1.1. Problem Statement

Cholesterol is the most abundant lipid found in the human body including the brain, spinal cord, serum, and other tissues. Cholesterol plays important physiological functions as well as structural roles in a cell; as an essential component of cell plasma membranes, neurotransmitters, and hormones. In human body, the biosynthesis of cholesterol takes place in the liver. Moreover, certain dietary sources of cholesterol include some animal foods such as eggs, meat, fish, milk, and almost all dairy products. The U.K. government, as per the 1992 Health of The Nation Report (The Health of The Nation, 1992) and the United States as per the recommendations of the National Research Council (National Research Council, 1989) have recognized the need to limit dietary cholesterol intake.

On the other hand, 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 (Rose and Shipley, 1980), gall bladder stones (Bouchier, 1991; Carey, 1993), Alzheimer's disease (Sun et al., 2015), 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. Consequently, the requirement for the determination of accurate serum cholesterol has stimulated wide spectrum work in the development of methods for the estimation of cholesterol that are fast enough for routine use, simple, and reproducible. The serum cholesterol analysis is usually performed using a three enzyme assay (Cholesterol esterase, Cholesterol oxidase (Chox), and Peroxidase) and an indicator dye; method originally devised by Richmond et al. (Richmond, 1973).

Chox is one of the key enzymes used in the estimation of cholesterol concentration in human serum. In recent times, the medical application of Chox is mainly focused on the field of clinical diagnostics for the detection of cholesterol concentrations in human serum (Lolekha et al., 2004; Srisawasdi et al., 2006) and the development of Chox based biosensors (Gholivand, 2014). Moreover, Chox has also proved to be an important biotechnological tool in pharmaceutical industries, for the commercial production of precursors of the steroidal hormones from cholesterol and its derivatives (Kazandjian et al., 1986; Khmelnitsky et al., 1988; Mahato and Garai, 1997).

Despite their potential medical and pharmaceutical applications, the commercial cost of Chox remains very high. The major underlying reasons accounted are the production of Chox exclusively through the microbial fermentation process (no plant and animal sources have been documented), inducible expression of Chox by producer microorganisms, the pathogenicity of most of Chox producing microorganisms limits the commercially available strains. Besides the downstream processing strategies adapted for Chox so far, mainly consists of conventional purification methods (ion-exchange chromatography, affinity purification) which causes loss of enzyme activity at each step resulting in lower yield and suffering the shortcomings of being a time-consuming and labor-intensive process.

The commercial production of Chox demands the search for new and better yielding microbial strains as well as economically viable bioprocesses for its large-scale production. Over-production of Chox through various approaches has been a matter of current interest amongst researchers worldwide. Optimization of nutrients and process parameters may be one of the primary strategies applied for maximizing yield in the fermentative production of Chox. Effective downstream processing may help reduce the final cost of the enzyme.

1.2. Cholesterol oxidase: an overview

Cholesterol oxidase (Chox) (EC 1.1.3.6) is a bifunctional (Flavin Adenine Dinucleotide) FAD-dependent enzyme that catalyzes the oxidation of cholesterol (5-cholesten-3-ol) to an intermediate 5-cholesten-3-one with the reduction of molecular oxygen to form hydrogen peroxide (Smith and Brooks, 1974; Doukyu, 2009). The isomerization of 5-cholesten-3-one forms 4-cholesten-3-one by the conversion of Δ^5-bond to a Δ^4-bond (Salva et al., 1999; Yamashita et al., 1998). Chox is a flavoenzyme that belongs to the family of oxygen oxidoreductases and it acts on the CH-OH group of donors where oxygen is the electron acceptor. In food industries, Chox is used for the detection of cholesterol concentrations in food samples for the quality control assay. In pharmaceutical industries, Chox is used for the bioconversion of 3-β-hydroxysteroids where some cholesterol biodegradation intermediates such as 4-androstene-3,17-dione and 1,4-androstadiene-3,17-dione are used in the commercial production of precursors of the steroidal hormones from cholesterol and its derivatives (Kazandjian et al., 1986; Khmelnitsky et al., 1988; Mahato and Garai, 1997). In the medical field, it has been used for the detection of cholesterol concentrations in blood serum (Allain et al., 1974; Lolekha and Jantaveesirirat, 1992). In agricultural biotechnology, the Chox gene has been cloned and expressed in tobacco plants for developing transgenic crops, playing a vital role in pest control due to its insecticidal activity against boll weevil larvae (Purcell et al., 1993; Cho et al., 1995). As all these applications studies have been done using Chox produced by *Streptomyces* sp., it seemed to be a commercially promising culture for Chox production.

Many microorganisms including bacteria, actinomycetes, and fungi produce Chox. Many Chox producing microorganisms have widely been isolated including *Nocardia rhodochrous* (Buchland et al., 1975), *Arthrobacter simplex*(Liu et al., 1988), *Pseudomonas*

sp.(Noriyaku and Rikizo, 1998), *Actinomyces lavendulae* (Petrova et al., 1981; Shirshova et al., 1992), *Streptomyces fradiae* (Yazdi et al., 2001). Most of them produce cell-bound Chox which are often difficult for isolation and also some are pathogenic in nature. Apart from other microbial sources, microbial flora belonging to the group of *Actinomycetes* have been reported to produce high levels of extracellular Chox (Doukyu and Aono, 1998; Fukuda et al., 1973; Inoye et al., 1982; Lartillot and Kedziora, 1990; Varma and Nene, 2003) easier for both isolation and purification than the intracellular and cell-bound enzymes. *Streptomyces* is the largest genus of the phylum *Actinobacteria*; characterized by complex secondary metabolism, melanoid pigment (light brown, brown-black, or distinct brown) formation in the medium. They are gram-positive and spore-forming organisms (Sharma, 2014). *Streptomyces* are potent producers of valuable antibiotics and are also non-pathogenic. Chox is an inducible enzyme having low yield which increases its cost at the commercial level. Chox from *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 (Lolekha and Jantaveesirirat, 1992).

The production of Chox in shake flask culture has been studied in various microorganisms including bacteria, actinomycetes, and fungi (Buchland et al., 1975, Noriyaku and Rikizo, 1998, Yazdi et al., 2001). The metabolic processes of the microorganisms are very much influenced by media components and culture conditions. Therefore, it is desirable to optimize the environmental conditions to reach maximum enzyme production. The classical method viz. one variable at a time (OVAT) optimization strategy consists of varying one variable at a time keeping the other constant (Rathi et al., 2001). The OVAT is often time-consuming as it needs to perform a large number of experiments and also fails to study the interaction of different process variables involved,

which affects the final yield (Wahid and Nadir, 2013). Therefore, nowadays optimization strategies based on statistical tools (Response Surface Methodology, RSM) and machine learning (Artificial Neural Network, ANN) are preferably used for quick screening of a large experimental domain and studying the interactive effect of each variable (Sim and Kamaruddin, 2008; Bas and Boyaci, 2007). RSM optimizes the parameters using factorial design, regression analysis, and analysis of variance (ANOVA). RSM suffers the limitation of assuming a quadratic linear correlation for optimizing the parameters. ANN is a more accurate modeling technique as compared to RSM for prediction, data fitting ability, and modeling non-linear relationships (Bas and Boyaci, 2007; Haykin, 2009).

Aqueous Two-Phase System (ATPS) have emerged as an effective green extraction technique for purification and recovery of a wide range of biological products such as enzymes, proteins, nucleic acids, amino acids, cellular organelles, and microorganisms from their respective contaminants and impurities (Rahimpour et al., 2016, Souza et al., 2015, Xavier et al., 2015). ATPS may provide an appropriate alternative to the conventional method for purification of enzymes by preferentially partitioning the enzyme in one phase and the interfering substances into another phase. ATPS contains about 80-90% water which provides a favorable environment for cells, cellular organelles, and biologically active substances like enzymes. The purified form of Chox is required to study most of its medical and biotechnological applications.

1.3. Rationale of the study

Based on the above facts, the present research work aims the development of a process for the production of Chox in shake flask fermentation using a potent Chox producing *Streptomyces* strain. Different optimization strategies were used to optimize reaction conditions, culture conditions, and nutrient media components to achieve

maximum production of Chox in the shake flask. In addition to these, the effort for reducing the cost of the Chox assay was done by immobilizing the horseradish peroxidase onto graphene oxide-coated magnetic chitosan beads. Chox from *Streptomyces olivaceus* MTCC 6820 was purified by partitioning of Chox in different PEG-Salt-Water aqueous two-phase systems composed of PEG X (X = 4000, and 6000) and salts (dipotassium phosphate, sodium citrate, ammonium sulfate, and sodium sulfate). A suitable PEG-Salt system was chosen for enhanced Chox partitioning. The biochemical characterization studies of the enzyme including pH optima, temperature optima, pH stability, thermo-stability, and kinetic parameter were determined. The purified enzyme was then used to study its applicability for serum cholesterol determination.