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Stainless Steel Sponge as a Carrier for Immobilization of *Rhizopus nigricans* Isolate used for Biotrnsformation of Progesterone to 11α-hydroxprogesterone

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ABSTRACT

Biotransformation is an important tool to provide the intermediate for steroid manufacturing. Stainless steel sponge was used as a carrier to immobilize an isolate of *Rhizopus nigricans* which can convert progesterone to 11α-hydroxyprogesterone (11α-HP). The conversion ability of the isolate was measured. The immobilized cells showed the ability to withstand the stress of the biotransformation process. Different parameters were studied to achieve the optimum conditions for 11α-HP production. The immobilized cells were reused for seven successive runs and maintained high bioconversion abilities during the first two runs then lost the conversion ability in seventh run.

Keyword: Biotransformation, *Rhizopus nigricans*, 11α- hydroxyprogesterone

INTRODUCTION

The Decrease in raw materials supplies of steroid and the increase in demand for anti-inflammatory agents, contraceptives**,** and sex hormones resulted in the development of alternative sources and new steroid modification methods. The presence of an oxygen atom at 11α- position of a steroid's nucleus is an obligatory requirement for the hormonal activities as carbohydrate regulation for the adrenal steroids: corticosterone, 11-dehydrocorticosterone, 17-hydroxycorticosterone, and 11-dehydro-17 -hydroxycorticosterone [1, 2]. Immobilized microorganisms have received increasing attention over the last years for the biotransformation of steroids [3, 4]. The cell immobilization has the same operational advantages as those inherent in immobilized enzymes. In addition, they offer advantages that it decreases the costly enzyme isolation and keep the enzyme in its natural environment. Compared with a system of free growing cells, the immobilization technique offer a multitude of advantages, such as high biomass**,** high metabolic activity and strong resistance to the toxic materials [5, 6, 7, 8, 9, 10, 11, 12, 13]. Moreover**,** the cells immobilization could be cost effective since they can be reused several times without significant loss of activity [14, 15] The support, carrier, selection is one of the crucial decisions to be made during the immobilization process [16]. Several natural and synthetic materials can be used as immobilization matrices. The common methods of immobilization depend on adsorption of the cells on the carriers, covalent bonding between the cells and the carries or entrapment of the cells in porous carriers. Entrapment of the cells within a porous support can be achieved under ambient conditions [17]. Stainless steel sponge has a porous structure and is a completely safe material of no toxic effect for the living cells. The aim of this study is to investigate the suitability of the stainless steel sponge as immobilization carrier during biotransformation process, 11α-hydroxylation of progesterone. An isolated strain of *Rhizopus nigricans* has a good transformation ability was used during the work.

MATERIALS AND METHODS

Microorganisms.

Seven fungal strains were isolated from soil sample with three other bacterial strains. The fungal strains were identified after comparison of its abilities to transform progesterone to 11α-hydroxyprogesterone. The identification depends on its cultural and microscopic characteristics according to Gilman, [18] and Moubasher, [19]. The experimental fungal stains were maintained as pure cultures on slants of potato dextrose agar (PDA) and incubated at 30°C for 5 days. The slants were kept refrigerated and subcultured at constant intervals (30 days).

Biotransformation by free cells.

Two milliliters of the spore suspension of each fungal strain were inoculated in fraction of 50 ml of sterilized potato dextrose (PD) in 250 ml conical flasks. The flasks were incubated on a rotary shaker at 30ºC for 48h at 150 rpm. Thereafter, 10 mg of progesterone dissolved in 1 ml of ethanol was added to each flask and the incubation conducted for 24h.

Immobilization on stainless steel sponge.

Cuttings of stainless steel sponge (made in china) of irregular size were boiled in distilled water prior to use, washed and dried at room temperature. The supports were autoclaved at 121º C for 20 min. Two milliliters of spore suspension were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose and 5 g of the sponges cuttings as an immobilization matrix. The inoculated flasks were incubated at 30ºC and shaken at 100 rpm. After 5 days of incubation the immobilized biomass of *Rhizopus*

nigricans were harvested from the medium, washed twice with distilled sterile water and stored at 4ºC to be used later.

Biotreansformation by the immobilized cells.

The reaction mixture is composed of citrate buffer at pH 6 and 10 % glucose. The biomass immobilized on stainless steel sponge was placed in 100 ml of the reaction mixture in 250 ml conical flasks, then 10 mg of progesterone dissolved in 1m ethanol was added. The flasks were incubated at 30º C for 24 hour or as specified.

Analysis of the products.

The content of each flask was extracted three times with double of its volume of chloroform. The extracts were then washed with sodium bicarbonate (2%) and dried over anhydrous sodium sulfate. The extracts were evaporated under reduced pressure till dryness and the residues is considered test materials. The transformation products present in the test materials were detected by thin layer chromatography on silica gel plates. Methanol soluble substances from the residues were subjected to dryness and dissolved in chloroform**.** The clear solutions were applied at TLC plates, then the plates were developed using solvent system (Chloroform: ethanol: water ***94:5.5:0.5)***v/v). The developed plates were then air dried at room temperature. The transformation products were identified by comparison of the specific Rf value and the colour with their corresponding authentic, both in day light and under UV rays **.** One ml of the prepared test material was evaporated till dryness in a water bath at 70ºC. The residues were dissolved in acetonitril and injected in HPLC apparatus (HP-Agilent). The apparatus runs under the following conditions : wavelength 241 nm, Flow rate 0.8 ml/min, Solvent system acetonitril : water 70:30 (v/v) **(** Shimadzu, PL Hi-Plexpb column).

The percent of the products were estimated as follows:

$$\text{HP (\%)} = \frac{\text{HP (mg)}}{\text{Totla added progesteron (mg)}} \times 100$$

$$\text{Residual Prog.} = \frac{\text{residual prog.(mg)}}{\text{Total added progest. (mg)}} \times 100$$

RESULTS AND DISCUSSION

Screening for active microorganism

Seven fungal and three bacterial isolates were screened for their ability to transform progesterone to 11α- hydroxyprogesterone. After screening the bacterial strains showed low conversion activities. The fungal strains were identified according to their morphological and microscopic characteristics. As shown in Fig. (1) *Rhizopus nigricans* was able to convert progesterone to 11α-hydroyprogesterone in higher ration than the other fungal strains. *Rhizopus* is member of order mucorales which has a common conversion ability [20].

The pH value.

To determine the most suitable pH value for the progesterone conversion the pH value of the growth medium (PD) was changed using HCl / NaOH. The pH values were changed over range of 4.5 – 7.5. The data in Fig (2) indicate the obtained results. It is clear that the optimum pH was found to be around 6. Shifting the pH near alkalinity resulted in a decrease in product formation. Sukhod et al., [21] and Ahmed et al., [22] recorded that, the optimum pH for hydroxylation reaction lies near neutrality.

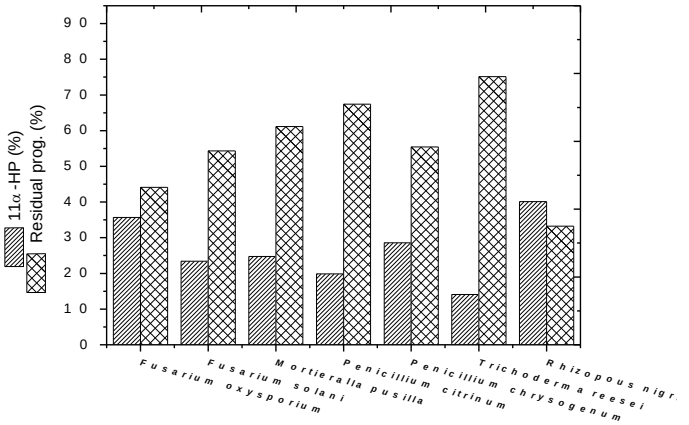

Fig (1) Bioconverion of the progesterone to 11α-HP by the isolated fungal strains

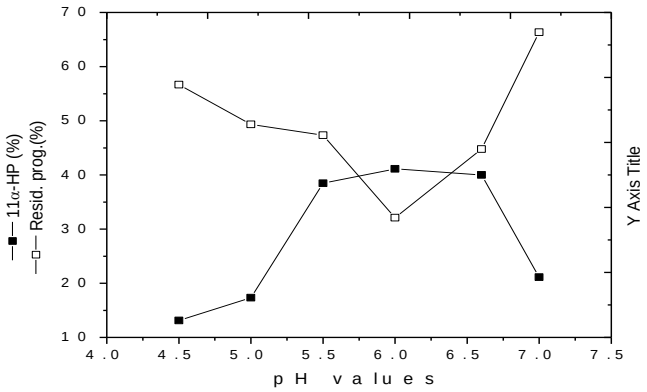

Fig. (2) Response of the 11α- HP formation to the change of the pH value for the growth medium using the free cells

Type of buffer solution.

To investigate the influence of the type of the buffer solution in the reaction mixture on the biotransforamation process, the citrate buffer was replaced by citrate-phosphate buffer and maleate buffer. pH of each buffer was adjusted at 6 (Fig. 3). There was no significant differences between the tested buffer solutions with respect to their effects on the product formation. Citrate-phosphate buffer was selected to be used in subsequent experiment where the product yield was 56.17%.

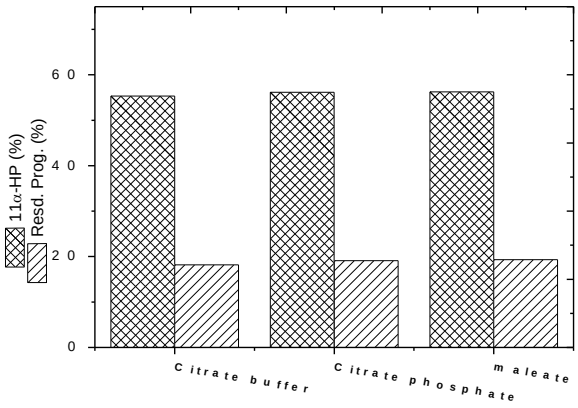

Fig (3) Effect of the type of the buffer in the reaction mixture on the 11α-HP formation using *Rhizopus nigricans*

The optimum reaction time.

By carrying out the bioconversion process at different periods of time it was found that the optimum time is 24h (Fig. 4). This results agree with that reported by Maddox et al [23] who found that the best time for production of 11α-HP was after 24h.

Consumption of glucose.

Glucose is a preferable carbon source for the living cells. Different glucose concentrations were added to the reaction mixture in order to support the metabolic activities for the immobilized cells (Fig. 5). The results have proven the negative effect of the increased glucose concentration on the product (11α-HP) formation. Glucose is preferable fast metabolized carbon source and this could affect the bioconversion of the foreign molecules. However, low concentration of glucose can support the metabolic activities for the cells and help the cells to resist the toxic effect of the substrate. αcause increase in production of 11α- HP by *R. nigricans* and to maintain the transformation ability, the cells should be kept with slow growth rate and generally with low metabolic activity by lowering the concentration of carbon source.

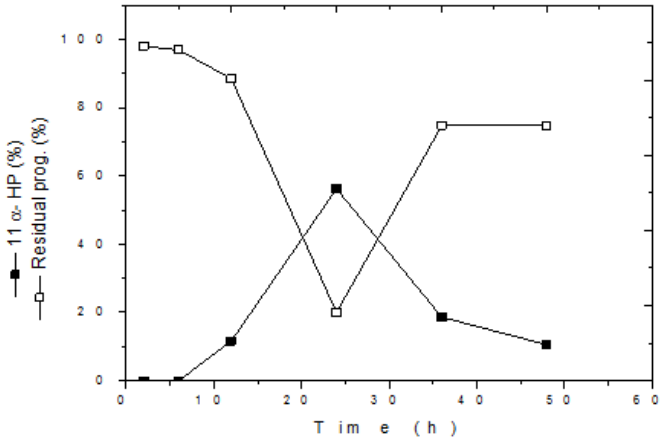

Fig. (4) production of 11-α HP at different periods of time using *Rhizopus nigricans*

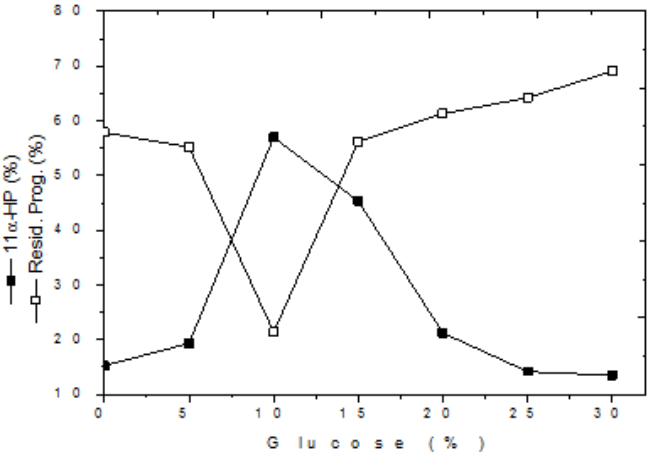

Fig. (5) The conversion of progesterone to 11-αHP after changing the glucose concentration in the reaction mixture by *Rhizopus nigricans*

Substrate concentration.

One of the difficulties of the biotransformation process is the toxicity of the substrate and the products for the living fungal cells. The cells convert the substrate as defense mechanism against its toxicity. To measure the resistance of the tested immobilized cells for the substrate toxicitym the concentration of the progesterone was changed from 5 mg/100 ml to 100 mg/100 ml as shown in Fig (6). It was noticed that raising the progesterone concentration above 15 mg/ml led to a gradual decrease of the products formation. This can be a direct result of the product/substrate toxicity. However, the immobilization matrix added a diffusion barrier allowed only by lower substrate concentration to be in direct contact with the immobilized cells. According to Perez, et al., [25] and Donova, [26] the steroids can suppress the metabolic enzyme and the growth of the microbial cells.

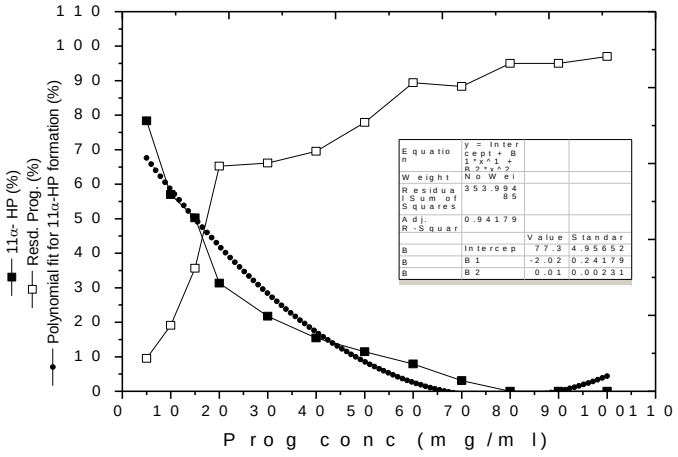

Fig. (6). The conversion of different concentrations of progesterone by *Rhizopus nigricans* in reaction mixture.

The surface active substances

The steroids very poorly soluble in aqueous media, the progesterone was added to the reaction mixture as ethanolic solution. To increase the accessibility of the substrate to the cells in the aqueous reaction mixture some surface active substances were added by concentration (0.1%). The results in Fig (7) showed that, the addition of surface active substance can improve the product yield. Tween 80 was selected as surface active substance which will be added to the reaction mixture. However, by changing the concentration of the tween 80 from 1% to 2.5% the product yield decreased from 71% to 25% (Fig.8). Simth, et al. [27] found that the surface active substance (Tweens) can increase the solubility of the steroids while high concentrations of these surface active substances can affect the permeability of the cell membrane and the protein structure of the cell enzymes and may cause the cell death and decline in the product formation.

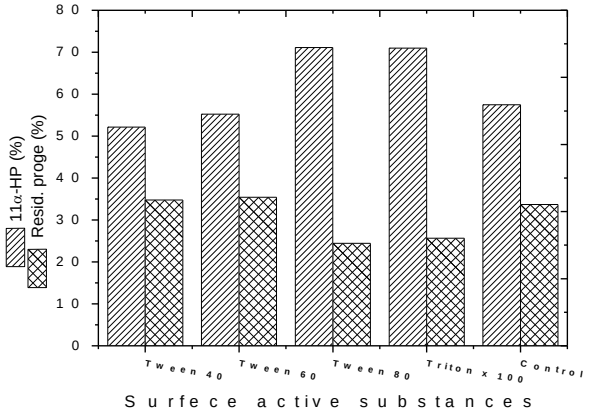

Fig. (7) The percent of the formed 11α-HP in presence of surface active substance using *Rhizopus nigricans*

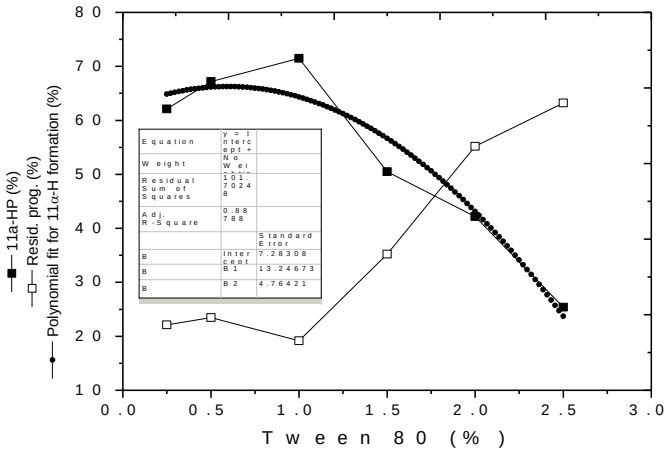

Fig. (8) Difference in 11α-HP formation by *Rhizopus nigricans* after variation of the concentration of the Tween 80

Successive reuse.

The immobilized cells were reused for seven successive cycle (Fig. 9). It was found that the immobilized cells maintained 100% of their bioconversion efficiency after two cycles. While the cells completely lost the bioconversion capacity at seventh cycle.

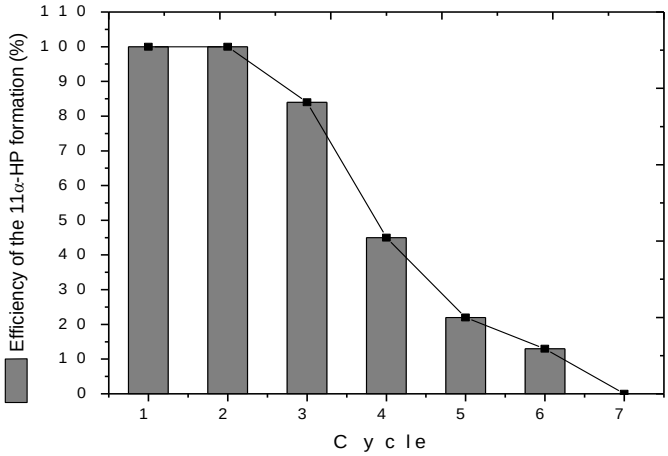

Fig. (9) Efficiency of the conversion process on successive reuse.

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