ISSN: 0975-766X
CODEN: IJPTFI
Available Online through
Research Article
www.ijptonline.com

ISOLATION, EXTRACTION AND PARTIAL PURIFICATION OF CHOLESTEROL OXIDASE FROM *STENOTROPHOMONAS* SP.

Rajesh Kanna.G, Florentyna Rodrigues, Palani Perumal*
Email: palanii7@unom.ac.in

Received on 14-07-2014 *Accepted on 28-07-2014*

Abstract

Samples of soil were collected from Varanasi, India. From these samples, 75 different bacterial isolates were obtained. These isolates were screened for the production of cholesterol oxidase enzyme. Two isolates tested positive for the production of Cholesterol oxidase and the isolate with the higher enzyme production was chosen for this study. The bacterium (isolate GS-14) was identified as *Stenotrophomonas maltophila.* The bacterium grew on nutrient medium, with cholesterol substrate. Cholesterol oxidase (EC 1.1.3.6), which catalyzes cholesterol into 4-cholesten-3-one, was evidenced from the strain. The conditions for the growth of the bacterium were optimized for extracellular enzyme production, which then reached around 1900 UL^{-1} within 20 hours of culturing. The enzyme was partially purified from the spent medium of the strain it was run on SDS-PAGE, and characterized. According to this technique, its molecular mass was found to be 20.5 kDa. Cholesterol oxidase showed an optimum activity at pH 7.0.The optimum temperature was found to be 50^{o}C and it retained more than 70% activity at 50°C after 60 min of heat treatment. The enzyme is pH dependent with maximum activity at around 7.0. Enzyme activity was found to be stable for at least 60 min at pH 7.0 by exhibiting more than 80% activity.

Key words: Cholesterol oxidase, Stenotrophomonas, Bacteria, production

Introduction

The ability of degrading cholesterol is quite prevalent among microbes. Cholesterol decomposition ability is wide spread among prokaryotic microorganisms that have been explored as extracellular, free and immobilized cells or as enzymes source in steroid bio transformations. However, knowledge about the degradation pathway of cholesterol is still very limited (Gilliland et al. 1985). *Nocardia* Cholesterol oxidase is a well known enzyme to convert cholesterol to 4-

cholesten-3-one (Smith and Brooks 1976). The bacterial degradation of cholesterol has been known to occur by a cholesterol oxidase, (cholesterol: oxygen oxidoreductase, EC 1.1.3.6). It has been reported that cholesterol oxidase dehydrogenates cholesterol to produce the intermediate 5-cholestane-3-one, in which isomerization (Δ5–6 to Δ4–5) then occurs to form 4-cholestane- 3-one (Motteran *et al.,* 2001). Cholesterol (5-cholesten-3-ol) has been linked with the induction of colon cancer and potent angiotoxic effects of several cholesterol oxides have led researchers to hypothesize a likely role in cardiovascular diseases (Kaunitz 1978).

Dietary cholesterol can raise blood cholesterol but generally is not as important as saturated fat and total fat in the diet. High total blood cholesterol levels (Hypercholesterolemia) and LDL (Low density lipoprotein) cholesterol levels increase risks of cardiovascular diseases such as atherosclerosis and myocardial infarction (heart attack). The bacterial degradation of cholesterol in cholesterol-containing foods may be beneficial for human health (Watanabe et al. 1986). Cholesterol oxidases are found as intrinsic membrane bound enzymes found extracellular, as in *Nocardia rhodochrous, Nocardia erythropolis,* and *Mycobacterium* species, or can be isolated from culture filterate as in *Streptomyces violascens, Brevibacterium sterolicum, Rhodococcus equi, Streptoverticilium cholesterolicum, Mycobacterium* ATCC 19652 and *Rhodococcus erythropolis.* Majority of strains isolated belonged to the genus *Rhodococcus* (Watanable *et al*., 1986; Kreit *et al*., 1994; Sojo *et al*., 1997). The main objectives of this study were to isolate a bacterial organism from soil or water samples from the shores of the Ganges and to obtain an efficient cholesterol oxidase producer, to optimize culture conditions for high yield production of cholesterol oxidase and to characterize the same.

Materials and Methods

Isolation, Screening and Selection of Bacteria

Soil samples were collected from the banks of the river Ganges in Varanasi at five different locations. Isolation was carried out by plating dilutions of soils in saline solution (0.5% NaCl) on nutrient agar and incubating the same at 37°C for 48 hrs. Individual colonies of bacteria which varied in shape and color were picked up and purified by streaking on nutrient agar. Soil isolates obtained were inoculated on medium containing agar (1.5%) and cholesterol (0.5%) as the sole source of carbon. This was incubated at 37°C for 4 days. The production of cholesterol oxidase by bacteria was qualitatively analyzed by the appearance of clear zones on the plates; the plates were stained with Sudan III solution (Fig. 1). The bacterial isolate which showed highest enzyme activity quantitatively, was selected for further study.

Cholesterol oxidase assay

Cholesterol oxidase activity was measured quantitatively at 240 nm by Richmond (1973). The volume of reaction mixture composed of 3ml of 0.1M sodium phosphate buffer (pH 7) with 0.05% Triton X-100, 0.05ml 6mM cholesterol in 2-propanol and 0.05 ml crude enzyme. The enzyme unit of cholesterol oxidase was defined as the amount of enzyme oxidizing 1 μ mole of cholesterol to 4-cholesten-3-one per min at 30^{o}C. Total protein was estimated using Bradford's method (1976).

Preparation of crude enzyme extract

The bacterial cultures were centrifuged at 10,000 rpm for 15 minutes to remove the bacterial cell debris, the supernatant obtained served as a crude source of the enzyme.

Optimization of culture media and Substrate

Three different media namely Nutrient broth NB, Luria Bertani broth (LB) and Tryptone soya broth (TSB) along 5 % of substrate were used for optimization of media. pH ranging from 6.0 to 9.0. was used to determine the best pH range. Substrate concentrations ranging from 1.0% to 2.0% were amended to the medium. Finally, Time and Temperature were also assessed for optimum enzyme production by observing it over a period of 48 hours at one hour intervals. Temperatures from 20ºC to 40ºC were maintained to determine the optimum. An alternative cheap source of cholesterol is tallow. Beef fat was boiled down filtered and cooled .This contains high levels of cholesterol and is called tallow. Different concentrations of tallow were amended to the medium to determine the best concentration.

Identification of bacterium

The identification of bacterial isolate was carried out by Bergey's Manual of Systematic Bacteriology (Williams et al., 1989). Biochemical characterization and 16S r-RNA sequencing was carried out to identify the bacterium.

Purification of COD Enzyme

Concentration of the Cholesterol was accomplished by TPP (Roy *et al*., 2005). The supernatant (20ml) was mixed with 20 - 90% ammonium sulfate (W/V) and 1-butanol (V/V). The mixture was vortexed and incubated at 2-6°C for 5 min, then centrifuged at 10,000 rpm for 5 min. The mid-layer, concentrated proteins was collected and dissolved in a minimal volume of 0.1 M (pH 7.0) of phosphate buffer, this was followed by dialysis against several changes of the same buffer. This dialysate served as a partially purified enzyme.

Characterization of Cholesterol oxidase

Temperature and pH

The optimum temperature and pH of the crude enzyme was determined by carrying out the enzyme assay in the temperature range from 10°C to 80°C and pH ranges from 6.0 to 9.0. Enzyme activity without incubation is taken as 100% for both the analysis. The pH stability of the cholesterol oxidase was determined by pre incubation of enzyme with buffer of optimum pH for 1 h at 30^0C and measuring residual activity.

Molecular weight

The molecular weight of cholesterol oxidase enzyme was determined by running SDS-PAGE (SDS-Polyacrylamide gel electrophoresis).

Results

A total of 25 bacterial isolates were obtained from the collected samples from the banks of river Ganges. From the overall screening of quantitative production of COD, the bacterial strain GS2 and GS-14 showed peak in cholesterol oxidase production 0.99 and 1.33 U ml^{-1} respectively (Fig. 2). Strain GS-14 showed promising level in production of cholesterol oxidase and showed clear zone in qualitative plate assay (Fig. 1). Thus, GS-14 was selected for further analysis of the work. The culture media was optimized. Nutrient broth with pH 7.0, optimum temperature, time and substrate were 30ºC, 20hrs of incubation with vigorous shaking and 0.6% respectively, yielded the highest amount of enzyme production (Fig. 3, 4, 5, 6). The cheap cholesterol substrate tallow were optimized that 0.8% is required for cholesterol oxidase production (Fig. 7).

Identification of bacterial strain GS-14

The bacterial strain GS-14 was identified as *Stenotrophomonas maltophilla* based on biochemical tests (Table 2), morphological characterization (Table 1) and 16S rRNA sequencing.It was submitted in GeneBank, under the accession number KJ958487

Partial purification of COD from *Stenotrophomonas sp.*

Partial purification of cholesterol oxidase involves, Ammonium sulphate and Butanol treatment recovered the enzyme with a specific activity of 6.7 Uml^{-1}, while the yield was 82.7%.

Characterization of partially purified COD

The effect of temperature and pH on activity and stability of extracellular enzyme was assessed under a standard condition. The optimum temperature was found to be 50°C and it retained more than 70% activity at 50°C after 60 min. of heat treatment and the enzyme is pH dependent with maximum activity at around 7.0 (Table 3). Enzyme activity was found to be stable for at least 60 min at pH 7.0 by exhibiting more than 80% activity (Table 4). The molecular weight of the obtained Cholesterol oxidase was concluded to be 20.1 kDa (Fig. 8).

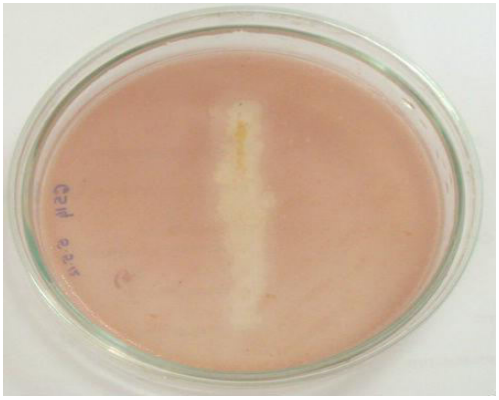

Fig. 1: GS-14 shows clear zone by utilizing the substrate in Sudan III stain.

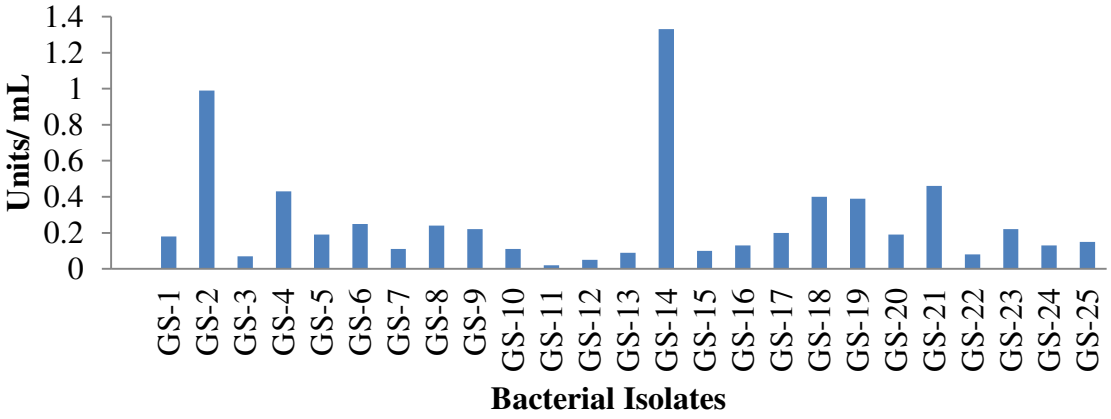

Fig. 2: Screening for COD from soil isolated bacterial strains

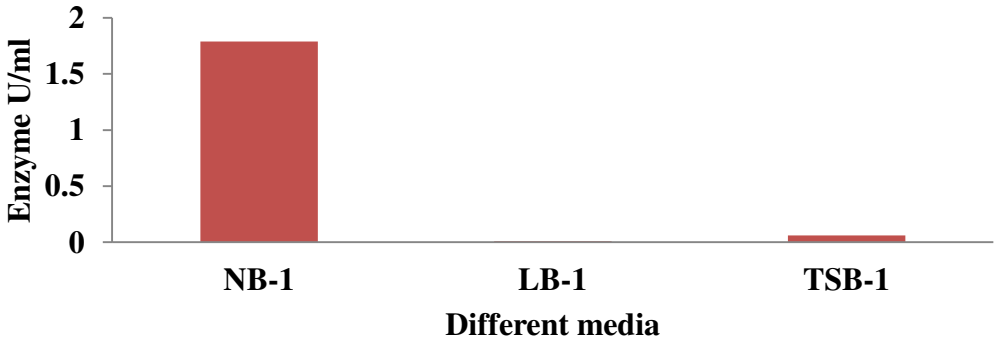

Fig. 3: Optimization of different media for COD production

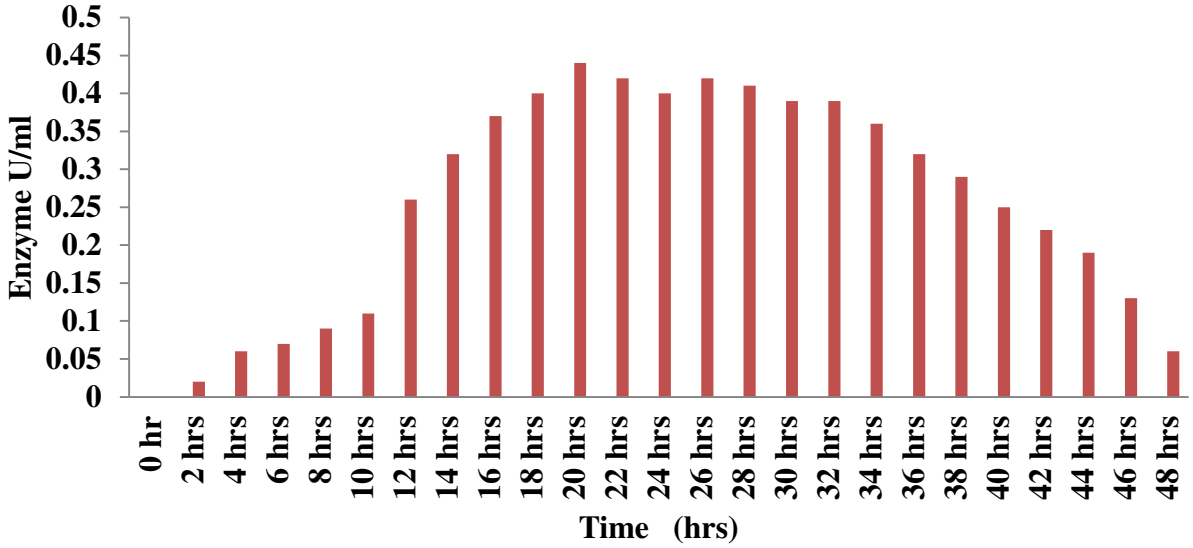

Fig. 4: Optimization of Time for COD production

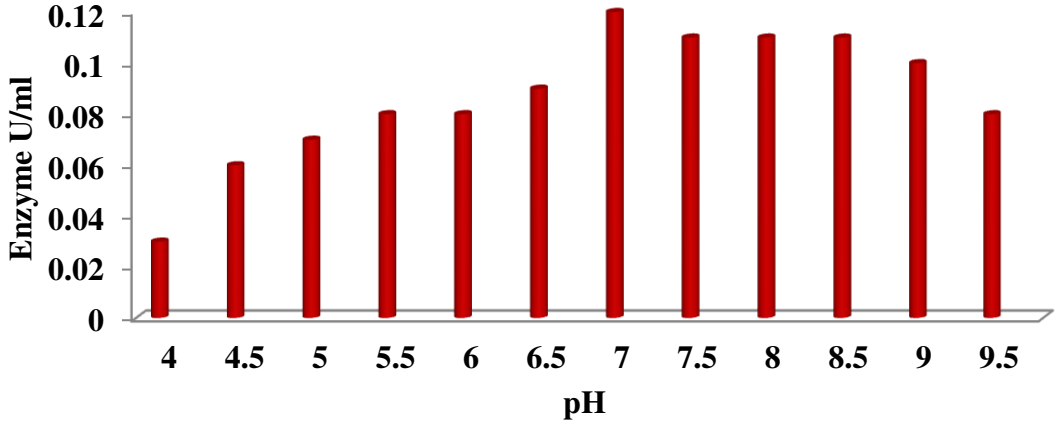

Fig. 5: Optimization of pH for COD production

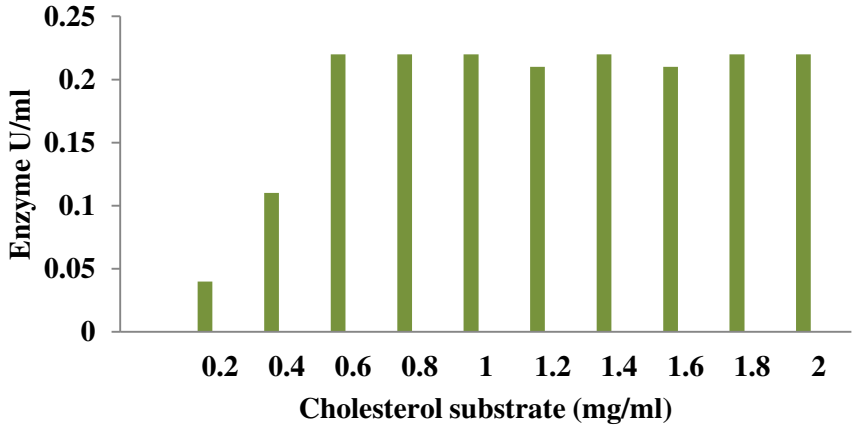

Fig. 6: Optimization of Substrate concentration

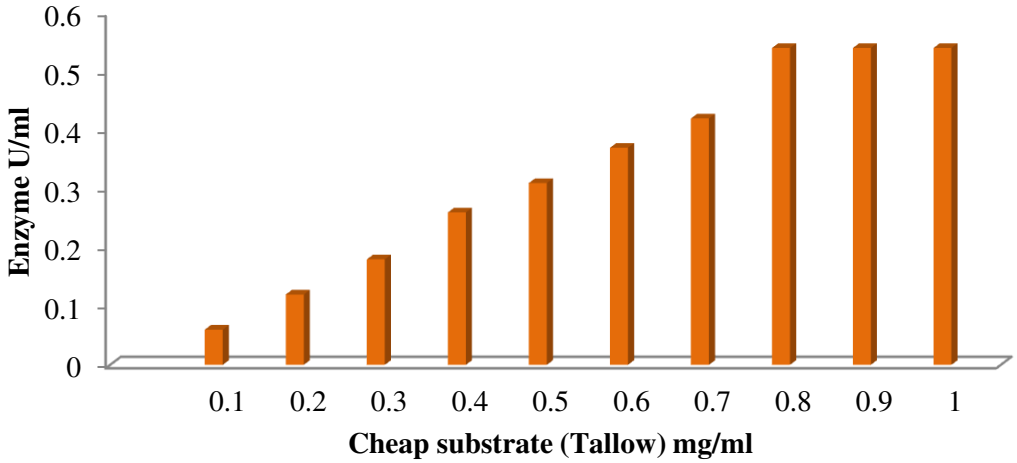

Fig. 7: Optimization of a cheap substrate- Tallow

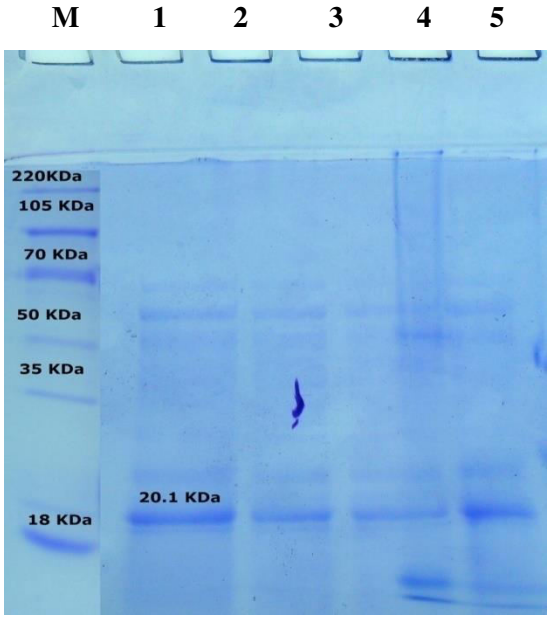

Fig. 8: SDS-PAGE analysis of partially purified COD from GS-14, M-marker, 1-5 crude COD

Table-1: Colony Morphology of GS-14.

S.no	Colony Morphology	Result
1.	Shape	Punctiform
2.	Elevation	Convex
3.	Colour	Yellow
4.	Shape of Edge of Margin	Entire
5.	Surface Texture	Smooth, Shiny

Table-2: Biochemical Characterization of GS-14.

S.No	Biochemical Test	Result
1.	Gram's Staining	-
2.	Catalase	+
3.	Spores	-
4.	Motility	+
5.	Urease	-
6.	Glucose	+
7.	Lactose	+
8.	Sucrose	+
9.	Maltose	+
10.	Starch Hydrolysis	-
11.	Gelatin Hydrolysis	-
12.	Citrate Utilization	-
13.	Nitrate Reduction	-
14.	Oxidase	+
15.	Methyl Red	-
16.	Vogues Proskauer Test	-
17.	Indole Production	-
18.	Hydrogen Sulphide	-

Table-3: Effect of temperature on COD enzyme activity and stability.

S.No	Temperature ºC	Enzyme Units/mL	Protein mg/mL	Enzyme Stability %
1.	10	0.5	362	100
2.	20	0.5	356	100
3.	30	0.5	353	100
4.	40	0.5	367	100
5.	50	0.5	351	100
6.	60	0.34	247	70
7.	70	0.27	192	53
8.	80	0.22	158	44

Table-4: Effect of pH on COD enzyme activity and stability.

S.No	pH	Enzyme Units/mL	Protein mg/mL	Enzyme Stability %
1.	6.0	0.30	336	60
2.	6.5	0.36	350	72
3.	7.0	0.42	369	84
4.	7.5	0.41	365	82
5.	8.0	0.31	347	62
6.	8.5	0.28	321	56
7.	9.0	0.17	313	34

Discussion

Cholesterol oxidase is an enzyme of good commercial value widely employed by laboratories routinely devoted to the determination of cholesterol in food, serum and other clinical samples (Sojo *et al*., 1997). Hence, a diversity of micro-organisms, which are capable of producing high levels of this enzyme have been isolated.

Soil samples brought from Varanasi served as the source of bacterial isolation. The bacterial strain GS-14 was sequenced to 16s rRNA and has 97% similarity with *Stenotrophomonas sp.* observed in the phylogenetic tree at a maximum distance of 0.05. Thus GS-14 was identified as *Stenotrophomonas maltophila.* Taking into account the extracellular production, its efficient recovery, pH tolerance and a good thermal stability, cholesterol oxidase produced by *Stenotrophomonas sp.* should prove to be an industrially important enzyme. Our preliminary work led to the conclusion that *Stenotrophomonas sp.* might be considered as potentially interesting source of extracellular cholesterol oxidase for clinical and commercial purposes. This appears to be the first report on production of cholesterol oxidase by *Stenotrophomonas sp.* isolated from Varanasi, India.

The extra-cellular enzyme was concentrated by a Three Phase Partition (TPP) method. In some other reports for concentrating Cholesterol oxidase, methods such as precipitation by an organic solvent and by ammonium sulfate have been used efficiently for enzyme concentration (Richmond, 1973; Inouye *et al*., 1982). However, these precipitation methods did not yield satisfactory amounts of enzyme. This might be due to the presence of hydrophobic patches on the Cholesterol oxidase molecule. These patches increase the solubility of the enzyme in organic solvents. This may cause

low efficacy of the ammonium salt precipitation method. However, some authors have reported good results in Cholesterol oxidase concentration using ammonium sulphate precipitation for some sources (Lee *et al*., 1997; Fujishiro *et al*., 2002). A higher recovery rate of cholesterol oxidase can be obtained easily, using TPP in comparison to the classical ammonium sulphate method.

Thermal stability of an enzyme is an important property for industrial applications. The effect of temperature on activity and stability of extracellular enzyme was assessed under a standard condition. It is seen that optimum temperature was found to be 50^{o}C and it retained more than 70% activity at 50°C after 60 min of heat treatment These values are higher than the optimum temperature for the enzyme from *Rhodoccocus equi.* and *Corynebacterium cholesterolicum*, with maximum activity at 47^{o}C and 40^{o}C, respectively (Terezinha *et al*., 1999) and are similar to earlier reports for *Streptomyces fradiae* and *Brevibacterium* sp. which produced *cholesterol oxidase* with optimum activity and stability for 30 min at 50°C and 53°C respectively (Tabatabaei *et al*., 2001; Fujishiro *et al.*, 2002).

It was observed that the enzyme is pH dependent with maximum activity at around 7.0. Enzyme activity was found to be stable for at least 60 min at pH 7.0 by exhibiting more than 80% activity. The optimum pH for cholesterol oxidase for *Stenotrophomonas* sp. was similar to that found in literature, for the same enzyme from other micro-organisms (Doukyu, 2009). Generally, optimum pH for enzyme activity is between 7.0 and 8.0, as can be seen for enzymes from *Actinomyces lavendulae* mycelium, *Corynebacterium cholesterolicum*, *Streptoverticillium cholesterolicum*, *Rhodococcus equi* and *Streptomyces violascens* (Lashkarian *et al*., 2010).

It is widely acknowledged that high levels of blood cholesterol are detrimental to human health. Therefore, the biotransformation of dietary cholesterol to other harmless or even wholesome compounds is of great importance. Cholesterol oxidase can be used as a biocatalyst in serum cholesterol determination or for biotransformation of sterols and stanols to useful ketosteroids. It is worthy to report that 4-cholesten-3-one can be used to control obesity and to treat liver diseases, or to prevent the skin from keratinization. In the present study, cholesterol was converted to 4-cholesten-3-one by growing cells of strain B4. This appears to be the first report on production of cholesterol oxidase by ***Stenotrophomonas sp.*** isolated from Varanasi, India and the cholesterol-transforming capability of strain GS-14 could be optimized for industrial application.

References:

1. Bradford, M. M., (1976)," A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding" *Anal Biochem,* 72 pp. 248–254.
2. Doukyu, N., (2009), "Characteristics and biotechnological applications of microbial Cholesterol oxidases" . *Appl Microbiol Biotechnol,* 83 (5) pp. 825–837.
3. Fujishiro, K., H. Uchida, K. Shimokawa, M. Nakano, F. Sano, T. Ohta, N. Kayahara, K, Aisaka, and T. Uwajima (2002). Purification and properties of a new Brevibacterium sterolicum cholesterol oxidase produced by E. Coli. MM294/pnH10. FEMS Microbiology letters Vol. 215: 243-248.
4. Gilliland, S. E., C. R. Nelson and C. Maxwell (1985). Assimilation of cholesterol by Lactobacillus acidophilus. Appl. Environ. Microbiol. Vol. 49: 377-381.
5. Inouye, T., K. Taguchi, A. Fujii, K. Ishimaru, S. Nakamura and R. Nomi (1982). Purification and characterization of extracellular 3-hydroxysteroid oxidase produced by Streptoverticillum cholesterolicum. Chem. Pharm. Bull. Vol. 30: 951-958.
6. Kaunitz, H. (1978). Degradation of cholesterol oxidase by Bacillus subtilis SFF34 in flatfish during fermentation. Lipids. Vol. 13: 373-375.
7. Kreit, J., G. Lefebvre and P. Germain (1994). Membrane-bound Cholesterol oxidase from Rhodococcus sp. cells. Production and extraction. J. Biotechnol. Vol. 33: 271-282.
8. Lashkarian, H., J. Raheb, K. Shahzamani, H. Shahbani and M. Shamsara (2010). Extracellular Cholesterol oxidase from Rhodococcus sp.: Isolation and Molecular Characterization. Iranian Biomedical Journal. Vol. 14, No. 1-2: 49-57.
9. Lee, M. T., W.C. Chen, C.C. Chou (1998)," Maximization of Cholesterol oxidase production by *Rhodococcus equi no*. 23 by using response surface methodology" *Biotechnol Appl Biochem*, 28 (Pt 3) pp. 229–233.
10. Motteran, L., M. S. Pilone, G. Molla, S. Ghisla and L. Pollegioni (2001). Cholesterol oxidase from Brevibacterium sterolicum the relationship between covalent Flavinylation and redox properties. The Journal of Biological Chemistry. Vol. 276: 18024-18030.

11. Richmond, W., 1973. Preparation and properties of a cholesterol oxidase from *Nocardia* sp. and its application to the enzymatic assay of total cholesterol in serum. *Clinical Chemistry*., 19: 1350-1356.
12. Roy, I., A. Sharma and M. Gupta, 2005. Recovery of biological activity in reversibly inactivated proteins by three phase partitioning. *Enzyme Microbiology. Tech*., 37: 113-120.
13. Smith, A. G., C. J. W. Brooks (1967). The substrate specificity and stereochemistry , reversibility and inhibition of the 3-oxo steroid- isomerase component of cholesterol oxidase. Biochem. J. Vol. 167: 121-129.
14. Sojo, M., R. Bru, D. Lopez-Molina, F. Garcia-Carmona, J. C. Arguelles (1997). Cell-linked and extracellular Cholesterol oxidase activities from Rhodococcus erythropolis, Isolation and physiological characterization. Appl. Microbiol. Biotechnol. Vol.47: 583-589.
15. Tabatabaei, M., M. Zahraei, K. Aghaepour and N. Kamranpour (2001). Purification and partial characterization of a Cholesterol oxidase from Streptomyces fradiae. Enzyme Microb. Technol. Vol. 28: 410-414.
16. Terezinha, J. G. Salva, A. M. Liserre, A. L. Moretto, Marco, A. T., G. Ventrucci, T. J. B. Menezes (1999). Some enzymatic properties of Cholesterol oxidase produced by Brevibacterium sp.. Revista de Microbiologia Vol. 30: 315-323.
17. Watanable, K., H. Shimizu, H. Aihara, R. Nakamura, K. I. Suzuki and K. Momagata (1986). Isolation and identification of cholesterol degradation Rhodococcus strains food of animal origin and their cholesterol oxidase activities. J. General and Appl. Microiol. Vol. 32: 137-147.
18. William, S. T., M. Goodfellow and G. Alderson (1989). Genus Streptomyces Waksman and Henrici 1943, 339AL. In: Williams, S. T., Sharpe, M. E., Holt, J. G., (eds) Bergey's manual of systematic bacteriology. Vol. 4. Williams and Wilkins, Baltimore: 2452-2492.

Corresponding Author:

Palani Perumal*

Email: *palanii7@unom.ac.in*