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Original Article

Metabolic profiling of cholesterol transformation by *Pseudomonas aeruginosa* strain choltrans (MK782058), an isolate from the rhizosphere of *Aloe vera* (L.)

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Abstract

Cholesterol, integral to cellular membranes, has vital biological functions but poses environmental and health risks. The rhizosphere, rich in diverse microbial communities, serves as a niche for isolating specialized bacteria with unique metabolic capabilities. This study screened potent cholesterol-transforming bacteria from the rhizosphere of *Saccharum officinarum* L., *Aloe vera* (L.) Burm. f., *Cymbopogon citratus* (DC.) Stapf, *Ocimum sanctum* L., and *Allium cepa* L., and identified them using biochemical characterization, including analytical profile index determination as well as 16S rRNA gene sequencing and phylogenetic analysis. As supported by GC-MS analysis, the efficient bacterium *Pseudomonas aeruginosa* strain Choltrans showed potential for bioremediation and health applications. The GC-MS analysis revealed the presence of metabolites, including cholestane-3,5-diol, 5-acetate, (3 beta, 5 alpha); cholest-5-ene, 3-methoxy-, (3 beta); cholesta-3,5-diene; cholest-7en-3-ol, 14-methyl-, (3 beta); cholesta-7,14-diene; and cholesteryl formate. Bacterial ability to metabolize cholesterol into valuable intermediates offers industrial prospects for steroid production. This study emphasizes exploring isolate potential for sustainable solutions.

Keywords: cholesterol degradation, GC-MS, rhizosphere bacteria

1. Introduction

Cholesterol, a vital component of cell membranes and a precursor to steroid hormones and bile acids is ubiquitous and plays a crucial role in various physiological processes. However, excessive accumulation of cholesterol can lead to health complications such as atherosclerosis and cardiovascular diseases, highlighting the importance of cholesterol metabolism in both health and disease. The current understanding of the cholesterol degradation pathway remains limited in the scientific literature (Arima, Nagasawa, Bae, & Tamura, 1969). Microorganisms, particularly bacteria, have been recognized for their ability to degrade cholesterol, offering potential avenues for environmental remediation, biotechnological applications, and insights into microbial metabolism (Afful, Ling, Lin, Yang, & Addotey, 2021; Khiralla, 2015; Wali, Rehman, Umar, & Ahmad, 2019). Exploiting microorganisms, in conjunction with either isolated or engineered enzymes, offers an eco-friendly and sustainable approach to yield high-value compounds from natural sources, eliminating the necessity for traditional chemical synthesis.

The rhizosphere, the soil region influenced by plant roots, harbors a rich diversity of microorganisms with specialized metabolic capabilities. Rhizospheric micro organisms are essential in plant health, nutrient cycling, and soil ecosystem functioning. Many studies have explored the potential of rhizospheric bacteria for cholesterol degradation (García, Uhía, & Galán, 2012; Kumari & Kanwar, 2015). Medicinal plants, known for their diverse secondary metabolites and intricate root exudates, present an intriguing environment for the isolation of novel cholesterol-degrading

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microorganisms (Valentová, 2023). Microbial degradation of cholesterol yields various intermediates and principal products with significant applications in environmental, biotechnological, and medical fields. Cholestenone (cholest-4-en-3-one), formed through the oxidation of cholesterol, plays a crucial role in steroid biosynthesis (de las Heras, Perera, & Llorens, 2014). Androstenedione (AD) is essential for synthesizing anabolic steroids and hormone therapies, acting as a precursor for testosterone and estrone (Donova & Egorova, 2012). Additionally, *Nocardia* sp. converts cholesterol to 1,4-androstadiene-3,17-dione (Sharma, Slathia, Somal, & Mehta, 2012). In *Mycobacterium tuberculosis*, cytochrome P450 catalyzes C26-hydroxylation and initiates sterol side-chain degradation in *Rhodococcus jostii* RHA1 (Rosłoniec *et al.*, 2009).

The present investigation aims to isolate, identify, and characterize bacteria with the ability to degrade cholesterol efficiently. By identifying novel bacterial strains, researchers can expand the understanding of the mechanisms involved in cholesterol metabolism by identifying cholesterol-derived products using the GC-MS technique. Overall, the study of cholesterol degradation by novel bacteria serves multiple objectives, ranging from basic scientific inquiry to potential practical applications in environmental, biotechnological, and medical fields.

2. Materials and Methods

2.1 Isolation and screening of cholesterol degrading bacteria

2.1.1 Collection of soil samples

The five soil samples from the rhizosphere were collected using sterile gloves from diverse plant specimens including sugarcane (*Saccharum officinarum* L.), Ghritkumari (*Aloe vera* (L.) Burm. f.), lemongrass (*Cymbopogon citratus* (DC.) Stapf), tulsi (*Ocimum sanctum* L.), and onion (*Allium cepa* L.). Each sample was carefully transferred into sterile polythene bags and subsequently conveyed to the laboratory for analysis. Upon arrival at the laboratory, the samples were promptly refrigerated at a temperature of 4°C until required for further investigation.

2.1.2 Enrichment of the cholesterol degrading bacteria

The soil suspension was prepared with 1 g of soil in 10 ml of sterile distilled water in a clean test tube. For enrichment, 5 ml of soil suspension was aseptically inoculated into a 250 ml conical flask containing 100 ml sterile modified minimal medium (MMM) broth. The composition of MMM broth per 100 ml was as follows: KH_2PO_4 0.3 g, Na_2HPO_4 0.6 g, NaCl 0.5 g, NH_4Cl 0.2 g, $MgSO_4$ 0.01 g, cholesterol 0.1 g, 0.01% $FeSO_4$ 0.5 ml, 0.01% $CaCl_2$ 0.5 ml, pH 7.0 using 1N HCl/NaOH. Cholesterol served as the sole carbon source. Cholesterol and sodium dodecyl sulfate (SDS) were combined in a molar ratio of 1:1 for 30 min on a vortex mixer to enhance cholesterol solubility in water. The flask was incubated on a rotary shaker at room temperature (30°C ±2°C) for 48 to 72 h.

2.1.3 Isolation of cholesterol degrading bacteria

Following the incubation period, the enriched sample was employed for the isolation of cholesterol degrading bacteria. The enriched soil suspension was streaked onto modified minimal medium (MMM) agar plates, supplemented with 0.1% cholesterol, using the four-quadrant streak plate method. The plates were then incubated at room temperature (30°C ±2°C) for 24 to 48 h to obtain primary isolates.

2.1.4 Screening of cholesterol degrading bacteria

The primary isolates were further transferred on different sterile modified minimal medium (MMM) agar plates having 0.2% and 0.4% cholesterol concentration, and plates were incubated at 30°C (±2°C) for 24 to 48 h. Isolates growing at high cholesterol concentrations were selected as key isolates.

2.2 Detection of cholesterol degradation

After 4 days of incubation at 30°C (±2°C) in MMM broth containing 0.2% cholesterol on a shaker at 100 rpm, each key isolate was separated by centrifugation at 4,000 rpm for 15 min, and the respective supernatant was employed for the extraction and detection of cholesterol degrading potency of each key isolate by thin layer chromatography (TLC). The extract of the potent isolate was then used for the detection of degradation products by gas chromatography - mass spectrometry (GC-MS) analysis, after the identification of the potent isolate.

2.2.1 Thin layer chromatography (TLC)

The culture supernatant of each key isolate was extracted with chloroform at a supernatant to chloroform ratio 10:3.5. The extract was then concentrated by evaporation, and subsequently applied to silica gel plates. These plates were placed in a chromatography glass chamber containing a fresh solvent system of chloroform: benzene: ethyl acetate (1:3:6). After the development, the plates were dried, sprayed with 0.3 M sulphuric acid (H_2SO_4), followed by heating at 100°C until the appearance of spots (Saranya, Shekinah, Rajagopal, Vijayakumar, & Ponmanickam, 2014).

2.3 Identification of isolates

2.3.1 Morphological, physiological, and biochemical characterization

The selected isolate grown on sterile modified minimal medium (MMM) agar was examined for colony characters, cell morphology, Gram character, motility, and presence of endospores and capsules under a light microscope. Each isolate was inoculated into separate tubes of nutrient broth and incubated at various temperatures for 24 h to determine its optimal growth temperature. Similarly, the isolates were inoculated into nutrient broth tubes with different pH levels and incubated at 30°C (±2°C) for 24 h to

ascertain the optimum pH for growth. Turbidity, indicative of growth, was observed and quantified using a colorimeter at a wavelength of 520 nm post-incubation.

Biochemical characterization followed standard protocols outlined in Bergey's Manual of Determinative Biology (Bergey & Holt, 1993). Tests included IMViC (indole, methyl red, Voges-Proskauer, and citrate utilization), urea hydrolysis, oxidase, gelatin hydrolysis, nitrate reduction, hydrogen sulfide production, and carbohydrate utilization tests, particularly those of glucose and mannitol. Additionally, the analytical profile index (API) of each isolate was determined using an ID-64 GN card and automatic reader mini API instrument (Biomerieux VITEK® 2) according to the manufacturer's manual, aiding in further identification of the bacterial biochemical fingerprint.

2.3.2 16S rRNA gene sequencing

Genomic DNA extraction from the potent isolate was performed using Sigma's GenElute Bacterial Genomic DNA Kit. PCR was conducted using the primer combination 27F-1492R, known for amplifying a 1.5 kb fragment universal to eubacteria. The PCR mixture consisted of ddH_2O (12.0 µl), 10 X PCR buffer (2.0 µl), 200 µM dNTPs (2.0 µl), 2 pmol forward primer (0.4 µl), 2 pmol reverse primer (0.4 µl), 1 U Taq DNA Pol. (0.2 µl), and approximately 10 ng template DNA (3.0 µl). PCR products were subjected to cycle sequencing reaction using only the forward primer (27F). Subsequently, the samples were purified and loaded onto the sequencer (Avant 3100 Gene Analyzer) for further analysis. PCR was conducted using 27F and 1492R primers to amplify the 16S rRNA gene. The PCR conditions were: initial denaturation at 94°C for 3 min, followed by 32 cycles of denaturation at 94°C for 45 sec, annealing at 51°C for 1 min, extension at 72°C for 1.30 min, and final extension at 72°C for 10 min. PCR products were precipitated using PEG 6000, washed with 70% ethanol, and dissolved in Tris HCl (10 mM, pH 8.0). Sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Sequences were compared with EZTAXON, submitted to NCBI, and analyzed using BLAST tools for identification. Phylogenetic analysis was conducted using MEGA X software with the neighbor-joining method.

2.4 Gas chromatography - mass spectrometry (GC-MS)

Qualitative analysis of formed metabolites was done by using GC-MS analysis after 4 days of incubation. Cholesterol-degraded products from the supernatant were extracted with chloroform at a supernatant to chloroform ratio of 10:3.5. The supernatant was subjected to three sequential extractions with 20 ml, 10 ml, and 5 ml of chloroform, respectively by centrifugation at 4,000 rpm for 5 min at room temperature. The chloroform extract was then concentrated by evaporation and a 1 ml aliquot of concentrated sample was refluxed at 58°C, for 1 h with 15 ml of 95% ethanol and 3 to 4 drops of concentrated H_2SO_4 to derivatize cholesterol-derived products making them more amenable for GC-MS analysis. After completion of the reaction, the mixture was concentrated up to 1 ml in a boiling water bath. To this 5 ml of diethyl ether was added and mixed well with 5 ml $NaHCO_3$ and filtered to get a filtrate that was dried in a thermostat. The dried residue was then dissolved in 1 ml chloroform and transferred into a glass vial for GC-MS analysis.

The GC-MS analysis was conducted with the assistance of the Central Instrumentation Facility (CIF) at Savitribai Phule Pune University, Pune, using a Shimadzu TQ 8030 mass spectrometer with an ionization voltage of 70 eV. Gas chromatography utilized a Resteck column (0.25 mm × 30 mm; XTI 5) in temperature programming mode. The initial column temperature was 40°C for 4 min, followed by a linear increase at 10°C min^{-1} to 270°C, held for 4 min. The injection port temperature was set to 275°C, and the GC-MS interface was maintained at 300°C. Helium served as the carrier gas at a flow rate of 1 ml min^{-1}, with a total runtime of 30 min. Compound identification was accomplished through mass spectrum analysis and comparison with the NIST library integrated with GC-MS software. If the NIST search returned multiple hits, the structure with the highest confidence level was chosen based on the similarity score and key diagnostic ions specific to that structure (Makin, Honour, Shackleton, & Griffiths, 2010; Mali, 2014).

3. Results and Discussion

3.1 Isolation and screening of cholesterol degrading bacteria

Among the 24 primary isolates, four key isolates growing at a high cholesterol (0.4%) concentration were identified (Table 1), denoted by 1S, 2A, 3L, and 4T based on their respective plant origins: sugarcane (*Saccharum officinarum* L.), *Aloe vera* (L.) Burm.f., lemongrass (*Cymbopogon citratus* (DC.) Stapf), and tulsi (*Ocimum sanctum* L.).

TLC was utilized to detect cholesterol and its derivatives in chloroform-extracted samples from cultures grown in MMM broth with 0.2% cholesterol. Standard cholesterol (control) exhibited a violet-colored spot on the TLC plate (C). By comparing the developed spots of cholesterol-transforming products on TLC plates, cultures 1S, 2A, 3L, and 4T produced 6, 5, 4, and 2 spots, respectively (Figure 1). Notably, a faint spot of cholesterol was discerned in the TLC plate of the 2A culture, suggesting the highest level of degradation, compared to other cultures. Consequently, the 2A culture was identified as the most potent isolate, further confirmed by 16S rRNA gene sequencing and phylogenetic analysis, with its products identified via GC-MS analysis.

3.2 Identification of isolates

The key isolates were Gram-negative, rod-shaped organisms measuring 0.5-1 µm x 1.5-3 µm. They were non-spore-forming, observed individually or in pairs under the compound microscope. Colonies of cultures 1S and 2A developed bluish-green diffusible pigmentation on nutrient agar. All key isolates exhibited growth across a pH range of 5, 7, 9, and 11, with optimal growth at pH 7. Maximum growth occurred at 30°C (optimal) followed by 37°C. Microscopic, cultural, and biochemical characterization results are summarized in Table 2. According to Bergey's Manual of Determinative Biology, key isolates 1S and 2A were

Table 1. Isolation and screening of cholesterol degrading bacteria

Rhizosphere soil sample	No. of isolates in MMM (agar/broth)				Labeling to key isolates	Identification	Based on TLC	Identification
	0.1% Chl.	0.1% Chl.	0.2% Chl.	0.4% Chl.				
Saccharum officinarum L.	Enrichment in broth	6	3	1	1S	Morphological, physiological, biochemical analysis, API-determination	-	-
Aloe vera (L.) Burm. f.		5	2	1	2A		2A	16S rRNA gene sequencing and phylogenetic analysis
Cymbopogon citratus (DC.) Stapf		7	3	1	3L		-	-
Ocimum sanctum L.		3	1	1	4T		-	-
Allium cepa L.		3	1	-	-	-	-	-
Total no. of isolates		24[a]	10[b]	4[c]	4[c]	4[c]	1[d]	1[d]

Note: 'Chl.' = Cholesterol; 'a' = Pre-primary isolates; 'b' = Primary isolates; 'c' = Key isolates; 'd' = Potent isolate

Table 2. Microscopic, cultural, and biochemical characterization

S. No.	Biochemical test	1S	2A	3L	4T
1	Gram nature	-	-	-	-
2	Motility	+	+	+	-
3	Spore formation	-	-	-	-
4	Capsule formation	-	-	+	+
5	pH range (Optimum)	7-9	7-9	7-9	7-9
6	Temperature range (Optimum)	30°-37°C	30°-37°C	30°-37°C	30°-37°C
7	Indol test	-	-	-	+
8	Methyl red	x	x	-	-
9	Voges Proskauer test	x	x	+	+
10	Citrate test	-	-	+	+
11	Catalase	+	+	+	+
12	Oxidase	+	+	-	-
13	Gelatinase	+	+	-	-
14	Urease	-	-	-	+
15	H_2S	-	-	-	-
16	Nitrate reduction	+	+	+	x
17	Sugar utilization:				
	1. Glucose	+	+	+	+
	2. Mannitol	+	+	+	+

Note: '+' = positive; '-' = negative, and 'x' = not determined

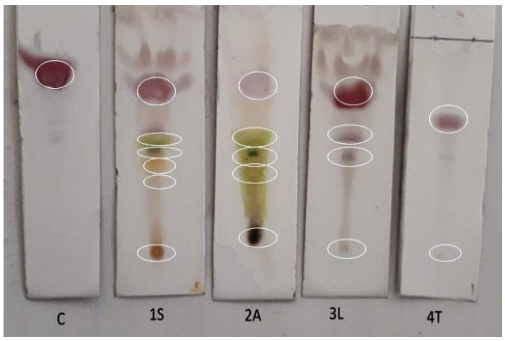

Figure 1. TLC of chloroform extracts derived from key isolates

identified as members of the genus *Pseudomonas*, based on their production of diffusible bluish-green pigment, ability to reduce nitrate, and positive oxidase test. Isolates 3L and 4T were identified as members of the genera *Enterobacter* and *Klebsiella*, respectively, based on their common traits of being oxidase-negative, catalase-positive, and their ability to ferment glucose with the production of acid and gas. *Klebsiella* species were further distinguished from *Enterobacter* species by their non-motile nature, indole production, and urease activity.

Following API analysis, key isolates 1S and 2A were identified as *Pseudomonas aeruginosa*, isolate 3L as *Enterobacter cloacae*, and isolate 4T as *Klebsiella oxytoca*. Results of the biochemical tests from API determination are detailed in Table 3.

The potent isolate (2A), identified as *Pseudomonas aeruginosa* through physiological, and biochemical tests, and confirmed by 16S rRNA gene sequencing, underwent phylogenetic analysis. Its gene sequence was compared with the GenBank database via BLAST, revealing close relatedness to *Pseudomonas aeruginosa* strain DSM50071. The 16S rRNA gene sequence was deposited in GenBank under accession number MK782058, and the isolate was designated as *Pseudomonas aeruginosa* strain Choltrans. The phylogenetic tree depicting the isolate's relationship is shown in Figure 2.

Table 3. Biochemical tests under API determination

Well	Test	Symbol	mg/well	Key isolates showing results			
				1S	2A	3L	4T
2	Ala-Phe-Pro-Arylamidase	APPA	0.0384	-	-	-	-
3	Adonitol	ADO	0.1875	-	-	-	+
4	L-Pyrrolydonyl Arylamidase	PyrA	0.018	-	-	-	+
5	L- Arabitol	lARL	0.3	-	-	-	+
7	D- Cellobiose	dCEL	0.3	-	-	+	+
9	β- Galactosidase	BGAL	0.036	-	-	+	+
10	H_2S Production	H2S	0.0024	-	-	-	-
11	β-N-Acetyl-Glucosaminidase	BNAG	0.0408	-	-	+	-
12	Glutamyl Arylamidase Pna	AGLTp	0.0324	+	+	-	+
13	D-Glucose	dGLU	0.3	+	+	+	+
14	Gamma-Glutamyl- Transferase	GGT	0.0228	+	+	-	-
15	Fermentation/ Glucose	OFF	0.45	-	-	+	+
17	β- Glucosidase	BGLU	0.036	-	-	+	+
18	D- Maltose	dMAL	0.3	-	-	+	+
19	D-Mannitol	dMAN	0.1875	+	+	+	+
20	D- Mannose	dMNE	0.3	+	+	+	+
21	β-Xylosidase	BXYL	0.0324	-	-	+	+
22	β- Alanine Arylamidase PNA	BAlap	0.0174	-	+	-	-
23	L-Proline Arylamidase	ProA	0.0234	+	+	-	-
26	Lipase	LIP	0.0192	-	-	-	-
27	Palatinose	PLE	0.3	-	-	+	+
29	Tyrosine Arylamidase	TyrA	0.0276	+	+	+	+
31	Urease	URE	0.15	-	-	+	+
32	D-Sorbitol	dSOR	0.1875	-	-	+	+
33	Saccharose/ Sucrose	SAC	0.3	-	-	+	+
34	D-Tagatose	dTAG	0.3	-	-	-	-
35	D- Trehalose	dTRE	0.3	+	-	+	+
36	Citrate (Sodium)	CIT	0.054	+	+	+	+
37	Malonate	MNT	0.15	+	+	-	+
39	5-Keto-D- Gluconate	5KG	0.3	-	-	-	+
40	L-Lactate Alkalinization	lLATk	0.15	+	+	+	+
41	Alpha-Glucosidase	AGLU	0.036	-	-	-	-
42	Succinate assimilation	SUCT	0.15	+	+	+	+
43	β-N-Acetyl Galactosaminidase	NAGA	0.0306	-	-	+	-
44	Alpha- Galactosidase	AGAL	0.036	-	-	+	-
45	Phosphatase	PHOS	0.0504	-	-	+	-
46	Glycine Arylamidase	Gly A	0.012	-	-	-	-
47	Ornithine Decarboxylase	ODC	0.3	-	-	+	-
48	Lysine Decarboxylase	LDC	0.15	-	-	-	+
53	L-Histidine Assimilation	lHlSa	0.087	-	-	-	-
56	Coumarate	CMT	0.126	+	+	-	-
57	β- Glucoronidase	BGUR	0.0378	-	-	-	-
58	O/129 resistance (comp. vibrio)	O129R	0.0105	+	+	+	+
59	Glu-Gly-Arg-Arylamidase	GGAA	0.0576	-	-	-	-
61	L- Malate Assimilation	lMLTa	0.042	+	+	-	-
62	Ellman	ELLM	0.03	-	-	-	-
64	L- Lactate assimilation	lLATa	0.186	+	+	-	-

Note: '+' = positive; '-' = negative

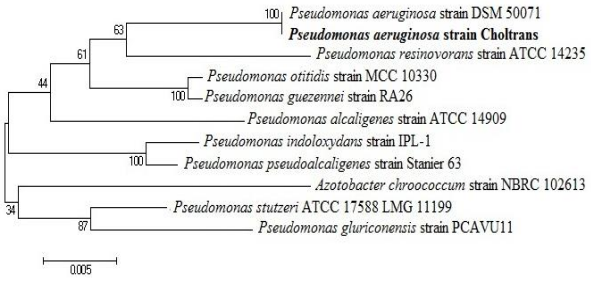

Figure 2. Phylogenetic tree of *Pseudomonas aeruginosa* strain Choltrans

3.3 GC-MS analysis

The GC-MS analysis of chloroform-extracted esterified samples unveiled six prominent cholesterol derived products attributed to *Pseudomonas aeruginosa* strain Choltrans, the most potent isolate in this study. Table 4 displays these products alongside their molecular formulae, molecular weights, and retention times (RT) in minutes. Figure 3 depicts a gas chromatogram featuring standard cholesterol and associated mass spectra, while Figure 4 showcases the mass spectra of the cholesterol-derived products. The X- and Y-axis titles for the mass spectra in Figures 3 and 4 are m/z (mass-to-charge ratio) and relative

Table 4. GC-MS profile of *Pseudomonas aeruginosa* strain Choltrans

S. No.	Name of compound	Molecular formula	M.W.	Retention time (RT) in min.
1	Cholesteryl formate	$C_{28}H_{46}O_2$	414	24.28
2	Cholesta-7, 14-diene	$C_{27}H_{44}$	368	24.67
3	Cholest-7en-3-ol, 14-methyl-, (3 beta)-	$C_{28}H_{48}O$	400	24.87
4	Cholesta-3,5-diene	$C_{27}H_{44}$	368	25.42
5	Cholest-5-ene, 3-methoxy-, (3 beta)-	$C_{28}H_{48}O$	400	25.43
6	Cholestane-3,5-diol,5-acetate,(3 beta, 5 alpha)-	$C_{29}H_{50}O_3$	446	25.70

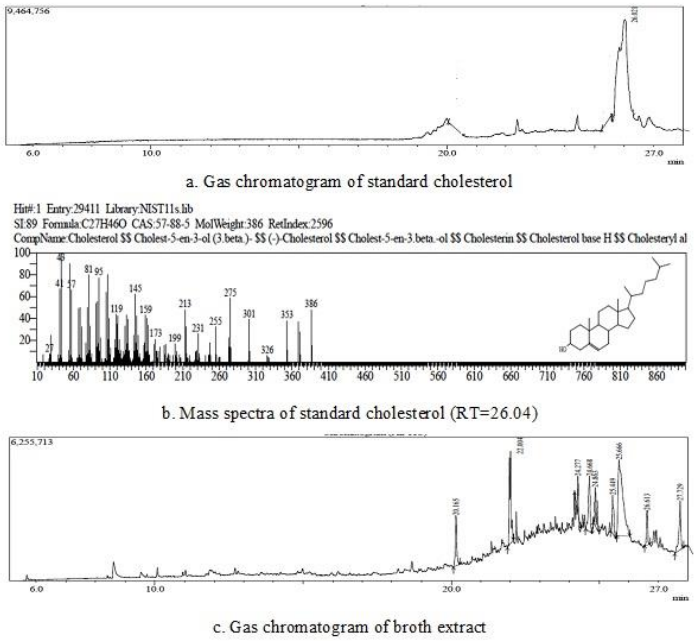

Figure 3. Gas chromatograms of standard cholesterol and broth extract

intensity, while the gas chromatogram in Figure 3 has retention time (minutes) and absorbance or abundance. Figure 5 represents cholesterol transformation reactions due to enzymatic reactions shown by *Pseudomonas aeruginosa* strain Choltrans.

All compounds such as cholesteryl formate (Synonym: cholest-5-en-3-ol (3β)-, formate); cholesta-7, 14-diene; cholest-7en-3-ol, 14-methyl-, (3 beta); cholesta-3,5-diene; cholest-5-ene, 3-methoxy-, (3 beta) and cholestane-3,5-diol,5-acetate, (3 beta, 5 alpha) (Synonym: 3-hydroxy cholestan-5-yl acetate) are derived from cholesterol and hold importance in the chemical synthesis of novel cholesterol derivatives. The bacterial biotransformation of cholesterol to cholesteryl formate presents a knowledge gap, with limited information available regarding the specific enzymes and bacteria involved in this process. It is conceivable that multiple enzymatic steps catalyzed by steroid-degrading bacteria are necessary, likely involving esterification enzymes capable of attaching a formate group to the cholesterol molecule. Further research is warranted to elucidate this aspect of bacteria-mediated cholesterol metabolism. Mycobacterium and Rhodococcus were found to transform cholesterol to cholesta-7,14-diene through the action of cholesterol oxidase and additional enzymes like dehydro genases or desaturases, according to Van der Geize *et al.* (2000a) and Najle, Nusblat, Nudel, & Uttaro (2013). Strains of *Rhodococcus, Mycobacterium, Nannocystis exedens*, and other steroid-degrading bacteria were observed to convert cholesterol to cholest-7en-3-ol by producing cholesterol oxidase enzymes, as reported by Bode *et. al.* (2003) and Wei, Yin & Welander (2016). Additionally, the conversion of cholesterol to cholest-7en-3-ol, 14-methyl-, (3 beta) likely entails the participation of methyltransferases, as highlighted by Horinouchi & Hayashi (2021). Chiang, Ismail, Müller, and Fuchs (2007) elucidated the role of steroid-degrading bacterial cholesterol oxidases in catalyzing the dehydration of cholesterol to cholesta-3,5-diene. The formation of cholera-3,5-diene from cholesterol occurred through dehydration, leading to the creation of a double bond at C-3 by the elimination of the hydroxyl group from C-3 and a hydrogen atom from C-4. The conversion of cholesterol to cholest-5-ene, 3-methoxy-,(3 beta) involves several key reactions including initial oxidation of the 3 beta -hydroxyl group of cholesterol to form a 3-keto intermediate (cholest-4-en-3-one), then reduction of 3-keto intermediate to form cholest-5-en-3-ol (desmosterol). The final step involves the methylation of the 3-hydroxyl group to form cholest-5-ene, 3-methoxy-, (3 beta). Enzymes, including cholesterol oxidase and steroid

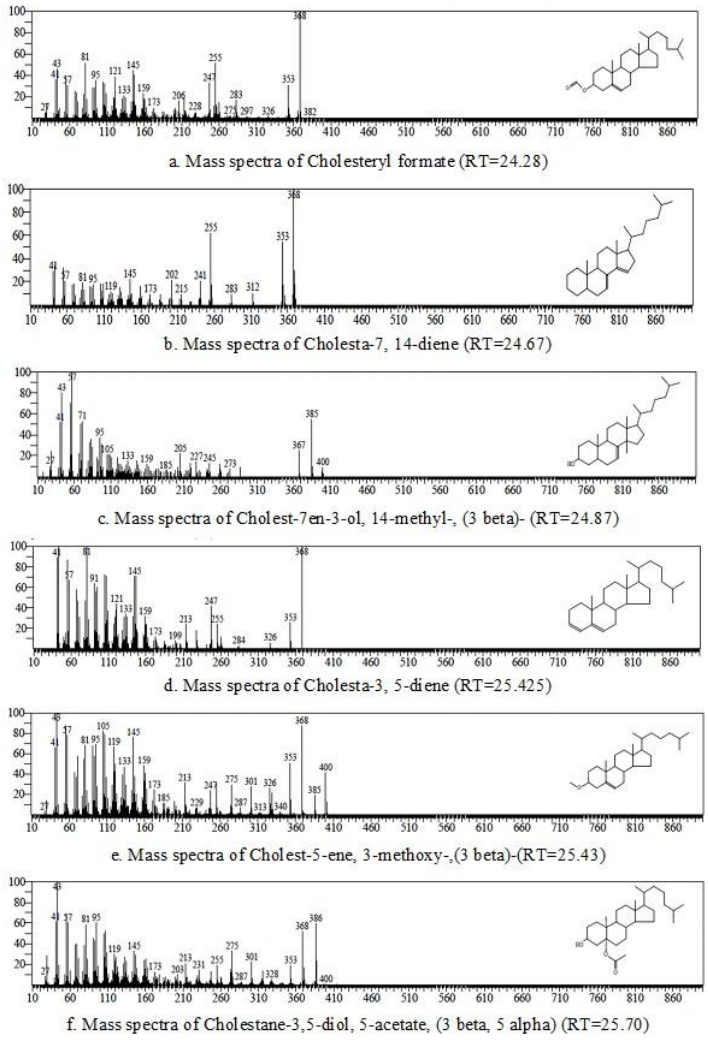

Figure 4. Mass spectra of cholesterol derived products

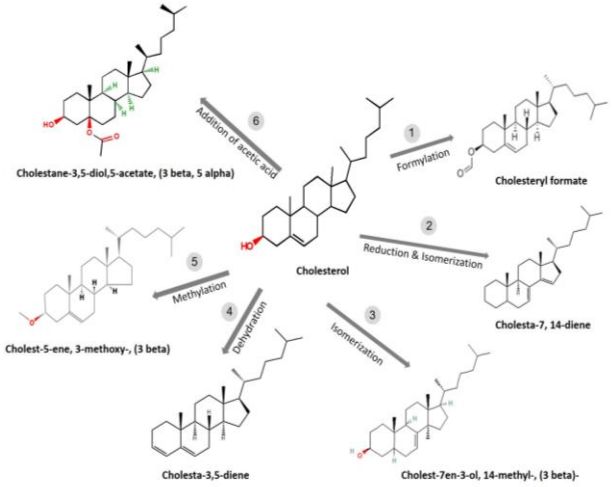

Figure 5. Cholesterol transformation reactions

methyltransferase, produced by steroid-degrading bacteria such as *Brevibacterium, Micrococcus, Mycobacterium,* and *Rhodococcus* mediate this transformation (Brown, Olagunju, Giner, & Welander, 2023; Horinouchi & Hayashi, 2021; Kumari & Kanwar, 2012; Nakakuni, Yamasaki, Yoshitake, Takehara, & Yamamoto, 2019; Pollegioni, Piubelli, & Molla, 2009; Yamada *et al.*, 2019). The biotransformation of cholesterol to cholestane-3,5-diol, 5-acetate, (3 beta, 5 alpha) constitutes a hydroxylation process, wherein hydroxyl groups (-OH) are incorporated into the cholesterol molecule by either a cholesterol oxidase or a steroid hydrolase. This kind of conversion is facilitated by steroid-degrading bacteria such as *Mycobacterium, Rhodococcus,* and *Sterolibacterium,* as documented by Van der Geize *et al.* (2000a, 2007b). In this study, we hypothesize that cholesterol-degrading *Pseudomonas aeruginosa* strain Choltrans might produce analogous metabolites by employing similar enzymatic machinery.

However, the bacterial biotransformation of cholesterol to cholesteryl formate presents a knowledge gap,

with limited information available regarding the specific enzymes and bacteria involved in this process. It is conceivable that multiple enzymatic steps catalyzed by steroid-degrading bacteria are necessary, likely involving esterification enzymes capable of attaching a formate group to the cholesterol molecule. Further research is warranted to elucidate this aspect of bacteria-mediated cholesterol metabolism.

The diverse array of cholesterol-derived compounds detected in our study holds significant promise for various pharmaceutical applications. Cholesteryl formate, in particular, emerges as a versatile precursor with potential roles in drug synthesis, lipid-based drug delivery systems, diagnostic agents, and as a tool for investigating cholesterol metabolism and related diseases. Further exploration into its therapeutic potential and utilization in drug discovery and development is warranted, as supported by previous studies (Markovic, Ben-Shabat, Aponick, Zimmermann, & Dahan, 2020; Misiak, Markiewicz, Szymczuk, & Wilczewska, 2020). Similarly, compounds such as cholesta-7,14-diene (desmosterol) and cholest-7en-3-ol, 14-methyl-,(3 beta) (lanosterol) offer promising avenues in pharmaceutical research due to their roles as cholesterol biosynthesis precursors and potential therapeutic targets. Investigations into their pharmacological properties, including anti-inflammatory, neuroprotective, and antioxidant effects, highlight their potential applications in the development of novel therapeutics for various diseases (Chan, Spike, Trowbridge, & Schroepfer, 1979). Moreover, structural modifications of cholest-5-ene, 3-methoxy-,(3 beta)- and its derivatives present opportunities for drug development, particularly in cancer therapy, warranting further mechanistic and clinical investigations (Dembitsky, 2023; Gupta, Sathish Kumar, & Negi, 2013; Jin, Tan, Wu, & Ren, 2023).

Steroidal compounds like cholestane-3,5-diol, 5-acetate, (3 beta, 5 alpha) exhibit anti-inflammatory and neuroprotective properties, making them potential candidates for the development of drugs targeting inflammatory and neurodegenerative diseases (Hu *et al.*, 2014; Zhan *et al.*, 2023). Compounds such as cholesta-3,5-diene and cholesta-7,14-diene have been reported as biomarkers for disorders of cholesterol metabolism and as intermediates in the synthesis of steroidal hormones (Albuquerque, Santos, & Silva, 2019; Al-Hassan *et al.*, 2020). Compounds identified in our study may have similar potential applications as those mentioned by previous researchers. In conclusion, our study enhances the current understanding of bacterial cholesterol metabolism and its implications. Further research is needed to elucidate the enzymatic pathways responsible for cholesterol transformation by the studied bacterium and to investigate the physiological effects of cholesterol-derived products on host health and disease.

4. Conclusions

In this investigation, we delved into the biosynthesis of cholesterol-derived metabolites by *Pseudomonas aeruginosa* strain Choltrans, isolated from the rhizosphere of *Aloe vera* (L.) Burm. f.. Our focus encompassed a range of compounds, including cholestane-3,5-diol, 5-acetate, (3 beta, 5 alpha); cholest-5-ene, 3-methoxy-,(3 beta); cholesta-3,5-diene; Cholest-7en-3-ol, 14-methyl-,(3 beta); cholesta-7,14-diene and cholesteryl formate. Remarkably, these compounds were collectively explored within a single bacterial culture for the first time, showcasing the degradative capabilities of *Pseudomonas aeruginosa* strain Choltrans on cholesterol-derived substrates. Prior to this investigation, individual reports documented the production of these compounds by diverse bacteria under disparate culture conditions. Our findings underscore the versatile metabolic repertoire of the bacterium under the present studies in converting cholesterol into chemically diverse metabolites through intricate enzymatic processes. Future inquiries are warranted to unveil the specific enzymatic pathways governing cholesterol transformation by bacteria and to decipher the physiological implications of cholesterol-derived products on host health and disease. Moreover, exploration into the potential application of these metabolites in steroidal hormone synthesis presents promising avenues for further investigation.

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