COMMUNICATION TO THE EDITOR

Strain Improvement of *Aspergillus* Sp. and *Penicillium* Sp. by Induced Mutation for Biotransformation of α-Pinene to Verbenol

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Abstract: Variants of *Aspergillus* sp. and *Penicillium* sp. obtained after treatment with colchicine, ethyl methanesulphonate (EMS), or ultraviolet (UV) irradiation indicated varying levels of significant increases in their efficiency to transform α-pinene to verbenol. In case of *Aspergillus* sp. the UV-induced variant was the best performer with a 15-fold increase in biotransformation efficiency compared to the wild type. In case of colchicine and EMS-induced variants the biotransformation increases were 2- and 8-fold, respectively. The UV-induced variant of *Penicillium* sp. was capable of eight fold increase in efficiency while the colchicine- and EMS-induced variants were 1.5- and 2-fold, respectively. The variants were characterised with respect to changes in colony morphology, spore dimension, DNA content, and products formed, viz. verbenol and verbenone. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **63**: 249–252, 1999.
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INTRODUCTION

Verbenol (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol) with the pronounced camphor and mint flavour notes is used as a food flavourant. It has been shown to have insecticidal properties and hence has potential for use in agriculture (Repp et al., 1987). Verbenol costs US$ 1750 kg^{-1} and is currently obtained from pine and eucalyptus sources. Production of verbenol by biotransformation of the readily available and inexpensive precursor compound, α-pinene, a byproduct of orange peel oil, is expected to be economical and advantageous owing to the recently available tools in microbial technology and separation techniques (Abraham et al., 1994; Hagedorn and Kaphammer, 1994). α-Pinene is commercially available at US$ 0.0075 kg^{-1}. Some microbial isolates were found capable of biotransformation of α-pinene to verbenol. However the rate and direction of conversion are not often adequate for the process to be economical. This calls for improvement of the strains. Induced mutation has proven successful in number of several other microbial processes for the production of useful end products (Andreoni et al., 1995; Kirimura et al., 1988, 1992; Toyama and Toyama, 1990a,b). In the present work we have employed UV irradiation, ethyl methanesulphonate, and colchicine to mutagenise *Aspergillus* sp. and *Penicillium* sp. In some of the mutants, substantial increases in the biotransformation capability of α-pinene to verbenol have been found.

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MATERIALS AND METHODS

Aspergillus sp. and *Penicillium* sp. strains were isolated from garden soil and maintained on potato dextrose agar. Colchicine, ethyl methanesulphonate (EMS), and standard DNA and diphenylamine reagent for DNA analysis were purchased from Sisco Research Laboratory, India. Potato dextrose agar and broth media were purchased from the Hi-media Pvt. Ltd., India. Verbenol and verbenone were from Aldrich Chemical Company (St. Louis, MO).

The scanning electron microscope (SEM) used was Model No. LEO 435 VP (U.K.), and the UV illuminator was Model No. 3-3000-2 (Photodyne, USA). Verbenol was analysed by gas chromatography using GC Model 14 B (Shimadzu, Japan), with an SE-30 column (Shimadzu, Japan), 3 m; N_2 30 mL/min with temperature programme 60–160°C, 2°C per min. The gas chromatography mass spectra of verbenol was obtained using GCMS, Model 2P-5000 (Shimadzu, Japan), SE-30 column (Shimadzu, Japan) 30 m, He 1 mL/min, 40 kPa. The standard mass spectra were from Noever et al. (1988). DNA content of the mycelium was determined after isolation according to Bignon and Martin (1992) and by spectrophotometry using diphenylamine re-

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agent (Burton, 1995). The mycelial biomass was determined gravimetrically by drying after washing with 0.5% saline to constant weight in an oven at 120°C and expressed as mg/100 mL dry weight. For biotransformation of α-pinene to verbenol the mycelial pellet (200 mg dry wt) grown in potato dextrose broth to late-exponential phase (16.5 h) was taken in phosphate buffer (100 mL; 0.05 *M;* pH 7.0) and incubated with α-pinene (20 mg) for 6 h, and supernatant was incubated with verbenol (2 mg). The product formed was extracted in dichloromethane twice (50 mL) for complete extraction. The product was analysed by GC and identified using GCMS spectral data; verbenol formed was quantified by comparison with standard verbenol by GC.

Induced mutations were carried out using ethyl methanesulphonate, colchicine, and UV irradiation. Typically, the fungal culture was grown on PDA for 3 days and spores were harvested, washed, resuspended in appropriate buffer, and subjected to mutagenic treatments. For colchicine treatment the method of Toyama and Toyama (1990a,b) was followed, and for ethyl methanesulphonte treatment that of Shathamma et al. (1972) was followed. For UV irradiation the procedure according to Rupp et al. (1971) was followed.

Verbenone and verbenol were the two products monitored in the biotransformation. The formation of verbenol reflected the activity of α-pinene hydroxylase and the formation of verbenone that of verbenol dehydrogenase (Prema and Bhattacharyya, 1962; Jonathan et al., 1989).

The mycelial mass after growth of the culture in PDA to exponential phase was suspended in phosphate buffer (0.05 *M*) pH 7.0. and sonicated for 1 min. The sonicate was centrifuged at 100,000*g* for 30 min. The pellet and supernatant fractions were separated. Supernatant was assayed for verbenone using verbenol as substrate, and the pellet was suspended in 0.05 *M* phosphate buffer, pH 7.0, for verbenol using α-pinene as substrate (20 mg/100 mL). The activities were calculated on the amount of product formed μg/100 mL in supernatant and pellet fractions due to the enzyme activities.

RESULTS

The data in Fig. 1 pertains to the kill pattern of *Aspergillus* sp. and *Penicillium* sp. as a function of dose of the mutagens. For both cultures, the LD_{50} for colchicine was 0.75 mg mL^{-1}. The kill pattern of cultures due to treatment with 14 μg mL^{-1} of EMS allowed for different periods of incubation time under shaken conditions is also indicated. The LD_{50} in terms of contact time of the chemical for both the cultures was 2 h (Fig. 1). The LD_{50} in terms of exposure time to UV irradiation in the case of *Aspergillus* sp. was 5 min, and in the case of *Penicillium* sp. it was 4.5 min. In comparision to *Aspergillus* sp. and *Penicillium* sp. showed resistance to the chemical mutagens at lower doses.

The variants of both the strains were located on plates based on visual morphological features of the colonies Tables I and II. Around 20 variants arbitrarily selected from each treatment were examined. The variants were characterised on the basis of DNA content and products of biotransformation. Data for one culture representing each category are given in Tables I and II. Marked variations were noticed in the morphological features of colony and spore size of both the cultures subjected to mutagenic treatment. The colchicine-treated variant of *Aspergillus* sp. produced colonies on potato dextrose agar which were curled from the periphery to the centre whereas the wild type produced circular, planar colonies (Table I). The colchicine variant was also significantly larger in diameter and produced spores which were also larger than the wild type spores. The EMS-treated *Aspergillus* sp. were similar in appearance to the wild type but were almost two times larger than the wild type colonies. Its spores were also larger. In the case of UV-treated *Aspergillus* sp. no significant change in the mor-

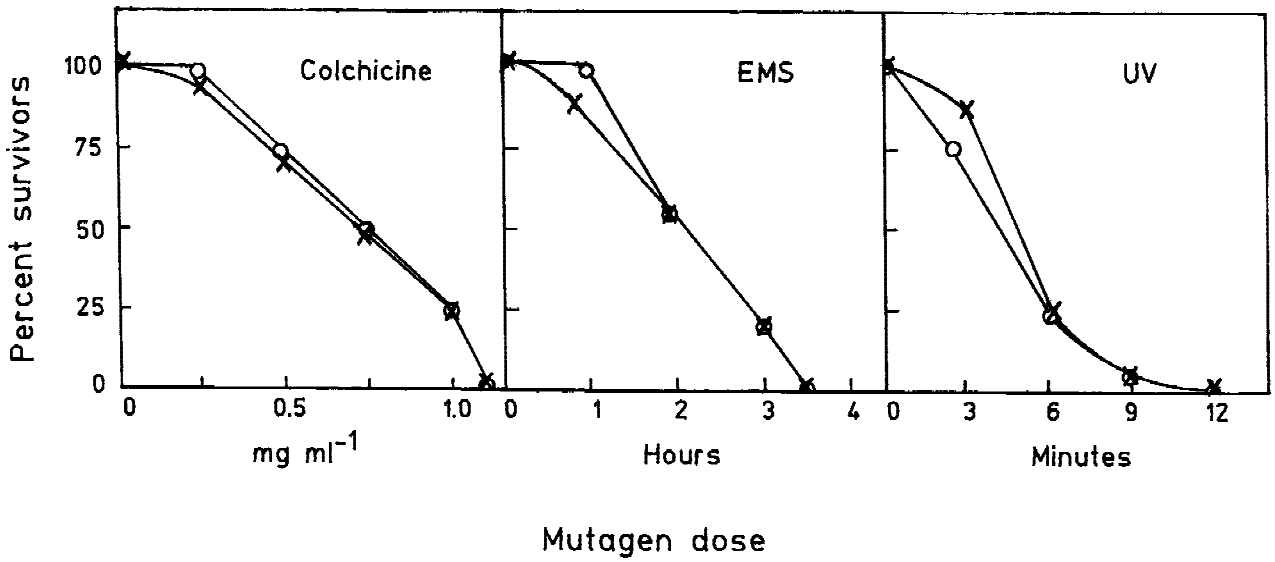

Figure 1. Survival pattern of *Aspergillus* sp. (×) and *Penicillium* sp. (○).

Table I. Characteristics of wild type and Variants of *Aspergillus* sp.

Strain	Morphology	Biomass* (mg 100 ml^{-1} dry wt.)	DNA* (μg 100 mg^{-1} biomass)	Product of biotransformation	
				verbenol (μg 100 ml^{-1})	verbenone (μg 100 ml^{-1})
Wild type	Black colony, 1.3 to 1.7 cm dia. spore, 2.84 μm dia.	96 (12.0)	182 (16.1)	300 (14.5)	1000 (115.8)
Colchicine treated	Thick curled black colony, 2.5 cm dia. spore, 3.10 μm dia.	76 (8.2)	226 (14.7)	652 (16.3)	85 (10.2)
EMS treated	Black colony, 3.5 cm dia. spore, 3.17 μm dia.	100 (6.9)	105 (24.9)	2500 (124.7)	72 (9.5)
UV treated	Black colony, 1.5 to 2.2 cm dia. spore, 2.46 μm dia.	78 (5.2)	100 (12.4)	4566 (68.2)	62 (8.2)

*Estimations (biomass, and DNA) done after 16.5 h of culture in Potato dextrose broth.
Each value is mean of three replicates.
Values in parenthesis indicate ± S.D.

phological features were evident. Since all the cultures, namely the wild type *Aspergillus* sp. and its variant referred above, were cultured under similar conditions for the same period of 16.5 h, marked differences in the biomass indicate the changes in the growth rates. The DNA content of the colchicine variant appeared to be indicative of an aneuploid like situation. On the other hand the DNA contents of the EMS- and UV-treated cultures were significantly reduced to nearly half the value.

Characteristics of the wild type and variants of *Penicillium* sp. are given in Table II. In this case, marked differences in the morphological features of the mutagen-treated variants of *Penicillium* sp. were also evident. The colchicine-treated variant produced darker colonies of much larger diameter compared to the wild type colonies, and its spore size was slightly larger. The EMS variant of *Penicillium* sp. was remarkably different in that it formed a pink colony in contrast to the grey colonies of the wild type.The UV variant also produced darker colonies of slightly larger diameter compared to the wild type. The data on biomass also indicated that the growth rates of the colchicine variant and UV variant also changed. In this case the DNA content of the colchicine variant had changed but was more like duplication of the chromosome by increasing the DNA content twofold.

There was considerable reduction in the DNA content of the UV variant. Data are given in Tables I and II to indicate that both the wild type cultures were better producers of verbenone than verbenol. The biochemical pathway leading to flavour compounds verbenol and verbenone from α-pinene is indicated in Fig. 2.

In the colchicine variants the verbenol production increased more than 1.5-fold, while the verbenone formation was substantially reduced. The improvements in verbenol

Table II. Characteristics of wild type and Variants of *Penicillium* sp.

Strain	Morphology	Biomass* (mg 100 ml^{-1} dry wt.)	DNA* (μg 100 mg^{-1} biomass)	Product of biotransformation	
				verbenol (μg 100 ml^{-1})	verbenone (μg 100 ml^{-1})
Wild type	Greyish colony, 1.2 cm dia. spore, 1.94 μm dia.	140 (3.7)	120 (13.9)	440 (10.2)	1500 (98.6)
Colchicine treated	Greyish black colony, 2.7 to 3.7 cm dia. spore, 2.13 μ dia.	188 (8.0)	243 (22.2)	720 (16.3)	105 (15.2)
EMS treated	Pink wrinkled and thick colony, 1.2 cm dia.	140 (7.2)	109 (10.6)	944 (17.9)	92 (18.7)
UV treated	Dark black colony, 2.0 cm dia. spore, 1.94 μm dia.	190 (7.5)	80 (20.5)	3698 (35.2)	80 (12.5)

*Estimations (biomass, and DNA) done after 16.5 h culture in Potato dextrose broth.
Each value is mean of three replicates.
Values in parenthesis indicate ± S.D.

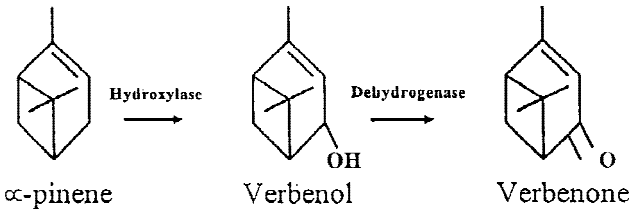

Figure 2. Biotransformation scheme for conversion of α-pinene to verbenol and verbenone.

formation in EMS-treated and UV-irradiated cultures were exceedingly high, a 15-fold increase in the case of *Aspergillus* sp. and an 8-fold increase in the case of *Penicillium* sp. The shift in product formation in all the variants reflects enhanced activity or level of α-pinene hydroxylase and perhaps a diminished activity of verbenol dehydrogenase that has resulted in higher level of verbenol formation than the verbenone. The precise biochemical or genetic causes for this is unknown. Modifications in the regulations of either enzyme activities or enzyme biosynthesis or both may have occurred.

DISCUSSION

Data obtained in this study suggested that the mutagenic treatments have caused genetic changes leading to variations in the biochemical capability of the cultures as seen in the changed pattern of biotransformation products. It was also seen that in some other traits also, such as growth rate, morphological features and DNA content, the variants differed from the wild type cultures. Detailed in-depth characterization would be necessary to precisely pinpoint the biochemical and genetic modification incurred in the variants. Nevertheless, from the point of view of developing a process for production of flavour end products, the present study is significant in the isolation of variants which produced predominantly verbenol. In several earlier studies the authors have found mixtures of oxygenated biotransformation products (Prema and Bhattacharrya, 1962) starting from α-pinene as the substrate which may not be desirable for developing an economical process for the production of flavour end products. Repp et al. (1988) patented a process where in *Acetobacter methanolicus* was employed to give 47% yield of *trans*-verbenol out of 30 g of α-pinene in 100 min. Repp et al. (1988) have also patented a process for the biotransformation of α-pinene to verbenol employing methanol utilizing acidophilic or neutrophilic bacteria or yeast. As per the patent information a strain of *A. methanolicus* cultured in medium containing methyl alcohol and glucose under nitrogen limiting conditions in a fed batch process yielded 7 g of oil from 25 g of α-pinene. The oil was composed of 70% *trans*-verbenol, 10% verbenone, 5% *trans*-pinocarveol, 5% *trans*-sobrerol, and 5% α-campholene aldehyde. The yield of verbenol was 39%, the synthetic rate being 4.1 g verbenol kg^{-1}. In the present study 20 mg α -pinene was used in 100 mL of buffer, yielding 4.5 mg/100 mL in *Aspergillus* sp. and 3.6 mg/100 mL in *Penicillium* sp. The bioconversion efficiency was 23% and 18%, respectively. When a process based on fungal culture is developed employing the variants obtained in the present study, many process advantages could be expected for reasons mentioned above. Also expected is a facile separation of the biomass from the reaction mixture to facilitate the purification of the products obtained in the biotransformation.

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