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Construction Of New Mutants Of *Mucor Racemosus* To Improve Progesterone Biotransformation

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Abstract: The use of *Mucor racemosus* for biotransformation of progesterone has stimulated the biotechnological market. The objective of this work was to select superior mutants with high ability to convert progesterone to 11α-hydroxyprogesterone. The results obtained after fermentative screening of mutant strains, Out of 34 tested strains, four superior mutant's strains were able to convert progesterone (P) into high amount of -11α-hydroxyprogesterone (11α-HP). The highest superior mutant was No. 23/12 which exhibited 68.02 % higher of 11α-HP than- *M. racemosus* (original strain). Furthermore, thin-layer chromatography (TLC) was carried out to detect 11α-HP produced by some selected superior mutants in comparison with the original strain. The results of TLC suggested that, the improvement of the produced 11α-HP was achieved. The application of RAPD technique on some selected superior mutants in comparison with original strains lead to correlate the genetic characteristics of some superior mutants with the results obtained from the biotransformation process. Some differences were noticed in mutant strains in comparison with original strain. These differences in RAPD profiles confirmed the evidence of genetic variations of mutants and *M. racemosus* genome after UV-mutagenesis. Finally, when tested the effects of transformation times, pH and some activators additives on 11α-hydroxylation process. The results showed that the best 11α-hydroxylation of progesterone was obtained after 24 h, the best bioconversion activity was obtained at pH 6.3. Zinc (Zn) was stimulated the bioconversion activity of the original strain but Cobalt (Co) was the best one with all superior tested mutants.

Key words: Optimization, Progesterone biotransformation, *Mucor racemosus*, UV-mutants, HPLC, TLC, RAPD-PCR.

INTRODUCTION

The manufacture of many commercial steroids industrially consists of transforming readily available basic steroids microbiologically to important intermediates , which then converted chemically to the final products. Alternative methods are to perform the necessary chemical changes first and then carry out the final step using microorganisms (Berger, 2009). The members of the genus *Mucor* have recently attracted increasing interest in consequence of such reasons as the ability of some species to produce valuable microbial products, e.g. industrially used extracellular enzymes (Godtfredsen, 1990) or the pharmaceutically important polyunsaturated fatty acids (Immelman *et al.*, 1997). They also find application in the formation of various groups of organic compounds by biotransformation (Madyastha and Joseph 1994).

The production of steroid drugs and hormones is one of the best examples of the successful application of microbial technology in large scale industrial process. The most important part in microbial transformation is that concerned with synthesis of hormones of the adrenal gland and their powerful therapeutically selective synthesis analogs (Fernendes *et al.,* 2003). The filamentous fungi were the first known hydroxylators of progesterone at the 11α- position, a microbial step in the industrial production of corticosteroids (Znidarsic *et al.,* 1998, Lisurek *et al.,* 2004, Roglic *et al.,* 2007). Moreover, studies carried out so far clearly indicate the versatile nature of Mucorales for introducing hydroxyl functions at different positions in the progesterone molecule (Singh *et al.,* 1967, Madyastha and Srivatsan,1987, Bihari *et al.,* 1988). Progesterone (P), which is a lipophilic drug, is an important precursor of 11α-hydroxyprogesterone (11α-HP) production. 11α-HP is used for the production of many other important steroidal drugs.

The fermentative screen after mutation induction can be performed in order to confirm the- superior strains for biotransformation process. As examples, Abd-El Salam and Khattab (2005) production of antrostendione (AD) and androstadienedione(ADD) using genetically improved *Fusarium* sp. strain through ultraviolet (UV) and ethylmethansulfonate (EMS) mutagenesis as well as protoplast fusion. Biotransformation of flavanones by one wild strain and three UV mutants of *A. niger* resulted in the reduction of carbonyl group and dehydrogenation at C-2 and C-3 positions; reduction of carbonyl group and hydroxylation at C-7; and dehydrogenation at C-2 and C-3 and hydroxylation at C-3 producing flavonol (Kostrzewa-Suslow *et al.,* 2006).

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Wenyu *et al.,* (2007) developed a new method for breeding the hydrocortisone overproducing strain *Curvularia lunata* by screening ketoconazole-resistance mutant. A hydrocortisone overproducing mutant *C. lunata* KA-91 with ketoconazole-resistance marker was obtained from protoplasts treated with ultraviolet radiation. Cornelia and Rita (2008) used random mutagenesis by PCR for improved progesterone conversion to about 1.5-fold and used mutagenesis by site-directed mutagenesis for improved in two mutant generations by more than a factor of four. In addition, also the conversion of P improved in these mutants. Kollerov *et al.,* (2010) obtained a stable mutant (M_4) of *C. lunata* with high 11β-hydroxylase activity. Although there are several successful examples of combing classical mutation and resistant selection method in *Streptomyces* species improvement (Hesketh and Ochi 1997; Hu, and Ochi 2001), reports about the resistant mutation selection for steroid biotransformation are rarely seen in literatures.

The random amplified polymorphic DNA (RAPD) technique has been shown to be extremely useful in evaluation of genetic variability of microbial strains. Several authors cite the RAPD as ideal in the study of genomic polymorphism. This method has been used to compare intra- and interspecific differences in bacteria. The purified DNA or the cell extracts cultivated in agar can be used (Williams *et al.,* 1990; Ikeh 2003).

Within our breeding program for isolation of mutants capable of highly transforming of progesterone into hydroxyprogesterone, a strain of *Mucor racemosus* was irradiated with UV-light. The products have been purified chromatographically and characterized using HPLC. The objective of the present study was to adopt of mutation and RAPD techniques to improve the biotransformation process using *Mucor racemosus*. The effect of some factors affecting the 11α-hydroxylation process such as transformation times, pH and some activators additives were also studied.

MATERIALS AND METHODS

Chemicals:

progesterone and 11α-hydroxy progesterone were purchased from Sigma Chemical Co. (St Louis, MO, USA). Silica Gel G 60 was used for preparative TLC, all the other chemicals were obtained from Merck (Darmstadt, Germany). All reagents and solvents used were of analytical grade.

Microorganism and Cultivation:

The microorganism used in the present study was *Mucor racemosus* NRRL 3639 which obtained from the Agricultural Research Service Culture Collection (NRRL), Peoria, IL, USA. The strain was maintained at 4 ^{o}C on Dox's medium g/L (Sucrose 30, $NaNO_3$ 2, KH_2PO_4 1, $MgSO_4$ 0.5, $FeSO_4.7H_2O$ 0.001 and agar 20) and freshly sub-cultured before using in the transformation experiments. The organism was transferred to a fresh medium every two months.

Biotransformation process:

Erlenmeyer flasks 250 ml -containing 100-ml of the production medium g/L (Glucose 40, peptone 1, yeast extract 1, $MgSO_4$ -1, KH_2PO_4 0.75, L-Asparagine 0.7, pH 6.3) according to Abdel Salam, 2009. The flasks were inoculated by 2 ml spore suspension- of the tested strain and continued to grow at 200 rpm for 48 h. After incubation for 48 h, 10 mg progesterone/100 ml medium was added and the fermentation process was continued for another 48 h (transformation time) at 200 rpm and 30^{o}C.

Extraction and identification of biotransformation products:

Extraction: At the end of the fermentation period equal volume of chloroform was added and maintained on rotary shaker for 24 h. Then the solvent layer was separated and evaporated to give semisolid residue (test material).

Qualitative analysis: the test materials were dissolved in chloroform: methanol (1:1, v/v). The transformation products in the test material were detected by TLC using Silica Gel G 60. Cyclohexane: chloroform: isopropanol (10:5:2, v/v) was used as developing solvent system. The transformation products were identified after comparison with a standard product.

Quantitative analysis: the transformation products (11α–HP and P) in 1ml of sample (test materials) were assayed spectrophotometrically, and the results were confirmed using HPLC (HP, Agilent series 1100, USA). HPLC analysis was performed under the following conditions: Hi Plex Pb column (Schimadzu, PL, USA), detection at 254 nm, flow rate 1 ml/min and methanol: water (70:30, v/v) as solvent system. Minor side products were neglected. All experiments were carried out in duplicate and the averages were taken.

UV-mutagenesis:

Spore suspension from 5 day old culture of *Mucor racemosus* was prepared in saline solution (0.85%, w/v, NaCl containing a drop of Tween 80) and irradiated with Philips TUV- 30-W 245 nm lamp, No. 57413-P/40 for 4, 8 and 12 min at a distance of 20 cm. The treated spores were kept in dark for 2 hr to avoid photoreactivation

repair, then treated spores were diluted and spread onto Dox's medium containing different concentration of metal ions and 0.1% (w/v) Triton x-100 as a restriction factor for radial colonies growth at 28ºC for 5 days. The growing colonies were transferred into slant and used to inoculate the growth medium for transformation process of progesterone.

Isolation of total DNA from mutant strains:

Total DNA was isolated according to Khattab (1997) with using (10 mg/ml) Novozyme 234 in addition of Snail enzyme. The quantity and purity of the obtained DNA were determined according to the UV-absorbance at 260 and 280 nm using spectrophotometer (Shimadzu UV-VIS model UV-240) according to Sambrook *et al.,* (1989).

Molecular analysis of new superior mutants by PCR:

PCR-GOLD Master-Mix Beads (BIORON, Germany, Cat. No. 10020-96) were used for PCR experiments. Each bead contains all of the necessary reagents, except primer and DNA template, for performing 25 µl PCR amplification reactions. Two different primers were used in the present study. The first primer sequence was 5'- GTA GAC CCG T-3'. The second primer sequence was 5'- AGG GGT CTT G-3'. All primers were supplied by Operon Technologies Company, Netherlands. To each PCR bead, 12 ng of the used random primer and 40 ng of the purified DNA sample were added. The total volume of the amplification reaction was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows: Denaturation at 95^{o}C for five min. Thirty-five cycles each consists of the following segments: Denaturation at 95^{o}C for one min; primer annealing at 35^{o}C for two min. according to GC ratio of each primer and incubation at 72^{o}C for two min. for DNA polymerization. At the end, hold the PCR at 4^{o}C till analysis. The amplified DNA products from RAPD analysis were electrophorated on 1.5% agarose gel and 1 X TBE buffer at consistent 100 volt for about 2 hrs. The different band sizes were determined against 100 bp ladder (Pharmacia Biotech.) and the separated bands were stained with 0.5 µg/ml ethidium bromide and photographed using Gel Documentation System with UV Transeliminator.

Effect of transformation times, different pH and some activators additive on 11α-hydroxylation process:

Different fermentation times (12, 24, 48, 72 and 96 h) and different pH values (4.3, 5.3, 6.3, 7.3 and 8.3) were used. Also, some metal ions were used as activator additives. The concentration of the metal ions (Cr^{+2} , Zn, Co and Fe) was 0.1 mM.

RESULTS AND DISCUSSION

The strain used in this work was selected based on results reported in the literature related to its ability to transform progesterone.

Screening for improved UV-mutants:

The screening experiments confirmed that some mutants strains were able to perform the desired biotransformation of progesterone. Table (1) presents the 11α-HP productivity of 34 selected mutants after exposure of the original strain to UV-light for different times. compared with parental strain productivity. The obtained results showed out of 34 strains tested, four superior strains No. (8/4, 12/8, 23/8 and 31/12) were able to convert progesterone into 11α-hydroxyprogesterone. Moreover, 14 mutants produced 11α-HP in amounts less than their original parental strain. The remaining 20 mutants were of enhanced 11α-HP production. The highest 11α-HP producer mutants was No. 23/8. It was 39.5 percent (168.02% enhanced production compared with original strain).

Also, from the results in (Table 1), it could be concluded that increase resistance to heavy metal ions and antifungal agents can be successfully used as a selection procedure to improve the biotransformation capacity of the original strain. Most of the mutants resistant to Cd, Hg, C and Pb enhanced in 11α-HP production. So, it could be used as a positive selectable markers for detection of superior mutants of Mucor racemosus to improve progesterone biotransformation. On the other hand, most of the mutants resistant to As, Cr, N, B and Ba decreased in 11α-HP production. So, it could be used as a negative selectable markers for progesterone biotransformation.

Resistance to the heavy metal ions has been used as a criterion to select thiol-compounds (Miersch *et al.,* 2001), oxalic acid (Clausen and Green 2003) and lipase (Abel *et al.,* 1999) over producer mutants. On the other hand, antifungal agents can be successfully used as a selection procedure to improve the biotransformation capacity of the original strains (Wenyu *et al.,* 2007).

Table 1: Bioconversion of progesterone after 48 h by UV- mutants obtained after selection under different types of metal ions and antifungal agents.

Mutant No.	Phenotype	Transformation product 11α– HP(%)	RP(%)
Original strain	$Hg^{-}Cd^{-}As^{-}Li^{-}Pb^{-}Cr\text{-}Ba^{-}Cs^{+}Se^{+}Co^{+}B^{-}G^{+}N^{+}C^{-}$	23.5	62.1
2/4	As^{+}	24.5	67.7
3/4	As^{+}	16.3	75.4
4/4	As^{+}	21.5	75.2
5/4	As^{+}	17.6	66.7
6/4	Cr^{+}	21.7	71.2
7/4	Cr^{+}	25.7	61.5
8/4	Cd^{+}	35.7	43.9
9/4	Hg^{+}	31.2	51.7
10/4	N^{+}	17.5	68.5
11/4	N^{+}	25.1	55.7
12/8	Cd^{+}	37.9	44.2
13/8	N^{+}	21.5	55.7
14/8	N^{+}	23.6	70.7
15/8	C^{+}	25.9	64.7
16/8	C^{+}	27.9	72.3
17/8	Cr^{+}	21.5	64.9
18/8	Cr^{+}	17.6	66.7
19/8	B^{+}	18.5	54.9
20/8	B^{+}	21.5	66.2
21/8	Cd^{+}	27.3	55.6
22/8	Cd^{+}	30.1	53.7
23/8	Cd^{+}	39.5	35.7
24/8	Pb^{+}	28.1	43.7
25/8	Pb^{+}	21.3	65.6
26/8	Pb^{+}	25.3	54.2
27/8	N^{+}	21.2	61.7
28/12	C^{+}	31.2	52.9
29/12	Hg^{+}	28.1	59.2
30/12	Hg^{+}	31.6	47.5
31/12	Hg^{+}	34.2	52.2
32/12	Pb^{+}	30.9	45.9
34/12	Ba^{+}	21.5	61.2
37/12	Ba^{+}	32.9	35.7
40/12	Ba^{+}	22.5	49.7

11 α–HP (11alpha-hydroxyprogesterone), RP (Residual progesterone),+ (resistant), -(sensitive), Hg,mercury(100ppm); Cd, cadmium(250ppm); As, arsenate (250ppm); Li, lithium(500ppm); Pb, lead(750ppm); Cr, chromium(250ppm); Ba, barium(500ppm); Cs, cesium(200ppm); Se, selenium (100ppm); C0, cobalt(750ppm); B, benomyl(5µg/ml); G, griseofulvin (500µg/ml); N, Nystatin (50 µg/ml); C, cyclohexamide (90 µg/ml).

Analysis of the transformation products by TLC:

In addition to the effects UV- mutagenesis on the production quantities of 11α-HP, it was also proposed that genetic alterations could affect the quality and quantity of the by-products produced by the isolated mutants. Therefore, TLC was carried out to detect the types of 11α-HP which could be produced by some selected mutants in comparison with the original strain and also with the standard materials of 11α-HP and P.

Results obtained in Figure (1) showed a close similarity between 11α-HP produced by the wild type strain from one side and those produced by all mutants on the other side depending on the basis of RF values. All mutants exhibited the highest intensity of the 11α-HP spot (RF, 0.8) in comparison with the original strain and the standard material. Moreover, no differences in RF value could be detected for the first spot (RF, 0.08) between all mutants and the original strain(lane 1). Also, no differences in RF value could be detected for the second and the fifth spots between all mutants and the standard of 11α-HP (RF, 0.8) and P (RF, 0.187). On the other hand, all mutants exhibited two additional faint spots (RF, 0.293 and RF, 0.34) which disappeared completely in the original strain. Furthermore, mutant No.8/4 (lane 2) exhibited the highest intensity of the spot (RF, 1) in

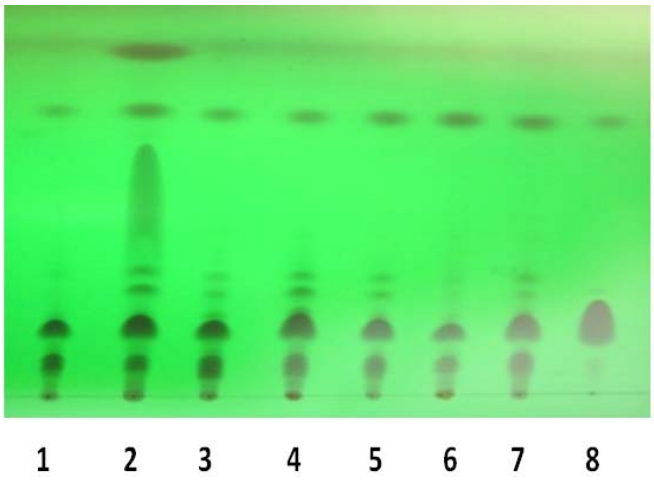

Fig. 1: Chromatographic analysis of 11α-HP spot produced by *M. racemosus* wild type strain (lane 1) and six selected mutants No. 8/4, 12/8, 23/8, 30/12, 31/12, 37/12 (Lanes: 2 to 7) on Silica Gel plates in comparison of pure materials of 11α-HP and P (lane 8).

RAPD Analysis of some superior mutants:

Based on the results obtained in the fermentative screening, the RAPD technique was used in order to test if genetic characteristics could be correlated with the microbial capacity of progestrene bioconversion. These experiments used the two primers from Operon Technologies. The amplification reactions with the two primers generated a total of 27 distinct fragments, 8 (29.63%) of them being polymorphic.

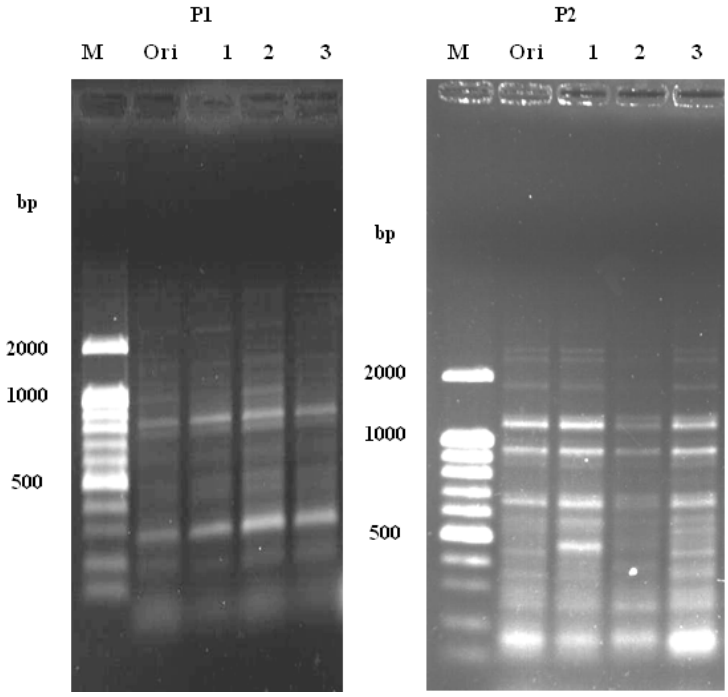

Fig. 2: Photographs of DNA amplified banding patterns based an RAPD for three superior mutants compare with original strain (Ori) and 100 bp ladder marker (M) using primer No. 1 (P1) and No. 2 (P2). Mutants sequence as follows: (Lanes 1 to 3), 8/4, 12/8, 23/8.

The fragments size ranging from ~50 to ~2200 bp (Fig. 2). Figure 2 shows the variability observed for different mutants analyzed using the two primers. The obtained results was agreement with those reported by Schlick *et al.,* (1997) they demonstrated that some differences and similarities in RAPD profile between mutants

in *Trichoderma harzianum* obtained by gamma radiation. Moreover, Barcelos *et al.,* (2011) reported that, RAPD markers were useful for detecting genetic variability among isolates of *C. lindemuthianum*.

Effect of different fermentation times on 11α-hydroxylation process:

Analysis of the transformation products were investigated at different fermentation time (12, 24, 48, 72 and 96 hrs). The results presented in Table (2) showed that the tested mutants have various activity. Mutants Nos. (8/4 and 23/8) showed the best 11α-HP outputs (44.2%, 47.3%) were obtained after 24 h, respectively. On the other hand, the isolate No. 12/8 showed a considerable yield at (37.2 %) at 48h. In accordance to our results (Farooq *et al.,*1994 and Polona *et al.,* 1998) showed that the best 11α- hydroxylation of progesterone was obtained after 24 h, while Abdel Salam, (2009) showed that the best yield was obtained at 48 h.

Table 2: Effect of different transformation time on the bioconversion of progesterone by some selected superior mutants.

Mutant No.	Transfromation time (hr)	Transformation product	
		11α– HP(%)	RP(%)
Original strain	12	21.6	67.9
	24	25.7	61.5
	48	23.5	62.1
	72	22.6	54.5
	96	20.1	42.7
8/4	12	26.9	61.5
	24	44.2	31.6
	48	35.7	43.9
	72	31.6	33.7
	96	24.5	31.6
12/8	12	21.2	53.7
	24	33.3	45.7
	48	37.9	44.2
	72	29.5	36.7
	96	24.3	31.6
23/8	12	31.6	43.6
	24	47.3	32.6
	48	39.5	35.7
	72	30.1	31.5
	96	22.2	30.7

Effect of different pH values on 11α-hydroxylation process:

The transformation process was investigated at different pH value(4.3, 5.3, 6.3, 7.3 and 8.3) the results presented in Table (3) revealed that all the tested isolate s require the same pH to show a high transforming capacity. The best 11α-HP output (47.3%) was obtained by the isolate No. (23/8) at pH 6.3. In accordance to these results Sabina *et al.,* 2008, who stated that the best bioconversion activity was obtained at pH 6.3.

Table 3: Effect of different pH values on the bioconversion of progesterone by some superior mutants after 24 h.

Mutant No.	pH	Transformation product	
		11α– HP(%)	RP(%)
Original strain	4.3	17.4	70.9
	5.3	22.7	63.7
	6.3	23.5	61.7
	7.3	22.5	45.4
	8.3	21.7	50.1
8/4	4.3	20.6	56.3
	5.3	32.7	43.9
	6.3	44.2	31.6
	7.3	31.3	35.7
	8.3	27.6	39.6
12/8	4.3	19.5	61.1
	5.3	24.2	54.2
	6.3	37.9	44.2
	7.3	29.6	35.7
	8.3	24.2	51.6
23/8	4.3	21.2	55.7
	5.3	32.9	41.6
	6.3	47.3	32.6
	7.3	35.6	44.5
	8.3	27.3	51.6

Effect of some activators additive on 11α-hydroxylation process:

Metal ions are known to play a role as cofactors for enzyme activities, and often act as salt or ion bridges between two adjacent amino acid residues. In this study, various metal ions such as Cr^{+2} , Zn, Co and Fe at 0.1 mM concentration were tested for progesterone bioconversion activation/inhibition effect. The results presented in Table (4) showed that the heavy metals used varied in their activity to the transformation process. The addition of Zn showed stimulation effect (34.5%) compared to the control. A remarkable induction was obtained by the addition of Co(56.6%) . The bioconversion of all mutants was reduced in the presence of Zn and Fe. This result is accordance with that obtained by Farooq, *et al.,* (1994), Abel *et al.,* (1999) and Kubicek (2001).

Table 4: Effect of different additive activators on the bioconversion of progesterone some superior mutants after 24 h.

Mutant No.	Additives	Transformation product 11α– HP(%)	RP(%)
Original strain	Control	25.7	61.5
	Cr^{+2}	30.1	53.7
	Zn	34.5	22.5
	Co	26.5	60.1
	Fe	27.5	58.2
8/4	Control	44.2	31.6
	Cr^{+2}	53.4	23.4
	Zn	34.5	31.5
	Co	56.6	24.5
	Fe	42.1	23.5
12/8	Control	37.9	44.2
	Cr^{+2}	44.7	33.4
	Zn	34.5	21.8
	Co	45.5	22.7
	Fe	33.5	34.1
23/8	Control	47.3	32.6
	Cr^{+2}	46.3	24.5
	Zn	34.8	22.9
	Co	49.4	21.2
	Fe	38.5	23.8

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