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Full Length Research Paper

11 β-Hydroxylation of cortexolone using immobilized *Cunninghamella elegans* protoplasts

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Transformation of cortexolone to cortisol and prednisolone by the filamentous fungus *Cunninghamella elegans* protoplasts as a research tool was studied. The immobilized protoplasts of the fungus hydroxylated cortexolone at 11 β-position had significantly higher activity than the free protoplasts. Sucrose as an osmotic stabilizer was found to be best at 0.6 M level. Relatively higher cortexolone bioconversion was obtained on using 3 ml of protoplasts (1.2×10^7 protoplasts/ml) entrapped in 3% calcium alginate gel after 18 h incubation. The addition of some supplements was also investigated. The highest bioconversion efficiency was recorded with sodium citrate (1 g/L) and sodium benzoate (1 g/L). Similarly, the bioconversion process was fairly enhanced in the presence of Tween 20, 60 and the tested vitamins supplements. The entrapped protoplasts proved to be able to maintain the bioconversion process for repeated 13 cycles over 252 h. Sodium citrate and sodium benzoate, folic acid, riboflavin, menadione, vitamin B_{12} and ascorbic acid relatively were able to increase bioconversion efficiency.

Key words: Cortexolone, 11β-hydroxylation, immobilization, *Cunninghamella elegans*, protoplasts.

INTRODUCTION

Steroids are very useful pharmaceutical preparations for treating and preventing different diseases. In this sense, the manufacturing of steroid drugs and hormones is obtained combining microbial technologies and chemical synthesis (Donova, 2007). The hydroxylation processes involve the direct oxidation of C-H bond. These reactions may take place at various points on the molecule, especially hydroxylation of non-activated centers that are difficult to be achieved using classical chemical methods. Microbial transformations have proven to be an efficient alternative to chemical methods (Borges et al., 2009). This has led to the development of microbial biotransformation processes for production of different steroidal compounds (Arima et al., 1969; Pinherio et al., 1993; Dias et al., 2002; Fernandes and Cabral, 2007; Malaviya and Gomes, 2008; Oda and Isshiki, 2008).

The 11 β-hydroxylation of cortexolone by fungi belongs to transformations of biotechnological importance. It is a direct way to obtain cortisol, a pharmaceutical corticesteroid of considerable commercial value (Paraszkiewicz and Dlugonski, 1998). Fungal protoplasts have been a subject of great interest in various fields of research. They are useful model for investigation of cell wall synthesis and also serve for isolation and characteristics of fungal cell organelles (Peberdy, 1989). Additionally, they also can be used to investigate metabolic as well as biotransformation processes in which the tough fungal cell wall impairs substrate access to the enzyme or those which take place in intracellular organelles (Dlugonski et al., 1984).

Different species of *Cunninghamella* and *Curvularia* have been reported to perform the 11 β-hydroxylation reaction, mostly using the free cells of these cultures. (Chen and Wey, 1990; Dlugonski et al., 1992, 1997;

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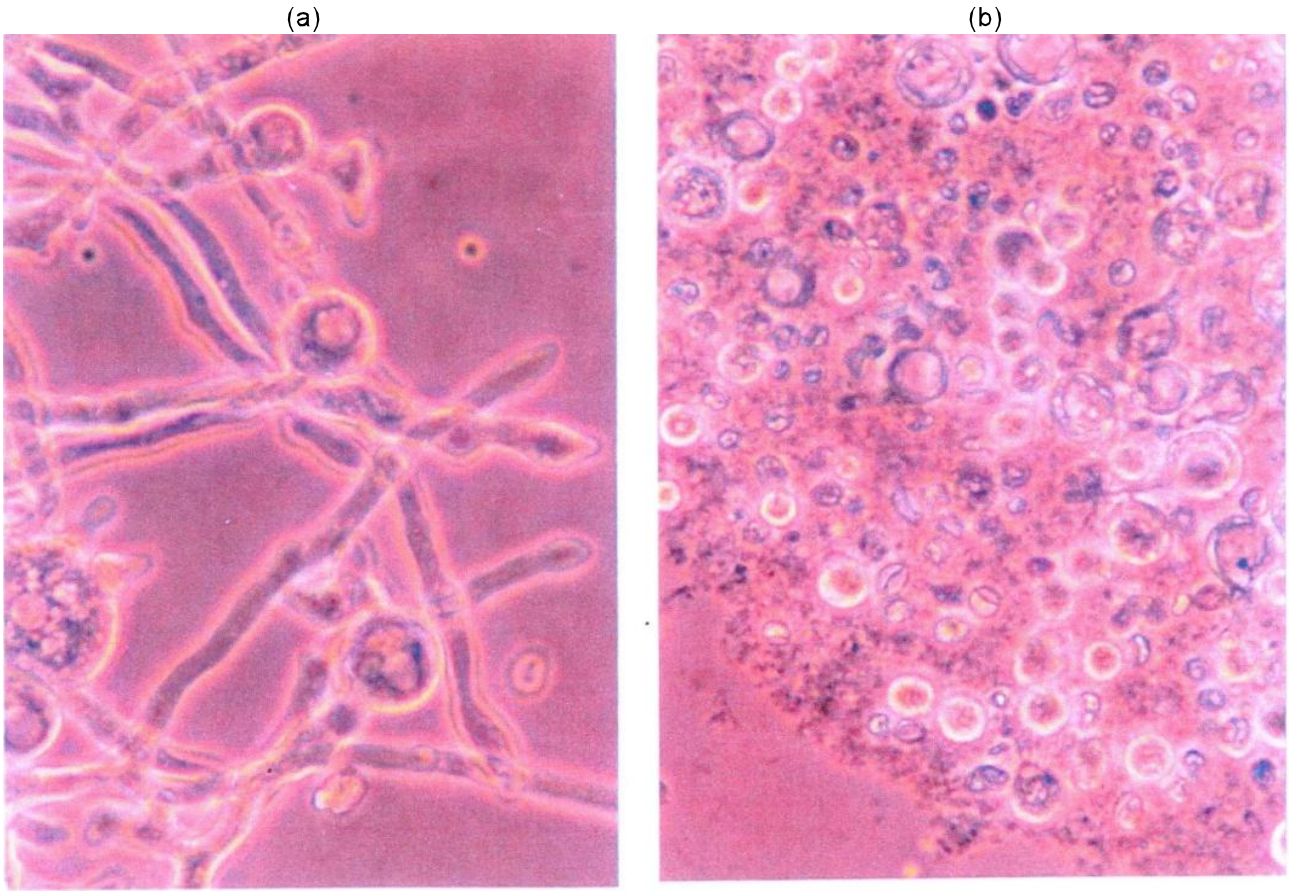

Figure 1. Micrographs of mycelia and protoplasts of *C. elegans*. (a) Branched germ tubes of *C. elegans* after 6 h of incubation in the growth medium; (b) protoplasts released from young *C. elegans* hyphae after the action of lytic enzymes.

Paraszkiewicz and Dlugonski, 1998; Feng et al., 2005; Lu et al., 2006; Manosroi et al., 2006, 2007; Joing et al., 2007). This work was devoted to optimize the conversion of cortexolone to cortisol and prednisolone by immobilized protoplasts of *Cunninghamella elegans*. The transformation process was investigated under different conditions, including the utilization of the immobilized protoplasts for different repeated cycles.

MATERIALS AND METHODS

Microorganism

The tested microorganism, namely *C. elegans* RCMB 012001 was kindly obtained from the Regional Centre of Fungi, Al-Azhar University, Cairo, Egypt.

Preparation of the fungal protoplasts

C. elegans was grown for 9 days on solidified Sabouraud medium dispensed in Roux flasks inoculated with spore suspension of 7-day cultures. Sporangiospores from the Roux flasks were collected (the contaminating fragments of mycelia were removed by filtering through glass wool), washed twice and suspended in sterile distilled water. Spore suspension (5 ml) containing 1.5-2.0×10^7 spores/ml was transferred to 45 ml of liquid medium composed of (g/L): yeast extract, 5; bacto-peptone, 5; glucose, 20; NaCl, 5; K_2HPO_4, 5 (Chickolkar et al., 1995). The medium was then induced by adding 0.5 mg of cortexolone dissolved in 0.3 ml ethanol then incubated at 28°C on a reciprocal shaker (0.81 × *g*) until first branches of the growth hyphae appeared (usually 6 to 7 h). The culture was filtered, washed and suspended in water which was removed by centrifugation (894.6 × *g* for 10 min). The fungus growth (1 g) was transferred to 50 ml of 0.8 $MgSO_4$ in citrate-phosphate buffer, at pH 4.2. Lytic enzyme (2.5 mg/ml) was added, the digestion mixture was incubated for 15 to 18 h, and filtered through nylon net to remove the mycelial debris. The obtained protoplasts suspension was centrifuged (3000 × *g*, 10 min). Depending on the nature of the osmotic stabilizer, protoplasts were collected in the resulting sediment or in the top layer of the stabilizer. Protoplasts were resuspended in a small portion of the stabilizer until the required concentration of protoplasts was reached (Figure 1) (Dlugonski et al., 1984).

Immobilization of protoplasts

Briefly, 3 ml suspension of 1.2×10^7 protoplasts/ml were suspended in 0.8 M sucrose, thoroughly mixed with 7 ml of 3% (w/v) sodium alginate and then add to 2% $CaCl_2$ was added drop-wise to form beads of approximately 2 mm in diameter.

Table 1. Effect of different osmotic stabilizer using free fungus protoplast on the bioconversion of substance.

Osmotic stabilizer concentration (M)	Cortisol (mg/50 ml)	Prednisolone (mg/50 ml)	20β-hydroxy cortisol (mg/50 ml)	Residual substrate (mg/50 ml)	TBE (%)
KCl (0.6)	0.60	0.52	0.9	2.1	22.4
MgSO4 (0.8)	0.42	0.38	0.9	2.6	16
Mannitol (0.8)	0.70	0.60	1.0	1.7	26.2
Sucrose (0.8)	0.80	0.74	0.90	1.6	31

The values were calculated as the average of 3 parallel determinations displaying a variation co-efficient lower than 5%.

Transformation of cortexolone using immobilized *C. elegans* protoplasts

The alginate beads of a total volume of 10 ml were transferred to 50 ml of 0.8 M sucrose containing 5 mg of cortexolone dissolved in 0.3 ml ethanol. The transformation process was carried on a rotary shaker at 28°C for 18 h.

Qualitative analysis

At the end of the transformation period, the steroidal transformation products were extracted by 100 ml of chloroform which was added to each flask. The extraction was repeated three times. The combined chloroform extracts were washed with equal volumes of distilled water, dried over anhydrous sodium sulphate and evaporated to dryness in vacuo to give solid residue (test material) (Paraszkiewicz and Dlugonski, 1998). The test material was dissolved in a measured volume of chloroform: methanol (1:1, v/v). The steroid substances present in the test material were identified by thin-layer chromatography (TLC) in comparison with authentic steroid references using Liebermann-Burchard color reagent (Waldi, 1965). Spots appeared after heating the plates at 110°C for 5 min in a dry oven.

Quantitative analysis

The tested materials (total transformation mixture) were dissolved in a measured volume of chloroform: methanol (1:1, v/v). A known volume of the prepared solution was streaked at the bottom of the TLC plate. The plate was then developed with the solvent system. After development, the area of each product and residual substrate were marked, scrapped from the plate and quantitatively determined by HPLC for residual cortexolone, cortisol, prednisolone and 20β-hydroxycortisol.

RESULTS AND DISCUSSION

Role of osmotic stabilizers in the bioconversion process using free fungal protoplasts

Fungal protoplasts are very convenient system for genetic manipulation, cell wall biosynthesis and metabolic studies (Peberdy, 1989; Ras et al., 2003). The protoplasts are also found to be useful for steroid biotransformation, as the fungus cell wall physically impairs steroid entry into the cell.

This work was started by investigating the biotransformation process using the free fungus protoplasts. The application of free protoplasts was however limited because of the sensitivity of cells lacking a cell wall to mechanical stress. Therefore, samples of the free protoplasts were separately suspended in 50 ml of different osmotic stabilizer namely KCl, 0.6 M, $MgSO_4$, 0.8 M, mannitol, 0.8 M and sucrose, and 0.8 M in citrate-phosphate buffer at pH 4.2. The results in Table 1 show that although $MgSO_4$ has good stimulating effect in protoplasts liberation from different microorganisms yet it impaired steroid transformation resulting in inferior cortexolone bioconversion. This may be correlated to the fact that steroid hydroxylases are metal-containing enzymes inhibited by a number of inorganic compounds (Nagasawa et al., 1970). Therefore, it was convenient to liberate protoplasts in the presence of 0.8 M $MgSO_4$ and then transfer them to the other tested stabilizers which do not interfere with the activity of steroid-hydroxylating enzymes. Obviously, it was found that sucrose treatment stimulated the cortexolone bioconversion (31%).

Role of osmotic stabilizers in the bioconversion process using immobilized protoplasts.

To find out a stabilizing system which would prevent destruction and regeneration of protoplasts due to fragility of the cell wall- less protoplasts, the protoplasts-Ca-alginate beads of total volume 10 ml (100 beads) were transferred to 50 ml of different osmotic stabilizers (0.6 M KCl, 0.8 M $MgSO_4$, 0.8 M mannitol, 0.8 M sucrose; in citrate-phosphate buffer, pH 4.2). The data given in Table 2 indicates that sucrose supported relatively better bioconversion of cortexolone (50%) than that of free protoplasts. This may be due to the protective effect of alginate against protoplast destruction (Komel et al., 1985; Dlugonski and Sedlaczek, 1988). In this respect, Dlugonski et al. (1984) indicated that immobilized protoplasts have been shown to retain full steroid 11-hydroxylase activity and not to change the transformation pattern as compared with the mycelium which is often the case in changed conditions.

Effect of different concentrations of sucrose

The effect of varying concentrations of sucrose (osmotic stabilizer) from 0.2 to 1.2 M on the biotransformation

Table 2. Cortexolone hydroxylation by entrapped *C. elegans* dispersed in different osmotic stabilizers.

Osmotic Stabilizer concentration (M)	Transformation mixture (mg/50 ml)				TBE (%)
	Cortisol	Prednisolone	20β-hydroxy cortisol	Residual cortexolone	
KCl (0.6)	0-93	0.86	1.0	2.1	36
$MgSO_4$ (0.8)	0.7	0.65	0.8	2.3	27
Mannitol (0.8)	1.2	1.1	1.1	1.2	46
Sucrose (0.8)	1.3	1.1	1.1	0.93	50

The values were calculated as the average of 3 parallel determinations displaying a variation co-efficient lower than 5%.

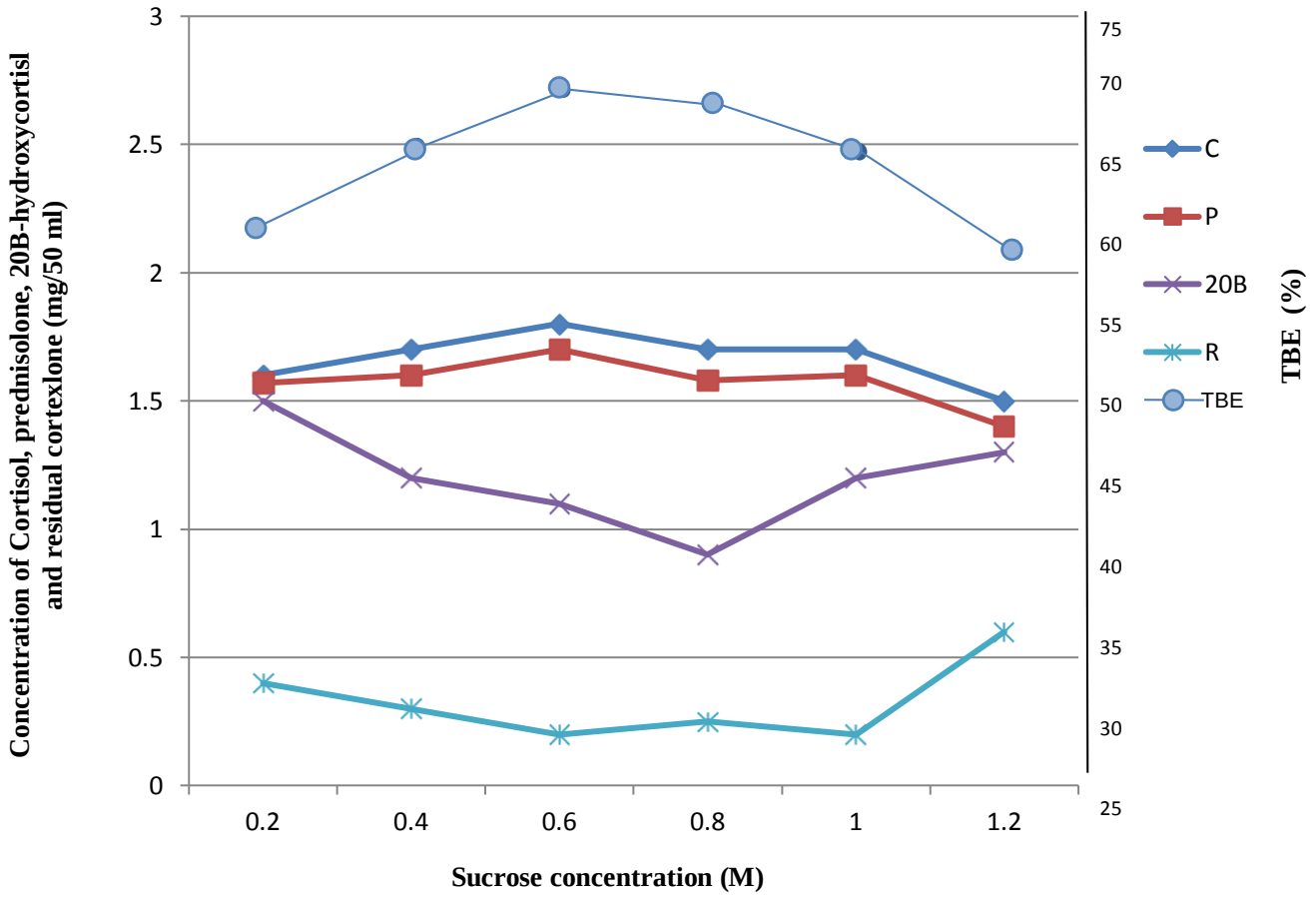

Figure 2. Effect of different concentrations of sucrose (osmotic stabilizer).

process indicated that the bioconversion efficiencies positively responded to the increase of the sucrose concentration (Figure 2). 0.6 M sucrose allowed the maximum bioconversion efficiency (70%). On the other hand, the increase or decrease in the sucrose concentration than 0.6 M was accompanied by the gradual depression of the biotransformation activity. This may be due to the considerable loss of protoplasts. Moreover, stability of protoplasts released in the presence of increasing sucrose was much less than that in other concentrations. Similar results were obtained by Dlugonski and Sedlaczek (1988) who used the same concentration of sucrose for the bioconversion of cortexolone to hydrocortisone and epihydrocortisone.

Role of immobilized protoplasts concentrations

Different amounts of the prepared *C. elegans* protoplasts (1 to 5 ml) suspended in citrate phosphate buffer (pH 4.2), were immobilized separately in 100 beads of calcium alginate. The beads were then transferred to 50 ml of 0.6 M sucrose prepared in citrate-phosphate buffer (pH 4.2) and charged with 5 mg of cortexolone. Figure 3

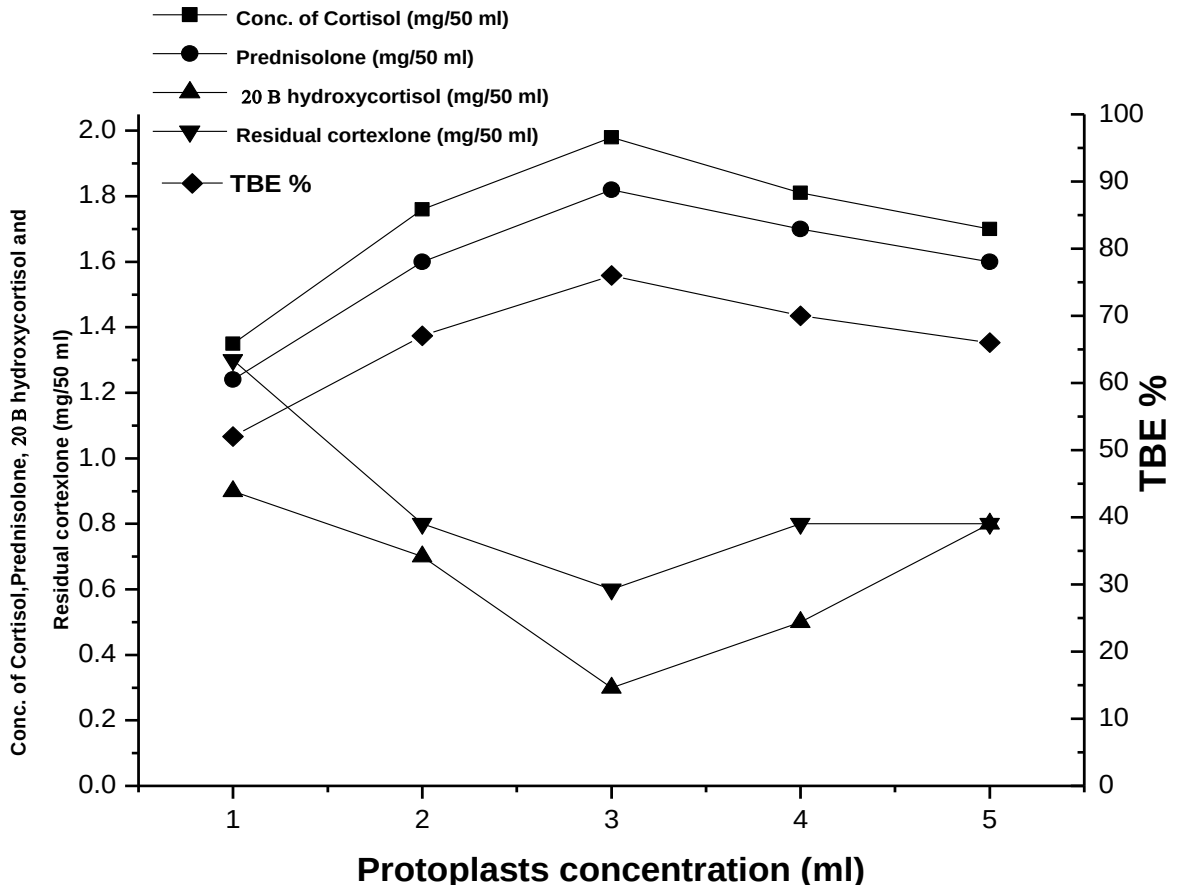

Figure 3. Different concentrations of *C. elegans* protoplasts on cortexolone bioconversion.

Table 3. Effect of different concentrations of sodium alginate.

Sodium alginate concentration (%w/v)	Cortisol (mg/50ml)	Prednisolone (mg/50ml)	20β-hydroxycortisol (mg/50ml)	Residual substrate (mg/50ml)	TBE (%)
1	1.3	1.17	0.8	1.15	49
2	1.83	1.7	0.7	0.6	70
3	2.1	1.8	0.26	0.4	78
4	2	1.8	0.52	0.5	76
5	1.7	1.5	0.8	0.9	65

Spore suspension concentration $1.6x10^7$ cell/ml; conc. of protoplasts = $1.2x10^7$ cell/ml; Substrate concentration = 5mg/ 50ml. The values calculated as the average of 3 parallel determinations displaying a variation co-efficient lower than 5%.

clearly indicates that loading of alginate beads with *C. elegans* protoplasts corresponding to 3 ml of concentration 1.2×10^7 protoplast/ml supported the highest bioconversion activities (76%). Similar results were obtained by Wilmanska et al. *(*1992) when they used 1×10^7 concentrations for the same reaction.

Effect of sodium alginate concentrations

1, 2, 3, 4 and 5% (w/v) concentrations of sodium alginate were used for immobilization of fungal protoplasts to select the most suitable concentration. Each 50 ml of 0.6 M sucrose was inoculated by aliquots of 10 ml of immobilized beads (100 beads) containing 3 ml of protoplasts. The results in Table 3 indicated that the transformation capacity of the immobilized protoplasts proved to be dependent on the concentration of the entrapping gel. Entrapping *C. elegans* protoplasts in 3% calcium alginate gel appeared to be the most suitable treatment for suitable substrate conversion (78%). Higher concentrations of gel were accompanied by a decrease in

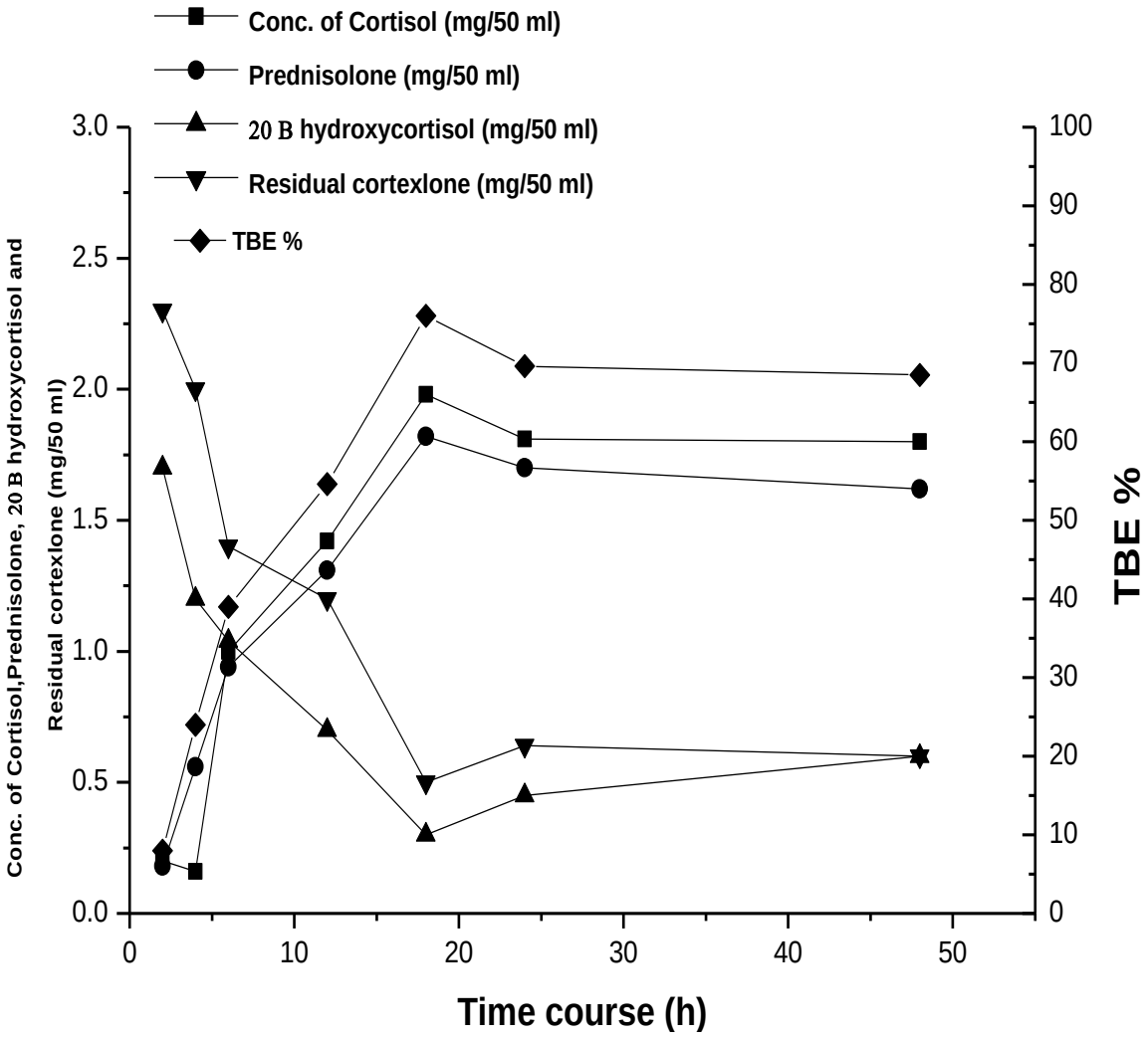

Figure 4. Effect of bioconversion time course on cortexolone bioconversion using immobilized *C. elegans* protoplasts.

bioconversion efficiency. This may be attributed to the higher concentration of calcium alginate which act as a barrier and hinders steroid permeation into the beads (Dlugonski et al., 1997).

Effect of biotransformation periods

The proper time at which the highest cortisol and prednisolone could be obtained was studied. Samples were taken for assay at different time intervals (Figure 4). The transformation of cortexolone linearly increased with the increase of the transformation period. The highest total conversion value (80%) was achieved after 18 h transformation.

This time course was less than that obtained in the case of free cells (48 h) and immobilized spores (72 h) which may be due to the lack of cell wall (physically impairs steroid substrate entry into the cell). Dlugonski et al. (1997) found that maximum cortexolone bioconversion to 11β-hydroxy derivative was obtained after 11 h using 0.9 M sorbose as an osmotic stabilizer.

Role of some supplements

Organic acid supplements

The sodium salts of the tested organic acid (citrate, acetate, benzoate and oxalate) were individually added in concentration of 1 g/L. The addition of sodium citrate and benzoate exceptionally increased the amount of the formed cortisol and prednisolone, wherein the efficiency of cortexolone conversion reached maximum. Sodium acetate and oxalate exerted a relatively inhibitory effect on the cortisol and prednisolone production (Table 4). Similar results were obtained by Sallam et al. (1995) and Adham et al. (2003).

Table 4. Role of some organic acids.

Additives	Cortisol (mg/50ml)	Prednisolone (mg/50ml)	20β-hydroxy cortisol (mg/50ml)	Residual cortexolone (mg/50ml)	TBE (%)
Sodium citrate	2.2	2.0	0.2	0.3	84
Sodium acetate	1.9	1.8	0.3	0.4	74
Sodium benzoate	2.2	2.0	0.2	0.4	84
Sodium oxalate	1.8	1.6	0.4	0.9	69

Spore suspension = $1.6x10^7$ spore/ml; protoplast suspension = $1.2x10^7$ cell/ml; the values calculated as the average of 3 parallel determinations displaying a variation co-efficient lower than 5%

Table 5. Effect of some supplements on the conversion of cortexolone to cortisol by *C. elegans* protoplasts.

Surface active agent	Cortisol (mg/50ml)	Prednisolone (mg/50ml)	20β-hydroxy cortisol (mg/50ml)	Residual substrate (mg/50 ml)	TBE (%)
Tween 20					
10 mg	2.2	2	0.2	0.3	84
20 mg	2.1	1.9	0.3	0.3	80
30 mg	2`	1.8	0.4	0.5	76
Tween 60					
10 mg	2.1	2	0.2	0.4	83
20 mg	2	1.9	0.3	0.4	78
30 mg	2	1.8	0.3	0.6	77
Tween 80	2	1.8	0.41	0.53	77
10 mg					
20 mg	2	1.8	0.4	0.5	76
30 mg	1.8	1.7	0.3	0.8	71

The values were calculated as the average of 3 parallel determinations displaying a variation co-efficient lower than 5%.

Surface active agent supplements

Since transformation occurs when dissolved steroid diffuse through the fungal cell wall into the enzyme rich interior (Sedlaczek, 1988), it was important to evaluate the role of some surface active agents on cortexolone bioconversion. The data in Table 5 shows that the addition of 10 mg of Tween 20 and 60 exerted a slight increase in the rate of cortexolone transformation. Treatments using Tween 80 decreased the rate of biotransformation process (Azab, 1987; Abu El-Hawa et al., 1993).

Vitamin supplements

The transforming enzyme system of cortexolone into cortisol and prednisolone responded differently to the tested vitamins and auxinic compounds (Figure 5). Biotin, thiamine and inositol proved to be of inhibitory action on cortexolone bioconversion. Similarly, pyridoxine and choline showed less inhibitory action. Conversely, folic acid, ascorbic acid, riboflavin, vitamin B_{12} and menadione activated the cortexolone 11β -hydroxylase enzyme. These results may reflect the requirement of flavines as a co-factor in the enzymatic oxidation-reduction reactions of cortexolone (Yusef, 1991). Additionally, menadione seems to serve as an external electron acceptor (Pinheiro and Cabral, 1992).

Repeated utilization of the Ca-alginate immobilized protoplasts

The loaded Ca-alginate protoplasts beads (100 beads) were inoculated in 50 ml of 0.6 M sucrose (as a stabilizer). Briefly, 5 mg of cortexolone substrate were added and transformation process was performed for 18 h. The loaded gel beads of the first batch were then washed thoroughly with normal saline solution and then reused for the next 18 h batch. The transformation process was successively repeated for different 14 cycles. The highest cortexolone bioconversion was obtained at the end of the second cycle (88%). Figure 6

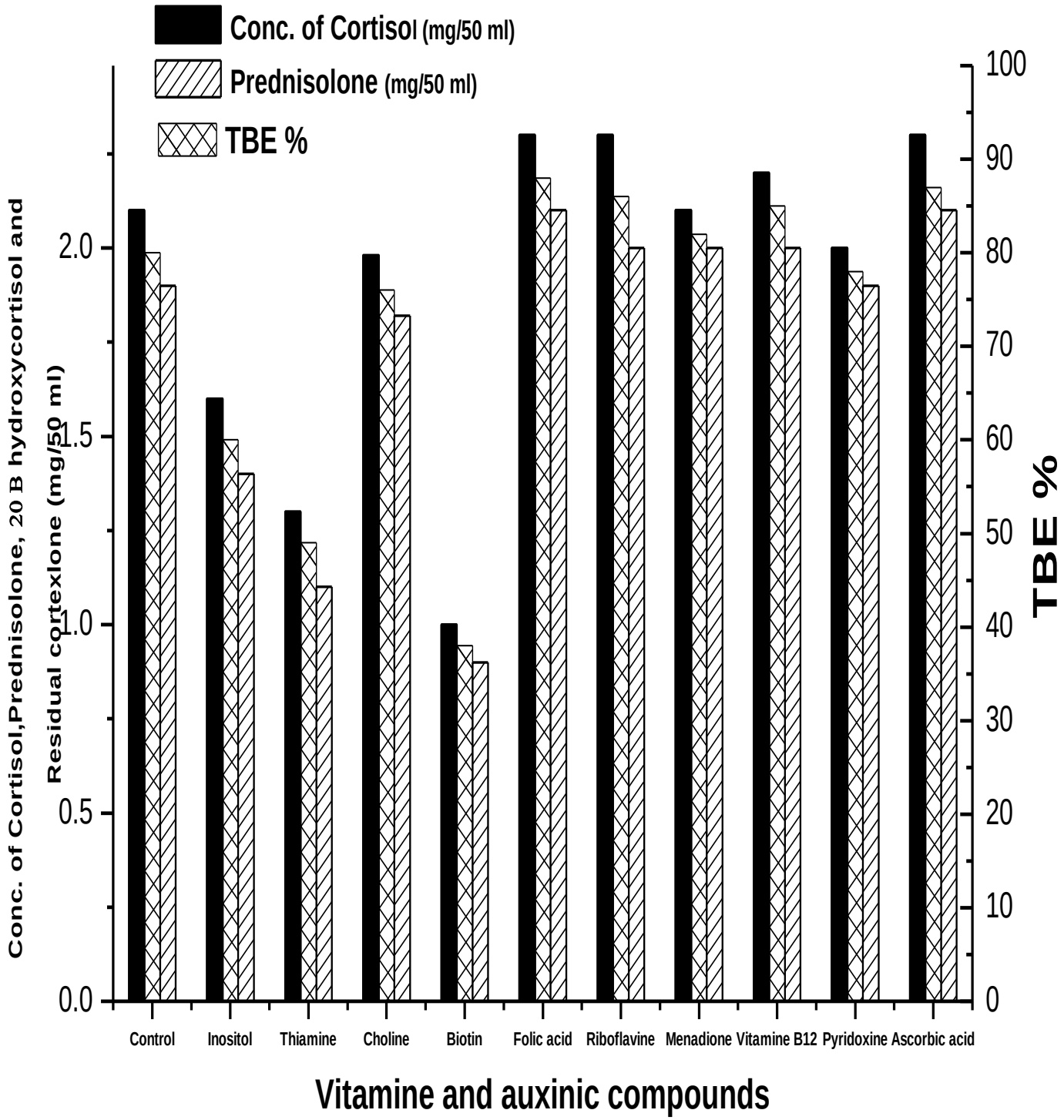

Figure 5. The role of some vitamin and auxinic compounds on the bioconversion of cortexolone.

shows that the repeated use of immobilized protoplasts caused the decrease of the products yields. This may be either due to the lysis of the formed beads or the cell wall re-synthesis which hinders steroid permeation into the cell (Jaworski et al., 1984; Sedlaczck, 1988). The immobilized protoplasts were used for 14 cycles over 252 h. Similar results were previously obtained by Dlugonski et al. (1997); they reutilized the immobilized protoplasts for 13 cycles over 247 h under the optimal conditions for the same reaction.

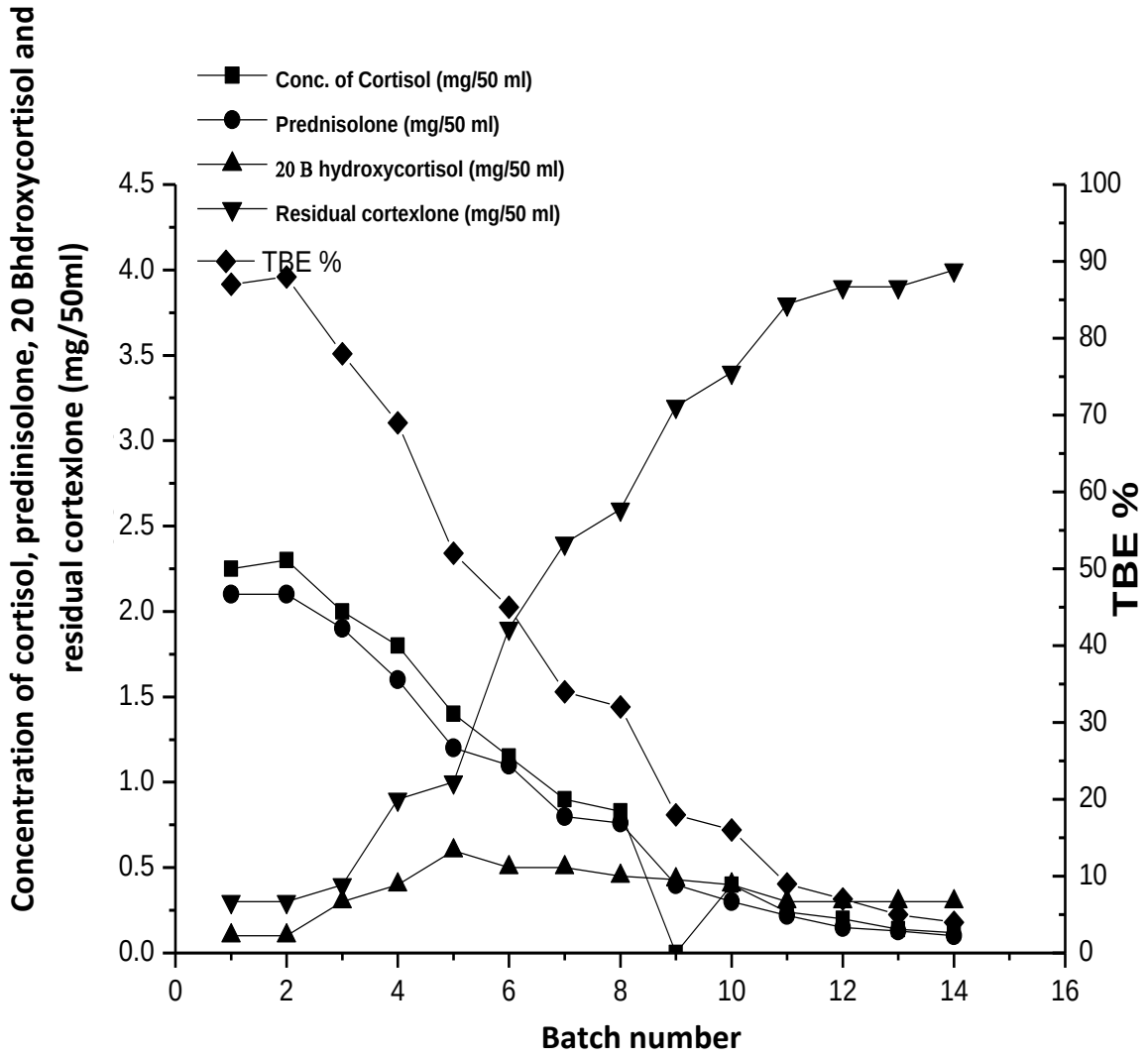

Figure 6. Re-cyclization of the entrapped *C. elegans* protoplast in Ca-alginate.

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