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Evaluation and Optimization of a Method for Production of Methandienone Using PTFE Immobilized Fungal Cells

E. M. ahmed*[1] and M. I. Ammar[2]

[1] Chemistry of Natural and Microbial Products, National Research Center, Dokki, Cairo, Egypt, P.O.12622.,

[2] Plant Pathology Research Institute; Agriculture Research Center; Giza Egypt.

*Corresponding author: E. M. ahmed, E-mail: eahmed98@hotmail.com

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ABSTRACT

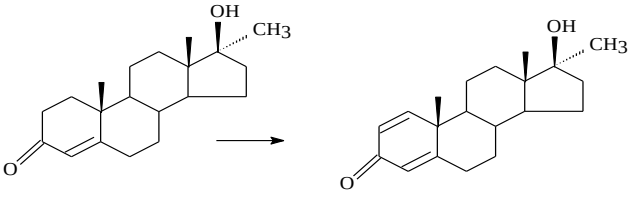
A cell immobilization technique using polytetrafluoroethylene (PTFE) was employed in biotransformation process. Conversion of methyltestosterone to methandienone was achieved using the immobilized cells. An optimization strategy included two levels factorial model and three levels model was used. The most significant factors, glucose concentration, shaking speed and reaction time, were determined after Plakett-Burman screening study. Thereafter, the three levels quadratic model determined the optimum measures for thses significant factors and predicted the maximum product yield. The optimization led to improvement of the methandienone yield, from 45 % to 85% using the immobilized cells. The immobilized cells were reused for six runs with a good conversion outcomes and retained its metabolic activities after storage at 4ºC for one month. To the best of our knowledge, no previous work on this topic, the optimization and production of methandienone using the immobilized cells. The isolated fungus, *Circinella mucoroides*, was not previously used in similar work.

Keyword: Biotransformation, immobilization, PTFE, methandienone.

INTRODUCTION

Biotransformation provides converting the foreign substrate to less toxic substances. Microbial transformation is one of the most attractive approaches for introducing functional groups into various positions of organic compounds. Microbial transformation of steroid has been studied by almost every type of microorganisms including fungi [1,2,3,4,5,6]. Fungal biotransformation of steroids is among the earliest examples of biocatalysis for producing stereo- and site-specific products. Fungi that grow in simple conditions and having no harmful effects on human health are of importance in this field. Variety of steroids are widely used as anti-inflammatory, diuretic, anabolic, contraceptive, anti and ergonic, progestational, and anticancer agents as well as other application [7]. Methandrostenolone (methandienone) is described as, anabolic, and causes weight gain [8]. It is also used for post-menopausal osteoporosis and prevents bone loss. However, the increased bone mass above initial value is less certain [9, 10]. Methandienone is one of the most highly effective mass building steroids, ever treated. In addition, study on osteoporosis showed that the methadienone is more effective than calcium supplementation in reducing osteoptic activity; rather it was shown to increase muscle mass more effectively [11]. The growing market and the continuous demand for the steroid encourage searching for new methods of improvement and increasing the production. A suitable reaction mixture was adopted and the potential of the isolated fungal strain was investigated. Utilization of the immobilized microbial cells has many advantages over the free cells. At least immobilization can support the mechanical strength for the immobilized cells. It is well known that PTFE has a high melting point, is chemically inert and strongly hydrophobic. The ePTFE membrane has high strength and a smooth surface and each square centimeter contains billions of continuous superfine fibrils interconnected according to the method of preparation the porous structure can be controlled [12]. Compared with conventional suspension system, the immobilized microorganism technology offer a multitude of advantages, such as high biomass, high metabolic activity and strong resistance to toxic chemicals [13,14].

This work includes the use of cell immobilization for the production process. The process involved the optimization of different parameters controlling the biotransformation and affecting the cell immobilization process. The optimization determine the optimum conditions and predict the maximum product outcome. Methandienone production was taken as a pattern for medical important biotransformation process.



Methyltestosterone Methandienone

MATERIALS AND METHODS

Microorganisms.

Eleven fungal strains were isolated from marine water. The plates of malt extract agar media containing Chloramphenicol were seeded with 1.5 ml of water from Red sea and Mediterranean Sea. Thereafter, these plates were incubated for three days at 25º C, the growing culture were purified and transferred to new plates. After purification the fungal isolates were maintained refrigerated on Sabroud's dextrose agar media. The purified cultures for the fungi were identified on the basis of its taxonomical characteristics [15, 16].

Biotransformation using culture media

Potato dextrose media was used as growth medium and as a culture medium for the transformation process during screening. Two milliliters of spore suspension ($6x10^5$ spore / ml) were used to inoculate fraction of 50 ml of the sterilized media in 250 ml Erlenmeyer flasks. The inoculated flasks were then incubated on shaker at 150 rpm and 27º C for 48h then 1mg of methyltestosterone dissolved in 0.5 ml of absolute ethanol was added to each flask for induction. After 24h, 10 mg of methyltestosterone dissolved in 1ml of ethanol were added to the flasks and the biotransformation process was conducted for anther 48h.

Analysis.

After completion of the biotransformation process, the contents of each flask were extracted with double of its volume of ethyl acetate. The organic layer was separated and dried over anhydrous sod. sulphate then evaporated using rotavapour till dryness. The residual substances after evaporation were dissolved in 5 ml of methanol. These residuals were considered as the test materials. Amount of 1 ml the test materials containing the transformation products were applied to TLC plates that were developed using solvent system composed of n-hexane- ethyl acetate (85:15 v/v). In each case, transformation products were identified by comparison of the specific Rf value (0.4 for methandienone and 0.5 for methyltestosterone) and the color with their corresponding authentic in day light, metandienone, orange-brown, methyltestosterone, orange and under UV rays. The quantitative analysis was done using HPLC analysis, Shimadzu, PL Hi-Plexpb column. One ml of the prepared test material was evaporated till dryness in a water bath at 70ºC. The residues were dissolved in acetonitrile and injected in the apparatus. The apparatus runs under the following conditions: Wavelength 240 nm, -Flow rate 0.8 ml /min, Solvent system acetonitrile : water 70:30 (v/v) (Fig.1).

Estimation of the methandienone yield.

All results given in this work were the arithmetical means of at least three different replicates. The experimental values of the same treatment which showed a difference exceeding ±5% were routinely discarded and the treatment were repeated again

Where:

$$RT = \frac{\text{amount of residual methyltestosterone}}{\text{Total amount of the charged methyltestosterone}} x100$$

$$MT = \frac{\text{amount of detected Methadienone}}{\text{Total amount the added methyltestosterone}} x100$$

Immobilization procedure

After screening the strain which form the highest amount of MT was subjected to the immobilization process. The fungal mycelia were separated by filtration after growth on PDA and washed thoroughly with saline solution (0.5%). The desired cell weight was suspended in saline. A slurry of PTFE particles (0.05-0.5 µm) in emulsion (approx. 60%by weight) was added dropwise to suspension of cells to a final concentration of 30% (w/w). The formed mixture was then centrifuged at 6000 rpm for 20 min. After discarding the supernatant, the mixture was washed with saline phosphate buffer (SPB) and centrifuged. The mixture was placed in a Petri dish and pressed carefully with a rubber mill in different directions to form a thick membrane of approximately 3 mm thickness. The formed thick membrane was cut into pieces. The pieces were kept refrigerated in SPB to be used. The resulted membrane had a porous structure (Fig.2).

Biotransformation using the immobilized cells.

The immobilized cells were placed in 50 ml of sterile citrate phosphate buffer at pH 6 in 250 ml Erlenmeyer flasks (the reaction mixture). Then of 10 mg of methyltestosterone dissolved in 1 ml ethanol were added to the reaction mixture. The biotransformation process was conducted at 25ºC for 24h or as specified. The extraction and the analysis were performed according to the described methods.

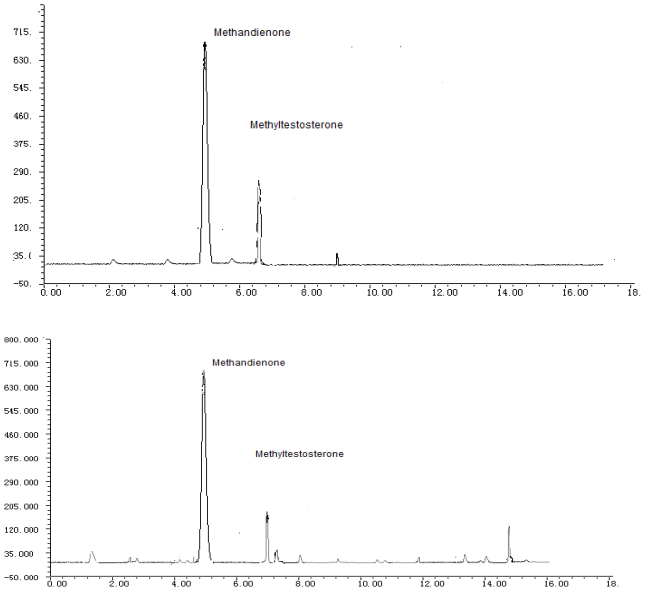


Fig (1) The chromatogram of the biotransformation products. The chromatogram for the authentics (above) and the chromatogram for the test material (blew)

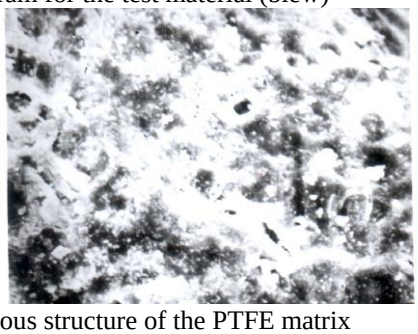


Fig (2) Porous structure of the PTFE matrix

Evaluation and optimization

Two steps have been performed in order to determine the most significant factors and the optimum conditions for these significant factors. In first step regular two factorial design was used to determine the significant factors while in the second step three level quadratic design Box-Behnken [17] was used to investigate the optimal for the significant factors and to predict the maximum product yield. The equation for the model as follows:

$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{11}X_{21}+b_{22}X_{22}+b_{33}X_{23}$

Where Y is the predicted MT (%), X_1, X_2 and X_3 are the factors. b_0 is a constant, b_1, b_2, and b_3 are the linear coefficients, b_{12}, b_{13}, and b_{23} are the coefficients of interactions among the variables, and b_{11}, b_{22}, and b_{33} are the quadratic coefficients.

RESULTS AND DISCUSSION

Screening.

Eleven fungal isolates were identified and screened for their ability to transform methyltestosterone to methandienone, only the most potent was selected to be used for immobilization and in the subsequent experiments (Fig.3). The identification depends on the colonial morphology and the microscopic characterization. The most potent fungal strain was identified as *Circinella mucoroides*, which was immobilized and was used for the biotransformation process. This agrees with the published data, many fungal species of order mucorales were reported to have biotransformation activities [4]. The methandienone (MT) yield was 48.11% with residual methyletestosterone (RT) 33%.

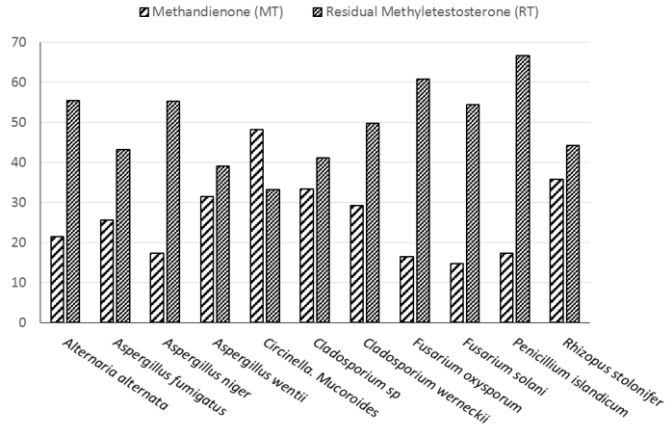


Fig (3) The isolated fungal species and their biotransformation abilities to transform methytestosterone to methandienone

pH Relation.

Changing the pH value of the reaction mixture from 4.4 to 7 resulted in variation of the MT yield (Fig. 3). Lowering the pH value to 4.4 led to a drop in MT formation from about 56% to 13.12% also increasing the pH value to 7 the MT yield fall to 45.21%. The values of pH 5.5-6 appeared to be suitable for the MT formation as a major product of biotransformation. Above and blew these pH values the product formation was depressed. On the other hand, to investigate the effect of the type of buffer, different buffer systems were used as in Fig. (4). Citrate buffer, Citrate phosphate buffer, phosphate hydroxide, succinate and disod. hyd. phathalate /Sod. dihyd. orthophosphate were used separately as buffer systems in the reaction mixture. It was found that there is no difference between these systems of buffer. Accordingly, any of these buffers can be used in subsequent experiments.

The carrier concentration.

Different amounts of PTFE, 25, 30, 40, 50, 60, 75 and 100% were used to immobilize 2g of fresh mycelia. It was found that increasing the PTFE to 60% improved the production formation to 61.33%, while concentration over 60 % decreased MT formation (Fig. 5). At PTFE content (100%), the MT yield was 45.96%. Although the matrix may, support the mechanical strength of the cells, but it is could form a barrier to reduce the accessibility of the substrate for cell and mobility of the product from inside to the surrounding reaction mixture. The effect of this diffusion barrier may explain the reduction of the product formation at high matrix concentrations.

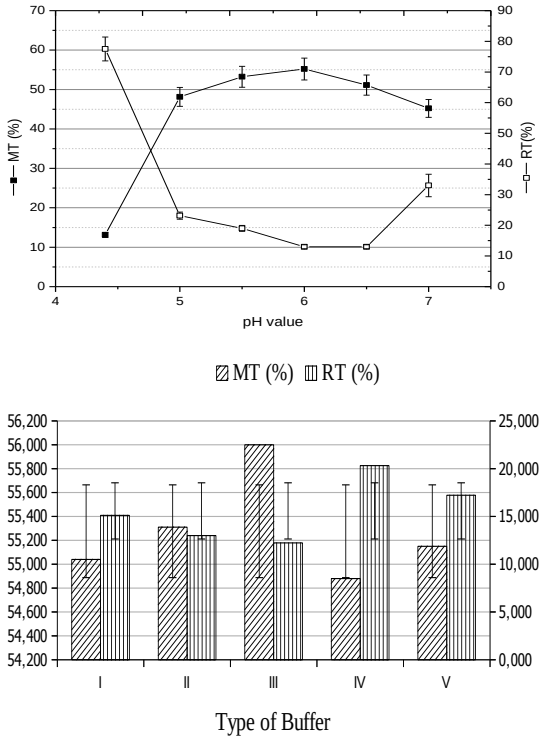


Fig (4) The relation of the pH value and the type of buffer with the methanedienone formation

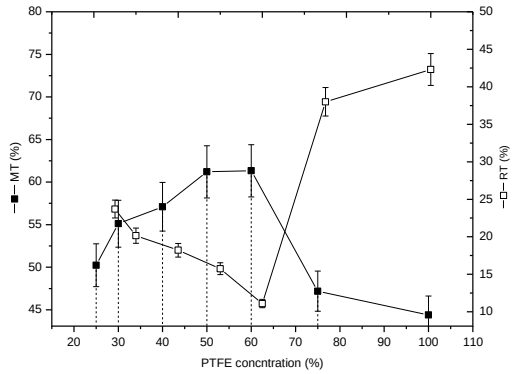


Fig (5) Effect of the carrier concentration on the biotransformation process.

The solubilizing agents.

Steroids are insoluble in aqueous environment solvents are added to increase the solubility of the substrate (Smith 1984). The effect of some different solvents that are used to solubilize the substrate was investigated, ethanol, acetone, acetonitrile, methanol and propanol were used. The results indicated the suitability of all these solvents for the biotransformation process (Fig. 6). Any the above mentioned solvent could be used during the biotransformation process. Furthermore, to increase the solubility and the accessibility of the substrate to the cells some surface-active substances were added separately (Fig.4). Each of Tween 40, 60, 80, triton x100 and triton x200 was added and caused an increase in the MT formation. The effect of surface active on the cell permeability was reported by Williams and Fieger [18], Tween 40 acted on lipoprotein of cytoplasmic membrane of the living cells, this will affect the permeability of the cellular Membrane. Generally, Tweens are able to make physical and chemical changes in the cell wall changing the cell permeability [19]. Changing the cell permeability may facilitate the mobility of the substrate into the cells and the product to outside the cells. However, high concentrations of these surface-active materials will disrupt the secondary and tertiary structure of the enzyme leading to decrease in product formation. Vieira et al. [20] reported the toxic effect of surface-active substances on the microbial cells.

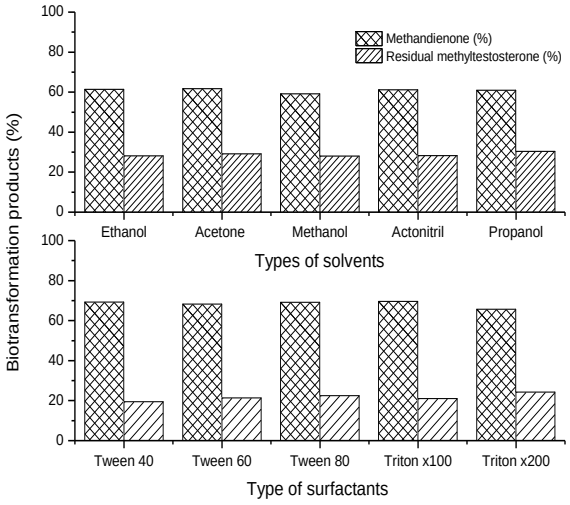


Fig. (6) Effect of the solubilizing agents, Solvents and Surface-active substances, on MT production

Supplementation with carbon sources.

The carbon sources were added to the reaction mixture to support the metabolic activities of the resting immobilized cell (Fig. 7). The results depicted that glucose was the most appropriate carbon source to be added to the reaction mixture if it compared with the other ones. Moreover, the variation of the glucose concentration was investigated and it was found that, the addition of glucose improved the MT formation to reach 73%. However, addition of excess glucose above 20 g/l led to a reduction of MT yields with a parallel increase in residual glucose. Glucose may support the metabolic activities of the immobilized cell under the stress of the biotransformation and after the immobilization process itself. In spite of this positive effect, addition of excess glucose as a fast and easy metabolized carbon source can hinder the transformation activities. Vezina *et al*. [21] reported that, the requirement for glucose might be tied to the generation of NADPH, which is essential for steroid transformation reaction.

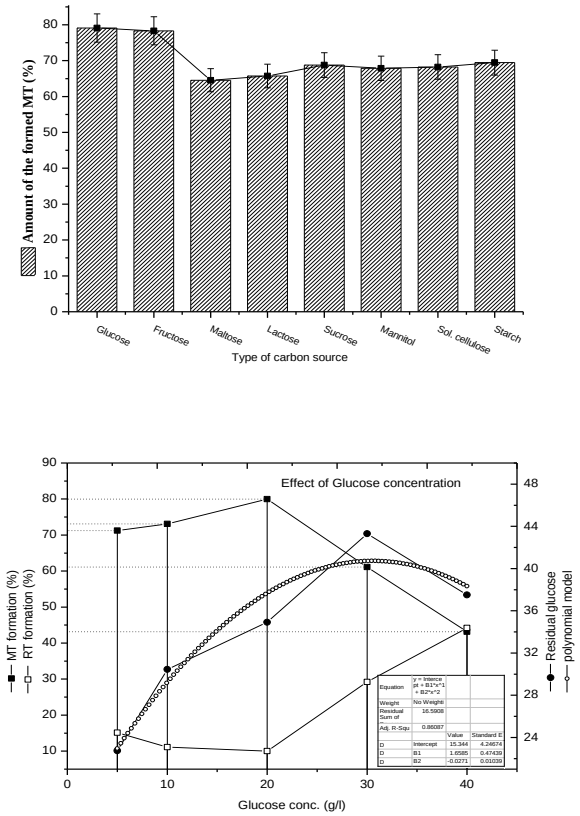


Fig (7). Supplementation the reaction mixture with carbon sources

The amounts of the added substrate.

To measure the biotransformation load of the immobilized cells different amounts of substrate concentration were used. The gradual increase in substrate to 50 mg was explored (Fig. 8). Gradual increase of the substrate concentration resulted in a gradual decrease of the MT formation. This may be attributed to the toxicity of the substrate to the microbial cells. This agrees with that reported by Breskvar [4] and Smith [22] who recorded the toxic effect of the steroids on the microbial cells.

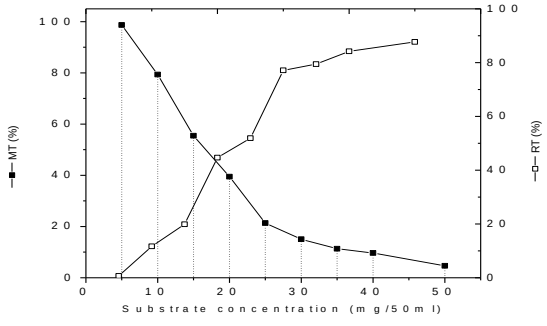


Fig (8) Biotransformation capacity of the immobilized cells for different substrate concentrations

Improvement of the product production.

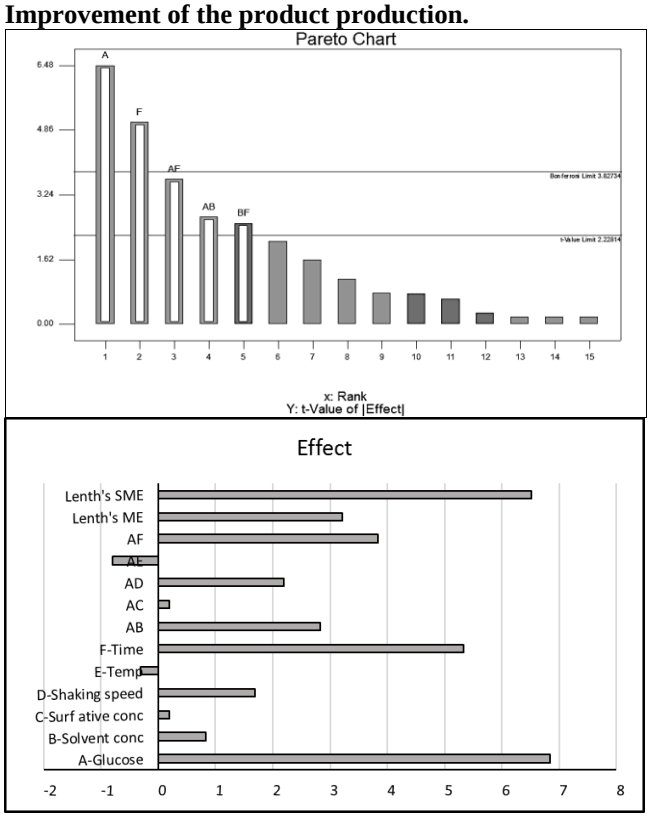


Fig (9) Pareto chart and effect chart for the two level factors model.

The two level factorial model consisted of a totally sixteen runs for six factors of different conditions. The results of these experiments are indicated in Table (1). Based on the statistical analysis, glucose, shaking speed and time are the significant factors. P values of these factor are less than 0.05. In the Pareto chart (Fig. 9), the maximal effect was presented in the upper portion and then progress down to the minimal effect. Accordingly, the most significant factors determining the MT formation are glucose, shaking speed and time. The three factors were subjected to three levels quadratic model of Box-Behnken where the total number of runs reached 17 (Table 2). This model can predict the maximum yield after determining the optimum conditions. The model was examined by the coefficient of determination, R2, which was found to be 0.97, indicating that the sample variance of 97.00% was attributed to the variables (Table 2). A regression model having R^2-value higher than 0.97 was considered as having a very high correlation [23]. Therefore, the present R^2 -value reflected a very good fit between the observed and predicted responses, and implied that the model is reliable for MT production in the present study. The adjusted determination coefficient (R–Sq = 94%) was also satisfactory to confirm the significance of the model. The equation of the model as follows:

MT (%) = +82.03+4.44.A+0.76. B-1.65. C+0.12. A. B.-0.67. A. C-1.08. B.C.-7.27. A2 -0.80. B2- 1.31. C2

Where, A is glucose, B is shaking speed and C is the time.

The model also showed statistically insignificant lack of fit (P= 0.47), so the model was supposed to be adequate for prediction within the range of the variables (Table 2). The combined effects in Fig (10) illustrate the effect of the three factors. The product formation, increase when glucose near to its middle value and shaking at its high value and time at its lowest value. Therefore, the optimum condition for these three factors after solution of the model is, glucose at 25 g/l, shaking speed at 200 rpm after 24h of reaction time. High shaking speed allows a good contact between the cells and the substrate serving a better transformation.

Table (1) The results and statistical analysis for regular two level factorial model

Runs	Glucose (g/l)	Solvent conc. (ml)	Surfactant Conc. (ml)	Shacking speed (rpm)	Temp. (ºC)	Time (h)	MT (%)
1	20	2	1.5	150	30	48	80.11
2	20	2	0.5	150	20	12	75.00
3	0	0.5	0.5	50	20	12	66.00
4	20	2	0.5	50	20	48	75.55
5	0	0.5	0.5	150	20	48	68.00
6	0	2	1.5	150	20	48	65.00
7	0	2	0.5	150	30	12	66.00
8	20	0.5	1.5	150	20	12	67.00
9	20	2	1.5	50	30	12	69.00
10	0	0.5	1.5	150	30	12	65.00
11	0	2	1.5	50	20	12	65.00
12	20	0.5	0.5	50	30	12	63.00
13	20	0.5	1.5	50	20	48	77.00
14	0	0.5	1.5	50	30	48	70.00
15	20	0.5	0.5	150	30	48	78.00
16	0	2	0.5	50	30	48	65.00

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	453.3176	8	56.66	36.34	< 0.0001
A-Glucose	186.7322	1	186.73	119.75	< 0.0001
B-Solvent conc.	2.772225	1	2.77	1.77	0.2242
D-Shaking speed	11.4921	1	11.491	7.37	0.0300
F-Time	113.7422	1	113.74	72.94	< 0.0001
AB	32.09223	1	32.09	20.58	0.0027
AD	19.2721	1	19.27	12.35	0.0098
AF	58.75223	1	58.75	37.67	0.0005
BF	28.46223	1	28.46	18.25	0.0037
Residual	10.91483	7	1.55		
Cor Total	464.2324	15			

Table (2) The matrix and the statistical analysis for the Box-Behnken model

Run	Glucose (g/l)	Shaking speed(rpm)	Time (h)	MT (%)
1	40	150	48	75.45
2	40	150	24	79.79
3	25	150	36	83.11
4	25	150	36	83
5	10	200	36	69.33
6	10	100	36	69.16
7	25	100	24	79.33
8	10	150	24	70.1
9	25	200	24	84.1
10	25	200	48	78.33
11	25	150	36	80.12
12	40	100	36	78.34
13	40	200	36	79
14	10	150	48	68.45
15	25	150	36	83.23
16	25	100	48	77.88
17	25	150	36	80.67

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	432.14	9	48.01	27.49	0.0001
A-Glucose	157.88	1	157.88	90.39	< 0.0001
B-Shak.speed	4.575	1	4.57	2.61	0.1496
C-Time	21.81	1	21.81	12.48	0.0095
AB	0.06	1	0.06	0.03	0.8582
AC	1.80	1	1.80	1.03	0.3427
BC	4.66	1	4.66	2.67	0.1462
A^2	222.26	1	222.26	127.25	< 0.0001
B^2	2.71	1	2.71	1.55	0.2526
C^2	7.25	1	7.25	4.15	0.0809
Residual	12.22	7	1.74		
Lack of Fit	3.18	3	1.06	0.46	0.72
Pure Error	9.04	4	2.26123		
Cor Total	444.36	16			

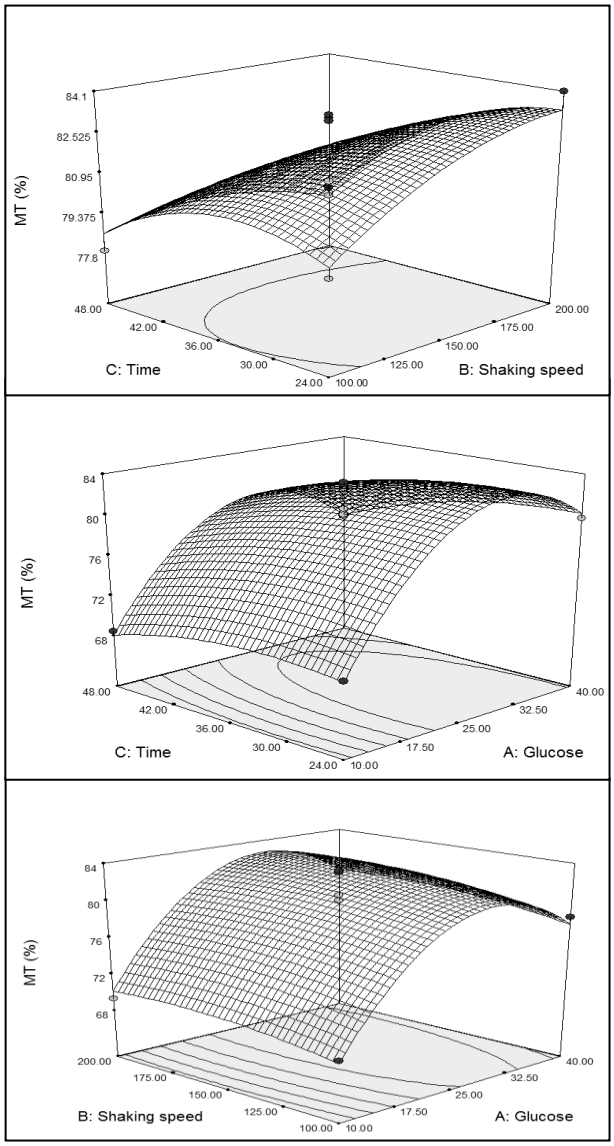


Fig (10). The campaigned effects of the three factors, glucose, shaking speed and time on the MT formation.

Recycling.

The reuse of the immobilized cells is one of the targets for which the immobilized cells were utilized. It was shown that the immobilized retained acceptable biotransformation efficiency after 6 runs and the lowest activities were recorded after run number 11 and run number 12 (Fig. 11). It may be the resting cells undergo metabolic exhaustion upon repeated use. However, reusability represents an advantage for using immobilized cells.

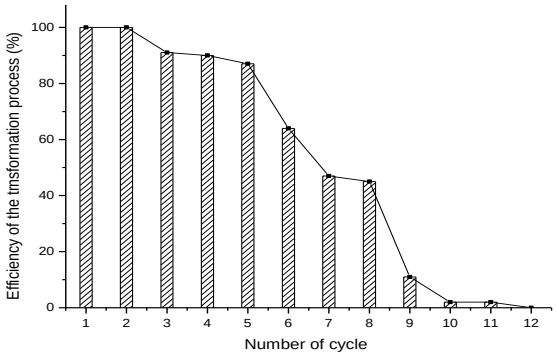


Fig (11) Repeated use of the immobilized cells

Storage.

The ability of the cells to resist long-term storage was measured. The immobilized cells were successfully stored in 4º C, 25º C and 30º C for 7, 14, 20 and 30 days. It was found that the storage at 4º C is more suitable than storage at 25 or 30º. At 4º C the cells retained 71.5 % of its biotransformation efficiency after 20 days (Fig. 12).

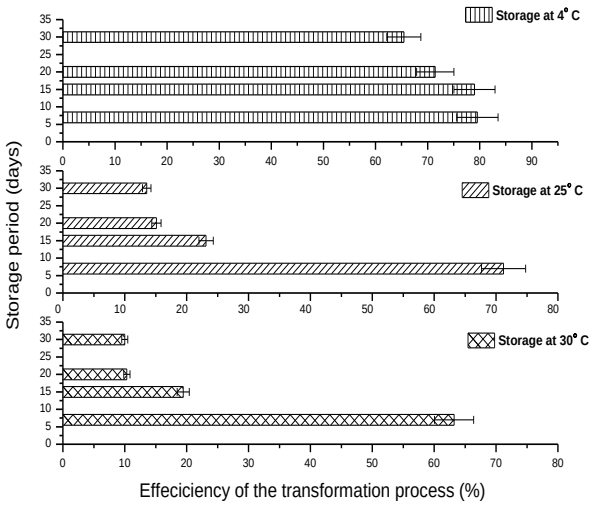


Fig (12) Efficiency of the immobilized cell for biotransformation after long time storage.

CONCLUSION

The immobilized cells on PTFE was successfully used for the transformation of the methyltestosterone to methandienone. The immobilized cells were recycled and stored for one month and showed a good transformation ability. The optimization of some controlling factors led to a significant improvement in the production yield.

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