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Effect of some abiotic stresses on the biotransformation of α-pinene by a psychrotrophic *Chrysosporium pannorum*

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ABSTRACT

Biotransformation of hydrophobic terpenes is limited by their toxicity and low bioavailability to biocatalyst cells. The metabolic activity of microorganisms, which is strongly dependent on environmental parameters, may also be affected by stressful conditions. The influence of pre-incubation of the fungus *Chrysosporium pannorum* in different stress conditions (organic solvents, medium pH and temperatures) on its activity in the oxidative bioconversion of α-pinene to verbenone and verbenol was examined. The total bioconversion activity increased over 2-fold after 15-min pre-treatment with 1.4-dioxane as an abiotic stress factor. Also, the change of the medium pH from the optimal 5.6 to 2.0 and 10 for 1 h before biotransformation enhanced product yield nearly 1.5-fold. Moreover, the use of dioxane, chloroform and ethanol and thermal stress at 50 °C caused changes in the oxidation product profile (predominance of *trans*-pinocarveol over *trans*-verbenol). A maximum increase in the yield of verbenol was observed when the biocatalyst was subjected to 15-min dioxane stress before 24-h biotransformation of 1% (v/v) pinene. The significance of the data presented is that abiotic stresses may improve the bioconversion activity or create changes in the proportion of the main biotransformation products.

1. Introduction

Monoterpenes, beside sesquiterpenes, are the main constituents of essential oils of many plants, including fruits, vegetables and herbs. They contain two isoprene units, i.e. ten carbon atoms and sixteen hydrogen atoms per molecule ($C_{10}H_{16}$). Monoterpenes prevent carcinogenesis at both the initiation and promotion/progression stages [1,2]. Terpenoids such as limonene and perillyl alcohol have been shown to prevent mammary, liver, lung and other cancers [3]. Biotransformation products of α-pinene, i.e. *trans*-verbenol and verbenone, have been recently shown to exert biological effects against human colon tumor cells [4]. Apart from their use in medicine, terpenoids are also applied in many branches of industry as flavor and fragrance components [5–8].

Desired compounds of a high commercial value can be selectively obtained through enzymatic [9,10] or microbial transformation of monoterpenes by both bacterial and fungal strains [7,11–15]. Some of these monoterpenic substrates, for example α- and β-pinene, *p*-cymene and *R*-(+)-limonene are not expensive and are abundantly produced by a variety of plant species. Unfortunately, the biotransformation of terpenes involves some problems, the most important of which are toxicity to the microorganisms resulting in inhibition of their metabolism, and poor solubility in aqueous media. α-Pinene is practically insoluble in water (2.49 mg/L at 25 °C); it only shows a slightly higher water solubility at 20 °C (18 mg/L) [13,16,17]. To improve the availability of a water-immiscible substrate to the biocatalyst, hydrophilic co-solvents, such as EtOH, MeOH or acetone, are often used [18,19].

The relative effectiveness of bioconversion depends on the characteristics of the microbes used and their enzymes, the physiological conditions, such as nutritional and environmental parameters (T, pH, pO_2), exposure to chemicals which can inhibit or induce enzyme levels, and effects of extreme environments (*e.g.*, non-aqueous media) as well as the kind of product isolation techniques used [18,20–25]. According to literature data, most biotransformations of terpenes by eukaryotic microorganisms involve cytochrome P-450-dependent monooxygenases, which are considered to be coupled with primary metabolism but compete with the respiratory chain for cofactors NAD(P)H [18,26].

In a previous study, we showed that the final concentration of perillyl alcohol produced by *Mortierella minutissima* 01 in a culture medium could be increased to over 120 mg L^{-1} by optimizing some biotransformation conditions (including composition and pH of the growth and bioconversion media, substrate concentration and temperature and time of biotransformation) [27]. Further experiments (involving alteration of oxygen and hydroperoxide concentrations, addition of an organic solvent and optimization of the time of fungal cultivation and biotransformation together with

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substrate concentration) resulted in the improvement of the bioconversion yield of perillyl alcohol up to 260 mg L^{-1} [28]. In another work, gradual addition of the substrate during 3 d of biotransformation of α-pinene at a low temperature resulted in a significant increase in the production of *trans*-verbenol and verbenone [29] (Scheme 1).

Psychrophiles and psychrotrophs have a considerable potential in biotechnology, enzymology, food industry and medicine [30,31]. They offer numerous advantages, such as high microbial growth rates and high enzymatic activities at temperatures from 0 to 25 °C, which, in turn, give energy-saving benefits, prevent microbial contamination and minimize undesirable chemical reactions that can occur at higher temperatures [18,32,33]. Microorganisms isolated from cold environments have received little attention in the field of biotransformation reactions. As far as we know, only few reports on bioconversion of toxic organic compounds by psychrotrophic fungi and bacteria have been published to date [27,28,34,35].

Microorganisms have evolved adaptive networks to face the challenges of changing environments and to survive under conditions of stress. All adaptive responses, whether to changing nutrients or to various stresses encountered in minimal processing, involve a series of genetic switches that control the metabolic changes taking place [22,36,37]. The response of yeasts to environmental stresses involves ~900 genes, whose expression is stereotypically altered when the cells are in stressful environments. Regulation of the expression changes of these genes is gene-specific and condition-specific, which means that initiation of the program is precisely controlled in response to each new environment [38]. Other mechanisms may be based on modifications of the cell membrane, such as changes in its stiffness/fluidity, lipid composition and fatty acid profile [39–41].

Many organic solvents are toxic to living organisms because of their devastating effects on biological membranes [21]. Some efforts have been made to explain the mechanisms responsible for solvent tolerance in some bacteria, also in the context of using solvent-tolerant cells for biotransformation of hydrophobic compounds in biphasic systems. Up to date, various adaptation mechanisms have been described [40,42,43].

As far as we know, in eukaryotes the mechanisms of cell tolerance to organic solvents have not yet been discovered, except for the yeast *Saccharomyces cerevisiae*, strain KK-211. Several factors contributing to organic solvent tolerance of this strain have been identified, such as Pdr1p and ABC transporters. It has also been found that a one point mutation (R821S) of Pdr1p causes organic solvent tolerance [42,43]. Overexpression of genes encoding proteins effective for tolerance to specific organic solvents would enable enhanced tolerances for practical use [43]. So far, the impact of stress with organic solvents on the biocatalytic activity of microbes is also unknown. It has been found that some hydrophilic solvents, such as acetone, increase the permeability of the bacterial cell membrane towards the alkane substrate, thereby increasing its mass transfer rates [44,45].

The aim of the present study was to investigate the effect of different abiotic stresses on the biotransformation of α-pinene to verbenol and verbenone by *Chrysosporium pannorum* A-1.

2. Materials and methods

2.1. Fungus and media

The origin of the psychrotrophic fungus *C. pannorum* A-1 used in this study and details of the methods of its storage and culture have been described in our previous paper [29]. Fungal cultivation was conducted in a liquid basal medium (BM) consisting of malt extract 1%, peptone 0.5%, glucose 1% and yeast extract 0.5%. The final bioconversion reactions were carried out using the same fresh BM medium.

2.2. Conditions of abiotic stresses

After 3 d of culture (25-mL BM in 50 mL Erlenmeyer flasks), the mycelia of *C. pannorum* (about 5.1 g of dry mass per L) were uniformly and aseptically transferred to media (50 mL) with various stress factors. The conditions in the particular stress media were as follows: *organic solvent stress:* 15–60-min treatment of the mycelium with 20% (v/v) water solutions of organic solvents (methanol, chloroform, *n*-decane, 1.4-dioxane, ethanol or 2-propanol); *thermal stress:* 1-h incubation at 20, 30, 40, 50 and 60 °C in 0.1 M McIlvaine buffer, pH 5,6; *acid/alkali stress:*1-h incubation of the mycelium in BM media with pH 1, 2, 3, 5.6, 8.5 and 10.

With the exception of the temperature stress experiments, the samples were incubated at 20 °C on a rotary shaker (200 rpm). Finally, the mycelia were harvested, washed twice with sterile 0.1 M McIlvaine buffer, pH 5.0, and aseptically suspended into 25 mL of sterile BM to be used for 1-d biotransformation of 1% (v/v) α-pinene.

2.3. Oxygen uptake measurements

Fixed amounts of the pre-grown mycelia of *C. pannorum* were exposed to 20% (v/v) organic solvent solutions in water for 1 h. After that, the biomasses were filtrated, washed twice with sterile 0.1 M McIlvaine buffer, pH 5.0, and then transferred uniformly to fresh BM in order to measure the relative metabolic activity of living mycelia, expressed as oxygen consumption rates.

The samples were aerated to saturation with dissolved oxygen (DO), sealed from atmospheric oxygen and incubated with magnetic stirring (150 rpm) under a controlled temperature (20 °C). The rate of oxygen uptake was measured in a 250-mL reactor (6.5 × 18.0 cm) using an Ingold electrode (Mettler-Toledo Inc., Columbus, Ohio, USA).

According to the method reported previously [28,29], the electrode was calibrated at the beginning of each experiment by measuring its signal in the air-saturated media before adding the mycelium. The values of the readings were expressed as a percentage of the initial DO level. In all experiments, an effort was made to obtain representative samples of equal pellet sizes and mycelium weights.

2.4. Biotransformation analysis

After 48 h of biotransformation, 500 μL of a 0.1% internal standard (IS) solution in hexane was added to the medium. The biomass was harvested by filtration, and the liquid for product recovery was extracted twice by an equal volume of diethyl ether in a separatory funnel. The ether fraction was separated, dried over anhydrous sodium sulfate and concentrated on rotary vacuum evaporators at a water bath temperature of 40 °C. The residues obtained were dissolved in 4 mL of hexane and used for GC and GC–MS analyses conducted according to the method reported previously [28].

Products (*trans*-verbenol, verbenone and *trans*-pinocarveol) were identified by fitting their mass spectra to those from the NIST 2004 and MassFinder 3 libraries and by an additional comparison to the GC retention indexes of standard compounds. The terpenoids were quantified by comparing with the internal standard (IS) added, using a calibration curve of peak area ratios (analyte/IS) vs. amount ratios (analyte/IS) from standard authentic samples. 0.1% solutions (w/v) of *n*-decane (for the substrate) and linalool (for oxidation products) in hexane were used as internal standards for gas chromatography