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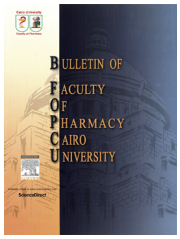

ORIGINAL ARTICLE

LC–MS/MS based-comparative study of (S)-nicotine metabolism by microorganisms, mushroom and plant cultures: Parallels to its mammalian metabolic fate

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Abstract The metabolic fate of nicotine using the cell cultures of microorganisms, mushroom and plants was explored using LC–MS/MS analysis. The study demonstrated parallels to phase I mammalian metabolism of nicotine and reported that nicotine was biotransformed into its *N*-oxide by *Streptomyces fradiae* culture. Moreover, it was metabolized in *Pleurotus ostreatus* culture into nornicotine, norcotinine and *β*-nicotyrine; whereas, cotinine and its 3′-hydroxylated derivative were the identified nicotine metabolic products in *Pimpinella anisum* cell culture. However, the microbial culture of *Agaricus bisporus* bioconverted nicotine into one of its derived-carcinogenic nitrosamines which is "4-(methylnitrosamino)-4-(3-pyridyl)butanal". Mushroom and plant cell cultures were thus proven to be competent to microbial cultures in bioconverting nicotine into many of its previously reported metabolites. Convincingly, the obtained results highlighted the prospect of utilizing other species which are intrinsically-endowed with unique biocatalytic systems, such as mushrooms and plants, in the drug metabolic studies.

1. Introduction

The inherent enzymatic bioconversion process in mammals known as "metabolism" involves the biotransformation of a wide range of xenobiotics into specifically-modified structures via both oxidative and conjugative biotransformation pathways.[1–3] Analogously, the biocatalytic systems of other species

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such as microorganisms and plants were reported to produce chemical changes on compounds that are not their natural substrates.[4] There are several reports that highlighted the intrinsic potential of microbes to mimic the mammalian drug metabolism due to the involvement of comparable monooxygenase systems.[5,6] Moreover, it was found that the enzymatic system of plant cell cultures has the potential to perform special biotransformation reactions to produce its characteristic secondary metabolites. Such cultures also retain the ability to transform exogenous compounds into new products that are hardly obtained by chemical means.[7]

Regarding the nicotine metabolism, it is mostly carried out in the liver through its cytochrome P450 isoenzymes (such as CYP2A6), aldehyde oxidase and flavin monooxygenase (FMO).[8] In humans, 70% of nicotine is oxidized to cotinine by cytochrome P450 and aldehyde oxidase, 4% is oxidized differently of which only a small fraction of nicotine is metabolized to nornicotine, 9% is excreted unchanged in the urine, while the metabolic fate of the remaining 17% is still unidentified.[9,10]

It is worth noting that previous nicotine metabolic studies were aimed to explore its metabolic fate using various liver microsomal preparations and microbial cultures.[11–13] However, there is a scarce nicotine biotransformation work using plant suspension culture. Biotransformation of nicotine was studied using some *Nicotiana* species such as *Nicotiana tabaccum* cv. Wisconsin-38 in which the conversion of nicotine into nornicotine was optimized to 100% efficiency.[14]

The present study was designed to explore the bioconversion efficiency of some microorganism cultures as well as the mushroom and plant cell suspension cultures toward a selected naturally-occurring alkaloid "nicotine". The obtained results were correlated with the previously reported nicotine mammalian metabolites.

2. Experimental

2.1. Materials and reagents

(S)-nicotine was purchased from Sigma–Aldrich Chemie GmbH, Buchs, Switzerland. HPLC-grade acetonitrile (Scharlau Chemie S.A., Sentmenat, Spain), HPLC-grade methanol, formic acid and ultra-pure water (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) were used. All other chemicals were of analytical grade.

2.2. Tested microbial and plant cultures

The preliminary screening study involved the use of about fifteen microbial strains, namely: *Cunninghamella blakesleeana* MR 198, *C. blakesleeana* NRRL 1369, *Penicillium chrysogenum* ATCC 10002, *P. vermiculatum* NRRL 1009, *P. purpureus* U_1 193, *Streptomyces fradiae* (lab isolate), *Aspergillus niger* NRRL 328, *A. flavus* NRRL 501, *A. ochraceous* NRRL 405, *A. flaviceps* ATCC 11013, *A. alliaceous* NRRL 315, *Saccharomyces cerevisiae* NRRL Y-12632, *Rhodotorula rubra* NRRL Y-1592, *Gymnascella citrina* NRRL 6050 and *Rhizopus* species ATCC 36060 as well as the mycelia cultured from the fruit bodies of two edible mushroom species: *Pleurotus ostreatus* (oyster mushroom) and *Agaricus bisporus* (button mushroom). The microorganisms were obtained from two sources: American Type Culture Collection, Maryland, USA (ATCC) and Northern Regional Research Laboratories, Department of Agriculture, Peoria, Illinois, USA (NRRL).

Besides, several plant cell suspension cultures were also examined for their biotransformation capability of nicotine, which are the cell suspension cultures obtained from leaves of *Ocimum basilicum* L., *Echinacea purpurea* and *Vinca minor* L., besides, fruits of *Coriandrum sativum* L. and *Pimpinella anisum*.

2.3. Biotransformation protocol

2.3.1. For microbial cultures

Two week-old microbial slants were used to inoculate sterilized liquid culture medium consisting of 10 g dextrose, 10 ml glycerol, 5 g yeast extract, 5 g peptone, 5 g K_2HPO_4, 5 g NaCl and 1000 ml distilled water. The pH was adjusted to 6.0 before sterilization at 121 °C for 15 min. The microbial cultures were incubated at 25 °C with rotary shaking at 150 rpm for 48 h[3]. Nicotine was then added in a concentration of 10 mg/flask (250 ml) using acetone as a solvent. The incubation was allowed to continue for 15 days. After which, the cultures were exhaustively extracted with chloroform after basification with ammonium hydroxide. Both drug and culture controls were treated similarly. The formed metabolites were detected by TLC analysis using a solvent system of chloroform–ethanol–methanol–0.5 M NaOH (30:15:2:1.5, v/v) and Dragendorff's reagent to visualize the alkaloidal spots.

2.3.2. For mushroom cultures

Mycelia from mushroom stock culture maintained on Potato Dextrose Agar (PDA) petri-dishes were transferred into autoclaved Mushroom Complete Medium (MCM) consisting of 20 g/L glucose, 2 g/L yeast extract, 2 g/L peptone, 0.46 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$ and 1000 ml distilled water at pH 5.5, by punching out 5 mm of the agar plate culture with self-designed cutter. The mushroom cultures were then incubated at 25 °C for 24 h in the dark.[15] Nicotine was added using the same solvent and concentration mentioned before. The incubation was then allowed to continue for 7 days at 25 °C with rotary shaking at 150 rpm. The culture extraction and TLC analysis were performed as mentioned before.

2.3.3. For plant cell suspension cultures

The callus tissues belonging to the different plants under study were grown on MS static media supplemented with 2,4-dichlorophenoxyacetic acid (1.0 ppm) for 3 weeks and then transferred into appropriate liquid MS medium supplemented with 30 g/L sucrose and benzyladenine (1.0 ppm). The cultures were grown under 16 h photoperiod at 23 ± 1 °C on a gyratory shaker at 100 rpm and subcultured every 4 weeks. The same concentration of nicotine was added to the cultures and periodical samples were withdrawn.[16] At the end of the experiment, the biotransformation cultures and their controls were lyophilized. The lyophilized cultures (200–250 mg) were then homogenized with 3 ml MeOH and extracted with 10 ml of 3% sulfuric acid in an ultrasonic bath for 45 min. The extracts were centrifuged at 4000 rpm, for 10 min, the supernatant layer was extracted with chloroform. The aqueous