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ORIGINAL PAPER

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New approaches to augment fungal biotransformation

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Abstract The possibility of using solid supports and intermittent substrate feeding to manipulate biotransformation by fungi was examined, with amoxapine as a model compound. *Cunninghamella elegans* ATCC 8688a grown as free cells in six-well plates showed 7-hydroxyamoxapine as the major metabolite of amoxapine biotransformation. However, when cells were grown in the presence of activated carbon, *N*-formyl-7-hydroxyamoxapine was formed as the major metabolite. Intermittent feeding of amoxapine also favored the formation of *N*-formyl-7-hydroxyamoxapine.

Keywords Amoxapine · Biotransformation · *Cunninghamella* · Solid support · Substrate feeding

Introduction

Biotransformation is an attractive approach to generate structural diversity in a chemical library [1, 6, 8]. Fungi are often used as whole cell biocatalysts because of their ability to mediate many different reactions, including oxidative, reductive, and hydrolytic transformations of a wide range of substrates [2]. Recently, microtiter plates have been introduced to aid rapid screening of microbial cultures for biotransformation activities [4, 12]. However, these studies have been confined to screening eubacteria and have not been evaluated for fungi. Fungi in liquid media can grow either as free mycelia in a filamentous form or remain aggregated in a pellet/floc form. These morphological patterns strongly regulate metabolite production and have been exploited extensively for the production of secondary metabolites [5]. Filamentous fungal growth is likely to be challenged in microwell plates because of limited surface area, mixing, and aeration, thereby affecting oxygen availability, which is imperative for growth and biotransformation activities. The effects of these fungal morphological variations on biotransformation remain obscure.

This study was initiated to evaluate fungal biotransformation in a six-well plate. Different types of solid supports were introduced with the view that these solid matrices would provide the fungus with additional surface area for growth. We chose amoxapine, a tricyclic antidepressant developed by Wyeth as Asendin, as a model compound for biotransformation by *Cunninghamella elegans*. Moody et al. [9] had previously reported that 7-hydroxyamoxapine was the major metabolite of amoxapine biotransformation by *C. elegans*. In this work, the influence of growing fungi in the presence of solid supports to enhance biotransformation efficiency and create new metabolites was investigated. In addition, the effects of intermittent feeding of substrate on biotransformation were also studied.

Materials and methods

Fungal culture, media and chemicals

C. elegans strain ATCC 8688a was obtained from the American Type Culture Collection (Manassas, Va.) and maintained on Sabouraud agar (Becton and Dickinson, Sparks, Md.). Sabouraud-dextrose broth (Becton and Dickinson) was used for biotransformation studies.

Amoxapine (Asendin) [2-chloro-11-(1-piperazinyl)dibenz-(b,f) oxazepine] was purchased from Sigma-Aldrich (St. Louis, Mo.) The following solid supports were used in biotransformation studies: glass bead (3-mm diameter), cellulose pulp (ash-free; Schleicher and Schuell, Keene, N.H.), acid-washed activated carbon (Baker, Phillipsburg, N.J.), florisil (100–200 mesh) (Baker) and celite 545 (Baker).

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Inoculum preparation

Frozen fungal stocks were plated on Sabouraud dextrose agar and incubated for 4–7 days at room temperature. Three 8-mm diameter agar plugs bearing surface growth were extricated from the plates and inoculated into 50 ml Sabouraud dextrose broth in 250-ml Erlenmeyer flasks, which were then incubated at 25°C at 200 rpm with a 5-cm throw. After 7 days of incubation, the mycelium was harvested by filtration through several layers of sterile cheesecloth. The mycelial mass was washed three times with sterile distilled water (100 ml per wash) and the mycelia resuspended in 50 ml sterile distilled water and blended for 5 min on ice using a homogenizer (Omni International, Warrenton, Va.) under aseptic conditions. The blended mycelium was used as inoculum at 10% (v/v).

Biotransformation conditions

Biotransformation experiments were conducted either in a six-well plate (Becton Dickinson, Franklin Lakes, N.J.) containing 5-ml Sabouraud dextrose broth or in 10 ml in a 25 ml Erlenmeyer flask. Where indicated, pre-weighed and autoclaved activated carbon, celite, cellulose pulp, florisil or glass beads at 1% (w/v) were added and subsequently inoculated with the fungus. Wells with medium alone, medium + cells, medium + autoclaved cells, medium + substrate, medium + solid supports, and medium + substrate + solid supports served as controls. The six-well plates were sealed with Parafilm and incubated at 25°C at 150 rpm. After 48 h of incubation, filter-sterilized substrate was added to the pre-determined wells to give a final concentration of 0.5 mg/ml. Aliquots were aseptically removed at various times during incubation and the samples were extracted with equal volumes of ethyl acetate, concentrated, resuspended in methanol and analyzed by LCMS.

LCMS analysis

LCMS analysis was performed on the extracts of the culture broth of amoxapine-biotransformed cultures using a model 1100 HPLC system (Agilent, Wilmington, Del.) consisting of a G1322A degasser, G1313A autosampler, G1311A quaternary pump and a G1315A photodiode array UV detector. The HPLC system was coupled to an LCQ ion trap mass spectrometer (ThermoFinnigan, West Palm Beach, Fla.) equipped with an electrospray (ESI) probe. The compounds were eluted using a linear gradient from 5% to 95% acetonitrile in water with 0.025% formic acid over 25 min at a flow rate of 1 ml/min. A 3.5-μm Zorbax Eclipse XDB-C18 column (4.6×50mm; Agilent) was used. The metabolites observed were confirmed as related to amoxapine by UV data from the PDA chromatograms and identified using mass spectral data from positive mode ESI base peak chromatograms.

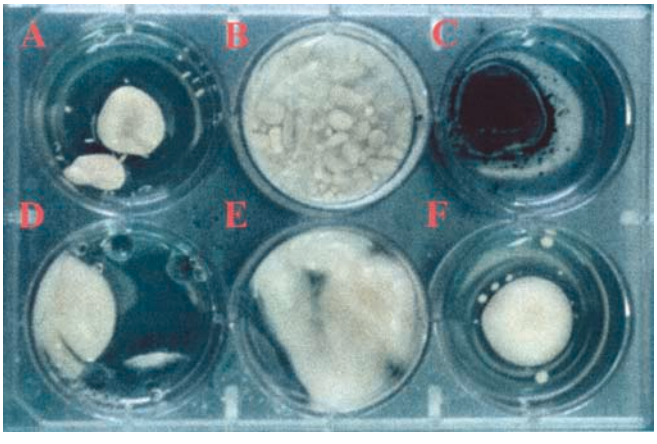


Fig. 1A–F Growth of *Cunninghamella elegans* on different solid supports in a six-well plate after 7 days of incubation. **A** Free-growing cells. **B** Cellulose pulp. **C** Activated carbon. **D** Glass beads. **E** Florisil. **F** Celite. The plates were incubated at 25°C and 150 rpm

Intermittent feeding of substrate

For substrate feeding studies, the experimental design described above was followed except that the substrate amoxapine was added intermittently to the growing culture at different incubation times. Amoxapine was added in increasing increments to the growing culture at each 24 h time point to give a total of 10% at 24 h after inoculation, 30% at 48 h incubation, 60% after 72 h, and 100% at 96 h.

Results and discussion

Fungal morphological variations when grown in the presence of solid supports

Growth of *C. elegans* in the presence and absence of solid supports is presented in Fig. 1. In general, the fungal mycelium tended to aggregate as one big pellet. This morphological form appeared to be common when grown without any solid support or in the presence of glass beads and celite (Fig. 1A, D, F). However, in the presence of florisil, the fungus grew as a mat (Fig. 1E). Interestingly, with activated carbon, the mycelium sequestered the resin, and fungal growth encompassed the particulate matter (Fig. 1C). In contrast to the morphological forms observed with other solid supports, *C. elegans* formed pellets of various sizes when grown in the presence of cellulose pulp (Fig. 1B). Fungi have been grown in microtiter plates for other applications [7, 10] but there is no mention of the morphology of the cells or variations thereof. *Penicillium brevicompactum* grown in the presence of celite 535 and 545 in a shake-flask culture was reported to form smaller pellets than when grown as free cells and showed enhanced production of mycophenolic acid [11].

Biotransformation of Amoxapine

Amoxapine (Fig. 2), a tricyclic antidepressant drug that belongs to the dibenzoxazepine class, is the *N*-deme-

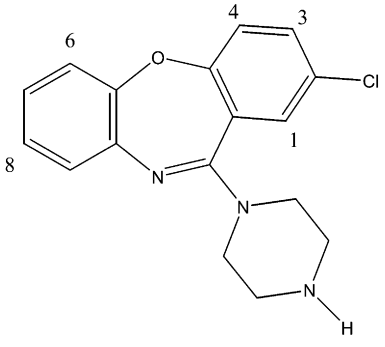
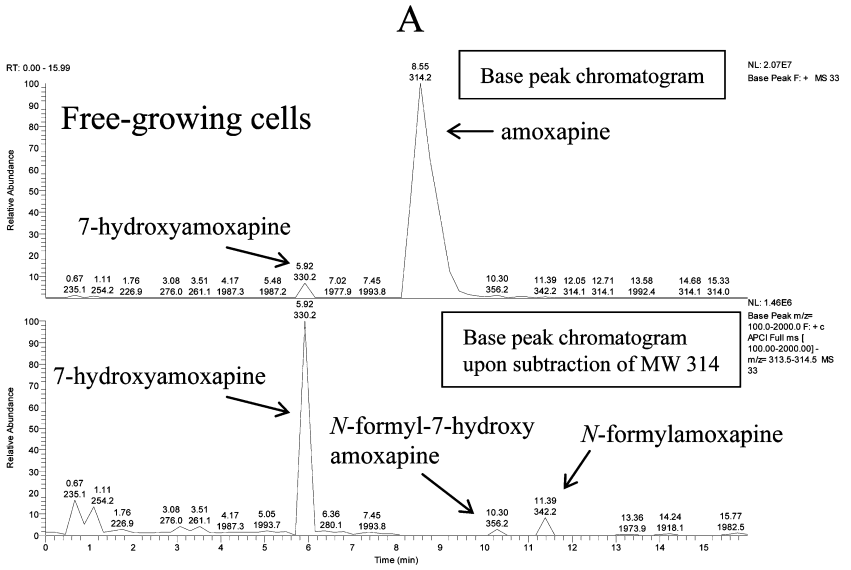
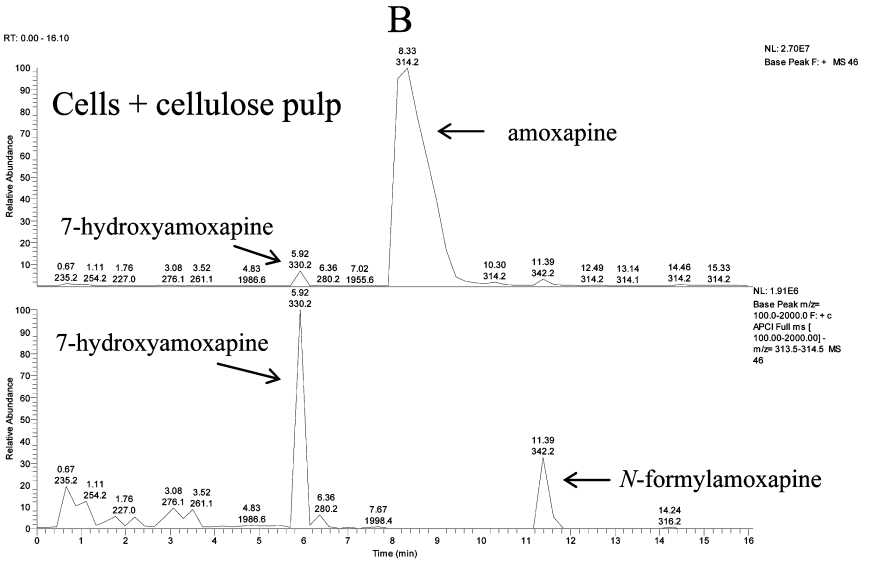
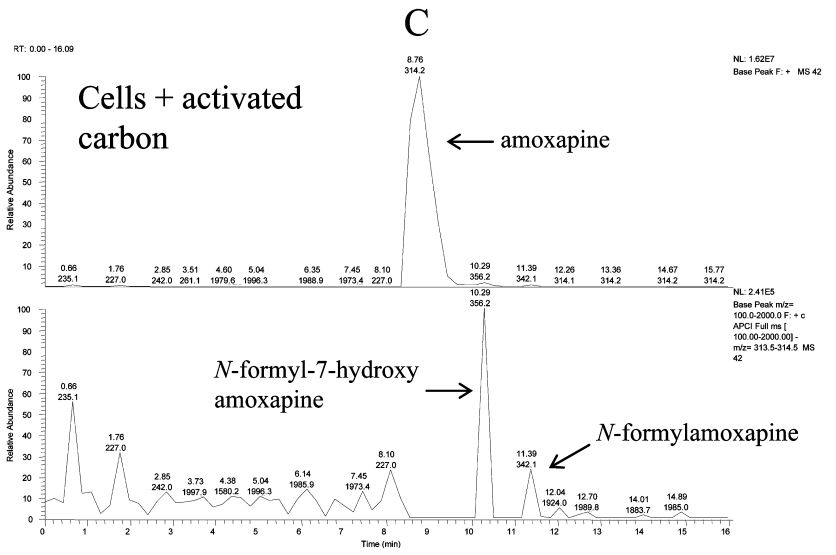
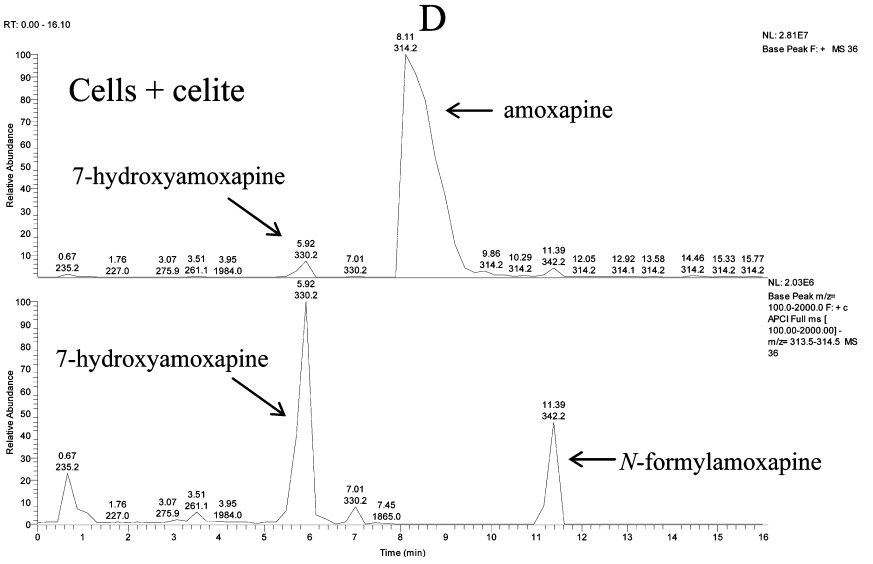


Fig. 2 Structure of amoxapine


A
Free-growing cells
Base peak chromatogram
amoxapine
7-hydroxyamoxapine
Base peak chromatogram upon subtraction of MW 314
7-hydroxyamoxapine
N-formyl-7-hydroxy amoxapine
N-formylamoxapine
Time (min)


B
Cells + cellulose pulp
amoxapine
7-hydroxyamoxapine
7-hydroxyamoxapine
N-formylamoxapine
Time (min)


C
Cells + activated carbon
amoxapine
N-formyl-7-hydroxy amoxapine
N-formylamoxapine
Time (min)


D
Cells + celite
amoxapine
7-hydroxyamoxapine
7-hydroxyamoxapine
N-formylamoxapine
Time (min)

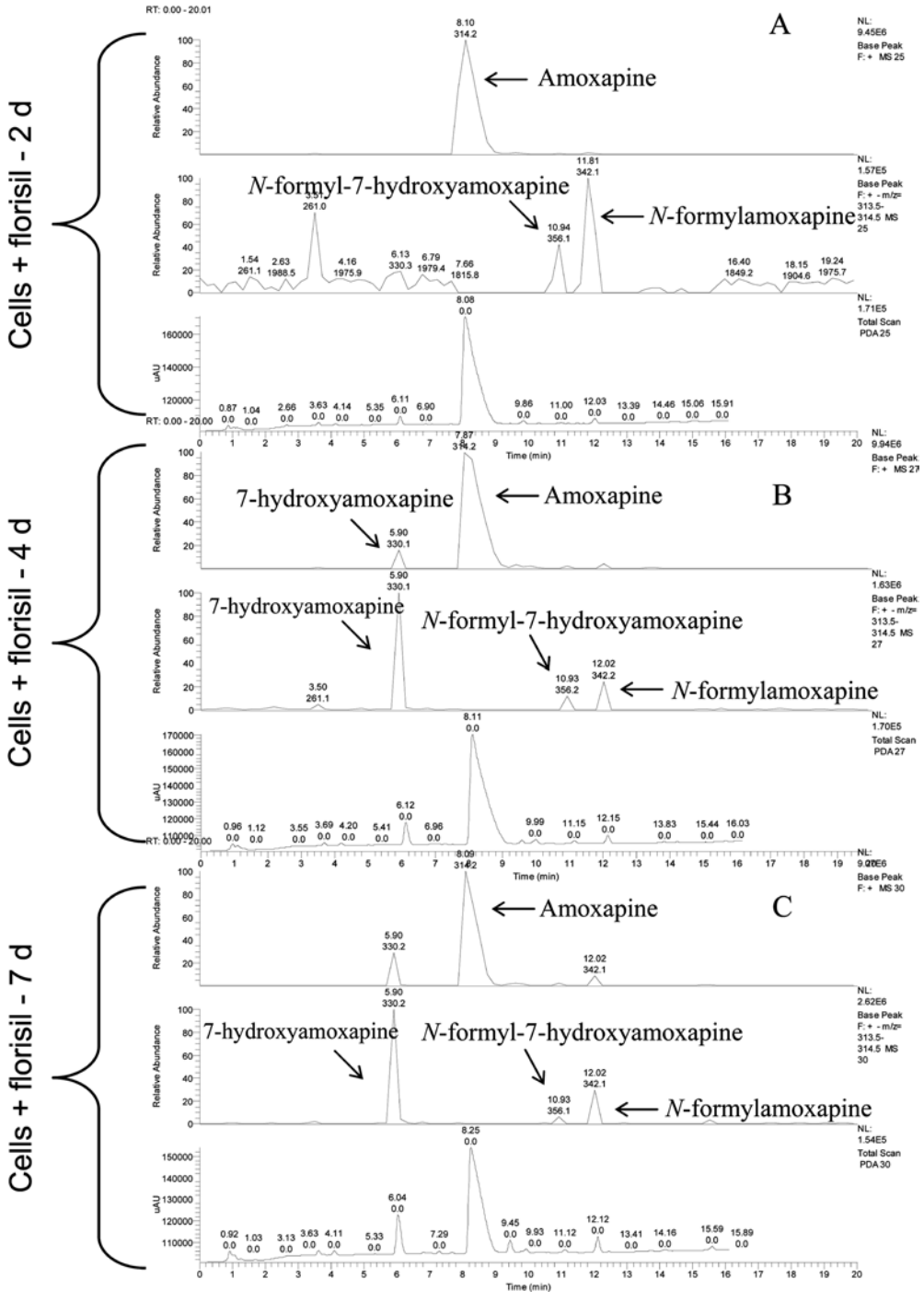


Fig. 4A–C LCMS profile of the temporal biotransformation pattern of amoxapine by *C. elegans* grown in the presence of florisil. Cells were grown in six-well plates. **A**, **B**, **C** 2, 4, 7 days of incubation, respectively. *Top panels* Base peak chromatograms, *middle panels* peaks seen on subtraction of amoxapine (molecular mass 314) from the top panel, *bottom panels* UV

thylated derivative of the neuroleptic compound loxapine, developed by Wyeth as Asendin. This compound was chosen as a model to investigate the effects on biotransformation of growing *C. elegans* with and without solid supports in a six-well plate and intermittent feeding of substrate.

Moody et al. [9] previously reported that 7-hydroxyamoxapine was the major metabolite of amoxapine biotransformation by *C. elegans*. *N*-Formylamoxapine and *N*-formyl-7-hydroxyamoxapine were the other metabolites of biotransformation. In accordance with their finding, we also observed 7-hydroxyamoxapine (molecular mass of 330) as the major metabolite in cells grown without any solid supports (Fig. 3A) on day 7 of

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Fig. 3 LCMS analysis of amoxapine biotransformation by *C. elegans*. Cells were grown in six-well plates at 25°C, 150 rpm, as free-growing mycelia without any solid supports (**A**), or in the presence of cellulose pulp (**B**), activated carbon (**C**), or celite (**D**). The top panel in **A**–**D** is the base peak chromatogram and the bottom panel represents peaks seen on subtraction of amoxapine (molecular mass 314) from the top panel

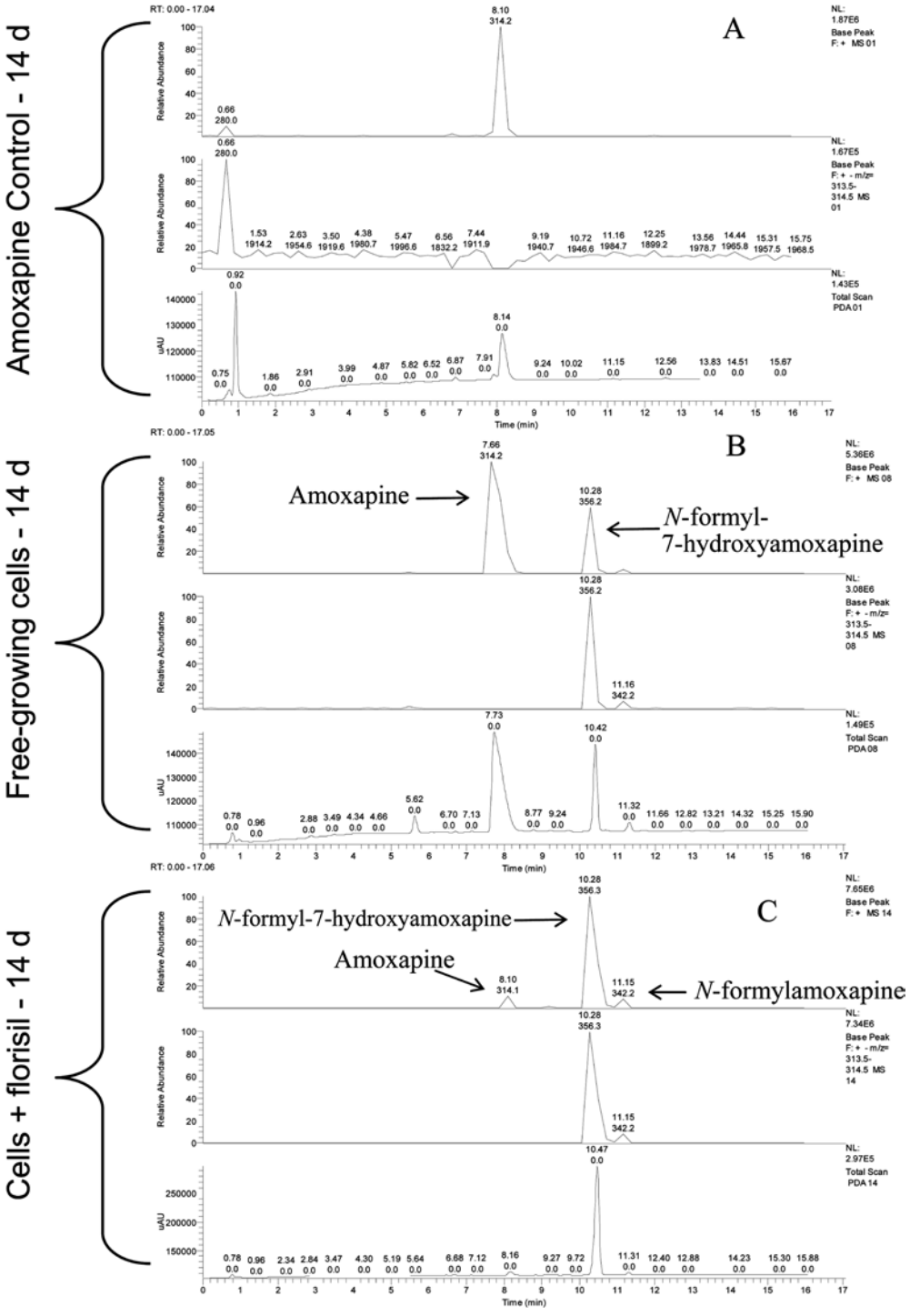

Fig. 5A–C LCMS profiles showing effect of intermittent feeding of amoxapine on biotransformation by *C. elegans* on day 14. **A** Control containing medium and amoxapine. **B** Free-growing cells with amoxapine. **C** Cells grown in the presence of florisil

incubation. Cells grown in the presence of cellulose pulp and celite also showed 7-hydroxyamoxapine as the major metabolite (Fig. 3B, D) and also showed *N*-formylamoxapine. *N*-Formyl-7-hydroxyamoxapine was not detected in these cultures. In contrast to these observations, cells grown in the presence of activated carbon (Fig. 3C) produced *N*-formyl-7-hydroxy-amoxapine as the major metabolite and showed no formation of 7-hydroxyamoxapine. Fungal morphology when grown in activated carbon may possibly be a factor that regulates the production and/or activity of these enzymes. Alternatively, this adsorbent may have selectively immobilized the enzyme and/or substrate, favoring formation of certain metabolites. Temporal biotransformation studies with cells grown in the presence of florisil initially showed low levels of *N*-formyl-7-hydroxyamoxapine (Fig. 4A) and *N*-formylamoxapine, but in subsequent days only 7-hydroxyamoxapine was observed to accu-

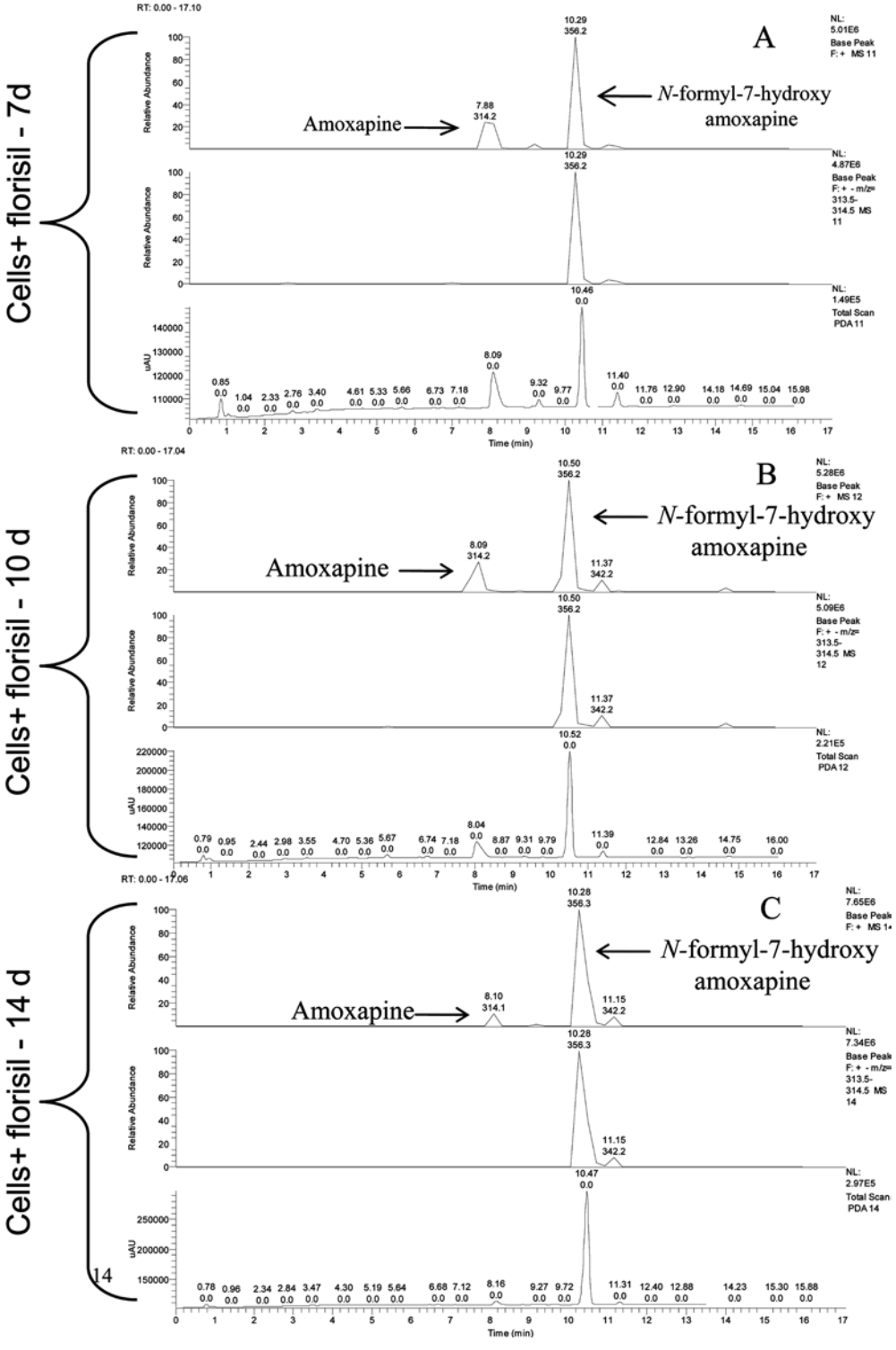

Fig. 6A–C Intermittent feeding of amoxapine. LCMS profiles showing temporal analysis of biotransformation activities on days 7 (**A**), 10 (**B**), and 14 (**C**)

mulate (Fig. 4B, C). It is possible that more than one hydroxylase enzyme is involved — one presumably acting on *N*-formylamoxapine to form *N*-formyl-7-hydroxyamoxapine and another that hydroxylates amoxapine to 7-hydroxyamoxapine. There is also the possibility that a deformylase is being induced later in the growth cycle that may explain the accumulation of 7-hydroxyamoxapine.

Intermittent feeding of substrates is commonly used in microbial fermentation to help regulate nutrient level in the medium and is an effective strategy to maximize production of microbial metabolites [3]. In addition, intermittent feeding can be an attractive approach in the following scenarios: (1) the substrate is toxic or inhibitory to growth of the cells at the desired final concen-

tration, (2) the solvent used to dissolve the substrate is likely to affect cell growth, (3) a desired concentration of substrate induces the enzyme(s) associated with biotransformation, or (4) there is a significant amount of substrate that is not used, resulting in low biotransformation efficiency.

Feeding amoxapine intermittently to *C. elegans* cells grown without any solid supports or in the presence of florisil showed the formation of *N*-formyl-7-hydroxyamoxapine as the major metabolite (Fig. 5B, C). This is in contrast to the observation of 7-hydroxyamoxapine as the major metabolite when amoxapine was provided in a single addition (Figs. 3A, B, D; 4). Conversion of amoxapine to *N*-formyl-7-hydroxyamoxapine on day 14 seemed to be favored by the presence of florisil as evidenced by amoxapine peak heights (Fig. 5B, C). Furthermore, a progressive decrease in amoxapine with a concomitant increase in the formation of *N*-formyl-7-hydroxyamoxapine was observed in cultures grown in the presence of florisil that were fed intermittently with amoxapine (Fig. 6). Although several plausible explanations can be proposed for the observed results, one possibility could be that substrate concentration mediates regulation of the enzyme(s) associated with biotransformation. The lower concentrations of amoxapine may favor induction of formylase while repressing it at higher concentrations. On the contrary, since 7-hydroxyamoxapine was not observed in intermittent feeding studies, it is possible that the concentration of amoxapine was inadequate to induce the hydroxylase(s). Unfortunately, there is limited information on the regulation of enzymes associated with biotransformation in filamentous fungi. Use of solid supports such as activated carbon and florisil seems to not only enhance biotransformation efficiency, as observed with amoxapine, but also promote formation of a greater diversity of metabolites, as was observed with other compounds (data not shown). Feeding substrates intermittently also seems to favor fungal biotransformation. In conclusion, the two approaches investigated in this study have proven to be useful tools in manipulating fungi for their successful application in biotransformation of interesting compounds.

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