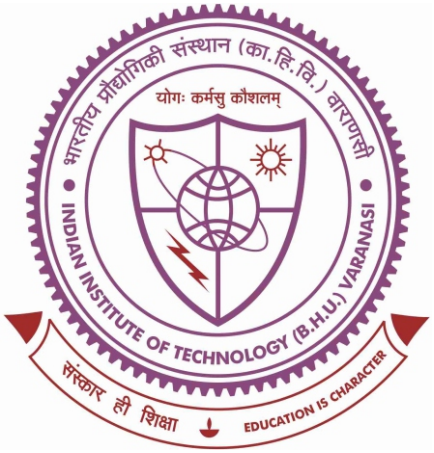
STUDIES ON MICROBIAL PRODUCTION OF CHOLESTEROL OXIDASE AND ITS MEDICAL APPLICATIONS



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By

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Cholesterol is the most abundant lipid found in the human body and it plays important physiological functions as well as structural roles in the cell; as an essential component of cell plasma membranes, neurotransmitters, and hormones. Cholesterol biosynthesis takes place in the liver; it is also taken up by certain dietary sources. The elevated level of serum cholesterol puts human life to certain life-threatening risks and plays a major role in the onset of serious diseases like atherosclerotic plaque formation, hypertension and coronary heart disease, gall bladder stones, Alzheimer's disease, etc. With the increasing risk of these diseases, monitoring the total serum cholesterol on a routine basis is the only way to stay away from these lifestyle disorders. The requirement for the determination of accurate serum cholesterol has stimulated wide spectrum work in the development of its assay methods that are fast enough for routine use, simple, and reproducible. The serum cholesterol analysis is usually performed using a three enzyme assay (Cholesterol esterase, Chox, and Peroxidase) and an indicator dye.

The present work aimed to study the production and purification of Chox; one of the key enzymes used in the serum cholesterol analysis. Besides, Chox is also widely used in the food and pharmaceutical industry. Chox is a bacterial flavoenzyme that catalyzes the oxidation of cholesterol to 4-cholesten-3-one with the simultaneous reduction of molecular oxygen to hydrogen peroxide. Due to its growing applications in diverse fields, Chox has been produced by various microorganisms and purified. *Actinomycetes* are the producers of high levels of extracellular Chox. *Streptomyces* species for serum cholesterol assay has been reported to be superior to those from other microorganisms, due to lower cost of production and longer shelf life.

Hence, the first objective of this research work was to select a potent microbial strain of *Streptomyces sp.* with non-pathogenic and high-yielding properties, which could

meet the demand of relatively high production of Chox. Six different species of *Streptomyces* viz. *S. niger* MTCC 4010, *S. fradiae* MTCC 4002, *S. olivaceus* MTCC 6820, *S. hygroscopicus* MTCC 4003, *S. annulatus* MTCC 6818, and *S. clavifer* MTCC 4150 were procured from Microbial Type Culture Collection, IMTECH, Chandigarh, India. *S.olivaceus MTCC 6820* was selected as the most potent Chox producing strain among the six *Streptomyces sp.* studied. The enzymatic assay method involving Chox coupled with H_2O_2 is extremely simple, specific, and highly sensitive, which indicates the relative concentration of cholesterol indirectly by the measurement of H_2O_2. Coupling of H_2O_2 with chromogen like 4-aminoantipyrine and o-dianisidine in the presence of peroxidase yields an adduct; quiononeimine, (exhibits highly absorbing chromophore) that allows more sensitive measurement of cholesterol than any other method. In our study, this assay method was selected as the best method for the estimation of Chox. The assay conditions for the Chox produced by *S. olivaceus MTCC 6820* was optimized using a combined Response Surface Methodology and Artificial Neural Network modeling based approach, resulting in enhanced enzyme activity attaining unbiased optimum values for each assay parameter optimized. The developed ANN model was successful in predicting the Chox activity of *S. olivaceus MTCC 6820*. ANN technique helped minimize the labor, cost and enhanced the Chox activity to a greater extent. The enzyme activity was enhanced by 1.71 folds after optimization of reaction conditions viz. pH of the reaction mixture (8.0), cholesterol concentration (0.6 %w/v), 4-aminoantipyrine (1.5 mM), crude Chox volume (100 µl), and horseradish peroxidase (10.0 U/ml). The generated ANN model would work as a template for the prediction, modeling, and estimation of Chox from other *Streptomyces sp.* or other microorganisms using similar reaction conditions studied in this work.

The second objective was to enhance the Chox production by the optimization of process parameters. The growth of microorganism and production of enzyme is significantly affected by variation in the medium components and culture conditions; hence it becomes necessary to optimize them. The effect of different carbon and nitrogen sources on Chox production was investigated. D-sorbitol and soyabean meal were found to produce maximum Chox as compared to other sources studied. The effect of several surfactants were also studied. Tween – 20 was found to be the best surfactant as they increased the cell membrane permeability and helped in the solubilization of cholesterol in the medium thereby helping the microbial cells for the easy uptake of cholesterol from the production medium leading to enhanced Chox production. The effect of suitable inducer concentration was studied, which showed 0.25% cholesterol concentration was optimum for Chox production. Physical parameters for Chox production were performed using RSM and ANN. Both the RSM and ANN were employed to model the Chox production (U/ml) as a function of six independent variables and their optimum conditions were determined. A comparative performance evaluation of RSM and ANN techniques was done. The verification of the optimization results and accuracy of the model was accomplished by performing the experiments thrice under optimized culture conditions i.e., pH of the media (7.5), inoculum age (48 h), inoculum size (11.25 %), fermentation period (72 h), incubation temperature (30°C) and shaking speed (175 rpm). Under these culture conditions, the maximum Chox produced was 4.2 ± 0.51 U/ml, which corresponds very well to the value predicted by the ANN model.

It was concluded that ANN can be considered as an effective tool to model and predict optimum parameters for Chox production as the ANN model provided more accurate predictions than RSM with higher regression coefficient (R^2), greater Pearson

correlation coefficient (r), and lower AAD values. The Chox production was enhanced by 2.2 fold after optimization of the culture conditions with the maximum Chox activity reaching up to 4.2 U/ml.

Downstream processing is an integral part of the fermentation process and plays a very crucial role in the commercial production of enzymes. The third objective was to purify the Chox produced by *S. olivaceus MTCC 6820* from the fermentation broth. The conventional purification technique is a complicated multi-step process that suffers the limitation of time-consuming, labor-intensive, and reduced recovery of the desired enzyme causing low yield due to the loss of enzyme activity at each step. Aqueous Two-Phase System consisting of PEG-salt-water was used for the purification of Chox from the crude extract (supernatant of the fermentation broth). Eight different systems were designed using PEG 4000 and 6000 with four inorganic salts viz. dipotassium phosphate, sodium citrate, sodium sulfate, and ammonium sulfate. Two-phase systems were prepared by titration of PEG against salt and the phase diagrams were prepared for all the systems and were characterized by binodal curve. Selection of the best two-phase system was done based on the value of the partition coefficient. PEG-4000-ammonium sulfate-water two phase system was found to be the suitable system for Chox partitioning. The Chox partitioned on the top phase was recovered using chitosan beads activated with glutaraldehyde.

The physicochemical properties of the purified Chox were determined. The molecular weight of the Chox purified from *S. olivaceus* MTCC 6820 determined through SDS-PAGE was found to be nearly ~60 kDa. The pH and temperature optima of the Chox were 7.0 and 35˚C respectively. The effect of increasing substrate concentration on the reaction velocity of the Chox obeyed the Michaelis-Menten kinetics. The Km and Vmax

values of the COX were found to be 0.36µM and 1.62µM min^{-1} respectively. The enzyme was stable between pH 6-8.5 and retained 98-100% of residual activity while it retained 69-71% of its residual activity between pH 9.5-12 for 24 h. This showed that the Chox was stable over a wide range of pH, which makes it suitable for various applications. Thermostability studies showed that the enzyme retained 90-95% of its residual activity between temperatures 25-50°C after 30 min of incubation at each temperature. At higher temperatures beyond 60°C, 69-79% residual activity was retained at temperatures 70, 80, and 90°C for about 30 min. The Chox was stable in the presence of various organic solvents including dichloromethane, ethyl acetate, ethanol, methanol, toluene, and non-ionic detergents Tween-80 and Triton X-100. It proves that Chox from *S. olivaceus MTCC 6820* is an organic solvent and detergent tolerant enzyme, which makes it a potential enzyme for various biopharmaceutical applications.

The use of soluble peroxidase in the estimation of Chox by the enzymatic endpoint method makes this assay method expensive due to its poor reusability and low stability. So, the fourth objective of this research work was the immobilization of Horseradish Peroxidase onto Graphene oxide/Magnetic chitosan beads to reduce the cost of this Chox enzyme assay by increasing the reusability and storage stability of soluble peroxidase. The Graphene oxide coated Fe_2O_3/chitosan beads were prepared and activated by crosslinking with glutaraldehyde treatment. The characterization study was performed by the various state of the art techniques including SEM, FTIR, XRD, and VSM. These beads were used as carrier support for Horseradish Peroxidase immobilization by covalent binding technique, and the application of these Graphene oxide coated magnetic chitosan beads for increasing the reusability of peroxidase in Chox assay. The immobilization of HRP was successful with ~80% immobilization efficiency and the maximum amount of bound

protein was 0.08 mg per bead. The immobilized HRP retained ~90% residual activity after being used for up to 12 cycles of Chox assay, which gave a more satisfying performance as compared to the previous reports, thus providing an economic advantage for the fermentative production and purification of Chox in this research work. By increasing the reusability of HRP we are the pioneer to use low-cost technology for HRP mediated estimation of Chox.

Apart from these, some optimal operational conditions for immobilized peroxidase were determined and the stability of immobilized peroxidase under various conditions was studied. The effect of pH on the activity of free and immobilized peroxidase was studied by carrying out the reactions with different buffers in the pH range 4 – 9 at 30˚C. The optimum pH for the activity of free and immobilized peroxidase were 6.5 and 7.0 respectively. The optimum temperature of the free and immobilized peroxidase was determined by measuring the enzyme activity at variable temperature ranging from 25˚C to 85˚C at pH 7.0. An elevation in the optimum reaction temperature of the free peroxidase (50˚C) as compared to the immobilized peroxidase (55˚C) was observed. The thermal stability of free and immobilized HRP was studied at three different temperatures 35˚C, 55˚C, and 75˚C for 2 h. The immobilized HRP was stable for 90 min at temperatures 35˚C and 55˚C and retained 80% and 70% of residual activity respectively. The stability of immobilized peroxidase was investigated in the presence of various detergents and organic solvents and it showed ≥ 150% relative activity in the presence of triton X -100 and tween – 20. The relative activity of immobilized peroxidase increased in the presence of methanol, ethanol, and isopropanol, which proved that peroxidase immobilized beads are highly suited for the estimation of Chox.

The purification of Chox from the ATPS technique was first time in this thesis, no other report was found to the best of our knowledge. The recovery of Chox from the partitioned enzyme-ATPS mixture still a cumbersome task, which was achieved by the direct immobilization of Chox from the top-phase of Chox and it was again a novel approach reported in this thesis and has not been reported in any literature.

The fifth objective was to investigate the applicability of purified Chox for total cholesterol estimation in human serum using three enzyme assay viz cholesterol esterase, Chox, and peroxidase. More than 70% of the cholesterol present in human serum is esterified; cholesterol esterase was used in the reaction mixture to generate free cholesterol. Chox coupled with horseradish peroxidase was used for the estimation of total cholesterol. The linearity assessment of Chox from S. olivaceus was done in the range of 2.6 to 25.9 cholesterol mmol/l; the linear range was found to be between 2.6 to 15.5 mmol/l. The Chox purified from *S. olivaceus MTCC 6820* was suitable for application in the serum assay.

Future scope

The recovery of the Chox from the top-phase of PEG-Ammonium sulfate-Water ATPS in the form of immobilized chitosan beads of Chox would be further investigated for the use of Chox in the diagnostic tests in the immobilized form. We wsih to develop it in the form of cholesterol biosensor. Also, this new recovery method of Chox from ATPS, in future may be investigated for cost-effective purification and recovery from ATPS.