Novel bacterial isolates used for the bioconversion of some agriculture wastes into important steroid hormones

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Background and objectives
Microbial bioconversion of phytosterols produces many compounds especially steroid intermediate hormones. One of the most important transformation reactions is side chain degradation of sterols especially phytosterols β-sitosterol to androstenedione (AD), and androstadienedione (ADD). These compounds are considered to be critical intermediates in the preparation of testosterone, estrogen hormones. The main objective is to study the bioconversion of some agriculture wastes as a cheap source of phytosterols to more valuable products AD and ADD.
Materials and methods
In the present study, phytosterols of some agriculture products especially soybean, rice bran, and wheat bran were used as a substrate for the production of both AD and ADD using locally isolated bacterial strain. Different physiological and biochemical factors were tested as well as qualitative and quantitative estimation of the transformation products were carried out according to a previously recorded method.
Results and discussion
The results showed that screening experiments were carried out to investigate the ability of 12 bacterial isolates to transform plant agriculture wastes phytosterols into steroid hormone intermediates AD and ADD. The results also indicated that only four strains possess this ability. One of which was selected to complete this study according to its high AD and ADD productivity. Different physiological and biochemical tests involving catalase, oxidase, coagulase, indole production, urease, citrate and voges-proskauer, type of the agriculture waste, moisture content, parentage of the waste as well as some additives were tested. The results showed that the best bioconversion (3.98 and 3.37 mg/100 ml of both AD and ADD, respectively) were obtained by using soybean – what bran mixture at a ratio of 1 : 1. Qualitative and quantitative analyses of the transformation products were investigated. The phylogenic analysis was carried out and the results indicated that the new strain referred to is *Ochrobactrum anthropi*, which is first recorded to be a phytosterol transformer.
Conclusion
The study has indicated that the newly isolated bacterial strain *Ochrobactrum* is first recorded to perform the side chain degradation of phytosterols presented in soybean and wheat bran to AD and ADD under the above-selected fermentation conditions.

Keywords:
agriculture waste, androstenedione, androstadienedione, bioconversion, phytosterols

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Introduction

Phytosterols, which include plant sterols and stanols, are steroid compounds that occur in plants and are similar to cholesterol in animals, but vary only in carbon side chain and/or in the presence of a double bond [1,2].

Agriculture waste mixtures act as a fermentation medium on which the selected microorganism can grow and degrade the cellulosic and lignin components as well as other compounds present in the wastes. Phytosterols are constituents of the agriculture waste and are converted to important steroid intermediate hormones, androstenedione (AD) and androstadienedione (ADD). These microbial step reactions are of great economic significance to produce C_{19} and C_{22} steroid hormones. It is the most notable industrial reaction for the manufacture of corticosteroids [3].

Modern solid-state fermentation for future biotransformation occurs to exploit the vast complexity of the technology to convert biomass, which produced by the agriculture and food industry to valuable byproducts, through the microbial bioconversion process [4].

Soybean is rich in phytosterols and has become the main source of commercial phytosterols. β-sitosterol, stigmasterol, campesterol, and brassicasterol are the four major types of soybean sterols [5,6].

The current study aims at studying the bioconversion of some plant phytosterols presented in some agriculture wastes to AD and ADD using locally isolated bacterial strains.

Material and methods

Material

Microorganisms

Fifteen waste water samples were collected from the waste water plant in El-Qalyubia Governorate, and from sewage water, El-Beheira Governorate, Egypt. Ten soil samples from El-Qalyubia Governorate, Egypt and five samples from air were collected. The obtained pure bacterial colonies were purified and identified according to the standard microbiological methods [7]. The stock cultures were maintained at 4°C, and were preserved in 20% glycerol under −80°C.

Chemicals

The authentic steroids samples AD and ADD and sterols, β-sitosterol were provided by Sigma Aldrich Company, Missoui, USA. Yeast extract was obtained from Biolife (biolife Viale Monza 272 Milano, Italy) and Bacto Peptone was purchased from Difco Laboratory (Missoui, USA). Thin layer chromatography plates (Fluka, Silica gel G-60 of, thickness 0.2 nm were used for qualitative analysis. All solvents used in the current study (chloroform, acetone, ethanol, and benzene) and the other solvents were obtained from El-Nasr Pharmaceutical Chemical Company (ADWIC), Qalyubia, Egypt. All chemicals and solvents used in thin layer chromatography and gas chromatography-mass analyses were of HPLC grade.

Methods

Screening biochemical identification of bacterial isolates

Investigations were done according to the method described by Kicenam [8], which includes, Gram stain, indole test, voges-proskauer test, citrate utilization, urease test, catalase test, and coagulase test.

Preparation of inoculums

A 250 ml Erlenmeyer flask contains 50 ml of the following sterilized medium (g/l) glucose 10, K_2HPO_4 0.75, KH_2PO_4 3, $(NH_4)_2SO_4$1, $MgSO_4.7H_2O$ 1, and 8 hydroxyquinoline 0.8; the pH was initially adjusted to 6.5 and prepared according to Spencer and Ti brook [9]. The inoculated flasks were incubated at 150 rpm, 30±2°C on a rotator shaker for 3 days.

Screening experiment

The isolated bacterial strains were tested for their ability to transform plant sterols (phytosterol) to the important steroid hormone (AD and ADD, respectively). The most potent strain was selected.

Transformation process

A 250 ml Erlenmeyer flask contains sterile (100 ml) of the transformation medium (g/l) glucose 10, K_2HPO_4 0.75, KH_2PO_4 3, $(NH_4)_2SO_4$1, $MgSO_4.7H_2O$ 1, 8 hydroxyquinoline 0.8 was inoculated by 3.0 ml from the previously prepared inoculums at pH that was initially adjusted at 6.5. The culture flasks were agitated at 200 rpm for 24 h, then induced by 1.0 mg β, sitosterol/100 ml after which 10 mg of sterol was added to each flask, and the bioconversion process was continued. Due to the limited solubility of the sterol of the nutrient solution, it was dissolved in the minimal volume of ethyl alcohol. Upon using the waste the above-mentioned medium was replaced by the waste after moisturized by distilled water and the transformation process continued.

Extraction

The transformation products were extracted according to the method described by Sallam *et al.* [10], by using equal volume, and repeated three times, the solvent layer was evaporated till dryness and the residue dissolved in 5 ml chloroform for subsequent analysis.

Analysis of transformation mixture

Both qualitative and quantitative analyses and gas chromatography analysis of the reaction mixtures were carried out according to Sallam *et al.* [10].

Identification of bacterial isolates by DNA amplification

Perform PCR using the recommended thermal cycling conditions outlined below:

The PCR clean-up is done to the PCR product using the GeneJET PCR Purification Kit (Thermo K0701). Finally sequencing is done to the PCR product on GATC Company using the ABI 3730xl DNA sequencer by forward and reverse primers [11].

Only by combining the traditional Sanger technology with the new 454 technology, genomes now can be sequenced and analyzed in half the usual project time, with a considerable reduction in the number of coatings and gaps. In addition, considerable cost advantages now make genome sequencing with the 454 technology accessible to the research community.

Results

Screening experiments of bacterial isolates for androstenedione and androstadienedione production

The objective of this experiment was to estimate the ability of pure 12 bacterial isolates to produce AD and ADD and to choose the most potent one. The tested bacterial isolates at a concentration of (2×10^6 cell/ml) were grown on a suitable medium (g/l) glucose 10, K_2HPO_4 0.75, KH_2PO_4 3, $(NH_4)_2SO_4$1, $MgSO_4.7H_2O$ 1, 8 hydroxyquinoline 0.8. The isolates were screened for the active transformer of β-sitosterol; the results in Table 1 showed that only four isolates (2, 4, 8, 10) produced AD and ADD.

The results in Table 2 show that the best bioconversion efficiency (38.63 and 35.73%) was obtained using isolates 2 and 4, respectively. On the other hand, isolates 10 and 8 were considerably of low efficiency (25.14 and14.64%, respectively).

Table 1 Qualitative screening experiment for the active transformer of β-sitosterol

Isolate no.	Transformation Residual sterols	Mixture mg % AD	ADD
1	+++	–	–
2	+	++	+
3	++	–	–
4	+	++	+
5	–	–	–
6	++++	–	–
7	++++		
8	+	++	+
9	–	–	–
10	+	++	+
11	++++	–	–
12	+++	–	–

+, positive; –, negative; AD, androstenedione; ADD, androstadienedione.

Identification of isolated strains

Identification

Microorganisms that convert β-sitosterol into AD and ADD were isolated, purified, and transferred to maintenance media (g/l) glucose 10, K_2HPO_4 0.75, KH_2PO_4 3, $(NH_4)_2SO_4$1, $MgSO_4.7H_2O$ 1, 8 hydroxyquinoline 0.8. Four bacterial strains were isolated named 2, 4, 8, 10 were subjected to biochemical tests and Gram staining (catalase, oxidase, coagulase, indole production, urease, citrate, and voges-proskauer) were accomplished for the identification of these isolates. The results in Table 3 show the data of various biochemical reactions.

Application of different agriculture products and wastes on the bioconversion of β-sitosterol

This experiment was carried out to test the bioconversion of different agriculture products and wastes (wheat bran, soybean, rice bran, wheat straw, and olive waste) to AD and ADD. The data presented in Fig. 1 and Table 4 indicated that the yields of AD was improved at soybean and rice bran which give 3.26 and 2.80 mg/100 ml, respectively. On the other hand, by using wheat straw the production of AD decreased (1.22 mg/100 ml).

Effect of different moisture contents of agriculture wastes on the bioconversion of their phytosterol to androstenedione and androstadienedione

The effect of different moisture agriculture wastes (5, 10, 15, 20, and 25%) on the production of AD and ADD were tested. The results are given in Fig. 2 and Table 5 showed that at a moisture content of 15 and 20% the out puts of AD increased to 3.98 and 3.37 mg/100 ml, respectively. On the other hand, at a lower moisture content of 5% a remarkable decrease (2.45 mg/100 ml) was recorded and also at higher moisture contents (25%) a low AD output was recorded.

Effect of different agriculture waste mixtures on the bioconversion of β-sitosterol

The aim of this experiment was to investigate the effect of different agriculture waste mixtures (wheat

Table 2 Quantitative analysis for β-sitosterol transformation using selected bacterial isolates

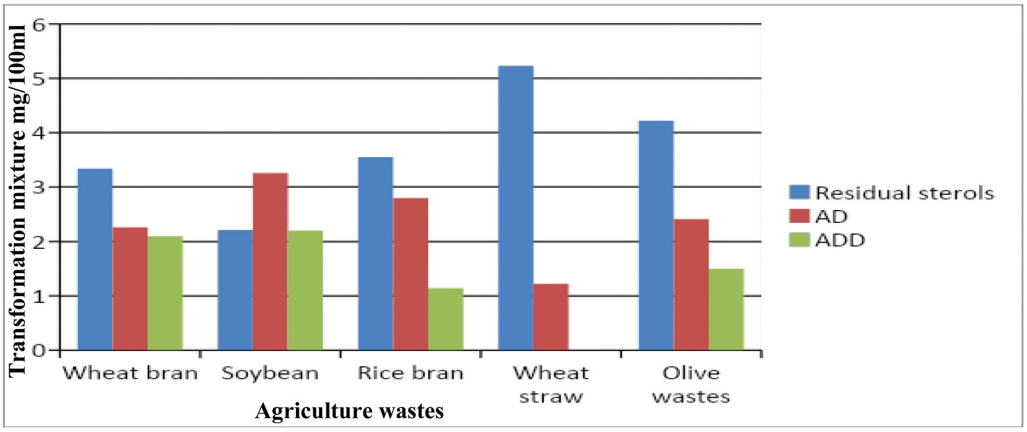
Isolate no.	Transformation Residual sterols	Mixture mg/100 ml AD	ADD	Bioconversion efficiency %
2	5.36	2.13	3.65	38.63
4	344	3.53	3.22	35.73
8	4.53	2.34	1.23	14.64
10	5.21	1.44	2.37	25.14

AD, androstenedione; ADD, androstadienedione.

Table 3 Preliminary biochemical identification of isolated strains

Test	Isolates			
	2	4	8	10
Gram stain	–ve rods	+vecocci	–ve rods	–ve rods
Growth on MacConkey agar	+ve	–ve	+ve	+ve
Lactose fermentation on MacConkey agar	+ve (pink)	–ve (no growth)	+ve (pink)	+ve (pink)
Growth on nutrient agar	Green	Yellow	White	Gray
Catalase	+ve	+ve	+ve	–ve
Citrate	–ve	+ve	–ve	+ve
Oxidase	+ve	–ve	+ve	+ve
Coagulase	+ve	–ve	+ve	–ve
Urease	–ve	+ve	–ve	–ve
Indole production	–ve	-ve	–ve	+ve
Voges-proskauer	+ve	+ve	+ve	–ve

Figure 1



Effect of using different agriculture wastes on the bioconversion of β-sitosterol.

Table 4 Descriptive statistics

	N	Minimum	Maximum	Mean	SD
Residual sterols	5	2.21	5.23	3.7100	1.11613
AD	5	1.22	3.26	2.3900	0.76013
ADD	4	1.14	2.20	1.7350	0.50289
Valid *N* (listwise)	4				

AD, androstenedione; ADD, androstadienedione.

Table 5 Descriptive statistic

	N	Minimum	Maximum	Mean	SD
Residual sterols	5	2.21	4.89	3.3740	1.00106
AD	5	2.45	3.98	3.2320	.54961
ADD	5	1.35	2.41	1.9520	.42892
Valid N (listwise)	5				

AD, androstenedione; ADD, androstadienedione.

bran–soybean, wheat bran–rice bran, wheat bran–wheat straw and wheat bran–olive waste) on the production of AD.

The results given in Fig. 3 and Table 6 showed that the production of AD was affected by adding wheat bran–soybean and wheat–olive waste mixtures, which increased the output of AD (4.26 and 3.57 mg/100 ml, respectively). The yield of AD was decreased at wheat bran–wheat straw and wheat bran–rice bran mixtures and give 2.10 and 2.43 mg/100 ml, respectively.

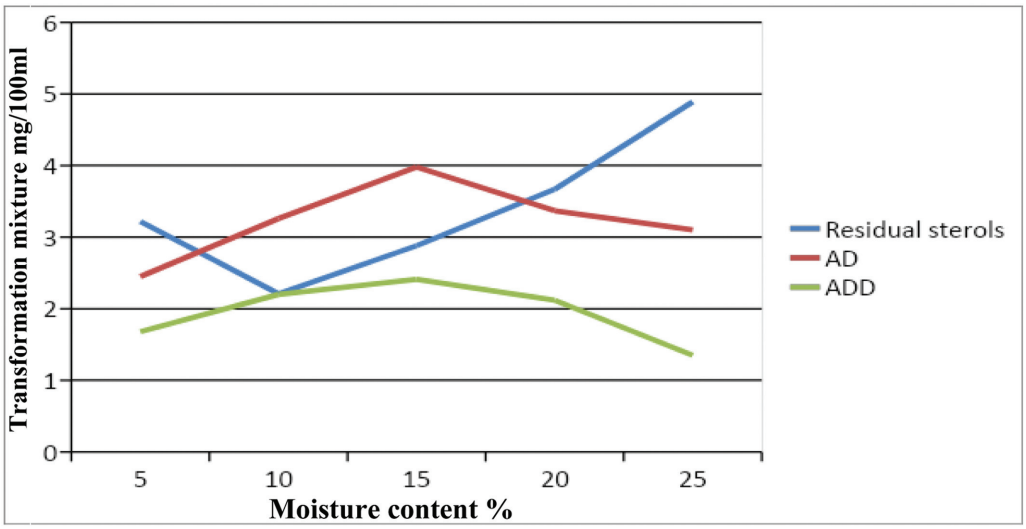
Effect of using different ratios of wheat bran and soybean mixture on phytosterol bioconversion

This experiment deals with studying the effect of different (wheat bran and soybean) ratios (1 : 1, 1 : 2, 1 : 3, 2 : 1 and 3 : 1) on the bioconversion of β-sitosterol. The data presented in Fig. 4 and Table 7 showed that the best yield of AD was 4.78 and 4.26 mg/100 ml at 1 : 2 and 1 : 1 %, respectively. At ratio 3 : 1 and 2 : 1, the production of AD was reduced to 1.67 and 2.78 mg/100 ml, respectively.

Phylogenetic identification of bacterial isolates by DNA amplification

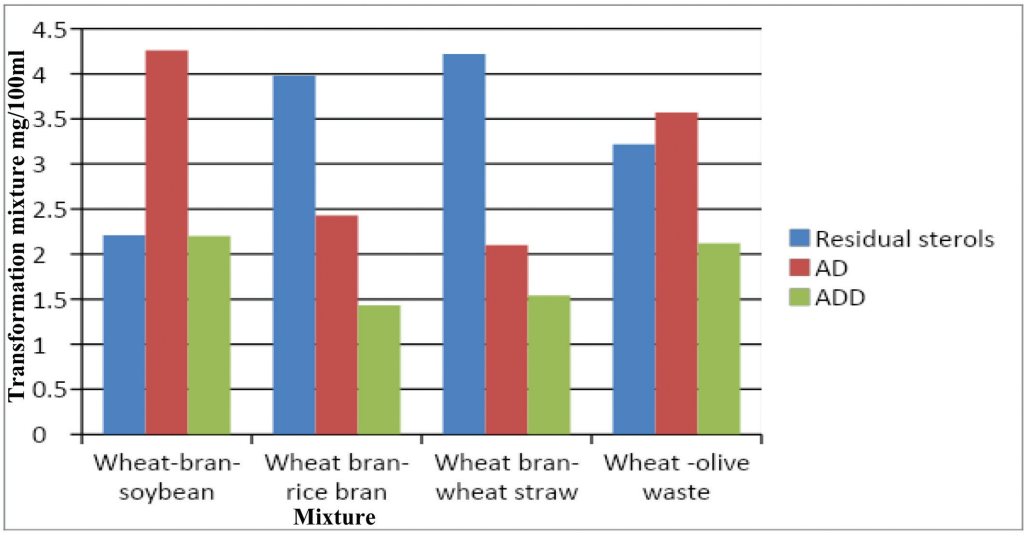
The two isolates (isolate no. 2 and no. 4) which produce the highest amount of AD were identified

Figure 2



Effect of using different moisture content of agriculture wastes on the bioconversion of β-sitosterol.

Figure 3



Effect of different agriculture waste mixtures on the bioconversion of β-sitosterol.

Table 6 Descriptive statistics

	N	Minimum	Maximum	Mean	SD
Residual sterols	4	2.21	4.22	3.4075	.90500
AD	4	2.10	4.26	3.0900	1.00250
ADD	4	1.43	2.20	1.8225	.39365
Valid N (listwise)	4				

AD, androstenedione; ADD, androstadienedione.

by PCR as follows: the nucleotide sequences identified in this study were deposited into GenBank Data Library. The GenBank accession numbers for the *Ochrobactrum anthropi* are ATCC49188T CP000758.1–CP000763.1 (Fig. 5).

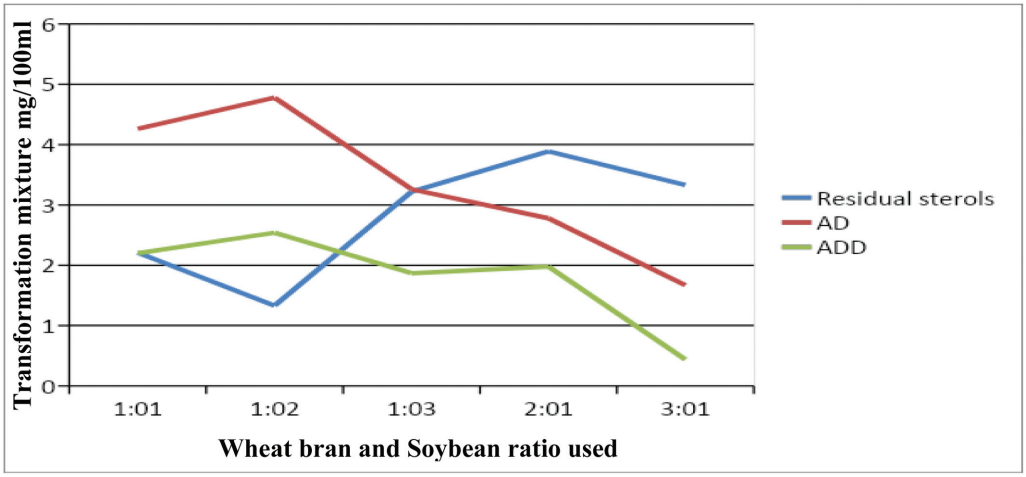
Gas chromatography-mass spectrometry analysis and identification of transformation product samples

Analyses of the obtained data for the transformation products of the used phytosterols to AD and ADD were recorded. The results presented in Fig. 6 and Table 1 reveal the intermediate compounds and the different steps between sterol degradation and the formation of the desired products (Table 8).

Characterization of the different transformation products using gas chromatography-mass spectrometry analysis

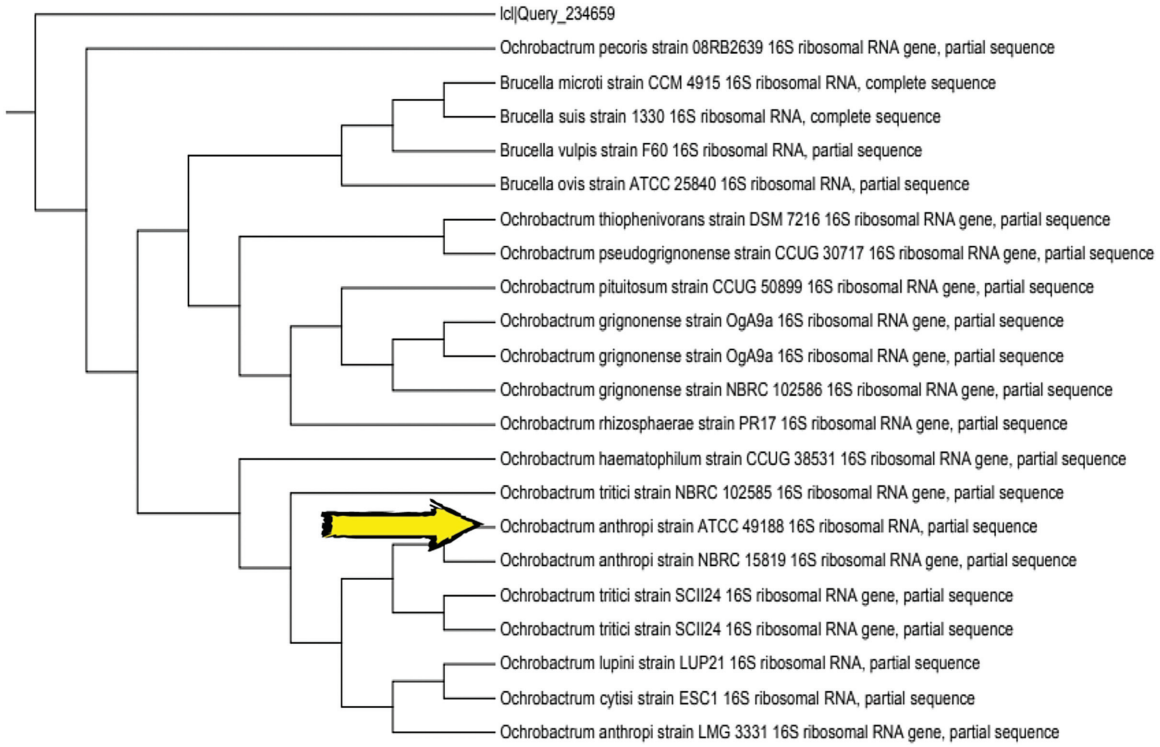
Gas mass spectrometry was used for the identification of products and standard steroids; the results presented

Figure 4



Effect of using different waste mixture ratios on phytosterol bioconversion.

Figure 5



Phylogenetic tree showing detailed genotype of *Ochrobactrum anthropi* strain ATCC 49188 16 S ribosomal RNA, partial sequence.

Table 7 Descriptive statistics

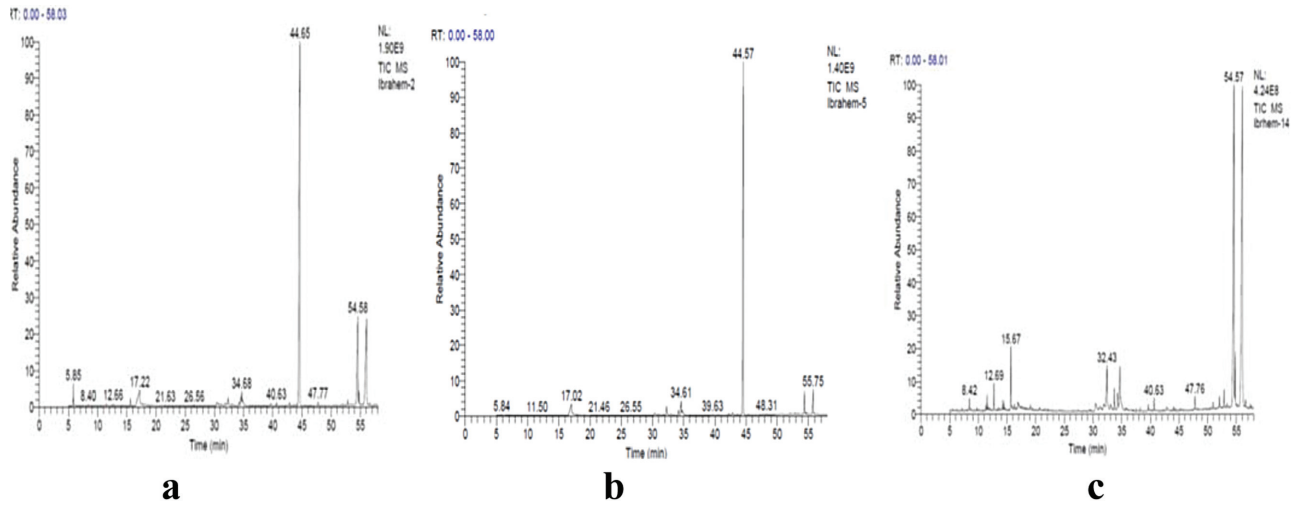
	N	Minimum	Maximum	Mean	SD
Residual sterols	5	1.33	3.89	2.7960	1.01911
AD	5	1.67	4.78	3.3500	1.22764
ADD	5	.44	2.54	1.8060	.80534
Valid N (listwise)	5				

AD, androstenedione; ADD, androstadienedione.

in Table 6 reveal that a number of different steroid derivatives were obtained due to the high chromatographic resolution capacity and reproducible ionization efficiency of the derivatization procedures used before injection of the product sample obtained after extraction.

Brandenbusch and Sodowski [11] reported that derivatives can be time consuming; it permits the profiling of additional compounds by allowing both polar and nonpolar steroid substances to be successfully and easily separated. From the above results, we found

Figure 6



(a–c) Structure elucidation of some transformation products.

that the transformation products were 3-hydroxy Androst-2-en-17-one and 3-hydroxy Androst-5-en-17-one as well as Androst-5-ene-3,17-diol and pregnenolone, which is considered to be a precursor of AD and ADD.

Discussion

The pharmaceutical industries have a long history of converting phytosterols to therapeutic steroid hormones by microbial transformation. Steroids are terpenoid lipids of specific structures that contain the nucleus of four cycloalkane rings. In the body, cholesterol leads to the formation of steroidal hormones [12].

AD being one of the most important steroids which has a high demand in the pharmaceutical industry as it is a precursor for a widely marketed drug category known as steroids. Therefore, the industrial production of this compound will greatly benefit the biomedical sector.

Our results on screening of bacterial isolates for AD and ADD production cleared that the tested bacterial isolates are grown in a 250 ml Erlenmeyer flask containing 100 sterilized fermentation medium. This medium helps in growth of the bacterial isolates that produce AD and ADD. These results agreed with those of Xiong *et al*. [13], where the used media and culture conditions were the same as the previously described conditions [14].

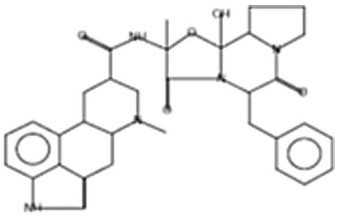
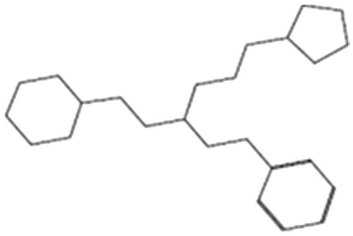
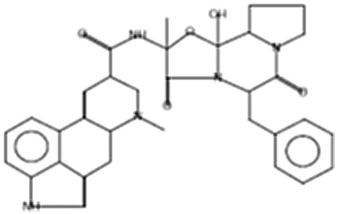
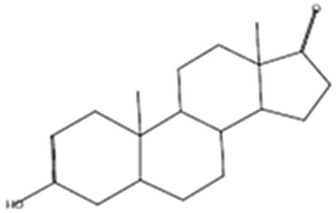
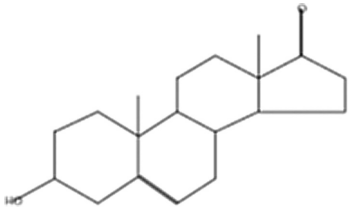
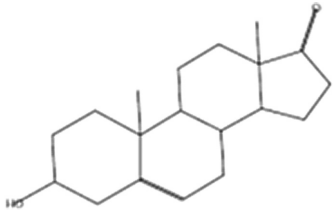
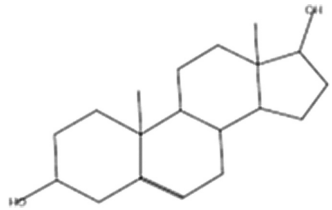
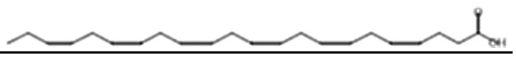
Identification of the bacterial isolates that produce AD and ADD was carried out using Gram staining and biochemical tests (catalase, oxidase, coagulase, indole production, urease, citrate and voges-proskauer, and DNA amplification using the PCR technique; these steps of identification agreed with those of Xiong *et al*. [13] where they used biochemical tests and PCR for the identification of the bacteria that produce AD and ADD.

The results on the identification of bacterial isolates by DNA amplification cleared that the two isolates (isolate no. 2 and no. 4), which produce the highest amount of AD were identified by PCR as isolate no. 2. *O. anthropi* strain ATCC 49188 16 S ribosomal RNA, partial sequence our results agreed with those of Palermo *et al*. [15], where they indicated the PCR method is the best method for identification of bacterial RNA nucleotide sequences and the main bacterial isolates that produce AD is the *O. anthropi*.

Our results on the phylogenetic tree show the detailed genotype of *O. anthropi* strain ATCC 49188 16 S ribosomal RNA, partial sequence [15,16]. Rep-PCR analysis is a technique aimed at defining clonal relationships, and its ease of use and faster turnaround time as compared with PFGE (Unified Pulsed-Field Gel Electrophoresis) makes it a rapid method of screening outbreaks of *O. anthropi* and therefore allows timely implementation of control measures. Our results on the effect of different agriculture wastes on the bioconversion of β-sitosterol cleared that the yields of AD were improved for soybean and rice bran which gave 3.26 and 2.80 mg/100 ml, respectively.

These results attributed to their effect on the bacterial growth as it is considered as a good source of energy

Table 8 Molecular weight and structure of some steroid intermediates

Peak	Name of the intermediate compounds	Molecular formula	Molecular weight	Peak area %	Molecular structure
24.64	9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl) Ergotaman-3',6',18-trione	$C_{35}H_{37}N_5O_5$	583	0.01	
28.58	[3-(2-cyclohexylethyl)-6-cyclopentylhexylBenzene	$C_{25}H_{40}$	340	0.01	
29.34	12'-hydroxy-2'-methyl-5'-phenylmethyl Ergotaman-3',6',18-trione	$C_{33}H_{35}N_5O_5$	581	0.02	
31.59	3-Hydroxy Andrrost-2-en-17-one	$C_{19}H_{30}O_2$	288	0.01	
33.72	3-Hydroxy Andrrost-5-en-17-one	$C_{19}H_{30}O_2$	288	0.03	
35.01	3-Hydroxy Andrrost-5-en-17-one	$C_{19}H_{30}O_2$	288	0.06	
45.43	Andrrost-5-ene-3,17-diol	$C_{19}H_{30}O_2$	290	0.05	
45.43	Doconexent	$C_{22}H_{32}O_2$	328	0.05	

that activated the bacteria for the production of AD and ADD [16]. On the other hand, with wheat straw the production of AD was decreased (1.22 mg/100 ml). Also, our results on the effect of different moisture agriculture wastes on the bioconversion of β-sitosterol cleared that at concentration moisture content percentage (15 and 20), the outputs of AD increased (3.98 and 3.37 mg/100 ml, respectively). On the other

hand, the output of AD decreased at 5% which give 2.45 mg/100 ml.

The results agreed with those of Pandey [17] where they reported that at a moisture level of 15% there is increasing level of biotransformation and formation of AD. Our results on the effect of different agriculture waste mixtures on the bioconversion of β-sitosterol showed that the production of AD was affected by adding wheat bran–soybean and wheat–olive waste mixtures, which increased the output of AD (4.26 and 3.57 mg/100 ml, respectively).These results agreed with those of Wrońska *et al.* [18], where they reported that the sources of energy and carbon such as wheat–olive waste are considered as energy sources for the activation of bacteria and improving the biotransformation and production of AD. Meanwhile, our results on the effect of different waste mixture percentage on the bioconversion of β-sitosterol showed that the best yield of AD was 4.78 and 4.26 mg/100 ml at 1 : 2 and 1 : 1 ratio, respectively [19]. Also, our results cleared that at 3 : 1 and 2 : 1% the production of AD reduced to 1.67 and 2.78 mg/100 ml, respectively.

Conclusion

This study has concluded that microbial transformation of phytosterols to therapeutic steroid hormones is of economic value for the pharmaceutical industry especially for the formation of AD and ADD. The type of bacteria used and the fermentation medium conditions are very important and must be adjusted for maximum AD and ADD formation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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