Malaysian Journal of Microbiology, Vol 9(3) 2013, pp. 237-244
http://dx.doi.org/10.21161/mjm.49213

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Biotransformation of progesterone to hydroxysteroid derivatives by whole cells of *Mucor racemosus*

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Received 7 December 2012; Received in revised form 20 March 2013; Accepted 21 March 2013

ABSTRACT

Aims: The possibility of using *Mucor racemosus* cells in the biotransformation of progesterone to industrially important hydroxysteroid derivatives in one-step biotransformation process was investigated in this study
Methodology and results: The fungal strain was inoculated into the transformation medium which supplemented with PR as a substrate (5-50 mg). The transformation products were separated and characterized on the bases of their GC/MS analysis as 11α-hydroxyprogesterone (11α-HP) as main product (I); 4-pregnen-18-al-11β,21-diol-3,20-dione (Aldosterone) (II) and 20-hydroxy-pregnan-18-oic acid (III) as minor products. The organism was tested for PR bioconversion at different transformation periods (6-96 h), as well as optimization of the basal medium through the addition of different concentrations of yeast extract and peptone (0.5 to 4 g/L) at various pH values (4-9). The optimal biotransformation conditions for maximum production of these PR derivatives were observed using 0.2 g/L of PR, 3 g/L of yeast extract and 3 g/L peptone after 48 h at pH value 5.5. Under these optimal conditions, cells total bioconversion efficiency reached about 96% of the original added PR.
Conclusion, significance and impact of study: Under these optimum conditions, *M. racemosus* has the ability to biotransform PR to 11α-HP (I), Aldosterone (II) and 20-hydroxy- pregnan-18-oic acid (III) with total bioconversion efficiency of 96 ± 1.77%. These results may be of industrial importance because compounds II and III had not been previously recorded as biotransformation products of PR.

Keywords: Mucor racemosus, progesterone, biotransformation, steroid

INTRODUCTION

The pharmaceutical industry has great interest in the biotransformation of steroids for the production of steroid hormones. Steroid hormones and their derivatives have been used for a wide range of therapeutic purposes. Beside the established utilization as immunosuppressive, anti-inflammatory, anti-rheumatic, progestational, diuretic, sedative, anabolic and contraceptive agents, recent applications of steroid compounds include the treatment of some forms of cancer, osteoporosis, HIV infections and treatment of declared AIDS (Fernandes *et al*., 2003; Znidarsic and Plazl, 2010). Nowadays steroids represent one of the largest sectors in pharmaceutical industry with world markets in the region of US$ 10 billion and the production exceeding 1,000 000 tons per year (Schmid *et al*., 2001; Bureik and Bernhardt, 2007; Donova and Egorova, 2012).

Microbial transformations have been used to produce oxygenated derivatives of steroids which have been difficult to synthesize by chemical means. These reactions usually proceed with high regiospecificity, stereoselectivity and efficiency (Holland and Diakow, 1978; Sedlaczek, 1988). Introduction of a hydroxyl group to a steroid molecule is one of the most important steps in the preparation of various steroidal derivatives. In microbial hydroxylation, hydroxylase enzyme can introduce a hydroxyl group to various positions of the steroid molecule. In fact, several positions in the steroid molecules can be hydroxylated by various microbial strains (Manosroi *et al*., 2008).

Steroidal hydroxylase system of filamentous fungi is usually presented by monooxygenase of microsomal localization which contains cytochrome P-450 (Cyt P-450) as a terminal oxidase. The cytochrome P-450 binds a substrate and flavoprotein NADP(H)-P450-reductase that provides an electron transport from reduced coenzyme to

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Cyt P-450 enzyme (Kelly *et al*., 2003; Bernhardt, 2006; Lah *et al*., 2008; Kollerov *et al*., 2010).

Fungi are widely used in steroid microbial transformation studies (Fernandez *et al*., 2003), since their versatile enzymatic reservoir allow them to modify a wide range of steroids (Carlile *et al*., 2001). *Mucor racemosus* is one of the most common species of the genus *Mucor* and is widely distributed in nature (Domsch *et al*., 1980; Faramarzi *et al*., 2008). Although fungi belonging to the genus *Mucor* have widely been applied in steroid transformation studies (Mahato *et al*., 1989; Mahato and Garai, 1997); very limited studies have been done using *M. racemosus*. It has been used in the transformation of some steroid substances such as pregnane (Mahato and Majumdar, 1993), cardenolide (Charney, 1976), and dehydroepiandrostane (Li *et al*., 2005). In addition, it has been exploited as a microbial model of drug metabolism, e.g. 19-norsteroid substances (Lacroix *et al*., 1999). Moreover, it has the ability to modify androst-1, 4-dien-3, 7- dione (Faramarzi *et al*., 2009).

In the present study, the ability *of M. racemosus* to introduce some enzymatic modifications of progesterone (PR) is demonstrated, producing main product 11α-hydroxyprogesterone (11α-HP) and other hydroxy derivatives. In many cases, hydroxy derivatives are characterized with much higher biological activity than the less polar substrate (Janeczko *et al*., 2009; Donova and Egorova, 2012). For example, 11α-HP is one of the main steps in the production of corticosteroid hormones (corticosterone, cortisone and hydrocortisone) (Hanisch *et al*., 1980; Znidarsic *et al*., 2000). Furthermore, 11α-HP affords interesting possibilities for the preparation of bioactive derivatives (prednisone, prednisolone and triamcinolone) (Vezina, 1987; Borges *et al*., 2009). Aldosterone induces the urinary excretion of K^+ and renal reabsorption of Na^+ (Marver, 1980; Winter *et al*., 1999); pregnan-18-oic acid, having an oxygenated function in the C_{18} position of the steroid molecule, is a valuable intermediate in the synthesis of aldosterone (Muller *et al.*, 1965). Characterization of the encountered steroid derivatives as well as optimization of the bioconversion process was outlined.

MATERIALS AND METHODS

Chemicals and instruments

Progesterone and 11α-hydroxyprogesterone standards were supplied by Sigma Chemical Co. (St. Louis, USA), potato dextrose agar (PDA) was obtained from Biolife Italiana S.R.L. (Milano, Italy). TLC silica gel 60 F254 plates were from Merck (Darmstadt, Germany). N, O-bis-(trimethysilyl)-trifluroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The organic solvents were analytical grade.

GC/MS analysis of the sample was performed using a GC (Agilent Technologies 7890A, USA) interfaced with a mass-selective detector (MSD, Agilent 7000, USA) equipped with a polar Agilent HP-5ms (5% phenyl methyl poly siloxane) capillary column (30 m × 0.25 mm i. d. and 0.25 µm film thickness). The carrier gas was helium with the linear velocity of 1 mL/min. The following temperature program was used: The oven temperature was set at 100 °C for 2 min and then ramped to 270 °C a 5 °C/min and then finally to 300 °C (hold for 2 min) at 2 °C/min. The injector and detector temperatures were 150 °C and 250 °C, respectively. Sample injections (2 µL) were made in 1:5 split modes. The ionization mode selected was the electron ionization (EI) mode. The MS operating parameters were as follows: ionization potential 70 eV, interface temperature 250 °C, and the mass spectrometer was set to full scan, m/z 40-600.

The volatile constituents were analyzed by GC/MS, and identified by comparing their mass spectra and retention time with those of the authentic compounds and by computer matching with NIST (Nat. Inst. St. Technol., USA) and WILEY (Wiley Inst. USA) library as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature.

Microorganism

The strain of *M. racemosus* was kindly obtained from the Center of Cultures of Natural and Microbial Products Chemistry Department, National Research Center, Cairo, Egypt. The experimental cultures were inoculated with descendants from a single slant of the pure experimental organisms maintained on potato dextrose agar medium. All the slants were stored at 4 °C with regular transfer every month.

Inoculum preparation

Seven days old spores were washed from slants with distilled water containing 1.0 g/L Tween-80 was inoculated in 500 mL Erlenmeyer flask containing 100 mL of the growth medium, g/L (w/v): glucose 20, peptone 1, yeast extract 1, L-asparagine 0.7, KH_2PO_4 0.74, $Mg_2SO_4 \cdot 7H_2O$ 1, pH of the medium was adjusted to 6.5. The flask were incubated on a rotary shaker at 150 rpm, 30 ± 2 °C for 24 h and used as a primary culture with inoculums concentration of 2×10^6 spores/mL. The obtained fungal mass was used in the biotransformation process. The concentration of spores was monitored using a haemocytometer.

Biotransformation process

Two milliliters, corresponding to 0.24 g cell dry weight, of the primary culture were inoculated into 250 mL Erlenmeyer flask, under aseptic conditions, containing 48 mL of the biotransformation medium. The biotransformation medium used was the same as that in growth medium. Biotransformation was performed under controlled operational conditions as follows: agitation speed 150 rpm and temperature 30 ± 2 °C for 16 h. Afterwards PR (0.5 mg) dissolved in 0.3 mL 96% ethanol was added to each flask as inducer. The flasks were then incubated for further 8 h. PR (5 mg) dissolved in 96%

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

ethanol was added to each flask. The biotransformation process was allowed to proceed for further 48 h. The experiment was performed in triplicate.

Extraction and determination of transformation products

The biomass was removed from the reaction mixture and the filtrate was extracted twice using chloroform as described by Vitas *et al*. (1995). The resulting extracts were evaporated under reduced pressure. The residues were dissolved, loaded and chromatographed on preparative TLC using a solvent system of benzene/ethyl acetate/acetone (4:1:1, v/v/v), and detected under UV lamp. The semi-solid compounds were purified by hot washing several time using suitable solvent and dried in vacuum drying pistol (Abderhalden's drying pistol), to free samples from traces of water, containing sodium hydroxide (Chai and Armarego, 2003). The produced compounds were determined using GC/MS.

Quantitative analysis was estimated using high pressure liquid chromatography (HPLC). A 10 µL sample was injected into an HPLC C-18 Novopak column, 50% methanol, 50% water as a mobile phase, 1 mL/min flow rate. Absorbance detection was at 254 nm and steroid concentrations were determined by comparison with standards and the data was statistically analyzed.

Statistics

All experiments were repeated three times. The data shown in the corresponding tables and figures were the mean values of the experiments, and the relative standard deviations were shown.

Molar yield (%) =

$$\frac{\text{[yield of product (mg) / MW* of product]}}{\text{[amount of added PR (mg) / MW of PR]}} \times 100$$

Total bioconversion efficiency (TBE) =

compound I (%) + compound II (%) + compound III (%).

*MW is the molecular weight

Sample preparation for GC/MS analysis

All manipulations were carried out using schlenck-line technique under a stream of argon gas dried over phosphorous pentaoxide (P_2O_5). Chloroform was dried by stirring with P_2O_5. The sample was dried by evaporation at 40 °C under a stream of argon then kept in a vacuum desiccator over P_2O_5/KOH for at least 30 min. The dried residue was reconstituted with a volume of 200 µL of derivatizing reagent, BSTFA (1%), TMCS, at 70 °C for 60 min using an ultrasonic water bath (Daihan Lab Tech, 40khz, South Korea). The excess of the silylated reagent was removed under a stream of argon to dryness. A volume of 400 µL dried chloroform was added and a 2 µL sample was injected for GC-MS analysis (Shama *et al*., 2008; Bowden, *et al*., 2009).

Time course experiment and the effect of substrate concentration, modification of basal medium and pH

Cells of *M. racemosus* were transferred into a 250 mL Erlenmeyer flask containing 50 mL medium supplemented with 5 mg of PR dissolved in 0.3 mL of absolute ethanol and then the incubation continued for 4 days. Sampling was carried out every 24 h. Controls were similarly processed except that no microorganisms were added.

Studies were performed to determine the maximum amount of substrate which could be transformed as well as optimization of the basal medium and the pH value. The tested substrate concentrations ranged from 0.1 to 1.0 g/L. The amount of yeast extract and peptone of the basal medium were varied from 0.5 to 4.0 g/L. The effect of pH value on biotransformation process was studied within the range 4-9 using HCl and NaOH. For each experiment only one parameter was changed at a time.

RESULTS AND DISCUSSIONS

Characterization of the different transformation products

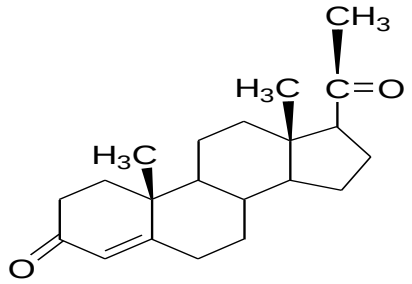
The enzymatic activities of *M. racemosus* on PR in 4 days led to the formation of the main product 11α-HP (I) and other two hydroxy-derived PR compounds (compound II and III). No transformation occurred in the control media. Three PR derivatives were characterized through the application of GC/MS technique (Table 1). Gas chromatography /mass spectrometry (GC/MS) is well-suited for the identification of a large number of potential steroids and metabolites due to its high chromatographic resolution capacity and reproducible ionization efficiency of the derivatization procedures employed prior to injection of the sample. Derivatization alters functional groups in an effort to make the compound more amenable to standard GC/MS analysis, by increasing the volatility and /or thermal stability of a compound. Although derivatization can be time-consuming, it permits the profiling of additional compounds by allowing both polar and non-polar steroids to be successfully separated (Bowden *et al*., 2009). These investigations revealed the following results:

The main product (Rt 7.27) exhibited a molecular ion peak 330 m/z corresponding to the molecular formula of 11α-hydroxyprogesterone, $C_{21}H_{30}O_3$ (NIST) (Figure 1, compound I), other ions appeared at 312, 288, 207, 189, 163, 124 m/z. The second product (Rt 4.92) showed molecular ion peak at 360 m/z which matches with the molecular formula of Aldosterone, $C_{21}H_{28}O_5$ (NIST) (Figure 1, compound II) , other ions could be detected at 314, 299, 283, 255, 219, 213, 163, 145 m/z. The third product (Rt 5.67) showed molecular ion peak at 334 m/z which stands for the molecular formula of 20-hydroxy-pregnan-18-oic acid, $C_{21}H_{34}O_3$ (NIST) (Figure 1,

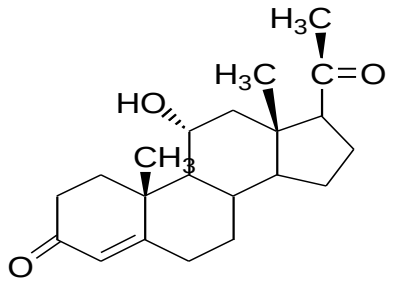
 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 1: GC/MS analysis of the isolated compounds.

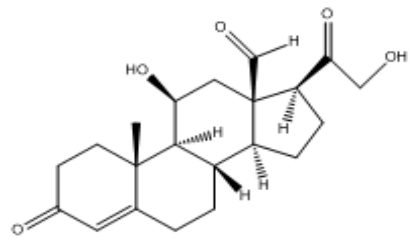
Isolated compounds	Rf (min)	Molecular weight	Molecular formula
4-pregnen-18-al-11β,21-diol-3,20-dione (II)	4.92	360	$C_{21}H_{28}O_5$
20-hydroxy-pregnan-18-oic acid (III)	5.67	334	$C_{21}H_{34}O_3$
11α-hydroxy-4-pregnene-3,20-dione (11α-HP)(I)	7.27	330	$C_{21}H_{30}O_3$



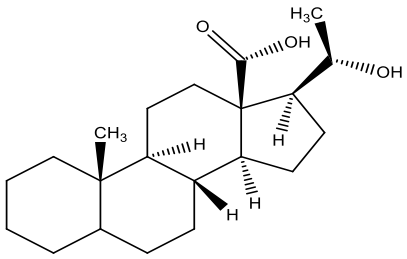
Progesterone



(I)



(II)



(III)

Figure 1: The chemical structures of progesterone and the isolated products; (I) 11α-hydroxyprogesterone, (II) aldosterone, (III) 20-hydroxy-pregnan-18-oic acid.

compound III), other ions were appeared at 231, 207, 121, 95, 83, 63 m/z.

The formation of these PR derivatives indicated that *M. racemosus* has multienzyme systems which catalyze the oxidation, reduction and isomerisation reactions. Kieslich, (1984) reported that most reactions mediated by microorganisms are oxidation, reduction, hydrolysis, condensation, side-chain degradation and formation of carbon-carbon bonds, several reactions can be done simultaneously due to the enzyme compatibility.

Optimization of the bioconversion of progesterone by *M. racemosus*

Optimization of the environmental conditions for microbial bioconversion process on a laboratory scale is important to achieve information for scaled up production of the target product in a large-scale fermentor. Of the parameters assessed, bioconversion time course, PR concentration, peptone and yeast levels and medium pH value were those that mostly affected overall bioconversion efficiency.

Effect of transformation period and inoculum size

Shuvalova *et al*. (2001) reported that the intensity of product formation is mainly determined by the structure of the steroid substrate and, to a certain degree, depends on the age of mycelium and transformation duration. The data illustrated in Figure 2 shows the effect of bioconversion time (6-96 h) on PR bioconversion by *M. racemosus*. The bioconversion of PR to compounds I, II and III increased gradually to reach a maximum (62.95 ± 4.28%) after 48 h. Compounds II & III were maintained at low level with a slow increase in comparison with compound I. Compound I rose sharply from 30.69 to 54 % between 36 and 48 h. There was a decrease in the products yield with further incubation, probably due to further metabolism of the products. The current results indicate that the 11α–hydroxylase enzyme is far more active than C_{21} and C_{18} hydroxylase enzymes initiating the production of compound II and III. Bihari *et al*. (1984) found that maximum production of 11α-HP was obtained after 36 h and further incubation resulted in decrease in 11α-HP formation due to its conversion to 6β, 11α-dihydroxyprogesterone. Increasing the inoculum size of

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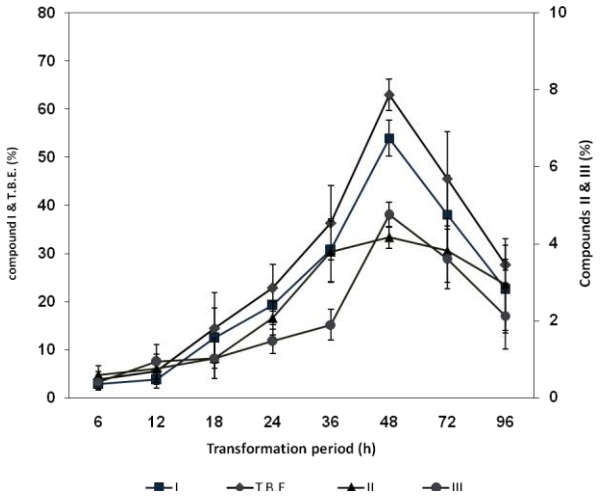

Figure 2: Time course of PR biotransformaion by *M. racemosus.* The cultivation medium was inoculated with 2 mL inoculums, PR (5 mg/50mL) at pH 6.5, 150 rpm and 30 °C ± 2. Total Bioconversion Efficiency (T.B.E), 11α-hydroxyprogesterone (I), 4-pregnen-18- al - 11β, 21- diol - 3, 20- dione (II), *20-Hydroxy- pregnan-18-oic acid* (III). The results are the average of three replicates with indicated standard deviations.

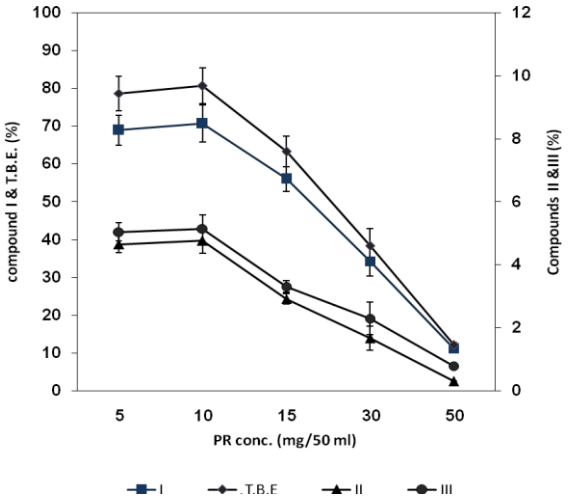

Figure 3: Effect of substrate concentration on the production of different products from PR by *M. racemosus*. The results are the average of three replicates with indicated standard deviations.

the microorganism from 2.0 to 5.0 mL /50 mL medium led to increasing the bioconversion efficiency of the products by 5.7%. By further increasing the concentration of the inoculums the biotransformation process was retarded (data not shown). This may be due to the bulky formation of the fungal cells which reduce aeration and inhibit the oxygen exchange.

Effect of substrate concentration

The utilization of high amount of the steroidal substrate is one of the most important factors affecting the economy of the transformation process. Different concentrations of PR were supplemented to the biotransformation medium (5-50 mg /50 mL medium) after 48 h growth. The data in Figure 3 shows that the total bioconversion yield slightly increased with increasing PR concentration, from 5 mg/50 mL medium to 10 mg/50 mL medium. Thus the maximum bioconversion efficiency (80.74 ± 4.76%) was obtained at substrate concentration of 10 mg/50 mL. By increasing the substrate level over 10 mg/50 mL medium led to a remarkable decrease in the bioconversion activity. The lowest products yield (12.33 ± 0.58%) was obtained at substrate concentration of 50 mg/50 mL medium. This may be attributed to the toxic effect of the substrate on the activity of tested fungus as well as, the instantaneous accumulation of the substrate and its adsorption to the surface of mycelium decreased the substrate dissolution (Adham *et al*., 2003; Lu *et al.*, 2006). According to Manosroi *et al*. (2007) higher concentrations of the substrate that led to a decrease in the bioconversion activity, might be due to either the poor solubility of the substrate in the aqueous media or the limited activity of the enzyme.

Effect of peptone and yeast extract concentrations

Peptone and yeast extract at 3 g/L for each proved to afford the best nitrogen sources concentrations for the biotransformation of PR. Under these conditions, the maximum total bioconversion efficiency of *M. racemosus* was 90.98%±2.67 and 95.32%±1.62 for yeast extract and peptone, respectively (Table 2). This can be due to the fact that peptone and yeast extract contain different amino acids, vitamins (especially B group) together with amounts of different essential elements (Fe, Mg, P, Mn) which are necessary for many enzymes systems and in order to maintain high transformation activity, a combination of glucose with peptone are required (Ahmed, 2003). The bioconversion outputs were decreased substantially with increasing or decreasing the concentration of peptone and yeast extract. This might be due to the formation of small, very light and fluffy pellets of the experimental fungus at the lowest nitrogen concentrations, as well as the formation of clumpy growth at higher nitrogen concentrations that limits the oxygen transfer in the liquid medium which has a negative effect on the biotransformation process.

Effect of pH value on PR bioconversion

Regulation of the pH value of the biotransformation medium is essential for the efficient biotransformation of PR. The effect of initial pH on the production of compounds I, II and III was studied in the range from 4.0

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 2: Effect of different yeast extract and peptone concentrations on the production of different products from PR by *M. racemosus*.

Yeast (g/L)	Conversion %			T.B.E. (%)
	I	II	III	
0.5	67.23±3.66	4.25±0.23	5.73±0.31	77.22±4.2
1	71.52±2.12	5.97±0.17	6.89±0.20	84.4±2.5
2	73.75±0.78	6.27±0.06	7.84±0.08	87.88±0.93
3	75.33±2.21	6.58±0.19	9.06±0.26	90.98±2.67
4	66.93±5.3	4.94±0.39	7.93±0.62	79.81±6.32
Peptone (g/L)				
0.5	73.35±3.2	4.5±0.19	7.05±0.29	84.91±3.69
1	75.32±1.03	6.58±0.09	9.06±0.12	90.98±1.25
2	76.81±2.48	6.84±0.22	9.41±0.30	93.08±3.01
3	76.21±1.29	8.28±0.14	10.81±0.18	95.32±1.62
4	72.89±4.30	5.36±0.31	8.23±0.48	86.5±5.1

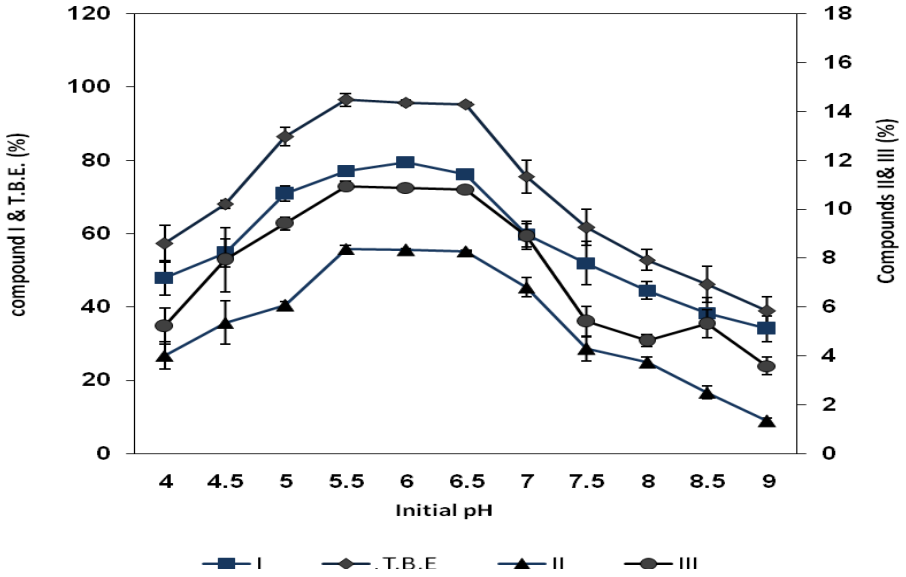

Figure 4: Effect of different pH values on PR biotransformation by *M. racemosus*. Culture medium was supplemented with 10 mg PR /50 mL medium for 48 h at 150 rpm and 30 °C ± 2. The results are the average of three replicates with indicated standard deviations.

to 9.0. As shown in Figure 4, the maximum yields of compounds I, II and III (77.14%±1.41, 8.38%±0.15 and 10.94%±0.2, respectively) were observed at pH 5.5. At lower or higher pH values, the bioconversion yields gradually decreased. Our results are in agreement with that obtained by Zanidarsic *et al*., (2000) who reported that the highest biomass activities were in all cases obtained at pH 5 or 6 which were beneficial in the repeated batch biotransformation of PR. Lu *et al*. (2006) suggested that the pH regulation can maintain the stability of the cytochrome P450 activity which is responsible for the oxygen insertion in the steroid substrate molecule. Moreover, Manosroi *et al*. (2007) reported that the optimal pH value for the activity of hydroxylase in the culture should be in the acidic range.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

CONCLUSION

From this study, we can conclude that the transformation of progesterone using *M. racemosus* whole cells yielded valuable transformation products. These products were separated and characterized on the bases of their GC/MS analysis.11α-HP was obtained as a major compound; in addition there were another two compounds which have been detected namely; aldosterone and 20-hydroxy-pregnan-18-oic acid (lower yield). According to the available literature, compounds II and III had not been previously recorded as biotransformation products of PR. The optimum conditions to obtain maximum transformation of progesterone were 48 h transformation period, using 5 mL inoculums. The biotransformation medium was modified, where; 3 g/L of yeast extract and 3 g/L peptone were used under pH value 5.5. Under these optimum conditions, the tested fungus was found to be able to convert PR to 11α-HP (77.14±1.41%), compound II (8.38±0.15%), and compound III (10.94±0.20%) with total bioconversion efficiency equal to 96.48±1.77%. These results may be of considerable industrial importance in view of the formation of important derivatives from PR in a single step biotransformation process.

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