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Exploring Calcium Alginate Entrapped Fungal Mycelia for Progesterone Transformation

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Abstract: ***The influence of calcium alginate immobilized mycelia of Rhizopus oryzae on the biotransformation of progesterone (1) was investigated. Bioconversion resulted in hydroxylation of 1 at 11α- and 6β, 11α- positions. All the transformation products, namely 11α-hydroxyprogesterone (1a) and 6β, 11α-dihydroxyprogesterone (1b) were purified by chromatography techniques and identified on the basis of spectroscopic methods.***
Keywords: Biotransformation, Immobilization, Calcium alginate, Rhizopus oryzae

I. INTRODUCTION

Structurally diverse steroids are extensively used in medicine and industrial applications [1-4]. Steroids with different functional groups usually show variation in their biological activities. Consequently, modification of steroids by derivatization or introduction of new functional groups in basic steroidal skeleton is well known approach to improve their biological activities. Steroids have multi-cyclic structure with number of inactive carbons; therefore modification of steroids at specific position is a tedious process [5], [6]. Traditional synthetic route for derivatization usually involves use of hazardous chemical reagents and protection, deprotection steps which leads to overall low yield of the process. On the other side, biotransformation using various micro-organisms provides an easier and greener way for production of steroid derivatives. The whole cell biotransformation of steroids is a well established and widely studied technique [7], [8] but suffers some limitations such as recycling of biomass, difficulties in separating metabolites from fermentation media etc. In this context, immobilization of micro-organisms has received increasing attention over the last several years [9-11]. Utilization of immobilized cells overcomes few limitations of suspended cell culture biotransformation such as simple metabolites extraction, reuse of biomass, high cell concentration, flexibility in reactor design.

Many methods, namely adsorption, covalent bonding, cross-linking, entrapment and encapsulation are widely used for the immobilization of bacteria, yeast cells and fungal spores [10-12]. However, immobilization of fungal mycelia is difficult due to mycelial structure and often results in subsequent loss of many metabolic activities. Consequently, limited study has been carried out for transformation of steroids using immobilized fungal mycelia. In few earlier reports, entrapment of fungal mycelia using different media such as polyvinyl alcohol (PVA), polytetrafluoroethane (PTFE) was evaluated [13], [14]. These methods involved addition of polymeric material such as PTFE, PVA into mycelial suspension. The resulted pellets of immobilized mycelia were cut into small pieces and used for the transformation. Immobilization of fungal mycelia using polyacryl amide involved polymerization of acrylamide monomer in the broth containing fungal mycelia [10]. In 2012, Patrice Peart and co-workers have trapped fungal mycelia into the matrix of calcium alginate [15]. The comparative studies showed that calcium alginate gives a better retention in the cellular activity [16].

Considering the promise of immobilization to improve the bioconversion process, we planned to examine the transformation of progesterone (**1**) by immobilized mycelia of Rhizopus oryzae. The mild conditions and more efficiency associated with alginate method emphasized us to investigate the bioconversion utilizing alginate entrapped Rhizopus oryzae mycelia. The results of biotransformation are communicated in the present study.

II. MATERIAL AND METHODS

A. Chemicals and Microorganism

Inorganic salts and fermentation media components were purchased from Merck (India) and Himedia (India). Analytical grade solvents were obtained from Merck (India). Progesterone (**1**) was procured from Sigma-Aldrich. *Rhizopus oryzae* used in the present

study was obtained from the National Collection of Industrial Microorganisms (NCIM), Pune. It was maintained and propagated as reported earlier [17].

B. Preparation of mycelia

The fungal mycelia of Rhizopus oryzae were grown as reported earlier [17] in the modified Czapek Dox medium. In brief, 20 flasks (500 mL) each containing 100 mL sterile medium were inoculated with a spore suspension from well grown (3-5 days old) culture of Rhizopus oryzae on a potato dextrose agar slants and incubated at 30 °C on a rotary shaker at 200 rpm for 36 h. At the end of the incubation period the cells were harvested by filtration and washed with DI water to remove traces of fermentation media.

C. Immobilization of mycelia in Calcium alginate

The washed mycelia was homogeneously suspended in 50 ml of 4% sodium alginate slurry (0.25 g of mycelia/mL) at 35 ºC using a glass rod. The resulted slurry was added drop by drop into stirred 2% calcium chloride solution (500 mL). The sodium ions in sodium alginate were replaced with calcium ions and the mycelia got trapped in calcium alginate. The mycelial alginate beads were incubated in same $CaCl_2$ solution for 1 h at room temperature for hardening of beads. After 1 h the alginate beads were transferred to fresh 2% $CaCl_2$ solution and stored at 4 ºC until used.

*D. Biotransformation of progesterone (**1**) using Rhizopus oryzae trapped in alginate beads*

Fermentation was carried out at large scale for the isolation and purification of metabolites. 35 mg of progesterone (**1**) dissolved in 0.1 ml tetrahydrofuran was added into each flask containing 50 mL of 2% glucose solution and 10 g of alginate immobilized beads of Rhizopus oryzae. 20 such flasks were then incubated at 30 ºC on a rotary shaker at 200 rpm for 6 days. Control experiments were also performed with the substrate, but without microorganism and with the organism but without substrate. Reusability of alginate bead was checked by repeating the same biotransformation process using recycled beads.

For the time course experiment, 35 mg of **1** per flask was added to six flasks containing 10 g of fresh immobilized beads suspended in 50 mL sterilized DI water supplemented with 2% glucose and the reaction mixture was incubated at 30 ºC on a rotary shaker. On every alternate day, one flask was withdrawn and beads were separated from the broth by filtration. Metabolites were extracted from the filtrate and the level of transformation was monitored by HPLC at every 24 h for 12 days. The quantitation and levels of different metabolites formed were determined on the basis of area under the respective peak in chromatogram obtained using HPLC and comparing with the standard graphs obtained for individual metabolite. All the experiments were carried out in triplicates and under identical conditions.

E. Extraction of metabolites

At the end of the incubation period, the contents from all flasks were pooled and mycelial beads were separated by filtration. The filtrate was extracted with ethyl acetate (500 mL × 4). The organic layers were combined, dried over anhydrous sodium sulfate and concentrated under reduced pressure on a rotary evaporator. The crude residue obtained was subjected to column chromatography to isolate the metabolites in pure form.

F. Chromatographic procedure

The crude extract was subjected to column chromatography over silica gel (200-400 mesh) and the metabolites were eluted with methanol/dichloromethane (CH_2Cl_2) gradient (1% to 10% of methanol in CH_2Cl_2) mixture. Compounds were analyzed by thin-layer chromatography (TLC) using 5% methanol in CH_2Cl_2. TLC was performed on pre-coated silica gel 60-F254 plates (0.25 mm) and compounds were visualized by spraying with a solution of 3.2% anisaldehyde, 2.8% sulfuric acid, 2% acetic acid in ethanol followed by heating. High-performance liquid chromatographic (HPLC) analyses of metabolites was carried out on Waters 600A HPLC instrument, with X-bridge C_{18} column (4.6 × 100 mm) and methanol/water gradient at 1 mL/min, monitoring at 245 nm wavelength.

G. Spectral studies

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and CD_3OD on Bruker AC-400 at 400.13 and 100.03 MHz or on a Bruker DRX-500 spectrometer at 500.13 and 125.78 MHz respectively. Chemical shifts are reported in parts per million with respect to TMS (tetramethylsilane) as internal standard. Optical rotations ($[\alpha]_D$) were recorded using Jasco, P-1020 polarimeter and are reported in deg/dm and the concentration (c) is given in g per 100 mL in the specified solvent. Melting points were recorded on Buchi M-560 melting point instrument. Mass spectra were recorded on Q Exactive Orbitrap (Thermo Scientific).

III. RESULT AND DISCUSSION

Immobilization of fungal strain is difficult due to mycelia nature and usually involves the loss in activity during the maceration process. Considering the ease associated with calcium alginate entrapment, transformation of **1** (0.7 g/L) was carried out using Rhizopus oryzae mycelia immobilized in alginate, as described in the experimental section. The crude extract (0.83 g) obtained after incubation for six days upon TLC and HPLC analyses (Fig. 1) revealed the presence of two metabolites along with of substrate **1**. Since the transformation was carried out in the water supplemented with glucose and without any fermentation media, HPLC analysis showed that the crude extract contained minimum amount impurities.

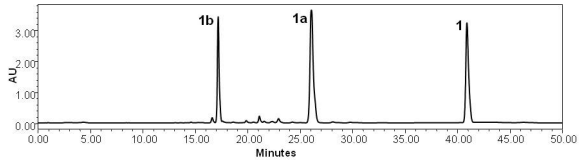
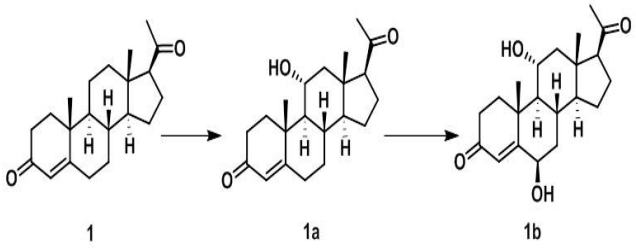


Figure 1. Transformation of progesterone (**1**) with immobilized cells of *Rhizopus oryzae*

The purification of crude extract (0.83 g) furnished two purified metabolites 1a, 1b (286 mg, 129 mg, R_f: 0.47, 0.34, R_t: 25 min, 16.5 min respectively). From HRMS and ^1H, ^{13}C NMR spectral data analyses, the compounds were characterized as 11α-hydroxyprogesterone (1a) and 6β,11α-dihydroxyprogesterone (1b) (Scheme 1). The spectral data for these compounds agreed well with earlier reports [18], [19]. 11α-hydroxyprogesterone (1a): Colorless crystals (crystallized from chloroform/methanol), mp: 165 ºC, $[\alpha]_D^{22}$: +177.86 (c=1.8, MeOH), ^1H NMR (500 MHz, CD_3OD, Me_4Si): δ 0.69 (3H, s, H-18), 1.35 (3H, s, H-19), 2.14 (3H, s, H-21), 3.96 (1H, dt, J =4.6, 10.7 Hz, H-11β), 5.72 (1H, s, H-4); ^{13}C NMR (125 MHz, CD_3OD, Me_4Si): δ 211.6 (C-20), 203.0 (C-3), 175.3 (C-5), 124.7 (C-4), 69.3 (C-11), 64.3 (C-17), 60.0 (C-10), 56.5 (C-14), 50.9 (C-12), 45.2 (C-13), 41.4 (C-9), 38.6 (C-1), 36.2 (C-8), 34.9 (C-2), 34.7 (C-6), 33.0 (C-7), 31.4 (C-21), 25.2 (C-15), 23.8 (C-16), 18.6 (C-19), 14.7 (C-18); HRMS: *m/z* 353.2065 $[M+Na]^+$ [calcd-*m/z* 353.2093].



Scheme 1. Transformation of progesterone (**1**) by Rhizopus oryzae immobilized in calcium alginate

6β,11α-dihydroxyprogesterone (**1b**): Colorless crystals (crystallized from ethyl acetate); m.p: 231-232 ºC, $[\alpha]_D^{22}$: +148 (c=0.35 MeOH), ^1H NMR (500 MHz, CD_3OD, Me_4Si): δ 0.67 (3H, s, H-18), 1.46 (3H, s, H-19), 2.10 (3H, s, H-21), 3.97 (1H, dt, J =4.6, 10.7 Hz, H-11β), 4.22 (1H, t, H-6α), 5.74 (1H, s, H-4); ^{13}C NMR (125 MHz, CD_3OD, Me_4Si): δ 211.7 (C-20), 203.6 (C-3), 171.8 (C-5), 127.2 (C-4), 73.8 (C-6), 69.3 (C-11), 64.3 (C-17), 60.0 (C-10), 56.4 (C-14), 50.8 (C-12), 45.3 (C-13), 40.6 (C-7), 40.3 (C-9), 38.9 (C-1), 35.2 (C-2), 31.4 (C-21), 29.7 (C-8), 25.2 (C-15), 23.8 (C-16), 20.3 (C-19), 14.7 (C-18); HRMS: *m/z* 369.2038 $[M+Na]^+$ [calcd-*m/z* 369.2042].

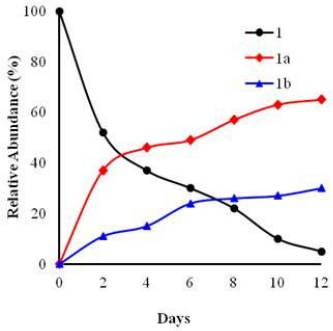


Figure 2. Time course study of progesterone (1) transformation using calcium alginate immobilized cells of Rhizopus oryzae: (●) progesterone (1), (♦) 11α-hydroxyprogesterone (1a), (▲) 6β, 11α-dihydroxyprogesterone (1b)

From the HPLC analysis of crude extract, it was revealed that after six days of incubation the entrapped Rhizopus oryzae transformed 1 into hydroxylated metabolites which are identical to that with suspended cell culture. The retention of transformation activity up to 70% was also observed (Fig. 1). During the standardization of immobilization procedure, it was observed that the suspension of mycelia into sodium alginate can be obtained more easily at slightly higher temperature of about 35 ºC without loss in enzyme activity. In addition, the transformation study carried out using the beads stored at 4 ºC for different time interval revealed that there was no considerable change in the transformation activity of immobilized beads even after one month storage of beads at 4 ºC.

Time course experiment carried out using 1 as substrate with the immobilized mycelia revealed that, during early stages of incubation (two days) nearly 50% of 1 was transformed into metabolites 1a and 1b. When the incubation period was extended for 12 days, 11α-hydroxyprogestrone (1a) was obtained as a major metabolite (65%), with only 5% unconsumed 1 left out in the fermentation flask (Fig. 2). With immobilized mycelia, the percentage of dihydroxy derivative 1b has increased only up to 30%, even after an increase in the incubation period to 12 days.

In conclusion, we have standardized the procedure for the transformation of progesterone using mycelia of Rhizopus oryzae entrapped in calcium alginate and it could be extend further for production of different hydroxy steroids on a large scale as well as over a wide range of mycelial fungal strains.

IV. ACKNOWLEDGEMENT

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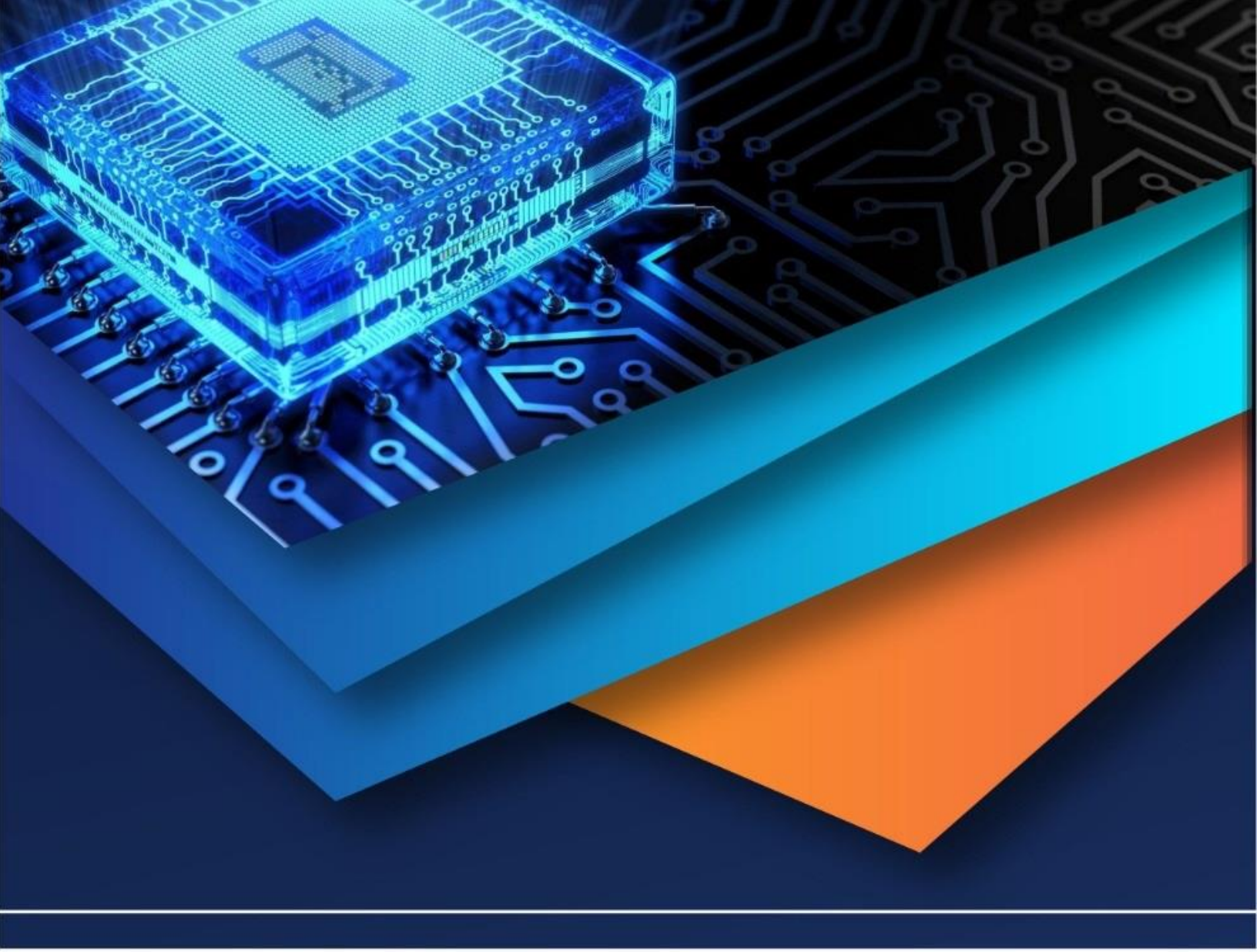

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