Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.6154

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (1):** 713-720 (2018)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Isolation and Characterisation of Cholesterol Biodegrading Bacteria from Industrial Waste, Partial Purification of Cholesterol Oxidase, its Bioreactor and Application Studies

Athul Sanjeev*, Kavyamol K. Nair, Nija R. J., Arya T. Dharan, Nahida Nazeer, Ananthavishnu S. G. and P. Roopa

Department of Biotechnology and Biochemical Engineering, Mohandas College of Engineering and Technology, Thiruvananthapuram, kerala, India

*Corresponding Author E-mail: drathulsanjeevtvm@gmail.com

Received: 7.01.2018 | Revised: 10.02.2018 | Accepted: 15.02.2018

ABSTRACT

Cholesterol oxidase (CHO) is a monomeric bi-functional FAD-containing (flavoenzyme) enzyme belongs to the family of oxidoreductases those catalyses the oxidation of cholesterol into 4-cholesten-3-one. Cholesterol oxidase has various clinical and industrial applications. . Recently, microbial cholesterol oxidase have received a great attention for their wide usage in medicine. The present study aims at isolation and characterization of microorganisms which possess ability to produce CHO when grown in a medium rich in cholesterol. The presence of CHO producing microorganism was confirmed by CHO indicator plates. Biochemical tests and molecular characterization (i.e.) 16s rRNA sequencing was performed to identify the strain as Bacillus sonorensis. Optimum production was observed at 35°C and pH 7.0 on cholesterol minimal medium. The product obtained during fermentation is partially purified by ammonium sulphate precipitation and Dialysis. The purified cholesterol oxidase enzyme was electrophoresised and Molecular weight of this enzyme was estimated at 55 kDa. Antimicrobial activity of purified enzyme was tested. Also the effect of the enzyme on serum cholesterol was studied.

Key words: *Bacillus sonorensis, Cholesterol oxidase, Cholesterol, 4-cholesten-3-one, CHO indicator plates.*

INTRODUCTION

Some microorganisms present in the soil are capable of growing by utilizing cholesterol as sole carbon source. These Cholesterol degrading microorganisms generally produce an enzyme known as cholesterol oxidase (CHO) which catalyzes the conversion of cholesterol into 4-cholestene-3-one[1,2].

Cholesterol oxidase (CHO), a bi-functional FAD-containing (flavoenzyme) enzyme belongs to the family oxidoreductases that catalyze the first step in cholesterol catabolism. This bifunctional enzyme oxidizes cholesterol to cholest-5-en-3-one in an FAD-requiring step,

Cite this article: Sanjeev, A., Nair, K.K., Nija, R.J., Dharan, A.T., Nazeer, N., Ananthavishnu, S.G. and Roopa, P., Isolation and Characterisation of Cholesterol Biodegrading Bacteria from Industrial Waste, Partial Purification of Cholesterol Oxidase, its Bioreactor and Application Studies, *Int. J. Pure App. Biosci.* **6(1):** 713-720 (2018). doi: http://dx.doi.org/10.18782/2320-7051.6154

Which is then isomerized to cholest-4-en-3-one with the release of H_2O_2 [20]. Many microorganisms have been reported with ability to produce CHO. Knowledge on the isolation and characterization of cholesterol-degrading microorganisms and evaluate their potential for degradation of cholesterol has diverse applications.

In the present study, *Bacillus sonorensis* strain was isolated from soil and its biochemical and molecular characterizations were elucidated. Then the optimization of cholesterol oxidase production by *Bacillus sonorensis* strain using different pH and Temperature were performed in order to increase the cholesterol oxidase production. The cholesterol oxidase was produced in pilot scale in a fermenter providing optimized conditions and the purification of cholesterol oxidase was performed. Then the genome was isolated using whole genome isolation kits from HiMedia. The genome was subjected to 16S rRNA sequencing to authenticate the microorganism. Application of cholesterol oxidase obtained from Bacillus sonorensis was also studied.

MATERIALS AND METHODS

Sample collection

Soil samples containing oil waste and effluents from soap industry were collected. These soil samples were chosen, since they contain oil for prolonged period of time and have turned into medium for the microorganisms to thrive on by degrading the fat thereby using cholesterol content as a source of carbon. Soil samples were collected from the regions were oil from industries have been dumped for years[21].

Medium preparation

Cholesterol containing minimal medium of below mentioned composition was prepared and autoclaved[1,2,21].

1. NH_4NO_3 – 0.5%
2. K_2HPO_4- 0.00025%
3. $MgSO_4$ $7H_2O$- 0.00025%
4. NaCl- 0.005%
5. $FeSO_4$ $7H_2O$- 0.0005%
6. Cholesterol- 0.5%
7. 1% Agar

Isolation of microorganisms

Cholesterol containing minimal medium thus prepared was inoculated with soil samples and effluent samples of dilutions (10^{-1}) pour plate techniques and was allowed to solidify. The plating was done in laminar air flow chamber in order to avoid presence of contaminants and ensure sterile conditions. This was further incubated under specific conditions for about 15 days at 28 – 29 °C. From that we conclude that the bacterial species was able to degrade cholesterol. Colonies thus obtained were sub-cultured on agar slants. Cholesterol containing broths were also inoculated under similar sterile conditions. Broth was then incubated in a shaking incubator.

Gram staining

Gram staining is performed to identify if the organism is gram positive or negative. The glass slide containing microorganism is stained with crystal violet and washed with iodine that act as a mordant. Followed by this the glass slide is washed with 95% alcohol and is stained with safranine and is washed off with distilled water. Gram positive bacteria take up crystal violet and appear violet in colour whereas gram negative appears red in colour when observed under a microscope.

Biochemical tests:

Glucose fermentation

Two culture tubes are added with methyl red indicator. The colour changes from red to yellow in acidic pH range. Sterilized Durham's tube is inverted to observe gas production.

Indole test

Addition of 0.5ml Kovac's reagent to the culture tubes pre inoculated and incubated at 37° C for 24 - 48 hours.

MR test:

To pre inoculated culture tubes add few drops of methyl red and test is positive if colour of broth turns red.

VP test:

To the test tubes containing microorganism add 10 drops of Barrit's reagent. VP test is positive if colour changes to crimson red.

Indicator plate assay

CHO is able to convert Cholesterol into Cholest-4-en-3-one and hydrogen peroxide. CHO producing colonies were plated on cholesterol oxidase indicator plates. These plates were prepared by adding 1.0 g. Cholesterol, 1.0 g Triton X-100, 0.1g o-dianisidine and 1000 Units of peroxidase to 1 litre of agar medium. Bacterial colonies were cultured on these plates and incubated at 30° C. Cholesterol penetrates into bacterial cells where it can be converted into hydrogen peroxide by Cholesterol oxidase. Reagents that exist in the medium react with hydrogen peroxide (H_2O_2) to form azo compound which turns the colour of medium into intense brown colour. The supernatant and pellets obtained as a result of partial purification were inoculated into the above media.

OPTIMIZATION

The factors like cholesterol concentration, pH and temperature enhance the production of cholesterol oxidase by the isolated strain.

Effect of cholesterol concentration

Broth media were prepared at different concentrations of cholesterol such as 0.5 mg, 0.75 mg and 1 mg and were inoculated with isolate. This was done to find out the optimum concentration of cholesterol required for the growth of cholesterol degrading micro organisms.

Effect of pH

In order to investigate the effect of pH on cholesterol oxidase production, the selected bacterial isolate was cultivated with different pH such as 6, 7.0, 8.0 in shake flask culture. The amount of enzyme produced was determined by the enzymatic assay.

Effect of temperature

The influence of different temperature on cholesterol oxidase production was investigated using different temperature such as 30°C, 35°C, 40°. The amount of enzyme produced was determined by the enzymatic assay.

Pilot scale production and purification of cholesterol oxidase

The isolate was cultured in a 3L fermentor providing optimized conditions for 15 days. The fermantor broth was subjected to ammonium sulphate precipitation followed by dialysis. SDS PAGE was performed and Protein bands on the gel were detected by silver staining method.

Bacterial genomic DNA extraction

Chromosomal DNA was prepared using a modified protocol which was previously described[14]. Bacteria from 5 ml aliquots of a stationary phase broth culture were cultured in LB broth medium containing NaCl, 5 g/l; yeast extract, 5 g/l; Tryptone, 10 g/l (pH 7.2) and then precipitated by centrifugation at 6000 rpm for 20 min. DNA was extracted using bacterial Genomic DNA extraction kit (HiMedia) according to the manufacturer's instructions.

Phylogenic analysis of isolated strain

For final assessment of isolated bacteria, a partial sequence of 16s rRNA was amplified by PCR using two sets of primers (Reverse 1492: 5'-TACGGYTACCTTGT TACGACT-3' and Forward 27: 5'- AGAGTTTGA TCMTGGCTC-3). These primers were designed based on universally conserved sequences. Reaction mixture for PCR amplification was prepared as follows : 1µl DNA template, 1µl of Taq DNA polymerase, 5 µl of Taq polymerase assay buffer 10×, 2 µl of each primers, 2 µl of dNTP Mix and 37 µl of nuclease free water in a final volume of 50 µl. Mixed the components gently. A short spin of 10000 rpm for 20 seconds was given. Placed the PCR tubes in thermal cycler machine.

Thermal cycling PCR program was as follows: initial denaturation of DNA at 95^{o}C for 5 min, then denaturation at 94^{o}C for 45 s, annealing at 58^{o}C for 45 s, extension at 72^{o}C for 90 s in 30 cycles. Final extension was performed at 72^{o}C for 10 min. A volume of 4 µl of resulting PCR products was analysed by electrophoresis on 0.8% gel agarose. Purified DNA was sequenced by Rajiv Gandhi Centre for Biotechnology (India). Resulting sequence was then compared with the non-redundant sequence database in NCBI.

Application of purified CHO:

Effect on serum cholesterol

The initial serum cholesterol value was determined. Then the serum was treated separately with 5µl of the purified enzyme

sample and kept for incubation at 35°C for 10 min. After completion of incubation period the amount of residual cholesterol was estimated.

Antimicrobial activity

Antimicrobial activity was tested by well diffusion method. The solidified nutrient agar plates were swaped with the sufficiently turbid suspension of the organisms *Escherichia coli*, *Staphylococcus aureus*, *saccharomyces cerevisiae*.

Then the 6 mm wells were dug in the centre of agar plates and 25µl enzyme sample was poured into them and kept at 4°C for 1h for efficient diffusion. The plates were then incubated at 35°C for 24 h and resulted inhibition zones were measured.

RESULTS AND DISCUSSION

Isolation of microorganism

White chalk powder colonies were observed in cholesterol minimal media plates (Figures 1 and 2)

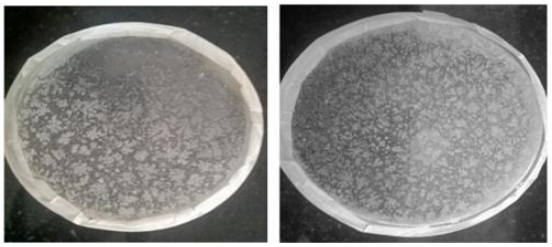

Fig. 1 & 2: colonies in petriplate inoculated with soil sample 1(from industrial oil waste containing samples) and soil sample 2 (from effluent containing samples)

Biochemical Test

The sample was biochemically identified as Gram positive, motile rods which give positive glucose fermentation, methyl red and voges-proskauer test (Figure 3 and 4). The indole test gave a negative result (Figure 5).

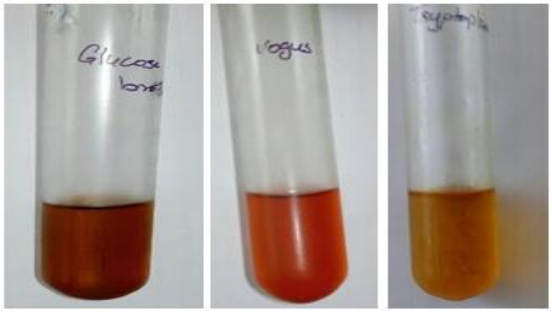

Fig. 3, 4 & 5: positive glucose fermentation test, positive MRVP test, Negative Indole test

Indicator plate assay

Indicator plate's media turns into intense brown colour confirming presence of CHO (Figure 6).

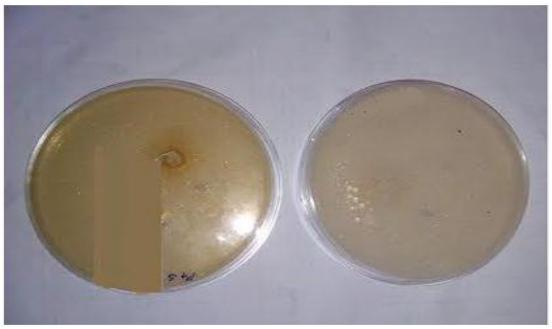

Fig. 6: indicator plate (showing intense brown colour) and control plate

Optimum pH

The maximum cholesterol degradation was observed at 7 pH (Figure 7).

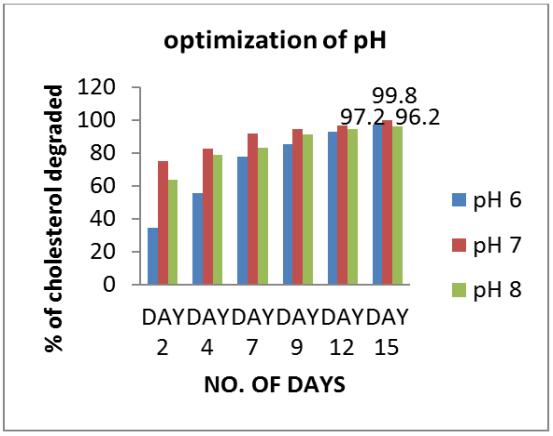

Fig. 7: graph showing % of cholesterol degradation on different pH media

Optimum Temperature

The maximum cholesterol degradation was observed at 35ºC temperature (Figure 8).

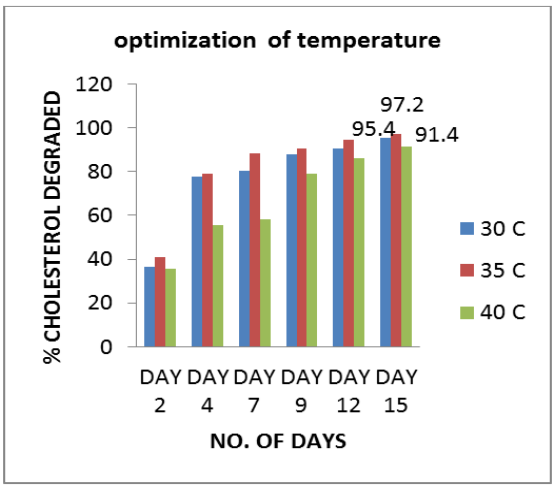

Fig. 8: graph showing % of cholesterol degradation on different Temperature

Cholesterol concentration

The maximum growth of the microorganism was observed on 0.5mg cholesterol concentration (Figure 9).

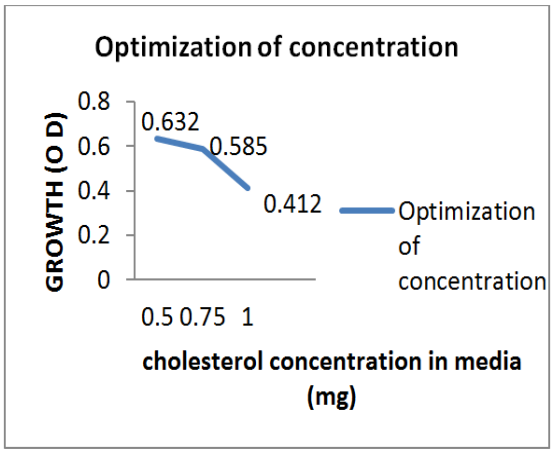

Fig. 9: graph showing growth of microorganism at different cholesterol concentration

Purification of enzyme

The cholesterol oxidase enzyme was purified from the fermentation broth using Ammonium sulphate precipitation followed by dialysis. A 55 kDa molecular weight band was observed on 10% polyacrylamide gel after purification of the cholesterol oxidase enzyme. (Figure 10)

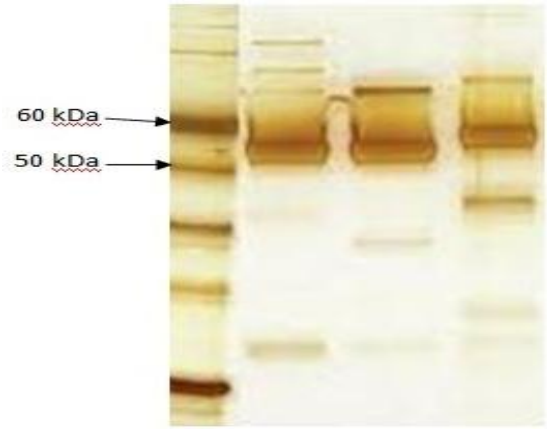

Fig. 10: SDS PAGE showing bands, lane 1: protein marker, lane 2: sample before precipitation, lane 3: sample after precipitation, lane 4: sample after dialysis

GenBank accession number

The 16s rRNA sequence was deposited in GenBank with accession number MF574324.

Phylogenic Analysis

BLAST results indicate that the obtained 16s rRNA sequence show maximum similarity with the organism *Bacillus sonorensis.*

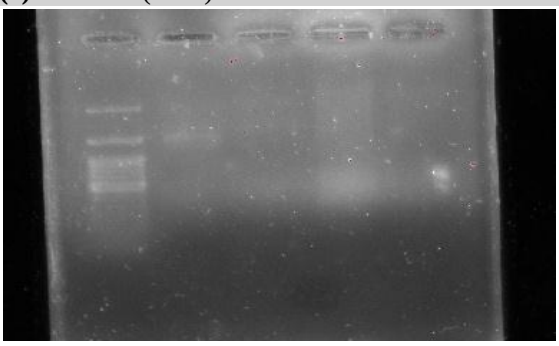

Fig. 11: PCR products subjected to electrophoresis. In lane 2 a prominent band was observed (1600 bp). This PCR product is subjected to sequencing

Effect on serum cholesterol

The effect of enzyme on the serum cholesterol, in vitro was studied and it was found that reduction in serum cholesterol level was 31.57%.

Antimicrobial Activity

The antimicrobial activity assay the purified enzyme showed 5 mm and 7 mm zone of inhibition against *E. coli* and *Saccharomyces cerevisiae* (Figure 12 & 13), while for *Staphylococcus aureus* no zone appeared.`(Figure 14)

Fig. 12 & 13: 5mm zone of inhibition was observed for E. coli , 7mm zone of inhibition was observed for Saccharomyces cerevisiae

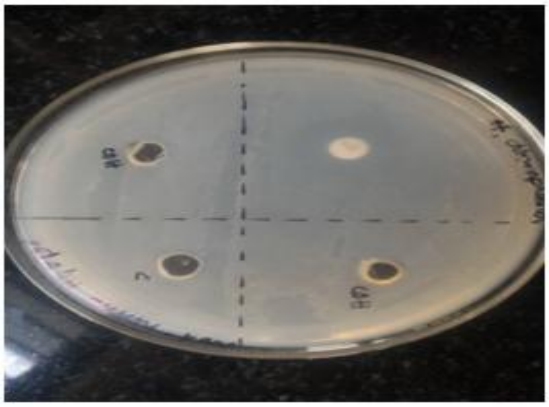

Fig. 14: No inhibition was observed for Staphylococcus aureus

Biochemical and molecular tests showed that this CHO producing Gram-positive bacterium belongs to *Bacillus* genus. Several studies have used various methods to confirm the production of CHO enzyme by different bacteria[12.] In the present study CHO indicator plates were used for this purpose. A protein band of molecular weight 55 KDa on SDS-PAGE was the evidence for the presence of CHO enzyme. The acquired CHO molecular weight is similar to that produced by Brevibacterium[24], *Rhodococcus* sp. PTCC 1633 [9] and *Rhodococcus erythropolis*[13]. It must be noted that molecular weight of CHO protein produced by other bacterial strains has a range of 30-61 kDa. However, Sojo et al. [14] have estimated molecular weight of CHO at 55 and 56 kDa from Rhodococcus erythropolis and 56 kDa from Rhodococcus equi no. 23 [10]. In 1982, Cheetham *et al.*[22] noted that optimum pH for CHO derived from Nocardia and Rhodococcus is 7.5. In our study the optimum pH for activity of the enzyme was 7.0, which is similar to that for Watanabe[9] studies. According to our results the CHO has maximum activity at 35^{o}C and Yazdi *et al.*[20] showed, CHO enzyme is active between temperature range of 4-50^{o}C. Molecular characterization i.e. 16s rRNA sequencing (phylogenic analysis) was carried out to confirm isolated bacterium. BLAST results showed 99% homology with *Bacillus sonorensis* bacteria. The CHO enzyme produced from bacteria has much clinical and industrial application but its production in large scale for industrial usage is expensive and time consuming. Recently many studies have been focusing on cloning bacterial CHO genes into a suitable expression vectors and proper hosts[3,23,24]. Further studies are required for cloning this gene into an expression vector and evaluating applicability of recombinant cholesterol oxidase for industrial purpose.

CONCLUSION

The presence of CHO producing microorganism was confirmed by CHO indicator plates. Biochemical tests and molecular characterization (i.e.) 16s rRNA sequencing was performed to identify the strain as *Bacillus sonorensis*. Optimum production was observed at 35°C and pH 7.0 on cholesterol minimal medium. The product obtained during fermentation is partially purified by ammonium sulphate precipitation and Dialysis. The purified cholesterol oxidase enzyme was electrophoresised and Molecular weight of this enzyme was estimated at 55 kDa. Antimicrobial activity of purified enzyme was tested. Also the effect of the enzyme on serum cholesterol was studied.

Acknowledgement

The Authors is thankful to the Biotech department of Kerala University, karyavattom campus for providing necessary help. This project was funded by Kerala State Council for Science, Technology & Environment (KSCSTE) and MCET R&D cell.

REFERENCES

1. Saranya, S. Shekinah, T. Rajagopal, J. Vijaykumar, Isolation and characterization of cholesterol degrading bacteria from soap and vegetable oil industrial wastes , *Ind J of biotech* **13:** 508-513 (2014).
2. Lee S-Y, Ree , Tae W-C, Shin J-C, Park B-K, Purification and characterization of cholesterol oxidase from *Pseudomonas* sp and taxonomic study of the strain, *Appl Microbial Biotechnol* **31:** 542-546 (1989).
3. Yazdi, M. T., Yazdi, Z. T., Ghasemian, A., Zarrini, G., Olyaee, N. H., Purification and characterization of extracellular cholesterol oxidase from *Rhodococcus sp*. PTCC 1633, Biotechnology, **7:** 751-756 (2008).
4. Parekh S N & Desai P B, Isolation and characterization of extracellular cholesterol oxidase producing *Microbacterium* sp. from waste of regional oil mill, *Int J Adv life Sci*, **6:** 87-92 (2013).
5. Bholay, A. D., Gadekar, D. J., Sawant, S.K. and Sonawane, S.M., Bacterial extracellular cholesterol oxidase and its pharmaceutical perspectives, *Int J Curr Microbial Appl Sci* , **2:** 19-28 (2013).

6. Lashkarian, H., Raheb, J., Shahzamani, K., Shahbani, H. and Shamsara, M., Extracellular cholesterol oxidase from *Rhodococcus sp*. :Isolation and molecular characterization, *Iran Biomed J*, **14:** 49-57 (2010).
7. Richmond, W., Preparation and properties of a cholesterol oxidase from nacardia sp. And its application to the enzymastic assay of total cholesterol in serum, *Clin chem.*, **19:** 1350-1356 (1973).
8. Yazdi, M.T., Malekzadeh, F., Zarrini, G., Faramarzi, M.A., Kamranpour, N., Production of cholesterol oxidase by a newly isolated *Rhodococcus* sp., *World J Microbiol Biotechnol*, **17:** 731-737 (2001).
9. Watanabe, K., Aihara, H., Nakagama, Y., Nakamura, R. and Sasaki, T., Properties of the purified extracellular cholesterol oxidase from *Rhodococcus equi* No 23, *J Agri Food Chem*, 37: 1178-1182 (1989).
10. Mac Lachlan, J., Wotherspoon, A.T.L., Ansell, R.O. and Brooks, C.J.W., Cholesterol oxidase: sources, physical properties and analytical applications. J. Steroid. *Biochem. Mol. Biol.* **72(5):** 169-195 (2000).
11. Takagi, T., Yoshida, and Taguchi, H., "Effect of Oleic Acid Adsorption onto Cell Surface on Cholesterol Oxidase Production by Schizophyllum commune," *J Fermentation Technol*, **60(1):** pp. 31-35 (1982).
12. Nishiya, Y., Harada, N., Teshima, S., Yamashita, M., Fujii, I., Hirayama, N. and Murooka, Y., Improvement of thermal stability of Streptomyces cholesterol oxidase by random mutagenesis and a structural interpretation. *J. Pro. Eng.* **10 (3):** 231-235 (1997).
13. Lata Kumari, Shamsher S. Kanwar, Cholesterol Oxidase and Its Applications, *Adv in Microbiology* **2:** 49-65 (2012).
14. Sambrook, J., Molecular cloning. a laboratory manual. Cold Spring harbor Laboratory Press. Dream Books Co, Aurora, CO, USA. Garcia J L, Uhia I & Galan B, Catabolism and *bacteria, Microb Biotechnol*, **5:** 679-699 (2012).
15. Sojo, M., Bru, R., Loppez, M.D., Garcia, C.F. and Arguelles, J.C., Cell-linked and extracellular cholesterol oxidase activities from Rhodococcus erythropolis isolation and physiological characterization. J. Appl. Microbiol. Biotechnol. **47 (5):** 583-589 (1997).
16. Ahmad, S., Garg, S. K. & Johri, B.N., Biotransformation of sterols: Selective cleavage of the side chain, *Biotechnol Adv*, **10:** 61-67 (1992).
17. Naghibi, F., Yazdi, M. T., Sahebharani, M. and Dalioii, M.R.N., Microbial transformation of cholesterol by *Mycobacterium smegmatis*, *J Sci (Iran)*, **13:** 103-106 (2002).
18. Doukyu, N. and Aono, R., Purification of extracellular cholesterol oxidase with high activity in the presence of organic solvents from *Pseudomonas* sp. Strain ST-200, *Appl Environ Micobiol*, **64:** 1929-1932 (1998).
19. Kim, K-P, Rhee, I-K & Park, H-D, Degradation of cholesterol by *Bacillus subtilis* SFF34 in flatfish during fermentation, *J Microbiol*, **41:** 284-288 (2003).
20. Andhale, M.S. and Sambrani, SA, Cholestrol biotransformation in monophasic systems by solvent tolerant *Bacillus subtilis* AF333249, *Indian J Biotechnol*, **5:** 389-393 (2006).
21. Yazdi, M. T., Malekzadeh, F., Khatami, H. & Kamranpour, N., Cholestrol degrading bacteria: Isolation, characterization and bioconversion, *World J Microbiol Biotechnol*, **16:** 103-105 (2000).
22. Salva, T.J.G., Liserre, A. M., Moretto, A. L., Zullo, M.A.T., Ventrucci, G., Some enzymatic properties of cholesterol oxidase produced by *Brevibacterium* sp., *Rev Microbiol*, **30:** 315-323 (1999).
23. Cheetham, P.S., Dunnill, P. and Lilly, M.D., The characterization and interconversion of three forms of cholesterol oxidase extracted from Nocardia rhodochrous. *J. Biochem*. **201(3):** 512-521 (1982).

24. Ghasemian, A., Tabatabaei, Y.M., Sepehrizadeh, Z., Tabatabaei, Y.Z. and Zarrini, G.H., Overexpression, one-step purification, and characterization of a type II cholesterol oxidase from a local isolate Rhodococcus sp. PTCC 1633. *World J. Microbiol. Biotechnol.* **25(5):** 773-779 (2009).
25. Fujishiro, K., Uchida, H., Shimokava, K., Nakano, M., Sano, F., Ohta, T., Nakahara, N., Aisak, K. and Uwajima, T., Purification and properties of a new Brevibacterium sterolicum cholesterol oxidase produced by E. coli MM294/pnH10. J. FEMS. Microbiol. Lett. **215(2):** 243-248 (2002).
26. Loredano Pollegioni, Luciano Piubelli, Gianluca Molla, Cholesterol oxidase: biotechnological applications, *The FEBS Journal*, **276:** 6857-6870 (2009).