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# UTILIZATION OF CONCRETE AS A CARRIER FOR BACTERIAL CELLS DURING BIOCONVERSION OF SOME STEROLS

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## ABSTRACT

Concrete is a heavy waste, which has porous structure. To great extent concrete is safe and biologically inactive. This can make this material be used as a carrier for the biocatalyst. Androst-4-ene-3, 17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD) are important intermediate in synthesis of wide range of pharmaceutical steroids, Cells of bacterial strain isolated from olive mill and identified as *Alkalibacterium olivoapovliticus* showed a good ability to convert sterols to AD an ADD. The cells of this stain was entrapped within blocks of concrete. Different parameters were investigated to obtain the maximal products. Study of these parameters nearly doubled the AD formation (from about 56% to over 90%) and the ADD production (from about 35% to about 55%). Furthermore, the immobilized cells possessed a good capacity for recycling and withstand all the bioconversion stresses for about ten successive cycles. Absolutely, this is the first time for the concrete to be used in such studies also *Alkalibacterium olivoapovliticus* is a recent species and this is the first time to be tested with such important biotransformation application.

**Key words**: Concrete, Immobilization, *Alkalibacterium olivoapovliticus*.

## INTRODUCTION

It is well known that phytosterols (PSs) are suitable raw materials for microbial degradation to 17-ketosteroids because of low cost and easy availability[1]. As two important pharmaceutical steroid precursors, androst-4-ene-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD), which belong to 17-ketosteroid family can be further used to produce a wide range of pharmaceutical steroid derivatives[2].

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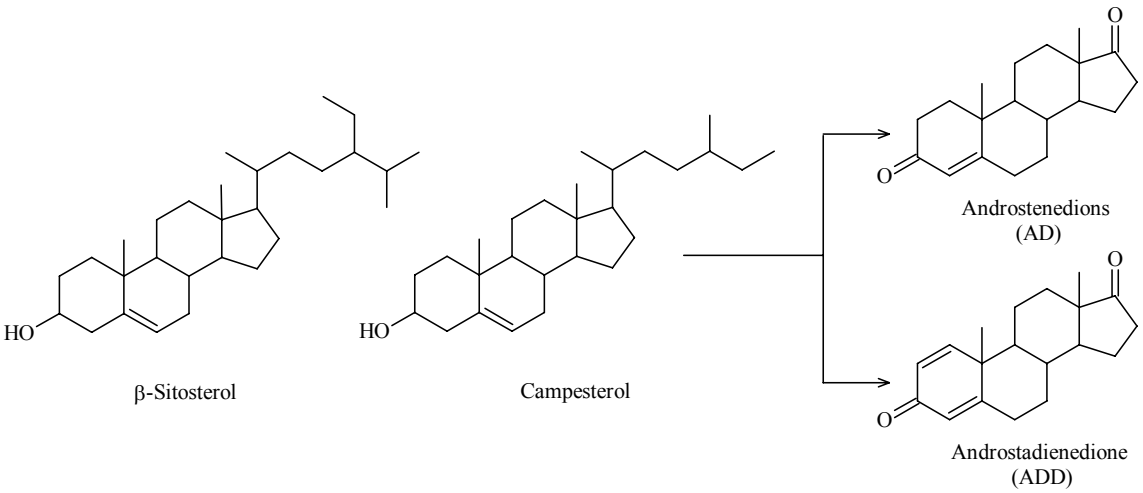
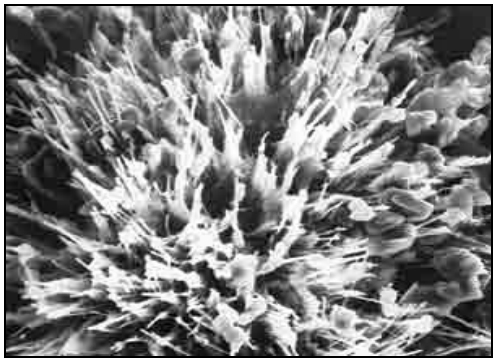



**Bioconversion of some sterols to ketosteroid**

Biotransformation serves as an important defense mechanism to the toxic xenobiotics and the wastes will be converted into less harmful compounds[3]. The olive wastewater has phytotoxic effect[4]. So the bacteria that present in this environment may be able to withstand the toxicity of the xenobiotic.

On the other hand, concrete can be available with low cost during building demolition and construction. This material is usually unwanted and may be considered as useless waste. The relative biological inertia and the porous structure of this material make it a candidate and safe carriers for immobilization the living microbial cells.



**Porous structure of the concrete**

The current work was devoted for testing the efficiency of the concrete, waste material, as a carrier for the immobilization of the microbial cells. Furthermore, natural oil

was used for production of important intermediate products, AD and ADD, which are commonly used in steroid synthesis. Additionally, to the best of our knowledge no previous works did biotransformation using the new alkliphelicstrain of bacteria, *Alkalibacterium olivoapovliticus*. These bacteria were isolated from olive mill and identified on the basis of its morphological, physiological and molecular characters.

## EXPERIMENTAL

### Microorganism

The bacterial strains were isolated from olive oil wastewater and olive oil mill. Isolation of bacteria involved dilution plating on a solid medium consisting of diluted olive wastewater (60% V/V) and 2% (W/V) bacteriological agar (BDH). The isolated bacteria were then maintained on medium composed of 0.5% yeast extract, emulsion of (10%) olive oil, 0.1% Mag. sulfate, 0.1% Amm. sulfate and 2% agar (BDH). The pH was adjusted at 9 using Tis/HCl buffer.

### Identification of the bacterial strain

The most potent bacterial strain for biotransformation was identified according to its cultural and physiological characteristics. In addition, the 16 srDNA sequence determination and phylogenic analysis were carried out. The DNA was isolated as described by Moniatis et al.[5] (5-GGAGAGTTAGATCTTGGCTCAG-3) and (5-AGAAAGGAGGTGATCC AGCC-3) were used for amplification of the complete 16S rDNA sequence. BioRad thermal cycler (iCycler) was used during the PCR. The amplified products were analyzed using ABI prism 3100 genetic analyzer. Homology search was achieved using the Blast program against NCBI database.

### The Substrate

A hundred grams of olive oil were saponified[6,7]. Only the unsaponified portion was separated and dissolved in ether. The ether soluble portion was washed three times with distilled water then dried under vacuum and the residue was considered as test materials. The test material was analyzed for the sterols contents using Schimadzu HPLC under the following conditions: 2 µL of the sample was injected into the HPLC instrument Hewllet-Packard 6890 HPLC with flame ionization detector (FID). The column was ZB-5 Column (30 cm × 0.32 mm × 0.25 µm film thickness) eluted at a flow rate 1 mL/min with methanol/water/ acetic acid 75 : 25 : 0.4, v/v/v), pH 5 (adjusted with acetic acid) as mobile phase; the detection at a wavelength 220 nm.

**Table 1: Composition of the unsaponified portion from olive oil**

| Sterols | Percent (%) |
|---|---|
| β-sitosterol | 92 |
| Campsterols | 2.5 |
| Stigmasterol | 1.5 |
| $\Delta^7$ stigmasterol | 0.5 |

## Biotransformation

The two isolated stains were screened for their bioconversion capacities using a media composed of 0.5% yeast extract, emulsion of (10%) olive oil, 0.1% mag. sulfate, 0.1% amm. sulfate. portions of 50 mL medium were expensed in conical flasks 250 mL after serialization it was inoculated with 2 mL of cell suspension 7 x $10^4$ cell/mL. The cultures were allowed to grow at the rotary incubator for 24 h at 30ºC then 15 mg of the substrate dissolved in acetone were added to each flask. The biotransformation process was continued to 48 h. After analysis of the biotransformation products, the most potent strain was further purified and subjected to the identification process.

## Analysis

At the end of fermentation period, the culture broth was taken from the flasks and extracted twice by vigorous shaking with the same volume of chloroform. The extracts were washed twice with distilled water, dried over sodium sulfate anhydrous. Thereafter, samples were applied onto one TLC plate which was spread by silica gel GF254 (0.25 mm). The solvent system used for TLC was Cyclohexane: chloroform: isopropanol (5 : 2.5 : 1 v/v) and the compounds were visualized by iodine staining. Steroid products were observed as orange spots. The products were isolated by preparative TLC (silica gel GF254, 1.0 mm). The spots obtained were scraped off and dissolved in methanol. After centrifugation, the products were further analyzed spectrophotometerically and by HPLC (Shimadzu C18 column, 5 μm particles, and 250 mm × 10 mm). The mobile phase was composed of methanol and water (80 : 20, v/v), flow rate was 5 mL/min, and detection wavelength was 254 nm.

## Estimation of conversion capacity

$$\text{Percent of the product} = \frac{\text{The amount of the detected product (mg)}}{\text{The total amount of the substrate (mg)}} \text{ x } 100$$

### Cell immobilization

All the immobilization processes were performed under aseptic conditions. The cell pellets of *Alkalibacterium olivoapovliticus*, obtained in the logarithmic phase of growth, were collected by centrifugation (5000 rpm, 15 min) in a refrigerated centrifuge. Then, the wet cell pellets were suspended in 0.85% sterile saline and used for the cell immobilization.

### Adsorption on concrete

Two hundred grams of the concrete blockswere treated with 250 mL of 0.1% glutaraldehyde overnight. The carriers were collected and were washed with sterile distilled water to remove the excess glutaraldehyde. Then the cells obtained from culture of 500 mL volume were incubated with the carrier in 1 Liter of sterile saline solution. The carriers were collected, washed with sterile water and stored at 4$^{o}$C for further use.

### Bench scale biotransformation using the immobilized cells

The concrete blocks (200 g) were placed in 5 Liter conical flask containing 2 Liter of reaction mixture. The reaction mixture composed of 2% glucose, Tris buffer (pH 9). The conical flask was stirred with mechanical stirrer (GallenKamp) at 100 rpm at 30 C. The air was supplied to the medium at a flow rate (1 V/V). The reaction was conducted for 48 h and changed as specified in each experiment. 10 mL of the reaction mixture was extracted at the end of the biotransformation periods and analyzed as described above.

### Assay of glucose

The glucose concentration in the reaction mixture was determined according to the methods of Somgyi[8,9].

Calculation of glucose concentration

Absorbance corresponds to 0.1 mL of test = x mg of glucose

10 mL contains = x / 0.1 × 10 mg of glucose = % of reducing sugars

## RESULTS AND DISCUSSION

### Identification of the bacteria

The bacterial strain was motile gram-positive bacteria. After analysis of 16s rDNA sequence, the bacterial strain was identified as strain of *Alkalibacterium olivoapovliticus* WW2SN4c. The phylogenic tree was generated (Fig. 1).

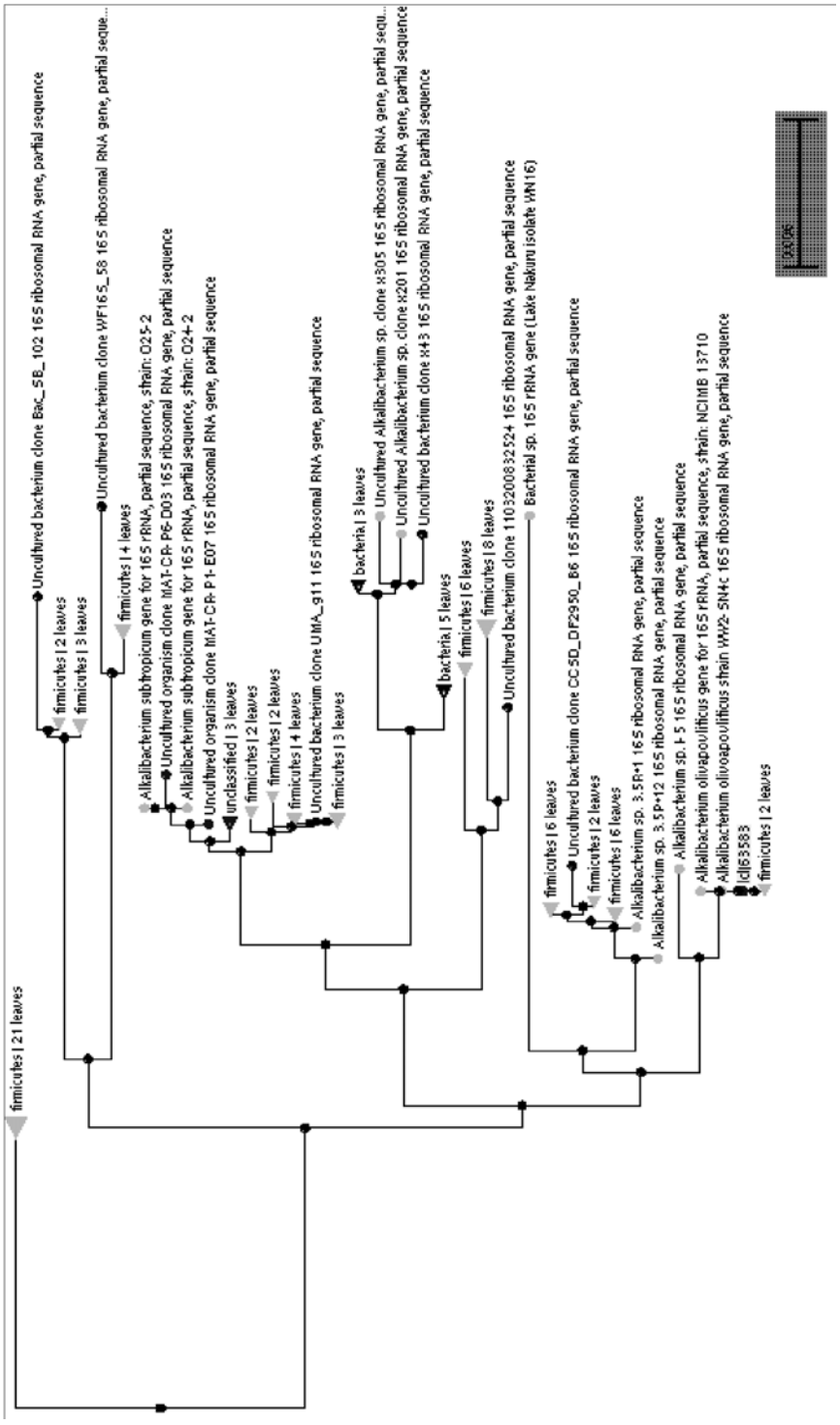



**Fig. 1: The phylogenic tree of the bacterial strai**

## Time course

The bioconversion was conducted at different time intervals 6, 12, 18, 24, 36, 48, 60 and 72 h (Fig. 2). The optimum time for the transformation process was at 24 h. Shorter and longer time caused the decrease of the transformation products. The theoretical calculations indicated that the optimum time for the products formation might be between 24 and 48 h. The experimental results determined the maximalat 24 h and this appear shorter than that recorded by Perez et al.[10] and Korycka-Machala et al.[11] The maximalwas 57% of AD and 43% of ADD[12].

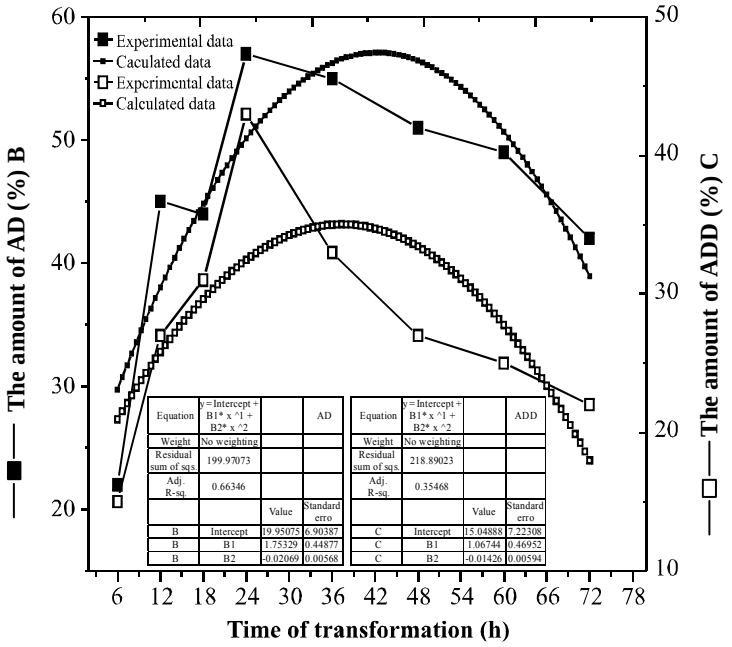



**Fig. 2: Time course for the bioconversion of the sterols using concrete immobilized *Alkalibacterium olivoapovliticus***

## pH relation

It was found that the optimum pH for the bioconversion processes using *Alklibacterium olivapovoliticous* from 8.5 to 10.5 (Fig. 3). Below this pH range a noticeable decrease of the amount of the transformation products was observed. Similarly, the calculations in Fig. 3 showed that the optimum pH values for the product formation could be from pH close to 8.5 to 10.5. This can be attributed to the nature of the bacterial cells that are alkaliphilic.

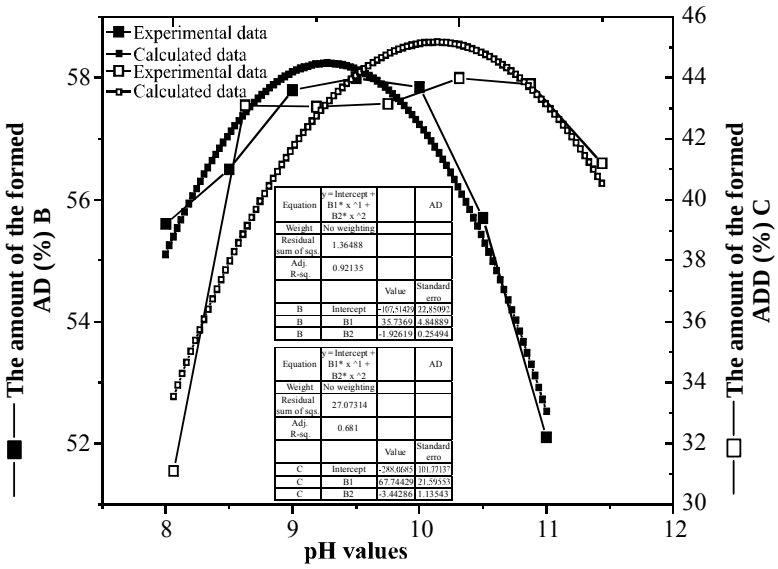



**Fig. 3: pH Relationfor the bioconversion of the sterols using concrete immobilized *Alkalibacterium olivoapovliticus***

## Solubilizing agents

To overcome the slight solubility of the sterols in aqueous solution different types of solvents were investigated to measure their suitability for concrete carries and this species of bacteria. The results in Fig. 4 show that there is no considerable difference between them with respect to their effect on the amounts of the produced AD or ADD.

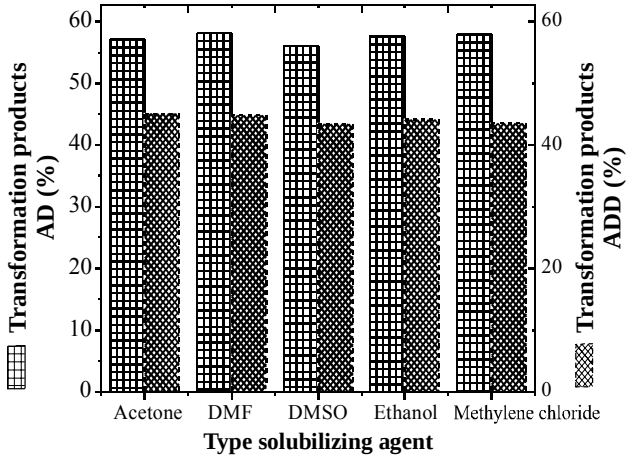



**Fig. 4: Role of solvents in Ad an ADD production from sterols using for the *Alkalibacterium olivoapovliticus* immobilized on concrete**

Accordingly, ethanol or acetone will be used throughout this work. Low concentration was used because of the toxicity of the solvent itself on the microbial cells[13].

## Carbon sources

A trial to use the reaction mixture contains only the substrate as a sole carbon source led to dramatic decrease in the bioconversion process. Meanwhile, different carbon sourceswere supplied to the reaction mixture (Fig. 5). The carbon source supported the transformation activities in different degrees. Glucose appeared to be the best carbon source for production of AD and ADD. It may glucose can provide an easier enzyme source for the resting bacterial cells and this will support the transformation activities. Vezina et al.[14] reported that, the requirement for glucose may be tied to the generation of NADPH, which is essential in the oxidation process.

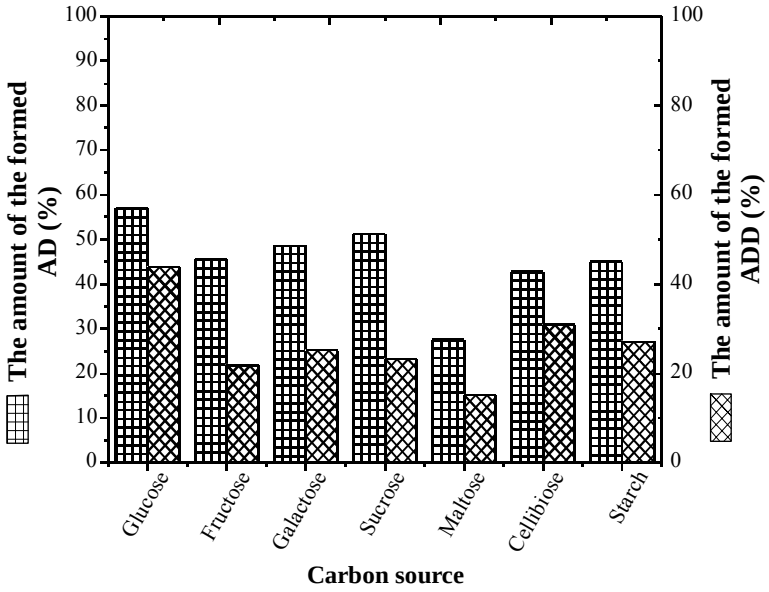
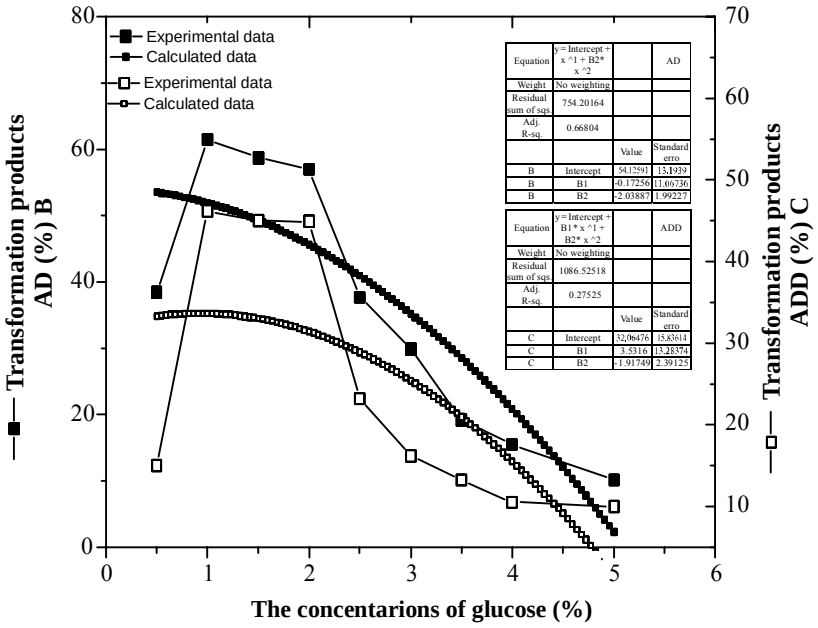



**Fig. 5: The suitability of the carbon souce for AD and ADD formation by for *Alkalibacterium olivoapovliticus* immobilized on concrete**

## Levels of glucose

Because of the crucial role of the glucose as have been proved in the above results, different portions of glucose were added to the reaction mixture. 0.5, 1.0, 1.5, 2.0, 2.5, 3, 3.5, 4 and 5%. The results showed that decreasing of the glucose concentration improved the transformation process (Fig. 6). Upon using 1% or 1.5% of glucose instead of 2% at the beginning of the bioconversion process, the AD and ADD increased from 57, 44% to 61%

and 46.5%, respectively. This can suggest that, although the importance of the glucose presence but the excess of glucose is of inhibitory effect for the biotransformation of the sterols. The calculated results in Fig. 6 also suggest that the gradual increase of the glucose concentrations could completely inhibit the bioconversion process to the level at which the products output could be zero. This may be explained on the basis of that glucose represents a favored carbon source to the cells if it compared with other sources of carbon in the media including the sterols.




| Equation | y = Intercept + x ^1 + B2* x ^2 | | AD |
|---|---|---|---|
| Weight | No weighting | | |
| Residual sum of sqs. | 754.20164 | | |
| Adj. R-sq. | 0.66804 | | |
| | | Value | Standard erro |
| B | Intercept | 54.12591 | 15.1939 |
| B | B1 | -0.17256 | 11.06736 |
| B | B2 | 2.03887 | 1.99227 |

| Equation | y = Intercept + B1* x ^1 + B2* x ^2 | | ADD |
|---|---|---|---|
| Weight | No weighting | | |
| Residual sum of sqs. | 1086.52518 | | |
| Adj. R-sq. | 0.27525 | | |
| | | Value | Standard erro |
| C | Intercept | 32.06476 | 15.83614 |
| C | B1 | 3.5316 | 13.28374 |
| C | B2 | -1.91749 | 2.39125 |

**Fig. 6: Effect of glucose concentartions on the AD an ADD formation by for *Alkalibacterium olivoapovliticus* immobilized on concrete carrier**

## Glucose-substrate relation

The concentrations of the glucose were measured at different time intervals in the presence and absence of the substrate. This is to determine the relation between the substrate and the glucose consumption as a carbon source. It was found that in both cases presence and absence of the substrate the residual glucose decrease gradually with the time until it reached a constant value (Fig. 7). Relatively, in the presence of the sterols as substrates the residual glucose concentrations appeared higher than that in the absence of these substrates. There is a minor effect of the substrate on glucose consumption. Glucose may be used by the resting cells to keep the metabolic activities. In addition to that, sterols are hard carbon source and can be metabolized slower than glucose.

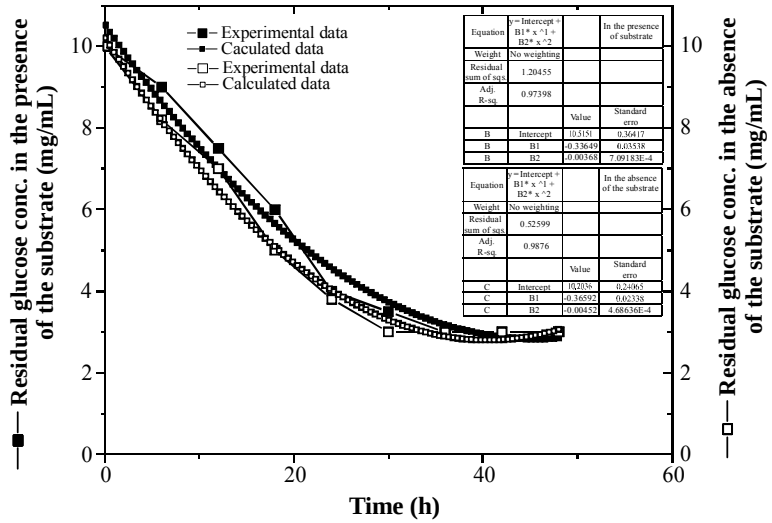



**Fig. 7: The concentration sof the residual glucose during the bioconversion process and its relation to the presence and absence of the substrate**

## Substrate load

To determine the tolerance of the immobilized cells to the substrate different quantities were added separately (5-200 mg/100 mL). Increasing the substrate concentrations hindered the biotransformation process hence the products decreased (Fig. 8).

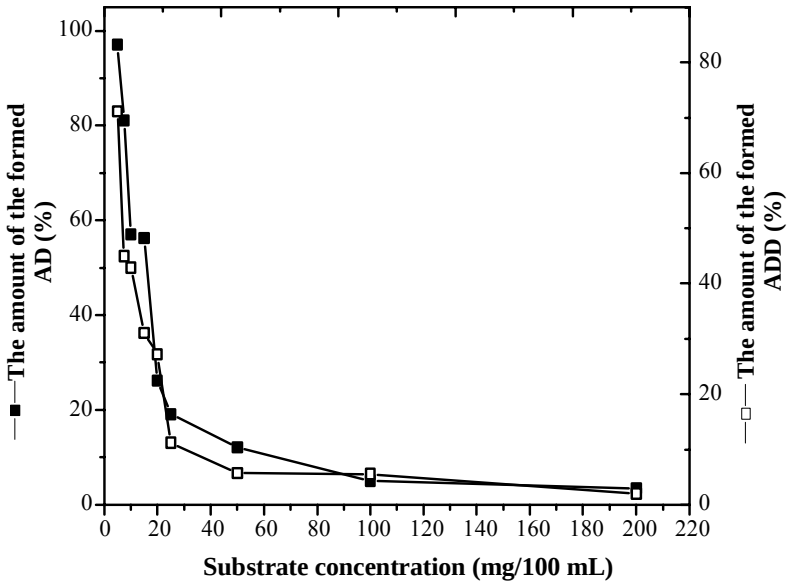



**Fig. 8: The tolerance of the immobilized cells to the substrate concentrations**

However, the results proved the presence of transformation activities at a substrate concentration equal to 200 mg/100 mL. The inhibition effect can be explained on the basis of the toxicity of the sterols substrates to the living microbial cells and the efforts of these cells to overcome this toxic effect[2,15].

## Addition of surfactants

Different surfactants were added to increase the accessibility of the substrate to the cells. Although the addition of the surfactants led to increase in the transformation products but there is no considerable difference between the effects of these surfactants (Fig. 9). However, Tween 80 relatively showed a better effect if it compared with the others (Triton x 100, Tween 20, Tween 40 and Tween 60). The experimental and the calculated data in Fig. 10 indicated that the best concentrations of Tween 80 should be between 0.75 and 1%, this is for both AD and ADD. Williams and Fieger[16] observed that tween 40 acted on the lipoprotein of cytoplasmic membranes of the living cells and thus affecting the permeability of those membranes. Wayman[17] reported that Tweens can cause physical/ chemical changes in the cell wall and this result in a change in the cell permeability according to our results, it is safe to conclude that, tween 80 affects the permeability barrier of the bacterial cells. Smith, et al.[18] found that high concentration (4.2%) of Tweens severely inhibited the products formation. They also reported that whereas high detergent concentration might be sufficiently to partly increase the solubility but the secondary and tertiary structure of the enzymemay be disrupted.

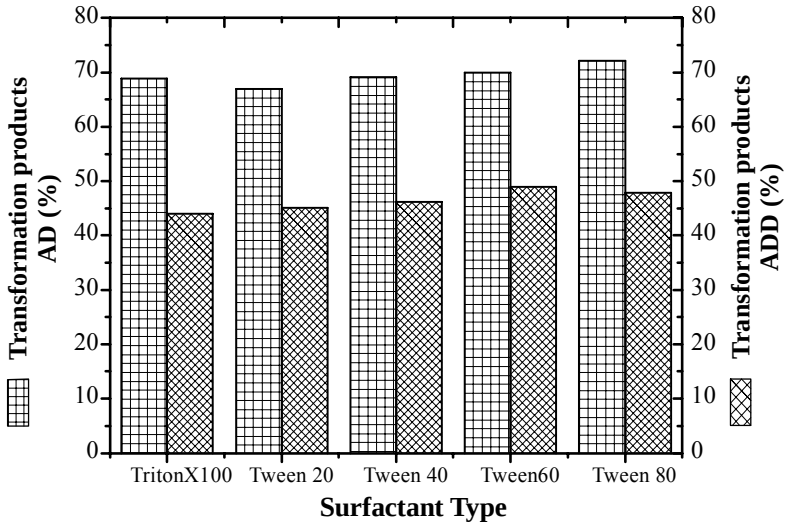



**Fig. 9: Effect of the surfactant on the produced AD an ADD by for the immobilized *Alkalibacterium olivoapovliticus***

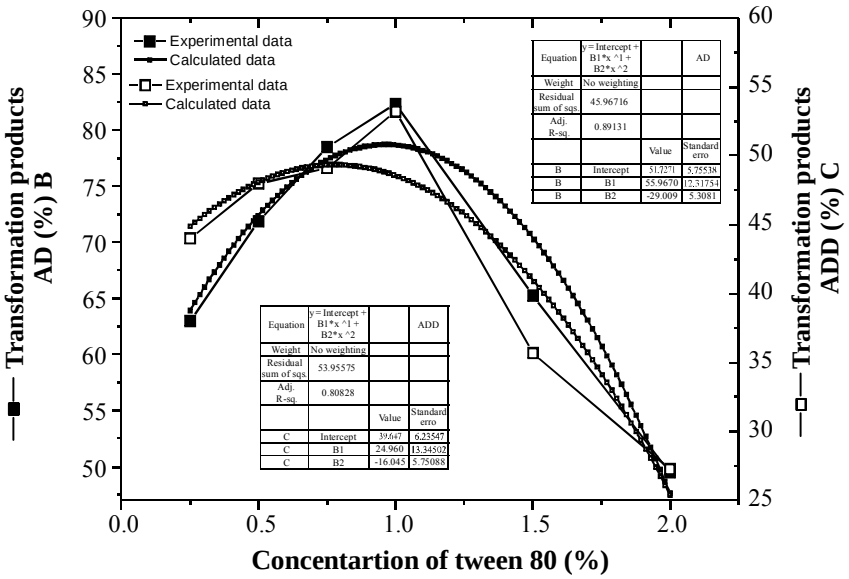



**Fig. 10: Adjustment of the surfactant concentrations**

## Air supply

The results in Fig. 11 indicate that reduction of air supply to 0.5 (V/V) resulted in a reduction in the bioconversion outputs.

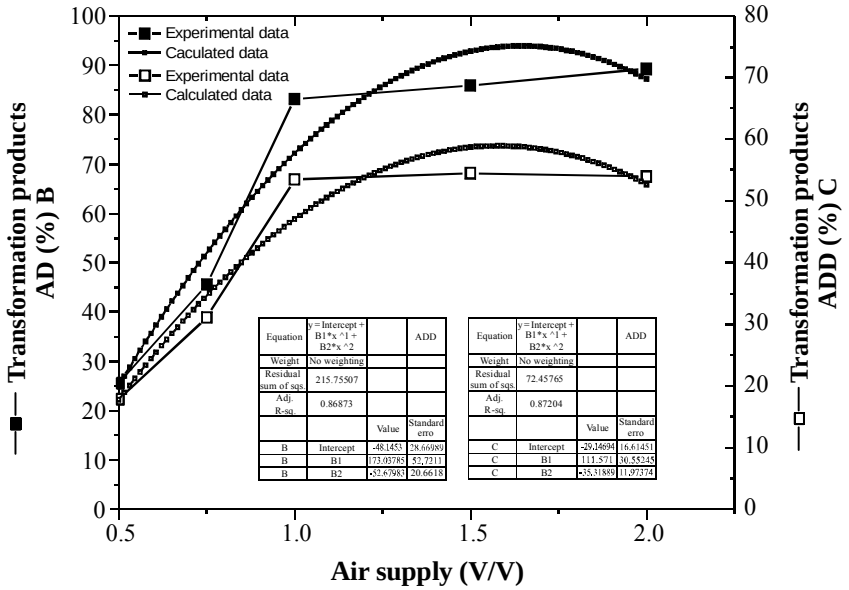



**Fig. 11: The Role of aeration rate in the product formation**

However, increasing the air supply to 1 (V/V) increased the products formation very close to their maximal. The further increase in air supply did not lead to a considerable advance and the products formation may be remained constant. The theoretical calculations predicted that, the excess of air may lead to reduction in AD an ADD formation. Smith[13] cited that the side chain degradation of sterol is an oxidation reaction hence depends on oxygen concentrations that are dissolved in the reaction media. This can explain the increasing of the products by increasing of air supply. Barberis and Segovia[19] suggested that oxygen could enhance metabolite formation if the enzymatic reaction of the product formation strongly depend on oxygen. In addition, Ingle and Boyer[20] and Milner et al.[21] observed that when the oxygen concentration falls below a critical level, cell respiration could be shifted from dissolved oxygen to the gaseous form and this decrease the growth rate.

## The catalyst reuse

Reusability of the carrier and the immobilized cells were investigated (Fig. 12). The carriers and the immobilized cells succeeded to with stand the stresses of the bioconversion process for about ten cycles. Meanwhile, the best results could be achieved during the first three cycles and gradually the cells became exhausted. Although the cell resist ten cycles of resuse but the cells may be became exhausted after repeated batches due to the toxicity of the substrate and aging of the cells.

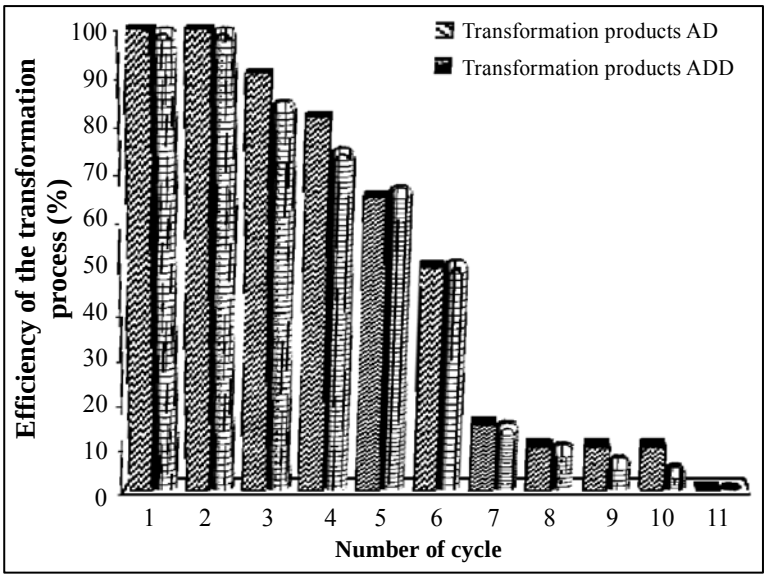



**Fig. 12: Reusability of the immobilized cells**

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