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Influence of whole microalgal cell immobilization and organic solvent on the bioconversion of androst-4-en-3,17-dione to testosterone by *Nostoc muscorum*

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The use of free, immobilized and reused immobilized cells of the microalga *Nostoc muscorum* was studied for bioconversion of androst-4-en-3,17-dione (AD) to testosterone in hexadecane. Among polymers such as agar, agarose, κ-carrageenan, polyacrylamide, polyvinyl alcohol, and sodium alginate that were examined for cell entrapment, sodium alginate with a concentration of 2% (w/v) proved to be the proper matrix for *N. muscorum* cells immobilization. The bioconversion characteristics of immobilized whole algal cells at ranges of temperatures, substrate concentrations, and shaking speeds were studied followed by a comparison with those of free cells. The conditions were 30 °C, 0.5 g/L, and 100 rpm, respectively. The immobilized *N. muscorum* showed higher yield (72 ± 2.3%) than the free form (24 ± 1.3%) at the mentioned conditions. The bioconversion yield did not decrease during reuse of immobilized cells and remained high even after 5 batches of bioreactions while Na-alginate 3% was used; however, reuse of alginate 2% beads did not give a satisfactory result.

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1. Introduction

Whole cell biocatalyst activity and stability during bioconversion reactions may be influenced by parameters such as temperature, shaking speed, and substrate concentration [1], which can be reduced with cell immobilization techniques. The main advantages claimed for applying immobilized whole cells include the higher reaction rates due to increased cell densities, possibilities for regenerating the biocatalytic activity, ability to conduct continuous operations at a high dilution rate without washout, easier control of the fermentation process, long-term stabilization of cell activity, reusability of the biocatalyst, and higher specific yields [2–5]. The combination of cell immobilization methods and organic solvent systems in biotransformation processes decreases the difficulties of the low solubility of high concentrations of lipophilic precursors and products, and provides environmental protection for biocatalysts [6].

The use of immobilized cells for bioconversion of steroids in organic media is a valuable method to increase the solubility of poor water-soluble steroid compounds. However, the critical point is the toxicity of solvents, which may lead to the disruption of the cell membrane, denaturation of membrane-bound enzymes, and cytolysis [4,5]. Although much work has been reported on the utilization of immobilized bacteria and fungi in organic media for the conversion of organic compounds [1,7,8], no data are available for the application of both techniques for the biotransformation of steroids by microalgae [9].

The use of algal biotechnology has received considerable attention in recent decades. These photosynthetic organisms have been used in food, cosmetics, aquaculture, and pharmaceutical industries [10]. However, the small size of microalgal cells implies a problem in the application of biotechnology processes to those organisms. Microalgal cell immobilization has been developed to solve the mentioned difficulties.

In the this study, we applied immobilized microalga *Nostoc muscorum* for biotransformation of androst-4-en-3,17-dione (AD) to testosterone in an organic medium. In our previous study, various organic solvents were examined to find out the proper organic media that caused no toxicity effect on *N. muscorum* cells [11]. Hexadecane and tetradecane proved to increase the solubility of the lipophilic steroid substrate and enhanced enzyme activity. To mask the inhibition effects of the solvent and substrate toxicity and provide the possibility of reusing the algal cells, we tried to immobilize *N. muscorum* by entrapment methods in different matrices. Appropriate bioreaction conditions such as temperature, agitation rate, and substrate concentration were also studied to maximize the bioconversion yield.

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2. Materials and methods

2.1. Chemicals and instruments

Androst-4-en-3,17-dione (AD) and κ-carrageenan were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Agar, agarose, polyvinyl alcohol, and polyacrylamide were obtained from Merck (Darmstadt, Germany). Sodium alginate was supplied by B.D.H. Chemical Ltd. (Poole, England). All other reagents and solvents were of the highest purity grade available (Merck, Darmstadt, Germany) unless otherwise mentioned.

The high-performance liquid chromatography (HPLC) apparatus consisted of a Jasco model PU-986 pump, a UV-1570 UV variable-wavelength detector, and an online degasser, all from Jasco (Tokyo, Japan). Samples were injected in a Jasco AS-950 injector system with an auto-injector. The data were acquired and processed with Borwin chromatography software (version 1.5) from Jasco (Tokyo, Japan). Chromatographic separation was achieved on a Finepack SIL C18 reverse-phase column (C18, 15 cm × 0.46 cm i.d., 5-μm particle size) from Teknokroma (Barcelona, Spain).

2.2. Algal strain and incubation condition

An axenic culture of *N. muscorum* [12,13] was grown in BG-11 medium [10] and maintained at 4 °C on BG-11 agar slants. The BG-11 medium contained (g/L) $NaNO_3$, 1.5; K_2HPO_4, 0.04; $MgSO_4{\cdot}7H_2O$, 0.075; $CaCl_2{\cdot}2H_2O$, 0.036; ferric ammonium citrate, 0.006; citric acid, 0.006; Na_2EDTA, 0.001; Na_2CO_3, 0.02; trace element solution, 1 mL; distilled water up to 1000 mL. The trace element solution contained (g/L) H_3BO_3, 2.86; $MnCl_2{\cdot}4H_2O$, 1.81; $ZnSO_4{\cdot}7H_2O$, 0.222; $Na_2MoO_4{\cdot}2H_2O$, 0.3; $CuSO_4{\cdot}5H_2O$, 0.079; $Co(NO_3)_2{\cdot}6H_2O$, 0.494; distilled water up to 1000 mL.

The alga was transferred to fresh medium every two months. To prepare adequate cell mass, *N. muscorum* was inoculated into two 500-mL Erlenmeyer flasks, each containing 100 mL of BG-11 liquid medium (pH 7.0), and incubated at 25 °C under continuous illumination from all sides at an irradiance of 60 μmol photons/m^2 s^1 with cool-white fluorescent lamps. Cells were harvested in the late exponential phase and centrifuged at 2.0×10^3 *g* for 5 min. Each 5-mL packed wet cell volume [14], which is equal to 100 mg dry cell weight, was used to inoculate a 100-mL Erlenmeyer flask containing 20 mL of organic solvent. The control aqueous biotransformation medium was prepared using the same procedure except that the organic system was replaced by 20 mL of BG-11 medium.

The viability of algal cells in the presence of hexadecane was proved by the cells appearance and re-culturing in fresh BG-11 medium. The optimum reaction temperature and substrate concentration were 30 °C and 0.5 mg/L, respectively.

2.3. Immobilization technique

According to a recent study [11], hexadecane with log $p_{octanol}$ 8.2 is the proper organic medium for biotransformation of AD to its single reductive derivative by the alga *N. muscorum*. Therefore, the following immobilization experiments in the selected matrices were carried out in hexadecane under the conditions mentioned above (see Section 2.2). In all experiments, free whole cells of *N. muscorum* were used as control. Before each immobilization experiment, algal cells were harvested in the late exponential phase, centrifuged at 2.0×10^3 *g* for 5 min and provided a 5-mL packed wet cell volume.

2.3.1. Cell entrapment in agar and agarose

The reaction mixtures were separately prepared by dissolving agar or agarose (2%, w/v) in distilled water at 100 °C, and then cooled at 40 °C and mixed with an equal volume of packed algal cell volume. The mixtures were placed in Petri dishes and allowed to solidify by standing at 4 °C. Hard gels were cut into small pieces and entrapped cells removed by washing with distilled water [15].

2.3.2. Alga entrapment in κ-carrageenan

A defined portion of *N. muscorum* cells was mixed with the same volume of κ-carrageenan solution (2%, w/v). The resulting mixture was pumped through a needle (30-mL syringe, fitted with a wide-bore needle approximately 2 mm diameter for droplet formation) into a 2% (w/v) potassium chloride solution. The formed beads containing algal cells were gently mixed with a magnetic stirring bar for 1 h at room temperature. The beads were washed with distilled water before use [16].

2.3.3. Acrylamide entrapment

N. muscorum cells (200-mg wet weight equal to double the packed cell volume) were added to 6 mL of Tris–HCl buffer, pH 7.8, containing 105 mg of acrylamide and 3 mg of bis-acrylamide. The mixture was added drop by drop using the needle mentioned before into 100 mL of mineral oil containing 84 mg of ammonium persulfate and 84 μL of tetramethylethylenediamine (TEMED). Polymerization was performed at room temperature for 24 h. The formed beads were washed with sterile water to remove undesired residue [17].

2.3.4. Sodium alginate immobilization

Algal cells were mixed homogenously with the same volume of sodium alginate solution (2%, w/v). The mixture was added into 0.2 M of cold calcium chloride solution by a syringe and left to harden for 1 h. The alginate beads were washed with distilled water before use [3].

2.3.5. Polyvinyl alcohol (PVA) immobilization

The reaction mixture was prepared by dissolving PVA (15%, w/v) in distilled water at 100 °C. After cooling down to 30–40 °C, one portion of PVA aqueous solution was mixed with an equal volume of packed algal cells. The resulting mixture was put drop wise into the saturated boric acid solution through a needle and gently stirred for 1 h to form spherical beads. The PVA beads were screened with a mesh, and then rinsed with tap water to remove the residual boric acid. The formed gel beads were transferred to a 1-M sodium phosphate solution pH 7 for 30 min to harden. The phosphorylated beads were separated by filtration and rinsed with distilled water [18].

2.4. Bioconversion

The prepared beads of the immobilized algal cells were separately suspended in 100-mL Erlenmeyer flasks; each containing 20 mL of BG-11 medium for the aqueous system and 20 mL of hexadecane for the organic mono-phase system. A defined amount of AD, 10 mg, dissolved in 100 μL of chloroform was added to each flask. Free whole cells of *N. muscorum* were used as control. The flasks were incubated on a rotary shaker at 30 °C with a shaking speed of 100 rpm. After 5 days, the samples were taken followed by AD and testosterone analysis by HPLC. To validate the reproducibility of the data, each bioconversion run was carried out in triplicate using different batches.

2.5. Quantitative analyses

HPLC was applied for quantitative studies to obtain bioconversion yield in aqueous and organic systems as well as time course study, influence of temperature, substrate concentration, and shaking speed. In this order, each 100 μL of samples was diluted with 400 μL of acetonitrile (ACN). A total of 10 μL was injected into

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