IV. RESULTS

Part I. Cultivation of *Myrothecium verrucaria* for bilirubin oxidase production

[1.] Cultivation and identification of *Myrothecium verrucaria*

Myrothecium verrucaria TISTR 3112 and TISTR 3225 cultivated at 25 °C for 14 days on the potato dextrose agar plates demonstrated the white to light yellow colonies. The more intense yellow pigments were observed on the subcolonies of strain TISTR 3225 than that of strain TISTR 3112 (Figure 4, 5, 6, 7). Morphological characteristics studied by a slide culture technique were concluded as follows (Figure 8).

- Hyphae : septate hyaline hyphae (1.2-2.5 μm in width)
- Phialides : columnar (1.0-2.0 μm x 10.0-17.0 μm)
- Conidia : spindle-shaped, bearing conidia terminally, fan-shaped substance at end (1.5-2.5 μm x 6.5-8.0 μm)

Since no sexual reproductive organ was observed, so both strains are the imperfect fungi, and could be classified as belonging to the genus *Myrothecium.*

[2.] The growth characteristics of *Myrothecium verrucaria*

Table 2 showed the growth characteristics of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 on the potato dextrose agar plate cultivated at 25°C for 7 days. Diameters of the colonies ($\bar{X} \pm SD$, triplicate study) of strain TISTR 3112 and TISTR 3225 were increased with the cultivation time and reached the maximum at 7 days of cultivation. As demonstrated in Figure 9, the rate of growth of strain TISTR 3225 was shown to be slower than the strain TISTR 3112 on this type of media used for cultivation.

Figure 4. Macroscopic appearance of *Myrothecium verrucaria* TISTR 3112 cultivated at 25 °C for 14 days. Upper view of colonies on potato dextrose agar plate.

Figure 5. Macroscopic appearance of *Myrothecium verrucaria* TISTR 3112 cultivated at 25 °C for 14 days. Under view of colonies on potato dextrose agar plate.

Figure 6. Macroscopic appearance of *Myrothecium verrucaria* TISTR 3225 cultivated at 25 °C for 14 days. Upper view of colonies on potato dextrose agar plate.

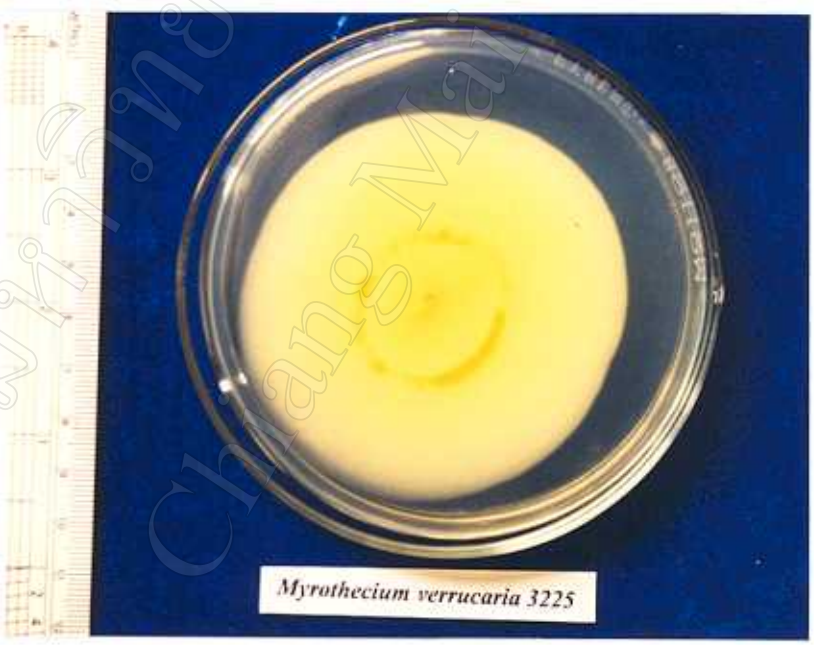


Figure 7. Macroscopic appearance of *Myrothecium verrucaria* TISTR 3225 cultivated at 25 °C for 14 days. Under view of colonies on potato dextrose agar plate.

Figure 8. Microscopic appearances of *Myrothecium verrucaria* (x400).

Table 2. Growth characteristics of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 on potato dextrose agar.

Day	Diameter of the colony (cm) (Mean ± SD)	
	Myrothecium verrucaria TISTR 3112	*Myrothecium verrucaria* TISTR 3225
1	1.00 ± 0.10	0.83 ± 0.06
2	1.33 ± 0.06	1.17 ± 0.06
3	2.03 ± 0.06	1.57 ± 0.06
4	2.50 ± 0.10	2.10 ± 0.10
5	2.97 ± 0.06	2.50 ± 0.10
6	3.53 ± 0.06	2.97 ± 0.06
7	4.17 ± 0.12	3.37 ± 0.12

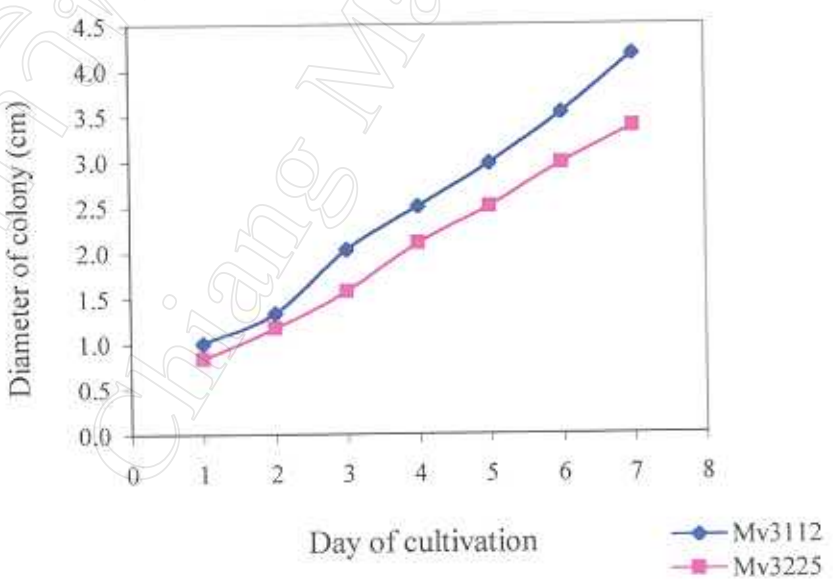


Figure 9. Rate of growth of microorganisms on potato dextrose agar.

[3.] Optimization conditions for bilirubin oxidase production by *Myrothecium verrucaria*

3.1 Optimization of cultivation temperature for bilirubin oxidase production

The effect of various temperatures on the growths of microorganisms and bilirubin oxidase productions cultivated according to that described in the method were shown in Figure 10 and 11. The mycelium dry weight which evaluates growth of the microorganism was maximum at 25 °C for the strain TISTR 3112 but more or less the same at 25 °C and 30 °C for the strain TISTR 3225 (Figure 10). The optimal cultivation temperatures for maximum enzyme production expressed as specific bilirubin oxidase activity; (U/g protein) by both strains were agreed with the mycelium dry weights. Total bilirubin oxidase activites in total volume of media were increased from 20 °C to reach maximum at 25 °C and dropped after the temperature of cultivation media was increased to 37 °C (Figure 11). Therefore, the optimal temperature for bilirubin oxidase production by both strains was selected at 25 °C for cultivation condition.

3.2 Optimization of cultivation time for bilirubin oxidase production

The effect of cultivation times on bilirubin oxidase productions at 25 °C by *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 were shown in Figure 12 and 13. Cell growths measured by the mycelium dry weights were increased with the increase of cultivation time (Figure 12). In agreement with results demonstrated in Table 1, cell growth of strain TISTR 3225 was lower than the other when the cultivation time was exceeded than 24 hrs. However, the mycelium dry weight of this strain was still increased in the same pattern as the strain TISTR 3112 which reached maximum at 72 hrs of cultivation time.

At different cultivation time and at 25 °C of cultivation temperature, the strain TISTR 3225 produced more bilirubin oxidase than that of the strain TISTR 3112 (except for at 6 hrs). Total enzyme (mU/total medium volume) and specific enzyme activities (U/g total protein) were increased and reached maximum at 48 hrs of the cultivation time (Figure 13). As compared with Figure 12, the enzyme

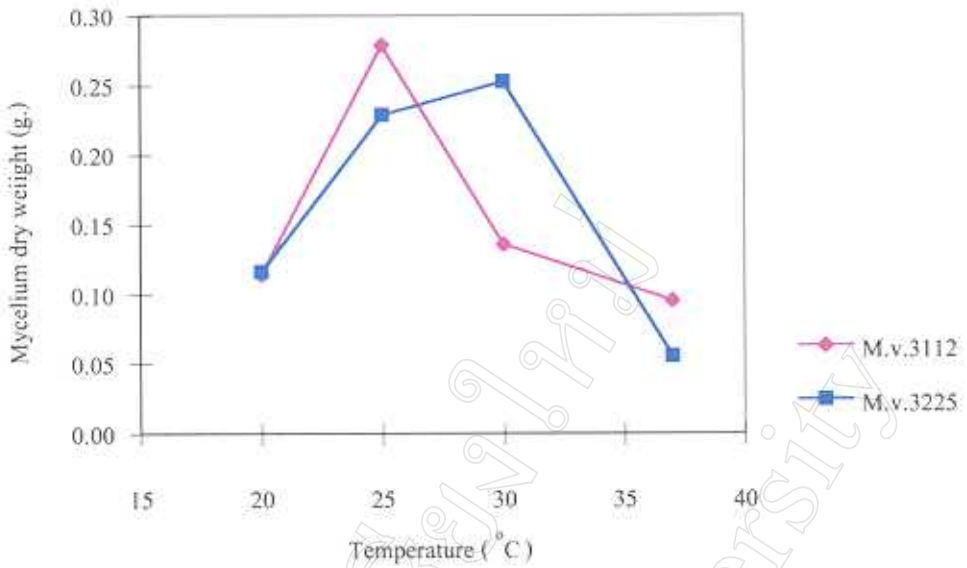


Figure 10. Effect of cultivation temperatures on growths of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225.

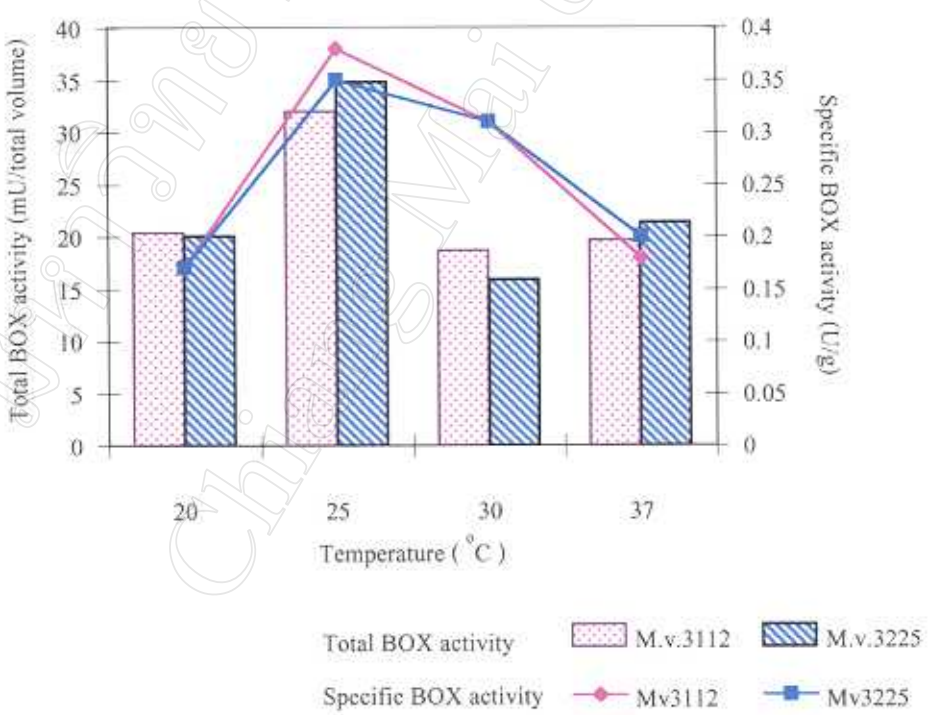


Figure 11. Effect of cultivation temperatures on BOX productions by *Myrothecium verrucaria* TISTR 3112 and TISTR 3225.

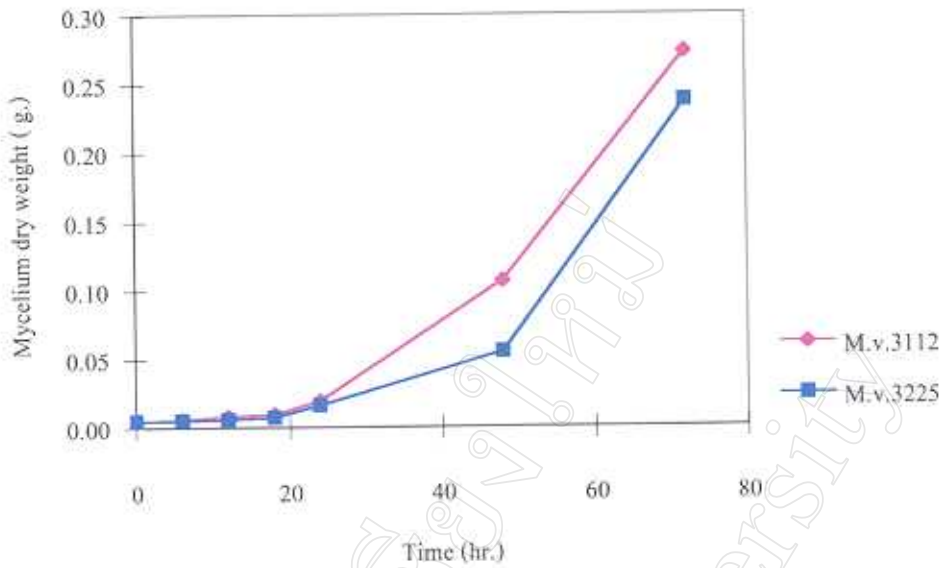


Figure 12. Effect of cultivation times on growths of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225.

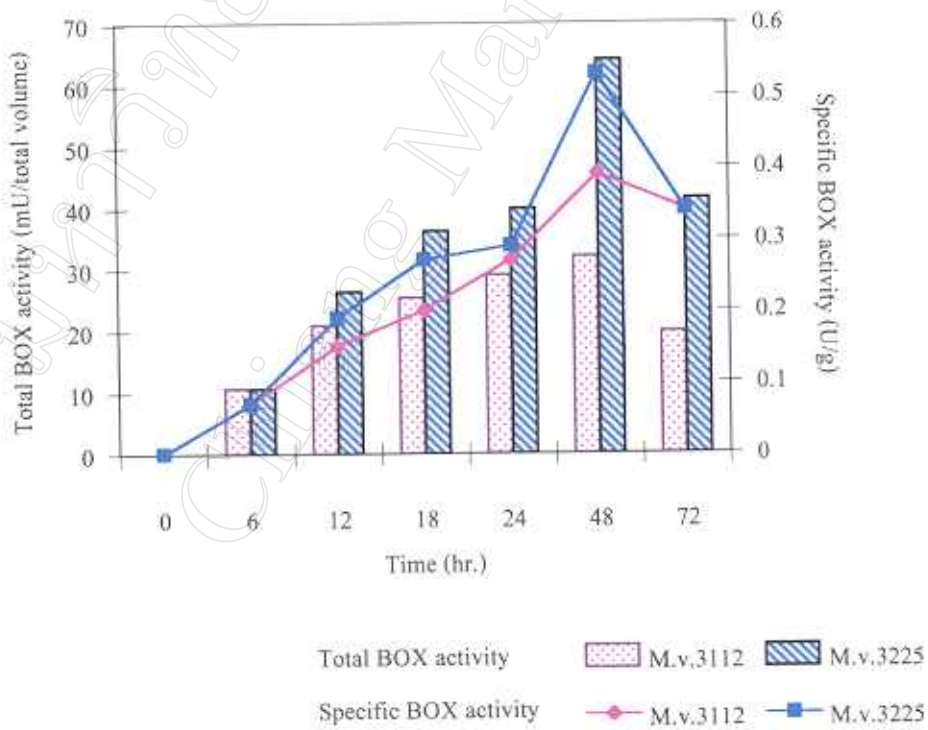


Figure 13. Effect of cultivation times on BOX productions by *Myrothecium verrucaria* TISTR 3112 and TISTR 3225.

productions by both strains were in the opposite direction of cell growth at 72 hrs of the cultivation condition.

It could be concluded that at 48 hrs of cultivation time was optimal for both microorganisms to produce maximum bilirubin oxidase and thus was chosen for further preparations.

3.3 Optimization of pH of cultivation media for bilirubin oxidase production

The growths of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 cultivated at 25 °C for 48 hrs in different pH of cultivation media were shown in Figure 14 and 15. The maximal growths of both microorganisms were observed at pH 8.0 of cultivation condition. The pH optimal for the enzyme productions were agreed with the mycelium dry weights. At different pH of cultivation media, bilirubin oxidase productions by both strains were not significant different.

In conclusion, the maximal bilirubin oxidase productions by *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 were obtained from cultivating of the microorganisms in a pH 8.0 cultivation medium at 25°C for 48 hrs. This condition was used to prepare the BOX enzyme for all purposes of studies.

Part II. Analytical methods for protein and bilirubin oxidase enzyme isolated from *Myrothecium verrucaria*

Since many heterologous proteins which successfully produced and secreted by *Myrothecium verrucaria* were released into the extracellular medium. Therefore, bilirubin oxidase enzyme production observed in a culture filtrate or purified eluated were evaluated by results calculated from the ratio of bilirubin oxidase produced (Unit) to total protein secretion (specific bilirubin oxidase activity) in every experiment of examination. The more bilirubin oxidase production, the higher specific bilirubin oxidase activity was obtained.

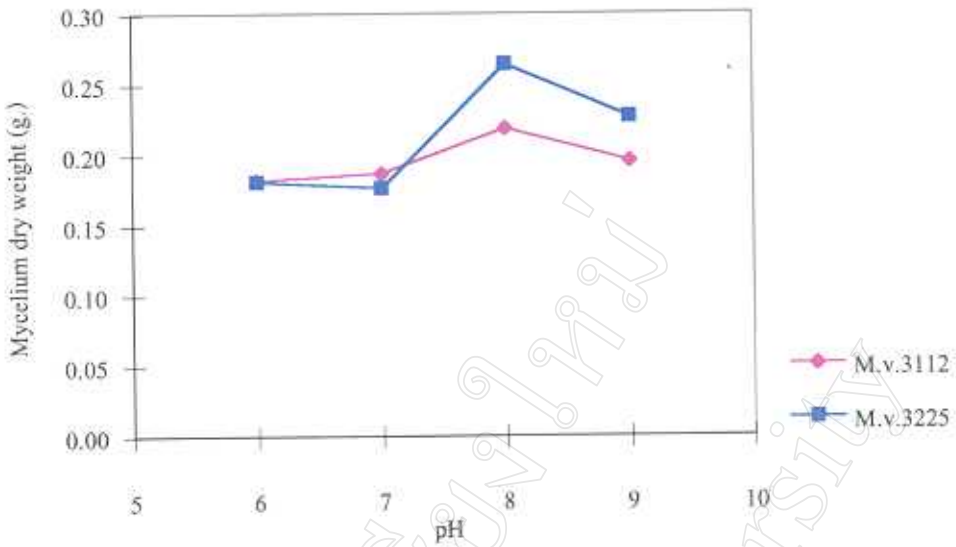


Figure 14. Effect of pH of cultivation media on growths of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225.

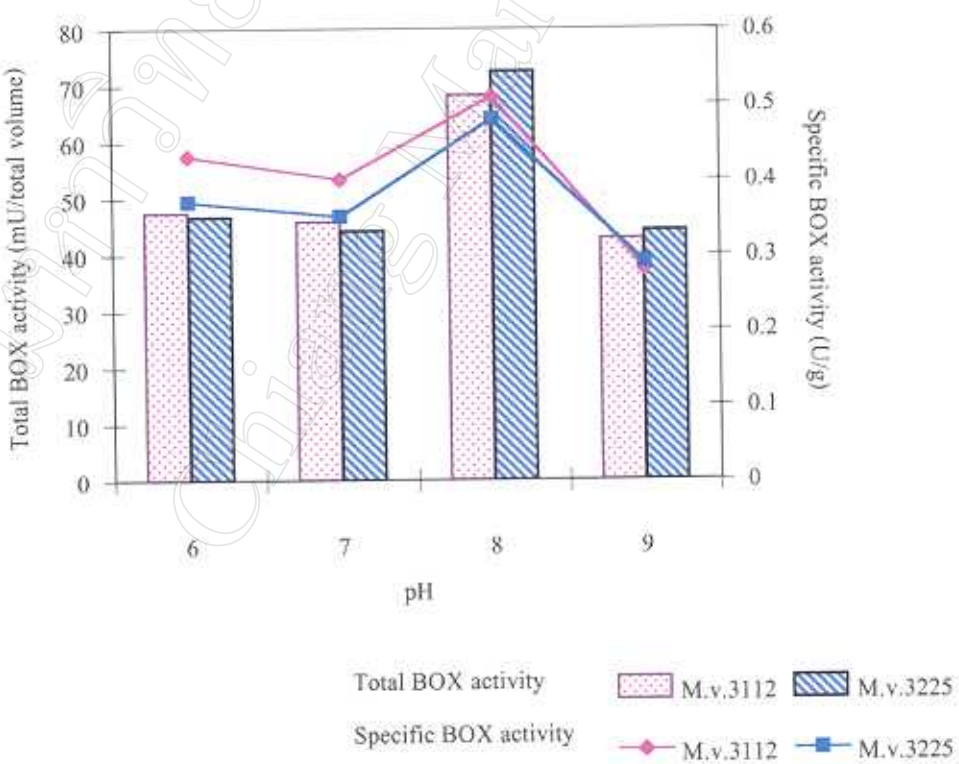


Figure 15. Effect of pH of cultivation media on BOX productions by *Myrothecium verrucaria* TISTR 3112 and TISTR 3225

Part III. Purification, identification and characterization of bilirubin oxidase obtained from a culture filtrate of *Myrothecium verrucaria*

[1.] Selection of the purification technique

1.1 Purification of bilirubin oxidase by ammonium sulfate precipitation

As seen in Table 3 that although the ammonium sulfate precipitation and color clarification process caused the increase in specific bilirubin oxidase activity, the yield recovery obtained from each step was very small. Charcoal treatment showed some losses of the enzyme from the purified solution. It is concluded that ammonium sulfate precipitation could not be used so far to purified bilirubin oxidase from the culture filtrate.

1.2 Clarification of bilirubin oxidase in the culture filtrates by adsorption on activated charcoal

From Table 4, it is seen that total bilirubin oxidase activity decreased whereas the specific bilirubin oxidase activity increased after some impurities in the culture filtrates were progressively adsorped on the activated charcoal surface. At final step of adsorption, the yields recovery of bilirubin oxidase activity was remained to approximately 70% of the original bilirubin oxidase in the culture filtrates. Obviously, the brownish-yellow color of cultivation media was also adsorped along with this purification process.

1.3 Purification of bilirubin oxidase on DEAE-Cellulose anion exchange chromatography

Culture filtrate of *Myrothecium verrucaria* applied to the DEAE-Cellulose column were eluted into three active protein peaks, bilirubin oxidase activities were detected approximately at the second protein peak area (Figure 16). It can be seen that, although a large amounts of protein were found to be eluted successfully from the DEAE-Cellulose column, the small yield of bilirubin oxidase enzyme was obtained (Figure 16).

Table 4. Clarification of bilirubin oxidase by adsorption on activated charcoal.

Organisms	Purification step	Total volume(TV) (mL)	BOX activity (U/L)	Total protein (g/TV)	Total BOX activity (U/TV)	Specific activity (U/g)	Activity increased (folds)	Recovery (%)
Mv TISTR 3112	Culture filtrate	330	11.38	1.043	3.755	3.60	-	100
	0.7%w/v Charcoal treatment	329	8.28	0.190	2.724	14.34	3.98	72.54
	0.2%w/v Charcoal treatment	324	8.36	0.108	2.708	25.07	6.96	72.12
Mv TISTR 3225	Culture filtrate	380	21.85	1.599	8.303	5.19	-	100
	0.7%w/v Charcoal treatment	378	15.47	0.282	5.847	20.73	3.99	70.42
	0.2%w/v Charcoal treatment	374	14.01	0.159	5.239	32.95	6.35	63.10

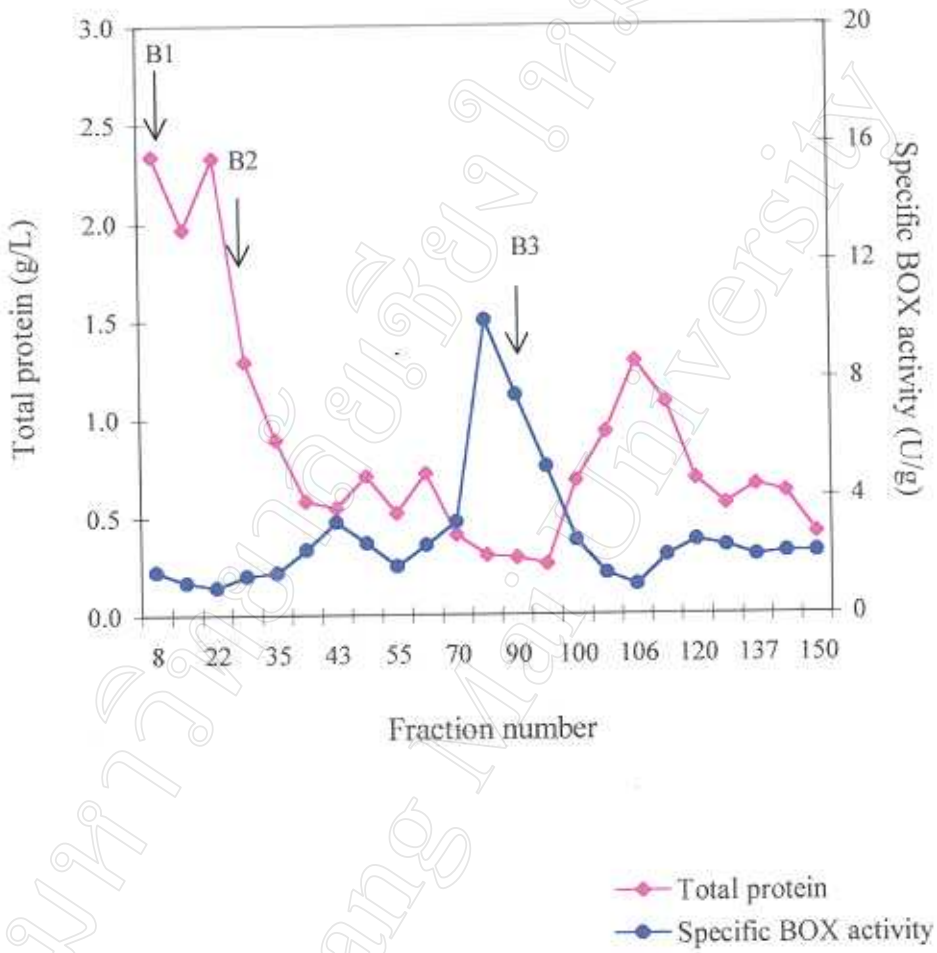


Figure 16. Purification of bilirubin oxidase from a culture filtrate of *Myrothecium verrucaria* by DEAE-Cellulose column chromatography. B1, B2 and B3 were 0.05, 0.1 and 0.2 mol/L Na_2CO_3-$NaHCO_3$ buffers, pH 9.2, respectively. The eluates were collected as a 2 mL fraction.

1.4 Purification of bilirubin oxidase on DEAE-Sepharose anion exchange chromatography

Figure 17 showed the elution patterns of protein eluted from culture filtrates of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225. The protein elution profiles of both strains demonstrated three identical peaks in which the bilirubin oxidase enzyme (U/g protein) were detected greatfully in the peak 3 of protein eluates. The bilirubin oxidase purified from strain TISTR 3112 was more eluted from the DEAE-Sepharose column than that of strain 3225 (Figure18). The active bilirubin oxidase fractions were pooled for further characterization.

1.5 Comparison of yield recoveries of bilirubin oxidase purified by different methods

Yield recovery of bilirubin oxidase obtained from different purification techniques were shown in Table 5. From results, it was elucidated that the highest yield of bilirubin oxidase was obtained after treating a culture filtrate with activated charcoal. DEAE-Sepharose column purification gave a sufficient amount of bilirubin oxidase but less than 60% of the starting material. The other methods were unsuitable for purification of bilirubin oxidase from the culture filtrates.

For further studies, adsorption on activated charcoal method was a selective method for preparation of enzyme solution for application uses, while the enzyme purified by DEAE-Sepharose column chromatographic method was used for the identification and characterization studies.

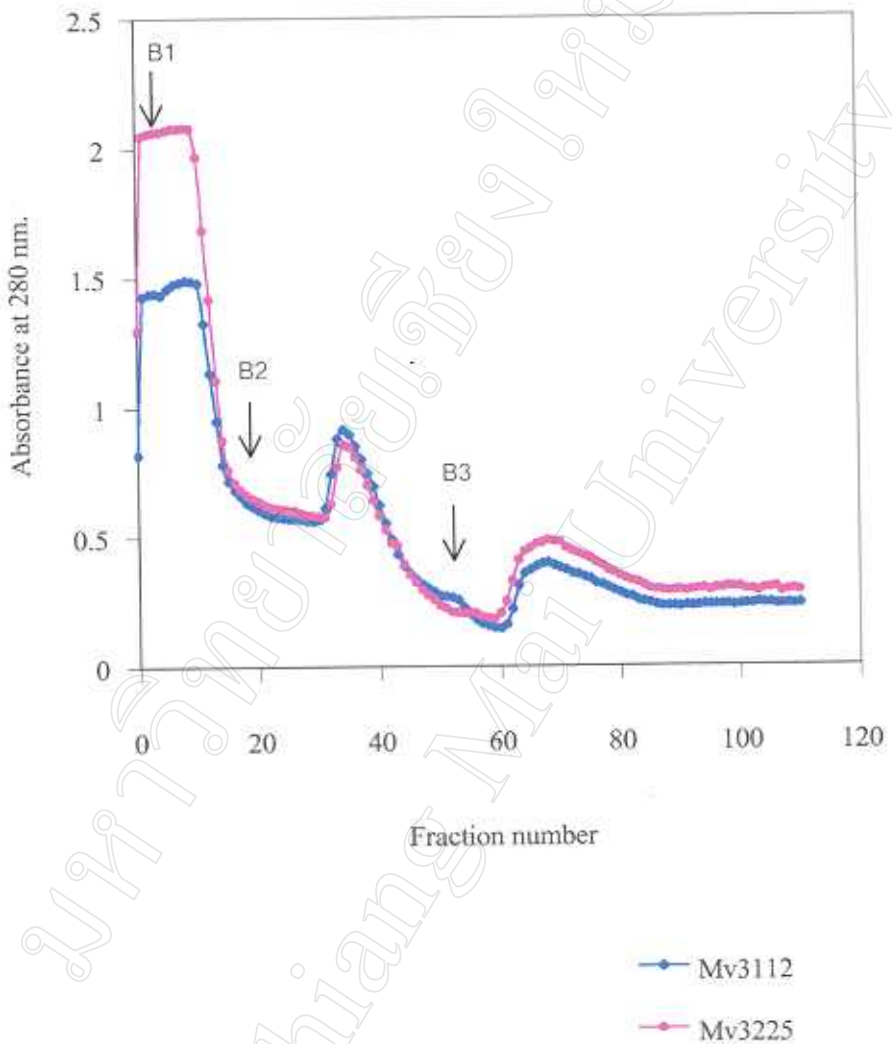


Figure 17. Elution profiles of protein purified from culture filtrates of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 by DEAE-Sepharose column chromatography.
B1, B2 and B3 were the elution buffers; 0.05, 0.1 and 0.2 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 9.2, respectively. The eluates were collected as a 2 mL fraction.

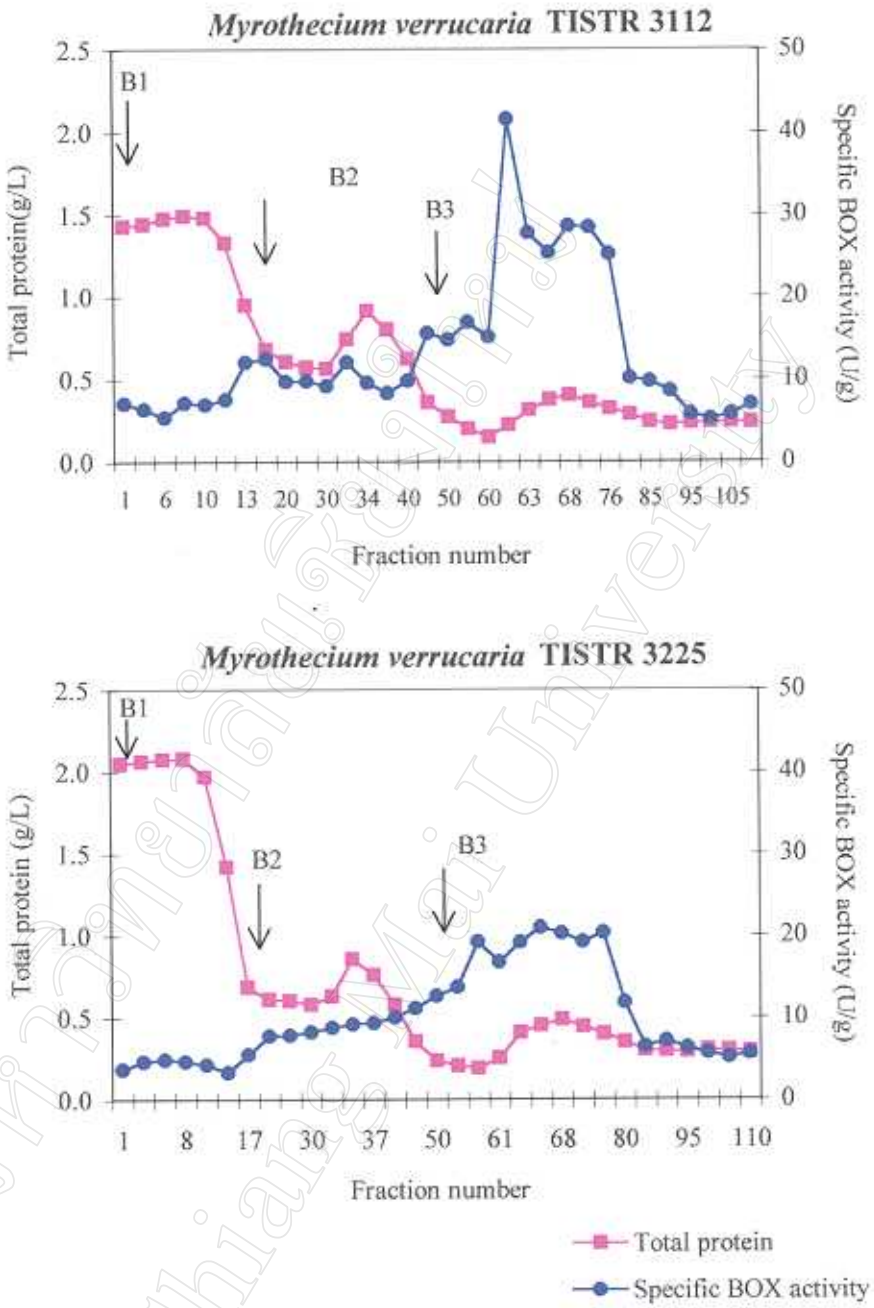


Figure 18. Demonstration of bilirubin oxidase enzyme elution as compared with its protein elution pattern. The purification of bilirubin oxidase from culture filtrates of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 were performed on DEAE-Sepharose column chromatography.

B1, B2 and B3 were 0.05, 0.1 and 0.2 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 9.2, respectively. The eluates were collected as a 2 mL fraction.

Table 5. Comparisons of yield recoveries of bilirubin oxidase purified by different methods.

Purification type	Starting culture filtrate				Purified enzyme solution				Activity increased (folds)	Recovery (%)
	Total volume (mL)	Total protein (g)	Total BOX activity (U)	Specific activity (U/g)	Total volume (mL)	Total protein (g)	Total BOX activity (U)	Specific activity (U/g)		
$(NH_4)_2SO_4$ precipitation	300	1.29	1.488	1.15	92	0.006	0.119	19.83	17.24	8.00
Adsorption on activated charcoal	330	1.04	3.755	3.61	324	0.109	2.709	24.85	6.88	72.14
DEAE-Cellulose column chromatography	350	1.13	1.960	1.73	34	0.021	0.065	3.10	1.79	3.32
DEAE-Sepharose column chromatography	120	0.43	0.647	1.50	46	0.023	0.368	16.00	10.67	56.88

[2.] Identification of purified bilirubin oxidase separated from a culture filtrate of *Myrothecium verrucaria*

The Mini Prep Cell electrophoretic technique was used to identify bilirubin oxidase from the culture filtrates of cultivated *Myrothecium verrucaria* TISTR 3112 and TISTR 3225. A crude commercial enzyme was used as a control for an electrophoretic separation. As shown in Figure 19, the elution patterns of proteins, obtained from each separate electrophoretic run, were all appeared in two peak characteristic. At the identical peak positions as compared with a commercial control peak elution, the enzyme separated electrophoretically from culture filtrates of both strains were identically as bilirubin oxidase. Bilirubin oxidase activities observed at the first and second peaks of protein elutions confirmed this identification(as 1.48 U/L and 1.12 U/L for *Myrothecium verrucaria* TISTR 3112; and 2.88 U/L and 1.77 U/L for the commercial bilirubin oxidase control, respectively).

[3.]Characterization of bilirubin oxidase purified from culture filtrates of *Myrothecium verrucaria*

3.1 Molecular weight determination of a purified bilirubin oxidase

Figure 20 showed the elution curve of bilirubin oxidase eluted from Sephadex G-100 column chromatography. (The enzyme solution applied on this type of column chromatography obtained from pooled active fractions of protein, previously purified by DEAE-Sepharose anion exchange chromatography). A single peak of enzyme obtained was compared with the elution peaks of the other enzymes which used as the molecular weight markers running on the column using the same procedure as the unknown enzyme. As seen from the plot of log of molecular weights versus relative elution volumes (Ve/Vo), the molecular weight of purified bilirubin oxidase was estimated to be approximately 49,000 (Figure 21).

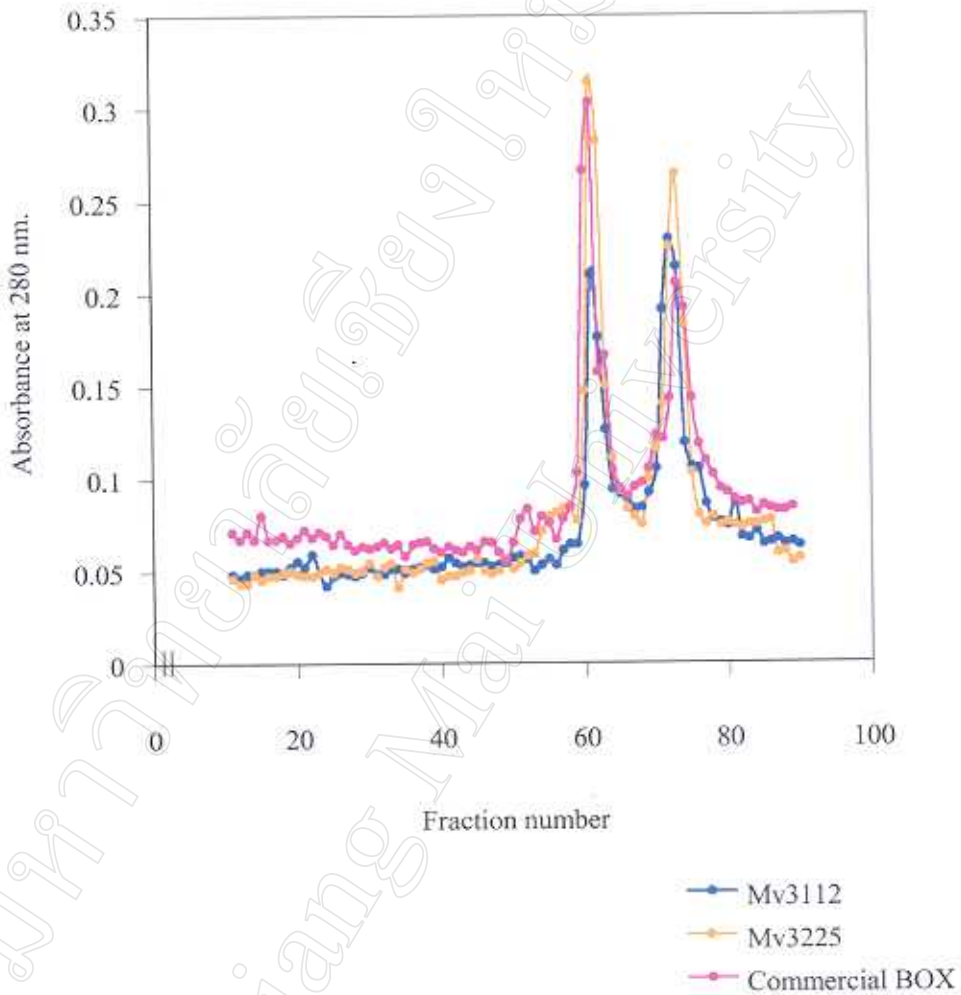


Figure 19. Elution profiles of bilirubin oxidase from separated culture filtrates of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 by Mini Prep cell electrophoresis. A crude commercial bilirubin oxidase from Sigma was used as a control for identification. The buffer eluted was 0.025 mol/L Tris-SDS, pH 8.3. The eluates were collected as a 1 mL fraction.

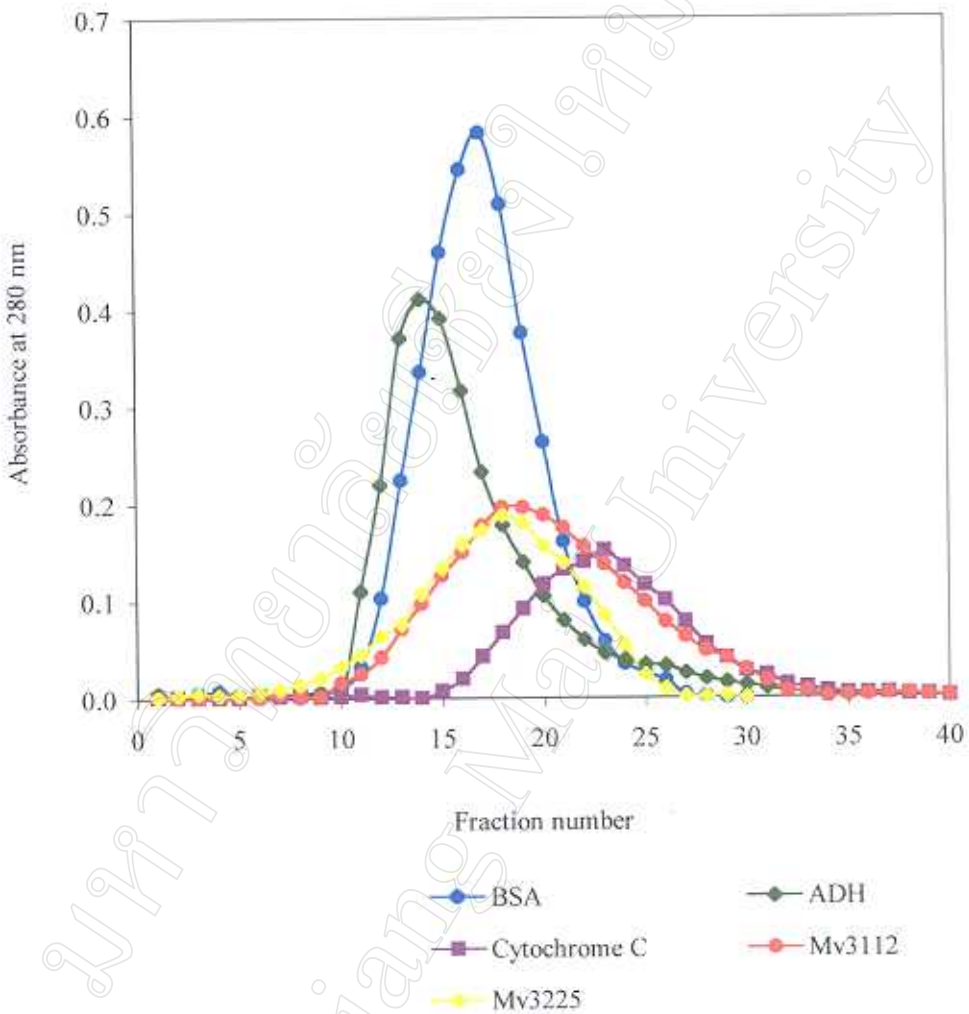


Figure 20. The elution curves of bilirubin oxidase and molecular weight enzyme markers obtained from applying of purified enzyme on Sephadex G-100 gel filtration column.
The buffer eluted was 0.02 mol/L Na_2CO_3-$NaHCO_3$ buffer (pH 9.2), and the eluates were collected as a 1 mL fraction.

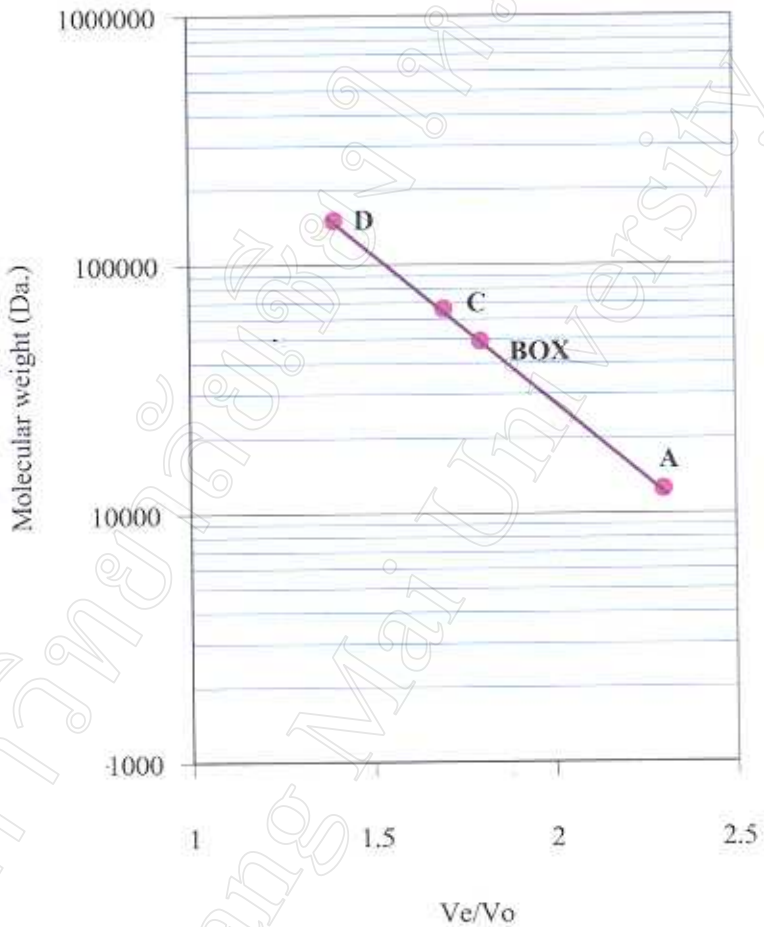


Figure 21. Estimation of the molecular weight of bilirubin oxidase on Sephadex G-100 gel filtration chromatography.

Protein markers used were : A,cytochrome C (12,400) ; C,bovine serum albumin (66,000) ; D,alcohol dehydrogenase (150,000).

3.2 Bilirubin oxidase kinetics

The rates of bilirubin oxidase enzyme utilized bilirubin substrates to form the biliverdin product were shown in Figure 22 and 23. A double reciprocal plot (Lineweaver-Burk) obtained from bilirubin oxidase oxidized various concentrations of total bilirubin substrate gave a straight line represented by the equation $y = 4.8145x + 0.0596$. The reciprocal values of substrate concentrations [S] and rates of bilirubin oxidase activity (V) were correlated significantly at $r = 0.9700$, ($p < 0.001$). K_m and V_{max} which calculated from the x and y intercept were 84.21 μmol/L and 17.5 μmol/min, respectively (Figure 22).

The kinetics of bilirubin oixidase enzyme using conjugated bilirubin substrate was also evaluated (Figure 23). The K_m and V_{max} which calculated in the similar way as the plot of total bilirubin were 135 μmol/L and 85 μmol/min, respectively. The straight line of Lineweaver-Burk plot was demonstrated as $y = 1.644x + 0.0077$ and $r = 0.9594$, ($p < 0.001$). The calculated "K_m" observed by this reaction was more than the above observation.

3.3 Effect of metallic ion and compounds on bilirubin oxidase activity

The effects of $ZnSO_4$, $CaCl_2$ and BSA on bilirubin oxidase activity were shown in Figure 24. The inhibition of bilirubin oxidase activity, expressed as % relative activity of the control reaction assayed without the inhibited ion or compounds, were shown in the same pattern. At the final of inhibitor concentrations (0.91 mmol/L for $ZnSO_4$ and $CaCl_2$ and 1.8 g/L for BSA), the bilirubin oxidase activities for all inhibited reactions were remained about 25 % .

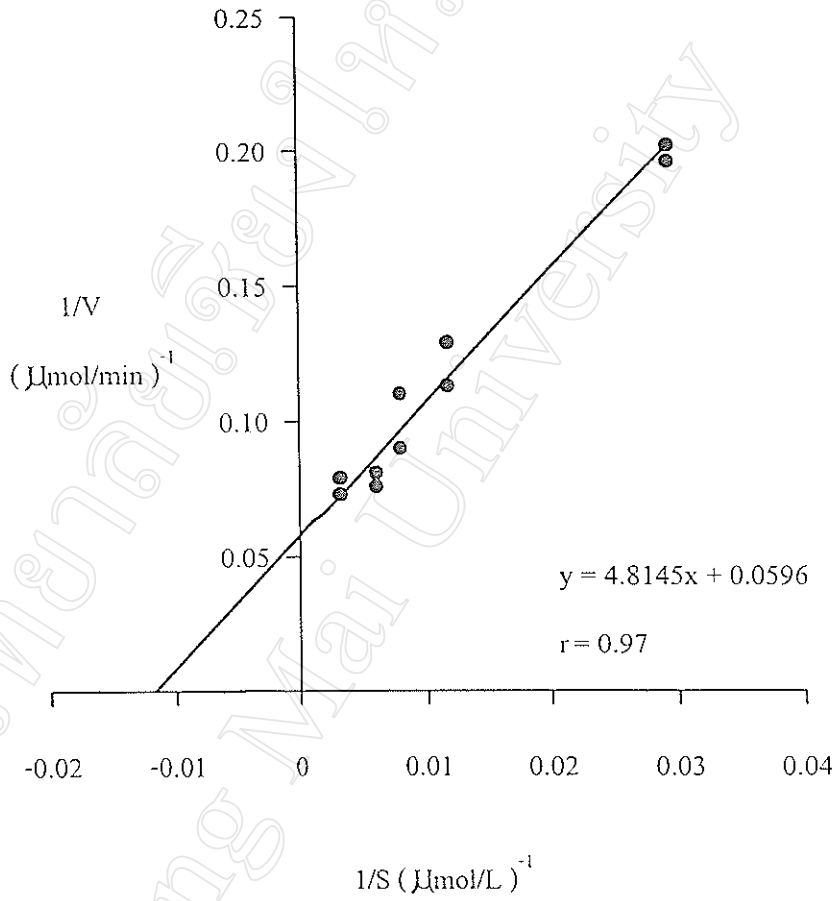


Figure 22. Lineweaver-Burk plot for BOX assayed in 0.1 mol/L Tris-SDS buffer, pH 8.0 (total bilirubin determination).
Calculated K_m and V_{max} values were 84.21 μmol/L and 17.5 μmol/min, respectively.

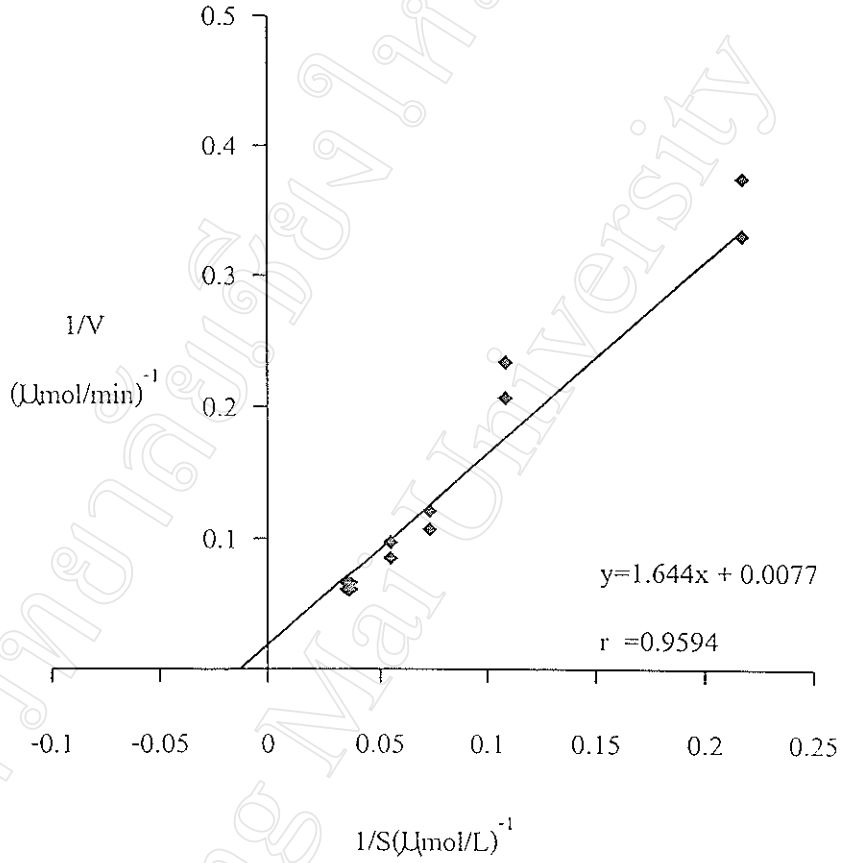


Figure 23. Lineweaver-Burk plot for BOX assayed in 0.1 mol/L Lactic acid buffer, pH 3.7 (conjugated bilirubin determination). Calculated K_m and V_{max} values were 135 μmol/L and 85 μmol/min, respectively.

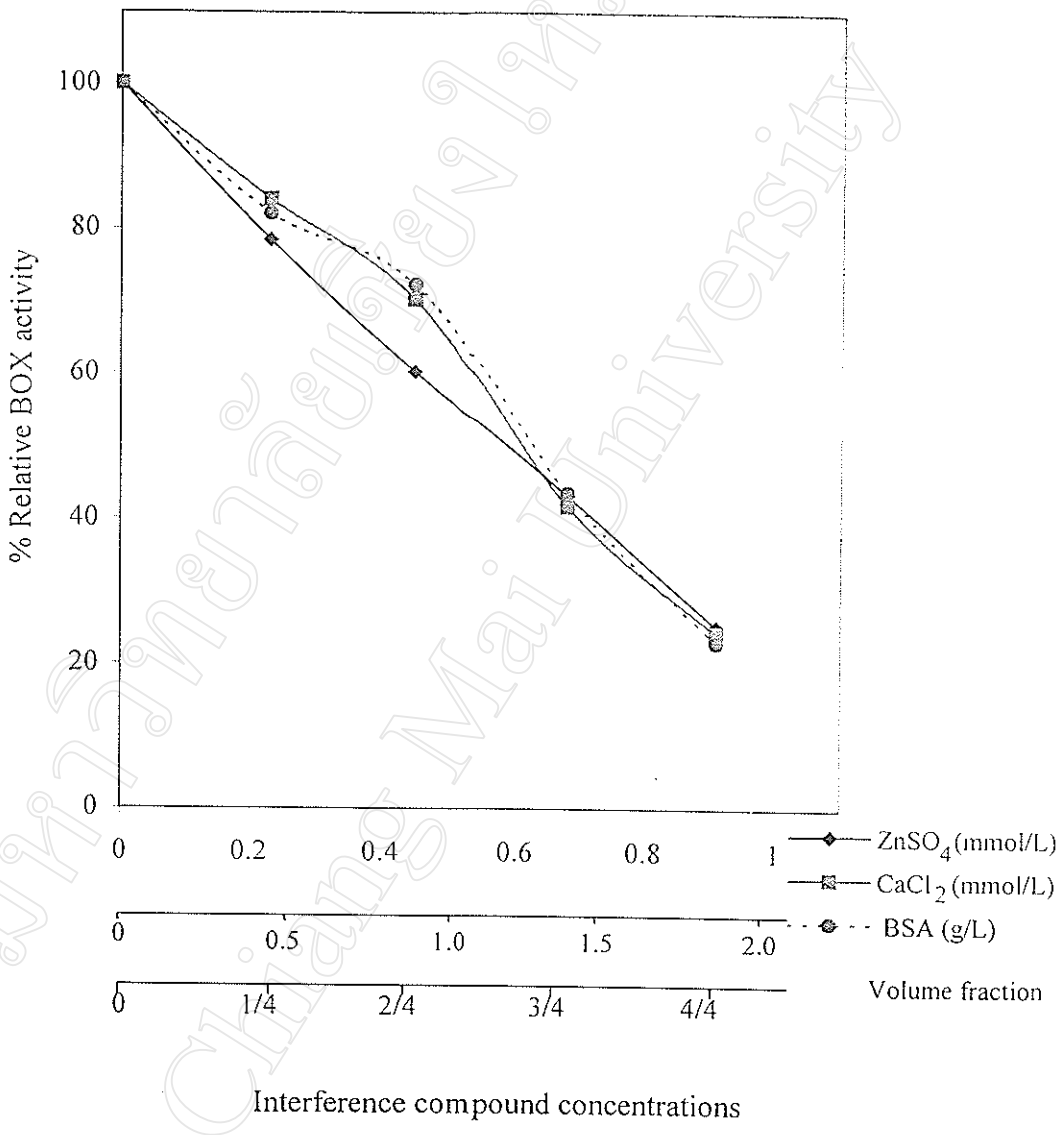


Figure 24. Effect of metallic ion and compounds on the bilirubin oxidase activity.

Part IV. Application and evaluation of bilirubin oxidase for the enzymatic determination of bilirubin in serum

[1.] The proposed enzymatic method developed for total and conjugated bilirubin determination in serum on an automated chemistry analyzer

Optimal bilirubin oxidase concentrations for serum total and conjugated bilirubin determination in a Beckman Synchron CX5 were 10-20 U/L and 5-15 U/L, respectively. Results of parameter setting were summarized as follows.

1.1 Proposed parameter for total bilirubin determination

Test name	TBOX	Calculation Factor	0
Reaction Type	[ENDPOINT 2]	Math Model	[LINEAR]
Reaction Direction	[NEGATIVE]	Cal Time Limit	336 hr
Units	[mg/dL]	No. of Calibrators	1
Decimal Precision	[X.XX]		

Primary Wavelength [470] nm Secondary Wavelength [520] nm

Sample Volume 10 μL

Primary Inject Reagent

B : 200 μL

CALIBRATORS

1 : 9.10

REAGENT BLANK			REACTION		
Start Read	:	330 sec	Start Read	:	40 sec
End Read	:	360 sec	End Read	:	70 sec
Low ABS Limit	:	-1.500	Low ABS Limit	:	-1.500
High ABS Limit	:	1.500	High ABS Limit	:	1.500

USABLE RANGE		SUBSTRATE DEPLETION	
Lower Limit	0.00	Initial Rate	-99.999
Upper Limit	99999.00	Delta ABS	1.500

1.2 Proposed parameter for conjugated bilirubin determination

Test name	DBOX	Calculation Factor	0
Reaction Type	[ENDPOINT 2]	Math Model	[LINEAR]
Reaction Direction	[NEGATIVE]	Cal Time Limit	336 hr
Units	[mg/dL]	No. of Calibrators	2
Decimal Precision	[X.XX]		

Primary Wavelength [470] nm Secondary Wavelength [520] nm

Sample Volume 10 µL

Primary Inject Reagent

B : 200 µL

CALIBRATORS

1 : 0.30

2 : 7.20

REAGENT BLANK			REACTION		
Start Read	:	250 sec	Start Read	:	120 sec
End Read	:	280 sec	End Read	:	150 sec
Low ABS Limit	:	-1.500	Low ABS Limit	:	-1.500
High ABS Limit	:	1.500	High ABS Limit	:	1.500

USABLE RANGE		SUBSTRATE DEPLETION	
Lower Limit	0.00	Initial Rate	-99.999
Upper Limit	99999.00	Delta ABS	1.500

[2.] Evaluation of the precision of total and conjugated bilirubin determination using bilirubin oxidase method

The within-run precisions (intra-assay) calculated as the percentage of coefficient of variation (%CV) of total bilirubin determination performed in three different levels of control sera in a Beckman Synchron CX5 autoanalyzer were between 5.18-11.38 %. The between-run %CVs (inter-assay) determined in the same control samples were ranged from 6.75 to 14.88 % , respectively (Table 6).

The within-run precisions of conjugated bilirubin determined in three different levels of control sera in the same automated analyzer were ranged from 0.72 to 5.07%. The %CVs of between-run precisions obtained were 1.06-7.73 %, respectively (Table 6).

Table 6. Analytical performances of the measurements of total and conjugated bilirubin oxidase in a Beckman Synchron CX5 autoanalyzer.

	Level of control material	Control material concentrations (mg/dL)	OCV (Intra - assay) n = 20		RCV (Inter - assay) n = 20	
			Mean±SD	%CV	Mean± SD	%CV
Total bilirubin	1	0.7-1.5	0.6905±0.0786	11.38	0.7035±0.1047	14.88
	2	1.7-2.9	1.4975±0.0950	6.34	1.4675±0.0991	6.75
	3	3.0-5.0	2.5540±0.1322	5.18	2.4485±0.1674	6.84
Conjugated bilirubin	1	0.3	0.2980±0.0151	5.07	0.3130±0.0242	7.73
	2	1.2	1.2825±0.0273	2.13	1.2915±0.0305	2.36
	3	7.2	7.2250±0.0520	0.72	7.2495±0.0767	1.06

Control materials (previously assayed by the reference method) ;

For total bilirubin = Control sera (Decision level 1, 2, 3 : Beckman Instruments)

For conjugated bilirubin = Pooled human sera

Figure 25 and 26 showed the distribution of analytical values of the between-run compared with its within-run variation of the total and conjugated bilirubin determination in each level of control serum analyzing by using Beckman Synchron CX5 autoanalyzer. Almost of the analytical performances were accepted according to the criteria recommened by WHO (Whitehead, 1977).

[3.] Evaluation of the accuracy of total and conjugated bilirubin determination by bilirubin oxidase method

Table 7 and 8 demonstrated the accuracy of bilirubin oxidase method for determination of total and conjugated bilirubin in serum in a Beckman Synchron CX5 autoanalyzer. The average percentages of recovery were 96.17% and 98.01% for total and conjugated bilirubin determination, respectively.

[4.] Linearity of total and conjugated bilirubin determination by bilirubin oxidase method

The dynamic ranges of total and conjugated bilirubin determination by bilirubin oxidase method in a Beckman Synchron CX5 autoanalyzer were reported in Figure 27 and 28, respectively. Total bilirubin values were linear as bilirubin in serum samples were ranged from 0 to 35 mg/dL. The linearity investigated by the analysis of conjugated bilirubin in the same instrument was lower than that of total bilirubin determination. The analytical range for conjugated bilirubin determination was shown to be 0-16 mg/dL.

[5.] The correlation of bilirubin oxidase method for total and conjugated bilirubin determination with the reference method

The correlation of total bilirubin concentrations analyzed (n =142) with bilirubin oxidase method as compared with the Jendrassik & Grof reference method was shown in Figure 29. Good correlation was shown with the regression equation and correlation coefficient (r) equalled to ; $y = 1.0554x - 0.0421$ and $r = 0.986$ ($p < 0.001$), respectively.

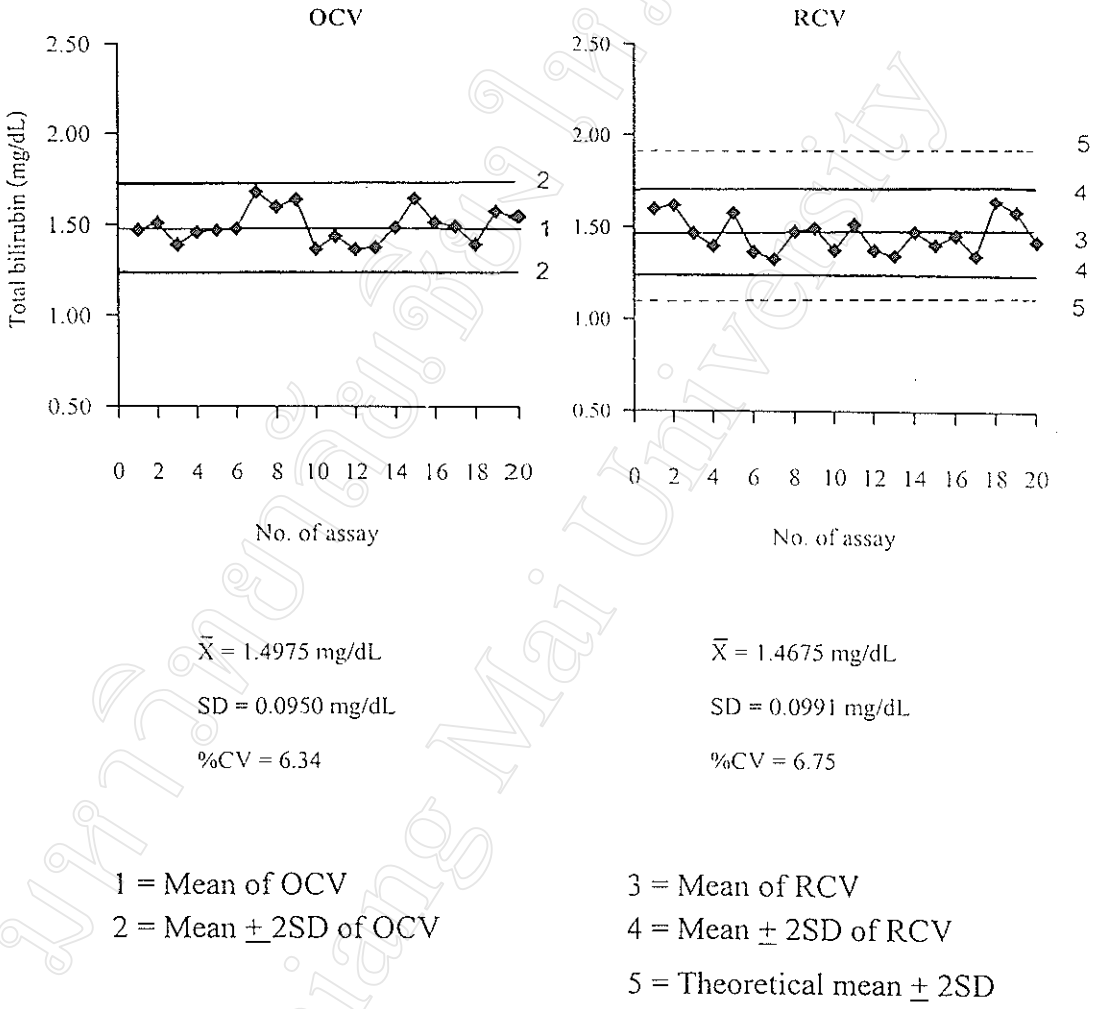
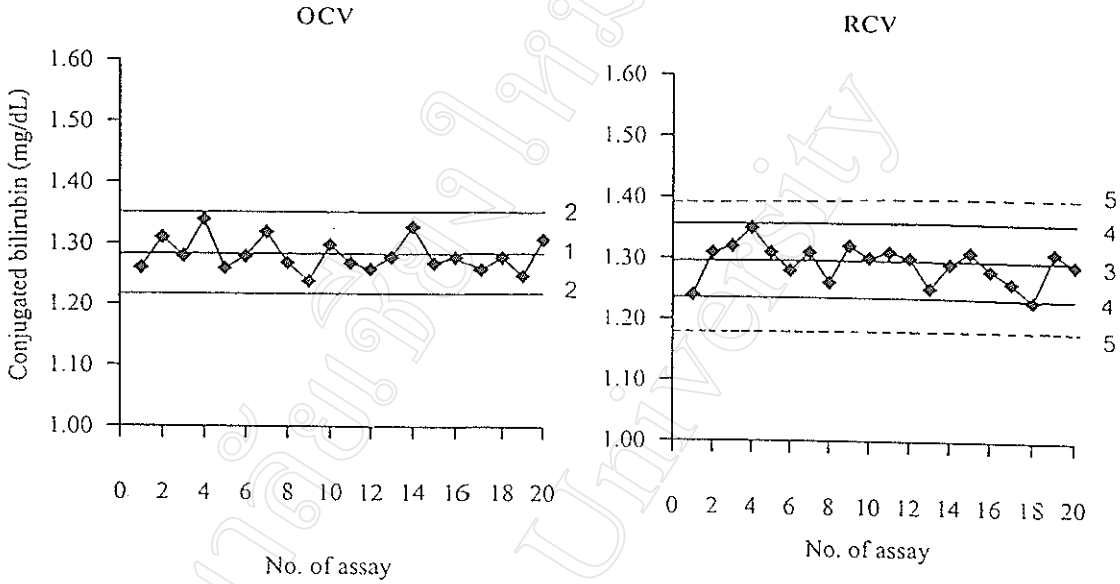


Figure 25. The quality control chart showing intra - and inter -assay precisions of total bilirubin determination using the bilirubin oxidase method .



$\bar{X}$ = 1.2825 mg/dL
SD = 0.0273 mg/dL
%CV = 2.13

$\bar{X}$ = 1.2915 mg/dL
SD = 0.0305 mg/dL
%CV = 2.36

1 = Mean of OCV
2 = Mean $\pm$ 2SD of OCV

3 = Mean of RCV
4 = Mean $\pm$ 2SD of RCV
5 = Theoretical mean $\pm$ 2SD

Figure 26. The quality control chart showing intra - and inter -assay precisions of conjugated bilirubin determination using the bilirubin oxidase method .

Table 7. The accuracy of total bilirubin measurement using enzymatic bilirubin oxidase method.

Samples	Total bilirubin in based serum (mg/dL)	Bilirubin standard added (mg/dL)	Expected values (mg/dL)	Assayed values (mg/dL)	Bilirubin recovered (mg/dL)	Recovery (%)
Serum 1*	0.53	2.34	2.87	2.65	2.16	92.31
		4.67	5.20	5.04	4.55	97.43
		7.01	7.54	7.26	6.77	96.58
		9.34	9.87	9.39	8.90	95.29
		11.68	12.21	11.67	11.18	95.72
Serum 2*	8.99	2.34	11.33	10.58	2.17	92.74
		4.67	13.66	12.92	4.51	96.57
		7.01	16.00	15.28	6.87	98.00
		9.34	18.33	17.59	9.18	98.29
		11.68	20.67	19.94	11.53	98.72
				Total average percentage		96.17

* See appendix

Table 8. The accuracy of conjugated bilirubin measurement using enzymatic bilirubin oxidase method.

Samples	Conjugated bilirubin in based serum (mg/dL)	Conjugated bilirubin added (mg/dL)	Expected values (mg/dL)	Assayed values (mg/dL)	Bilirubin recovered (mg/dL)	Recovery (%)
Serum 1*	0.57	0.95	1.52	1.43	0.94	98.95
		1.90	2.47	2.34	1.85	97.37
		2.85	3.42	3.27	2.78	97.54
		3.80	4.37	4.23	3.74	98.42
		4.76	5.33	5.21	4.72	99.16
Serum 2*	2.74	1.07	3.81	3.74	1.02	95.33
		2.13	4.87	4.76	2.04	95.77
		3.20	5.94	5.87	3.15	98.44
		4.26	7.00	6.95	4.23	99.30
		5.33	8.07	8.04	5.32	99.81
				Total average percentage		98.01

* See appendix

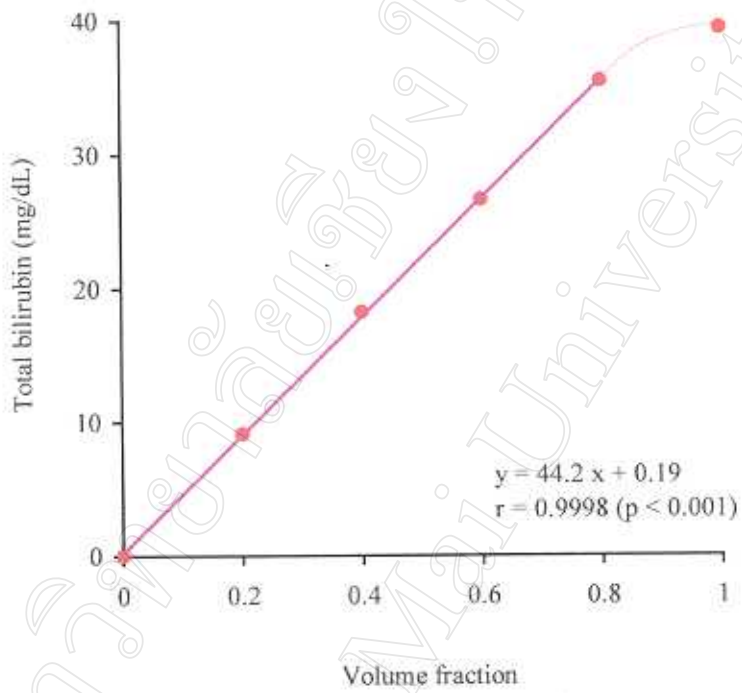


Figure 27. Linearity of total bilirubin determination by BOX method.

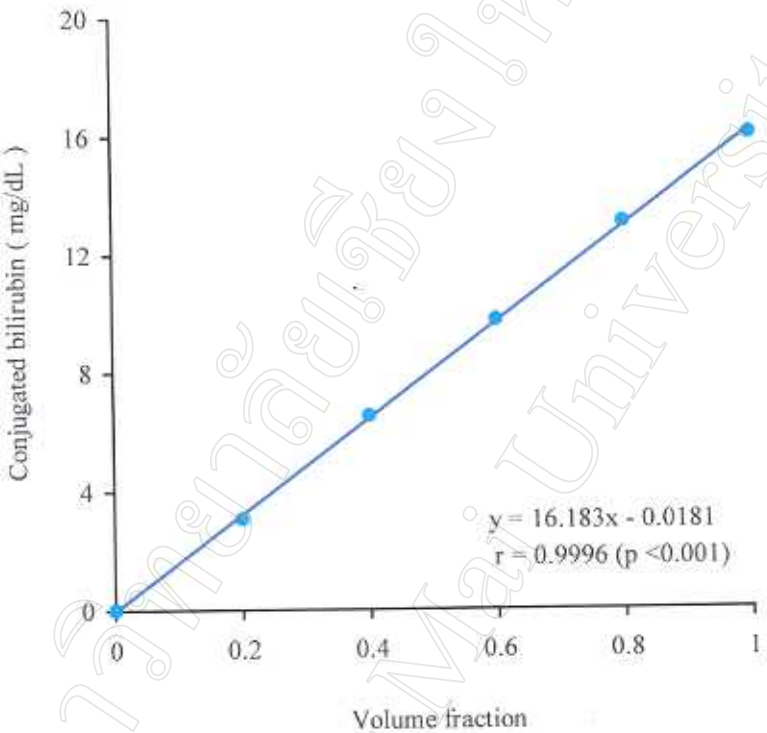


Figure 28. Linearity of conjugated bilirubin determination by BOX method.

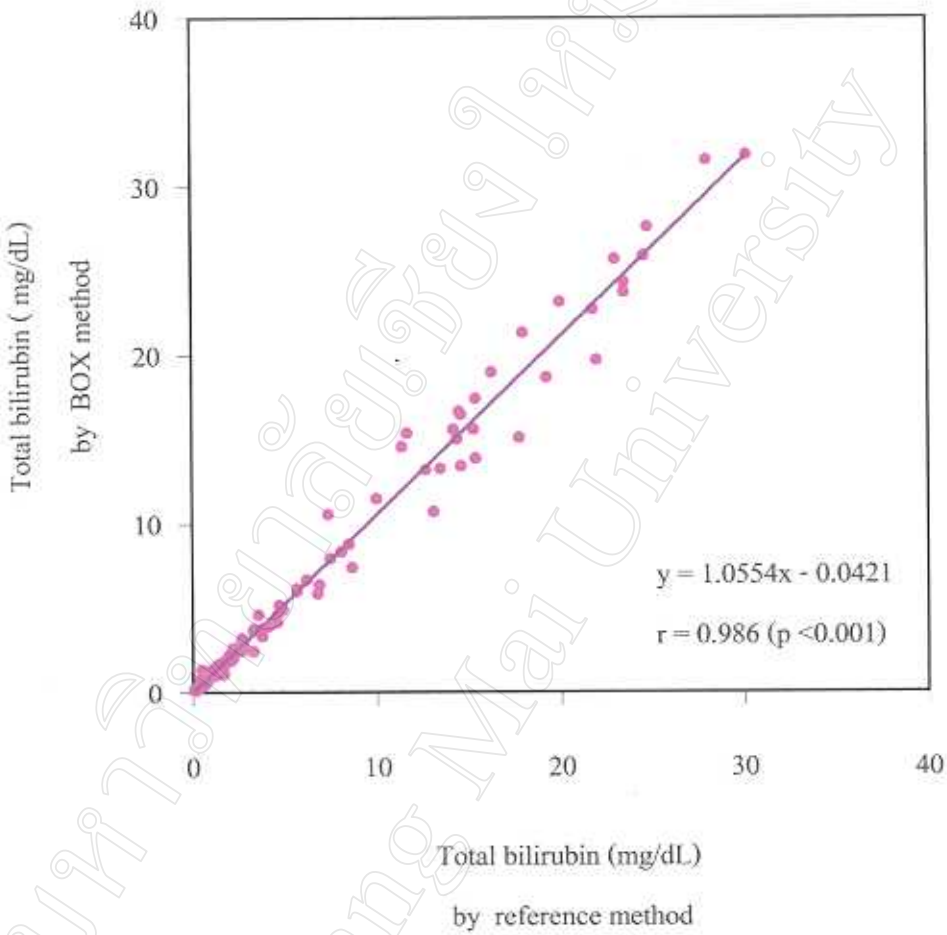


Figure 29. Comparison of total bilirubin values in serum determined by the BOX and reference methods (n =142).

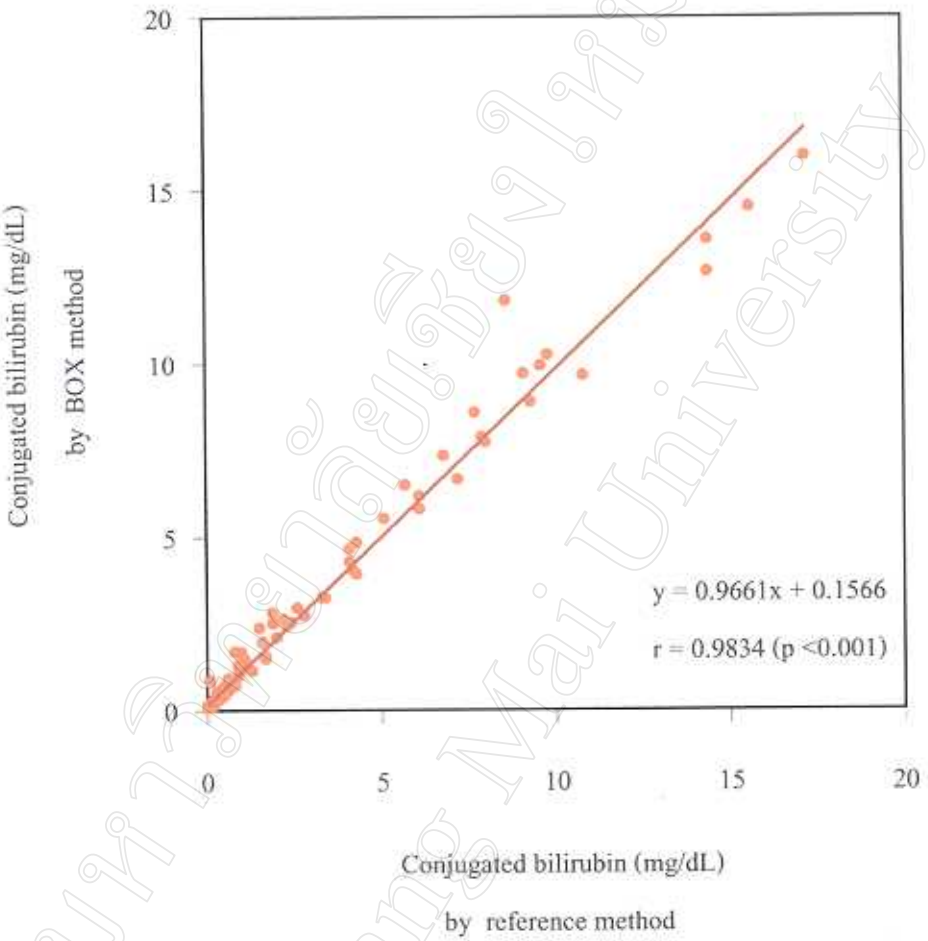


Figure 30. Comparison of conjugated bilirubin values in serum determined by the BOX and reference methods (n =142).

The agreement of bilirubin oxidase method and Jendrassik & Grof reference method (n = 142) for conjugated bilirubin determination in serum was shown in Figure 30. These two methods were correlated at the significant level of $p < 0.001$ and $r = 0.9834$. The equation represented the linear regression line was $y = 0.09661x + 0.1566$.

[6.] Hemoglobin interfering effect on total and conjugated bilirubin determination by bilirubin oxidase method

The interfering effects of hemoglobin on total and conjugated bilirubin determination by bilirubin oxidase method were shown in Figure 31. At low level of conjugated and total bilirubin concentrations in serum (0.32 and 0.87 mg/dL, respectively), hemoglobin at the concentration of 0.8 g/L interfered significantly with bilirubin determinations by the bilirubin oxidase method. The interfering effects of hemoglobin on high levels of both fractions of bilirubin in serum were slightly seen even when the concentration of hemoglobin in pooled serum was raised to 2 g/L.

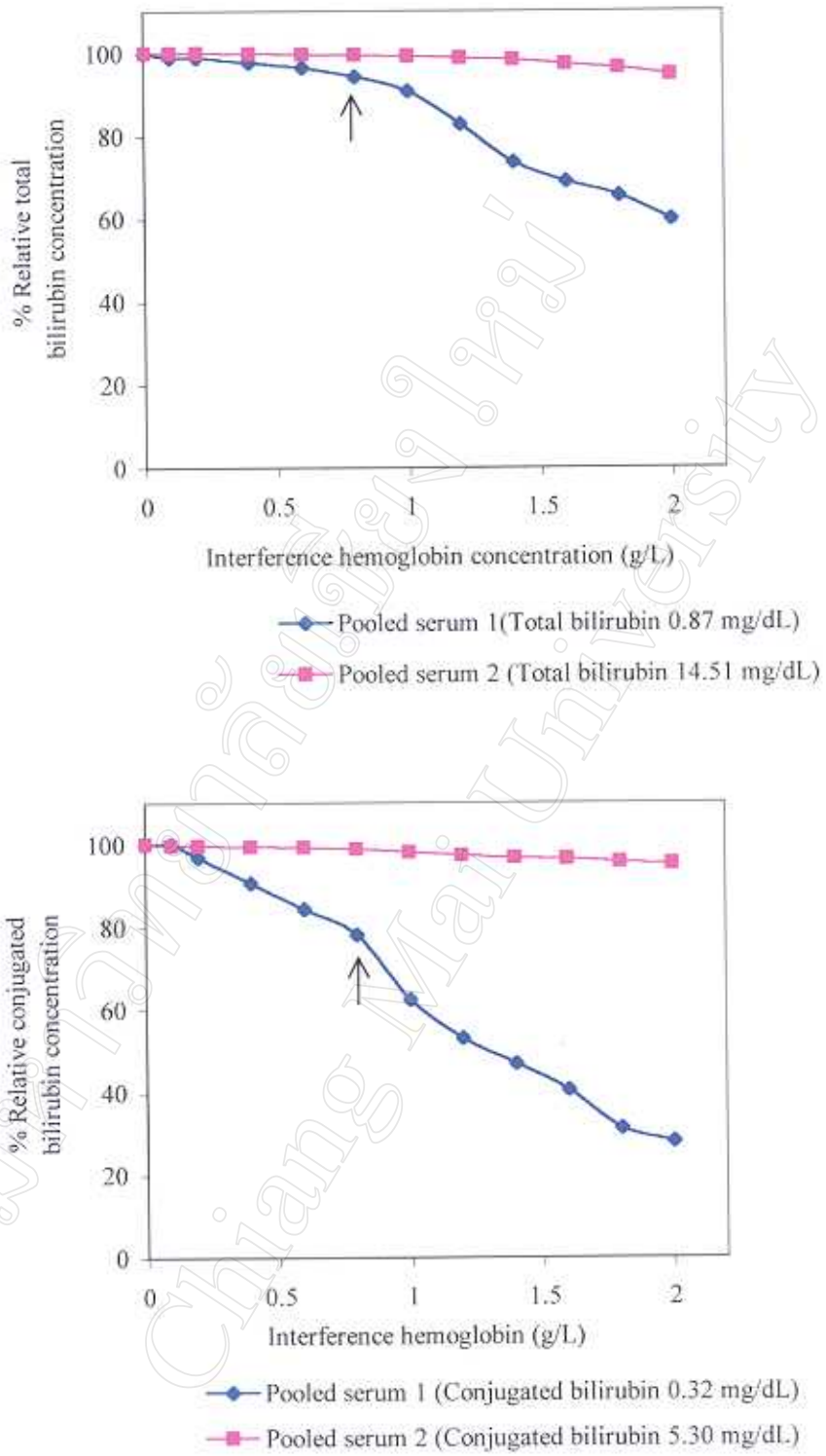


Figure 31. Interference by hemoglobin in determination of total (upper figure) and conjugated (lower figure) bilirubin using bilirubin oxidase method. (↑ demonstrated the starting level of hemoglobin concentration that interfered with bilirubin determination)