III. MATERIALS AND METHODS

A. Materials

1. Specimens :

One hundred-seventy serum samples having normal (0.5 mg/dL) to high levels (>40 mg/dL) of total bilirubin concentrations were obtained from Clinical Chemistry Laboratory, Central Laboratory Division, Maharaj Nakorn Chiang Mai Hospital. They were kept at -20 °C until used.

2. Microorganisms :

Myrothecium verrucaria TISTR 3112 and TISTR 3225 were obtained from Bangkok MIRCEN (Atthasampunna *et al.*, 1995).

3. Instruments :

Instruments used in this study were :

- Adjustable automatic pipette, DLALAB p20, p200, p1000, DLALaboratory, Switzerlands.
- Analytical balance, Mettler H10, Mettler Instrument, Switzerlands.
- Bio-Hazard protection, BH 2000 Series, Cylde Industries, Australia.
- Clinical chemistry autoanalyzer, Synchron CX5, Beckman, USA.
- Fraction collector, Buchler Fractomette Alpha 200, Buchler Instruments, New Jersey, USA.
- Incubator, SL Shel Lab Model 2005, Sheldon Manufacturing Inc., USA.
- Magnetic stirrer, Thermolyne Co., USA.
- Microscope, Olympus, Olympus optical Co., Japan.
- Mini Prep Cell Electrophoretic apparatus, Bio-Rad, USA.
- Motorized automatic pipette, Rainin Instrument Co., USA.
- pH Meter, Model 3560, Darmstadt, Beckman, USA.
- Power supply, Darmstadt, Beckman, USA.

- Shaker incubator, Orbital, Amarex Instruments, USA.
- Stopwatch, Tanita, Fujeribio Inc., China.
- UV-visible recording spectrophotometer UV-160 A, Shimadzu Co., Japan.
- Vortex mixer, Scientific Industries, New York, USA.
- Water bath, Hetobirkerod, Denmark.

4. Chemicals and Reagents :

Chemicals and reagents used were all of the analytical grade. They are listed as follows:

- Acrylamide (Sigma Chemical Co., USA, No. A-8887)
- Albumin, Bovine Serum (Sigma Chemical Co., USA, NO. A-8531)
- Albumin, Bovine, Fraction V (Sigma Chemical Co., USA, No. A-4503)
- Alcohol dehydrogenase (Sigma Chemical Co., USA, No. A-8656)
- Ammonium persulfate crystals : APS (J.T. Baker Inc., USA)
- Ammonium sulfate (Merck, Darmstadt, Germany)
- Bilirubin oxidase (Sigma Chemical Co., USA, No. B-0390)
- Bilirubin standard (Sigma Chemical Co., USA, No. B-4126)
- Blue Dextran (Pharmacia Biotech, USA, No. 17-0442 E)
- Calcium choride anhydrous (Sigma Chemical Co., USA, No. C-4901)
- Charcoal activated powder (Merck, Darmstadt, Germany)
- Citric acid (Merck, Darmstadt, Germany)
- Cytochrome C (Sigma Chemical Co., USA, No. C-7150)
- DEAE-Cellulose (Sigma Chemical Co., USA, No. D-0909)
- DEAE-Sepharose (Sigma Chemical Co., USA, No. DCL-6B-100)
- DL-Lactic acid (Sigma Chemical Co., USA, No. L-1250)
- Glycerol (Biomedicals Inc., USA)
- Glycine (BDH Chemicals, Poole., England, No. 28458)
- Hydrochloric acid (Merck, Darmstadt, Germany)
- N,N,-methylene-bis-acrylamide (Sigma Chemical Co., USA, No. M-7256)
- N,N,N',N'-Tetramethyl-ethylene diamine : TEMED (Sigma Chemical Co., USA, NO. T-8133)

- Potato dextrose agar (Difco Laboratories, USA)
- Potato dextrose broth (Difco Laboratories, USA)
- Sephadex G-100 (Sigma Chemical Co., USA, No. G-100-120)
- Sodium carbonate anhydrous (Merck, Darmstadt, Germany)
- Sodium dodecyl sulphate (Sigma Chemical Co., USA, No. L-5750)
- Sodium hydrogen carbonate (Merck, Darmstadt, Germany)
- Sodium hydroxide (Merck, Darmstadt, Germany)
- Tris [hydroxymethyl] aminomethane (Sigma Chemical Co., USA, No.T-1503)
- Zinc sulphate (BDH Chemicals, Poole., England, No. 10299)

B. Methods

Part I. Methods for cultivation of *Myrothecium verrucaria* for bilirubin oxidase production

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

Myrothecium verrucaria TISTR 3112 and TISTR 3225 were cultured aerobically for 14 days at 25 °C on potato dextrose agar plate (see appendix for media preparation). After incubation, colonies were cultured again on potato dextrose agar to obtain a single pure colony of the microorganism. After that they were identified by the slide culture technique as described by the key in "Illustrated Genera of Imperfect Fungi" (Barnett and hunter, 1972).

[2.] Growth characterization of *Myrothecium verrucaria*

The growth characteristic of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 cultivated on potato dextrose agar plates were investigated. One piece of the pure culture (0.6 cm diameter) prepared by using a cork borer was placed on potato dextrose agar plate and incubated at 25 °C for 7 days. The diameter of colony was measured everyday during the cultivation time. The growth studies were taken in triplicates.

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

3.1 Optimization of incubation temperature for bilirubin oxidase production

The effect of temperature on the enzyme production was investigated by transfering two pieces of mycelium discs (0.6 cm diameter) to a 125 mL Erlenmeyer flask containing 25 mL potato dextrose broth (see appendix for media preparation). The cultivation flasks were kept shaking in a rotary shaker incubator at 20°C, 25°C, 30°C and 37°C for 72 hrs with the shaking speed of 200 rpm. After cultivation peroids, the cultured were squeezed through a preweighted whatman paper no.1 to remove the mycelium. The crude enzyme was collected and the volume was measured. The amounts of protein were determined in the filtrate fractions by reading the absorbances at 280 nm in a Shimadzu UV-160A double beam spectrophotometer. Bilirubin oxidase activities were also assayed. The mycelia separated from the culture media were dried on the whatman paper in an oven at 60°C until constant weights were obtained (Griffin, 1994). In each temperature condition, the cultivation was taken in triplicates. Finally, the optimal temperature was selected for further studies.

3.2 Optimization of cultivation time for bilirubin oxidase production

The effect of time on the enzyme production was examined by transfering two pieces of mycelium discs (0.6 cm diameter) to a 125 mL Erlenmeyer flask containing 25 mL potato dextrose broth and incubated at 25°C in a rotary shaker incubator shaken at 200 rpm for 6, 12, 18, 24, 48 and 72 hrs, respectively. After cultivation periods, the filtrate fractions were collected and the volumes were measured. The protein contents and bilirubin oxidase activities were determined. The constant mycelium dry weights were obtained after drying them at 60°C on the preweighted whatman paper no.1. At different incubation conditions, the cultivations were taken in duplicates. The optimal cultivation time was finally selected for further studies.

3.3 Optimization of pH of the media for bilirubin oxidase production

In this study, the enzyme production was examined by transfering two pieces of mycelium discs (0.6 cm diameter) to a 25 mL of potato dextrose broth in a 125 mL Erlenmeyer flask. The initial pH of media used for cultivation were adjusted to 6.0, 7.0, 8.0 and 9.0, respectively. All flasks with different pH cultivation conditions were incubated at 25°C for 48 hrs in a rotary shaker incubater at the shaking speed of 200 rpm. At the end of cultivation peroids, the fungal mycelia were separated from the culture broth. The mycelium dry weights, the volumes of culture filtrate, the amounts of protein and bilirubin oxidase activities were determined the same way as before. At different pH conditions, the cultivations were taken in duplicates. The optimal pH of cultivation medium was selected for further studies.

For all optimization conditions, the mycelium dry weights were calculated by subtracting the weight of whatman paper from the constant dried weight of mycelium on that of preweighted whatman paper.

Part II. Methods for analysis of protein and bilirubin oxidase enzyme isolated from *Myrothecium verrucaria*

[1.] Protein determination

Protein concentrations in a culture filtrate and eluates were determined by characteristic absorption of UV light at 280 nm using Shimadzu UV-160A spectrophotometer assuming that $E^{1\%}_{1cm}$ at 280 nm was 10.0 (Tanaka and Murao, 1982 ; Johnstone and Thorpe, 1987).

[2.] Methods for determining bilirubin oxidase activity

Standard method (Modified Kosaka *et al.*, 1987)

Bilirubin oxidase activity was determined on a double beam UV-visible spectrophotometer (Shimadzu UV-160A) using the program CPS kinetic at wavelength 450 nm at 37°C. The enzyme activity was assayed as follow ; 2.0 mL of 0.1 mol/L Tris-SDS buffer, pH 8.0 previously

mixed with 100 μL of 10 mg/dL bilirubin standard was added in a cuvet. After warming at 37°C for 5 min in the cell compartment, 100 μL of bilirubin oxidase solution was added into the mixture and mixed thoroughly. The activity of the enzyme was followed at 450 nm, every 10 second interval for 6 cycles. The rate of change of absorbance against time (ΔA/min) was measured and bilirubin oxidase activity was then calculated, using data obtained from a linear portion of the reaction progress-curve.

One Unit of enzyme activity was defined as the quantity of enzyme catalyzing the oxidation of 1 μmole of bilirubin to biliverdin per minute under pH 8.0 at 37°C.

Calculation

The activity of enzyme was calculated from the following formula :

$$\text{Bilirubin oxidase (U/L)} = \frac{\Delta A/\text{min} \times 10^3 \times V \times 10^3}{\varepsilon \times S \times b}$$

where $\Delta A/\text{min}$ = Absorbance change per minute at 450 nm.
10^3 = Conversion of mL to L
V = Total volume of reaction ; 2.2 mL
10^3 = Conversion of millimole to micromole
ε = Molar absorptivity of bilirubin at 450 nm, measured in 0.1 mol/L Tris-SDS buffer pH 8.0, 51058 $L.mol^{-1}.cm^{-1}$(See appendix)
S = Sample volume in mL ; 0.1 mL
b = Light path in cm ; 1 cm

$$\text{U/L} = \frac{\Delta A/\text{min} \times 10^3 \times 2.2 \times 10^3}{51058 \times 1 \times 0.1}$$
$$= \Delta A/\text{min} \times 431$$

Part III. Methods for purification, identification and characterization of bilirubin oxidase from a culture filtrate of *Myrothecium verrucaria*

[1.] Selection of the purification technique

After cultivation, the culture filtrate was centrifuged and the clear supernatant was used as the starting material for purification.

1.1 Ammonium sulfate precipitation (Harlow and Lane, 1988)

Ammonium sulfate precipitation is one of the most commonly used methods for removing proteins from solution. Protein in a culture filtrate form hydrogen bonds with water through their exposed polar and ionic groups. After adding a high concentrations of the ammonium sulfate, the small, highly charged ions such as ammonium or sulfate dissociated compete with the proteins in culture filtrate for binding to water. This removes the water molecules from protein and decreases its solubility, resulting in precipitation.

In this experiment, 182 g of finely powdered ammonium sulfate was added to a 300 mL of the culture filtrate to 80% saturation under stirring and the mixture allowed to stand overnight at 4°C. Then, the mixture was centrifuged at 3,000 g for 30 min at 4°C. The precipitates were collected and resuspended with 40 mL of distilled water. The precipitated solution was dialyzed against three changer of 1 L of the 10 mM Na_2CO_3-$NaHCO_3$ buffer, pH 9.2 for 24 hrs at 4°C under stirring and then clarified by centrifugation at 3,000g for 10 min at 4°C. The supernatant was carefully removed and collected for protein and bilirubin oxidase activity determination. The dialyzed solution was further decolorized by treatment with 0.7% (w/v) and 0.2% (w/v) of activated charcoal powder, respectively. After protein concentrations and bilirubin oxidase activities were determined in the clear enzyme solutions, the calculated yield bilirubin oxidase recovery was compared with that obtained from the ammonium sulfate precipitation step.

1.2 Clarification by adsorption on activated charcoal

The culture filtrate obtained from cultivation of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 were centrifuged at 1,000g for 10 min. The clear supernatant was used for bilirubin oxidase purification.

In this experiment, a 330 mL of the supernatant was added to 2.31 g of activated charcoal powder (0.7% w/v) which previously weighed in a 1,000 mL Erlenmeyer flask. Under stirring condition, the mixture was allowed to stand for 10 min at room temperature. After that, the mixture was filtered through a whatman paper no.1 and the colorless filtrates collected were investigated for the protein contents and bilirubin oxidase activities. Then, this collected enzyme solution was repeatedly treated with the 0.2% w/v activated charcoal using the same precedure as before. The amount of protein and bilirubin oxidase activity per total volume of the filtrate in the treated solution were dertermined. The specific activity calculated as the ratio of unit of enzyme activity per gram of total protein content of each charcoal treatment step was compared.

1.3 DEAE-Cellulose anion exchange chromatography (Modified from Guo *et al.*, 1991)

Ion exchanger chromatography separates proteins according to their net charges. Using this purification technique, the proteins in a culture filtrate with a high net negative charges have a high affinity for DEAE-Cellulose, are bound strongly to the column. The protein with low net negative charges which bound loosely are thus eluted earlier as the ionic strength of the eluate is raised.

Preparation of DEAE-Cellulose column

DEAE-Cellulose, which is a weak tertiary amine resin, was treated sucessively with 0.5 mol/L HCl and 0.5 mol/L NaOH for 20 min and then equilibrated with 0.05 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 9.2 overnight at 4°C. The DEAE-Cellulose was packed into a column (2.0 x 8.5 cm) and equilibrated with 0.05 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 9.2 for 3 hrs before used .

Chromatographic method

The culture filtrate of *Myrothecium verrucaria* (350 mL) was loaded on top of a DEAE-Cellulose column and let it flowed through a column at a flow rate of 2 mL/min. After that, the enzyme was eluted, at the flow rate of 1 mL/min, with a concentration gradient of Na_2CO_3-$NaHCO_3$ buffer, pH 9.2; progressively with 60 mL of 0.05 mol/L, 120 mL of 0.1 mol/L, and 300 mL of 0.2 mol/L Na_2CO_3-$NaHCO_3$ buffer, respectively. The eluates were collected as a 2 mL fraction by a fraction collector (Buchler fractomette Alpha 200). For identification of peak fraction, the absorbances at 280 nm of each eluate was measured and the bilirubin oxidase enzyme activity was determined. The active protein fractions were pooled as pooled fraction (PF)1, 2 and 3, respectively for the further second purification on a smaller DEAE-Cellulose column chromatography.

1.4 DEAE-Sepharose anion exchange chromatography (Modified from Guo *et al.*, 1991)

Preparation of DEAE-Sepharose column

The DEAE-Sepharose DCL-6B gel suspension was packed into a column (2 x 8 cm) and washed several times by 0.05 mol/L Na_2CO_3-$NaHCO_3$ buffer, pH 9.2 and then was equilibrated with the same buffer for 3 hrs before used.

Chromatographic method

A 120 mL of culture filtrate of *Myrothecium verrucaria* was mixed with 40 mL of 0.2 mol/L pH 9.2, Na_2CO_3-$NaHCO_3$ buffer before loading on the DEAE-Sepharose column. The enzyme was eluted with a concentration gradient of Na_2CO_3-$NaHCO_3$ buffer, 40 mL of 0.05 mol/L, 60 mL of 0.1 mol/L and 120 mL of 0.2 mol/L at the flow rate 1 mL/min, respectively. The eluates were collected in 2 mL fraction. Protein concentrations and bilirubin oxidase activities in the eluates were determined using the same procedure as before. Active protein fractions were pooled for further studies.

1.5 Comparison of purification techniques for bilirubin oxidase enzyme preparation

Four techniques used for bilirubin oxidase purification as stated in 1.1-1.4 were compared for yield of bilirubin recovery. The technique provided the highest quantity of enzyme was chosen to prepare the enzyme solution for further clinical application.

[2.] Identification of bilirubin oxidase enzyme by Mini Prep cell electrophoresis

In this study, Mini Prep cell electrophoresis was used for identification of bilirubin oxidase produced by *Myrothecium verrucaria* TISTR 3112 and TISTR 3225. The patterns of protein eluted were compared with that of a commercial bilirubin oxidase obtained from Sigma chemical, USA.

The Mini Prep cell purifies bilirubin oxidase from complex protein mixtures by continuous-elution electrophoresis. The procedure was summarized as follows.

1. Preparation of polyacrylamide gel tube for Mini Prep Cell electrophoresis

The gel tube was filled in from the bottom to the top, 9.0 cm height, with polyacrylamide gel solution. After polymerization the stacking gel was laid over approximately 1 cm height and let to polymerize for 1-2 hrs before equipping in a Mini Prep Cell apparatus.

The resolving gel solution, polyacrylamide gel mixture and the stacking gel were prepared according to the operation manual of Mini Prep Cell electrophoresis (Mini Prep Cell instruction manual).

2. Sample preparation

A 0.08 mL of culture filtrate was solubilized in a 0.120 mL sample buffer containing 0.5 mol/L Tris-HCl, pH 6.8, glycerol, 10% (w/v) SDS and 0.5% bromophenol blue. After the buffer reservoirs were filled in with the working electrode running buffer, 0.025 mol/L Tris-SDS, pH 8.3, the sample solution was gently loaded on the stacking gel surface using a submerged technique. As soon as the lid was covered and the cables were attached to the power supply, the Mini Prep Cell apparatus was prepared readily to perform the electrophoretic separation of bilirubin oxidase from the protein solution.

3. Electrophoretic separation of bilirubin oxidase

An electrophoretic separation of bilirubin oxidase was carried out with a constant voltage of 220 V for 2-3 hrs. For principle, the enzyme electrophoresed through a cylindrical gel. As molecules migrated through the gel matrix, they separated into bands. Individual bands migrated off the bottom of the gel where they passed directly into the elution chamber consisting of a thin polyethylene frit. A dialysis membrane, directly underneath the elution frit, trapped proteins within the chamber. A 0.025 mol/L Tris-SDS, pH 8.3, which used as an elution buffer entered the chamber around the perimeter and then was drawn radially inward to an elution tube. The purified enzyme molecules which drawn down and out through the elution tube by a peristalic pump were collected in a 1 mL fraction by a fraction collector. For identification of bilirubin oxidase enzyme, the absorbance at 280 nm of protein eluted in a 1 mL fraction and bilirubin oxidase activities containing in each eluted peaks were measured. The pattern of protein elutions separated from both strains were compared with the pattern of protein separated from a commercial enzyme obtained in a separate run.

[3.] Characterization of purified bilirubin oxidase enzyme

3.1 Molecular weight determination by gel filtration (Andrew, 1964; Andrew, 1965)

This method is principally based on a linear relationship between the relative elution volume of a substance and the logarithm of its molecular mass over a considerable molecular mass range (Voet *et al.*, 1995). If a plot is made for a particular gel filtration column using macromolecules of known molecular masses, the molecular mass of an unknown substances can be estimated from its position on the plot.

The determination of molecular weight of bilirubin oxidase was performed using Sephadex G-100 column chromatography. In this study, Sephadex G-100 gel filtration medium was treated with 0.2 mol/L NaOH and equilibrated with 0.02 mol/L Na_2CO_3-$NaHCO_3$ buffer (pH 9.2) overnight. The gel was packed into a column (1.1 x 40 cm) and then left it in the cold (2-5C°) until used. Five millilitres of pooled purified enzyme fraction from DEAE-Sepharose separation was applied to top of the Sephadex G-100. The enzyme was eluted with 0.02 mol/L Na_2CO_3-$NaHCO_3$ buffer (pH 9.2) at the flow rate of 0.5 mL/min and the eluates were collected in a 1 mL fraction. The absorbance at 280 nm of each fraction was measured and plotted for peak identification. Cytochrome C (12.4 kDa), bovine serum albumin (66 kDa), and alcohol dehydrogenase (150 kDa) were used as the reference proteins of known molecular mass. The void volume (Vo) was determined by blue dextran. The molecular weight of bilirubin oxidase was investigated from the plot of log of molecular weights versus the relative elution volumes (Ve/Vo) of three molecular weight markers.

3.2 Bilirubin oxidase kinetic studies

The kinetics of bilirubin oxidase were determined using total bilirubin standard as substrate. Bilirubin substrate concentrations were varied while keeping the bilirubin oxidase activity contant. The assay solution consisted of 2.0 mL of 0.1 mol/L Tris-SDS buffer, pH 8.0, 100 µL of bilirubin standard (See appendix), and 100 µL of bilirubin oxidase solution from the purified enzyme (23.27 U/L). The reaction at 37 °C was initiated by adding bilirubin oxidase solution and reading the decrease in absorbance at 450 nm in a double beam UV-visible spectrophotometer using the program CPS kinetic. The maximum

reaction rates (ΔA/min) were calculated from the initial linear region of the reaction curves. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of purified enzyme was calculated from the Lineweaver-Burk double reciprocal plot.

The kinetics of bilirubin oxidase using conjugated bilirubin substrate was also examined. The procedure was as that previously described except for the buffer used was 0.1 mol/L Lactic acid, pH 3.7 and bilirubin substrate was the conjugated bilirubin obtained from a pooled human serum (See appendix).

3.3 Effect of metallic ion and compounds on bilirubin oxidase activity

The effect of $ZnSO_4$, $CaCl_2$ and BSA on bilirubin oxidase activity were examined. In experiment, 1 mmol/L $ZnSO_4$ prepared in 0.1 mol/L Tris-SDS buffer (pH 8.0) was diluted to volume ratios of 1 : 4, 2 : 4, 3 : 4 and 4 : 4 with 0.1 mol/L Tris-SDS buffer, pH 8.0 to obtain 0.23, 0.45, 0.68 and 0.91 mol/L of $ZnSO_4$, respectively in the final 2.2 mL reaction mixtures (See appendix). After gently mixed, 2.0 mL of mixtures which previously mixed with 100 μL of 10 mg/dL bilirubin standard substrate was added in a cuvet and placed into the cell compartment of a double beam UV-visible spectrophotometer to warm at 37 °C for 5 minutes. After that, 100 μL of the purified bilirubin oxidase solution (BOX activity 23.27 U/L) was added into the mixture and mixed thoroughly. The activity of the enzyme was measured spectrophotometrically at 450 nm, every 10 second interval for 6 cycles. The remaining activity was then calculated as percentage of relative activity of the control (used 2.0 mL of 0.1 mol/L Tris-SDS, pH 8.0 without $ZnSO_4$ as the assayed buffer).

The effect of $CaCl_2$ or BSA on bilirubin oxidase activity was performed in the same manner as $ZnSO_4$.

Part IV. Application and evaluation of bilirubin oxidase methods 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

1.1 Optimization of bilirubin oxidase concentration for the oxidation reaction of bilirubin in serum

Optimization of bilirubin oxidase concentration used for the oxidation of bilirubin in serum was determined in a Beckman Synchron CX5 autoanalyzer. The bilirubin oxidase was assayed from the lowest to the highest level concentrations in a 210 μL final reaction mixture, using different calibrators and conditions as indicated in Table 1.

1.2 Calibrators for total and conjugated bilirubin determination, carried out on the Beckman Synchron CX5 autoanalyzer

A commercial calibrator, value of 9.1 mg/dL from Beckman Instruments was used to set the instrument for the determination of total bilirubin by the enzymatic and the reference diazo method (Jendrassik and Grof, 1938).

For enzymatic determination of conjugated bilirubin in serum, two calibrators, values of 0.3 mg/dL and 7.2 mg/dL, obtained from previous analysis of pooled human serum by the Jendrassik-Grof reference method in a Synchron CX5 was used to calibrate the instrument for every automatically run (Table 1).

1.3 Method for automation of bilirubin fractions in serum, using bilirubin oxidase isolated from *Myrothecium verrucaria* TISTR 3112 and TISTR 3225

Bilirubin oxidase isolated from culture filtrates of *Myrothecium verrucaria* were used to prepare analytical reagents for bilirubin determinations without further purification. The proposed enzymatic

procedures which performed on a Beckman Synchron CX5 (Beckman Instruments) were modified from Part II of the materials and methods. Parameters for the instrument setting were carried out along with the optimization of enzyme concentration for each bilirubin determination which also elucidated in Table 1. After setting the parameters and calibrating the instrument with the different calibrators selected for each bilirubin determination, the enzymatic methods for total and conjugated determinations in serum were evaluated for the precision, accuracy, linearity and correlation with the recommended reference method running in the same instrument.

1.4 Reference method (Beckman Instruments, Jendrassik-Grof procedures)

Sera containing both total and conjugated bilirubin previously assayed by the proposed enzymatic method were measured again for bilirubin concentrations by the reference method (Jendrassik and Grof, 1938) in the same Beckman Synchron CX5 autoanalyzer. The assay conditions were carried out according to the manufacturer's instruction manual (Beckman instrument operation manuals).

[2.] Evaluation of the precision of bilirubin oxidase method for total and conjugated bilirubin determination on a Beckman Synchron CX5 autoanalyzer

The within-run assay or optimal condition variation (OCV) of total bilirubin determination was studied by analyzing in 20 identical aliquots of three level control materials, obtained from Beckman Instruments. Control materials having low, medium and high total bilirubin values were assayed in a Synchron CX5 autoanalyzer. Between-run assay or routine condition variation (RCV) was determined using the same control materials as the OCV. The RCV were performed simultaneously along with samples in each automatically run in the same autoanalyzer. The precision of the assay was evaluated from the percentage of coefficient of variation (% CV) calculated by the formula below (Kringle, 1996) .

$$\% \text{ CV} = \frac{SD}{\bar{X}} \times 100$$

Table 1. Optimization condition studies for bilirubin determination using bilirubin oxidase enzymatic method in a Beckman Synchron CX5 autoanalyzer.

Conditional examinations	**Total bilirubin**	**Conjugated bilirubin**
Observed methods (Beckman Instruction manual)	Endpoint 1 Endpoint 2 Rate 2	Endpoint 1 Endpoint 2 Rate 2
Primary/secondary Wavelengths	470/670 470/520	470/520
Bilirubin oxidase concentrations	10-200 U/L* in 0.1 mol/L Tris-SDS buffer, pH 8.0	5-120 U/L* in 0.1 mol/L Lactic acid buffer, pH 3.7
Calibrators	-Beckman TB calibrator 9.1 mg/dL -Standard bilirubin 2-20 mg/dL	-Beckman DB calibrator 7.49 mg/dL -Pooled human serum DB calibrator (0.3, 1.7, 7.2, 9.6, 18 mg/dL) -Dade Moni-Trol DB 0.29 mg/dL
Sample volume	0.01 mL	0.01 mL
Reagent volume	0.2 mL	0.2 mL
Reagent blank reading -start/end read (seconds)	60/90 120/150 330/360	250/280 330/360
Reaction reading -start/end read (seconds)	40/70 60/150 120/150	30/60 120/150 40/70 240/270 90/120

* The lowest to the highest of the observed bilirubin oxidase concentrations.

The precision of conjugated bilirubin by bilirubin oxidase method was performed in 20 identical aliquots of three level pooled human serum controls in different buffer. The precision study was performed in the same manner as the precision of total bilirubin determination.

[3.] Evaluation of the accuracy of bilirubin oxidase method for total and conjugated bilirubin determination on a Beckman Synchron CX5 autoanalyzer

The accuracy of total and conjugated bilirubin were determined by adding known amounts of total and conjugated bilirubin, either from bilirubin standard or pooled human serum, to two different pools of human based sera. The methods of preparation were shown in appendix.

The accuracy of the assay was evaluated by the average percentage of recovery which was calculated according to the following formula :

$$\% \text{ Recovery} = \frac{C}{D} \times 100$$

C = Concentration (mg/dL) of bilirubin in standard or plooled human serum recovered, as measured by the bilirubin oxidase method.

D = Concentration (mg/dL) of bilirubin added obtained from calculation.

[4.] Evaluation of linearity of bilirubin oxidase method for total and conjugated bilirubin determination on a Beckman Synchron CX5 autoanalyzer

The linearity of the bilirubin oxidase methods for total and conjugated bilirubin determinations were evaluated by analyzing serial dilutions (1 : 5, 2 : 5, 3 : 5, 4 : 5 and 5 : 5) of patient's serum specimen with high total bilirubin concentration (39.88 mg/dL) and serial dilutions of patient's serum specimen with high conjugated bilirubin concentration (16.09 mg/dl), respectively (For preparations, see appendix). Results obtained were plotted against the volume fractions.

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

In correlation experiment, 142 serum samples were analyzed for total and conjugated bilirubin concentrations by the bilirubin oxidase method comparing with the reference method (Jendrassik and Grof, 1938). Results obtained were plotted for a linear regression line and calculated for correlation coefficient (r).

[6.] Interference of hemoglobin on bilirubin oxidase method for determination of total and conjugated bilirubin in serum

The interfering effects of hemoglobin on total and conjugated bilirubin determination were evaluated. By adding various known amounts of hemoglobin solutions, prepared by freezing and thawing erythrocytes in water, to two different pooled human sera (See appendix), the enzyme activity was assayed using total bilirubin standard or pooled human serum containing conjugated bilirubin fraction as substrates. Results obtained were plotted against the concentrations of hemoglobin in serum.

Part V. Statistical analysis

All data were shown as the average of duplicate or triplicate (mean $\pm$ SD). The correlation coefficients, the plot of linear regression equation and the comparison studies performed by the analysis of student unpaired t-test (Kringle,1996) were analyzed statistically using the statistical function in the Microsoft Excel 7.0 program on a Microsoft windows 97 in a personal computer.