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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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ABSTRACT

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.

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

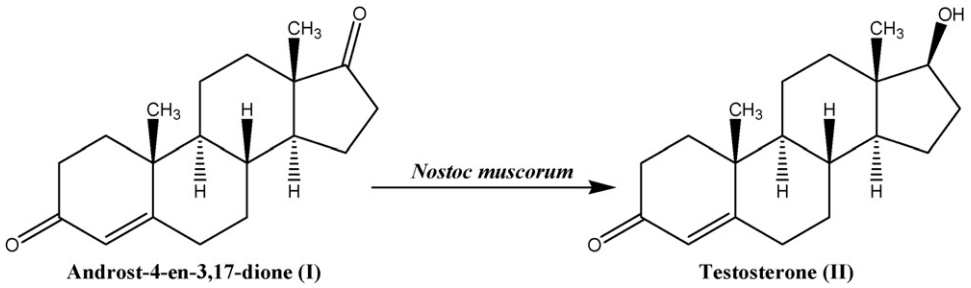

Fig. 1. Algal transformation of AD (I) to testosterone (II) by *N. moscurom*.

a C18 reverse-phase column. The mobile phase was water/ACN with a gradient of 0–100% ACN over 33 min and a flow rate of 1 mL/min. Detection was done by UV at 254 nm. The starting material and product were separated by HPLC with retention times of 21 and 23 min, respectively. No other metabolite was detected in the transformation medium. The validation method has been described previously [11]. The product's relative peak area was calculated and the amount of residual substrate was determined quantitatively by subtracting from the quantity of starting material.

2.6. Reuse of immobilized cell beads

The reusability of immobilized biocatalysts is often expected. Therefore, immobilized *N. muscorum* cells were examined for this purpose. After the maximum production of testosterone was attained in 5 days, the beads were harvested, washed with distilled water, and added to fresh medium that included 20 mL of hexadecane for the next use. The process was repeated for 7 batches until the product formation decreased. Each bioreaction was conducted in 20 mL of hexadecane supplemented with 10 mg of steroid substrate at 30 °C and an agitation rate of 100 rpm for 5 days.

3. Result and discussion

Bioconversion of the starting material (I) by immobilized *N. muscorum* cells led to accumulation of the sole bio-product (II) in the algal medium (Fig. 1).

3.1. Selection of immobilization method

Immobilized algal cells entrapped in the applied polymers of agar, agarose, κ-carrageenan, polyacrylamide, polyvinyl alcohol, and sodium alginate were compared for their bioconversion ratios. Table 1 shows the effect of each immobilizing carrier on testosterone production. Na-alginate entrapped cells could effectively convert AD with a yield of 72 ± 2.3%, whereas the other matrices showed lower yields. Since agar, agarose, κ-carrageenan, and polyvinyl alcohol gels (with bioconversion yields of 25 ± 1.8%, 19.8 ± 1.2%, 31 ± 3.1%, and 3.6 ± 0.6%, respectively) are formed by lowering the temperature, the strength of the resulting gels is somewhat lower. The additional disadvantage is due to the difficulty in the preparation of the beads, and many of the cells were lost or damaged due to the rather high temperature necessary to keep the gels molten. The toxicity of the acrylamide matrix affects algal cell viability, and most of them are lost (Table 1). In contrast, Na-alginate gel presents a higher suitable rigidity, and the preparation of the beads is quite easy. Thus, Na-alginate was selected for the entrapment of *N. muscorum*.

Table 1
Influence of type of matrices used for cell immobilization on the bioconversion yield. Bioconversion was carried out in a 100-mL Erlenmeyer flasks containing 20 mL hexadecane at 30 °C and 100 rpm for 5 days. The cell volume of immobilized alga was 5 mL packed cell volume and substrate concentration was 10 mg/20 mL. The conversion percentage is expressed as the value as testosterone produced per initial substrate.

Type of matrix	Testosterone concentration (g/L)	Residual AD (g/L)	Conversion (%)
Whole free cell	0.12	0.38	24 ± 1.3
Agar	0.125	0.375	25 ± 1.8
Agarose	0.099	0.401	19.8 ± 1.2
κ-Carrageenan	0.155	0.345	31 ± 3.1
Polyacrylamide	0.0	0.50	0.0
Polyvinyl alcohol	0.018	0.482	3.6 ± 0.6
Sodium alginate 2% (w/v)	0.36	0.14	72 ± 2.3

3.2. Optimum concentration of alginate beads

The concentration of utilized sodium alginate varied from 1% to 4%. As shown in Table 2, a yield of 72 ± 2.3% of testosterone was achieved when beads containing 2% alginate were applied. Higher concentrations resulted in yield reduction due to the difficulty of substrate accessibility to the immobilized cells. Beads containing 1% (w/v) Na-alginate also resulted in yield reduction due to the formation of weak beads, with the escape of the cells.

3.3. Culture condition for biotransformation with alginate immobilized cells

Bioconversion of AD to testosterone was studied under temperatures between 25 and 55 °C with an interval of 5 °C employing alginate immobilized *N. muscorum* cells. When the temperature was increased gradually from 25 to 55 °C, the highest yields of transformation were obtained at 30 °C for both immobilized and free cells. However, the immobilized cells were more stable at higher temperatures than the free cells (Fig. 2). This is probably due to significant water evaporation in the free wet cells compared to that of the immobilized cells. The decrease in the water layer surrounding bio-molecules, which is essential for their activity, may be deleterious to the cells.

Table 2
Influences of Na-alginate concentrations on the bioconversion of AD to testosterone. Bioconversion was carried out in a 100-mL Erlenmeyer flasks containing 20 mL hexadecane at 30 °C and 100 rpm for 5 days. The cell volume of immobilized alga was 5 mL packed cell volume and substrate concentration was 10 mg/20 mL. The conversion percentage is expressed as the value as testosterone produced per initial substrate.

Sodium alginate concentration (%, w/v)	Testosterone concentration (g/L)	Residual AD (g/L)	Conversion (%)
1	0.09	0.41	18 ± 1.6
2	0.36	0.14	72 ± 2.3
3	0.16	0.34	32 ± 1.5
4	0.105	0.395	21 ± 0.6

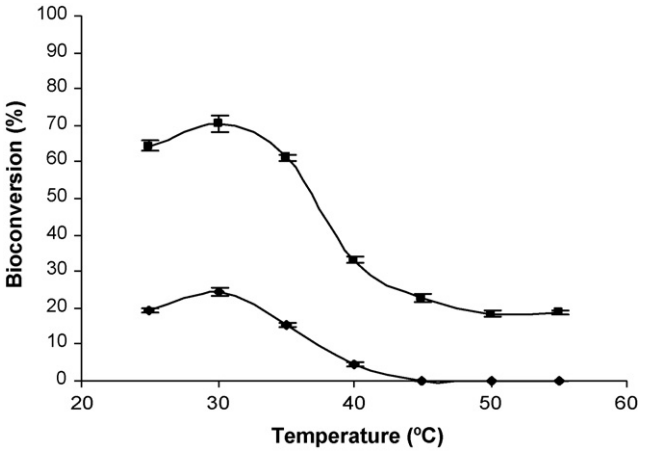

Fig. 2. Effect of temperature on production of testosterone by alginate (2%, w/v) immobilized *N. muscorum* cells. Bioconversion was carried out in 20 mL hexadecane supplemented with 10 mg steroid substrate at an agitation rate of 100 rpm for 5 days. (■) Immobilized cells and (▲) free cells.

A similar observation was found in the literature [19–23]. Entrapment is the physical confinement of enzymes within microspaces formed in matrix structures. In this method, enzymes do not chemically bond to polymeric matrices. Therefore, the three-dimensional structure of the enzymes may not be affected by the immobilization procedure, and thus, the optimum parameters of immobilized and free-form cells may be identical [21,23,24].

Similar to the free cells, the optimum substrate concentration that could be maximally biotransformed to product was found to be 10 mg/20 mL. Further increase in substrate concentration decreased the product formation; however, the immobilized cells appeared to be less affected than the free cells. It is probably due to immobilization protection against substrate toxicity (Fig. 3).

The optimum incubation time for the production of testosterone for immobilized cells was the same as for the free cells, and the highest yield of testosterone was achieved after 5 days in both the free and immobilized cells (Fig. 4).

External mass transfer limitation can be prevented by a suitable hydrodynamic condition. This experiment reflects, to a certain extent, the stability of the biocatalyst against the mechanical stress caused by shaking. An increase in the shaking speed from 50 to 100 rpm increased the yield from 44.3 ± 0.78% to 72 ± 1.8% (Fig. 5).

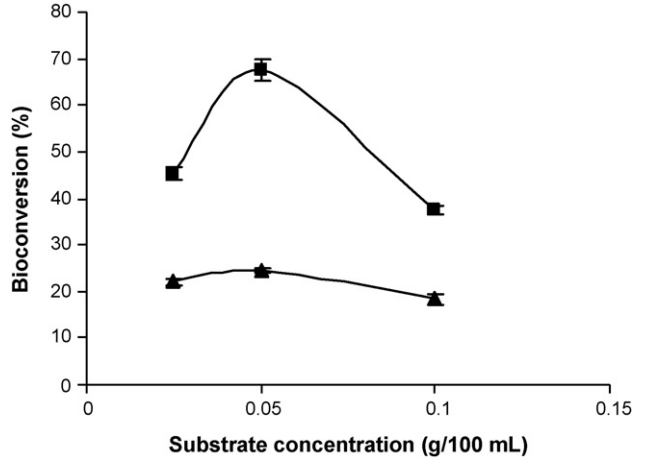

Fig. 3. Effect of AD concentrations on the production of testosterone by alginate (2%, w/v) immobilized *N. muscorum* cells. Bioconversion was performed in 20 mL hexadecane supplemented with steroid substrate at 30 °C and an agitation rate of 100 rpm for 5 days. (■) Immobilized cells and (▲) free cells.

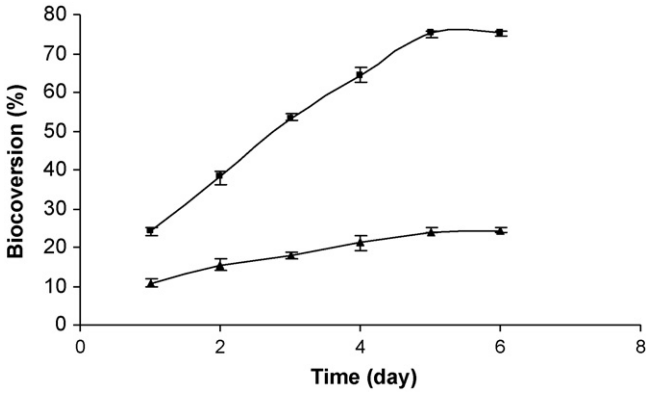

Fig. 4. Effect of incubation time on the production of testosterone by alginate (2%, w/v) immobilized *N. muscorum* cells. Bioconversion was carried out in 20 mL hexadecane supplemented with 10 mg steroid substrate at 30 °C and an agitation rate of 100 rpm. (■) Immobilized cells and (▲) free cells.

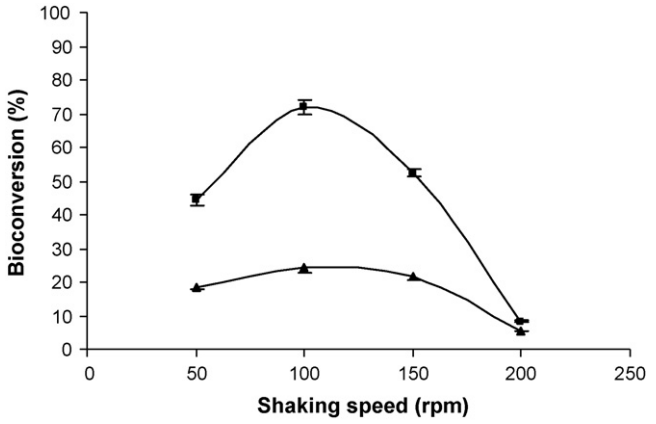

Fig. 5. Effect of shaking speed on the production of testosterone by alginate (2%, w/v) immobilized *N. muscorum* cells. Bioconversion was performed in 20 mL hexadecane supplemented with 10 mg steroid substrate at 30 °C for 5 days. (■) immobilized cells and (▲) free cells.

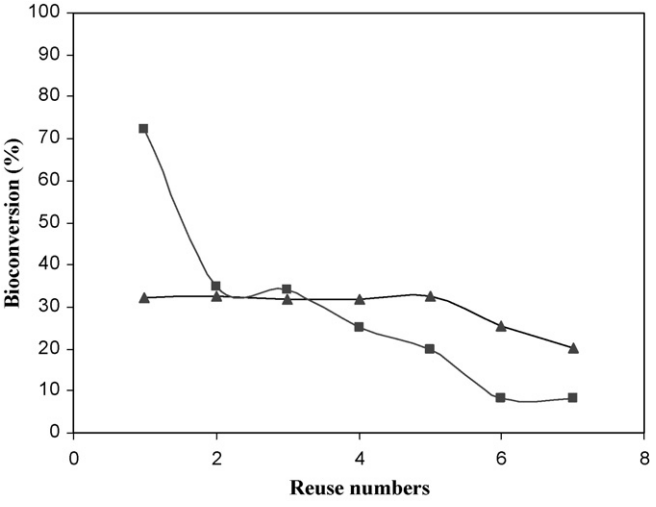

Fig. 6. Reusability of immobilized *N. muscorum* cells for 7 times. Each bioreaction was conducted in 20 mL of hexadecane supplemented with 10 mg steroid substrate at 30 °C and an agitation rate of 100 rpm for 5 days (■) Na-alginate, 2% and (▲) Na-alginate, 3%.

Further increase in the shaking speed caused a decrease in the amount of formed testosterone. Increasing the shaking speed up to 200 rpm probably caused cracks in the membranes. This phenomenon may be explained by the variation in the susceptibility of different microalgae to hydrodynamic forces caused by shaking and the spontaneous agglomeration of hydrogel particles in non-polar organic solvents [25]. The latter was previously reported for hexane, and considerable abrasion of the gel matrix was observed following the stirring or shaking used for the particles dissociations [26–28].

3.4. The reusability of immobilized algal cell beads

Immobilized cells on alginate beads (3%) were reused in five successive reaction cycles (each cycle lasted 5 days) without any loss of biocatalytic activity (Fig. 6). Reuse of Na-alginate 2% beads did not lead to satisfactory results. This is probably due to the influence of solvent toxicity on the alga when weaker beads of Na-alginate 2% are used. Freely suspended cells were difficult to handle in repeated cycling, and resulting in inconsistent recovery.

4. Conclusion

N. muscorum cells were immobilized in various gelling media in order to characterize the bioconversion of AD to testosterone. Among the polymers tested, Na-alginate was the proper material for whole cell immobilization of *N. muscorum*. Alginate 2% beads in the organic phase system effectively transformed AD to testosterone, and showed that immobilization of the *N. muscorum* cells improved the transformation percentage (ca. 72% vs. 24%). This observation was in agreement with Rao's [3] results in *Spirulina platensis* cultures, Mahmoud and El Sayed [29] results in the bioconversion of benzaldehyde to L-phenylacetyl carbinol by *Saccharomyces cerevisiae* cells immobilized in alginate beads with 90% yield in comparison with 10% yield from free cells, and Tripathi et al. [8] results in the biotransformation of phenylpropanoid compounds to vanilla-flavor metabolites in culture of *Haematococcus pluvialis*.

A major advantage of alginate gel is that the cells do not suffer extreme physicochemical stress during the immobilization process. In addition, the pH of the alginate solution is close to 7, and the polymerization process does not shift the pH inside the matrix, which is an important factor in the survival of microalgae. The permeability, null toxicity, and transparency of the formed matrix imply a very gentle environment for immobilized cells. Also, entrapment of cells in alginate is the simplest and cheapest method of immobilization. By checking different conditions we came to the point indicated that the immobilized cells are more stable at higher temperatures than free cells and more stable against mechanical stress [30,31]. The combined use of hexadecane as the organic medium and alginate as the immobilization matrix significantly increased the bioconversion yield in comparison with the use of free cells in hexadecane. This probably results from the protective effect of immobilization against organic solvent toxicity. Reuse of immobilized beads (5 times) gave satisfactory results without decreasing the enzyme activity and reduced the cost of cell recovery and recycling.

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