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SHORT CONTRIBUTION

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Bioconversion of alpha pinene to verbenone by resting cells of *Aspergillus niger*

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Abstract Resting cells of a locally isolated strain of *Aspergillus niger* caused the bioconversion of alpha pinene to verbenone. The formation of verbenone was raised from trace amounts (under screening conditions) to 3.28 mg/100 ml (equivalent to a molar yield of 16.5% conversion of the substrate) by amending the cultivation medium for the fungus. The optimal conditions were: 6 g/100 ml for the glucose concentration, a pH of 7.0, an alpha pinene concentration of 20 mg/100 ml, and a 6-h incubation period for the reaction.

Introduction

Flavouring compounds are of considerable importance to the food, perfumery and pharmaceutical industries (Abraham and Berger 1994; Chang et al. 1995; Gatfield 1988; Guenther 1966; Hagedorn and Kaphammer 1994; Molinari et al. 1995; Tripathi et al. 1997; Van Rensburg et al. 1997). The major sources of natural flavourings in general are plants, and some of these are also chemically synthesized. Recent developments in biotechnology have enabled the production of natural flavourings to be carried out economically and more efficiently. Although enzymes are used in the biotransformation, microbial whole cells have shown great potential for biotransformation owing to the ease with which the microorganisms can be cultivated and used in the bioreactors.

The objective of the present work was to select fungal cultures suitable for biotransformation of the inexpensive and readily available terpenoids (alpha pinene) to the highly priced flavouring compound verbenone. Verbenone is a major flavour constituent of strawberry, raspberry, dill, rosmarinus and spearmint flavour mixtures (Ravid et al. 1997); it has the flavour notes of camphor and mint.

Verbenone is in great demand in the food industry. The cost of verbenone is U.S. $ 3000/kg and is currently obtained by extraction from pine and eucalyptus sources. Production of verbenone by biotransformation of alpha pinene (which costs only U.S. $ 0.0075/kg) is expected to be economical and advantageous. A preliminary report on the possibility of biotransforming alpha pinene into verbenone was made earlier by Battacharyya et al. (1960), who used *Aspergillus niger* cultures, and this work provided a basic insight into the mechanism. Alpha pinene has to undergo two distinct biochemical reactions, i.e. a hydroxylation to produce verbenol and dehydrogenation, to form verbenone. Apart from this significant academic input, no serious attempt to develop a bioconversion process of technological interest has been reported. The present work was aimed at a selection of fungal cultures and the study of factors that influence the rate and efficiency of bioconversion of alpha pinene to verbenone.

Materials and methods

Chemicals

Potato-dextrose agar, potato-dextrose broth (PDB) and other medium components were purchased from Hi Media Laboratories Pvt Ltd. (India). 1R-Alpha pinene no. 26 807-0 and a verbenone standard were from Aldrich Chemical Company (St. Louis, Mo.). All other chemicals used were of analytical grade (from BDH, England).

Screening cultures for biotransformation

Twenty isolates of locally isolated *A. niger* and two standard *A. niger* strains (ATCC 326 and NCL 612) were screened for biotransformation of alpha pinene to verbenone. The culture was grown in PDB, using an active inoculum in the exponential phase (with an initial optical density of 1.3 at 660 nm), for a period of

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16 h (on a rotary shaker at 100 rpm at 30 °C and an initial pH of 5.75). The biomass was harvested by centrifugation at 10 000 rpm for 10 min; an amount equivalent to 100 mg dry wt was suspended in 100 ml sodium phosphate buffer (pH 7.0, 0.05 M) containing 25 mg 1R-alpha pinene and incubated for a period of 6 h. 1R-alpha pinene only, cells only, and boiled culture were each used as controls. This was extracted with dichloromethane three times with 20 ml each time. The pooled extract was dried over anhydrous sodium sulphate, concentrated to 1 ml and the verbenone estimated by using gas chromatography (GC) and GC/mass spectrometry (GC-MS) analysis. GC analysis was done on a Shimadzu gas chromatograph equipped with a stainless steel SE-30 column and a flame ionisation detector. The operating conditions were as follows: oven temperature, programmed 40–250 °C, carrier gas nitrogen, 30 ml/min. Identification was carried out using an internal standard and by GC-MS fragmentation patterns. For GC-MS data, a Hewlett-Packard 5995 GC-mass spectrometer (Hewlett-Packard, USA) was used with an HP-20M (Carbowax 20M) column (25 m × 0.32 mm); the temperature was programmed (60–250 °C, 4°/min) and the He gas flow rate was 1 ml/min. The verbenone content was calculated with respect to the area of standard compound and characterised by means of the GC-MS fragmentation pattern.

Growth and dry weight determination

The organism was grown on a rotary shaker at 30 °C for the period required. For the determination of the dry weight, the cell suspension was centrifuged and the pellet dried at 110 °C for 20 h and weighed.

Results

A limited screening of fungal cultures led to the selection of an *A. niger* strain (isolate no. 203 deposited in the culture collection of the Central Food Technological Research Institute, Mysore, India) that showed potential use in the development of a biotransformation process producing verbenone from alpha pinene. We had isolated the fungal cultures from soil underneath citrus trees, with the expectation that such cultures would be highly likely to show relevant biotransformation potential.

The screening procedure involved the following two steps: (i) culture development in a growth medium and (ii) use of the biomass (as resting cells) in a reaction mixture for the biotransformation. Employing the same two-step procedure, conditions were varied in the cultivation medium and reaction mixture which revealed remarkable influence on the bioconversion of alpha pinene to verbenone, increasing the efficiency by more than two orders of magnitude. Supplementation of the culture medium with urea, peptone or yeast extract, to enhance the supply of nitrogen and the growth factor did not have any significant effect. Variation in glucose content alone had tremendous effects on bioconversion (Table 1).

Although all the variables in the culture medium influenced growth rate and biomass production at the end of the 16-h cultivation period, the biomass produced in PDB with 6% glucose caused the maximum bioconversion (formation of 728 μg verbenone/100 ml. With 4% glucose the conversion efficiency was 30% less and with 3% or 2% (original concentration in PDB) glucose the efficiency was down by more than 95%. With slightly higher amounts of glucose (7%) the bioconversion was reduced by nearly 60% and with 10% glucose it was further reduced (data not shown in Table 1). Since the bioconversion reaction mixture contained the same amount of biomass (100 mg), the only difference to be considered was the physiological state of the fungal biomass cultivated in different levels of glucose. Studies with yeast culture have shown that glucose concentration in the cultivation medium influences not only the growth rate but also the aerobic and anaerobic routes of metabolism, through regulation of oxygen availability.

The variables adopted in the reaction mixture could have had a more direct effect on the bioconversion *per se* than the physiological state of the biomass. For all the variables in this case (e.g. pH and alpha pinene concentration), the biomass grown in 6% glucose medium for 16 h was employed. The pH effect in the reaction mixture was striking in that at the optimum of pH 7.0, 2840 μg verbenone/100 ml was produced. At pH values above or below this optimum, formation of verbenone was quite low (e.g. 728 μg verbenone/100 ml at pH 5.5 and only 120 μg/100 ml at pH 8.0). The concentration of alpha pinene was also found to influence its bioconversion to verbenone, the optimum being 20 mg/100 ml. It is noteworthy that above this alpha pinene concentration the bioconversion efficiency was drastically affected: for instance, at 30 mg/100 ml it was only 24 μg verbenone/100 ml and at 40 mg alpha pinene/100 ml there was no bioconversion detectable. A 6-h duration of incubation was found to be the optimum for the bioconversion. Beyond this period the product concentration was reduced.

The factors studied pointed to sharp optima in each case, which calls for careful manipulation of process conditions in scale-up. The precise cause of low yields with levels of alpha pinene higher than 20 mg/100 ml could have been product inhibition typical of enzyme-

Table 1 Factors in culture medium and reaction mixture influencing the bioconversion of alpha pinene to verbenone by *Aspergillus niger* strain no. 203

Variable	Range tested	Optimum	Maximum verbenone formation (μg/100 ml)
Culture medium			
Glucose (g/100 ml)	2–10	6	728
Reaction mixture			
pH	5–8	7	2840
Alpha pinene (mg/100 ml)	10–40	20	3280
Incubation period (h)	2–10	6	3280

catalysed reactions. Since we have employed resting cells it is reasonable to assume that the biotransformation is a two-step reaction involving two enzymes, i.e. a hydroxylase that converts alpha pinene to verbenol and a dehydrogenase that, in turn converts verbenol to verbenone. It cannot be confirmed, at this stage, if these enzymes are specific for the respective substrates mentioned. Several hydroxylases and dehydrogenases with somewhat loose specificities are known to exist in nature and thus enzymes such as these could have caused the reactions mentioned above. Prolonged incubation brought about reduced product yields that may have been due to further conversion of verbenone to other degradative products.

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