V. DISCUSSION

Bilirubin oxidase, used for enzymatic determination of bilirubin fractions in serum, was primarily isolated from *Myrothecium verrucaria* MT-1 (Murao and Tanaka, 1981). This strain produces more potent bilirubin oxidase enzyme than those of IFO 6113, IFO 6133, IFO 9531 (Murao and Tanaka, 1982) and the strain Mv 2, 1089 (Guo *et al.*, 1991), respectively. In this study, two strains of *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 which is collected at the Bangkok MIRCEN, Thailand, were used as available sources for producing bilirubin oxidase. The temperature and time for cultivation and pH of the medium were studied in order to select the most optimal conditions. From results, at 25 °C of cultivation temperature, growth of strain TISTR 3112 was best, while strain TISTR 3225 showed the highest growth at 30 °C. Results from mycelium dry weights were agreed with these observations. However, the cultivation temperature at 25 °C was selected for both strains because of the highest yield of bilirubin oxidase productions. This selected temperature was similar to that of the strain MT-1 (Murao and Tanaka, 1982).

During the cultivation, it is seen that the growth of microorganisms were maximum at 72 hrs. The protein productions were increased correspondingly with the growth rates. In distinguishness with the protein concentration, maximum bilirubin oxidase activity, determined as a unit of enzyme per total protein production or total bilirubin oxidase activities per total volume of a culture filtrate, were demonstrated at 48 hrs and then dropped down at 72 hr cultivation. From the previous study for time course of enzyme production by the strain MT-1, it was shown that the maximum enzyme production (U/mL of a culture filtrate) was approximately at 50 hrs whereas the dry cell weight was observed at 62 hrs of cultivation (Murao and Tanaka, 1982). From all studies, it could be suggested that bilirubin oxidase was synthesized, accumulated in the mycelium and then excreted into the medium at 48 hrs (or 50 hrs for Murao and Tanaka, 1982) of cultivation. The prolonged increasing of total protein synthesis at longer cultivation time may resulted from heterologous protein (enzyme) synthesis. For instance, cellulase was reported to produce remarkedly by the strains TISTR 3112 and TISTR 3225 which used as sources for bilirubin oxidase production in this study (Atthasampunna *et al.*, 1995).

The yields of bilirubin oxidase obtained from both strains were less as compared with the strain MT-1 and Mv 2, 1089 (Murao and Tanaka, 1982; Guo *et al.*, 1991). It could be discussed that strain TISTR 3225 and TISTR 3112, may have distinct metabolism from the strain MT-1 or Mv 2, 1089. Therefore, the other important environment condition, that is media for cultivation, should be examined to obtain excellent bilirubin oxidase formation. As shown in the previous studies, the strain MT-1 (Murao and Tanaka, 1981) and Mv 2, 1089 (Guo *et al.*, 1991) utilized different culture medium. The strain MT-1 produced maximum bilirubin oxidase in pH 5.5 medium containing 40 grams of potato and 1 gram of glucose in 100 mL distilled water whereas strain Mv 2, 1089 used 20% potato extract containing 0.25% glucose, 0.25% peptone, and 0.05% Triton X-100, pH 6.0 as a culture medium. Alternatively, there has been demonstrated that chosen strain may then be subjected to mutational influences in order to generate mutants which hyperproduce the bilirubin oxidase enzyme (Shimizu *et at.*, 1999).

Separation of the enzyme of interest from its biological environment requires a series of purification steps (Weelwright, 1989). Each step may lead to an increase in product purity but is accompanied with some loss of the target proteins leading to increase costs. In cases of Murao and Tanaka, 1982 and Shimizu *et al.*, 1999, the whole procedure schemes consisted of 3 steps. The first steps involved crude fractionation, clarification and concentration of bilirubin oxidase using processes of ammonium sulfate precipitation and adsorption on activated charcoal. The yield recovery from the first step was then taken through the second step of purification using the high resolution ion-exchange chromatography and the final step was the gel filtration in which the undesired products were removed and prepared the purified bilirubin oxidase for identification. Since the whole procedure was cost-effective, therefore Guo *et al.*, 1991 minimized the number of steps to purify bilirubin oxidase by subjecting a culture filtrate directly through a DEAE-Cellulose column chromatography. After the active fractions were pooled, the enzyme solution was placed onto a gel filtration column which was the final step of purification. In this study, each step of purification was evaluated separately in order to prepare the low cost of bilirubin oxidase product for clinical application.

Addition of high concentration of ammonium sulfate in the culture filtrate was expected to promote bilirubin oxidase salting out from the protein solution. Tanaka and Murao (1982) suggested to used a 80 % ammonium sulfate saturation for the first step purification. In this

present study, the Murao and Tanaka procedure was followed but small amount of bilirubin oxidase enzyme was recovered. This procedure was repeated several times but the same result was obtained. It was suggested that this precipitation technique usually gives a high yield of product purification (England and Sciefter, 1990). Thus low yield of bilirubin oxidase obtained in this experiment may be due to instability of the enzyme during the dialysis. In the other experiment, 2 steps of ammonium sulfate precipitation were made using a 50 % and 70 % saturation, respectively. The yield recovery was satisfactory enough to subject to further purification (Shimizu, 1999).

In adsorption chromatography, the nonpolar adsorbent such as activated charcoal has been used to purify and clarify the impurities from the protein mixture sample. In this study, activated charcoal was used to purify bilirubin oxidase in a culture filtrate. In addition to capturing some protein impurities, the color pigments were also removed. This purification step gave the highest yield of bilirubin oxidase as compared with the other purification techniques used in this study. Because of its simple technique, low cost preparation and the enzyme solution product was free from ionic species containing in the buffer used as eluent in chromatographic purification technique, therefore it was used as a method of choice in preparation of enzyme-buffer solution in this study.

Anion exchange chromatography has proven to be most popular technique to purify the protein of interest from the protein mixture. Guo *et al.* (1991) used two DEAE-Cellulose columns to purify bilirubin oxidase from a protein mixture. The large column used to separate bilirubin oxidase from a culture filtrate whereas the small column used to concentrate bilirubin oxidase in the purified enzyme solution. This chromatographic procedure could not be repeated by this present study because low of bilirubin oxidase was obtained from the second column purification (data not shown). The course of low yield elution may be due to the different in gel matrix and the column sizes from that of Guo's method (Guo *et al.*, 1991). These differences resulted in the lowering exchange of charges on the stationary phase and then fail to purify bilirubin oxidase from the protein mixture. Although, DEAE-Sepharose column was better than DEAE-Cellulose to purify bilirubin oxidase from a culture filtrate, but the efficacy of matrix is still less than QAE Sephadex which used to purify bilirubin oxidase by Murao and Tanaka (1982). The wet bead size (μ) and capacity of the exchange space (meq/g) of the DEAE-Sepharose is shown to be less than QAE Sephadex (Sigma-Aldrich).

By using Sephadex G-100 gel filtration chromatography, the final step of purification was carried out . The purified fractions from DEAE-Sepharose separation which subjected to this type of column chromatography was eluted in one homogenous peak. This result when plotted against the known molecular weight enzyme markers, the molecular weight of the bilirubin oxidase enzyme could be estimated. The molecular weight of 49,000 which obtained from bilirubin oxidase enzyme isolated from strain TISTR 3112 and TISTR 3225 was lower than 52,000 that observed from the strain MT-1 (Tanaka and Murao, 1982). The molecular weight determination of bilirubin oxidase by SDS-PAGE of strain MT-1 was differed and reported as 66,000 which also higher than 61,900- 62,700 of the strain Mv2, 1089 (Guo *et al.*, 1991). Discrepancies in molecular sizes of bilirubin oxidase purified from different strains may be derived from the different in posttranslational glycosylation (Bilirubin oxidase is an glycosylated protein, Shimizu *et al.*, 1999).

In this experiment, the identification of bilirubin oxidase isolated from *Myrothecium verrucaria* TISTR 3112 and TISTR 3225 were performed using minipreparative electrophoresis. This technique used to purify and prepare small amount of protein from the protein mixture such as antibodies (Mini Prep Cell instruction manual). By analyzing bilirubin oxidase from a culture filtrate of strain TISTR 3112 and TISTR 3225 and a commercial enzyme in a separate run by using the same procedure of electrophoresis, two elution peaks obtained were identical with each other. This result was agreed with the PAGE technique (Guo *et al.*, 1991), in which 2 bands of bilirubin oxidase were obtained from crude enzyme isolated from Mv 2, 1089.

Bilirubin oxidase isolated from strain TISTR 3112 and 3225 were evaluated for enzyme properties. It was shown that the enzyme used both forms of bilirubin substrate. K_m values obtained from kinetic studies were 84.2 μmol/L and 135 μmol/L for total and conjugated bilirubin substrates, respectively. These results were lower than 190 μmol/L obtained from strain MT-1 (Murao and Tanaka, 1982) and more or less the same as 94 μmol/L obtained from strain Mv 2, 1089 (Guo *et al.*, 1991). As stated in Text, K_m values is not a fix value, it may vary with the substrate structure, pH and temperature of reaction . The smaller K_m indicated a greater affinity of the enzyme for its substrate (Lehninger, 1970). This result was applied for the estimation of bilirubin oxidase concentration for determination of serum bilirubin using automated chemistry analyzer. As reported, as low as 15-20 U/L of

enzyme concentration in the final reaction was required for total and conjugated bilirubin analyzing in a Beckman Synchron CX5 autoanalyzer.

Apart from utilization of bilirubin substrate, bilirubin oxidase activity was evaluated for inhibitory effect of metal ion and compounds. The enzyme activity was found to inhibit by divalent ions such as Zn^{2+}, Ca^{2+} and compound such as BSA. As mentioned in the introduction that bilirubin oxidase is a metal containing enzyme. It contains multicopper in its molecule similar to laccase, ascorbate oxidase and ceruloplasmin (Shimizu, 1999). If the enzyme was incubated with these ions or compound, it inhibited enzyme activity by complexing with copper on the enzyme molecules and thus changed the enzyme conformation which lower binding sites for bilirubin substrate interaction (Tanaka and Murao, 1982). This is the valuable information for the application of enzyme in determination of bilirubin in serum. Other ions such as Fe^{+2}, Cu^{+2} ($CuSO_4$) and Hg^{+} (HgCl) has also been reported to inhibit bilirubin oxidase activity (Guo *et al.*, 1991). Patient's serum containing these ions more than 10 mmol/L resulted in underestimating of bilirubin concentration by the bilirubin oxidase method.

The application of bilirubin oxidase to use for bilirubin determination in serum in automated analyzer was primarily reported in 1986 (Perry *et al.*, 1986). The enzymatic method using bilirubin oxidase concentration 4.78 U/mL was applied on the Cobas-Bio centrifugal analyzer (Roche Analytical Instruments, Inc., Nutley, NJ 07110). Although the method was correlated well with the diazotization method but it was limited because it could determine only total bilirubin in serum. Conjugated bilirubin could be determined enzymatically in 1987 (Kosaka *et al.* and Doumas *et al.*) using the manual method in which very high concentration of bilirubin oxidase was required for the enzymatic reaction. In this study, an enzymatic assay for measuring total and conjugated bilirubin concentration in serum with used of bilirubin oxidase isolated from *Myrothecium verrucaria* TISTR 3112 or TISTR 3225 were demonstrated. The methods utilized bilirubin oxidase without chromatographic purification to prepare the buffer-enzyme solution for bilirubin assays in Beckman Synchron CX5 autoanalyzer. Both enzymatic methods for total and conjugated bilirubin were reproducible for precision, accuracy, linearity and correlation with the Jendrassik and Grof diazo method (Jendrassik and Grof, 1938). Low concentration of bilirubin oxidase was used for both determinations so that cost allowance was possible and thus methods could be introduced to be used in the

routine clinical chemistry laboratories. Hemoglobin at the concentration of 0.8 g/L was found to start influencing with the enzymatic reaction at low concentration of both forms of bilirubin substrate. However, the enzyme solution should be further concentrated or freeze dried to obtain a stable form of enzyme, keeping at 4 °C for the further uses.

The parameter setting for the enzymatic method of bilirubin determination in serum in automated analyzer was varied from one to another instrument. Most automatic methods used the end point reaction determination but varied with the buffer and enzyme concentration. The volume of reagent, the time for starting enzymatic reaction and the wavelength of reading were varied according to different kinds of automated instrument specification. Currently, the enzymatic method for bilirubin determination in serum using different automated instruments were published (Heinemann and Vogt, 1988 ; Nakayama, 1995 and Kurosaka, 1998). Hopefully, if the method could be fractionate all species of bilirubin in serum, then it will become a method of choice in the near future.