



ISOLATION AND OPTIMIZATION OF CHOLESTEROL OXIDASE PRODUCTION BY *STREPTOMYCES MARITIMUS*

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Received 7th July 2023; Revised 9th Aug. 2023; Accepted 9th Nov. 2023; Available online 15th Dec. 2023

<https://doi.org/10.31032/IJBPAS/2023/12.12.1013>

ABSTRACT

Microbial enzymes have unimaginable capabilities to catalyze biochemical reactions. Cholesterol oxidase is one such enzyme that converts cholesterol into cholest – 4-en-3-one, a precursor for the production of steroid hormones and pharmaceuticals. Due to its increased usage in determining blood cholesterol in laboratories and as a biocatalyst for the manufacture of many steroids, cholesterol oxidase has drawn a lot of attention. The present study aims to isolate and screen microbes from bio-dung samples for the production of cholesterol oxidase enzyme. The bacterial strain which showed maximum cholesterol oxidase activity was undergone morphological, biochemical and molecular identification and the strain was identified as *Streptomyces maritimus* by 16S rRNA sequencing. *Streptomyces maritimus* is a gram-positive bacteria belonging to Phylum Actinobacteria. Cholesterol oxidase production by *Streptomyces maritimus* was also optimized for various parameters. From the study, it was identified that *Streptomyces maritimus* was capable of producing the maximum level of cholesterol oxidase at an incubation temperature of 35°C, pH of 6, agitation rate of 200 rpm and inoculum size of 25%. Optimum enzyme activity was observed when the medium was supplemented with galactose as carbon source, yeast extract as nitrogen source and PO₄²⁻ as inorganic salt.

Keywords: Cholesterol oxidase, Cholest – 4-en-3-one, Cholesterol, *Streptomyces maritimus*, bio dung

1. INTRODUCTION

Microbial enzymes mediate the process of bioconversion of organic compounds into

its intermediate metabolites. They are economically viable, consistent, high yielding and susceptible to gene

modifications. The progress in recombinant DNA technology and enzyme engineering has given microbial enzymes a significant role in medicinal, pharmaceutical, and industrial applications [1]. Cholesterol oxidase is a bacterial monomeric FAD-possessing enzyme belonging to oxidoreductase family [2]. This enzyme is very efficient in transforming cholesterol into cholest-4-en-3-one which is a crucial intermediate metabolite in a number of steroid biotransformation reactions and it acts as the starting point for the production of several pharmacological metabolites, such as androst-4-ene-3,17-dione and androsta-1,4-dione-3,17-dione, that are important building blocks in the production of anabolic steroids and hormone contraceptives [3]. It works as a biosensor that tracks serum cholesterol levels, essential for detecting cardiovascular diseases and other lipid disorders [4]. It reveals high anticancer capabilities when studied on rhabdomyosarcoma and breast cancer cell lines [5]. Additionally, it functions as an insecticidal agent by disrupting the midgut epithelial membrane of the larvae of several lepidopteran pests [6].

Isolation and screening microbial strains capable of producing cholesterol oxidase from various possible sources are of utmost importance as it has an extensive array of applications and an elevated degree

of commercial value. A diverse group of microorganisms with different evolutionary histories produce this enzyme, and unexplored habitats usually offer microbes with a high potential for producing this enzyme. Since bio dung serves as a good source of beneficial microorganisms like bacteria, actinomycetes, fungi, and yeast, it could possibly be explored for the presence of cholesterol oxidase producing microorganisms. Considering their low toxic effects, efficiency, specificity and ecologically desirable properties, Actinobacteria are becoming more and more popular [7].

The current investigation aims to isolate and screen microbes from bio dung samples for the synthesis of cholesterol oxidase enzyme and to characterize the bacterial strain that exhibited maximum cholesterol oxidase production. The bacterial strain which expressed maximum cholesterol activity was optimized for various parameters like temperature, pH, inorganic salts, inoculum size, agitation rate, and carbon and nitrogen sources. To the best of our knowledge, no documented studies have been reported on cholesterol oxidase enzyme production and its optimization by the strain *Streptomyces maritimus* isolated from bio dung samples.

2. MATERIALS & METHODS

2.1 Collection of bio dung samples and isolation of bacteria

A total of 24 bio-dung samples were collected in sterile polythene bags from Kottayam, Pathanamthitta and Ernakulam Districts of Kerala, India. 1 g of the sample was suspended in 100 ml of distilled water and it was rapidly shaken for 1 hr in shaker incubator. 100 µl of the obtained supernatant was inoculated on M9 salt medium and was proceeded to incubate at 37°C for 48 hrs. The larger, quickly expanding colonies were sub-cultured on a secondary medium having cholesterol as the sole supply of carbon. Cultures were streaked on cholesterol coated M9 media plates, which were then incubated at 37°C for 7 days. The capacity of bacteria to make use of the available cholesterol was assessed by the growth of bacteria on these culture plates [8-9].

2.2 SCREENING FOR CHOLESTEROL-TRANSFORMING BACTERIA

2.2.1 Colony staining method

Colony staining test was carried out to verify the ability of the selected bacteria to produce cholesterol oxidase. Filter discs soaked into a mixture containing 6% phenol, 1.7% 4-aminoantipyrine, 0.5% cholesterol, and 3000 U/mL horse radish peroxidase (HRP) in 100 mM potassium phosphate buffer (pH 7.0) were fixed on fresh colonies and was left for incubation at 37° C for 24 hrs. The appearance of red indicates the production of cholesterol oxidase which is due to the formation of quinoneimine dye [9].

2.2.2 Cholesterol oxidase indicator plate study

Cholesterol oxidase-possessing strains were cultured on indicator plates having 1.0 g/L Triton X-100, 1.0 g/L cholesterol, 0.1 g/L o-dianisidine, and 1U/mL horse radish peroxidase of agar medium. The bacterial cultures were allowed to grow on the plates and maintained at 37°C. Bacterial cholesterol oxidase transforms cholesterol into hydrogen peroxide (H₂O₂), which turns the colour of the medium brown due to the generation of an azo compound [9].

2.2.3 Estimation of enzyme activity

The amount of hydrogen peroxide generated was used to determine the activity of cholesterol oxidase enzyme. The assay mixture (total 1 mL) contained 87 mM potassium phosphate buffer, 0.89 mM cholesterol, 64 mM sodium cholate, 1.4 mM 4-aminoantipyrine, 21 mM phenol, 0.34 % tween 80, and horseradish peroxidase 5 U/mL. The reaction mixture was allowed to incubate with 100 µL of the enzyme for 5 minutes at 35° C and the emergence of quinoneimine dye was monitored by measuring the rate of absorbance at λ500 nm.

$$\text{Units/ml enzyme} = \frac{(\Delta A_{500\text{nm/min Test}} - \Delta A_{500\text{nm/min Blank}})(3)(df)}{(7.5)(0.1)}$$

3 = Volume (in millilitres) of assay
df = 1
7.5 = Millimolar extinction coefficient of o-Dianisidine (Oxidized) at 500 nm
0.1 = Volume (in millilitres) of enzyme used

2.3 IDENTIFICATION OF THE ISOLATES

Bergey's manual of systematic bacteriology was used to identify the isolates that produce cholesterol oxidase according to their morphological, biochemical, and molecular properties [10].

2.4 SEQUENCE ANALYSIS OF 16S rRNA GENE

The bacterial strain analysis was done using 16S rRNA gene sequencing method as it is a highly conserved region in the bacterial genome that contains variable regions for strain identification. Two primers namely forward primer 5' – AGAGTTTGATCMTGG – 3' and the reverse primer 5' – ACCTTGTTACGACTT- 3' were selected for PCR amplification. PCR amplification was carried out with denaturation at 95°C for 5 minutes, followed by 30 cycles each at 94°C for 45 seconds, at 52°C annealing for 45 seconds and at 72°C extension for 45 seconds followed by final elongation at 72°C for 8 minutes.

2.5 OPTIMIZATION OF CHOLESTEROL OXIDASE PRODUCTION

2.5.1 Effect of Temperature

The bacterial isolate was incubated for a period of 24 hours at 15°C, 20°C, 25°C, 30°C, 35°C, 40°C and 45 °C to find out the ideal temperature preferred for the maximum enzyme activity [11].

2.5.2 Effect of pH

To figure out the ideal pH level favourable for the activity of the enzyme, the selected bacterial isolate was incubated from pH 3 to 9 for a period of 24 hours and level of enzyme production was monitored under standard conditions. Acetate buffer, Phosphate buffer and Tris HCl were employed for pH ranges of 3 to 5.5, 6 to 7 and 8 to 9 respectively [11].

2.5.3 Effect of Carbon source

The degree of enzyme production was analyzed by altering the medium with a number of carbon sources (0.5% w/v) like fructose, galactose, mannitol, glucose, sucrose, maltose and lactose.

2.5.4 Effect of Nitrogen source

The effect of multiple nitrogen sources (0.5% w/v) like Yeast extract, Peptone, Tryptone, Beef extract, KNO₃, NaNO₃ and Urea were checked for enzyme production.

2.5.5 Effect of Inoculum size

To estimate the optimum inoculum size favourable for maximum enzyme activity, bacterial inoculum sizes ranging from 5%-35% were used.

2.5.6 Effect of Agitation Rate

To optimize the rate of agitation on the production of cholesterol oxidase, a varying rate of agitation from 120 rpm to 240 rpm was carried out.

2.5.7 Effect of Inorganic salts

Inorganic salts such as Na⁺, K⁺, Cl⁻, Ca²⁺, Mg²⁺, SO₄²⁻ and PO₄²⁻ were

supplemented(0.2% w/v each) as individual components to the production media to check the effect of these sources on the enzyme production.

3. RESULTS & DISCUSSION

24 randomly collected bio dung samples were initially cultured on M9 salt medium. From the 24 sets of samples, 56 different bacterial strains were obtained. The obtained bacteria were again cultured on M9 salt medium containing 0.1% Cholesterol for analyzing its cholesterol degrading ability. Out of the 56 strains of bacteria, 28 strains were

successful in surviving on cholesterol-containing medium. Those 28 strains were further analyzed for the presence of cholesterol oxidase enzyme and 12 strains were found to possess the enzyme which includes CL3, CL11, CL16, CL17, CL24, CL32, CL37, CL44, CL49, CL50, CL54 and CL55. Out of the 12 strains, CL 54 strain was the one that showed maximum enzyme activity (1.816 U/mL) (**Table 1**) and it was chosen for further study. **Figure 1** shows the colony screening test and **Figure 2** shows the morphological features of the strain CL 54 respectively.

Table 1: Peroxidase activity in U/ml

| Peroxidase Screening Assay | | | |
|----------------------------|---|--|---------------------------------|
| Sample Name | $\Delta A_{500\text{nm}/\text{min}}$ Test | $\Delta A_{500\text{nm}/\text{min}}$ Blank | Peroxidase activity in Units/ml |
| CL3 | 0.416±0.009644 | 0.082 | 1.336 U/ml |
| CL11 | 0.357±0.0173 | 0.082 | 1.1 U/ml |
| CL16 | 0.331±0.0072 | 0.082 | 0.996 U/ml |
| CL17 | 0.305±0.0031 | 0.082 | 0.892 U/ml |
| CL24 | 0.485±0.0034 | 0.082 | 1.612 U/ml |
| CL32 | 0.428±0.0035 | 0.082 | 1.384 U/ml |
| CL37 | 0.459 ±0.0025 | 0.082 | 1.508 U/ml |
| CL44 | 0.359±0.0090 | 0.082 | 1.108 U/ml |
| CL49 | 0.392±0.0085 | 0.082 | 1.24 U/ml |
| CL50 | 0.354± 0.0051 | 0.082 | 1.088 U/ml |
| CL54 | 0.536±0.0064 | 0.082 | 1.816 U/ml |
| CL55 | 0.407±0.0096 | 0.082 | 1.3 U/ml |

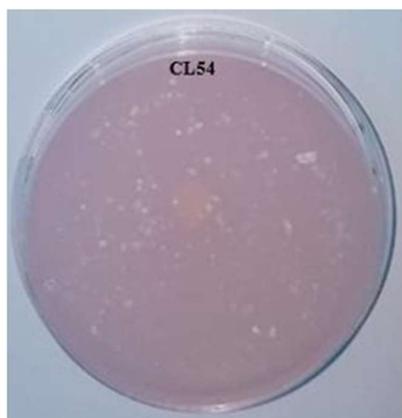


Figure 1: Colony staining test of strain CL54



Figure 2: Pure culture of Cholesterol oxidase producing bacteria (CL 54)

Morphological study indicates that CL 54 strain was a gram-positive, filamentous, white, medium, circular-shaped Actinobacteria and biochemical studies suggest that CL54 strain was positive for Methyl red, Oxidase and Catalyse test.

Genomic DNA was extracted from the bacterial strain and 16 S rRNA gene region was amplified and sequenced. The CL54 isolate was identified by PCR using primers, which were amplified on 1500 bp PCR product band. The band was visualized using Ethidium bromide staining on agarose gel. The bacterial strain was identified as *Streptomyces maritimus* using 16S rRNA sequencing. The sequence was added to GenBank NCBI and the Accession number for the sequence is OP855523.

3.1 Effect of temperature

The Optimum cholesterol transformation ability was shown at 35° C temperature by *Streptomyces maritimus* (Figure 3). The present study's findings were comparable with the previous works undertaken by Kumari and Kanwar [4]. Prior study reports that 35° C was the optimum temperature for *Bascillus licheniformis* [12] and *Rhodococcus sp.* [13]. Also, the optimum temperature for *Streptomyces fradiae* and *Brevibacterium sp.* were identified as 50° C and 53° C respectively [8 -14]. The ideal temperature needed for cholesterol oxidase synthesis by

Rhodococcus equi was 47° C and by *Corynebacterium cholestrolicum* was 40°C. [15].

3.2 Effect of pH

It has been identified that the *Streptomyces maritimus* demands an optimum pH 6 to have the highest level of enzyme activity (Figure 4). From the previously reported studies, bacterial strains like *Actinomyces lavendulae* mycelium, *Corynebacterium cholestrolicum*, *Streptoverticilium cholestrolicum*, *Rhodococcus equi* and *Streptomyces violascens* require a pH between 7.0 and 8.0 for their optimum enzyme production [9].

3.3 Effect of Carbon Source

Cholesterol oxidase production was optimum when cholesterol was supplemented by galactose (Figure 5). Earlier research disclosed that *Rhodococcus erythropolis*, showed an increased cholesterol oxidase activity when cultured in mineral medium having cholesterol as the only supply of carbon [16].

3.4 Effect of Nitrogen Source

Different sources of nitrogen were supplemented with the biotransformation media and it was identified that yeast extract was the most effective nitrogen source for *Streptomyces maritimus* (Figure 6). Previous studies reported that yeast extract in combination with (NH₄)₂HPO₄ gave a significant result on *Rhodococcus sp* [13].

3.5 Effect of Inoculum size

Streptomyces maritimus yielded cholesterol oxidase at a maximum inoculum size of 25% (**Figure 7**). Optimization of inoculum size is a crucial component of bioprocessing and is employed in a number of fields including biotechnology, food, beverage and environmental science. In biotechnology, optimization of inoculum size can lead to high yields of biofuels, pharmaceuticals and other value-added products

3.6 Effect of rate of agitation

The optimum agitation rate for the cholesterol oxidase production by *Streptomyces maritimus* was recorded to be 200 rpm (**Figure 8**). Optimization of agitation rate involves identifying the optimal speed for attaining the desired level of mixing, mass transfer and heat transfer in a given process.

3.7 Effect of inorganic salts

The optimum inorganic salts required for the biotransformation reaction of cholesterol were identified as PO42- (**Figure 9**).

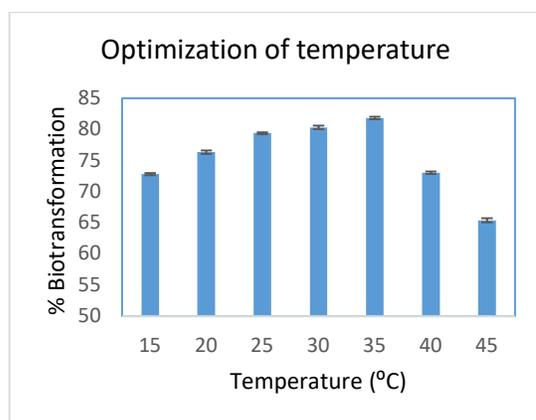


Figure 3: Effect of Temperature on enzyme activity

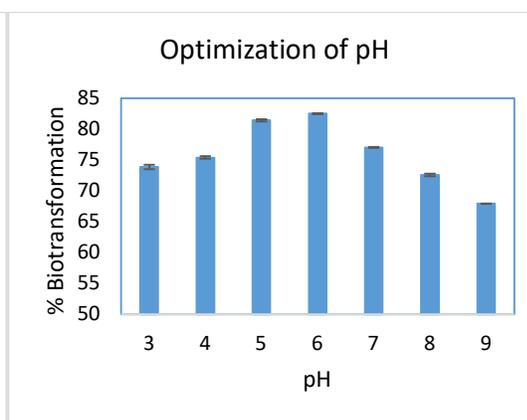


Figure 4: Effect of pH on enzyme activity

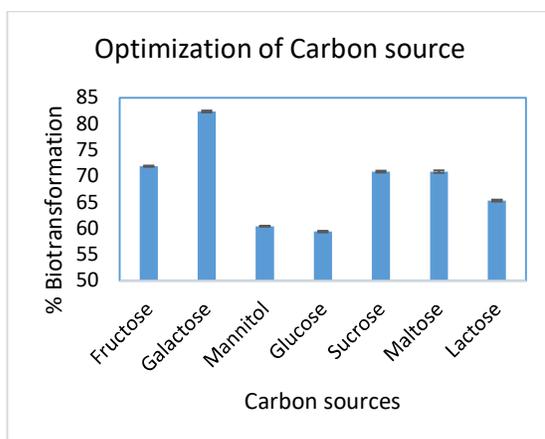


Figure 5: Effect of carbon source on enzyme activity

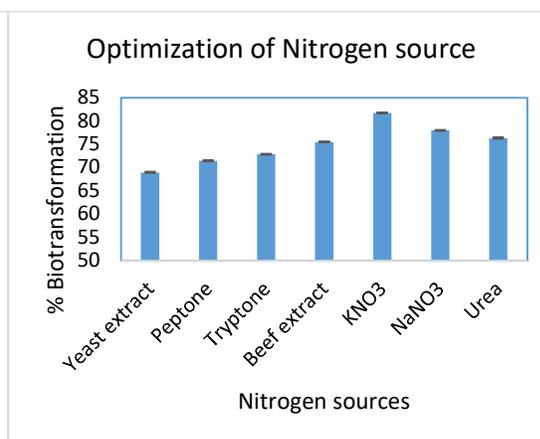


Fig 6: Effect of nitrogen source on enzyme activity

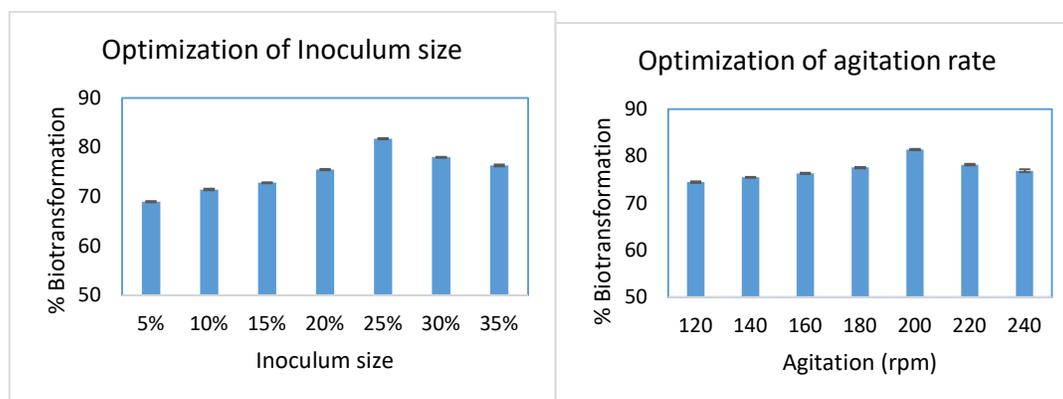


Figure 7: Effect of inoculum size on enzyme activity

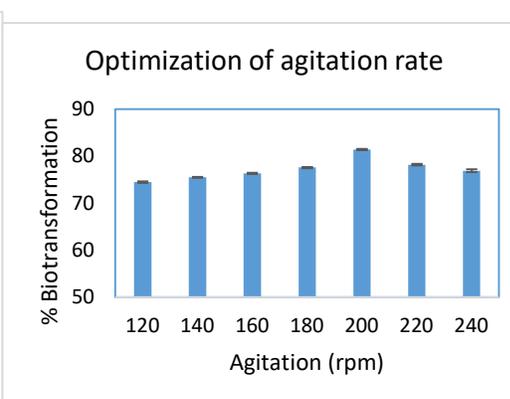


Figure 8: Effect of agitation rate on enzyme activity

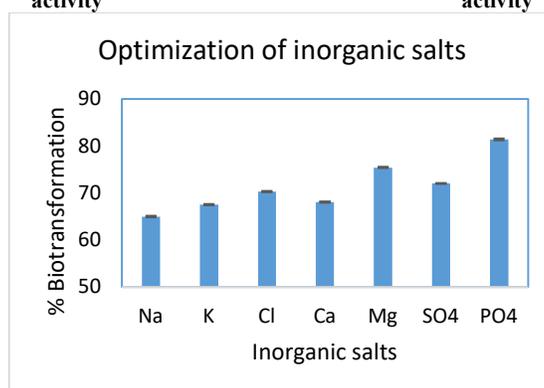


Figure 9: Effect of inorganic salts on enzyme activity

4. CONCLUSION

Since cholesterol oxidase is a potent enzyme with numerous applications, it is crucial to isolate and screen microbes from different environments to explore their potential for producing cholesterol oxidase enzyme. In the current investigation, a total of 56 microbial strains isolated from 24 bio-dung samples were screened for the production of cholesterol oxidase enzyme. The presence of cholesterol oxidase was detected in 12 bacterial strains, with bacterial strain CL 54 exhibiting the highest level of cholesterol oxidase activity. CL54 strain was identified as *Streptomyces maritimus* after being subjected to

morphological, biochemical and molecular examination. The culture parameters, including pH, temperature, carbon and nitrogen sources, inoculum size, agitation rate, and inorganic salts, were optimized to maximize the production of cholesterol oxidase by *Streptomyces maritimus*. According to the study, *Streptomyces maritimus* produces the highest concentration of cholesterol oxidase at an incubation temperature of 35°C, pH of 6, 200 rpm agitation rate, and 25% inoculum size. When the medium was supplemented with galactose as a carbon source, yeast extract as a nitrogen source, and PO4²⁻ as an inorganic salt, the enzyme activity was at its

peak. The results of the study point out that the bacterial strain *Streptomyces maritimus*, which has been isolated from bio dung sample collected from the Vypin Island, Ernakulam district, Kerala may serve as a potentially beneficial source of cholesterol oxidase enzyme for pharmaceutical and industrial usage.

5. REFERENCES

- [1] Gurung, N., Ray, S., Bose, S., & Rai, V. A broader view: microbial enzymes and their relevance in industries, medicine, and beyond, BioMed Research International, vol. 2013. (2013),1- 18.
- [2] Pollegioni L, Piubelli L, Molla G, Cholesterol oxidase: biotechnological applications, FEBS J 23 (2009): 6857-6870
- [3] Dogra N., Qazi G. Steroid biotransformation by different strains of *Micrococcus* sp., Folia Microbiol. 46 (2001), 17-20.
- [4] Lata Kumari, Kanwar S.S. Cholesterol Oxidase and its Applications. Advances in Microbiology, 2 (2012), 49 - 65.
- [5] El- Naggar NEA, Soliman HM, El-Shweihy NM. Extracellular cholesterol oxidase production by *Streptomyces aegyptia*, in vitro anticancer activities against *rhabdomyosarcoma*, breast cancer cell lines and in vitro apoptosis, Scientific reports, 8 (2018),
- [6] Purcell, J.P., J. T. Greenplate, M.G. Jennings, J.S. Ryerse, J. C Pershing, S.R. Sims, M.J. Prinsen, D.R. Corbin, M. Tran, R.D Sammons, and R.J Stonard, Cholesterol oxidase: a potent insecticidal protein active against boll weevil larvae. Biochem. Biophys. Res. Commun. 196 (1993), 1406 -1413.
- [7] Alam, A.A., Goda, D.A., Soliman, N.A., Production and Statistical Optimization of cholesterol oxidase generated by *Streptomyces* sp. AN strain, J SGenet Eng Biotechnol 20 (2022), 156.
- [8] Yazdi MT, Malekzadeh F, Zarrini GH, Faramarzi MA, Kamranpour N, Khaleghparast SH. Production of cholesterol oxidase by a newly isolated *Rhodococcus* sp., World J Microbiol Biotechnol 7 (2001), 731-737.
- [9] Lashkarian H, Raheb J, Shahzamani K, Shahbani H, Shamsaram M., Extracellular Cholesterol oxidase from *Rhodococcus* sp: Isolation and molecular characterization, Iran Biomed Journal,14 (2010), 49-57.
- [10] Bergey D, Manual of Determinative Bacteriology. 7th Ed. American Society of Microbiology. Williams and

- Willkins Co. Publishers, Baltimore, USA; 1957.
- [11] M.T Yazdi Z.T Yazdi, A. Ghasemian, G. Zarrini, N.H. Olyae and Z. Sephrizadeh. Purification and characterization of extracellular cholesterol oxidase from *Rhodococcus sp.* PTCC 1633. *Biotechnology*, 7 (2018), 751-756.
- [12] Bholay AD, Gadekar DJ, Sawant SK, Sonawane SM, Bacterial Extracellular cholesterol oxidase and its pharmaceutical perspectives, *International Journal of Current Microbiology and Applied Science* 2 (2013), 19- 28.
- [13] Kaur S, Kaur HP, Prasad B and Prasher M, Production and Optimization of Cholesterol Oxidase from *Rhodococcus sp.* *International Journal of Pharmacy and Pharmaceutical Sciences*, 7 (2015), 265 -268.
- [14] Fujishiro K, Uchida H, Shimokava K, Nakano M, Sano F, Ohta T, Purification and Properties of a new *Brevibacterium steolicum* cholesterol oxidase produced by E. Coli MM294/pn H10, *FEMS. Microbiology Letter* 2 (2002), 243-248.
- [15] Salva TJG, Liserre AM, Moretto AL, Zullo MAT, Ventrucci G, Menezes TJB. Some enzymatic properties of cholesterol oxidase produced by *Brevibacterium sp.* *Revista de Microbiologia*, 4 (1999), 315-323.
- [16] Sojo M, Bru R, Lopez –Molina D, Garcia – Carmona F, Arguelles JC. Cell linked and Extracellular cholesterol oxidase activities from *Rhodococcus erythropolis*. Isolation and physiological characterization. *Applied Microbiology and Biotechnology* 47 (1997), 583- 589.