GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

www.scienceandnature.org

INVESTIGATION OF A GRAM-NEGATIVE CHOLESTEROL DEGRADING BACTERIAL STRAIN FROM SOIL SAMPLE(S) CONTAMINATED WITH EFFLUENTS OF VEGETABLE OIL INDUSTRIES

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ABSTRACT
The present study investigated a gram negative cholesterol degrading bacterial species isolated form soil samples contaminated with vegetable oil effluents in Burdwan and Hooghly districts of West Bengal, India. Among the ten isolates that were randomly chosen from the colonies that had grown on Cholesterol-Tween 20 enrichment agar medium, one gram negative bacterial strain (designated as CB-8) was found to be most potent degrader of cholesterol as determined by the estimation of 4-cholestene-3-one as well as by kit method in which the cholesterol oxidase (CHO) enzyme activity of the bacterium was assessed spectrophotometrically. The cell-free supernatant of the Cholesterol-Tween-20 broth was used as the crude source of the CHO enzyme of the CB-8 isolate. The isolate was then subjected to a battery of biochemical and physiological characterizations. As interpreted from the result, the CB-8 isolate was found to be a non-coliform gram negative aerobic or facultatively aerobic bacterium and was a strong producer of gelatinase enzyme. The optimum temperature, pH and salt concentration for the growth of the bacterium was determined to be 28°C, pH 7.0 and 1% NaCl, respectively. As far as the antibiotic sensitivity profile was concerned, the CB-8 strain was most sensitive to ciprofloxacin (nalidixic acid) and was least affected by ampicillin that matched with its gram negative nature. The CHO enzyme of this bacterium has a promising scope to be used in various commercial cholesterol detecting kits if proper purification strategies are adopted. The bacterium could also be utilized for natural degradation of cholesterol and other steroid- like compounds from industrial wastes/effluents to get rid of the ecotoxicological effects of residual steroids that might be present in the food chain. Molecular characterization of the CB-8 isolate by 16srRNA sequencing and extraction and purification of the CHO enzyme could also be performed as future extensions of the current work.

KEY WORDS: Cholesterol degradation, steroid, ecotoxicology, characterization, enrichment media, cholesterol oxidase.

INTRODUCTION

Cholesterol (cholest-3 en-5-ol) is an essential compound for human body that belongs to the steroid family of molecules. It is the main sterol found in the cell membranes of eukaryotic organisms and also acts as a precursor for cholic acid and steroid hormone biosynthesis. The compound is present in human tissues as well either in free form or in combination with long chain fatty acids as cholesterol esters. Sterol compounds also constitute the bile salts that form detergents and aid in the digestion or solubilization of fats and lipid soluble vitamins (Wollam and Antebi, 2011). Cholesterol-like compounds are also found to be present in plant cells (called as phytosterols) as well as in bacterial cell membranes (called as hopanoids). But in humans too much cholesterol deposition in the body may lead to increased risk of heart disease inducing cerebrovascular, cardiac and peripheral vascular diseases. Therefore, elevated blood-cholesterol is considered as a major risk factor for coronary heart disease in individuals. Research also shows that the accumulation of cholesterol in different habitats, mainly as a component of pharmaceutical and edible oil industry spillages may have significant eco-toxicological effects including reproductive anomalies in fish and lower fertility in mammals (Lange *et al*., 2008; Colborn, 2004 and Lange *et al*., 2001). One of the potential remedies of detoxification of cholesterol from land would be the use of microbial oxidation or transformation. Factually, there are a wide range of species of gram-positive and gram-negative bacteria that modify, transform or partially assimilate cholesterol or other similar steroid compounds that are released into the environment (Hayakawa 1982; Fahrbach *et al*., 2006; Drzygza *et al*., 2009 and Kurisu *et al.*, 2010). Wild fish (roach, *Rutilus rutilus*) exposed to synthetic estrogen like compounds found in oral contraceptive pills was reported to be suffered from adverse reproductive effects. Male fish were born intersex with increased vitellogenin concentration. The life-cycle exposure to fathead minnows to EE_2 (Ethinylestradiol; a synthetic estrogen present in oral contraceptive pills) below 1nm/l caused a significant reduction in fertilization success and a decreased expression of secondary male characteristics. On the other hand, terrestrial animals like reptiles when exposed to environmental estrogen showed sex reversal and significantly altered secondary sex characteristics. According to many authors, the concentrations of cholesterol or estrogen-like steroid compounds detected in

the soil or water may not pose a direct threat to humans but there is a risk of biomagnification and bioaccumulation within aquatic organisms; thereby reaching the humans through the food chain or directly through drinking water. The bacterial degradation of cholesterol is known to occur by the enzyme cholesterol oxidase (cholesterol: oxygen oxido-reductase; E.C.1.1. 3.6.). The enzyme catalyzes the oxidation of cholesterol to 4-cholestene-3-one along with the reduction of oxygen to hydrogen peroxide (Smith and Brooks, 1976). This reaction is the first step of microbial degradation of cholesterol and other cholesterol derivatives. Bacterial cholesterol oxidase enzymes (CHOs) are commercially important because of their application in the quantification of serum and food cholesterol. It can be also used as precursors for the production of pharmaceutically important steroids. Moreover, it can also be applied to the degradation of dietary cholesterol if appropriate production and purification strategies are adopted. Microbial, mainly bacterial steroid degradation is a critical process in biomass decomposition in natural environments, for removal of important pollutants during industrial wastewater treatments. Several bacterial species like *Corynebacterium, Pseudomonas, Arthrobacter, Nocardia* and *Mycobacterium* are able to degrade cholesterol and many of these are soil-borne in nature. To date, microbial steroid degradation has been studied in relatively few model organisms while the ecological significance of bacterial steroid degradation remains largely unexplored. In this context, the present study aims to isolate and metabolically characterize cholesterol degrading soil bacterial flora from the proximal regions of edible vegetable oil refinery/packaging plants. The soil organisms capable of degrading cholesterol may be considered as the potent producers for the cholesterol oxidase enzyme. The CHO enzyme has wide clinical and industrial applications as described already. In addition, the isolated bacterial flora may also have implications to degrade the deposited cholesterol in soil or water which occur as an industrial contaminant that has been shown to have various eco-toxicological effects. Similar studies have been performed by Saranya S. *et al.* (2013) who isolated and characterized cholesterol degrading soil bacteria form vegetable oil waste (soil) and reported that among the fifteen isolated strains, only three were shown to have potent cholesterol degrading activity. In another such study, Merino E. *et al.* (2012) have isolated cholesterol and deoxycholate degrading bacterial flora from soil samples from Leon region of Spain. Yazadi M.T. *et al.* (1999) have also reported the isolation of a gram negative *Agrobacterium* strain from soil collected from the vicinity of factories producing animal and vegetable oils as well as from agricultural wastes and composts. The primary purpose of the current work is to isolate and characterize cholesterol degrading bacteria from soil samples collected from Burdwan and Hooghly districts of West Bengal, India and also to evaluate their cholesterol degrading ability.

MATERIALS & METHODS

a) Collection of Soil Samples: Five different soil samples were collected from Five different regions in proximity of vegetable oil (such as mustard oil, rice bran oil) refinery or packaging plants located within the Burdwan district (latitude 23° 13' 57.0468" N and longitude 87° 51' 48.3084" E) as well as in Hooghly district (latitude 22° 53' 60.0000" N and longitude 88° 23' 23.9964" E) of West Bengal, India. The samples were taken in air-tight screw-capped plastic tubes and were processed in lab within 12 hours of collection.

b) Isolation of cholesterol-degrading bacterial flora from the soil samples: For the isolation purpose, 1g of each of the five soil samples were suspended in10 ml of sterilized distilled water in separate test tubes. The samples were then serially diluted up to 10^{-5} dilution factor using sterile distilled water as the diluents. 1ml aliquot was taken from each of the 10^{-5} dilution tubes (one tube for each of the five soil samples) aseptically and was plated on selective Cholesterol-Tween 20 Agar medium (Nagasawa *et al.*, 1969) containing (g/l): NH_4NO_3, 17.0; K_2HPO_4, 0.25; $MgSO_4.H_2O$, 0.25; $FeSO_4$, 0.001; NaCl, 0.005; Tween-20, 0.1 ml; Cholesterol, 2.0 and agar 20.0) onto separate pre-sterilized petri plates with proper labels inside the Laminar Work Flow Station. All the ingredients were purchased either from Merck®, India or from Hi-Media®, India. All the plates were then incubated at 30°C for 4-5 days at inverted positions. After the completion of the incubation period, the plates were observed for the appearance of bacterial colonies. These colonies could be considered as cholesterol degrading species of bacteria. Two such isolates were randomly chosen from each of the five plates (a total of 10 isolates) and their colony features were studied followed by Gram-staining (by Schaeffer-Fulton Method). The developed selected colonies were further enriched as pure isolates by growing them in Cholesterol-Tween-20 broth media for 4 days in a shaker incubator at 30°C at a motion of 120 rpm in separate Erlenmeyer flasks. These pure isolates were subjected to the next round of experiments.

c) Estimation of 4-cholestene-3-one: The estimation of the first byproduct of cholesterol degradation by the activity of microbial CHO enzyme, *i.e*. 4-cholestene-3-one was performed in order to assay the cholesterol degrading activities of the selected isolates by the method as described by Jayachitra *et al.* (2012) with slight modifications. It is obvious that the more will the concentration of this product, the higher would be the degradation of cholesterol by the respective bacterial species. The cholesterol-Tween-20 broth media for all the ten isolates were centrifuged in individual centrifuge tubes with proper markings at 4°C temperature for a period of 10 minutes and the supernatant was collected. To 0.5ml of each supernatant, 0.5 ml of 100mM Tris-HCl buffer (pH 8.0) was added and the mixture(s) was incubated at water bath for a period of 5 minutes at 37°C temperature followed by the addition of 30 µl.of 25 mM cholesterol solution in isopropanol. The tubes were then incubated at room temperature (28-30°C) for a period of 30 minutes. After the incubation period, 2.5 ml of absolute ethanol was added to each tube and the concentration of 4-cholestene - 3-one in the reaction mixture(s) was determined by measuring the absorbance at 240 nm using a UV-Vis. spectrophotometer (instrument purchased from Systronics®, India; Model No. AU-2603). Among the ten

isolates, four bacterial species that yielded considerably higher absorbance were identified as potent cholesterol degraders and were subjected to next round of experiments. All the reagents used in this experiment were purchased either from Sigma-Aldrich® or from SRL®, India.

d) Confirmation of cholesterol degradation by the selected isolates: In order to confirm the degradation of cholesterol by the selected bacterial strains as well as to determine the percentage reduction of cholesterol, a standard was prepared containing 200µg/ml cholesterol and the cholesterol estimation assay was performed by using the cholesterol estimation kit (purchased from Sigma-Aldrich®, India) that exploits the principle of cholesterol estimation by colorimetric assay of cholesterol-oxidase/peroxidase enzymes. All the regents provided with the kit were mixed in different test tubes labeled as blank, standard and test as per the instruction. 1 ml. of supernatant obtained by the centrifugation of the Cholesterol-Tween-20 broth media as described above was obtained for each of the four selected bacterial strains which exhibited promising results in the earlier experiment. This supernatant was treated as the crude source of the CHO enzyme produced by the respective strains. The reaction mixture(s) were thoroughly mixed followed by incubation at 37°C temperature for 10 minutes. The absorbance in each of the test tubes was then read spectrophotometrically at 505 nm wavelength against appropriate blanks. The corresponding O.D values were then plotted onto the standard curve in order to determine the amount of cholesterol present in the reaction mixture. These concentrations were deducted from the initial concentration to obtain the percentage reduction in cholesterol concentration due to bacterial enzymatic activities.

Interestingly, it was found that the bacterial isolate (designated as CB-8) that showed maximum percent reduction in cholesterol was gram negative in nature. This isolate was next characterized by subjecting it to a battery of biochemical and physiological tests as described below.

e) **Biochemical Characterization of Gram negative isolate:**

i) **IMViC Tests:** Indole, Methyl Red, Vogues-Proskauer and Citrate Utilization Tests were performed for the bacterium under question according to the standard protocol (Dubey and Maheshwari, 2011) and the results were tabulated. All the regents/media required for the tests were purchased either form Merck®, India or SRL®, India and were of analytical grade.

ii) **Starch Hydrolysis Test:** Starch could be hydrolyzed by amylase, an extracellular enzyme produced by many bacterial species. In order to detect the starch degrading activity of the selected isolate, it was grown in Starch Agar medium (containing 1% starch; pH 7.0) and after incubation at 30°C temperature for a period of 48 hrs, the plates (in duplicates) were observed for the growth of the isolated bacterium. The hydrolysis of starch was detected by the appearance of a clear zone on the surface of the starch agar plates around the colonies after the addition of 2-4 ml.of 0.1 N iodine solution as an indicator (Aneja K.R., 2003) and the observation was tabulated.

iii) **Gelatinase Test:** This test is done to determine the ability of the test organism to produce gelatinase enzyme by which it can hydrolyze gelatin and utilize it as the source of carbon and /or nitrogen for its growth. Pure cultures of the selected bacterial species grown on Gelatin Agar medium (purchased from HiMedia®, India; containing 30g/L gelatin and 15g/L agar-agar; pH 7.0) and the tubes (in duplicates) were incubated at 30°C for 72 hrs. Following the incubation period, the culture tubes were subjected to low temperature treatment at 4°C for 30 minutes. The tubes which were positive for gelatin remain liquefied due to the production of gelatinase enzyme by the isolate while the other tubes become solidified at low temperature due to lack of gelatinase activity. The result was then tabulated accordingly.

iv) **Urease Test:** With an aim to detect the production of urease enzyme capable of hydrolyzing urea to release ammonia by the test bacterial strain, the isolate was grown in test tubes (in duplicates) with Urease Test Broth (purchased from HiMedia®, containing 20g/L urea and Phenol Red as the pH indicator dye) . The tubes were then incubated at 30°C temperature for 24-48 hrs. and were observed for the color change of the broth media from yellow to red that indicates a positive reaction.

v) **Carbohydrate Fermentation Profile:** In order to assay the fermentation of various carbohydrates viz. glucose, galactose, lactose, sucrose, mannitol and fructose, the test bacterial isolate was inoculated into appropriate fermentation media containing 1% of each of the carbohydrates in sterile test tubes with Durham's tubes to detect gas production. Phenol Red was used as the pH indicator dye that turns red under acidic condition (due to formation of mixed acids) and gives a positive reaction for acid production. Formation of gas could be detected by the presence of bubbles inside the small Durham's tubes. The result was recorded after 48 hrs. of incubation for all the culture tubes at 30°C temperature.

vi) **Catalase Test:** Catalase is an important enzyme that neutralizes the toxic hydrogen peroxide formed during oxygen metabolism in all aerobic bacterial species. The enzyme converts H_2O_2 into water and molecular oxygen. The test is performed simply by taking a loopful of the pure culture of the test isolate onto a clean and dry slide followed by addition of 4-5 drops of 30% hydrogen peroxide (purchased from Merck®, India). Occurrence of bubbles due to production of O_2 by the activity of catalase (effervescence) is an indicator of positive reaction. The observation was recorded and tabulated.

vii) **Oxidase Test:** This test detects the presence of Cytochrome c oxidase in the bacterial respiratory chain. When a loopful of pure bacterial culture was mixed with tetramethyl p-phylene diamine dihydrochloride (purchased form Sigma-Aldrich®, India) on a piece of dry filter paper, the color of the dye changes to blue due to reduction of the compound by the oxidase enzyme (Benson, 1994). This color change represents a positive reaction. The result for test bacterial strain for this test was recorded accordingly.

viii) **Nitrate Reduction Test:** To assay the production of the enzyme nitrate reductase by the bacterial isolate, 1 ml of pure culture in nutrient broth was inoculated in nitrate broth (Purchased from Sigma-Aldrich®, India; containing 5g/l peptone, 3g/l meat extract and 1g/l potassium nitrate;

pH 7.2) and the Erlenmeyer flasks (in duplicates) containing the inoculated medium were incubated at 30^0C temperature for a period of 48 hrs. After the incubation is over, 6-8 drops of sulphanilic acid (8g/l of 5N acetic acid) and equal amount of 1- naphthylamine (6g/l of 5N acetic acid) were added to the broth medium. If the color turns to red or pink within a few minutes of addition of the regents without the addition of zinc powder, the sample is considered as positive for nitrate reductase activity. The observed result for the isolate was tabulated.

ix) **Arginine Hydrolysis Test:** This test was performed to test the arginine decarboxylase (dihyrolase) activity of the isolate by inoculating the pure culture of the same into Arginine dihydrolase broth (with Bromo Cresol Purple as the pH indicator dye) and incubating the inoculated flasks (in duplicates) at 30°C temperature for 48hrs. Color change from purple to yellow and then back to purple indicates a positive result. The observation was made after the due time and was recorded.

x) **Test for Haemolytic Activity:** The haemolytic activity of the bacterial strain under study was done by culturing the same on Blood Agar medium (Nutrient Agar basal medium supplemented with 8% de-fibrogenated sheep blood) in duplicates and incubating the plates at 37°C temperature for 48 hours. The plates were then observed for the zone of haemolysis (clear zone) around the growth and the finding was recorded.

f) Physiological Characterization:

i) Effect of temperature on Growth: The effect of temperature on the growth pattern of the selected bacterium was studied by exposing the pre-inoculated LB broth media at specified temperatures (10°C, 28°C, 37°C, 45°C, 60°C and 80°C) for 15 minutes followed by incubating the flasks at 30°C for 48 hrs. The growth variation was then determined by measuring the absorbance spectrophotometrically at 540 nm wavelength.

ii) Effect of pH on Growth: To determine the effects of pH on the growth of the selected gram negative bacterial isolate CB-, LB broth media were prepared and the pH of the broth was adjusted accordingly (pH 4.0, 6.0, 7.0, 9.0 and 11.0) by using 1.5M NaOH and 5 N HCl. Separate Erlenmeyer flasks were used for each pH. All the flasks were incubated at 30°C for 48 hours and the growth was measured spectrophotometrically at 540 nm wavelength. The resulting data was recorded to depict the growth variation of the gram negative isolate at different pH ranges.

iii) Effect of NaCl on Growth: To study the variation in the growth pattern of the bacterial strain at different concentrations of NaCl (1%, 2%, 3%, 4% and 5%), the organism was grown in different LB broth media with respective salt concentrations in separate Erlenmeyer flasks. Following incubation at 30°C for 48 hrs, the absorbance was measured at 540 nm spectrophotometrically and the observed data was recorded.

g) Antibiotic Sensitivity Assay: The sensitivity of the CB strain against selected antibiotics (Streptomycin, Tetracyclin, Ampicillin, Ciprofloxacin and Kanamycin) was measured by Kirbey-Bauer Method (Disc-diffusion method). This was performed by spreading 0.5ml of pure broth culture (in LB Broth) of the isolate on the surface of the Muller-Hinton Agar plates and then by placing the commercially available discs of the said antibiotics (purchased form Bio-Rad®, India; disc content 30 µg of the specific antibiotic $disc^{-1}$) on the inoculated plates. All the plates were then incubated at 37°C temperature for 48 hrs. The sensitivity was measured in terms of diameter of the inhibition zones, i.e. the clear zones that appear around the discs due to the activity of the antibiotics.

RESULTS

a) Collection of Soil Samples: The five soil samples collected from either Burdwan or Hooghly Districts of West Bengal were labeled as presented in Table 1.

TABLE 1: Details of Collection of Soil Samples from different areas

Sample No.	Collection Area/District	Proximal Oil Plants
SB-1	Burdwan	Mustard Oil Packaging Plant
SB-2	Burdwan	Mustard Oil Manufacturing and Packaging Plant
SB-3	Burdwan	Vegetable Oil Refinery Plant
SH-4	Hooghly	Rice Bran Oil Packaging Plant
SH-5	Hooghly	Mustard Oil Refinery Plant

b) Isolation of cholesterol-degrading bacterial flora from the soil samples: The colony features and Gram nature of a total of 10 isolates (two colonies randomly selected from each of the five soil samples) which were able to grow on Cholesterol-Tween-20 enrichment medium and hence, were considered having cholesterol degrading activity were recorded in a tabular format as shown in Table 2.

c) Estimation of 4-cholestene-3-one: The absorbance values for spectrophotometric determination of 4-choestene-3-one, a product of bacterial cholesterol degradation were plotted for each of the ten selected isolates. The plot is represented in Figure 2. From the data obtained, it could be concluded that the bacterial isolates CB-2, CB-4, CB-8 and CB-10 produced higher amounts of the byproduct i.e.4-cholestene-3-one that indirectly reflected their potent cholesterol degrading capabilities. Among these four promising isolates, three were Gram positive rod-shaped bacteria and only one bacterial isolate was found to be Gram –negative rod in nature. This Gram negative strain of the soil-borne bacterium exhibited maximum cholesterol degrading potential and hence, considered to have highest cholesterol oxidase activity.

TABLE 2: Colony Features and Gram character of randomly selected cholesterol degrading isolates grown on Cholesterol- Tween-20 Enrichment agar medium:

Sample No.	Isolate No.	Colony Color	Colony Shape	Colony Margin	Colony Elevation	Colony Opacity	Consistency	Gram Nature
	CB-1	Whitish	Circular	Entire	Flat	Opaque	Smooth	Gram Positive, Rod-shaped
SB-1	CB-2	Whitish	Circular	Entire	Convex	Opaque	Smooth	Gram Positive, Rod-shaped
	CB-3	Whitish	Circular	Wavy	Flat	Translucent	Smooth	Gram Positive, Coccus
SB-2	CB-4	Whitish	Circular	Entire	Convex	Opaque	Smooth	Gram Positive, Rod-shaped
	CB-5	Whitish	Circular	Entire	Submerged	Opaque	Smooth	Gram Positive, Rod-shaped
SB-3	CB-6	Whitish	Circular	Entire	Flat	Opaque	Smooth	Gram Positive, Coccus
SH-4	CB-7	Whitish	Circular	Entire	Submerged	Opaque	Smooth	Gram Positive, Rod-shaped
	CB-8	Yellow	Circular	Wavy	Convex	Opaque	Smooth	Gram Negative, Rod-shaped
	CB-9	Whitish	Circular	Entire	Submerged	Translucent	Smooth	Gram Positive, Rod-shaped
SH-5	CB-10	Whitish	Circular	Entire	Submerged	Opaque	Smooth	Gram Positive, Rod-shaped

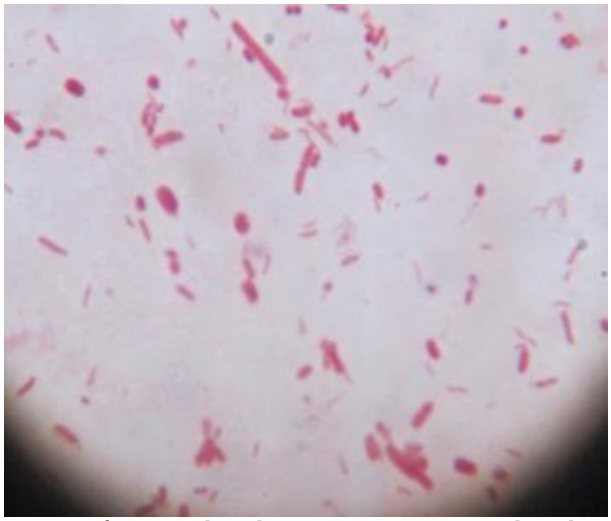


FIGURE 1: Gram Staining of CB-8 isolate showing gram negative rods under 100X magnification

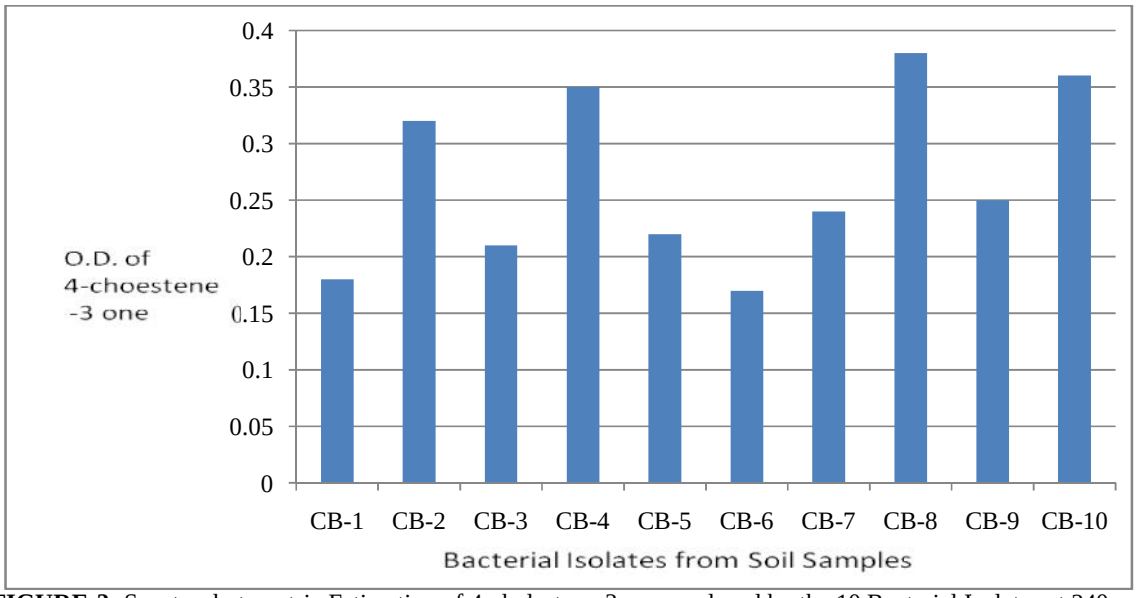


FIGURE-2: Spectrophotometric Estimation of 4-cholestene-3-one produced by the 10 Bacterial Isolates at 240 nm Wavelength:

d) Confirmation of cholesterol degradation by the selected isolates and determination of % reduction of total cholesterol: Further confirmation of the cholesterol degradation by the four promising isolates was performed by kit method available commercially and the amount of cholesterol present in the enrichment broth medium was determined as described above. The values were then used to assess the percentage reduction in total cholesterol that is recorded in table 3. The standard curve used for this estimation is also shown in Figure 3. The values thus

obtained showed that the isolate CB-8 *i.e.* the Gram-negative rod shaped bacterial species is the most potent degrader of cholesterol which could oxidize cholesterol up to more than 80%.This is in keeping with the result of the earlier experiment that measured cholesterol metabolism by means of production of 4-cholestene-3 one, a byproduct of the oxidative degradation pathway. This strain was then further studied for its biochemical, physiological as well as antibiotic sensitivity characters in the next course of the current work.

TABLE 3: Percentage Reduction in Total Cholesterol by enzymatic activities of the selected bacterial isolates:

Isolate No.	Total Cholesterol Conc. (µg/ml.)	Reduction In Cholesterol Conc. (µg/ml.)	Percentage Reduction In cholesterol conc.
CB-2	200	130.32	66.16
CB-4	200	152.25	76.12
CB-8	200	165.38	82.69
CB-10	200	157.27	78.56

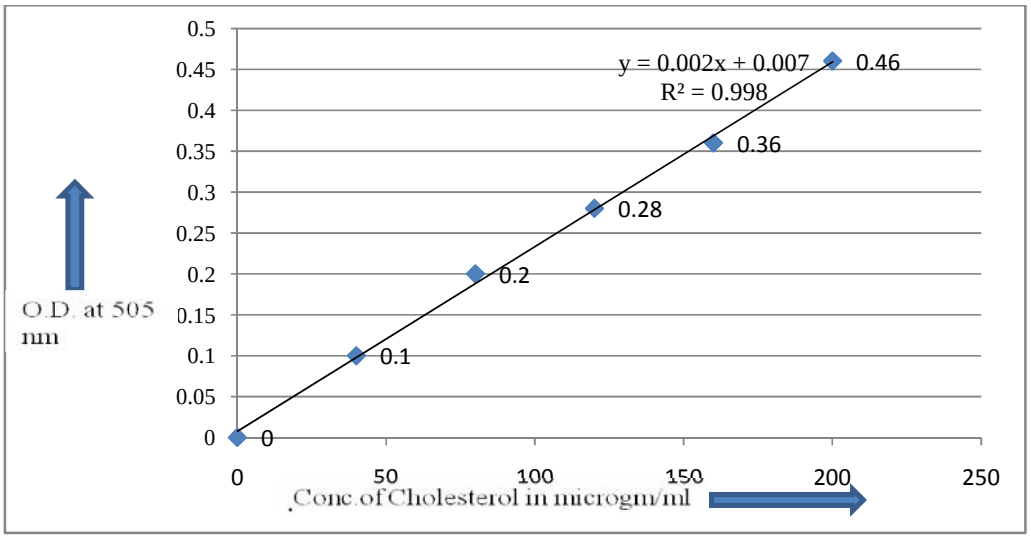


FIGURE-3: Standard Curve for Cholesterol Estimation by Kit Method (Cholesterol oxidase/peroxidase Assay)

e) **Biochemical Characterization of the Gram negative isolate CB-8:**

i) **IMViC Tests:** The results of IMViC tests (as presented in Table 4) clearly inferred that the Gram negative bacterial species is not *E. coli* or other coiliform but could be any other non-typical Gram negative bacteria as it is indole and citrate-negative, strongly positive for MR and weakly positive for V-P test.

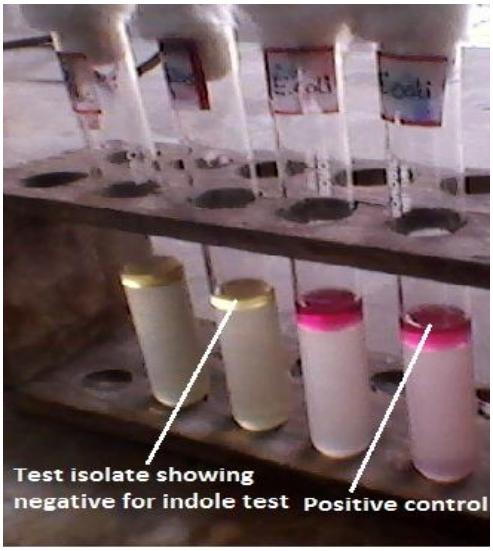


FIGURE-4: Result of Indole Test of the test isolate against a Positive Control

ii) **Starch Hydrolysis Test:** As evident from the result of starch hydrolysis test (included in table 4); the absence of clear zone around the growth on the starch agar media indicated that the CB-8 isolate cannot hydrolysis starch as its source of carbon.

iii) **Gelatinase Test:** The bacterial strain under study was found to hydrolyze gelatin as its carbon source since the gelatin remained liquefied in test tubes even at room temperature. This observation (Table 4) confirmed the production of gelatinase enzyme by the CB-8 isolate.

iv) **Urease Test:** The CB-8 isolate was found to be a weak producer of urease enzyme since the color of the Urease Broth turned to light red form yellow after 48 hours of incubation. The observation is as well recorded in Table 4.

TABLE 4: Observations for Biochemical Characterization: IMViC, Starch Hydrolysis, Gelatinase and Urease Tests:

Isolate No.	Indole Test	Methyl Red Test	V-P Test	Citrate Utilization Test	Starch Hydrolysis Test	Gelatinase Test	Urease Test
CB-8	--	++	+	--	--	++	+

- indicates negative, + indicates weakly positive and ++ indicates strongly positive result

v) Carbohydrate Fermentation Profile: According to the observations made (represented in table 5), the CB-8 isolate of the cholesterol degrading bacterial species was a strong fermenter of glucose, lactose and galactose producing both acid and gas but the same could not ferment sucrose, mannitol and galactose and thus, was not able to utilize these carbohydrates as its energy sources.

TABLE 5: Observations for carbohydrate Fermentation Profile:

Isolate No.	Glucose	Galactose	Lactose	Sucrose	Fructose	Mannitol
CB-8	++	++	++	--	--	--

- indicates negative (with no acid or gas) and ++ indicates strongly positive (with both acid and gas) results

vi) Catalase, Oxidase, Nitrate Reduction, Arginine Hydrolysis and Haemolytic Activity Tests: The results for these tests are documented in Table 6. The CB-8 isolate was found to be a strong producer of catalase enzyme as evidenced by the pattern of effervescence. It also exhibited positive result for nitrate reduction as evident by the change of the color of the Nitrate Broth to reddish pink without the addition of zinc powder. The organism was weakly positive for oxidase test inferred from the fact that the color of the dye turned into faint blue on the filter paper disc. As far as Arginine Hydrolysis is concerned, there was no color change observed in the broth after the incubation period and so, the bacterium was confirmed as negative for Arginine dihydrolase production. Interestingly enough, the bacterium was found to exhibit beta-haemolysis on blood agar. Hallow zone was observed around the growth on the blood agar surface indicating the production of haemolysin.

TABLE 6: Observations for Catalase, Oxidase, Nitrate Reductase, Arginine Hydrolysis and Haemolytic Activity Tests:

Isolate No.	Catalase Test	Oxidase Test	Nitrate Reductase Test	Arginine Dihyrolase Test	Haemolytic Activity On Blood Agar
CB-8	++	+	++	--	Beta-haemolysis

- indicates negative, + indicates weakly positive and ++ indicates strongly positive results

FIGURE 5: Colonies of CB-8 isolate exhibiting haemolysis (on Blood Agar Medium) as indicated by the clear hallows**:**

f) Physiological Characterization of the CB-8 bacterial isolate:

i) Temperature Variation Assay: The data recorded in the form of the plot (Fig.-6) revealed that the CB-8 isolate under investigation is a mesophilic bacterium exhibiting maximum growth at 28°C that matches with the temperature profile of typical soil-borne bacteria. High temperature (more than 50°C) drastically inhibits its growth as evident by the decrease in the A_{540}.

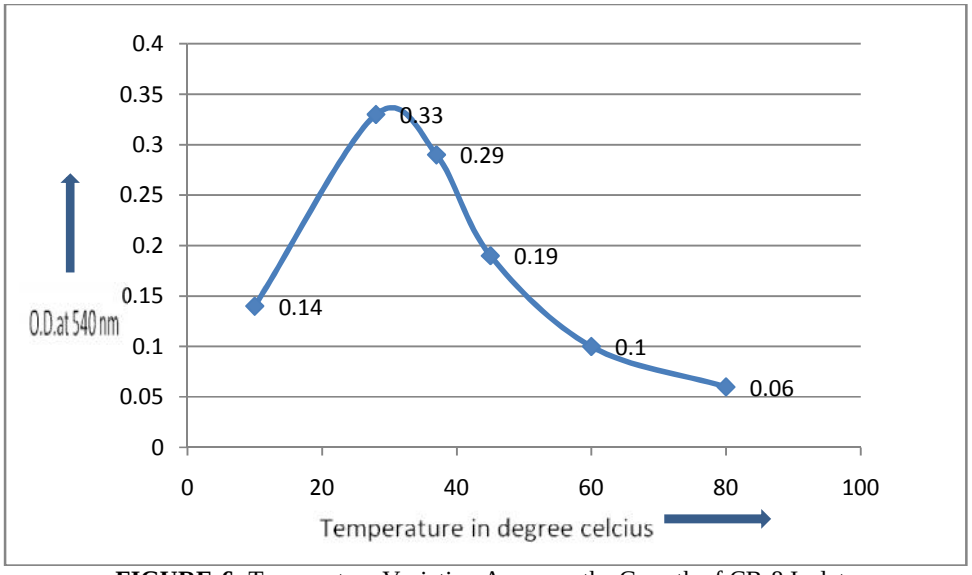


FIGURE-6: Temperature Variation Assay on the Growth of CB-8 Isolate

ii) pH Variation Assay: The plot connecting A_{540} values to different pH ranges (Fig.7.) suggested that the CB-8 isolate is a neutrophile as the growth is optimum at pH 7.0. pH higher or lower than this neutral point led to the decrease in the growth rate of the bacterium emphasizing its neutrophilic nature.

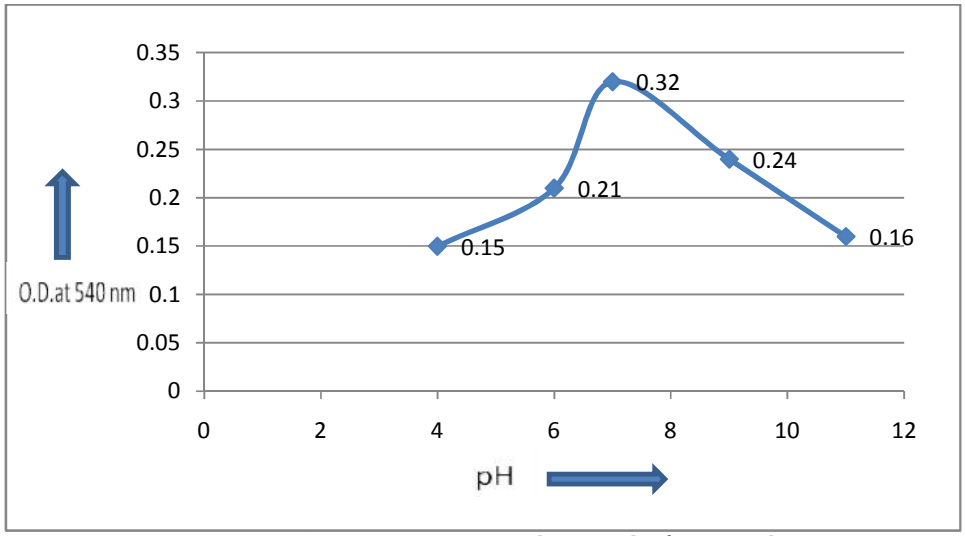


FIGURE-7: pH Variation Assay on the Growth of CB-8 Isolate

iii) NaCl Variation Assay: The growth of the bacterium was optimum only at 1% concentration of NaCl (Fig. 8). Hence, it could be interpreted that the organism is a non-halophile and non-halotolerant bacterium. Concentrations of NaCl higher than 1% were found to inhibit its growth in a consistent manner.

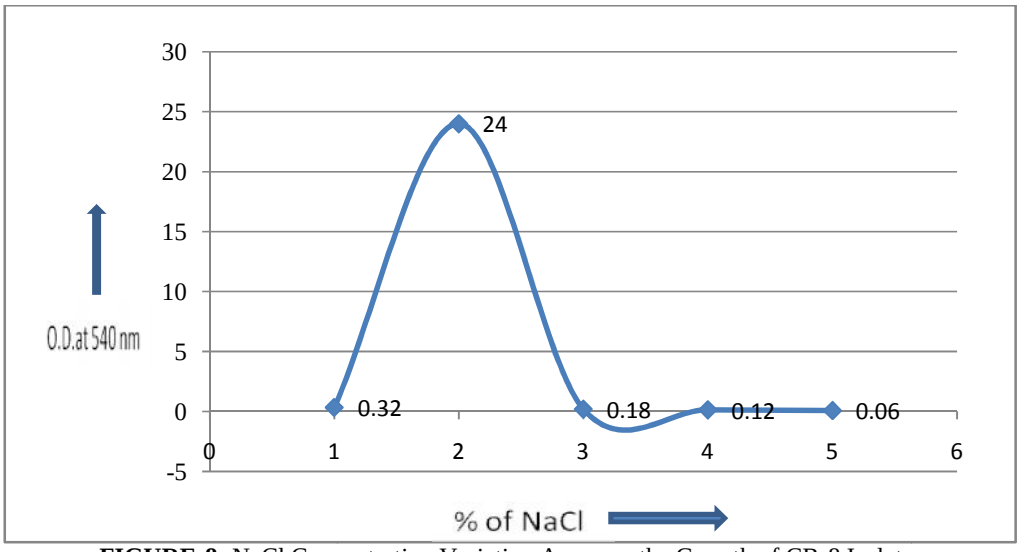
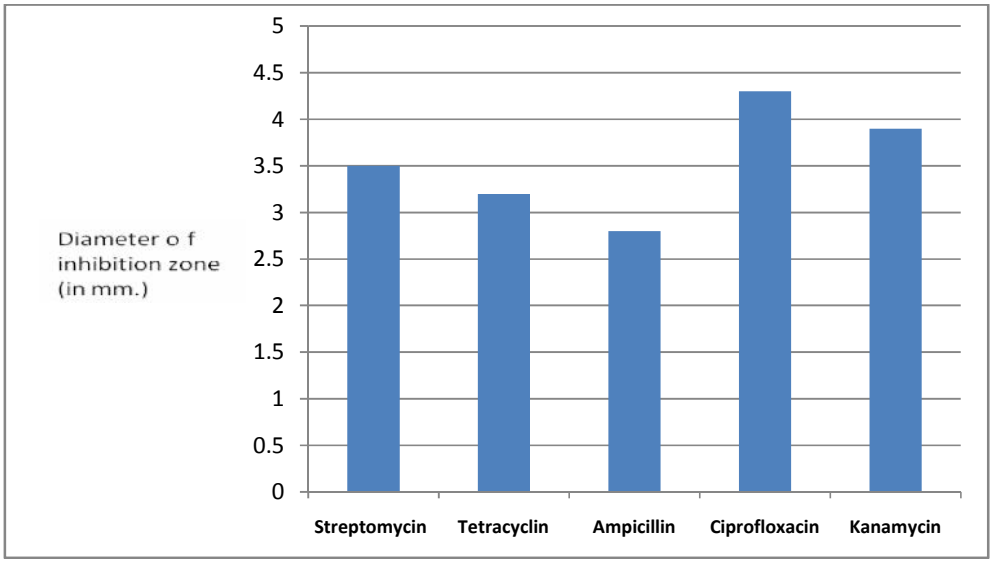


FIGURE-8: NaCl Concentration Variation Assay on the Growth of CB-8 Isolate:

g) Antibiotic Sensitivity Assay: Among the various antibiotics tested it was observed form the plot (Fig. 9.) that the CB-8 isolate which showed maximum cholesterol oxidase activity was most sensitive to the antibiotic ciprofloxacin which is a derivative of nalidixic acid and was least sensitive to ampicillin that acts on peptidoglycan biosynthesis This resistance pattern is in accordance with the gram negative nature of the isolated strain since it has a very thin layer of peptidoglycan layer on the cell wall structure.



DISCUSSION

By compiling all the documented observations it was inferred that among 10 randomly chosen soil isolates that had grown on the Cholesterol-Tween 20 Enrichment media, four exhibited potential cholesterol degrading activity as measured by both spectrophotometric assays of 4-cholestene -3 one and by CHO enzyme activity assay determined by kit method. As far as the results are considered, the isolate CB-8 showed maximum cholesterol degradation and cholesterol oxidase enzyme activity that resulted in greatest percent reduction in total cholesterol concentration *in vitro*. This strain was found to be gram negative in nature which is, however, also the only gram negative soil bacteria detected in the experimental set that comprised of 10 randomly chosen cholesterol degrading bacterial flora. It was also distinct from other isolates on the basis of yellow pigmentation on the enrichment agar medium while all other isolates produce non-pigmented colonies. On biochemical characterization, this organism was found to be a atypical gram negative strain whose features did not match with that of typical coliforms. The CB-8 strain did not hydrolyse starch but was found to have significant gelatinase activity. It was also able to ferment a few types of carbohydrates with both acid and gas production. The bacterium was mostly a facultative aerobic bacterium giving strongly positive result for catalase by weakly positive result for oxidase enzymes. It was able to produce urease enzyme and was also strongly positive for nitrate reductase activity. Physiologically, its optimum temperature and pH for growth were 28°C and

pH 7.0, respectively that are typical for a soil-borne species. Its growth rate was reduced at concentrations of NaCl higher than 1% thus proving its non-halophilic nature. Moreover, the CB-8 strain was most sensitive to ciprofloxacin and was least affected by ampicillin owing to its gram negative nature. As evident by its strong CHO enzyme activity, the enzyme could be exploited industrially for commercial applications in cholesterol determination kits widely used to measure serum and food cholesterol levels. Further investigation on the extraction and purification of the cholesterol oxidase from the CB-8 isolate remain to be done as a future extension of this work in order to evaluate its commercial application. Proper molecular identification of the strain by 16S rRNA sequencing studies could also be performed for more scientific and authentic characterization of the current strain under investigation. The strain could also find its potential application to remove the steroid deposits from the soils that are contaminated by pharmaceutical, vegetable oil manufacturing or leather processing industries. This is particularly important in connection to the eco-toxicological effects of steroid-like compounds present in the food chain as discussed earlier.

ACKNOWLEDGEMENT

The authors would like to acknowledge Durgapur College of Commerce and Science (DCCS), Rajbandh, West Bardhhaman, West Bengal-713212 and Triveni Devi Bhalotia (TDB) College, Raniganj, West Bardhhaman, West Bengal-713347 for providing the necessary laboratory facilities and infrastructure to carry out the present work.

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