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Isolation, molecular characterisation and sequencing of cholesterol degrading bacteria

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Abstract

Bacillus cereus from soil of agriculture waste using cholesterol-Tween-80 medium. Conformation of cholesterol degrading activity by using halo formation and estimation technique. Optimum growth was observed on 37°C, 7.0 pH on cholesterol-Tween-80 medium. Morphology characterization and molecular characterization (i.e.) 16s rRNA sequencing was performed to identify the strain. The cholesterol oxidase enzyme was purified and electrophoresised.This study was concluded that the isolate was confo rmed cholesterol degrading bacteria and produce the intermediate product 4-Cholesten-3-one by the enzyme cholesterol oxidase, it was proved by estimation and Halo formation. This *Bacillus cereus* was degrades cholesterol and takes as a carbon source for its growth.Cholesterol Oxidation occurs in both Oxic pathway and Anoxic pathway. But the Oxic pathway is the best for cholesterol oxidation. Cholesterol oxidase enzyme oxidize the cholesterol to 4-cholesten-3-one with the reduction of O_2 to H_2O.It is the first step in cholesterol degradation. The complete degrading pathway in cholesterol degradation has limited research. Thus, more molecular systematic studies need to be undertaken in this direction.

Key-Words: *Bacillus cereus*, Cholesterol, Halo formation, Cholesterol oxidase

Introduction

Cholesterol is a lipidic, waxy alcohol found in the cell membranes and transported in the blood plasma to establish proper membrane permeability and fluidity. Cholesterol is the principal sterol synthesized by animals, plants and fungi which responsible for causing atherosclerosis. Recently, cholesterol has also been implicated in cell signaling processes, assisting in the formation of lipid rafts in the plasma membrane[1]and it is the precursor molecule for many biochemical pathways . Cholesterol is an important precursor molecule for the synthesis of vitamin D and steroid hormones. In the present study cholesterol degrading bacteria was isolated from agricultural waste and its 16s rRNA gene sequence was analysed.

Cholesterol degrading Microorganisms and its function

Several bacterial genera, such an *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Pseudomonas* reportedly mineralize cholesterol in the presence of molecular oxygen.

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After intaking the dairy products fermented by *Lactobacillus acidophilus* [3] the level of cholesterol present in the serum was analysed. The bacterial degradation of cholesterol occurs via cholesterol oxidase which produces 4-cholesten-3-one along with the reduction of 0_2 to H_2O_2.

Material and Methods

Sample collection

Soil sample was collected from agriculture waste of Agriculture College, Madurai, Water sample from Leather factory, Dindugal and from Cheeses & Butter.

Isolation of cholesterol degrading microorganism

The samples were serially diluted using 9ml sterile saline. The bacterial species was isolated by spread plate techniques using cholesterol Tween-80 agar plates. 20 ml of cholesterol-Tween-80 agar containing different concentration of cholesterol was poured on to the plate and allowed solidify and then adding 100 µl of aliquot of appropriate dilution was added and spread out in to the each solidified cholesterol Tween-80 agar plates and then incubated in 37ºC incubator in inverted position. After 4 days incubation the plates were containing colonies. From that , we conclude that the bacterial species was able to degrade cholesterol.

Confirmation test

Halo formation

Cholesterol- Tween-80 medium was used to halo formation. The isolated culture was inoculated on the plate and incubated at 37° C for 4 days. The halo areas were examined.

Estimation of 4-cholesten-3- one

The supernatant from centrifuged broth at 10,000 rpm for 10 minutes at 5°C and take 0.4ml of 125 mM Tris-Hcl buffer, pH 7.5 was added 0.1ml of enzyme solution, and the mixture was incubated in water bath at 37°C. After 3 minutes, 25µl of 12 mM of cholesterol in isopropanol solution were added to mixture. After 30 minutes, 2.5 ml of absolute ethanol were added to reaction mixture the amount of 4-Cholesten-3-one was determined by measurement of the absorbance at 240 nm[4].

Biochemical and microbial characterization

50 ml 0f LB broth was prepared and 100 µl of culture was inoculated and kept in incubator for 2 days at 37ºC .After incubation it was used for following microbial and biochemical characterization y means of simple staining and gram's staining.

Motility determination- hanging drop experiment

A cover slip and a cavity slide were cleaned add a loop full of log phase culture was placed in the center of cover glass was inverted down carefully to make the drop hanging cavity and was observed in high power oil immersion microscope.

Triple sugar ion test

A loopful of culture was streaked in triplet sugar ion agar slants were kept for incubation for 37ºC.

Starch hydrolysis test

Starch agar plate was divided into two parts, isolates were streaked on the plate in a straight line and it was incubated at 37° for 24 hrs add a drops of Grams iodine and the result was observed.

Antibiotic test

Susceptibility to antibiotics was determined on Muller-Hinton agar and inhibition zone was noted after 24 hrs. The following antimicrobial agents were tested: Ampicillin , Methicillin , Kanamycin , Ciprofloxacin , Erythromycin , Nalidixic acid , Co-Trimoxazole , Clotrimazole , Cefazolin , Streptomycin , Tetracyclin , and Chloramphenicol.

Casein utilization test

Minimal salts agar with casein was prepared and divided into two parts and growth of isolates in casein agar plates was observed.

Hemolytic activity

Blood haemolysis was evaluated on Nutrient medium supplemented with 5% sheep blood which was incubated at 37° C for 4 days.

Indole test

A loopful of the culture in sterile Tryptone broth after incubation Kovac's reagent was added, cherry red colour indicates the positive whereas yellow colour denotes negative result.

Methyl red test

Culture were inoculated MR-VP broth and incubated for 1 day at 37° C and add 5-10 drops of methyl red solution .

Catalase test

A loopful of culture, grown on YEG plate for 24 hrs then placed in 1% H_2O_2 on a glass slide observed for the production of air bubbles.

Citrate test

A loop full of culture was streaked in Simmons citrate agar slants and it was kept incubation for 37ºC for 24 hrs.

Optimization

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

Effect of pH

Different pH (6, 7, and 8) was maintained in the media. Growth and activity were assessed for every day.

Effect of temperature

Different temperature (30°ºC, 37°ºC, and 57ºC) was maintained in the media. Growth and activity were assessed for every day.

Effect of concentration

Different concentration of cholesterol (0.05mg, 0.075mg, and 1mg) was maintained in the media. Growth and activity were assessed for every day.

Molecular identification

Separation of isolated genomic DNA by agarose gel electrophoresis and the DNA was visualized with the UV illuminator.

PCR amplification of 16s rRNA gene

Test: total volume 50µl

Genomic DNA =3µl
8F Primer =2.5µl (0.5µM)
1490R Primer =2.5µl
Genei master mix =25µl
Deionized water =17µl

Control: Total volume 50µl

Genomic DNA =3µl
Forward Primer =2.5µl
Reverse Primer =2.5µl
Deionized water =42µl

Separation of amplified pcr products

12 µl of amplified PCR product was loaded in the 1%Agarose gel electrophoresis.

Degradation analysis of cholesterol purification

Dialysis

This experiment was done to purify the cholesterol oxidase enzyme which is degrading the cholesterol. Sterile cholesterol Tween-80 broth was prepared in 100ml conical flask with cholesterol and 100µl of culture was inoculated and kept incubation for 4 days at 37 ºC. Then the broth with the bacterial culture was taken and centrifuged at 10,000 rpm for 10 minutes, to take supernatant. The supernatant samples were precipitated with Ammonium sulphate and the precipitated sample was dialyzed for over night at cool condition (4° c) to purify the enzyme. To evaluate the purify of active fractions, they were subjected to SDS-PAGE on a 10% gel slab.

Results and Discussion

Isolation

White, spherical shape colonies were observed using cholesterol- Tween-80 medium in Fig-1.

Halo formation

10mm of zone was observed as a halo by degrading the cholesterol in Fig-2.

Degradation of sheep cholesterol

Sheep cholesterol degrading was conformed by the observation of tubes with control in Fig-3.

Estimation of 4-cholen-3-one

The product was measured at 240 nm with the O.D units in Fig-11.

Antibiotic susceptibly test

Isolate was sensitive to Ampicillin, Amoxycillin, Chloramphenicol and Citrofloxacin shown in Fig-5a and 5b.

Optimization

pH - The maximum growth of the isolate was observed on 7.0pH in Fig-12

Temperature - The maximum growth of the isolate was observed on 37ºC temperature in Fig-13.

Cholesterol concentration - The maximum growth of the isolate was observed on 0.75mg in 50 ml concentration in Fig-14.

Purification of enzyme

The cholesterol oxidase enzyme was purified using Ammonium sulphate precipitation.

SDS-PAGE

55 kDa molecular weight bands was observed using 10% poly acrylamide gel after purification of the cholesterol oxidase enzyme in Fig 10

Out of four samples, soil of agriculture waste from Agriculture College, Madurai, Waste Water from Leather factory Dindugal, Butter, and Cheese, soil sample only exhibited the presence of Cholesterol degrading bacteria with high level activity. *Bacillus Subtilis*, isolated from Korean traditional fermented flatfish used for produce cholesterol oxidase in a medium containing 0.2% cholesterol. *Bacillus subtilis*, used in this study, could produce much higher level of cholesterol oxidase (3.14 Uml^{-1}) than other strains[5]. *Bacillus Subtilis* degrading Cholesterol was applied to reduce residual cholesterol content in Fermented flatfish. It has been reported that many cholesterol oxidase are produced during degradation of cholesterol in the presence of oxygen[6]. This concordance with Choleserol degradation by the *Bacillus cereus* was confirmed to degrade cholesterol. Hence the isolate was used for further studies. Halo formation on the agar medium containing cholesterol dependent on the conversion of cholesterol to 4-cholesten-3-one by the extra cellular cholesterol oxidase[7]. The amounts and percentages of the remaining cholesterol and the converted 4-cholesten-3-one in the vicinity of colonies of the strains grown on cholesterol-containing agar medium were obtained in comparison with the control. The *Bacillus cereus* experimental sample was biochemically identified as Gram positive, catalase positive rods, non motile, methyl red positive. The enzyme was conformed as a single band on an SDS-PAGE. The molecular weight of the purified cholesterol oxidase was estimated at 55 kDa. It has been estimated at 55 and 56 kDa from *Rhodococcus erythropolis* [9], 56 kDa from *Rhodococcus equi* [10] and 60 kDa from *Streptomyces fradiae* [11]. Discrete bands were observed in 1% agarose gel on UV illumination after electrophoresis of the Genomic DNA samples isolated from isolate. Discrete bands having size of ~1.5 Kb was observed in 1% agarose gel on UV illumination after loading the amplified PCR products. The PCR products were purified and loaded in agarose gel and band was observed as *Bacillus cereus*.

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Fig. 1: Isolation of cholesterol degrading bacteria

Fig. 3: Degradation of Sheep cholesterol

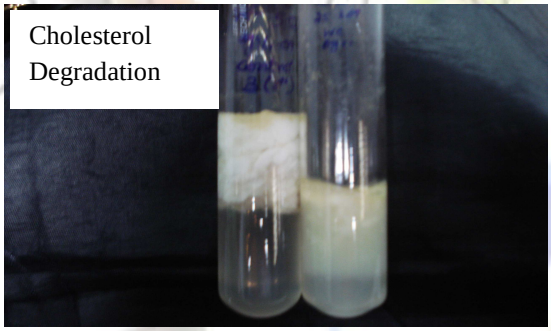

Fig. 2: Halo Formation of the *Bacillus cereus*

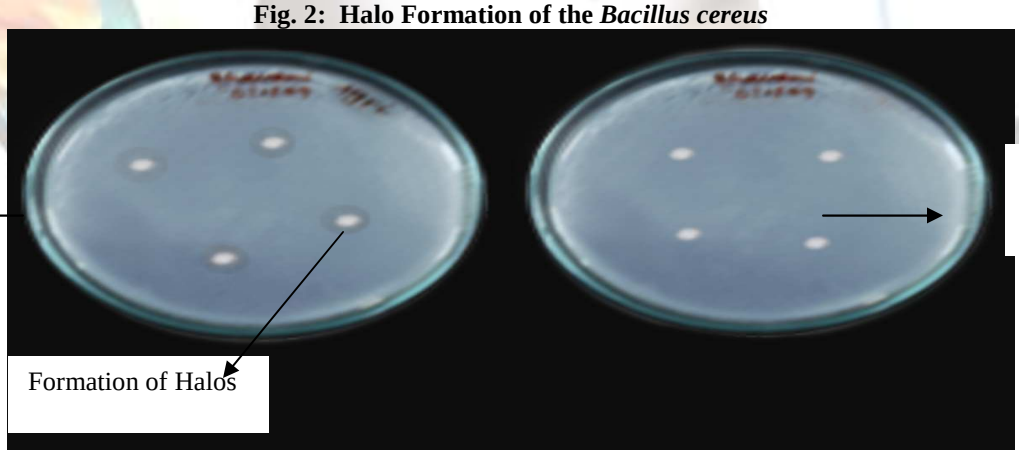

Fig. 4: Gram staining of the *Bacillus cereus*

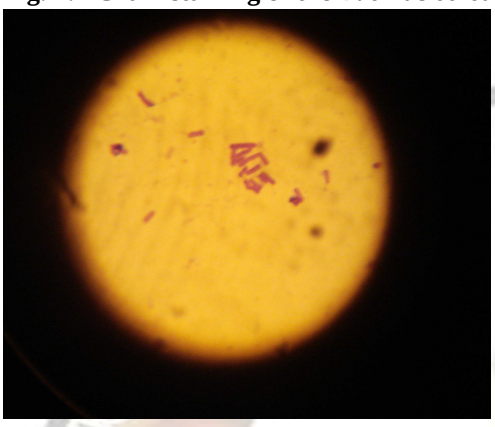

Fig. 5a: Antibiotic Test 5b

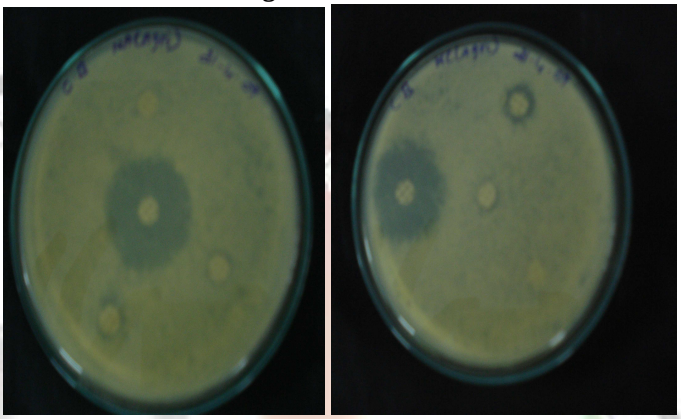

Fig. 6: Haemolytic Activity

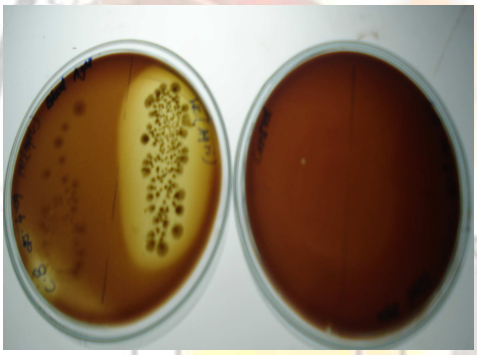

Fig. 7: Methyl red test

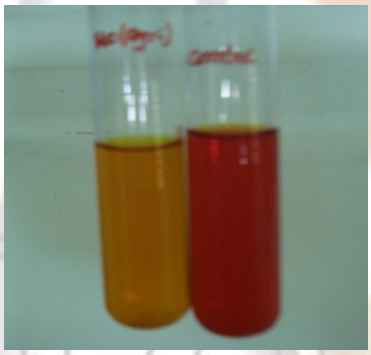

Fig. 8: Starch hydrolysis test

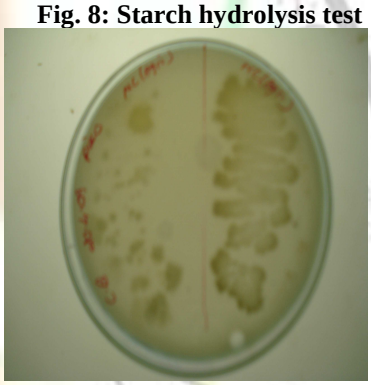

Fig. 9: Sereration of 16s r RNA product

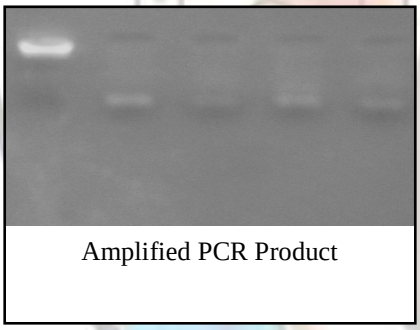

Fig. 10: SDS-PAGE

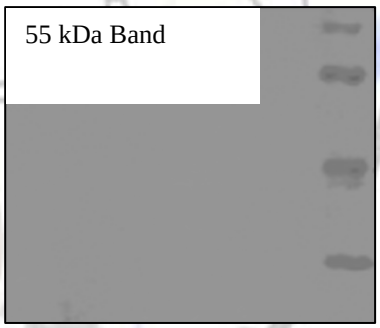

Fig. 11: Estimation of 4-cholesten-one

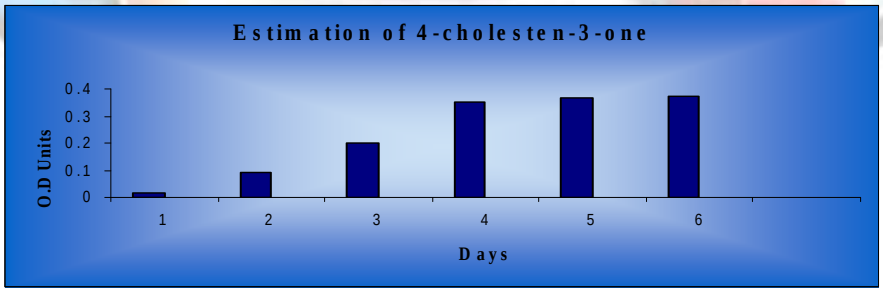

Fig. 12: Effect of pH on *Bacillus cereus*

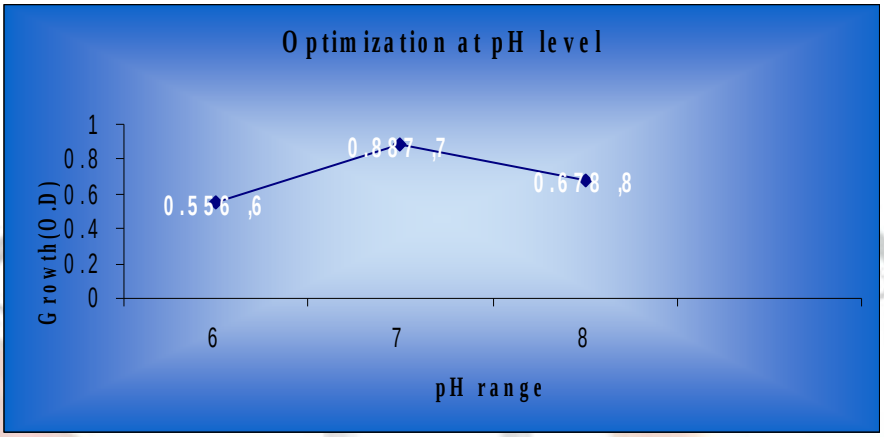

Fig. 13: Effect of temperature on *Bacillus cereus*

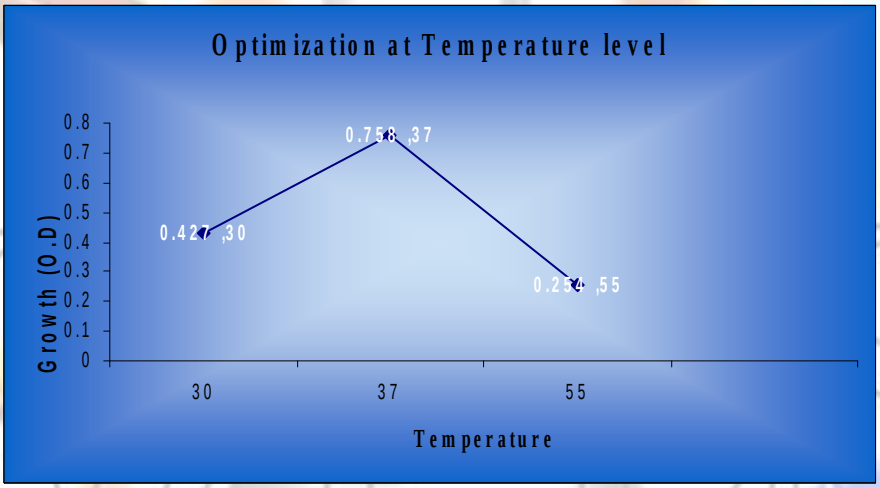

Fig. 14: Effect of cholesterol concentration on *Bacillus cereus*

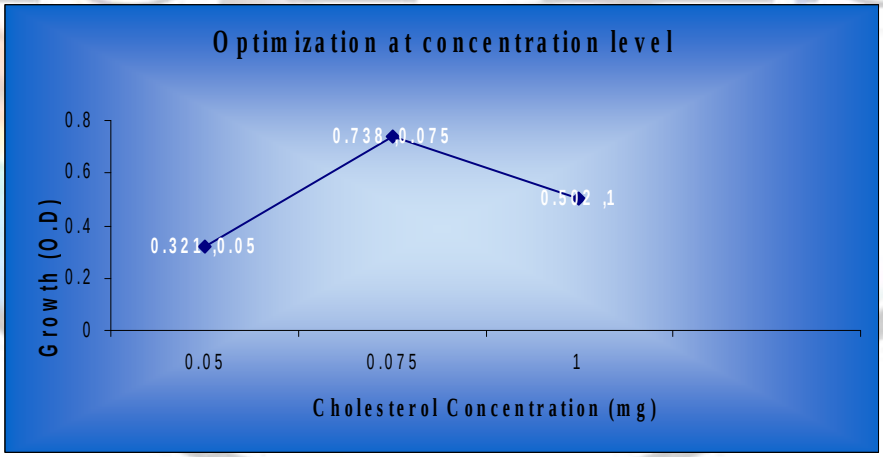

Table 1: Biochemical characteristic of the isolate

Gram stain	+
Morphology	Rod
Motility	+
Gasfromglucose	+
Catalase test	+
Methyl red test	+
Starch,Casein utization test	+

Table 2: Antibiotic susceptibility test-zone diameter disc diameter – 6mm

Antibiotics	Result
Ampicillin	Sensitive
Amikacin	Resistant
Amoxycillin	Sensitive
Bacitracin	Resistant
Cefazolin	Resistant
Ceftazidime	Resistant
Chloramphenicol	Sensitive
Citrofloxacin	Sensitive
Co-Trimazole	Resistant
Erythromycin	Resistant
Kanamycin	Resistant
Methicillin	Resistant
Nalidixic Acid	Resistant
Streptomycin	Resistant
Tetracycline	Resistant