Optimization of culture conditions for extracellular cholesterol oxidase production from *Castellaniella* sp. for cholesterol conversion to 4-cholesten-3-one

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Abstract
Cholesterol oxidase (CHOx) production by a new isolate characterized as Castellaniella sp. was studied in different fermentation conditions. The effects of various medium components and physicochemical conditions included production profile, inoculum size, inoculum age, carbon source, nitrogen source (organic and inorganic), surfactants, pH and temperature on extracellular CHOx production.

The maximum CHOx production was observed after 62 h of production time with 8.0%, v/v inoculum size with optimized fermentation medium containing cholesterol (0.1%, w/v), yeast extract (0.5%, w/v) and Triton X-100 (0.2%, v/v). The optimum pH and temperature for maximum CHOx production were found to be 8.0 and 35^{o}C respectively. The classical "one-factor-at-a-time" approach increased CHOx activity of Castellaniella sp. from 0.067 U/ml to 0.396 U/ml, indicating 5.9-fold enhancement. Cholesterol biotransformation of 73± 3.0% to 4-cholesten-3-one was achieved by using enzyme solution/ n-decane biphasic system after 3 h of reaction by using 0.2% (w/v) cholesterol. The bioconversion was confirmed by TLC, HPLC and FTIR analysis of synthesized 4-cholesten-3-one.

Keywords: Cholesterol oxidase, 4-cholesten-3-one, bioconversion, *Castellaniella* sp.

Introduction

Enzymes are natural macromolecular catalysts and today the use of enzymes is unlimited. Highly efficient biocatalysts enzymes are explored for industrial-scale catalysis due to their several diverse advantages ranging from operation in milder working conditions, remarkable product selectivity and to their lower physiological and environmental toxicity.[3] Cholesterol oxidase (3-β hydroxysterol oxidase, cholesterol:oxygen oxidoreductase, EC 1.1.3.6) is a flavin adenine dinucleotide (FAD)-dependent oxidoreductase catalysing the oxidation of cholesterol (cholest-5-en-3β-ol) to its 3-keto-4-ene derivative, 4-cholesten-3-one (cholest-4-en-3-one), with the reduction of oxygen to hydrogen peroxide.[6]

However, some cholesterol oxidases are reported to oxidize cholesterol to 6β-hydroperoxycholest-4-en-3-one (HCEO) instead of 4-cholesten-3-one (CEO).[9] CHOx is strictly of bacterial origin and is not produced in plant or animal systems.[16] The most common and versatile CHOx producers are bacteria of gram-positive origin including *Arthrobacter*[5], *Bacillus* spp.[21], *Bordetella*[23,] *Brevibacterium*[14], *Corynebacterium*[32], *Rhodococcus*[18] and *Streptomyces*.[11] The actinomycetes group is most common producer of CHOx. Few gram-negative bacteria including *Burkholderia*[7], *Chromobacterium*[9] and *Pseudomonas* sp.[15] are also reported for producing CHOx.

Screening and isolating new CHOx producing microorganisms are considered very important due to a range of applications that the enzyme holds and keeping it in focus, the enzyme has been isolated and purified from various microbial sources.[9] One of the well-established applications of CHOx is its use in the enzymatic assay carried out to detect and quantify the amount of cholesterol in food, serum and various other clinical samples.[27] The CHOx has also been stated to show promising results as an effective insecticidal and larvicidal agent.[30] CHOx can also play an important role as a biocatalyst for the production of a large range of valuable steroid intermediates such as 4-androstene-3, 17-dione (AD) and 1, 4-androstadiene-3,17-dione (ADD), which serve as starting materials in the synthesis of many anabolic drugs and steroid hormones.[15]

Cholest-4-en-3-one formed by catalysis of cholesterol by CHOx is an important synthetic intermediate in many steroid transformations and is found to be effective against obesity, liver disease and keratinization.[36] A previous study evaluated the functional effect of 4-cholesten-3-one in human cells and it was found that 4-cholesten-3-one generated after cholesterol oxidation restrained cell migration.[28]

The enzyme yield can be increased either by strain improvement or by optimization of production media components. This can be done by applying a wide range of methodologies ranging from classical "one-factor-at-a-time" to statistical and mathematical approaches. Medium optimization is still considered one of the most important phenomenons which are carried out before large scale enzyme production. The classical method is most preferred approach towards designing medium composition in initial stages because of its ease and convenience.[29]

In the study reported here, we studied the effect of different nutritional factors on the production of total CHOx by *Castellaniella* sp. using "one-factor-at-a-time" approach.

Also, CHOx from *Castellaniella* sp. was used for the bioconversion of cholesterol to 4-cholesten-3-one by using an aqueous/ organic biphasic system.

Material and Methods

Chemicals: The cholesterol, horseradish peroxidase and *o*-dianisidine were bought from Sigma (Bangalore, India); detergents and media components were obtained from HiMedia (Mumbai). All the chemicals were provided by local suppliers and were of analytical grade.

Microorganism and culture conditions: *Castellaniella* sp. strain COX (Accession number MF973093) isolated previously was used in this study. The microorganism was maintained and cultured in the cholesterol enriched (CE) agar/broth containing (g/l): cholesterol 2.0; NH_4NO_3 17.0; K_2HPO_4 2.5; NaCl 0.05; $MgSO_4.7H_2O$ 2.5; yeast extract 3.0; $FeSO_4$ 0.0001 and Tween 80 0.5 ml, pH 7.0± 0.1.

Assay for CHOx activity and protein estimation: The assessment of the CHOx activity in the cell-free broth was carried using cholesterol as a substrate by a previously reported colorimetric method.[25] One unit (U) of CHOx was defined as the amount of enzyme required for converting 1.0 µmol of cholesterol to 4-cholesten-3-one per minute at pH 7.5± 0.1 and temperature of 37ºC. The total proteins were assessed using Bradford's method applying bovine serum albumin (BSA) as the standard.

Culture conditions optimization for maximum CHOx production by *Castellaniella* sp.

Production profile of extracellular CHOx: A 24 h old seed culture of *Castellaniella* sp. was inoculated in sterile CE production broth (50 ml) taken in 250 ml flask. The extracellular CHOx activity as well as bacterial growth were assayed after every 8 h till 96 h at 30ºC under shaking (150 rpm).

Optimization of inoculum age and size for the extracellular CHOx production: To optimize the inoculum age of *Castellaniella* sp. for the CHOx production, a loopful of culture of bacterium was grown in CE broth and 4% (v/v) inoculum was withdrawn at various stages of incubation i.e. 8, 16, 24, 30 and 36 h and inoculated in CE production medium.

To determine optimum size of inoculum of *Castellaniella* sp. for CHOx production, varied amounts of 30 h old inoculum (2-12% v/v) were added in the CE production broth separately and the inoculated medium was incubated at 30ºC for 62 h under shaking (150 rpm) and cholesterol oxidase activity was assayed.

Optimization of carbon source for the extracellular CHOx production: Effect of various additional carbon sources viz. fructose, galactose, mannitol, glucose, xylose, sucrose, maltose, lactose and starch at 20 g/l, besides cholesterol (2.0 g/l) on extracellular CHOx production was evaluated. The CE broth (50 ml) was inoculated with 30 h old seed culture (8%, v/v) and incubated at 30ºC for 62 h under shaking. The CHOx activity was assayed thereof.

Optimization of cholesterol concentration for the extracellular CHOx production: To determine the effect of cholesterol concentration on extracellular CHOx production by *Castellaniella* sp., in 50 ml CE production broth, varying concentration of cholesterol (0.1, 0.2, 0.3, 0.4, 0.5 and 1.0% (w/v) was added and the extracellular CHOx activity was assayed after 62 h, incubating it at 30ºC under shaking.

Optimization of nitrogen source for the extracellular CHOx production: The effect of selected organic and inorganic nitrogen sources [viz. peptone, beef extract, yeast extract, urea, KNO_3, $NaNO_3$, NH_4NO_3 and $(NH_4)_2SO_4$; 0.5% (w/v)] on CHOx production was studied by adding selected nitrogen source individually to the production broth. The CHOx activity was assayed thereof in each case.

Effect of surfactant(s) on extracellular CHOx production: Surfactants increase the solubility of steroid substrates in the fermentation medium and also increase the secretion of proteins by enhancing the cell membrane permeability. Triton X-100, tween 20, tween 40, tween 60 and tween 80 were separately added in 50 ml CE production broth at a concentration of 0.1% (v/v). The effect of these surfactants on CHOx production was studied. Also, the effect of the selected surfactant i.e. Triton X-100 was evaluated by incorporating the surfactant to the production medium with different concentrations (viz. 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0% v/v). It was incubated at 30ºC for 62 h under shaking and the CHOx activity was assayed.

Optimization of production broth pH and incubation temperature for CHOx production: To study the effect of pH (4.0 to 11.0± 0.1) on the extracellular CHOx production by *Castellaniella* sp., the CE production broth(s) containing cholesterol (1.0 g/l), $NaNO_3$, (1%, w/v), yeast extract (0.5%, w/v) and Triton X-100 (0.2%, v/v) were individually set to above selected pH range followed by sterilization. The extracellular CHOx activity at each of the calibrated pH was assayed. To study the effect of incubation temperature on extracellular CHOx production, the culture was grown at different temperatures ranging from 25 to 55ºC (25, 30, 35, 40, 45, 50 and 55ºC) in 50 ml of optimized CE production medium. The optimum temperature was selected for further studies.

Bioconversion of cholesterol to 4-cholesten-3-one by using crude CHOx in aqueous/organic biphasic system: For the biotransformation of cholesterol to 4-cholesten-3-one, an aqueous/organic biphasic system was used and the conditions were as follows: cholesterol (0.1%), 65 ml aqueous/organic solvent mixture (10:3, v/v), 250 ml flask, 200 rpm, 30°C and 3 h. For the aqueous phase, supernatant

containing extracellular CHOx was used and for organic phase, different organic solvents viz. ethyl acetate, petroleum ether, *n*-hexane, *n*-heptane, *n*-decane and isoamyl alcohol were used. When the bioconversion reaction was complete, the products were extracted with equal volumes of ethyl acetate.

The extract was washed with water sufficiently to remove possible water-soluble substances such as Triton X-100 and enzyme. Then the organic layer was recovered and evaporated under vacuum for further use. The biotransformation rate of cholesterol to 4-cholesten-3-one was found by calculating the concentration ratio of accumulated 4-cholesten-3-one to initial cholesterol by measuring the absorbance at 240 nm at 25°C using ε_{240} = 14,000 $M^{-1}cm^{-1}$.[1,19]

Effect of initial cholesterol concentration on bioconversion: The effect of initial cholesterol concentration on the enzymatic conversion was studied in 250 ml rotary shaking flask which included 65 ml enzyme solution/n-decane solvent mixture (10:3, v/v) and the initial cholesterol concentration was varied from 0.05 to 1.0% (w/v). The reaction was performed at 250 rpm and 30°C for 3 h.

TLC, HPLC and FTIR analysis of synthesized 4-cholesten-3-one: The extracted product was dried under vacuum at 30°C. It was then dissolved in chloroform and applied to silica gel plates in a chromatography tank containing chloroform/benzene/ethyl acetate (6:3:1 v/v). The metabolites were visualised by spraying the dried plate with a solution of sulphuric acid/methanol (1:6 v/v). The 4-cholesten-3-one was analyzed by HPLC using a reverse phase C-18 column equipped with a Waters pump system (Waters 515 pump; Waters Corp, USA) and was detected by photo diode array detector (Applied Biosystems) at 240 nm. The mobile phase contained acetonitrile and iso-propanol in a ratio of 70:30 and samples were prepared in iso-propanol. The flow rate of mobile phase was 1 ml/min and temperature was kept at 30°C.

The FTIR of the purified 4-cholesten-3-one was used to determine functional groups and chemical bonds present and was done at Advanced Material Research Centre (AMRC), IIT, Mandi. The spectral measurements were carried out in absorbance mode. The spectrum was generated in the range of 400 to 4,000 cm^{-1} with a resolution of 4 cm^{-1} and processed with IR analytical software.

Results

Production profile of extracellular CHOx: The extracellular CHOx activities as well as bacterial growth were assayed after every 8 h till 96 h in the inoculated CE broth. The culture grew exponentially up to 62 h and then entered stationary phase. The maximum peak of activity was reached approximately at 62 h and after that activity decreased slowly (Fig. 1).

Optimization of inoculum age and size for CHOx production: The effect of age of inoculum of *Castellaniella* sp. on extracellular CHOx production was studied by inoculating the CE production broth with inoculum culture of varying age and estimating the CHOx activity after 62 h period of production. The maximum CHOx activity (0.075 U/ml; Fig. 2a) was observed with the 30 h old inoculum. Further the inoculum size of *Castellaniella* sp. was also optimized and 8% (v/v) inoculum was observed to result in maximum enzyme production (0.098 U/ml; Fig. 2b) after 62 h of production time.

Optimization of carbon source for CHOx production: Carbon is an important source to support growth and production of enzyme as it is one of the life supporting elements for the organism. Effect of various additional carbon sources viz. fructose, galactose, mannitol, glucose, sucrose, maltose, lactose and arabinose at 20.0 g/l, besides cholesterol (2.0 g/l) on extracellular CHOx was evaluated. The maximum CHOx activity was observed in medium containing cholesterol as sole carbon source (control, Fig. 3) while the presence of other studied carbon sources resulted in less production of CHOx.

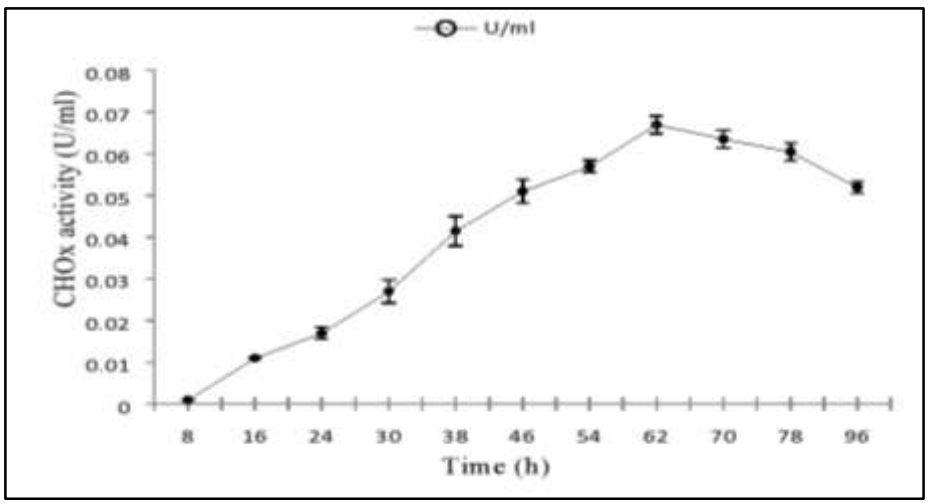

Figure 1: Profile of extracellular CHOx production by *Castellaniella* sp.

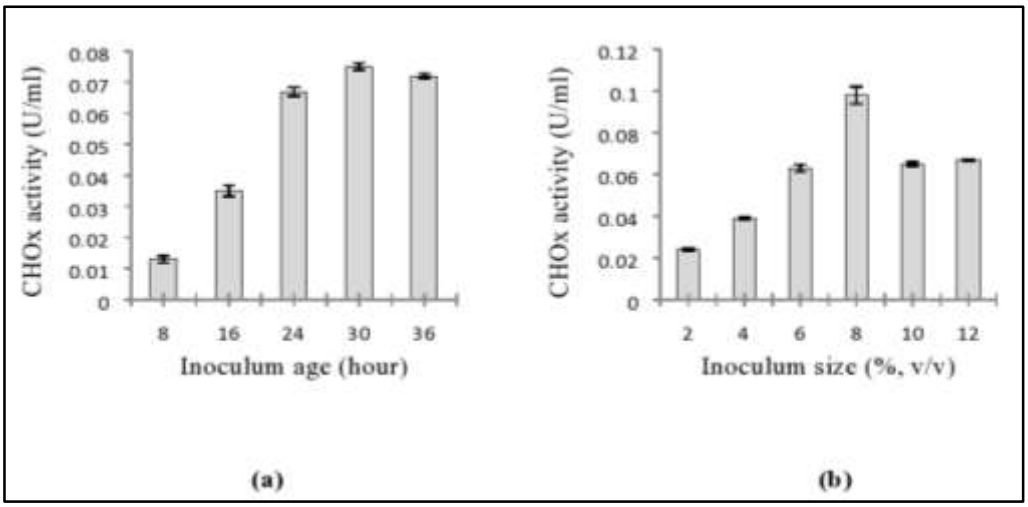

Figure 2: (a) Optimization of inoculum age and (b) size for extracellular CHOx production by *Castellaniella* sp.

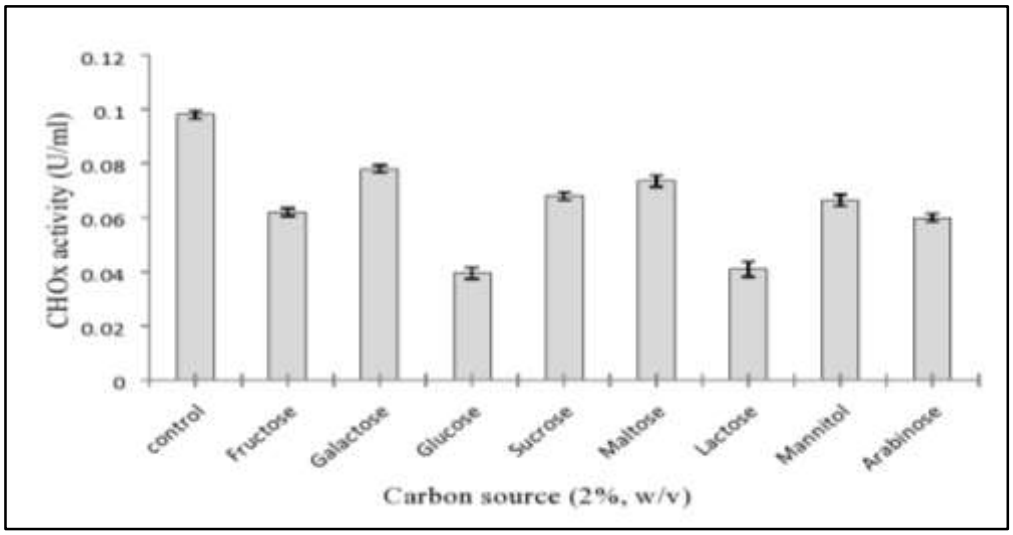

Figure 3: Optimization of carbon source for extracellular CHOx production by *Castellaniella* sp.

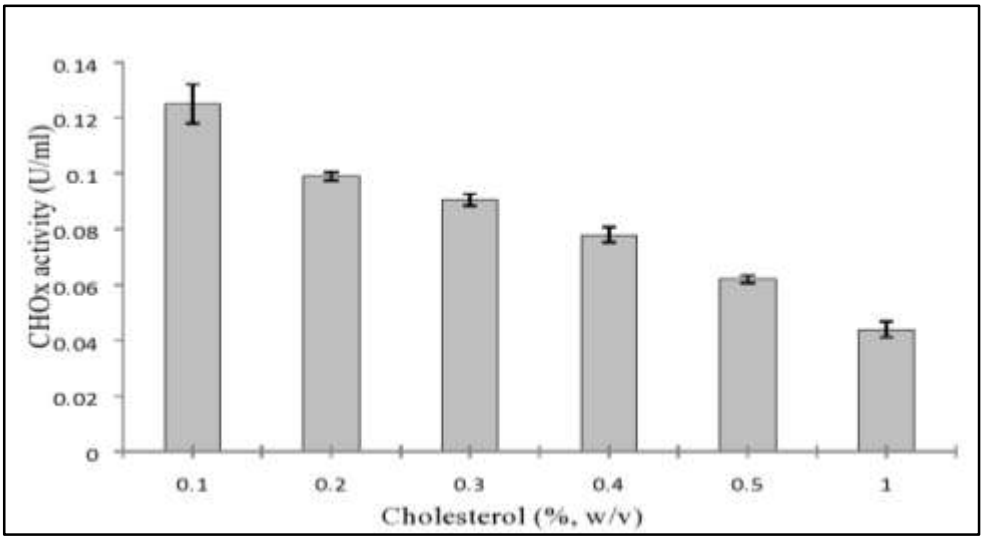

Figure 4: Optimization of cholesterol concentration for extracellular CHOx production by *Castellaniella* sp.

Optimization of cholesterol concentration for CHOx production: Carbon source optimization revealed that cholesterol is the best carbon source for CHOx production by *Castellaniella* sp. Further, the effect of cholesterol concentration on extracellular CHOx production was studied and maximum CHOx activity (0.125 U/ml) was observed in medium containing 0.1%

(w/v) cholesterol while higher amount of cholesterol inhibited the enzyme production (Fig. 4).

Optimization of nitrogen source for CHOx production: The effect of various organic and inorganic nitrogen sources on CHOx production by *Castellaniella* sp. was studied and the results showed maximum production of CHOx in the presence of 0.5 % (w/v) yeast extract.

Effect of surfactants on cholesterol oxidase production by *Castellaniella* sp.: The effect of various surfactants viz. triton X-100, tween 20, tween 40, tween 60 and tween 80 on CHOx production by *Castellaniella* sp. was studied. The maximum activity was observed in medium containing Triton X-100 (0.316 U/ml; Fig. 6a).

The effect of the concentration of selected surfactant, i.e. triton X-100 was also evaluated by incorporating the surfactant to the production broth at different concentrations. The maximum CHOx activity (0.324 U/ml; Fig. 6b) was observed in the presence of 0.2% (v/v) of Triton X-100 in CE production broth.

Optimization of production broth pH and temperature for CHOx production by *Castellaniella* sp.: The production of CHOx by *Castellaniella* sp. was studied in the CE broth at different pH and temperature. The optimum pH for production of CHOx enzyme was recorded to be pH 8.0 as maximum CHOx production (0.355 U/ml; Fig. 7a) was recorded at this pH and was considered as optimized to conduct further experiments. To study the effect of incubation temperature on extracellular CHOx production, the culture was grown at different temperatures ranging from 25 to 55^{o}C and the maximum CHOx activity (0.37 U/ml; Fig. 8) was observed at 35^{o}C.

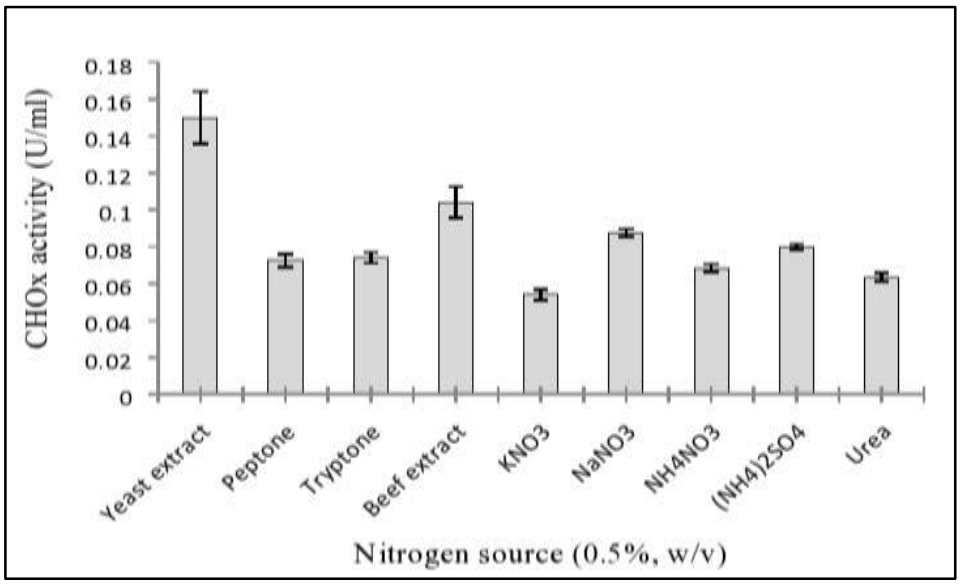

Figure 5: Optimization of different organic and inorganic nitrogen sources.

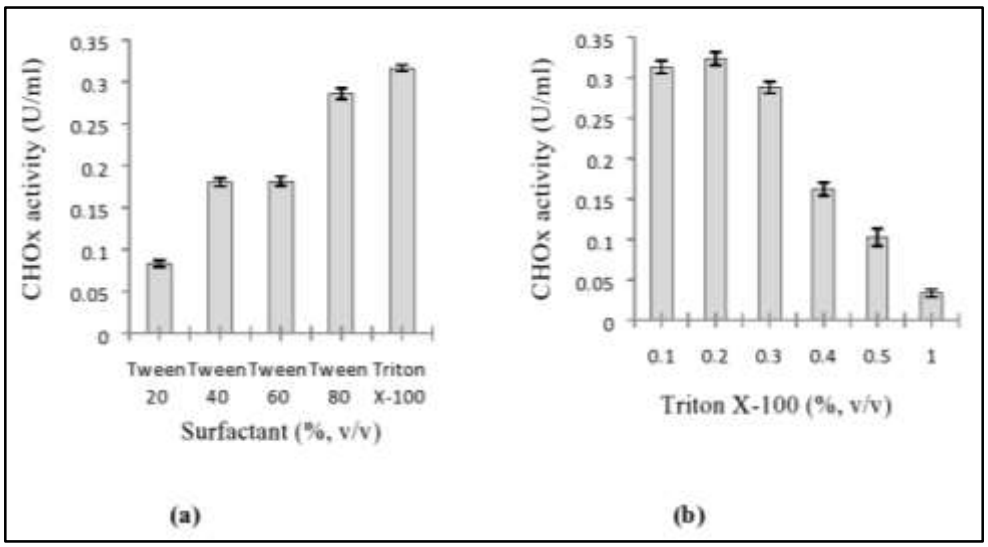

Figure 6: (a) Effect of different surfactants on the production of CHOx (b) Effect of Triton X-100 concentration on CHOx production by *Castellaniella* sp.

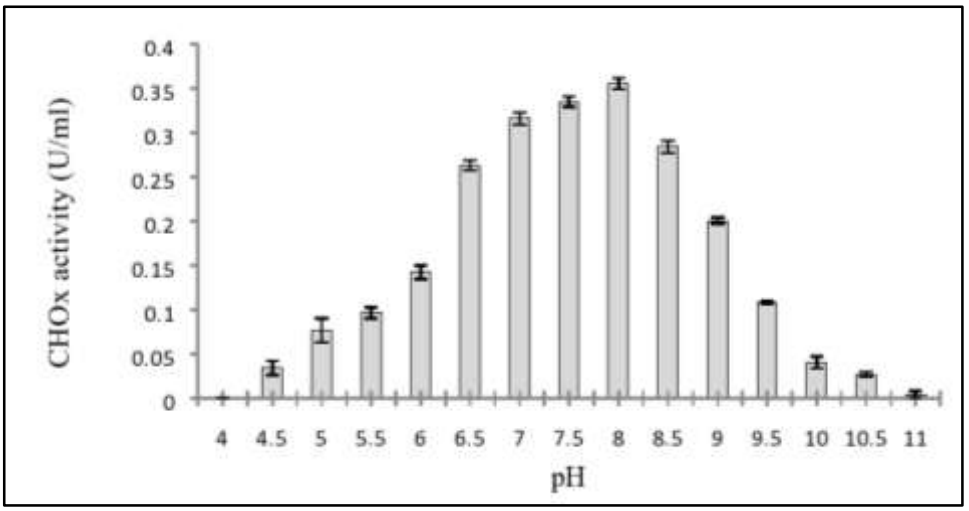

Figure 7: Effect of production pH on the CHOx production by *Castellaniella* sp.

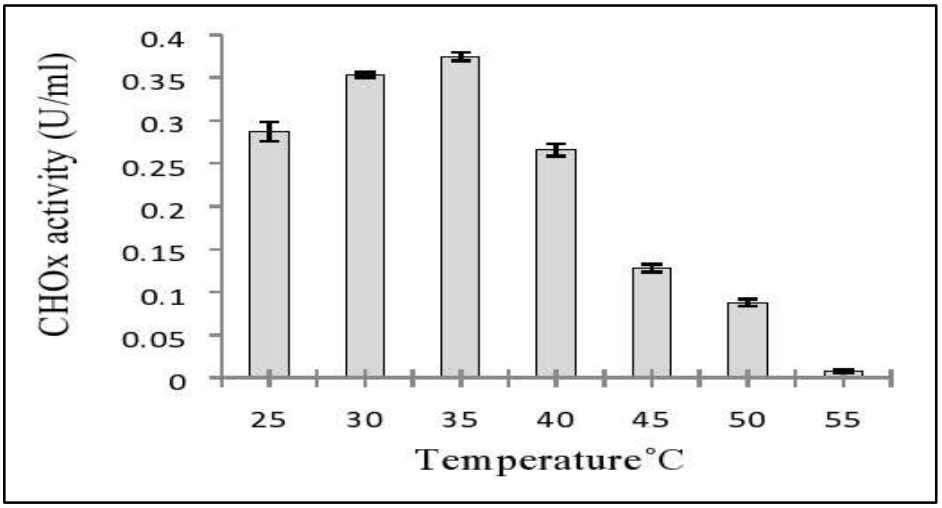

Figure 8: Effect of incubation temperature on the CHOx production by *Castellaniella* sp.

Bioconversion of cholesterol to 4-cholesten-3-one by using crude CHOx in aqueous/organic biphasic system: Different water-immiscible organic solvents were investigated for the conversion of cholesterol to 4-cholesten-3-one. Maximum biotransformation of 67± 3.0% was achieved in the presence of *n*-decane after 3 h of reaction by using 0.1% (w/v) cholesterol. The residual CHOx activity was about 71.0% after first bioconversion reaction. It was observed that in the presence of long-chain hydrocarbons bioconversion rate was higher in comparison to short chain organic solvents and also the enzyme was more stable (Fig. 9). Thus, *n*-decane was identified as the most promising water immiscible organic solvent for the biphasic conversion of cholesterol in shake flask cultures.

Effect of initial cholesterol concentration on bioconversion: To study the effect of cholesterol concentration on the conversion of cholesterol to 4-cholesten-3-one, varying concentrations of cholesterol were added to the biotransformation reaction medium. The maximum biotransformation (73± 3.0%; Fig. 10) was observed at 0.2% (w/v) cholesterol concentration by using enzyme solution/*n*-decane biphasic system. As the cholesterol concentration increased beyond 0.2% (w/v), a decrease in percent bioconversion of cholesterol to 4-cholesten-3-one was noticeable.

TLC, HPLC and FTIR analysis of synthesized 4-cholesten-3-one: Thin layer chromatography was performed for the detection and semi-quantification of reaction products after biotransformation of cholesterol to 4-cholesten-3-one. The metabolites were visualised by spraying the dried plate with a solution of sulphuric acid (1:6, v/v). Dark spots of cholesterol and 4-cholesten-3-one were observed on silica-gel plate (Fig. 11 a).

Further, the synthesized 4-cholesten-3-one was analysed by HPLC along with standard 4-cholesten-3-one. The retention time (RT) of standard 4-cholesten-3-one was 14.15 min while that of synthesized 4-cholesten-3-one was obtained as 14.07 min which confirmed the bioconversion of cholesterol to 4-cholesten-3-one (Fig. 11 b and c). Characterization of end-product of cholesterol biotransformation by FT-IR was also done. IR spectrum of the synthesized product was recorded which was identical with that of standard 4-cholesten-3-one. FTIR V_{max} of 1672.6 cm^{-1} suggested the

presence of a conjugated double bond and a C=O structure in the synthesized product further confirmed the bioconversion of cholesterol to 4-cholesten-3-one (Fig. 11d).

Discussion

CHOx is an enzyme having potential applications in medical and enzyme industries and is produced from a wide range of bacterial microorganisms with different enzymatic characteristics.[13] To increase the production of CHOx, different optimization experiments were carried out using the classical method by varying one parameter at a time and keeping the others constant at pre-determined levels. The development and management of the inoculum culture have a definite effect on the performance of enzyme production process as it directly influences the duration of the lag phase and specific growth rate.

Therefore, it is very important to investigate these variables in CHOx and other enzymes production.[35] In the current study, best result of 0.098 U/ml was obtained with 8.0% (v/v) inoculum size and 30 h of inoculum age of *Castellaniella* sp. after 62 h of enzyme production. Niwas et al[29] used 2.0% (v/v) of 55 h old culture of *Streptomyces* sp. for CHOx production and maximum CHOx activity was reported after 96 h of enzyme production.

CHOx production after 48 h of bacterial growth was reported optimum for *Streptomyces rimosus* with 40 h old and 3.0% (v/v) inoculum size[35] while 48 h old and 2.0% (v/v) inoculum was found optimum in case of *Streptomyces cavourensis* strain NEAE-42 which gave maximum CHOx production after 7 days of incubation period.[12]

CHOx production by *Castellaniella* sp. was studied by altering the CE production medium with different carbon sources besides cholesterol. The maximum CHOx activity was observed in medium containing 0.1% (w/v) cholesterol as a sole carbon source while the presence of other studied carbon sources resulted in less production of CHOx.

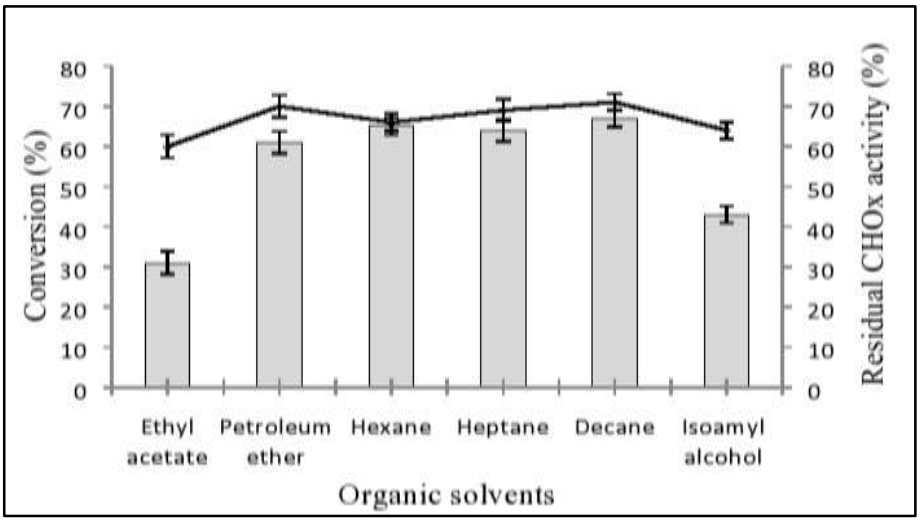

Figure 9: Bioconversion of cholesterol to 4-cholesten-3-one by CHOx from *Castellaniella* sp.

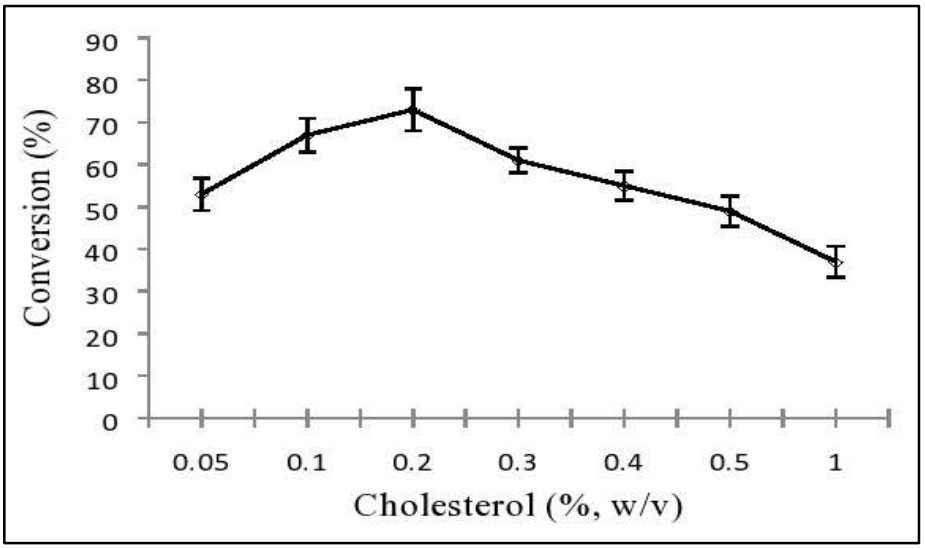

Figure 10: Effect of initial cholesterol concentration on bioconversion.

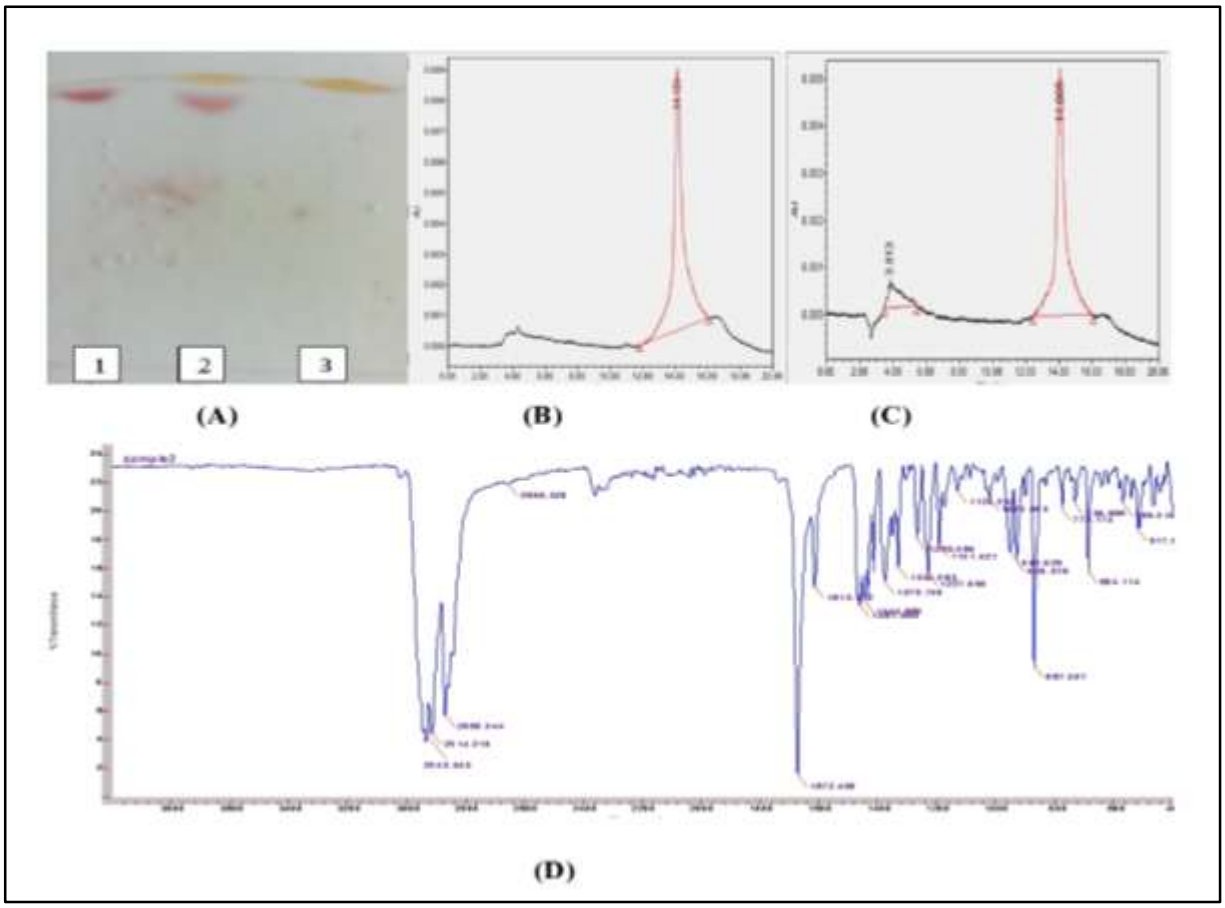

Figure 11: (A) Detection of reaction products of biotransformation by using TLC technique. Lane 1, standard sample of cholesterol; lane 2, end product of biotransformation; lane 3, standard sample of 4-cholesten-3-one. (B) HPLC chromatogram of standard 4-cholesten-3-one showing RT~14.15 min. (C) HPLC chromatogram of synthesised 4-cholesten-3-one showing RT~ 14.07 min. (D) FTIR spectrum of the synthesized product.

Yazdi et al[37] studied the effect of various steroid and sugar substrates on CHOx production and reported that cholesterol was best carbon source for CHOx production by *Rhodococcus equi* 2C. For the maximum CHOx production by *Streptomyces* sp. supplementation of 0.05 % of cholesterol as inducer was observed to be most suitable.[29]

In case of *Streptomyces rimosus*, it was observed that addition of starch or glucose in the production medium resulted in the increased CHOx production.[35] *Streptomyces cavourensis* strain NEAE-42 gave maximum extracellular production in the culture media containing glucose 10 g/l, starch 10 g/l and cholesterol 2 g/l.[12]

Yehia et al[38] reported that the cholesterol assimilation by cholesterol degrading bacteria is affected mainly by cholesterol concentration in culture media and maximum cholesterol assimilation (80.2 %) is observed at 1 g/l cholesterol concentration by the *Enterococcus hirae* strain. Maximum cholesterol assimilation was obtained by *Streptomyces fradiae* and *Rhodococcus erythropolis* ATCC 25544 at 2 g/l cholesterol concentration.[34,37]

Cholesterol is also reported as the best carbon source for CHOx production by *Pseudomonas aeruginosa*,[8,35] *Pimelobacter simplex*[31] and *Rhodococcus* sp.[2] Thus all these studies suggested cholesterol as the most suitable carbon source and inducer for CHOx production by different microbial sources.

Like carbon, the selection of most suitable nitrogen source along with its optimum concentration in the culture media also plays a critical role in enzyme production. Out of various studied nitrogen sources, maximum CHOx production by *Castellaniella* sp. was observed in the presence of yeast extract (0.15 U/ml) and $NaNO_3$ (0.175 U/ml). Further, when $NaNO_3$ (1.0%, w/v) and yeast extract (0.5%, w/v) were supplemented together in CE production broth, the CHOx production was increased to 0.238 U/ml.

El-Naggar et al[12] reported maximum enzyme production by *Streptomyces cavourensis* strain NEAE-42 in the presence of yeast extract 4.0 g/l; peptone 5.0 g/l; $(NH_4)_2SO_4$ 8.0 g/l. Another study also revealed that the combination of yeast extract and $(NH_4)_2HPO_4$ resulted in the maximum enzyme yield as compared to individual inorganic and organic nitrogen sources.[11] Further in another finding Moradpour et al[26] reported that an organic nitrogen source had a more significant effect on CHOx production than the inorganic nitrogen source. This could be because many organic

nitrogen sources contain almost all of amino acids and large variety of growth factors for the growth of enzyme producer, hence promoting higher CHOx production.[2] *Streptomyces rimosus* gave maximum enzyme production in the presence of yeast extract and inclusion of ammonium carbonate in production medium resulted in 30% increase in CHOx yield.[35]

Surfactants are surface-active agents which reduce the surface and interfacial tensions of liquids. Steroids are poorly soluble in water and need to be solubilized for better dispersion and availability for steroid assimilating microorganisms. To increase cholesterol solubility in culture media, various surfactants comprising of non-ionic detergents are used. The maximum CHOx production of 0.324 U/ml was observed in presence of 0.2% (v/v) of Triton X-100 in the production broth by *Castellaniella* sp.

In another study, the effect of various surfactants is studied on CHOx production and in one report, tween 80 and tween 60 were found to increase the enzyme production while in the presence of triton X-100, the enzyme production was nearly inhibited.[35] Moradpour et al[26] reported that after the addition of tween 20 to the culture medium, *Streptomyces badius* exhibited a substantial increase in CHOx production.

In contrary to this Doukyu et al[9] stated that CHOx production by *Pseudomonas* sp. and *Nocardia erythropolis* was significantly lowered in the presence of detergents. The positive effect that various surfactants exhibit on enzyme production might be due to the alteration of plasma membrane permeability, resulting in the enhancement of both uptake and exit of compounds from the cell. Furthermore, surfactants contribute to disperse cholesterol which is quite water-insoluble.[31]

Amongst the various physical parameters, pH and cultivation temperature play an important role by having a significant effect on microbial physiology and ultimately on enzyme production.[20] CHOx enzyme is found to perform best at a pH of 6.5–8.0.[8] The optimal pH and temperature for CHOx production by *Castellaniella* sp. in the optimized CE production medium were pH 8.0 and temperature 35°C. Enzyme production under these optimal pH and temperature conditions was 0.370 U/ml. El-Naggar et al[12] reported that initial pH along with nitrogen source was most important positive independent variable regulating CHOx production by *Streptomyces cavourensis* strain NEAE-42 and pH 8.0 was found to be optimum.

In another study by Yazdi et al[37] pH 8.0 was found to be most suitable for CHOx production by *Rhodococcus* sp. In another investigation, an initial pH of 7.5 was found to be most appropriate for CHOx production by *Streptomyces lavendulae*[4]. While in contrast for maximum CHOx production by *Streptomyces badius*, pH of 6.5 was found to be optimum.[26] For maximum CHOx production by *Streptomyces badius* and *Rhodococcus* sp., incubation temperature of 35°C was found to be optimum.[26] Optimum yields of CHOx were achieved at 30°C in case of *Bordetella* sp., *Rhodococcus* sp. and *Brevibacterium* sp.[22,23,33] The maximum CHOx activity from *Streptomyces anulatus* strain NEAE-94 was achieved at a temperature of 37°C and at an agitation speed of 150 rpm.[10]

The optimum enzyme production in the basal medium was at 30°C for *Rhodococcus* sp. NCIM 2891 and *Streptomyces cavourensis* strain NEAE-42.[1,12] In the present study higher CHOx production (0.396 U/ml) by *Castellaniella* sp. was achieved after optimizing the culture conditions which reflects 5.9-fold increase in CHOx productivity by applying classical "one-factor-at-a-time" approach.

Crude extracellular CHOx produced by *Castellaniella* sp. was used to catalyse cholesterol bioconversion to 4-cholesten-3-one by using an aqueous/organic two-phase system. The CHOx catalysed the transformation of cholesterol to 4-cholesten-3-one which was confirmed by TLC, HPLC and FTIR analysis. Maximum biotransformation of 73± 3.0% was achieved in the presence of *n*-decane after 3 h of reaction by using 0.2% (w/v) cholesterol. After the first cycle of bioconversion, ~29% of the CHOx activity was lost. It was observed that in the presence of long-chain hydrocarbons, bioconversion rate was higher and the enzyme was also more stable.

Liu et al[24] reported 94.6% cholesterol bioconversion to 4-cholesten-3-one by 6 g wet cells of *Arthrobacter* in 300 ml aqueous/carbon tetrachlorideride two-phase system containing 10.0% (w/v) cholesterol at 30˚C. The biotransformation of cholesterol (3.75 mM) with the *Rhodococcus* sp. NCIM 2891 CHOx immobilized on chitosan beads (3.50 U) resulted in ~88% mM bioconversion of cholesterol to 4-cholesten-3-one in 9 h reaction time at 25˚C.[1] In another study, cholesterol oxidase magnetic nanobioconjugates were used for the biotransformation of cholesterol and 7-ketocholesterol to 4-cholesten-3-one and 4-cholesten-3, 7-dione respectively.[17]

Wu et al[36] directly used the crude enzyme solution to catalyse the bioconversion of cholesterol to 4-cholesten-3-one and the conversion rate reached 4 g/l/h with 90% conversion by using the enzyme solution/petroleum ether system. In our study, about ~73% of bioconversion was achieved after 3 h long reaction time with ~71% of residual CHOx activity. The stability of the *Castellaniella* sp. CHOx can be improved after immobilizing it onto suitable support materials and hence this biotransformation method shows a great promise for the conversion of cholesterol to pharmaceutically important 4-cholesten-3-one.

Conclusion

Cholesterol oxidase is an important enzyme with applications in biosensors, pharmaceuticals, bioconversions and in agriculture. This study sought to optimize various

physicochemical parameters for the optimum extracellular CHOx production by *Castellaniella* sp.

The results obtained from the experiments suggested that the presence of cholesterol, yeast extract, $NaNO_3$ and Triton X-100 in optimum concentrations in production medium along with optimum pH and temperature is responsible for the enhancement of CHOx production by *Castellaniella* sp. Further, the crude enzyme was used successfully for the cholesterol biotransformation to 4-cholesten-3-one with ~73% bioconversion.

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