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Role of the biomass and pelleted forms of *Aspergillus ochraceus* ATCC 3150 on the 11α-hydroxylation of progesterone

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ABSTRACT

The formation of non-coagulative type of pellet could be induced in the submerged cultivation of *Aspergillus ochraceus* ATCC 3150. The results showed that the pellet size and form were found to be varied with inoculums size and agitation speed changes where, the maximum biomass was obtained at high inoculum ($1x10^7$ spore/mL) and high agitation (200 rpm). The differences in the pellets morphology results in changes in their physiology which reflected by its ability to transform progesterone to the 11α-hydroxyl derivative. The maximum 11α-hydroxy-progesterone out put (67.5%) could be obtained at inoculums size ($5x10^6$ spore/mL), agitation speed 150 rpm, and 28 °C at transformation time 48 h.

Keywords: biomass, pellet formation, *Aspergillus ochraceus*, 11α-hydroxylation

INTRODUCTION

The most important steroid biotransformation reaction is the hydroxylation reaction of the steroid nucleus. The highly important hydroxylation reaction is the 11α-hydroxylation of progesterone, which is one of the main steps required during the production of some medicinal and therapeutically useful corticosteroid compounds eg. cortisol derivatives (Hanisch *et al.*,1980; Shuvendu *et al*., 2002; Sabina *et al.*, 2008).

The growth characteristics developed during the submerged cultivation of filamentous microorganisms, can be either in a dispersed form or it conform aggregates of different forms. Spherical and stable mycelial pellets, were found to be a branched and intertwined network of hyphae (Braun and Vecht-lifshitz, 1991).

The filamentous fungi specially Mucorales were consider to be active hydroxylator. The biotransformation process depends on the type of the microorganism used, substrate, fermentation time as well as the forms of the organism used (spore, enzyme, mycelium). The improvement of the 11α-hydroxylation reaction could be achieved by induction techniqe or use of an immobilized biocatalyst (Hanisch *et al*., 1980). However, the alternative method for increasing the transformation yield is the reuse of biomass in a repeated batch process (Maddox *et al*., 1981).

The aim of the present investigation is to study the effect of the different growth conditions on the 11α-hydroxylation of progesterone using *A. ochraceus* ATCC 3150.

MATERIALS

Microorganism

The microorganism used in the current work *A. ochraceus* ATCC 3150 was obtained from the Natural and Microbial Products Chemistry Department, National Research center (NRC), Dokki, Cairo, Egypt.

Chemicals

The authentic steroids (progesterone, 11α-hydoryprogesterone) were obtained from Sigma Company. The other chemicals used in the current work were highly purified grade obtained from (Merck). All the solvents used were HPLC grade.

METHODS

Maintenance of the microorganism

The maintenance of the used microorganism was carried out using the following medium (g/L): glucose, 20; peptone, 6; yeast extract, 5.7; NaCl, 4; K_2HPO_4, 2 and Tween 80, 0.5 mL; pH was adjusted to 5.6 at 30 °C, with monthly intervals regenerated. Spore suspension was prepared by adding 5 mL of sterile 0.9% saline to each slant from which a given concentration was used.

Mycelium analysis

Biomass dry weight determination was performed by a standard procedure, described by Michel *et al*., (1992). The morphology of the culture was determined by examining aliquots on Petri dishes, replicate of cultures samples were photographed and weighed for biomass dry

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weight determination Trdic *et al.,* (1996). The average dry weight of a single pellet was determined from the dry weight and particle number of the photographed sample.

Transformation process

The progesterone bioconversion was achieved according to a modified method of Vitas *et al.*, (1994), where the microorganism incubated in 250 mL Erlenmeyer flasks containing 100 mL of phosphate buffer (0.75 mM Na_3PO_4; 0.75 mM NaH_2PO_4; 0.21 mM EDTA pH 5.5) and supplemented with 1% w/v glucose. Progesterone 0.1 g/L dissolved in absolute ethanol was added to each flask. The flasks were incubated on rotary shaker at different agitation speed (100, 150 and 200 rpm) were used at 28 °C for 48 h.

Analysis of steroid products

The biomass was removed from the reaction mixture by filtration and the filtrate was extracted using chloroform as described by Vitas *et al.*, (1995). The resulting solid extracts were re-dissolved in acetonitrile and assayed using HPLC (Rnauer, Berlin, Germany).

Five micro liter sample of authentic or sample were injected to C_{18} column employing a gradient elution of the mixture acetonitrile:water from 40:60 (v/v) at flow rate of 0.5 mL/min. Absorbance detection was at 254 nm and steroid concentration were determined by comparison with standards of progesterone and its 11α-hydroxyl derivative (Merck, Darmstadt, Germany).

Determination of growth kinetic

The biomass growth rate constant (K_c) was evaluated form the linear part of the curve according to Pirt, (1966) and Bailey and Ollis, 1986.

$$X_1 = X_o + K_c.t$$

Where, X_1 is biomass concentration and time, t (g/L). X_o is the biomass concentration at time t=0 and K_c is the biomass growth rate constant (g/L.h), and the constant regression coefficient R^2 was estimated according to the following equations.

$$R^2=X_1/(X_1-X_0)K_c$$

RESULTS AND DISCUSSION

Growth at different agitation speeds

The growth medium was inoculated with $3x10^4$ spore/mL and the cultivation were carried out at 30 °C and at different speed 100, 150, and 200 rpm. The microscopic observations revealed the spore germination with no agglutinated spores a few hours after inoculation at low agitation speed, loose and fluffy pellets are formed, while at the same inoculum size and faster shaking the agglomerates of hyphae were much more dense and smooth. The results presented in Figure 1 showed that the biomass yields were varied according to the agitation speed.

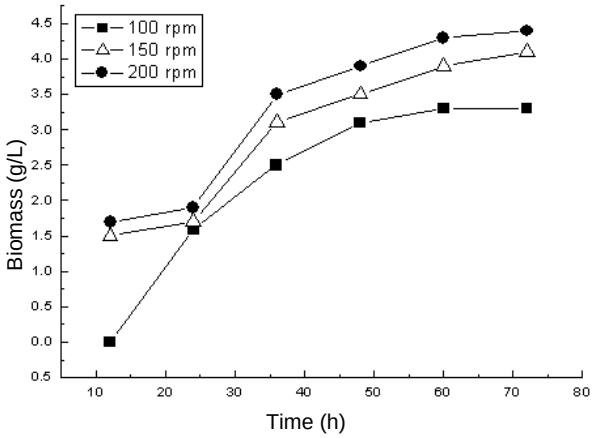

Figure 1: Growth of *A. ochraceus* at different agitation speed

At the low agitation 100 rpm, low biomass was obtained at 14 h in comparison to the yields at 150 rpm and 200 rpm. However, the biomass increases as the growth period increased at all agitation speed used until the beginning of the stationary phase (50 h) in our case. On the other hand, the longer periods accompanied with nearly steady growth was noticed at all the agitation speed used.

The biomass growth rate constant K_c was at 100 rpm agitation speed 0.045, R^2 (0.95), and were 0.033 (R^2= 0.92) at 150 rpm and 0.048 g/L.h (R=0.97 at 200 rpm) (Michel *et al.*, 1992). Furthermore, the duration of growth phase was longer at higher agitation resulted in higher biomass yield in the stationary phase.

2-Progesterone bioconversion in relation to biomass

A given number of spores from slants of the tested microorganism as stated in Table 1 were used to inoculate the growth medium and incubated on a rotary shaker at 30 °C and at the three agitation speeds for 72 h. The results showed that a direct proportional relationship between the spore concentration and biomass, which accompanied by a homogenous suspension of mycelium which confirmed the conclusion of non coagulative type of pellet formation. The coagulative type of pellets formation it were formed at low spore concentration (Metz and Kossen, 1977).

On the other hand, the results presented in Table 2 and Figure 2 clearly indicated that the bioconversion capability of the selected microorganism was affected by the inoculum size and the agitation speed. The maximum 11α-hydroxyprogesterone output (66.7%) was obtained at 5x106 spore/mL and agitation speed 150 rpm. These results correlated with that stated by Michel *et al.*, (1992), which indicated that the oxygen requirements for growth in relation to agitation speed was obviously affect on the pellets form and its physiological characteristics.

Sugar residual

The growth of microorganism depends on many factors involving the fermentation medium constituents used, i.e. pH, time as well as temperature. The composition of the fermentation medium affect on the microbial growth, the main target is the carbon source used (Abd-Elsalam, 2004).

The results represented in Figure 3 showed that variation on total sugar content differ according to the agitation used. At the agitation speed of 200 rpm, high sugar consumption rate was observed, which was reflected by a low residual sugar content. On the other hand, at lower agitation speed a reduction in the sugar consumption rate was observed, which was reflected by higher residual sugar. As the fermentation time increased more consumption of sugar appeared at all the agitation speed used until minimum value at the end of fermentation, these results agree with the finding by Trinci, (1970).

pH changes

The present study deals with the pH variations as a result of the microbial growth at different agitation speed. The results in Figure 4 showed that at the initial growth stages, noticeable changes in pH were recorded at all the speeds used (Abd-Elsalam, 2004). The pH increased as the fermentation process increased and reached to its maximum value at 48 h of fermentation at all agitation speed used. On the other hand, at the longer fermentation time a reduced pH changes were recorded and become overlapped at all agitation speeds.

Table 1: Relation of inoculum size and biomass

Inoculum size (spore/L)	Biomass (g/L)		
	100 rpm	150 rpm	200 rpm
$3x10^4$	3.1	3.5	3.6
$5x10^6$	4.5	4.7	4.9
$1x10^7$	5.1	5.0	5.3

Table 2: Bioconversion of progesterone at different inoculum size

Inoculums size (spores/mL)	Transformation products (%)					
	100 rpm		150 rpm		200 rpm	
	α-HP	Rp	α-HP	Rp	α-HP	Rp
$3x10^5$	37.5	60.1	42.5	47.0	40.0	3.5
$5x10^6$	55.0	30.0	66.7	25.1	51.0	40.0
$1x10^7$	42.7	51.5	55.7	31.0	32.0	37.5

% product = (concentration of substrate added - concentration of product)/concentration of substrate X 100

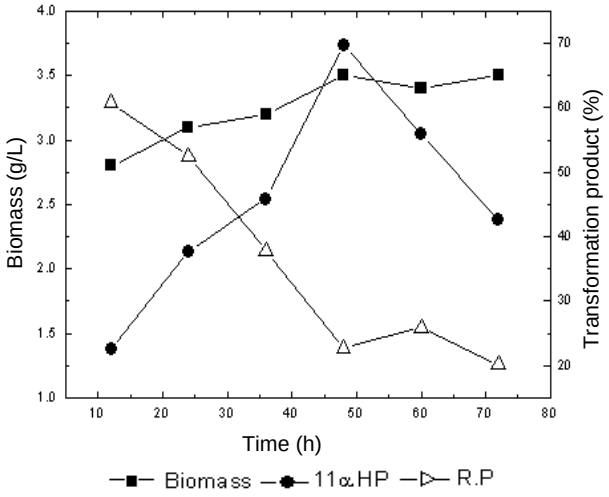

Figure 2: Relation of the fermentation time, biomass, and transformation products

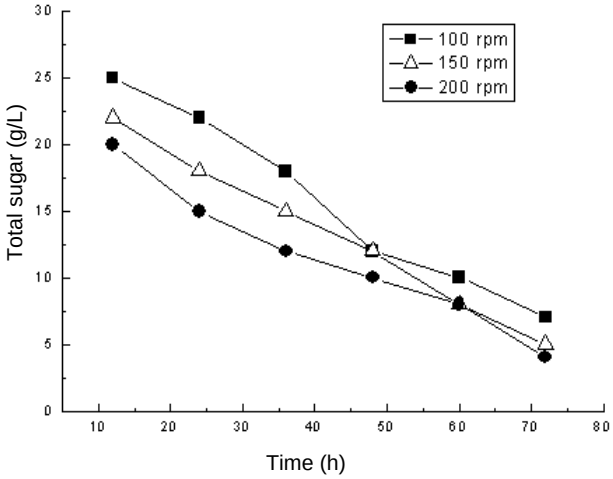

Figure 3: Total sugar changes with fermentation time

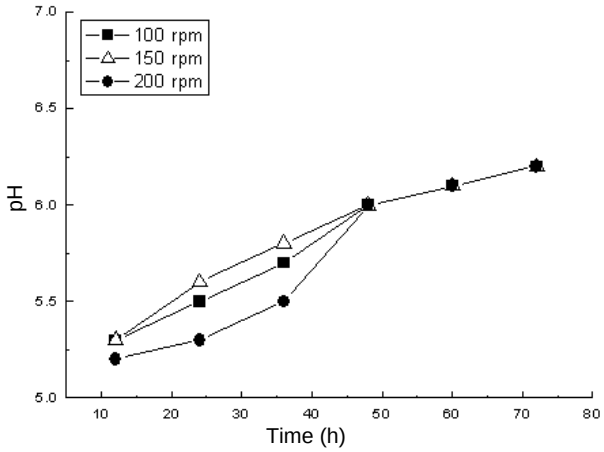

Figure 4: pH changes in relation to fermentation time

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