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Isolation And Characterization Of Cholesterol Degrading Bacteria From Vegetable Oil Industrial Waste

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Abstract

The cholesterol degrading bacteria were isolated and characterized from the vegetable oil industrial wastes of Buldhana District. thirteen strains were isolated by the primary screening. Among the thirteen, only two strains were identified as cholesterol oxidase enzyme producers. The morphological and biochemical characters revealed that the isolated microorganisms were *Pseudomonas* sp., and *Bacillus* sp. The isolated microorganisms were grown in a cholesterol medium and their degrading potency of cholesterol was determined by TLC. The cholesterol oxidase (CHO) extracted from the isolates was partially purified. The present study revealed that the isolates (*Pseudomonas* sp., *Bacillus* sp.) isolated from the industrial wastes had good cholesterol degrading potential and would be considered as good source of CHO for important medical applications.

Keywords: Bioconversion, cholesterol degrading bacteria, cholesterol oxidase, vegetable oil waste.

Introduction

In the natural environments various microorganisms play an important role in degradation (bio-conversion) and assimilation of cholesterol. These microorganisms are abundantly present in soil and are capable of growing on cholesterol as a sole carbon source. Microorganisms which are responsible for cholesterol degradation generally produces an enzyme known as cholesterol oxidase (CHO). This enzyme catalyzes the conversion of cholesterol in to 4-cholestene-3-one. (Lee S-Y, *et.,al.,* 1989, Yazdi M T, *et.,al.,* 2008). This reaction is the first step in the microbial degradation of cholesterol and its derivatives (Sojo M, *et.,al.,* 1997,

Lashkarian H, *et.,al.,* 2010). Cholesterol oxidase from microbes are commercially important because of their potential industrial and clinical applications. For instance, cholesterol oxidase is mainly used in the quantification of serum and food cholesterol (García J L, *et.,al.,* 2012, Richmond W,1973) it can be used as precursor for the production of pharmaceutical steroids (Watanabe K, *et., al.,* 1989). Moreover, it can be used in degradation and lowering the dietary cholesterol (Kaunitz H,1978), providing a diagnostic kit for degrading cholesterol (Yazdi M T, *et.,al.,* 2000, Bholay A D, *et.,al.,* 2013). Hence, research on microbial CHO has received much attention in recent years. Microbial conversions of cholesterol when compared with chemical reactions are more regio- and stereo-selective and have been used in the production of pharmaceutical products for a long time (Ahmad S, *et.,al*., 1992, Naghibi F. *et., al*., 2002). The most essential problem in preparing the steroid hormones from sterols is the selective cleavage of the side chain. Chemical approach is not effective because of its low conversion rate and has fallen far short of the economic advantage. This is due to low specificity of the reaction, causing considerable contamination by some undesired by-products (Naghibi F. *et.,al.,* 2002). These are the main reasons which prompted the use of the microbial degradation of cholesterol in the pharmaceutical industries. CHO producing ability has been reported in many microorganisms. For instance, it has been reported in Rhodococcus sp. (Naghibi F. *et.,al.,* 2008, Lashkarian H. *et.,al.,*2010), Pseudomonas sp.(Doukyu N & Aono R,1998) , Bacillus subtilis (Kim K-P *et.,al.,* 2003, Andhale M S & Sambrani S A, 2006), Microbacterium sp. (Parekh S N & Desai P B,2013) etc. The present study was aimed to isolate the potent cholesterol degrading bacteria from vegetable oil industrial wastes and evaluate their ability for degradation of cholesterol.

Materials and Methods

Isolation of Microorganisms from vegetable oil waste (soil) were collected from the local areas of Buldhana district of Maharashtra, India. The cholesterol degrading bacteria were isolated from the waste samples by an enrichment culture technique (Watanabe K. *et.,al.,* 1989). The medium used for isolation contained cholesterol as a sole carbon source and 1 L of medium contained: NH4NO3, 1 g; K2HPO4, 250 mg; MgSO4 7H2O, 250 mg; NaCl 5 mg; FeSO4.7H2O, 0.5 mg; and cholesterol, 1 g (Medium-A). The pH was adjusted to 7.0 with 10% NaOH prior to autoclaving. The waste sample was added and the mixture was incubated at 30°C on a shaker at 3000 rpm for 7-12 d. The growing cultures were transferred to the same medium 2 or 3 times. Finally, the broth was plated onto the nutrient agar, consisting of 10% peptone, 1% yeast extract, 0.5% NaCl, and 2% agar (pH 7.0) (Medium-B). Representative colonies were picked up onto an agar slant and were purified by repeated serial plating and used for morphological and biochemical tests for the identification of microbes. Confirmation of CHO Producing bacteria, to confirm the CHO producing strains, a colony staining method was performed on the grown colonies (Lashkarian H, *et.,al.,* 2010). The CHO producing colonies were selected on suitable indicator plates (Yazdi M, *et.,al.,* 2000). Detection of Cholesterol Degradation After (Yazdi M, *et.,al.,*2001) d of incubation at 30°C in a shaker at 80 rpm, the isolates were separated by centrifugation at 3000 rpm and the supernatant was used for purification of the enzyme and detection of degradation products by Thin Layer Chromatography (TLC) for determination of the cholesterol-degrading potency of each bacterium, the culture supernatant was extracted from the medium (Medium-A) with chloroform and was applied to the silica gel plates. These plates were then placed in a chromatography tank

containing chloroform: benzene: ethyl acetate (1:3:6). After chromatography, the plates were dried, sprayed with 0.3 M sulphuric acid followed by heating at 110°C until appearance of spots (Yazdi M T, *et.,al.,* 2000).

Characterization of CHO

Enzyme Isolation

The supernatant from the Medium-A centrifuged at 10,000 rpm for 10 minutes at 5°C was used as the extracellular CHO solution (Salva T J G, et.,al., 1999).

Enzyme Activity

CHO activity was measured by testing various pH (5 to 9) and temperatures (30 to 65°C) (Laemmli U,1970)

Results and Discussion

Thirteen strains of bacteria were isolated in the preliminary screening. Staining method was carried out to confirm the CHO enzyme producing microorganisms. The CHO indicator plates were used for further confirmation of the isolated bacteria. The CHO producing isolates changed the medium colour into intense brown (Fig. 1). Among 13 strains, only 2 strains were identified as the CHO enzyme producers. These strains were identified and were investigated further. According to the morphological and biochemical tests, these 2 isolates were identified as Pseudomonas sp., Bacillus sp. (Table 1). Maximum cholesterol degradation by the isolates occurred in the cholesterol medium

Table No. 1. Morphological and Biochemical characterization of isolates

Sr. No.	Charactristics	*Pseudomonas sp.*	*Bacillus sp.*
1	Gram staining	Gram negative rods	Gram positive rods
2	Motility	Motile	Motile
3	Indole	-ve	-ve
4	Methyl red	-ve	+ve
5	Voges Proskauer	-ve	-ve
6	Citrate	+ve	-ve
7	Catalase	+ve	+ve
8	Nitrate reductase	+ve	+ve
9	Oxidase	+ve	+ve
10	Starch hydrolysis	-ve	+ve

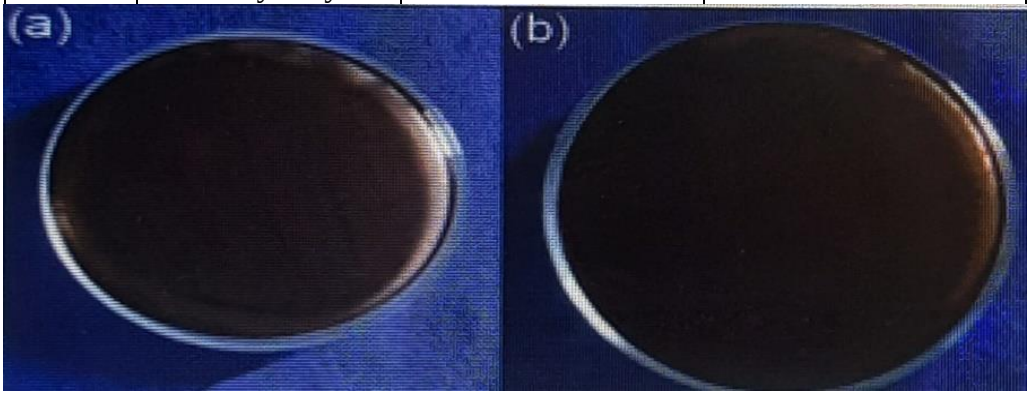

Fig. 1 CHO indicator plate: i) Pseudomonas sp., ii) Bacillus sp.

Fig.2 TLC of cholesterol degradation: Lane 1, *Pseudomonas* sp.; lane 2, *Bacillus* sp.

Table No. 2. Growth of Pseudomonas sp., and Bacillus sp. on cholesterol medium

Sr. No.	Day	*Pseudomonas sp. (CFU/mL)*	*Bacillus sp. (CFU/mL)*
1	0	6×10^5	3×10^6
2	1	3×10^6	2×10^7
3	2	4×10^9	2×10^9
4	3	7×10^8	7×10^9
5	4	5×10^9	6×10^9
6	5	7×10^9	6×10^8
7	6	7×10^8	8×10^7
8	7	8×10^7	7×10^7
9	8	7×10^7	4×10^7
10	9	5×10^6	4×10^6
11	10	4×10^5	3×10^5
12	11	3×10^5	2×10^5
13	12	3×10^4	3×10^4

Medium-A, where cholesterol (1 g/L) was supplemented with as a sole carbon source for 6 days Approx, 60-80% of the cholesterol was degraded by the isolates (Table 2). The isolated microorganisms were grown in the cholesterol medium and the growth rate and the cholesterol conversion rate were measured. The generation time of the isolates in the cholesterol medium had been 2 hrs. The number of viable cells decreased almost exponentially with prolonged incubation. The concentration of cholesterol in the culturing medium decreased slightly after 6 days of incubation. If the incubation was prolonged beyond 6 days, the viability of the isolates decreased but the concentration of the cholesterol remained the same. This indicates that the cholesterol degradation ability was lost due to the prolonged incubation. To determine the cholesterol utilization by the isolates. In the present study, TLC used. The metabolites involved in the cholesterol degradation by the isolates were detected by their Rf value (Fig. 2). The present study revealed that Rf value ranged between 0.5-0.7. The two isolates displayed only one spot. (Cheetham P S, *et.,al.,* 1982) reported that a single spot indicates that the isolates degrade cholesterol without accumulating appreciable amounts of the cholesterol intermediate products. The Rf value of 4-cholesten-3-one was reported as 0.6-0.9 and that for cholesterol 0.498. The present observations also showed somewhat the same Rf value in this range. Most probably the metabolite produced by the test microorganisms could be 4-cholesten-3-one. The isolated microorganisms appeared to have degraded the side chains in the cholesterol molecule, thereby releasing some metabolites. The concentration of cholesterol in the culturing medium decreased slightly after 6 days of incubation. If the incubation was

prolonged beyond 6 days, the viability of the isolates decreased but the concentration of the cholesterol remained the same. This indicates that the cholesterol degradation ability was lost due to the prolonged incubation. The present study revealed that the cholesterol degrading microorganisms from the vegetable oil industrial waste are having the ability to degrade cholesterol and are the very good source for CHO, which will be exploited for many therapeutic applications.

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