
- Filled up with distilled water to 1,000 mL.
- Stored at 4 °C.

(2.2) 0.1 mol/L Lactic acid-Sodium citrate buffer pH 3.7

- Dissolved 5.25 g of Citric acid and 1.15 g of Sodium hydroxide with 200 mL of 0.1 mol/L Lactic acid and mixed until well dissolved.
- Adjusted pH to 3.7 with 1 N NaOH or 1 N HCl.
- Filled up with 0.1 mol/L Lactic acid to 250 mL.
- Stored at 4 °C.

III. Buffer for elution of BOX from column chromatography (Tanaka and Murao, 1982)

(1.) 10 mmol/L Na₂CO₃

- Dissolved 1.06 g of Na₂CO₃ in distilled water and diluted to a 1,000 mL solution.
- Stored at 4 °C.

(2.) 10 mmol/L NaHCO₃

- Dissolved 0.84 g of NaHCO₃ in distilled water and diluted to a 1,000 mL solution.
- Stored at 4 °C.

(3.) 10 mmol/L Na₂CO₃-NaHCO₃ buffer pH 9.2

- Mixed 20 mL of 10 mmol/L Na₂CO₃ with 230 mL of 10 mmol/L NaHCO₃.
- Added distilled water to about 900 mL, mixed well.
- Adjusted pH to 9.2 with 1 N HCl or 1 N NaOH.
- Diluted to a 1,000 mL with distilled water.
- Stored at 4 °C.

IV. Reagents for purification of BOX by DEAE-Cellulose and DEAE-Sepharose anion exchange chromatography

- (1.) 0.5 mol/L HCl
 - Transfer 41.6 mL of 12.01 N HCl to a 1 liter volumetric flask.
 - Filled up with distilled water to 1,000 mL, mixed well.
 - Stored at room temperature.
- (2.) 0.5 mol/L NaOH
 - Dissolved 20.0 g of NaOH in distilled water and diluted to a 1,000 mL solution, mixed well.
 - Stored at room temperature.
- (3.) 0.2 mol/L Sodium carbonate (Na_2CO_3)
 - Dissolved 21.2 g of Na_2CO_3 in distilled water and diluted to a 1,000 mL solution, mixed well .
 - Stored at 4°C.
- (4.) 0.2 mol/L Sodium Bicarbonate (NaHCO_3)
 - Dissolved 16.8 g of NaHCO_3 in distilled water and diluted to a 1,000 mL solution, mixed well.
 - Stored at 4°C.
- (5.) 0.2 mol/L Carbonate buffer, pH 9.2
 - Mixed 20 mL of 0.2 mol/L Na_2CO_3 with 230 mL of 0.2 mol/L NaHCO_3 .
 - Added distilled water to about 900 mL , mixed well.
 - Adjusted pH to 9.2 with 1 N HCl or 1 N NaOH.
 - Diluted to a 1,000 mL with distilled water.
 - Stored at 4 °C.
- (6.) 0.1 mol/L Carbonate buffer, pH 9.2
 - Mixed 10 mL of 0.2 mol/L Na_2CO_3 with 115 mL of 0.2 mol/L NaHCO_3 .
 - Added distilled water to about 900 mL, mixed well.
 - Adjusted pH to 9.2 with 1 N HCl or 1 N NaOH.
 - Adjusted volume to a 1,000 mL with distilled water.
 - Stored at 4 °C.

- (7.) 0.05 mol/L Carbonate buffer, pH 9.2
- Mixed 5 mL of 0.2 mol/L Na_2CO_3 with 57.5 mL of 0.2 mol/L NaHCO_3 .
 - Added distilled water to about 900 mL, mixed well.
 - Adjusted pH to 9.2 with 1 N HCl or 1 N NaOH.
 - Adjusted volume to a 1,000 mL with distilled water.
 - Stored at 4 °C.
- (8.) 0.02 mol/L Carbonate buffer, pH 9.2
- Mixed 2 mL of 0.2 mol/L Na_2CO_3 with 23 mL of 0.2 mol/L NaHCO_3 .
 - Added distilled water to about 900 mL, mixed well.
 - Adjusted pH to 9.2 with 1 N HCl or 1 N NaOH.
 - Adjusted volume to a 1,000 mL with distilled water.
 - Stored at 4 °C.

V. Reagents for the study of effect of metallic ion and compounds on bilirubin oxidase activity

- (1.) 1 mmol/L ZnSO_4 in 0.1 mol/L Tris-SDS buffer, pH 8.0
- Dissolved 0.0086 g of ZnSO_4 in 0.1 mol/L Tris-SDS buffer, pH 8.0 and diluted to make 25 mL of solution, mixed well.
 - Stored at 4 °C.
- (2.) 1 mmol/L CaCl_2 in 0.1 mol/L Tris-SDS buffer, pH 8.0
- Dissolved 0.0033 g of CaCl_2 in 0.1 mol/L Tris-SDS buffer, pH 8.0 and diluted to make 25 mL of solution, mixed well.
 - Stored at 4 °C.
- (3.) 2 g/L Bovine Serum Albumin (BSA) in 0.1 mol/L Tris-SDS buffer, pH 8.0
- Dissolved 0.0605 g of BSA in 0.1 mol/L Tris-SDS buffer, pH 8.0 and diluted to make 25 mL of solution, mixed well.
 - Stored at 4 °C.

VI. Serum preparations for the recovery study of total bilirubin measurement using enzymatic bilirubin oxidase method.

(1.) Serum 1 preparation

No.	Volume mixed (mL)			Calculated total bilirubin concentrations (mg/dL)		Expected values (mg/dL)
	Serum 1.1*	Serum 1.2 added**	H ₂ O	Serum 1.1	Serum 1.2 added	
1	0.50	-	0.50	0.53	-	0.53
2	0.50	0.10	0.40	0.53	2.34	2.87
3	0.50	0.20	0.30	0.53	4.67	5.20
4	0.50	0.30	0.20	0.53	7.01	7.54
5	0.50	0.40	0.10	0.53	9.34	9.87
6	0.50	0.50	-	0.53	11.68	12.21

* Serum 1.1, Total bilirubin = 1.06 mg/dL

** Serum 1.2, Total bilirubin = 23.36 mg/dL

(2.) Serum 2 preparation

No.	Volume mixed (mL)			Calculated total bilirubin concentrations (mg/dL)		Expected values (mg/dL)
	Serum 2.1*	Serum 2.2 added**	H ₂ O	Serum 1.1	Serum 1.2 added	
1	0.50	-	0.50	8.99	-	8.99
2	0.50	0.10	0.40	8.99	2.34	11.33
3	0.50	0.20	0.30	8.99	4.67	13.66
4	0.50	0.30	0.20	8.99	7.01	16.00
5	0.50	0.40	0.10	8.99	9.34	18.33
6	0.50	0.50	-	8.99	11.68	20.67

* Serum 2.1, Total bilirubin = 17.98 mg/dL

** Serum 2.2, Total bilirubin = 23.36 mg/dL

VII. Serum preparations for the recovery study of conjugated bilirubin measurement using enzymatic bilirubin oxidase method.

(1.) Serum 1 preparation

No.	Volume mixed (mL)			Calculated conjugated bilirubin concentrations (mg/dL)		Expected values (mg/dL)
	Serum 1.1*	Serum 1.2 added**	H ₂ O	Serum 1.1	Serum 1.2 added	
1	0.50	-	0.50	0.57	-	0.57
2	0.50	0.10	0.40	0.57	0.95	1.52
3	0.50	0.20	0.30	0.57	1.90	2.47
4	0.50	0.30	0.20	0.57	2.85	3.42
5	0.50	0.40	0.10	0.57	3.80	4.37
6	0.50	0.50	-	0.57	4.76	5.33

* Serum 1.1 , Conjugated bilirubin = 1.13 mg/dL

** Serum 1.2 , Conjugated bilirubin = 9.51 mg/dL

(2.) Serum 2 preparation

No.	Volume mixed (mL)			Calculated conjugated bilirubin concentrations (mg/dL)		Expected values (mg/dL)
	Serum 2.1*	Serum 2.2 added**	H ₂ O	Serum 1.1	Serum 1.2 added	
1	0.50	-	0.50	2.74	-	2.74
2	0.50	0.10	0.40	2.74	1.07	3.81
3	0.50	0.20	0.30	2.74	2.13	4.87
4	0.50	0.30	0.20	2.74	3.20	5.94
5	0.50	0.40	0.10	2.74	4.26	7.00
6	0.50	0.50	-	2.74	5.33	8.07

* Serum 2.1 , Conjugated bilirubin = 5.48 mg/dL

** Serum 2.2 , Conjugated bilirubin = 10.65 mg/dL

VIII. Serum preparations for the study of linearity of bilirubin oxidase method for total and conjugated bilirubin determination in serum.

No.	Volume (mL)		Volume fraction	concentration (mg/dL)	
	Serum*	H ₂ O		Total bilirubin	Conjugated bilirubin
1	0.1	0.4	0.2	7.98	3.22
2	0.2	0.3	0.4	15.95	6.44
3	0.3	0.2	0.6	23.93	9.65
4	0.4	0.1	0.8	31.90	12.87
5	0.5	-	1.0	39.88	16.09

*Serum contained 39.88 mg/dL and 16.09 mg/dL of total and conjugated bilirubin previously assayed by Jendrassik & Grof diazo reagent method.

IX. Sample preparations for bilirubin oxidase kinetic study in 0.1 mol/L Tris-SDS buffer, pH 8.0 (total bilirubin determination).

No.	Volume (mL)		Final concentration	
	Bilirubin standard*	Dilutor **	mg/dL	μ (mol/L)
1	0.056	0.944	2.0	34.22
2	0.141	0.859	5.0	85.54
3	0.211	0.789	7.5	128.31
4	0.281	0.719	10.0	171.09
5	0.563	0.437	20.0	342.17

* 35.53 mg/dL bilirubin standard in 40 g/L BSA.

** 40 g/L BSA

X. Serum preparations for bilirubin oxidase kinetic study in 0.1 mol/L Lactic acid buffer, pH 3.7 (conjugated bilirubin determination) .

No.	Volume (mL)		Final concentration	
	Serum*	H ₂ O	mg/dL	μ mol/L
1	0.08	0.92	0.27	4.62
2	0.17	0.83	0.54	9.24
3	0.25	0.75	0.80	13.69
4	0.33	0.67	1.07	18.31
5	0.50	0.50	1.60	27.37

* Conjugated bilirubin in pooled human serum, value of 3.2 mg/dL.

XI. Serum preparations for the study of interfering effect of hemoglobin on the determination of total and conjugated bilirubin in serum by bilirubin oxidase method.

No.	Volume (mL)			Final Hb concentrations (g/L of serum)
	Pooled serum*	Stock Hb**	H ₂ O	
1	0.5	-	0.500	0
2	0.5	0.025	0.475	0.1
3	0.5	0.050	0.450	0.2
4	0.5	0.100	0.400	0.4
5	0.5	0.150	0.350	0.6
6	0.5	0.200	0.300	0.8
7	0.5	0.250	0.250	1.0
8	0.5	0.300	0.200	1.2
9	0.5	0.350	0.150	1.4
10	0.5	0.400	0.100	1.6
11	0.5	0.450	0.050	1.8
12	0.5	0.500	-	2.0

*Pooled serum

For total bilirubin :

-Pooled serum 1 (total bilirubin 0.87 mg/dL)

-Pooled serum 2 (total bilirubin 14.51 mg/dL)

For conjugated bilirubin :

-Pooled serum 1 (conjugated bilirubin 0.32 mg/dL)

-Pooled serum 2 (conjugated bilirubin 5.30 mg/dL)

** Stock hemoglobin, 4 g/L prepared by hemolysing red blood cells with distilled water, the concentration was determined using Drabkin's solution method.

XII. Bilirubin standard solution preparation

- Weighed out 20.0 mg of bilirubin of acceptable purity, transferred to a 100 mL volumetric flask.
- Dissolved by adding 1.5 mL of NaOH and 2.0 mL of 0.1 mol/L Na_2CO_3 .
- Diluted the solution to obtain 20 mg bilirubin standard per 100 mL of the bovine serum albumin diluent solution (40 g/L) previously adjusted to pH 7.4.
- Diluted 20 mg/dL bilirubin standard to obtain 2, 5, 10, 15 and 20 mg/dL with 40 g/L bovine serum albumin diluent.
- Kept in dark.

XIII. Determination of molar absorptivity (ϵ) of bilirubin standard

Molar absorptivity of bilirubin standard was determined by using the program photometric mode in a Shimadzu double beam UV-visible spectrophotometer. In this experiment, a 100 μL of 5, 10 and 20 mg/dL of bilirubin standard was added into 2.0 mL of 0.1 mol/L Tris-SDS buffer, pH 8.0. After thoroughly mixed, the absorbances of the mixtures were measured at 450 nm.

The absorbance measurements of all concentrations of bilirubin solution in 0.1 mol/L Tris-SDS buffer were performed in triplicates. The results obtained were calculated for molar absorptivity using the following formula:

$$\epsilon = \frac{A}{bc}$$

- where
- ϵ = Molar absorptivity of bilirubin at 450 nm.
 - A = Absorbance
 - b = light path in cm; 1 cm
 - c = concentration of bilirubin in mol/L.

The results obtained were shown in a table below.

Bilirubin concentration		No.	Absorbance	Absorptivity (ϵ) ($L \cdot mol^{-1} \cdot cm^{-1}$)
mg/dL	mol/L			
5	4.07×10^{-6}	1	0.209	51351
		2	0.211	51843
		3	0.213	52334
10	8.14×10^{-6}	1	0.413	50737
		2	0.420	51597
		3	0.415	50983
20	16.28×10^{-6}	1	0.819	50307
		2	0.813	49939
		3	0.821	50430
Total average (ϵ)				51058

XIV. The preparations of enzyme-buffer solutions for total and conjugated bilirubin determination in serum using a Beckman Synchron CX5 autoanalyzer

(1.) Enzyme-buffer solution for total bilirubin determination

- Dissolved 0.144 g of SDS (Sodium dodecyl sulfate) in a 80 mL of culture filtrate, previously treated with 0.7% and 0.2 % w/v activated charcoal respectively, mixed gently until well dissolved.
- Added 1.212 g of Trizma base [tris (hydroxymethyl) aminomethane] to the mixture and mixed well.
- Adjusted pH to 8.0 with 1 N NaOH or 1 N HCl.
- Filled up with a culture filtrate pretreated with activated charcoal to a 100 mL solution.
- This enzyme-buffer solution contained 14.01 U/L of bilirubin oxidase activity in 0.1 mol/L Tris-SDS buffer, pH 8.0 .

(2.) Enzyme-buffer solution for conjugated bilirubin determination

- Dissolved 2.94 g of tris-sodium citrate in a 80 mL of culture filtrate, previously treated with 0.7% and 0.2% w/v activated charcoal respectively, mixed gently until well dissolved.
- Added 0.692 mL of Lactic acid to the mixture and mixed well.
- Adjusted pH to 3.7 with 1 N NaOH or 1 N HCl.
- Filled up with a culture filtrate pretreated with activated charcoal to a 100 mL solution.
- This enzyme-buffer solution contained 10.56 U/L of bilirubin oxidase activity in 0.1 mol/L Lactic acid buffer, pH 3.7 .

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