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ROLE OF SOME FERMENTATIVE PARAMETERS FOR MICROBIAL TRANSFORMATION OF PROGESTERONE TO CORTEXOLONE AND/OR HYDROCORTISONE.

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ABSTRACT

Four strains belonging to the genus *Curvularia* were tested for their ability to transform progesterone to cortexolone and hydrocortisone. *Curvularia lunata* RCMB 019002 showed the greatest bioconversion efficiency (29.64%). In a medium containing (g/l); yeast extract 4 and malt extract 20, the substrate was converted after 48h to cortexolone and cortisol. The maximum bioconversion efficiency (37. 72%) was recorded when phosphate buffer was used at pH 7.0. The transformation pattern of progesterone markedly affected by the inoculum size and culture age. The bioconversion efficiency increased (63.2 %) when the substrate concentration was 5 mg/50ml fermentation medium. The addition of the surface active agents Tween 60 and Tween 80 to the medium at concentrations of 100 µl, 50 µl, respectively increased the bioconversion efficiency (66.70 and 68.64 %, respectively). Among the tested amino acids, L-asparagine enhanced the formation of cortexolone and cortisol.

Key words: *biotransformation, cortexolon, cortisol, Curvularia lunata, Progesterone.*

1. INTRODUCTION

Introduction of a hydroxyl group to a steroid molecule at position 11 is one of the most important steps in the preparation of various physiologically important steroidal derivatives and drugs (Manosroi *et al*. ,2008). In microbial hydroxylations, hydroxylase enzyme can introduce a hydroxyl group to various positions of the steroid molecule that are generally chemically inaccessible. Several positions in the steroid molecules can be hydroxylated by various microbial strains such as *Curvularia lunata* (Templeton *et al.*, 1987), *Mucor griseocyanus* (Krasnova *et al*., 1987), *Trichoderma* spp. (El-Kadi and Mostafa, 2004), *A .niger* (Fouad *et al.*, 2009) and *Rhizopus* spp. (Martin, 2010). The 11α, 11β, and 16α- hydroxylations have been achieved in the steroidal drug production by microbial transformations at high yield and controlled costs (Farooq *et al.*,1994 , Vitas *et al.*,1994).

Generally, steroidal drugs have therapeutic advantages such as increased potency, longer half lives in the blood stream and reduced side effects (Mohamed and Abd El- Hadi, 2010). Manufactured steroidal compounds have a wide range of therapeutic purposes, namely as anti-inflammatory, immunosuppressive, progestational, diuretic, anabolic and contraceptive agents (Fernandes *et al.*, 2003; Stephanie, 2006).

The aim of the present work was to evaluate the enzymic 11β hydroxylation of progesterone to cortexolone and cortisol by *Curvularia lunata* RCMB 019002 under different biochemical conditions.

2. MATERIALS AND METHODS

2.1. Microorganism and medium

Microorganisms used in this study (*Curvularia clavata* RCMB 019003, *Curvularia lunata* 59 and *Curvularia lunata* RCMB 019002) were kindly obtained from the Regional Centre of Fungi, Al-Azhar University while *Curvularia lunata* 2437 was taken from Assuit University Mycological Center (AUMC) and maintained on potato dextrose agar medium (PDA) g\l :PDA 39, agar 8. The fermentation medium contained (g\l): malt extract 20, yeast extract 4 at initial pH 6 .

2.2. Chemicals

The steroids used in this work namely: cortexolone (Reichstein's Substance S; 17α, 21-dihydroxy-pregn-4-ene-3,20-dione), cortisol (hydro-cortisone; Kendall's compound F; 11β, 17α, 21-trihydroxy-pregna-1,4-diene-3,20-dione) and progesterone were provided by Sigma Company, USA. Potato dextrose agar, yeast extract and Bacto peptone were purchased from Difco Laboratories (USA).

2.3. Bioconversion of progesterone by *Curvularia lunata* RCMB 019002

The organism was grown in 50 ml aliquots of the bioconversion medium using 250 Erlenmeyer flasks and each was inoculated with 5ml spore suspension, incubated at 28± 2^{o} C for 48h under shaking conditions (150 rpm).

The synthesis of the enzymes, 11β-hydroxylase, 17α- hydroxylase and 21 hydroxylase were induced by the addition of progesterone (0.5mg/50ml medium dissolved in 0.5ml absolute ethanol) for 24h. The bioconversion medium containing the fungal cells was supplemented with 10mg of progesterone dissolved in 1ml absolute ethanol and the fermentation was continued for 48h.

2.4. Analysis of steroid conversion

2.4.1. Extraction and qualitative determination of the transformation products

At the end of the transformation period, 100 ml chloroform were added to each flask containing free fungal cells. However, on using the immobilized cultures, chloroform was added to the filtrate. The extraction was repeated twice to ensure that none of the transformation products was left. The combined chloroform extracts were dried over anhydrous sodium sulphate and evaporated to dryness in vacuo to give semi - solid residue (test material), (Parasckiewicz and Dlugoński, 1998).

The steroid substances present in the test material were identified by thin-layer chromatography (TLC) in comparison with authentic steroid references , using the following solvent system: benzene: ethyl acetate: acetone (80: 20 : 10, v/v). The following colour reagent:Lieberman-Burchard: 5ml concentrated sulphuric acid were added slowly while cooling to 50 ml absolute ethanol then 5 ml acetic anhydride were dropped while cooling on the mixture (Waldi, 1965) this was used so spots appeared after heating the plates at 110°C for 5 min in a dry oven. The thin layer chromatographic pictures of the different steroids were encountered during the bioconversion process and compared with authentic samples.

2.4.2. Quantitative analysis

The tested material (total transformation mixture) was dissolved in a measured volume (5ml chloroform) and applied on TLC plate. The plate was then developed with the solvent system benzene : ethylacetate: acetone (80:20:10; v/v). The authentic steroid references dissolved in the same solvent were also applied as a narrow band along side the test material streak. The narrow band of the steroid references was sprayed with the colour reagent, while the rest of the plate (test material) was covered. The area at the same level of each steroid reference was marked, scrapped from the plate and quantitatively eluted with chloroform. The extract was separated by filteration and evaporated to dryness on a water bath. The residue was dissolved in 8 ml of the colour reagent composed of concentrated sulphuric acid: ethanol (45: 55; v/v). The obtained solutions were separetly heated for 20 min in a water bath, cooled and read at 420nm, 515nm and 445nm for cortisol, cortexolone and progesterone, respectively. The exact concentrations were calculated from prepared standard curves.

2.5.Optimization of transformation parameters

2.5.1. Screening for the most active organism

The four fungal strains were screened for their ability to transform progesterone to cortexolone and cortisol . The experimental microorganisms were cultured on the medium containing (g/l): malt extract 20 and yeast extract 4. Progesterone(10mg) was added and the transformation period was continued for 48h. The steroid products were extracted and the qualitative and quantitative analyses were made.

2.5.2. Bioconversion time course

Inoculated flasks containing fermentation medium (50ml) were shaken at 30^{o}C for 48h after which they were supplemented with 10mg of progesterone, the samples were then collected for analysis at 24, 48, 72, 96 and 120hr.

2.5.3. Bioconversion medium

The most potent microorganism was cultivated into five different media, each supplemented with 10mg/50ml media of progesterone dissoloved in 1ml of absolute ethanol. The inoculated media were incubated for 48h under shaking conditions at 28^{o}C , the initial pH was adjusted to 6 in all cases of the necessary analyses were carried out. The media utilized included (g/l) Medium I : glucose 40; peptone 1; yeast extract 1; $MgSO_4.7H_2O$ 1; potassium dihydrogen phosphate 0.74;L.asparagine 0.7 and pH adjusted at 6. Medium II consists of (g/l): malt extract 20; yeast extract 4, pH adjusted at 6. Medium III consists of (g/l) potato 300; glucose 20, pH adjusted at 5.6. Medium IV consists of(g/l) yeast extract 5; bacto peptone 5; glucose 20; sodium chloride5; potassium dihydrogen phosphate 5; pH adjusted at 6. Medium V consists of (g/l): soya flour 5; yeast extract 5; potassium dihydrogen phosphate 5; sodium chloride 5; glucose 20 and pH adjusted at 6.

2.5.4. pH Value

The effect of pH was studied either by adjusting the fermentation medium (No II) initially before autoclaving with (M NaoH or M HCl (pH 5.5- 8) or with phosphate buffer system after autoclaving in all cases. Flasks were inoculated , incubated under shaking conditions 48 h at 28^{o}C, and then progesterone(10mg) was added and the transformation period continued for 48h.

2.5.5. Inoculum size

Aliquots (50ml each) of medium II were inoculated with different amounts (1- 20ml) of spore suspension prepared from a 7- day old culture of the tested organism. The flasks were agitated at 150 rpm for 48h at 28^{o}C, supplemented with 10mg progesterone and the fermentation continued for 48h.

2.5.6. Culture age

Aliquots (50ml each) of medium II were inoculated with 15ml inoculums (0.178 g of cell dry weight) of *C.lunata* RCMB 019002 , incubation for 1, 2, 3, 5 and 7 days under shaking conditions (10mg each) of progesterone was separately added to the culture at each period, the transformation period was continued for 48h and the necessary analyses were carried out.

2.5.7. Substrate concentration

The substrate (progesterone) was added to the medium as separate batches of (5,7.5, 10, 15 and 20 mg/50ml after 48h and the necessary analyses were carried out.

2.5.8. Surface active agents

The effect of Tween 60 and Tween 80 were studied by supplementing the transformation medium with the composed at different concentrations (50, 100, 150µl / 50 ml medium). The transformation period continued for 48 h.

2.5.9. Amino acids

The effect of the amino acids L-asparagine, L.leucine and cysteine was tested where either was added individually at the concentration of 0.5mg/50ml medium. The tested amino acids were sterilized and supplemented to the medium under aseptic conditions at time of substrate addition.

3. RESULTS AND DISCUSSION

3.1. Screening for the most active organism

The results of the ability of different fungal cultures to carry out the 11β hydroxylation and/or 17α hydroxylation and/or 21 hydroxylation of progesterone to cortexolone and cortisol are given in Table (1). The amount of the formed cortexolone and cortisol with the active strain was further assayed in qualitative bases , it is evident in Fig.(1) that the different organisms are versatile in their potentiality to convert progesterone to cortexolone and cortisol. A comparable cortexolone & cortisol yield was obtained with all of the different fungi used. Among the tested fungal isolates *C.lunata* RCMB 019002 was clearly able to perform the hydroxylations reactions(29.64%). These results are in close agreement with the findings of others (Vitas *et al.* 1995, Manosroi *et al*. 2008,). Meggs *et al*. (1990) reported that steroid hydroxylations by filamentous fungi including 11β-hydroxylation are economically important for the production of corticosteroids. This may be due to the fact that the enzymes responsible are from the P450 superfinly (Nelson *et al.*, 1993).

Table (1): Thin layer chromatographic picture of the different steroids encountered during the transformation processes.

Compound	R_f x 100	Colour with Liebermann-Burchard Reagent in	
	With solvent system*	Day light	UV fluorescence
Cortexolone	25	Orange	Deep yellow
Cortisol	6	Pale brown	Green yellow
Progesterone	68	Brown	Brick

***Solvent system used: Benzene: ethylacetate: acetone (80:20:10,v/v)**

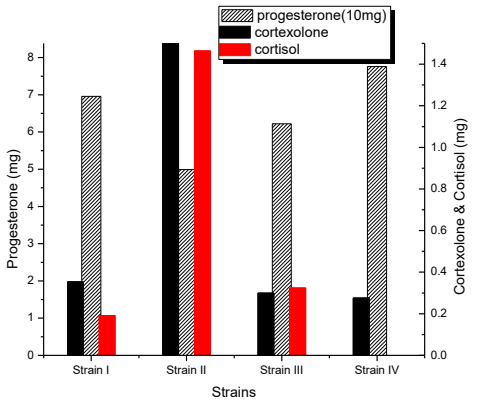

Fig.(1): Screening for the ability of different tested fungi to transform progesterone to cortexolone and cortisol.
Strain I *Curvularia clavata* ***RCMB 019003***
Strain II *Curvularia lunata* ***RCMB 019002***
Strian III *Curvularia lunata* ***59***

3.2. Bioconversion time course

The transformation of progesterone with the tested organisms was further analyzed at different intervals of the fermentation process. Optimal transformation period for cortexolone and cortisol

production was attained after 48h when the yield reached about (1.5,1.4mg of cortexolone & cortisol), respectively Fig.(2). As a result of this experiment, the study of progesterone transformation by the experimental organism was carried after 48h; similar results were obtained by El Kadi and Mostafa,(2004)and Mohamed and Abd El Hadi ,(2010).

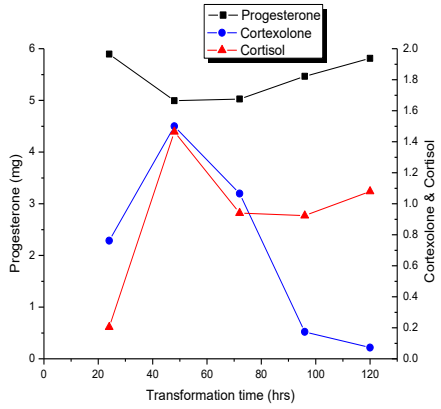

Fig. (2): Effect of the transformation time course of progesterone by *Curvularia lunata* **RCMB019002.**

3.3. Bioconversion medium

As an orientation step, selection of the proper fermentation medium for the performance of the hydroxylation of progesterone by *Curvularia lunata* RCBM was carried out.

Five media of different composition were tested for the propagation of the experimental organism catalyzing the transformation of progesterone. In all media tested, progesterone was added after 48h incubation.

The results shown in Fig.(3) indicated that medium II (Voigt *et al.*, 1993) proved to be the most favorable for the transformation of progesterone to cortexolone and cortisol .

The superiority of this medium may be due to the presence of yeast and malt extracts. The latter constituents may provide the necessary growth factors as well as factors and cofactors stimulating the biotransformation of progesterone to cortexolone and cortisol**.** Fouad *et al.* (2009) also reported a similar medium.

3.4. Effect of pH of the fermentation medium

The role of the initial pH or buffered pH values of the fermentation medium on the transformation of progesterone was conducted by adjusting, the pH of different aliquots of medium II with 1N of HCl or NaOH to cover pH ranges from 5.5to8.The highest bioconversion rates of progesterone were obtained at the pH range of 6-7 by using either unbuffered or buffered media. The bioconversion process was markedly retarded at pH below 6 or above 7.0. Acidic media below 5.5, or alkaline above 8 seemed unfavourable for hydroxylation reaction. The control of the reaction with buffer solution is essential for investigating the role of

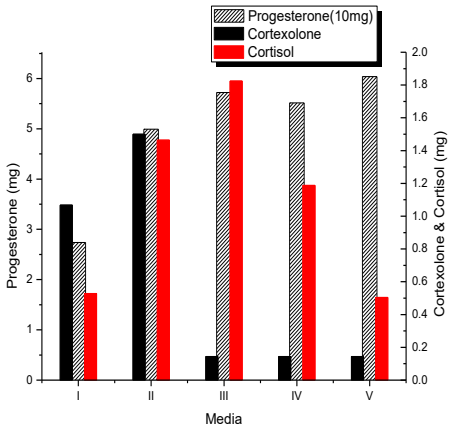

Fig.(3): Role of chemical composition of fermentation medium on progesterone bioconversion to cortisol and cortexolone.

pH value of the medium . Therefore, the aliquots medium II were adjusted to pH 5.5-8 using phosphate buffer. Data in Fig. (5) showed that cortexolone and cortisol yield were the highest at pH 7.0 similar to that obtained using initial pH (Fig. 4). Chinckolkar *et al.* (1995) found that pH 6.0 was the best optimal for progesterone biotransformation using *Cunninghamella blakseleana* NCIM 687.

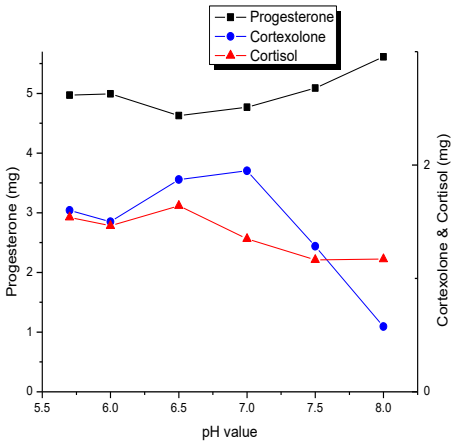

Fig. (4): Transformation of progesterone by *Curvularia lunata* **RCMB019002 as influenced by the initial pH value of the fermentation medium.**

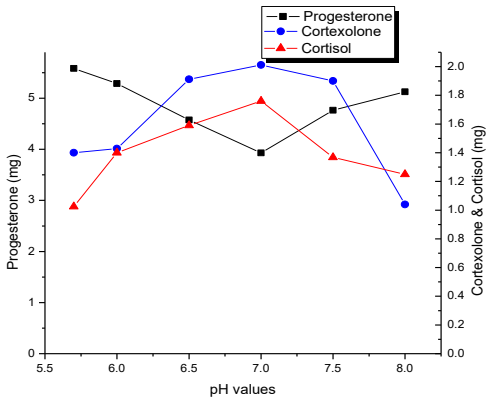

Fig.(5): Transformation of progesterone by *Curvularia lunata* **RCMB019002 grown on the fermentation medium adjusted with phosphate buffer to different pH values**.

3.5. Effect of inoculum size on progesterone bioconversion

A maximum cortexolone and cortisol yield (2.860 and 2.690 mg respectively) were obtained by using 15 ml of cell suspension equivalent to 0.1787g of cell dry weight (Table 2).These results were found to be in contrary with EL Refai *et al.* (1969) and Fouad *et al.* (2009) who used 2ml cell suspension of *Rhizopus nigricans* and *Asperigillus niger* respectively to transform progesterone to 11α-hydroxyprogesterone.

3.6. Effect of culture age on progesterone biotransformation

It was evident that, the bioconversion of progesterone by *Curvularia lunata* RCMB 019002 was affected by different growth ages. Table (3) showed that higher cortexolone and cortisol yield (58.65 %) was formed after 48h old cultures. These results run parallel with those reported by many authors (Mohamed and Abd El Hadi ,2010; El Kadi and Mostafa, 2004).

On the other hand, Fouad *et al*., (2009) found that 10 days were the best culture age for the biotransformation of progesterone.

3.7.Effect of substrate concentration on progesterone bioconversion

The utilization of high amounts of the steroidal substrates is one of the important factors affecting the economy of the transformation process. Increasing concentrations of progesterone to (20 mg/50 ml) retarded cortexolone and cortisol (Table 4). The optimum substrate concentration which gave the maximum bioconversion efficiency was 5 mg/50 ml medium when added in the batch. This may be due to the toxicity of the substrate (progesterone) on the activity of the microorganism which took place by using large amounts of substrate concentration dissolved in 1ml of absolute ethanol which in turn may inhibit the growth of the microorganism (Adham *et al*., 2003). The same phenomena were recorded by Constantinides(1980), Goetschel and Bar (1992)and Arinbasarova *et al.* (1996).

3.8. Effect of surface active agents

Since transformation occurs when the dissolved steroids diffuse through the fungal cell wall into the enzyme and rich interior, it was important to evaluate the role of some surface active agents on the hydroxylation of progesterone. The addition of Tween 60 at concentrations 50 and 100µl/50ml medium and Tween 80 at conc 50µl/50ml medium exerted an appreciable increase in the rate of progesterone transformation while treatments exerted no effect on the bioconversion process. These results are in disagreement with Mohamed and Abd El Hadi, (2010) who found that tween 20 increased the bioconversion efficiency to 80 % but Tween 60 and 80 decreased the bioconversion of progesterone. Similar results were recorded by Sallam *et al.(* 1995).

3.9. Amino acids

The amino acids : cysteine, L.asparagine and L.leucine were added individually to the fermentation medium to study their effect on the bioconversion process.Results illustrated in Fig. (6) showed that, L. asparagin increased the yield of cortisol to 66.36% compared to the control where bioconversion was 63.2%, while other amino acids showed inhibitory effects on the bioconversion process. Daba(2009) found that L.Leucine is the best amino acid for the bioconversion of cortexolone to the cortisol by *Curvularia lunata* RCMB 019001.

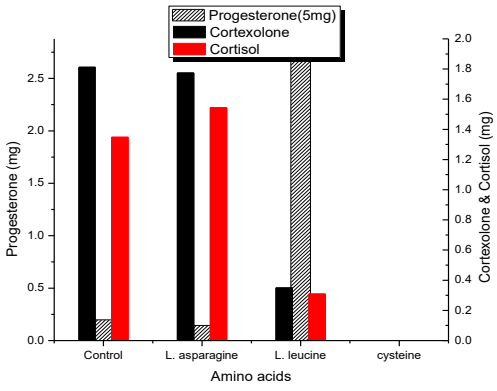

Fig. (6): Effect of amino acids on bioconversion of progesterone to cortexolone and cortisol.

Table (2): Effect of inoculum size of *Curvularia lunata* RCMB 019002 on bioconversion of cortexolone to cortisol.

Inoculum size (ml/50 ml medium)	Residual progesterone (mg)	Transformation products		
		Cortexolone (mg)	Cortisol (mg)	Bioconversion Efficiency (%)
1	5.506	1.122	0.14	12.62
2	4.88	1.272	0.80	18.77
3	5.76	1.536	0.341	20.72
4	4.54	1.5	1.184	26.84
5*	3.93	2.012	1.760	37.72
6	3.412	2.372	1.470	38.42
8	3.300	2.380	1.570	39.50
10	3.136	2.638	1.606	42.44
15	2.45	2.860	2.690	55.50
20	3.3	2.144	2.262	44.26

*control

Table (3): Effect of culture age of *Curvularia lunata RCMB* 019002 on bioconversion of progesterone to cortexolone and cortisol.

Culture age (Day)	Residual progesterone (mg)	Transformation product (Cortisol)		
		Cortexolon (mg)	Cortisol (mg)	Bioconversion Efficiency (%)
1*	2.450	2.860	2.690	55.50
2	2.421	2.992	2.873	58.65
3	2.884	2.650	2.084	47.34
5	3.541	2.224	1.063	32.87
7	5.886	2.008	0.516	25.24

*control

Table (4): Effect of different substrate concentrations on bioconversion of progesterone to cortexolone and cortisol by *Curvularia lunata*.

Substrate conc. (mg/50 ml medium)	Residual progesterone (mg)	Transformation products		
		Cortexolone (mg)	Cortisol (mg)	Bioconversion Efficiency (%)
5	0.198	1.812	1.348	63.2
7.5	0.311	2.61	3.124	61.2
10*	2.421	2.992	2.873	58.65
15	3.220	2.776	2.594	35.80
20	4.67	2.718	1.502	28.13

*control

Table (5): Effect of some surface active agents on the bioconversion of progesterone by *Curvularia lunata*.

Additive conc. In µl	Residual progesterone (mg)	Transformation Products		
		Cortexolone (mg)	Cortisol (mg)	Bioconversion Efficiency (%)
Control	0.198	1.812	1.348	63.64
Tween 60 : -				
50	0.173	1.820	1.425	64.9
100	0.166	1.886	1.449	66.70
150	0.333	1.540	1.400	58.80
Tween 80 : -				
50	0.154	1.890	1.592	68.64
100	-	-	-	-
150	-	-	-	-

*control (without additives)

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دور بعض العوامل فى التحول الميكروبي البروجيستيرون الى كورتيكسولون وهيدروكورتيزون

حسان امين عبد المجيد المنوفى- منى سيد شافعى - *محمد مدحت غريب - رانيا عبد الرازق عبد العال

قسم كيمياء المنتجات الطبيعية والميكروبية - المركز القومى للبحوث- الجيزة
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ملخص

اختبرت كفاءة اربع عزلات فطرية لتحويل مركب البروجيستيرون وثبت فاعلية الفطريات المختبرة لإجراء التفاعل المطلوب حيث تميز فطر *Curvularia lunata RCMB* 019002 بتكوين كمية مناسبة من الكورتيزول مقارنة بباقى الكائنات المختبرة حيث وصلت كفاءتها الى 29.64% فى مستنبت غذائى محتوى على (جم/لتر): 4مستخلص الخميرة، 20 malt extract الذى اعطى اعلى نسبة تحول بعد 48 ساعة من مركب الكورتيكسولون وكورتيزول حيث تأثرت عملية التحويل بدرجة الأس الهيدروجينى للمستنبت الغذائى المستخدم ووجد ان افضل عملية تحويل تم الحصول عليها باستخدام درجة أس هيدروجينى 7 باستخدام buffer solution حيث وصلت الى 37.72% وتأثرت ايضا كثافة اللقاح المستخدم، وتركيز المادة المضافة حيث وصلت كفاءة عملية التحويل إلى 63.2% عند إضافة 5mg/50ml من المستنبت الغذائى وبدراسة تأثير إضافة بعض المواد المساعدة لزيادة عملية التحويل مثل surface active agents ، الأحماض الأمينية وجد أن الحمض الأمينى L. asparagines قام بزيادة عملية التحويل إلى 66.36% من الكورتيكسولون والكورتيزول.

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (63) العدد الاول (يناير2012):88-95.