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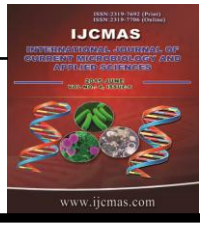

Original Research Article

Opmization of 11 α-Hydroxy-Progesterone Produced by Biotransformation of Progesterone with Immobilized *Penicillium auranticum* Cells on Poly Vinyl Alcohol (PVA) Beads using Central Composite Experimental Design

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Keywords

Central composite design; Penicillium auranticum; 11 α-hydroxy-progesterone; PVA beads

A B S T R A C T

The use of hydrophilic supports, as an immobilization matrix for *Penicillium auranticum* cells, was evaluated. Several homo- and co-polymers prepared by γ-irradiation were tested for cells surface adhesion. Immobilized *Penicillium auranticum* cells onto poly (vinyl alcohol) (PVA) and poly (Acrylamide- co-Acrylic) poly (AAm-co-AAc) acid copolymer hydrogels led to the best bioconversion estimates(59.8&43.9% respectively).The formation of gels having a good strength with the ability to retain a desirable amount of water in their three dimensional network can be achieved by using PVA polymer of composition (10%) and prepared at 20 kGy irradiation dose. At these conditions the prepared hydrogel is considered the most favorable one that gave the highest 11 α-hydroxy-progesterone yield, 64.1 %. 2^3Central composite design (CCD) was employed to optimize the conditions for the maximum biotransformation. 94.05 % biotransformation yield was achieved by incubating the PVA beads inoculated with 4ml of 48 h old inoculum contained $12X10^{-8}$ cell/ml in progesterone concentration of (30mg/50ml) for 72h with1.5 Fold increase in 11 α-hydroxy-progesterone yield. Moreover, PVA *Penicillium auranticum* beads could be used for 9 successive biotransformation cycles. The maximum product output obtained at 4th cycle was about 96 %. This work clearly highlights the ability of hydrogels to serve as an efficient immobilization matrix for *Penicillium auranticum* cells to convert progesterone to 11 α-hydroxy-progesterone.

Introduction

The importance of microbial biotechnology in the production of steroid drugs and hormones was realized for the first time in 1952 when Murray and Peterson of Upjohn Company patented the process of11α-hydroxy-progesteroneby a *Rhizopus* species (Murray and Peterson,1952). Since then, Microbial transformations of steroids provide an important method of obtaining new steroid derivatives of potential pharmaceutical activity, which additionally fulfills green chemistry principles (Huet

al.,1995; Mahato and Garai,1997;Mohamed andAbd El-Hadi, 2010). Progesterone plays an important role in repair of myelin sheath of damaged nerves(Fernandes *et al*.,2003).In many cases, hydroxyl derivatives are characterized with much higher biological activity than the less polar substrate.

Cell immobilization has been attempted to alleviate the inhibitory effects of the product and substrate (Flygare and Larsson, 1987; Lee and Liu, 1992)We have developed a novel and convenient method to immobilize microbial cells by the use of Gamma radiation cross linking of poly vinyl alcohol pre polymer . Microbial cells immobilized by this method have been successfully applied to the dehydrogenation of various steroids[Abd El-Hadi et al.,2004]. Hydrogels are polymeric networks, which absorb and retain water without dissolving. This property makes them interesting materials as carriers for immobilization of bioactive compounds as alternatives to others successfully used (Rosiak and Yoshii, 1999). PVA is a polymer with exceptional properties such as water solubility, biodegradability, biocompatibility, non-toxicity and non-carcinogenity that possesses the capability to form hydrogels by chemical or physical methods (Patachia *et al.*, 2009).

For several decades, statistical design-of-experiments techniques have been developed to obtain the optimal conditions by determining the effects of parameters and overcoming the disadvantages of factor interactions(Ren *et al*., 2006; Liu *et al*., 2013). The response surface method (RSM) is one such technique that is used extensively in the biotechnology industry (Wejse *et al*., 2003; Rui *et al*., 2009) . The RSM is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching for the optimum conditions. The RSM has become established as a convenient method for developing optimum processes with precise conditions that has also minimized the cost of production of many processes by efficient screening process parameters. The optimal conditions or the region that fits the operation specification can be determined by the RSM via a curvature approach(Elibol and Dursun, 2002).

The objective of the present work was to probe the feasibility of *Penicillium auranticum* cell adsorption onto homo hydrogel with the purpose of increasing the efficiency of progesterone transformation to 11α-hydroxy progesterone.

According to our knowledge, we are reporting for the first time the optimization of formation of 11α-hydroxy progesterone by using central composite design (CCD) by *Penicillium auranticum* cells immobilized onto radiation cross linked poly (PVA) hydrogel. Highoperational stability of the immobilized cells to be used in repeated batch process was also outlined.

Materials and Methods

Microorganism and medium

Penicillium auranticum strain was provided by maintained on potato dextrose agar medium (PDA)(g/L):Infused white potato 300, glucose 20 and agar 20 with an initial pH 6.8. The bioconversion medium contained(g/L):Glucose 20, peptone 1, yeast extract 1, KH_2PO_4 0.74, L-asparigine 0.7 and $MgSO_4$. 7H2O 1.0, the pH of themedium was adjusted to 6.5 the spore concentration was 3×10^6 spores/ mL.

Materials

Steroids were provided by Sigma Chemicals Company. Polyvinyl alcohol (PVA) with

degree of polymerization of 14000 and Poly (acrylamide-co-acrylic acid) poly (AA m-co-AAc) were purchased from Merck (Germany). 2-hydroxyethyl methacrylate (HEMA) was supplied by BDH (Poole, UK).The biochemical reagents were purchased from BDH Chemicals Ltd. All other chemicals were of analytical or high-performance liquid chromatography (HPLC) grade and were procured from various standard sources.

Preparation of hydrogels

Aqueous solutions of PVA (10%, w/v) was prepared in a water bath at 90 ^{o}C for 50 min. The aqueous solutions were poured in glass tubes.2-HEMA monomer was mixed with distilled water and ethanol (2:3, v/v) to yield a final concentration of 10%.1gm of AAm and 1ml of AAc were added to 8 ml distilled water for 24 hours and then every sample was stirred for 1 hour at (400 rpm) to ensure a very well mixing (Craciun *et al*., 2013).The nitrogen gas was passed through the solutions for 24 h to remove the dissolved oxygen. They were irradiated at -78"C with γ-rays from a Co^{60} source for 8h with a dose rate of 20 kGy/h . The resultant polymer carriers were cut into discs, approximately 3-5mm in diameter, and shaken with an excess amount of water for 2 days in order to be fully swollen. The swollen carriers were sterilized by autoclaving at 121"C for 40 min. The sterilized carriers were immersed separately into the nutrient medium for 2 days to imbibe the medium (Abd El-Hadi, 2003).

Cell growth and immobilization

Unless stated otherwise, the tested microorganism was grown either in the presence or absence of hydrogels by adding an inoculum to 250 ml Erlenmeyer flasks, containing 50 ml of bioconversion medium at 30^{o}Cfor 48 h. Cells were immobilized by adsorption onto hydrogel, added to the growth medium in a weight to volume ratio of 5g wet.wet/50 ml medium. Immobilized cells were harvested in an adequate sieve at the late exponential growth phase, thoroughly washed with pH 7 potassium phosphate buffer (0.2 M), and stored at -20^{o}C until use. Free cells were harvested by centrifugation (5 min, 4^{o}C, 5000 rpm) in the late exponential growth phase, thoroughly washed with pH 7 potassium phosphate buffer (0.2 M). The wet cell past (roughly120 mg dry cell wet/g) was either immediately used in bioconversion trials or stored at -20^{o}C until use (Staeblel *et al.*, 2004).

Evaluation of immobilized cells bioconversion

Unless otherwise stated, experiments were performed in 250 ml Erlenmeyer flasks, containing 50 ml of bioconversion medium supplemented with 10 mg solution of progesterone in 1 ml ethanol, and inoculated with free (2 g wet cell paste) or immobilized cells. Bioconversion was extended to 48 h, in a rotary shaker (150 rpm) at 30^{o}C. At the end of the transformation period, medium was extracted with twice its volume with chloroform. On occasion, when immobilized biocatalysts were used, solid and aqueous phase were recovered and extracted separately with chloroform. The extraction was repeated three times. The organic phase was collected and evaporated to dryness. The dried solids (test material) were then dissolved in chloroform and assayed for progesterone and11α-hydroxy progesterone using TLC and/or high performance liquid chromatography (HPLC).

Extraction and analytical procedure

For TLC, aliquots were applied to Kiesel gel 254 (Merck, Germany) plates. These were developed in chloroform/ acetone / ethyl

alcohol (40: 10: 0.7, v/v) and visualized under UV light (240 nm). 11α-hydroxy progesterone was visualized using spraying by 5% iodide solution .Sample preparation for HPLC analysis was carried out by extracting 5mL of fermentation culture with 5mL of extraction reagent (Chloroform: methanol, 1:1 v/v) in 50 mL Erlenmeyer flask and mixed for 10 min. After the clear separation of the organic phase from the aqueous phase, a certain fraction was withdrawn into 1.5 mL Eppendorf tube dried by exposure to the air. HPLC analysis. HPLC experiments was carried out on Jasco system consisting of a PU-980 HPLC pump, anAs- 950 sampler, a vv-975vv/visible detector, and an LG-980-02 gradient unit (Jasco, Gross-Umstadt, Germany).

Steroid standard solutions were 50 µg/ mL in methanol. Mobile phase (acetonitrile: water) were degassed before use. A reversed phase waters Novapak Nukleosil C18 (4µm, 3.9 × 150 mm) (waters, Milford, MA, USA) was used for these studies. Flow rate was 1.0 mL/min. 20 µL of a 50 µg/mL steroid solution in methanol was injected each time the column temperature was maintained at 25°C using the column thermostat BF0- 04 (w. o. Electronics,

Langenzersdorf, (Austria) the absorbance was measured at 240 nm. Each standard was analyses five times (Lisurek *et al*.2004).

Gel determination

In order to extract the insoluble parts of the hydrogels, that is, the gelled part, the prepared hydrogels were soaked in water for 48 hours at 100◦ C, then they were taken out and washed with hot water to remove the soluble part, dried and weighed. The gel percent in the hydrogel was determined from the following equation:

$Gel(\%) = \frac{WE}{Wg} \times 100$ (Murray and Peterson 1952)

Where *WE* and *Wg* are dry hydrogel weights after and before extraction, respectively (Lug˜ao *et al*.,1998).

Swelling measurement

The equilibrium swelling time of hydrogels depends on their gel content and cross linking density which are correlated to their preparation conditions. Therefore, the water content of the hydrogel formed by irradiation was determined by immersing the hydrogel in production medium at 30◦ C for 24 hours and then weighed. The water content was calculated based on the weight difference of the dry and swollen samples by using the following equation:

Sw (%) = $\frac{Ws-W\,0}{}$ ×100 (Hu *et al*.,1995)

*W*0,

where *W*0 and *Ws* are the weights of gel in the dry and swollen states, respectively (Savas *et al*., 2002).

Optimization of Biotransformation by Central composite design(CCD)

Substrate concentration, inoculum size and biotransformation time were selected for response surface methodology of central composite design (CCD). CCD proposed by (Box *et al*., 1987; Adinarayana *et al*., 2003). 2^3 factorial design with six star points and six replicates at the central points were employed to fit the second-order polynomial model, the experimental design consisted of 20 runs and the independent variables were studied at five different levels. The experimental design used for the study is shown in Table 3. All the experiments were done in triplicate and the average of activity

yield obtained was taken as the dependent variable or response (*Y*). The second-order polynomial coefficients were calculated and analyzed using the 'SPSS' software (Version 16.0) Second degree polynomials, Eq.(4), which includes all interaction terms, were used to calculate the predicted response:

$$Y_{Activity} = ß_0 + ß_1 X_1 + ß_2 X_2 + ß_3 X_3 + ß_{11} X_1^2 + ß_{22} X_2^2 + ß_{33} C_3^2 + ß_{12} X_1 X_2 + ß_{13} X_1 X_3 + ß_{23} X_2 X_3$$ Eq. 4

Where $Y_{Activity}$ was the predicted production of invertase (U/ml), and X_1, X_2 and X_3 were the independent variables corresponding to the concentration of Substrate concentration, Inoculum size and Incubation time respectively; $ß_0$ was the intercept, $ß_1$, $ß_2$, $ß_3$ were linear coefficients, $ß_{11}$, $ß_{22}$, $ß_{33}$ are quadratic coefficients, $ß_{12}$, $ß_{13}$, $ß_{23}$ are cross product coefficients. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficient of determination for each variable, the quadratic models were represented as contour plots (3D) and response surface curves were generated by using STATISTICA (0.6).

Results and Discussion

Bioconversion using γ - irradiated different monemers for *Penicillium auranticum* immobilization

Effect of different monomers as a carriers for cell immobilization was studied and it is shown in Fig.1. Higher bioconversion values were consistently observed when PVA and (AAm-co-AAc) were used as immobilization matrix, as compared to HEMA polymer. For non-ionic hydrogels such as PVA, and HEMA, swelling is controlled by the hydrophilicity of the polymers or monomers [24]. The hydrophilicity of PVA hydrogel is much higher than that of HEMA based hydrogel. The higher swelling of the hydrogels permitted the presence of more nutrient medium and cells inside of the hydrogel. Although the highest bioconversion yield (59.8%)was recorded using PVA homopolymer hydrogel. PVA is more preferable due to its higher mechanical strength, so that PVA hydrogelwas selected for progesterone bioconversion .

Effect of the degree of cross linking of PVA hydrogel prepared at different irradiation doses on the progesterone bioconversion

The effect of irradiation dose on the degree of cross linking and swelling property of PVA hydrogels was investigated and is shown in Fig.2.and Table.1.By increasing the irradiation dose, the gel content and cross linking degree increase resulting in a decrease inpolymer swelling property. As the irradiation dose increases, the polymer free radicals increase and consequently the degree of cross linking and gel content increase. Bioconversion of progesterone to its derivative 11 α-hydroxyprogesterone by immobilized cells is greatly affected by the total γ-irradiation dose adsorbed by PVA hydrogel. Fig.1. shows the relationship between progesterone bioconversion and 11 α-hydroxyprogesterone yield against PVA polymer prepared at different irradiation doses (10, 15, 20, 25 and 30kGy) . The11α-hydroxy progesterone yield reached the maximum value (64.1%) when γ-radiation of 20kGy was used for PVA polymerization process. However, by using PVA polymer prepared at 10 and 30kGy, the11α-hydroxy

progesterone yields were 42.0 and 24.0%, respectively. At low irradiation dose (10 kGy), the degree of crosslinking of the polymer matrix-entrapped cells is low, therefore the cells easily release from the polymer matrix to the medium giving the lowest progesterone bioconversion [Fernandes *et al*. 2003]. At the high irradiation dose (30kGy) cross linking degree, network density and gel content of PVA polymer hydrogel is high, these reduce the polymer swelling values and constantly the diffusion rate of substrate and product through the polymer matrix decreases resulting in reduction in the progesterone bioconversion(Abd El-Hadi,2003;Naim*et al*.,2003). Hence, 20kGy irradiation dose is considered the most favorable dose suitable for cross linking copolymerization process of PVA-entrapped cells, which gave the highest progesterone bioconversion (64.1%) and higher11α-hydroxy progesterone productivity (2.6 mg/L/h).

Factors affecting the bioconversion of progesterone by PVA immobilized *P. auranticum* were studied to establish the optimum conditions for bioconversion.

Optimization of progesterone bioconversion by immobilized *P. auranticum* on PVA beads by Central composite design (CCD)

Three variables (progesterone concentration, X_1; inoculum size, X_2 and incubation time, X_3), which significantly influenced 11α-hydroxyprogesterone production, were chosen to determine their optimum response region achieving maximum biotransformation yield. Table.2 represents the design matrix of the coded variables together with the experimental results of the 11 α-hydroxyprogesterone yield. All experimental trials were performed in triplicate and the average of the observations was used. The maximum11 α-hydroxyprogesterone yield was 94. 05 in run 15, while the minimum yield was 32.5 % obtained in run 10. This result reflected that the maximum11 α-hydroxyprogesterone yield produced by immobilized *P. auranticum* cells was obtained when the PVA beads inoculated with 4ml of 48 h old inoculum contained $12X10^{-8}$ cell/ml in progesterone concentration of (30mg/50ml) for 72h. The following regression equations obtained after the standard analysis of variance (ANOVA) presented the level of 11 α-hydroxyprogesterone yield, as a function of the initial values of progesterone concentration, inoculum size and incubation period. Regression analysis was used to analyze the data and thus a polynomial equation was derived from regression analysis as follows:

$$Y_{yield} = 27.181 + 2.835X_1 + 26.691X_2 - 0.359X_3 - 0.214\ 33.001\ X_1^2 - 4.452X_2^2 + 0.308X_1X_2 + 0\ .003X_1X_3 + 0.085X_2X_3$$

Where A_{yield} is the response variable11 α-hydroxy progesterone, X_1 is the coded value of progesterone concentration, X_2 is the coded value of inoculum size andX_3 is the coded value of incubation period. Table.3 showed a significant *F*-value (4.130) which implied the model to be significant. Model terms having values of Prob> F (0.006) are less than 0.05 which considered significant. The regression equation obtained after ANOVA indicating that the determination of coefficient (R^2) was calculated as 0.939 for11α-hydroxyprogesteroneyield (a value of R^2> 0.75 indicated the aptness of the model) which indicates the statistical model can explain 93.9% of variability in the response, in reasonable agreement with the adjusted R^2 of 0.881. The goodness of the model can be checked by the determination

of coefficient (R^2) and correlation coefficient (R). The R^2 value is always between 0 and 1. The closer the R^2 is to 1, the stronger the model and the better it predicts the response [Munk et al., 1963]. The value of R (0.886) for Eq. (2) being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. an overall 1.5 - fold increase in 11α-hydroxy progesterone yield was being predicted after validation of RSM.Table4.shows the regression results from the data of central composite designed experiments. The larger the magnitude of the *t*-value and smaller the *p*-value, the more significant is the corresponding coefficient (Aravindan and Viruthagiri, 2007) This implies that the variable with the largest effect was the linear effect of the inoculums size and the squared term of the inoculums size. The initial biomass (inoculum size) considered to be in the efficiency of immobilized system. Though immobilized cell systems are reported to enable the maintenance of high cell densities resulting in higher overall reaction rates and higher products yields, which is not possible with suspension cultures (Konsoula and Kyriakides, 2006). The decreased activity with increase in bed inoculum might be due to competition between cells because of which nutrient concentration available in the medium might not have been sufficient for optimal growth(Anisha and Perma, 2008). Furthermore, quadratic effect of the inoculums size and progesterone concentrations are more significant than inoculums size and incubation periods.

Three-dimensional response surfaces were plotted on the basis of the model equation, to investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum. (Figs. 4, 5 and 6) show that higher levels of the enzyme were attained with increasing of inoculums size and progesterone concentration. On the other hand, incubation period have less effect on the biotransformation of progesterone by immobilized *Penicillium auranticum* cells.

Verification of the model

Optimal conditions realized from the optimization experiments were verified experimentally and compared with the calculated data from the model. The estimated 11 α-hydroxyprogesterone yield was 94.05%, where the predicted value from the polynomial model was 87.44%. This verification revealed a high degree of accuracy of the model of more than 92.2%, which is an evidence for the model validation under the investigated conditions. There is a general practice of determining the optimal conditions or medium composition by varying one factor at a time.

However, this method does not depict the net effect of total interactions among the various variables (Rathi *et al*., 2000)Thus, the emphasis has shifted toward condition optimization using statistical methods. The factorial design of a limited set of variables is advantageous in relation to the conventional method of manipulation of a single parameter per trial, as the latter approach frequently fails to locate the optimal conditions for the process, due to its failure to consider the effect of possible interactions between factors. Moreover, the factorial design makes it possible to take advantage of practical knowledge about the process during the final RSM analysis (Kalil *et al*., 2000), For the best of our knowledge this work is the first study in optimization of progesterone biotransformation condition by using response surface methodology. 94.05% yield was achieved after the optimization by using (CCD) by *Penicillium auranticum immobilized* onto PVA hydrogel .

Table.1 Gel content and swelling behavior of PVA of (10%) composition (Wt/Wt) at different irradiation doses

Irradiation dose (kGy)	Water content (%)	Gel content (%)
10	1630	65
15	1450	85
20	1220	95
25	950	97
30	880	98

Table.2 Central composite design (CCD) consisting of 20 experiments for three experimental factors in coded and actual values for the production of 11 α-hydroxyprogesterone by immobilized *P. auranticumon* PVA beads

	Factors levels						11 α-hydroxy-progesterone Yield (%)	
Trial No.	Substrate concentration (mg/l) (X_1)		Inoculum size (ml) (X_2)		Incubation time (h) (X_3)			
	Coded	Actual	Coded	Actual	Coded	Actual	Experimental	Predicted
1[a]	-1	10	-1	2	-1	48	76.5	68.233
2[a]	+1	20	-1	2	-1	48	31.6	39.983
3[a]	-1	10	+1	6	-1	48	58	61.173
4[a]	+1	20	+1	6	-1	48	33.105	45.243
5[a]	-1	10	-1	2	+1	98	63.35	60.283
6[a]	+1	20	-1	2	+1	98	28.4	33.533
7[a]	-1	10	+1	6	+1	98	70.05	70.223
8[a]	+1	20	+1	6	+1	98	39.7	55.793
9[b]	-2	5	0	4	0	72	63.9	77.41
10[b]	+2	30	0	4	0	72	3.5	2.765
11[b]	0	15	-2	1	0	72	23.85	31.927
12[b]	0	15	+2	8	0	72	21.25	13.468
13[b]	0	15	0	4	-2	24	79.5	76.192
14[b]	0	15	0	4	+2	120	87.8	78.688
15[c]	0	15	0	4	0	72	94.05	87.44
16[c]	0	15	0	4	0	72	94.05	87.44
17[c]	0	15	0	4	0	72	94.05	87.44
18[c]	0	15	0	4	0	72	94.05	87.44
19[c]	0	15	0	4	0	72	94.05	87.44
20[c]	0	15	0	4	0	72	94.05	87.44

Table.3 Analysis of variance (ANOVA) test for central composite design (CCD)

Term	Response Y_1 11 α-hydroxyprogesterone Yield (%)
F value	4.130
P>F	0.006
R	0 .881
R^2	.9390
Adjusted R^2	0.886
Sundered Error of the Estimate	15.38325
Regression sum of square	8795
Residual sum of square	1183
Regression mean of square	9977.235
Residual mean of square	236.644
Regression degree of freedom	10
Residual degree of freedom	9

Table.4 Model coefficients estimated by multiples linear regression (significance of regression coefficients)

Term	Regression coefficient	Standard error	t- test	P-value
Intercept	27.181	97.596	0.279	0.792
X_1	2.835	5.319	0.533	0.617
X_2	26.691	16.572	1.611	-0.168
X_3	-0.359	1.369	-0.262	0.803
X_1^2	-0.214	.107	-2.005	0.101
X_2^2	-4.452	1.388	-3.208	0.024
X_3^2	0.000	.008	0-.020	0.985
X_1X_2	0.308	.544	0.565	0.596
X_1X_3	0.003	.043	0.059	0.956
X_2X_3	0.085	.109	0.778	0.472

Fig.1 Effect of different matrices for immobilization of *P. auranticum* on progesterone Bioconversion

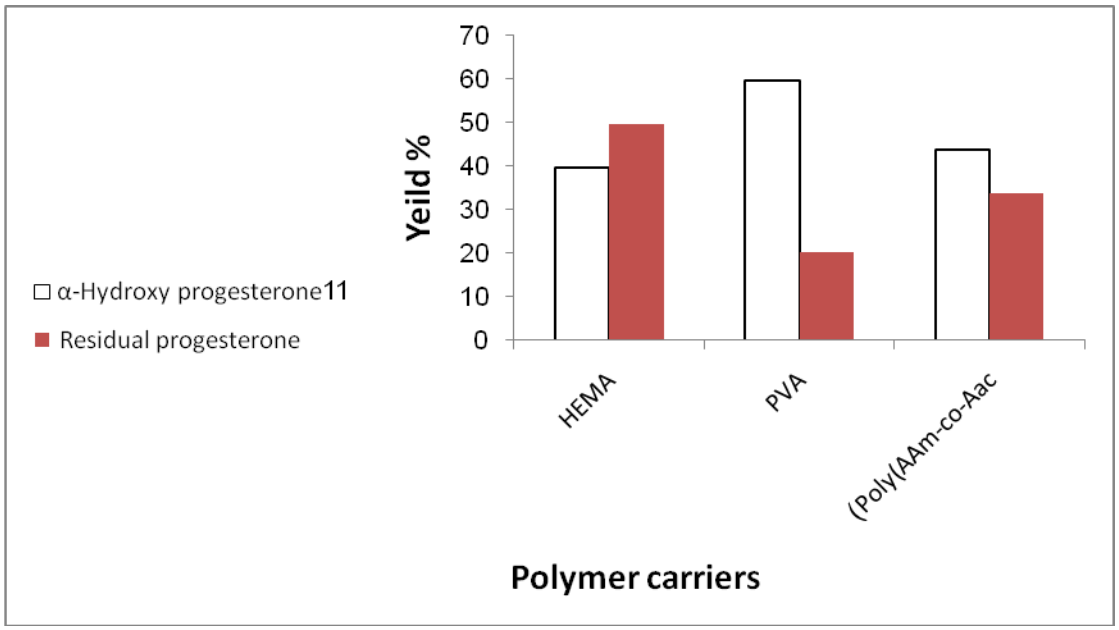


Fig.2 Effect of degree of γ-radiationcross linking of PVA on progesterone Bioconversion of immobilized *P. auranticum*

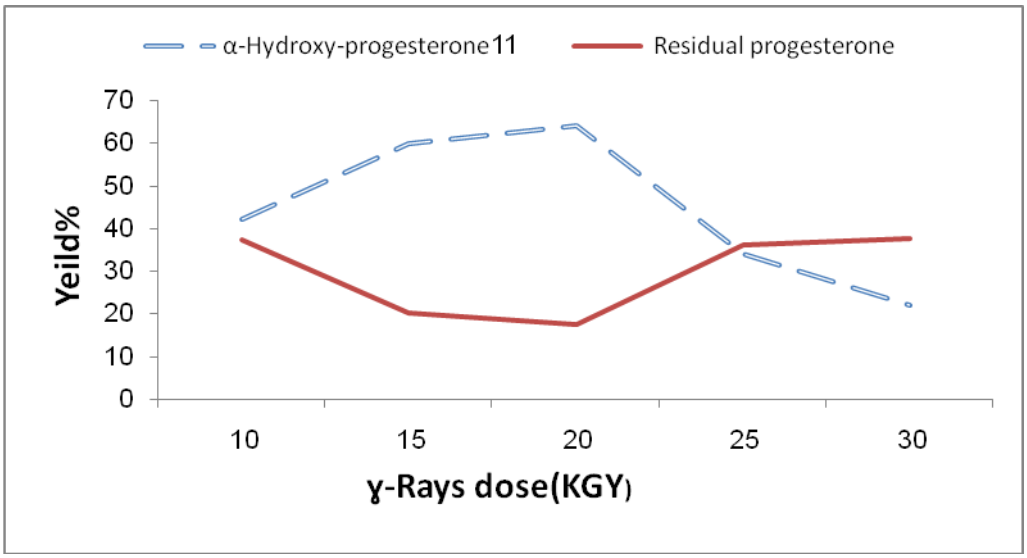
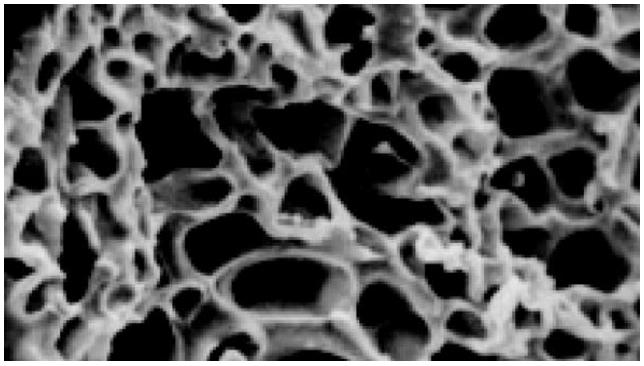


Figure.3 Represents the surface topography of PVA , prepared at a irradiation dose of 20 kGy after soaking it in the reaction medium for a long time (72 hours)

Fig(3a)



Fig(3b)

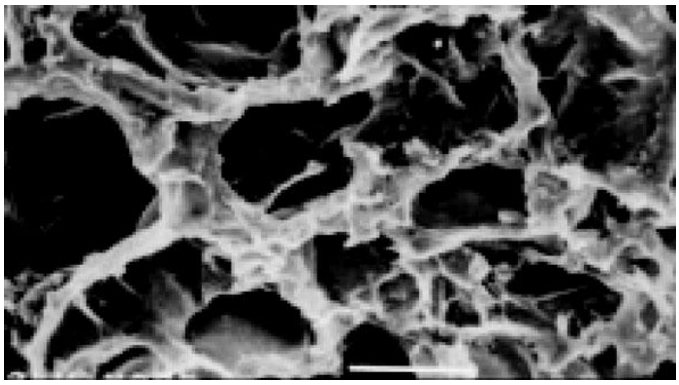


Fig.4 Response surface plot of 11 α-hydroxyprogesterone yield percent by produced by immobilized *P. auranticumon* PVA beads showing the Interactive effects of different progesterone concentrations and incubation period at X2

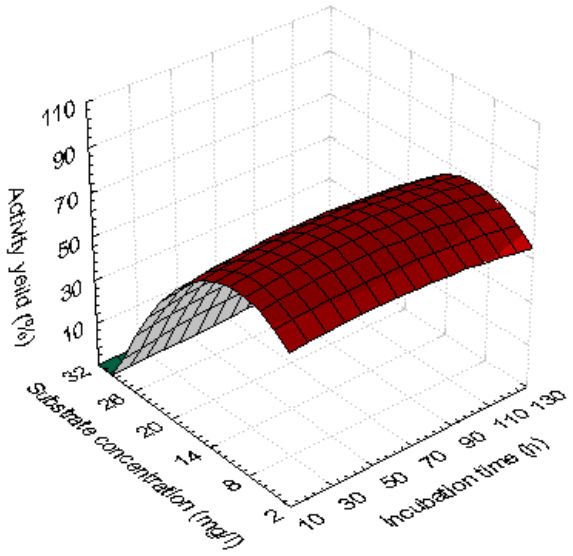


Fig.5 Response surface plot of 11 α-hydroxyprogesterone yield percent produced by immobilized *P. auranticumon* PVA beads showing the Interactive effects of different progesterone concentrations and inoculums size at X3

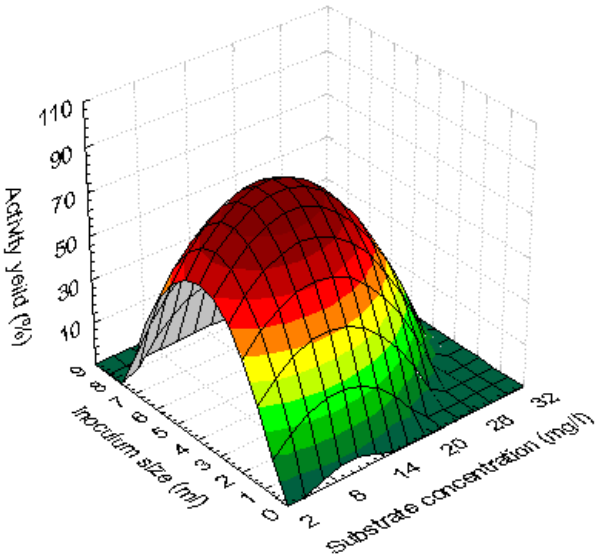


Fig.6 Response surface plot of 11 α-hydroxyprogesterone yield percent produced by immobilized *P.* auranticum on PVA beads showing the Interactive effects of different inoculums size and incubation period at X_1

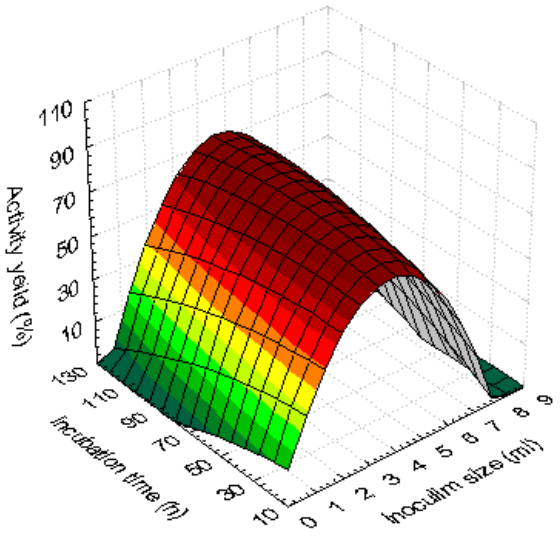
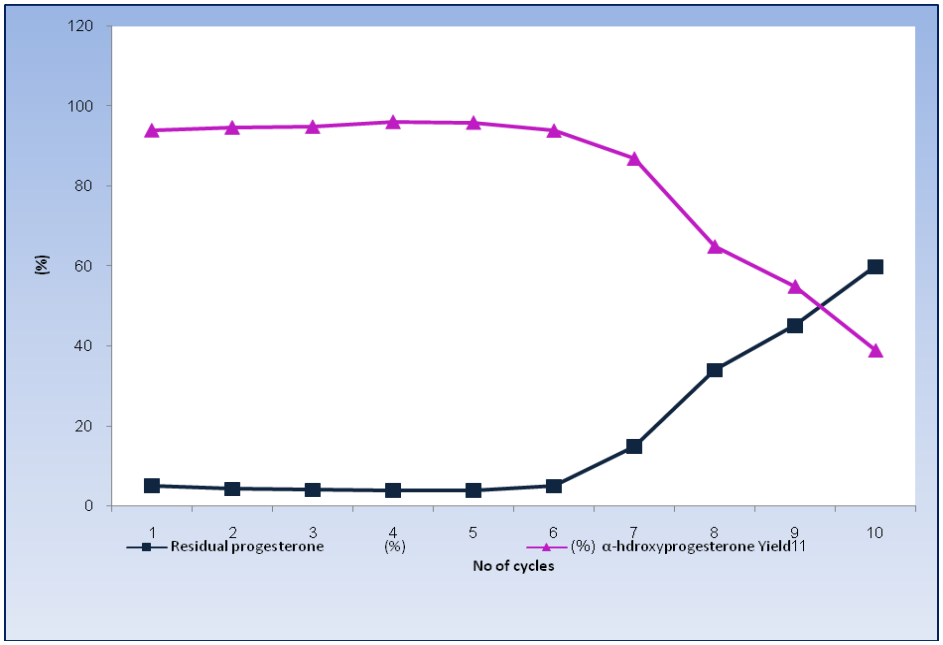


Fig.7 Reusability of immobilized *P. auranticum* in PVA hydrogel



Stability studies

Reusability of immobilized *Penicillium auranticumon* PVA hydrogel

The economics of an immobilized cell technology depends on the lifetime of the biocatalyst. The reusability of immobilized cells entrapped into PVA copolymer prepared at(10%) PVA polymer composition, and 20 kGy γ-irradiation dose was studied by using shaked flasks. The analyses of the sample (30mg/50mL medium) were carried out each 72 hours. The results presented in Table5.indicated that the transformation capacity of progesterone to 11 α-hydroxy progesterone highly increased by the repeated use of polymer for 4 times. This is accompanied by an increase in 11 α-hydroxyprogesterone yield to (96.2%). Meanwhile, the immobilized cells reused more than 4 times slightly decrease the 11 α-hydroxyprogesterone yield to (94 %) at the 6th cycles.

But the immobilized cells reused more than 6 times highly reduce the 11 α-hydroxy progesterone yield percentage as shown in the table to the 9th cycle. From the above results, we concluded that the cells probably grow in the gel so that the 11 α-hydroxyprogesterone yield percentage gradually increases. The repeated use of immobilized cells after the 4th cycle gradually decreases the 11 α-hydroxyprogesterone yield percentage and its productivity to (96–55%) and (3.8–1.7 mg/L/h), respectively, and this may be due to the lyses of the cells, and thus the density of the immobilized cells becomes lower and so led to lower in the cells multiplication and so decreases in the activity of the hydroxylase. On the other hand, it was found that by soaking the gel in the reaction medium for a long time(72 hours), its pore size increases (Figures 3a and 3b).This

observation explains the reduction of 11 α-hydroxyprogesterone yield after using the cell-entrapped copolymer system for several times. As the pore size increases, the ability for entrapped cell to scrape outside increases, resulting in a decrease in11 α-hydroxy progesterone yield(Silbiger and Freeman,1991).

Radiation crosslinking PVA copolymer hydrogels were successfully used for immobilization of *Penicillium auranticum* cells responsible for biotransformation of progesterone to 11 α-hydroxy progesterone with a sufficiently high activity. polymers of suitable cross linking degree, obtained by using PVA polymer of composition(10 %) and irradiation dose of 20 kGy, absorb a reasonable amount of water, prevent the microbial cells scarping, and increase 11 α-hydroxy progesterone yield.

The 11 α-hydroxy progesterone yield was also improved by usingCentral composite design where immobilized *P. auranticum* cells obtained when the PVA beads inoculated with 4ml of 48 h old inoculum contained $12X10^{-8}$ cell/ml in progesterone concentration of (30mg/50ml) for 72h gave the maximum11 α-hydroxy progesterone yield 94. 05 in run 15. Stability studies reflect the capability of the PVA polymer to retain the immobilized cells and form a stable system that can be used for regular transformation procedures of progesterone up to 4 times with an increase in 11 α-hydroxy progesterone yield to (96.2%).

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