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Cholesterol Biotransformation to produce Cholest-4-ene-3, 6-dione and Cholest-4-en-3-one Employing Organic Solvents: Focus on *Burkholderia cepacia*

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Abstract

Cholesterol biotransformation is a very crucial process industrially, as it guarantees the production of high medically/clinically valued products in fewer uncomplicated steps, compared with the classical or traditional synthesis. The biotransformation of cholesterol also yields a wide range of products that can be useful in many ways, even in the pharmaceutical industries. The enzymes from the microbes used to facilitate this transformation may have the capacity to be reused. This paper focuses on the use of *B. cepacia* to produce two main products; cholest-4-en-3-one and cholest-4-ene-3,6-dione. This paper also centers attention on the use of organic solvents and gives recommendations for the development of bioreactors to give room for even highly useful but harmful organic solvents to be employed without harmful consequences to the environment or the users.

Keywords: Cholest-4-en-3-one, organic solvents, *Burkholderia cepacia*, Cholesterol transformation, sterols.

Introduction

1.1 Structure and function of sterols

Sterols are steroids with a hydroxyl group attached to the four-ring core, and represent the most generally abundantwhich occur in all tissues of animals, green plants, fungi such as yeasts and may also be produced by certain bacteria. Steroids generally function as membrane components and also are cell signaling molecules, mainly hormones. Their molecules are derived from gonane by substituting a hydroxyl group in position 3 for a hydrogen atom. They are grouped into two main categories according to their source, whether from plants, called phytosterols or from animals, zoosterols. They comprise the common 3-monohydroxy steroids of the cholestane, ergostane, and stigmastane chains and corresponding methyl sterol biogenetic precursors: lanosterol, cycloartenol, and specific derivatives of these sterols, such as lophenol. Sterols such as cholesterol, which is the most common type is key to cell membrane structure,

cell growth and proliferation, secondary messenger in developmental signaling, occurring in cellular communication, general metabolism and plays a major role as a precursor to steroid hormones, bile acids and fat-soluble vitamins. Cholesterol, which is the major sterol in animals, is synthesized by animal cells. It is an amphipathic molecule, that is; it has polar (- OH) portion and a hydrophobic backbone, and plays a key role in membrane fluidity, cell growth and proliferation [1], [2]. Steroid hormones are known to regulate signal transduction pathways and influence various aspects of cell proliferation and tissue differentiation by binding to intracellular receptors that function as transcription factors to modulate specific gene expression. Steroidal compounds are widely used in pharmaceutical preparations as anti-inflammatory, diuretics, immunosuppressive, contraceptive and anticancer drugs, as well as in other applications [3]–[5]. Sterols are usually constituents of human oils [6], [7]. Phytosterols, for example, have been licensed by the US FDA for use as a food supplement after clinical trials showed that phytosterols have the ability to inhibit cholesterol absorption sites in the human intestine [8].

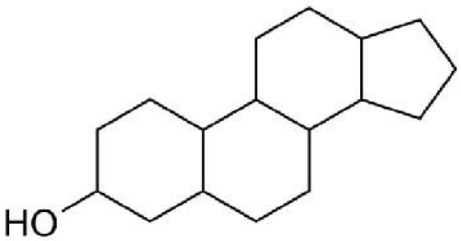


Figure 0-I

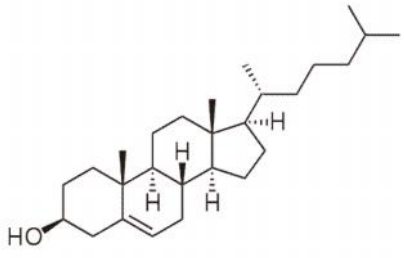


Figure 0-II

1. The structure of sterols comprising of a hydroxyl group attached to the steroidal four-ring core. The physiological activity of steroids depends on their structure, i.e., the type, number and regio- and stereo position of the functional groups attached the steroid core; the oxidation of the rings.

2. Graphical representation of a cholesterol molecule.

1.2 Research progress of the bioconversion of Sterols

The cumbersome and costly nature of classical methods of transformation has left the research industry more focused rather on the microbial transformation, which has rather proven more efficient. Attention to this research field was drawn by the advances made in genetic and metabolic engineering, whole cell biocatalysis in non-schematic media and process monitoring. The biotransformation of cholesterol yields a wide range of very useful products especially in the pharmaceutical industry, some of which have gained tremendous attraction in the research mainstream. Ongoing research is focused at finding novel biocatalysts and the development of biotechnological methods to exploit existing biocatalysts and microbial transformation reactions. Several microorganisms are capable of transforming cholesterol to various products yet the major pitfall has been low bioavailability of cholesterol which is due to the poor solubility of cholesterol in aqueous media. The use of surface-active agents such as Tween 80 or Triton X-100 and other sterol-solubilizing agents such as cyclodextrins have been deployed. However, the tendency of these surfactants to cause forming can be venomous to the microorganisms even at lower concentrations [9][10]. The use of lecithin in cholesterol biotransformation was reported by Wang et. al. to have improved both the productivity and product yield. The biotransformation with lecithin led to a maximum productivity of 0.127 g/L/day and maximum product yield of 59% (w/w), both higher than the productivity of 0.084 g/L/day and the product yield of 38% (w/w) obtained using Tween 80 as surfactant [10]. Compared with Tween 80, lecithin was more effective in dispersing cholesterol in water by forming micelles with the sterol [9]. However, the use of lecithin only improved yield from 38% for Tween 80 to 59% which is though significant yet leave room for more improvement. Some are achieved by the use of whole cells as biocatalysts, but the toxicity of organic solvents to the microorganisms tend to envenom the whole cell bioconversion. In order to remedy this shortfall, a biphasic aqueous/organic medium has been employed. However, bioconversion is more like to take place at the interface and with whole cells, the cell, being a carrier of the enzyme, which is the real activator seems to occupy a larger part of the interface, reducing the availability of

the substrate to the enzyme. This in turn causes lower bioconversion rate consequently leading to lower yield of products. Notwithstanding, some methods use surfactants and various metal ions to improve enzyme activity but this does not resolve the issue with substrate accessibility. In order to solve this problem, the use of enzyme solution has been evaluated and is very efficient. However, this method calls for cumbersome analysis of the enzyme responsible for the bioconversion and may not be cost effective. The use of 0microorganisms that are strong enough to withstand the organic solvent environment has been proposed, to enable the bioconversion to efficiently proceed in a single phase. This research centralizes focus on cholest-4-en-3-one and cholest-4-ene-3,6-dione.

2. Bioconversion products: cholest-4-ene-3,6-dione and cholest-4-en-3-one

2.1 Structural identification of Sterol conversion products

Organic compounds may be confirmed by mass spectrometry, nuclear magnetic resonance spectroscopy, infrared spectroscopy and ultraviolet absorption spectroscopy. Mass spectrum can determine the relative molecular mass of the carbide and infer the compound by observing the ions with different qualities. In the process of actual determination of the structure of compounds, it is usually used with high performance liquid chromatography – mass spectrometry (LC – MS) [11]. Infrared Spectroscopy (IR) plays an important role in the structural analysis of compounds.The role of the functional groups can be determined according to the position, shape and number of the sample absorption spectrum band, whether or not some functional group exists in the molecule [12]. In conjunction with other spectral methods, it can be used to judge the structure of a compound.

Nuclear magnetic resonance carbon spectroscopy (13C-NMR) and NMR (1H-NMR) are powerful tools for molecular structure determination of organic compounds. The two complement each other to provide structural information about the type, number, interconnection mode and surrounding chemical environment of hydrogen and carbon atoms in molecules [13].

When studying the structure of organic compounds, ultraviolet spectra are mainly used to provide molecular aromatic structures and conjugate systems. Because of the different structure of the compound, the wavelength range and absorption strength of the absorption band are different. Therefore, the measured value of UV spectra and maximum absorption wavelength (λ max) can be compared with the structural information of known compounds.

To further confirm the product structure, the data obtained can be compared with the relevant literature from the National Institute of Advanced Industrial Science library.

2.2 Cholest-4-ene-3,6-dione (CEDO)

2.2.1 Structure, functions and identification of cholest-4-en-3,6-dione

Cholest-4-en-3,6-dione is a derivative of cholesterol. It is solid, light yellow and has the same maternal structure as the C-17 and is indicated as a precursor for pharmaceutical preparations against colon cancer, obesity, diabetes, rheumatoid arthritis, hypertension, asthma, eczema, inflammation, metabolic disorders, neurodegenerative elderly diseases, central nervous system disorders, gynecopathy, anaphylactic shock. They are indicated as replacement agents in the treatment of adrenal insufficiencies, in the inhibition of HIV integrase, prevention and treatment of HIV infections and treatment of declared AIDS. They also play a role in cholesterol control, as well as cardiovascular and neuroprotective functions. [14]disclosed the application of Cholest-4-ene-3,6-dione in the preparation of drugs for the treatment or prevention of neuron damage. The authors selected different neuron damage models for experiments, and found that CEDO can reduce the oxidative stress damage of hippocampal neurons caused by glutamate, and can reduce the apoptosis damage of small brain granule neurons caused by potassium deprivation. At the same time, Cholest-4-ene-3,6-dione can be used in the brain of stroke rats. In the middle artery occlusion model, it can significantly reduce the infarct volume and has the potential to develop neuroprotective agents with multiple mechanisms.

Cholest-4-ene-3,6-dione has excellent physiological effects such as anti-obesity and improvement of lipid metabolism, which can be used in the treatment of obesity. It was reported by [15] that CEDO has antitumor activity.

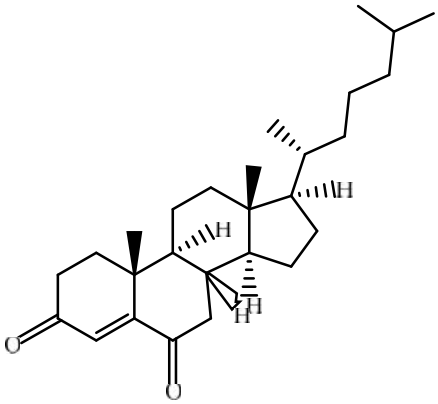


Figure 0-I: Graphical representation of Cholest-4-ene-3,6-dione

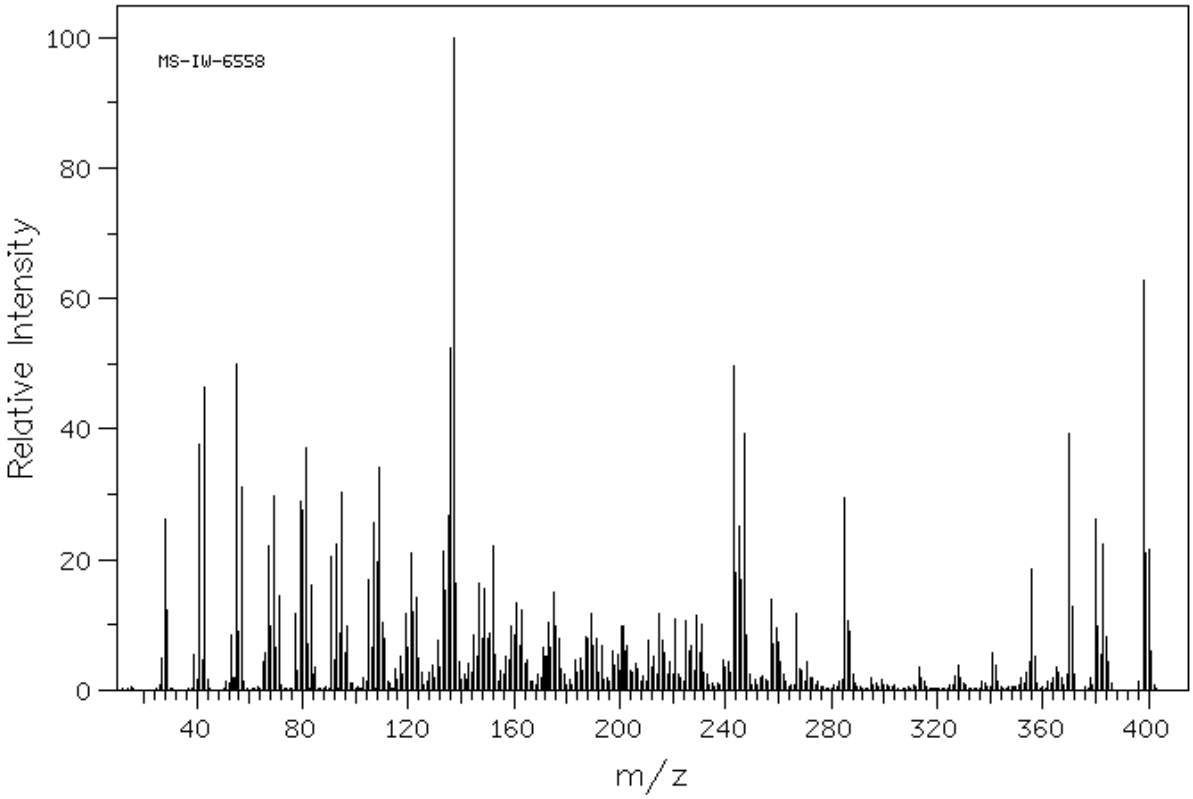


Figure 0-II: The MS spectra of cholest-4-3,6-dione (Adapted from Spectral Database for Organic Compounds, SDBS). Molecular weight: 398.6

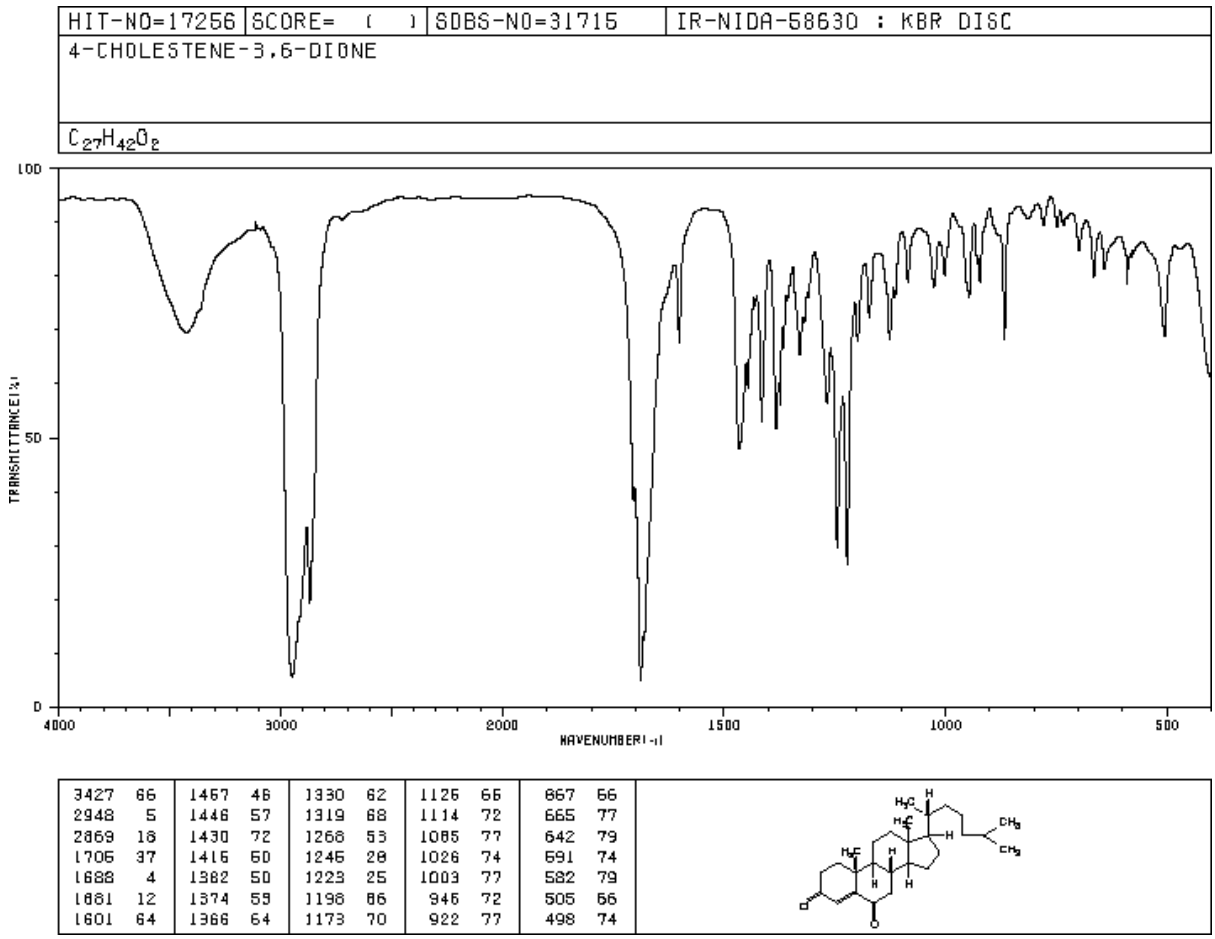


Figure 0-III: The IR spectra and data for cholest-4-en-3,6-dione (Adapted from Spectral Database for Organic Compounds SDBS)

2.2.2 Synthesis of Cholest-4-ene-3,6-dione

2.2.2.1 Chemical synthesis

Cholest-4-ene-3,6-dione has been identified to occur naturally and as a product of the autoxidation of cholesterol [16], [17]. [18] discovered and reported the identification of the compound in marine sponge *Geodia cydonium.* At present, Cholest-4-ene-3,6-dione is mainly synthesized from cholesterol by chemical method. [19] and [20] dissolved cholesterol (2 g) in acetone (250 ml), cooled to 0 ° C, and then added Jones reagent drop by drop until a lasting orange color was obtained. After stirring the reaction mixture at 0 ° C for 1-2 h, the reaction system was heated to room temperature. The reaction mixture was quenched with methanol, concentrated in vacuum, separated and purified, and Cholest-4-ene-3,6-dione was obtained in 86% yield. [21] proposed that cholesterol (5 g) be dissolved in anhydrous dichloromethane (50 ml) and pyridine chlorochromate (8.34 g), the mixture was stirred at room temperature for 3 days, pyridine chlorochromate (4.2 g) was added again, and then stirred at room temperature for another day, 150 ml of anhydrous ether was added for extraction, the upper organic phase was concentrated, separated and purified, and Cholest-4-ene-3,6-dione was finally obtained in 72% yield; [22] and others obtained Cholest-4-ene-3,6-dione through the oxidation of chromium trioxide pyridine and dichloromethane.

2.2.2.2 Biological synthesis

At present, there are few reports about microbial conversion of cholesterol to Cholest-4-ene-3,6-dione. [23] obtained Cholest-4-ene-3,6-dione in 16% conversion yield by cholesterol oxidase from *Pseudomonas fluorescens*. The transformation process is as follows: cholesterol oxidase first catalyzes cholesterol to sterol-5-en-3-one, then C-6 position is oxidized, and Δ 5 isomerization to Δ 4, resulting in 6 β - hydroperoxycholesteryl-4-en-3-one, which is an unstable compound.

With the extension of conversion time, it is converted to Cholest-4-ene-3,6-dione. The cholesterol oxidase from Pseudomonas sp. st-200 reported by [24] also experienced the above catalytic process to obtain Cholest-4-ene-3,6-dione. In the recovered catalytic products. In the recovered catalytic products. The weight percent of Cholest-4-ene-3,6-dione was 19.23%.

[25] pre dissolved cholesterol in the mixed organic solvent of p-xylene and alkane, added the organic solvent to the transformation system of Pseudomonas sp. at a volume ratio of 10%, converted at 30 for 7 days, and recovered the transformation products. Since Cholest-4-ene-3,6-dione was not separated from cholesten and cholest-4-en-3-one, the yield was 38%.

[26] and [27] reported the genus Rhodococcus (Rhodococcus sp.), Bordetella sp., arthrobacteria. Cholest-4-en-3-one is also the product of cholesterol transformation by *Arthrobacter simplex*, *Mycobacterium neoaurum* jc-12, etc.

2.3 Cholest-4-en-3-one

2.3.1 Structure, functions and identification of Cholest-4-en-3-one

Cholest-4-en-3-one is a valuable synthetic intermediate in sundry steroid transformations. Antecedent findings have revealed that it is potent against obesity, liver disease, and keratinization. Assessment of the functional effect of cholest-4-en-3-one in human cells by [28] unraveled that cholest-4-en-3-one generated by cholesterol oxidation repressed cell migration. Furthermore, cholest-4-en-3-one can serve as a precursor for the synthesis of other drug intermediates, such as androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione, which are significant starting materials for the synthesis of anabolic drugs and contraceptive hormones[29]. A developing novel drug, Olesoxime (cholest-4-en-3-one, oxime) which is its derivative, shows neuroprotective characteristics and medicinal properties in the treatment of spinal muscular atrophy.

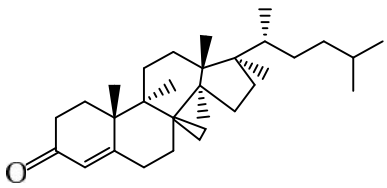


Figure 0-IV: Graphical structure of Cholest-4-en-3-one

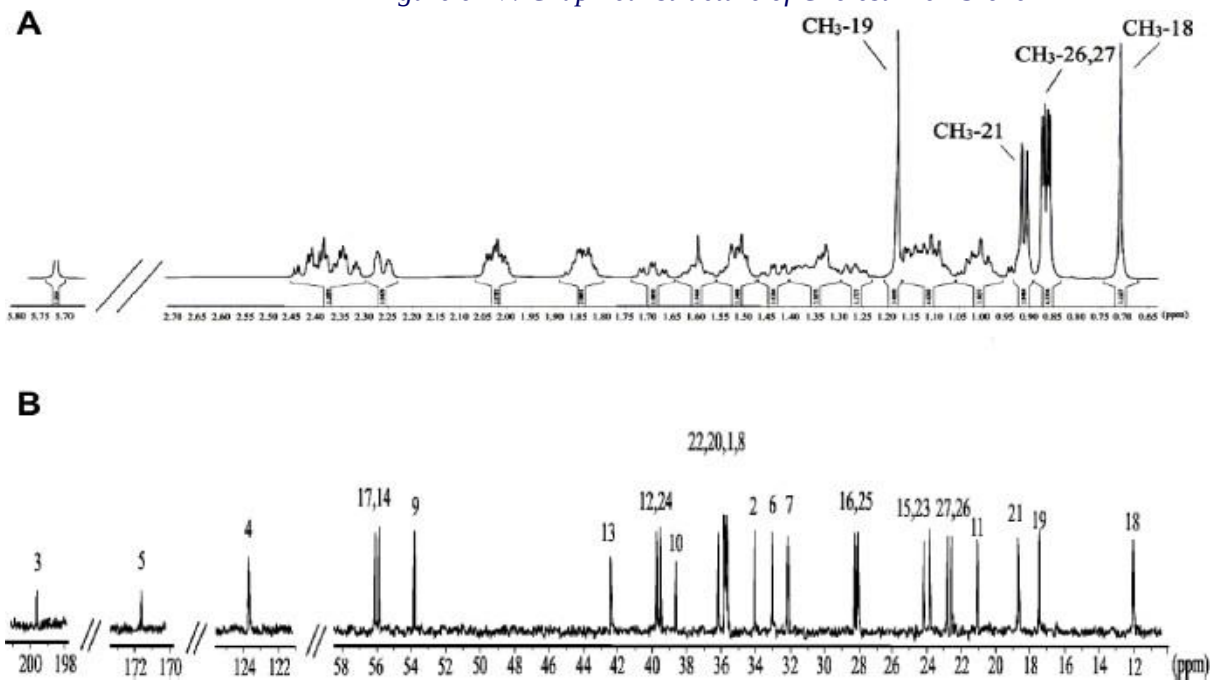


Figure 0-V: *1H-NMR (A) and 13C-NMR (B) of end product. The NMR spectra were recorded in CDCI3 as a solvent with TMS as internal standard. The number represents the carbon atom number in the nomenclature of choIest-4-en-3-one; the five methyl groups can be seen clearly in the 1H-NMR spectrum (A) and the 27 carbon atoms of cholest-4-en-3-one are also clearly shown in the 13C-NMR spectrum (B).*[30]

Table 0-I: The 13C chemical signals for the product (δ in ppm downfield from tetramethylsilane, in chloroform deuteride).

CARBON ATOM NO.	CHEMICAL SHIFT (PPM)a	CARBON ATOM NO.	CHEMICAL SHIFT (PPM)a
1	35.71	15	24.18
2	33.99	16	28.17
3	199.59	17	56.13
4	123.75	18	11.95
5	171.62	19	17.39
6	32.96	20	33.75
7	32.07	21	18.64
8	35.65	22	36.13
9	53.83	23	23.82
10	38.61	24	39.50
11	21.04	25	28.01
12	39.64	26	22.55
13	42.41	27	22.80
14	55.89		

(a) The chemical shifts data for cholest-4-en-3-one.Note: These chemical shifts data are in accordance with the spectral database for organic compounds (SDBS number 15235).

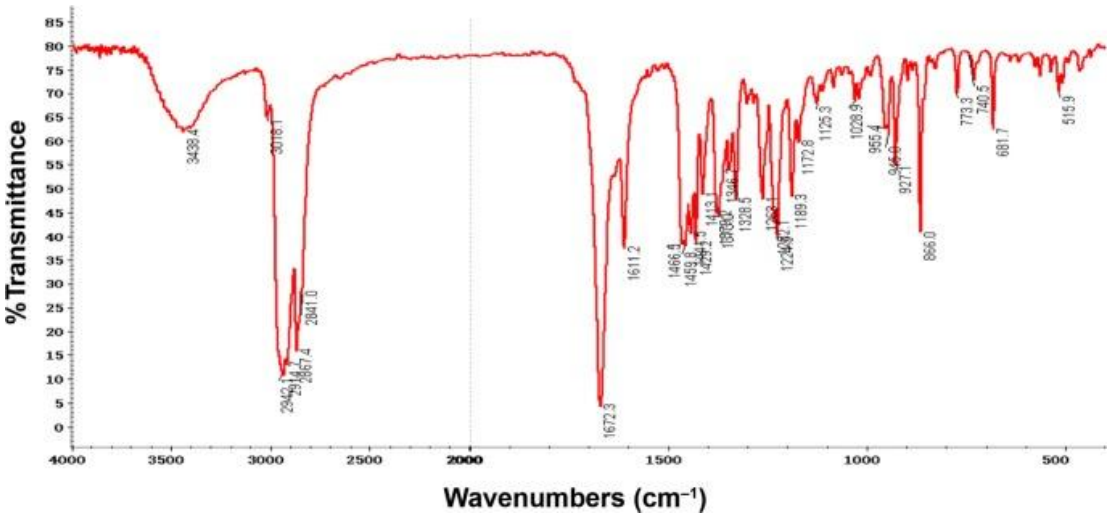
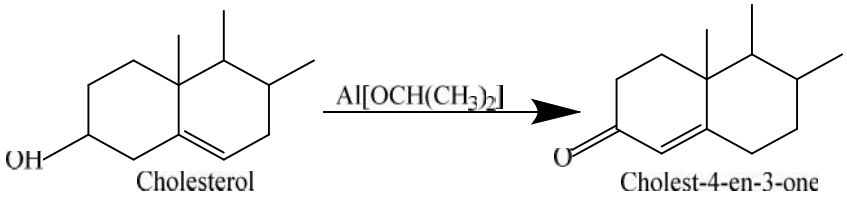


Figure 0-VI: *IR spectrum of Cholest-4-en-3-one (KBr disk)as recorded on an FT-IR spectrometer. The spectrum is identical with the standard IR spectrum of cholest-4-en-3-one in the Sadtler Standard Infrared Grating Spectra database.*[30]

2.3.2 Synthesis of Cholest-4-en-3-one

2.3.2.1 Chemical synthesis

Cholest-4-en-3-one may be synthesized chemically by methods such as the Oppenauer oxidation. Oppenauer Oxidation is the involves the conversion of secondary alcohols to their corresponding ketones by selective oxidation. The reaction is usually catalyzed by an aluminium alkoxide, accompanied by heat proceeding by oxidizing the secondary alcohol to its akin ketone. The hydroxyl group attached to the cholesterol is a secondary alcohol and therefore under the catalysis of an aluminium alkoxide accompanied by heat, will undergo oxidation to its corresponding ketone, producing cholest-4-en-3-one.



Equation 0- 1

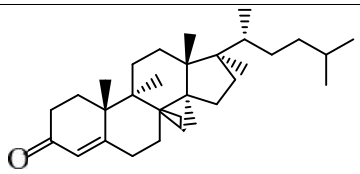
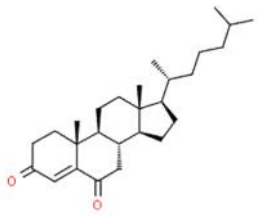
This can be problematic as high temperatures are employed, large quantities of ketone hydride acceptors and aldol condensation products produced with the hydride acceptors. The focus of research to develop more efficient Oppenauer-type oxidations that may proceed under milder condition have proven quite successful, with diisopropoxyaluminum trifluoroacetate efficiently catalyzing the oxidation of a variety of secondary alcohols in benzene at room temperature using 4-nitrobenzaldehyde as hydride acceptor. The complexity of these reactions, the use of harmful solvents such as benzene and the need for several steps in order to yield cholest-4-en-3-one accompanied with the long periods of time these processes take, necessitates the focus on new ways of achieving cholesterol oxidation to cholest-4-ene-3-one.

2.3.2.2 Biological synthesis

As at now, there are various reports on the increasing research on the biological synthesis of cholest-4-en-3-one. Numerous microorganisms such as Enterobacter, Arthrobacter, Mycobacterium and Gordonia are suitable for the transformation of cholesterol to cholest-4-en-3-one[26], [31]–[33]. The biotransformation of cholesterol has been achieved by various methods such as, the use of whole cells with the addition of metal ions and surfactants to ameliorate the activity of enzyme. The use of enhanced transformation conditions and the expression of cholesterol oxidase (COD) gene to improve bioconversion of steroids have achieved commendatory results in improve the activity of the enzyme.

Equation 0- 2 $\quad$ *Cholesterol* + $O_2 \xrightarrow{COD}$ *Cholest-4-en-3-one* + H_2O_2

Table 0-II.1 Comparison of cholest-4-en-3-one and cholest-4-ene-3,6-dione

Name	Chemical structure	Substrate action site	Microorganisms that can be used to transform	References
Cholest-4-en-3-one (C4EO)		Substrate cholesterol C3 hydroxyl oxidized to ketone group; Δ5 isomerization to Δ4	*Brevibacterium* sp.	[34]
			Rhodococcus sp.	[35]
			Arthrobacter simplex	[36]
			Bordatella sp.	[26]
			Pseudomonas sp.	[37]
			Mycobacterium	[27]
Cholest-4-ene-3,6-dione		Substrate cholesterol C3 hydroxyl, C6 Oxidized to ketone; Δ5 isomerization to Δ4	*Pseudomonas* sp.	[25]
			Pseudomonas fluorescens	[23]

3. Application of organic solvents and *Burkholderia cepacia* in transformation of sterols

There is no doubt about the significant progress made in the development of effective biocatalysts to enhance sterol microbial transformation, however, there is still a high demand to continue to search for cost-effective and economical biotechnologies to prompt invaluable products/derivatives[38]. As discussed earlier, one of the major problems of the microbial conversion sterols is low sterol solubility in aqueous media (between 1-100µM)[39]. Low solubility occasions poor availability of substrate to whole-cell biocatalysts, reduced cell dispergation; it is mostly also a reason for foaming at scaled fermentations. Attempts made to enhance bioconversions were based on micronization of substrate particles, surfactant-facilitated emulsification, application of two-phase systems with organic solvents, liquid polymers, or cyclodextrins, as well as combinations of different methods. There are other setbacks in microbial conversion technologies such as the inhibition effect, or even toxicity of steroid substrate/product for microbial cells in some cases, as well as undesirable degradation of steroid product by whole cells. The most efficient approaches used to solve these problems include selection of strains tolerant to high concentrations of toxic steroid product and in situ product recovery [40].

3.1 Application of organic solvents to improve sterol bioconversion

Sterol compounds being lipophilic, have better solubility in organic solvents. Therefore, in order to enhance the availability of sterols in a conversion system, sterol compounds can be pre-dissolved in minute volumes of organic solvents and then added to the conversion system to take part in the reaction process.

The organic solvent used to dissolve the substrate sterol and the transformation medium solution form a two-phase system. The microbial transformation of sterol is carried out at the interface of the two phases or in the aqueous phase. In the two-phase reaction system, the organic solvent can act as a repository for the substrate, increase the solubility of the sterol in the reaction system, distribute the substrate in aqueous

and non-aqueous media through the slow controlled release [37], increase the dissolution rate of substrate, improves contact efficiency between microorganism and substrate, and then promotes the forward progress of the transformation reaction; at the same time, the organic solvent can be used as the extractant of the conversion product and the feedback inhibition of the product can be reduced or even relieved.

Cholesterol can also be effectively transformed by dissolving in mixed organic solvents, as demonstrated by Rikizo Aono et. al[41], who dissolved cholesterol in p-xylene and diphenylmethane, (3:7, V/V) using Pseudomonas sp. ST-200 strain via dehydrogenation and oxidation; added mixed organic solvents containing cholesterol (final concentration of 2mg/ml) to the transformation system and the mixed organic solvents accounted for 10% of the total volume of the transformation system. After 8 days of culture, the final concentration of cholesterol was 0.04mg/ml, that is, 98% of cholesterol was transformed [25]. Malaviya and Gomes developed a chloroform microemulsion which allowed a maximum sitosterol solubility of 8g/L, and 4-Androsten-3,17-dione yield of 0.45g/L [42].

[30] performed enzymatic conversion from cholesterol to cholest-4-en-3-one by COD solution in an aqueous/organic biphasic system in a 0.5 L rotary shaking flask or 5 L fermenter, with reaction conditions as follows: 1 g cholesterol, 130 mL aqueous/organic solvent mixture (10:3, v/v), 0.5 L flask, 250 rpm, 30°C, 3 hours.

Table 0-I: *Comparison of bioconversion performance using different organic solvents. (Adapted from* [30])

Organic solvents	Conversion[a] (%)	Residual enzyme activity[b] (%)	Productivity ($g.L^{-1}\cdot h^{-1}$)
Glycerin	13.5	83.5 ± 4.1	0.45
Ethyl acetate	16.3	82.4 ± 3.8	0.54
Isoamyl alcohol	45.2	81.2 ± 3.2	1.51
Butyl acetate	47.6	85.7 ± 4.5	1.59
Petroleum ether	61.2	72.3 ± 3.4	2.04
Hexane	76.5	69.1 ± 3.1	2.55
Decane	87.3	64.4 ± 3.3	2.91
Heptadecane	88.7	63.3 ± 3.5	2.96

(a) The conversion system consisted of 100 mL enzyme solution, 30 mL organic solvent, and 1 g cholesterol; then the conversion was carried out at 30°C, 250 rpm for 3 hours. The long-chain hydrocarbons showed higher conversions, and petroleum ether possessed the best cost performance.
(b) The enzymatic activity is shown as relative activity with mean ± standard error. The absolute activity corresponding to 100% was 272.6 U/L. It showed that the residual enzyme activity decreased with the increase of conversion rate.

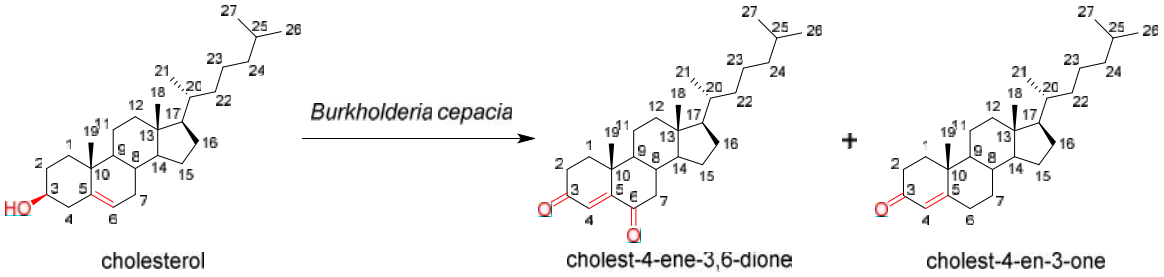
3.2 Burkholderia cepacia

The microbial conversion of water-insoluble substrates such as steroids has been stalled due to their low solubility in aqueous media. Organic solvents are usually applied to the solution to solubilize water-insoluble hydrophobic compounds. Meanwhile, most enzymes can be easily inactivated in the presence of organic solvents [43]. Organic-solvent-tolerant microorganisms are essential in screening for extracellular enzymes in the presence of organic solvents [44]. [45] isolated a cyclohexane-tolerant and cholesterol-oxidizing *Burkholderia cepacia* strain ST-200 which produces a fascinating extracellular cholesterol oxidase. This enzyme is vastly firm in the presence of organic solvents or detergents, and is also thermo-stable [46].

3.2.1 Establishment of subject basis

At present, the biotransformation of sterols focuses mostly on the modification of sterol at the C-3, C-9 and C-17, whilst there remain few studies on the ketylation of C-6. Specific strains of *Burkholderia cepacia* carry out the transformation of cholesterol and does not cause the degradation of C-17 branched chain, but carries out oxidation and isomerization at specific parts of A and B rings of sterol nucleus to obtain cholest-4-ene-3,6-dione (CEDO), a product with high medical value.

CEDO can be used to prepare drugs for the treatment of or prevention of neuronal injury, has antitumor activity [15], has excellent physiological effects such as anti-obesity, improves lipid metabolism [20].



Equation 0- 1 : Biotransfomation of cholesterol to cholest-4-ene-3,6-dione and cholest-4-en-3-one by B. cepacia.

4. Challenges and Further research

Most organic solvents are usually toxic to microorganisms and cause damage to the cells. Most microorganisms cannot grow in the medium containing large amount of organic solvent [25], which then requires that the strains used for biotransformation have good organic solvent tolerance.

At the same time, organic solvents cause damage to the human body. They are volatile and can cause damage to the respiratory system and the nervous system if exposed to its environment for a long period.

Furthermore, with the extension of transformation time, the organic solvent will decrease in the transformation system, resulting in the inhibition of the of products; organic solvents are flammable and explosive, therefore pollute the environment, also inconsistent with the purpose of green environmental protection[47].

Further research is required to provide insights into the development of efficient methods of biotransformation of cholesterol and other poorly water-soluble compounds, via limited enzymatic application and complex reaction systems.

It may also be required to provide insights into the progress of bioreactor development to facilitate the safe use of various organic solvents that may be harmful to the environment. Although, the use of bioreactors in biotransformation has been on the scene for decades now, of which [2] and [48] reported good results for two-phase bioreactors containing oleyl alcohol as a co-solvent for the degradation of benzene, toluene, xylene, and phenols by the Pseudomonas species. Approbatory results have also been shared for the application of silicone oil in two- 2 phase bioreactors for biodegradation of polycyclic aromatic hydrocarbons like pyrene, chrysene, benzo[a]pyrene and perylene[49].

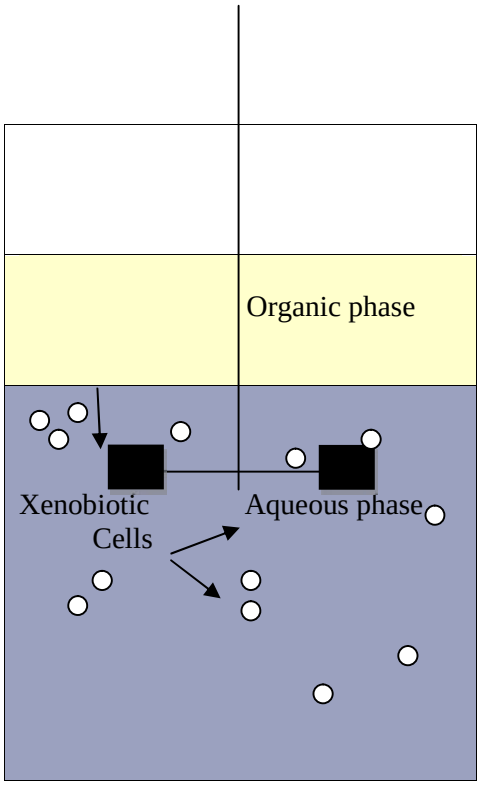


Figure 0-I: *Schematic diagram of a two-phase-liquid bioreactor (Adapted from* [50]*)*

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