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# Two rapid assays for screening of patulin biodegradation

S. A. I. Wright · D. V. de Felice · G. Ianiri ·
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**Abstract** The mycotoxin patulin is produced by the blue mould pathogen *Penicillium expansum* in rotting apples during postharvest storage. Patulin is toxic to a wide range of organisms, including humans, animals, fungi and bacteria. Wash water from apple packing and processing houses often harbours patulin and fungal spores, which can contaminate the environment. Ubiquitous epiphytic yeasts, such as *Rhodosporidium kratochvilovae* strain LS11 which is a biocontrol agent of *P. expansum* in apples, have the capacity to resist the toxicity of patulin and to biodegrade it. Two non-toxic products are formed. One is desoxypatulinic acid. The aim of the work was to develop rapid, high-throughput bioassays for monitoring patulin degradation in multiple samples. *Escherichia coli* was highly sensitive to patulin, but insensitive to desoxypatulinic acid. This was utilized to develop a detection test for patulin, replacing time-consuming thin layer chromatography or high-performance liquid chromatography. Two assays for patulin degradation were developed, one in liquid medium and the other in semi-solid medium. Both assays allow the contemporary screening of a large number of samples. The liquid medium assay utilizes 96-well microtiter plates and was optimized for using a minimum of patulin. The semi-solid medium assay has the added advantage of slowing down the biodegradation, which allows the study and isolation of transient degradation products. The two assays are complementary and have several areas of utilization, from screening a bank of microorganisms for biodegradation ability to the study of biodegradation pathways.

**Keywords** Apple · Desoxypatulinic acid · Mycotoxin

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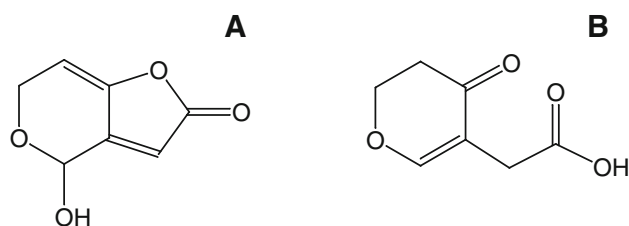e-mail: sandra.wright@unimol.it; saawrt@hig.se

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## Introduction

*Penicillium expansum* is the causal agent of blue mould in stored pome fruits, responsible for the accumulation of the mycotoxin patulin in processed products deriving from mouldy fruit (McKinley and Carlton 1991). Patulin, 4-hydroxy-*4H*-furo[3,2c]pyran-2(*6H*)-one (Fig. 1a), is a lactone that is toxic to humans and animals at very low doses. It causes the suppression of the immune response and targets numerous organs in the human body (Moake et al. 2005). A threshold level of 10 µg patulin/kg apple juice and other apple-based products for young children has been established by the EU [Commission Regulation (EC) No. 1881/2006; Commission Regulation (EC) No. 455/2004]. Acute symptoms include nausea and intestinal haemorrhage. Chronic symptoms that have been reported include genotoxicity, neurotoxicity, immunotoxicity, teratogenicity and carcinogenicity (reviewed by Moake et al. 2005). In many countries, the apples used for processed foods are usually second grade and can be infected by

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1388

Int. J. Environ. Sci. Technol. (2014) 11:1387–1398

Fig. 1 The structures of patulin (a) and desoxypatulinic acid (b)

postharvest pathogens, such as *P. expansum,* which is a common soil inhabitant. High-pressure water washes of apples after harvest are one of the most efficient ways of ensuring lower patulin content of the processed product (Acar et al. 1998; reviewed by Moake et al. 2005). Wash water from apple packing and processing houses can contain as much as $10^4$ conidia mL$^{-1}$ of *P. expansum* and often inadvertently end up in the ground near such facilities. The presence of fungi in drinking water is widespread (Sammon et al. 2010; Pereira et al. 2009; Hageskal et al. 2006). *Penicillium* species appear to be frequent inhabitants of tap water based on surveys carried out in several countries (reviewed by Siqueira et al. 2011); thus, they readily survive water treatment practices (Hageskal et al. 2006). The original source of these fungi is most frequently surface water (Hageskal et al. 2007; Pereira et al. 2009), which contains large amounts of organic material. Since patulin-producing *Penicillium* species also have been isolated from the marine environment (Vansteelandt et al. 2012), it is not clear whether the water from apple packing houses is directly responsible for the contamination of drinking water. Airborne spores may play an important role in contamination of water reservoirs (Sammon et al. 2011). Many different species of *Penicillium* are known to produce patulin. *P. expansum* is the most well-known species, due to its association with mouldy fruits. However, patulin production appears to be widespread in fungi. It appears to be synthesized even by species that are not phylogenetically close to *Penicillium* spp. (Steiman et al. 1989). *P. expansum* and *P. brevicompactum* accounted for almost half of all the filamentous fungi isolated from tap water in Portugal over the period of over a year (Gonçalves et al. 2006), suggesting the potential for patulin production in tap water. The presence of mycotoxins in surface water and drinking water has rarely been investigated. Several mycotoxins have been detected in the environment, that is, soil and water systems, and in drinking water (Aflatoxins B2 and G2, zearalenone, trichothecenes, fumonisins and ochratoxin A) (Gromadzka et al. 2009; Hoerger et al. 2009), which could potentially represent another way in which mycotoxins could threaten human health. There are, to our knowledge, no records of patulin contamination of drinking water. However, since *Penicillium* species are abundant in many studies of fungal composition of

drinking water, it is quite possible that patulin contamination has gone unnoticed. Patulin is toxic not only to humans and animals but also highly toxic to different microorganisms, including yeasts and bacteria (McKinley and Carlton 1991). Although *P. expansum* is the fungal species most commonly associated with the production of patulin (Castoria and Logrieco 2008), there are over 30 other different mould species, mainly of the genera *Penicillium* and *Aspergillus*, and two species of *Byssochlamys* that are known to produce patulin (Moake et al. 2005).

Since the days of patulin discovery and until the late 1970s, there were extensive studies on the modes of action and spectra of activity of patulin and other mycotoxins. Due to its large spectrum of antimicrobial activity, patulin was initially considered to be used as an antibiotic in humans, but was later found to be too toxic. In the 1960s, there was surge in interest for mycotoxins after the discovery of mycotoxicoses caused by aflatoxins (Ciegler 1978), and shortly afterwards, bioassays were developed and utilized to trace mycotoxin contamination in foods and feed. The bioassays were based on the sensitivity profiles of specific microorganisms to specific mycotoxins. In this connection, patulin was tested for the inhibition of a large number of microorganisms (Ciegler et al. 1971; Yates 1986). Filamentous fungi, such as species of *Alternaria, Aspergillus, Botrytis, Fusarium, Gloeosporium, Penicillium, Rhizoctonia, Sclerotinia* and *Verticillium*, could tolerate up to 100–300 μg/mL, whereas the Oomycetes, such as species of *Pythium* and *Phytophthora*, and Zygomycetes, such as species of *Rhizopus,* were more sensitive. Generally, they were inhibited by 3–12 μg/mL. Similarly, bacteria (*Bacillus stearothermophilus*, *Escherichia coli*, *Pseudomonas pyocyanea* and other species) were particularly sensitive to patulin (Yates 1986). *E. coli* is highly sensitive to patulin, to quantities as small as 6–10 μg/mL. Reiss (1975) developed a disc bioassay to monitor the presence of patulin, based on the sensitivity of spore germination of *Bacillus subtilis*. Different microorganisms were considered particularly suited for bioassays of specific mycotoxins. *Bacillus megaterium* was particularly suited for the detection of aflatoxins, *Bacillus cereus mycoides* was suited for the detection of ochratoxin A and B, and *Xanthomonas campestris* was suited for the detection of *Fusarium* toxin butenolide (reviewed by Reiss 1975).

Biotransformation of the biologically and economically most important mycotoxins by microorganisms has been well-documented and reviewed (Boudergue et al. 2009; Halász et al. 2009). In some cases, biotransformation leads to less toxic or non-toxic products and is considered to be a detoxification (He et al. 2010; Karlovsky 1999; Wu et al. 2009). The yeast *R. kratochvilovae* (formerly *Rhodotorula glutinis*) strain LS11 is an excellent biocontrol agent of *P. expansum* (Castoria et al. 2003, 2005; Lima et al. 2003,

2011). This strain is also able to grow in the presence of high concentrations of patulin and is very effective in biodegrading it in Lilly–Barnett medium in vitro and in a model system mimicking decaying apple tissue (Castoria et al. 2005). Over time, patulin disappears and two degradation products with $R_f$ 0.46 and $R_f$ 0.38, respectively, as detected by TLC, appear. The degradation product with $R_f$ 0.46 was purified by HPLC and identified by NMR as being desoxypatulinic acid (DPA) (Castoria et al. 2011) (Fig. 1b), a compound that is non-toxic to several different microorganisms that are sensitive to patulin (Scott et al. 1972). It is also non-toxic to human lymphocytes (Castoria et al. 2011). The methods currently used for monitoring patulin biodegradation involve the growth of the yeast starter culture, the subsequent adjustment of OD and a time-consuming biodegradation assay (10 days) in non-shaking, liquid culture (Castoria et al. 2005).

The aim of the present work was to develop more rapid methods for patulin biodegradation that use a minimum amount of patulin (experiment 2 and 3). These methods could be suitable for the simultaneous screening of a large number of patulin-degrading microorganisms. A combination of a small volume of medium and shaking was utilized to render the liquid culture bioassay more efficient (experiment 2). In addition, the possibility of executing the assay on a solid support was investigated for the first time (experiment 3). The validity of the biodegradation pathway on solid support was confirmed by quantitative HPLC of patulin and its biodegradation products (experiment 4). The high sensitivity of *E. coli* to patulin was utilized to develop a patulin detection system suitable for high-throughput screening. The experiments were carried out in the Dipartimento S.A.V.A. at the University of Molise, Italy, from mid-2007 until March 2010.

## Materials and methods

### Yeast and bacterial strains and growth conditions

The microorganisms used in this work were as follows: *Rhodosporidium kratochvilovae* strain LS11, a basidiomycetous yeast, isolated from olive fruits in Larino, Italy, by Dr. F. De Curtis (De Curtis 1998), and available from the culture collection of the Dipartimento di A.A.A., Università del Molise; and *E. coli* strain DH5, which was kindly provided by Dr. Leif Lundh at the University of Gothenburg, Sweden. Cells were maintained in 20 % glycerol at −80 °C. *E. coli* was grown at 37 °C in Luria–Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L). *R. kratochvilovae* strain LS11 was routinely grown at 23 °C in yeast peptone dextrose (YPD) (yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L) (Oxoid

Ltd, Basingstoke, Hampshire, UK) or in Lilly–Barnett (LiBa) medium (D-glucose 10.0 g, L-asparagine 2.0 g, $KH_2PO_4$ 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 mg, $ZnSO_4 \cdot 7H_2O$ 8.7 mg, $MnSO_4 \cdot H_2O$ 3.0 mg, biotin 0.1 mg, thiamine 0.1 mg) (Lilly and Barnett 1951). Lilly–Barnett medium was prepared by first dissolving glucose, $KH_2PO_4$ and $MgSO_4 \cdot 7H_2O$ in distilled $H_2O$, autoclaving the mixture at 121 °C for 20 min and allowing it to cool. A filter-sterilized (0.22 μm filters) asparagine solution (2 g dissolved in 100 mL of distilled water) was then added. Finally, 5 mL of a (200×) stock solution of $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, biotin and thiamine was added, and the volume was brought to 1 L.

### Patulin and desoxypatulinic acid

Commercial standards of patulin were purchased from Sigma-Aldrich (Milan, Italy) and from A.G. Scientific, Inc. (San Diego, CA, USA). Patulin stock solutions of 4 and 5 mg/mL (dissolved in ethyl acetate) were stored at 20 °C. For experiments, the appropriate aliquot of patulin was withdrawn and the ethyl acetate evaporated under nitrogen stream. The dry patulin was subsequently diluted in water (which had been acidified to pH = 4.0 through the addition of glacial acetic acid) to obtain working solutions at the desired concentrations, and filter-sterilized (0.2 μm), immediately prior to use. The stability of patulin increases with decreasing pH. Its pKa was estimated to be 11.686 (by calculation; the European Bioinformatics Institute website). The log $K_{ow}$ and water solubility at 25 °C were estimated to be −2.40 and $1 \times 10^6$ mg/L, respectively, using the US EPA Estimation Program Interface (EPI) Suite. Ver.4.0. Jan, 2009. The DPA standard was kindly furnished by Prof. Rosa Durán-Patrón, University of Cádiz, Spain, who purified this major product of patulin biodegradation by strain LS11, as previously described (Castoria et al. 2011). The log $K_{ow}$ and water solubility at 25 °C of DPA were estimated to be −0.75 and $7.681 \times 10^5$ mg/L, respectively, using EPI Suite. Ver.4.0. Jan, 2009.

### Patulin sensitivity assays based on growth inhibition of *E. coli*

The method described was set up to develop an alternative assay to detect the presence of patulin, an assay, which could be more efficient and less time-consuming than thin layer chromatography. A culture of *E. coli* strain DH5 grown in LB was allowed to reach $OD_{600} = 0.2$ (corresponding to $2.0 \times 10^7$ CFU/mL). Cells were centrifuged, resuspended in an equal volume of PBS[1], pH 7.4, and diluted tenfold in PBS. The soft agar LB mixture seeded

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[1] PBS, Phosphate buffered saline.

1390

Int. J. Environ. Sci. Technol. (2014) 11:1387–1398

with cells of *E. coli* consisted of melted LB soft agar (0.7 % w/v agar), cooled to 50 °C, and an aliquot of the suspension of bacterial indicator cells. Alternatively, the cell suspension was first mixed with a 2 % (w/v) solution of 2,3,5-triphenyltetrazolium chloride, syn. tetrazolium red (Sigma-Aldrich), in a ratio of 22:1 of cell suspension to tetrazolium red (2 %), before adding it to the melted and cooled LB soft (0.7 % w/v) agar. The volume of cell suspension added constituted one-tenth of the final volume. The soft agar components were mixed thoroughly and poured into Nunc<sup>TM</sup> plates of size 24.5 × 24.5 cm (M-Medical S.r.l., Milan, Italy) or into 6-well microtiter plates of 16 mL well volume (Barloworld Scientific, Milan, Italy). For the Nunc<sup>TM</sup> plate assay, 15 mL of cell suspension was added to 135 mL of the LB soft agar. For the 6-well microtiter plate assay, an equivalent of 0.25 mL cell suspension and 2.25 mL soft agar were mixed and poured in each well. The soft agar was allowed to solidify before 40 μL of culture supernatant of *R. kratochvilovae* LS11 that had grown in the presence of patulin, or aliquots of patulin standard were placed on the soft agar lawn. Alternatively, paper discs containing solidified soft (0.7 % w/v agar) LiBa medium supplemented with patulin were placed on the lawn (see section on semi-solid assay). Patulin diffused from the filter into the LB soft agar and could inhibit bacterial growth near the filter. The plates were subsequently incubated at 37 °C overnight, and the following day, zones of inhibition were visible. This method was a modification of the antibiotic assay described in Wright et al. (2001), in which the growth of *E. coli* was used to monitor the presence of pantocin A and B produced by a strain of *Pantoea agglomerans*. The diameters of inhibition zones were measured, and the areas of the zones calculated. Inhibition area was correlated with μg of patulin applied for three different concentrations of patulin. A correlation analysis was performed using the cor. test function in the R package for statistical computing (R Core Team, Vienna, Austria).

During the development of the *E. coli* bioassay, modifications of drop volumes of liquid and semi-solid LiBa medium, filter sizes and patulin concentration were tested (Table 1, part A), in order to obtain conditions that utilized a minimum of patulin whilst producing a clear inhibition zone in the *E. coli* lawn.

Thin layer chromatography (TLC) analyses

Qualitative analyses by TLC were performed as previously described (Castoria et al. 2005). The correspondence of the TLC method to detect patulin and its degradation products was compared to detection by the sensitivity of *E. coli* to patulin, as described in the previous section. Forty microlitres of the culture filtrates of LS11 grown in the presence

of patulin were loaded onto a silica gel 60 F$_{254}$ TLC plate with fluorescence indicator (Merck, Darmstadt Germany). Chromatography was performed at room temperature in glass tanks, by using toluene/ethyl acetate/formic acid 5:4:1 (vol/vol/vol) as the solvent system. Patulin was visualized by observing the corresponding spot under UV light ($\lambda = 254$ nm), when plates were used for the bioassays (see below). For analytical purpose only, plates were sprayed with a 0.5 % (w/v) solution of 3-methyl-2-benzothiazolinone hydrazone (MBTH) (Sigma-Aldrich Co., St. Louis, MO, USA) after development and heated at 120 °C for 15 min.

High-performance liquid chromatography (HPLC) analyses

HPLC analyses were performed as previously described (MacDonald et al. 2000) with slight modifications. The HPLC system was a Kontron (325 apparatus; Kontron, Milan, Italy). It was equipped with a 20 μL-loop, an autosampler (HPLC 360 Autosampler), a binary pump (325 System) and variable wavelength detector (HPLC 335 Detector) set at 276 nm. An analytical column Agilent Zorbax® Eclipse XDB-C18 250 × 4.6 mm 5 μm was used. The mobile phase was a mixture of water (acidified with 1 % acetic acid glacial)/methanol (95:5 v/v) with a flow rate of 1 mL/min.

Serial dilutions of standards of patulin and DPA in acidified water were injected, and peak areas were determined to generate standard curves for quantitative analyses.

Four experiments to optimize patulin biodegradation protocols for different laboratory ends

Four experiments were set up in order to obtain assays that could be useful in different situations, for example, for screening lots of library members or mutants for patulin biodegradation, or for use in gaining further insight into the pathway(s) of patulin biodegradation. The more assays that are available, the more flexible can the laboratory work be. Since the literature mentions that *E. coli* is sensitive to patulin but not to desoxypatulinic acid (Scott et al. 1972), experiment 1 was carried out to test this observation experimentally in our hands, and to see if it was possible to see the position of spots representing patulin and its biodegradation products on TLC whilst detecting their inhibitory action on *E. coli*. Experiments 2 and 3 aimed to develop and optimize alternative methods for patulin biodegradation (in liquid and semi-solid media, respectively). The sole aim of experiment 4 was to confirm that desoxypatulinic acid was being formed in the new assay described in experiment 3 at the same ratio as that observed in liquid culture (experiment 2).

Int. J. Environ. Sci. Technol. (2014) 11:1387–1398

1391

**Table 1** Conditions modified and tested during assay development

*A. Development of an* E. coli-*based assay for sensing the presence of patulin*

| | |
|---|---|
| 1. Patulin supplied as liquid drop | |
| Drop volumes (μL) | 5, 10, 20, 40, 50 |
| Concentrations of patulin (μg/mL) | 50, 100, 200, 250, 500 |
| 2. Patulin supplied via solid (filter) support | |
| Filter sizes (mm diameter) | 3, 6, 11 |
| Volumes (μL) of drops[a] placed on filters. | 1, 2, 3, 4, 5, 7.5, 10, 15 |

*B. Development of a patulin biodegradation assay in microtiter-well liquid culture (experiment 2)*

| | |
|---|---|
| Assay volume of LiBa[b]/well (μL) | 50, 100, 200 |
| Concentration of patulin (μg/mL) | 100, 200 |
| Patulin biodegradation monitored; days after start of culture | 0, 2, 3, 4, 6 |

*C. Development of a patulin biodegradation assay on solid support (experiment 3)*

| | |
|---|---|
| Percentage (w/v) agar in LiBa medium | 0.7, 2 |
| Position of yeast cells | On LiBa medium supplemented with patulin. Cells removed after 24–48 h by cotton swab and plates inverted over CHCl₃. |
| | In LiBa medium supplemented with patulin. |
| | In LiBa medium supplemented with patulin, placed as a drop on a filter |
| Filter disc size (mm) | 3, 6, 11 |
| Volumes (μL) of patulin (2 mg/mL) placed on filters | 1, 2, 2.5, 3, 5, 7.5 |
| Assay time; incubation of filters in moist chambers (days) | 0, 1, 2, 3, 4 |

[a] Drops consisted of semi-solid (0.7 w/v agar) Lilly–Barnett (LiBa) medium, supplemented with 2 mg/mL of patulin

[b] LiBa, Lilly–Barnett medium (Lilly and Barnett 1951)

## Experiment 1: TLC plate overlaid with Luria–Bertani soft agar seeded with *E. coli*

This experiment was carried out in order to test whether it was possible to simultaneously detect the presence of patulin biodegradation products and to test their toxicity to *E. coli*. Cells of *R. kratochvilovae* LS11 were grown in LiBa liquid medium in the presence of four different concentrations of patulin (50, 100, 250 and 500 μg/mL) on an orbital shaker at 160 rpm over 3 days. After each day of incubation, aliquots were filter-sterilized by means of 3-mm-diameter cellulose acetate filters (0.2 μm) (Iwaki Italia s.r.l., Paderno Dugnano, Milan, Italy), and 40 μL filtrates were analysed by TLC as

described above. The TLC plates that were destined for the overlay study were examined under the UV light after development. The overlay method of TLC plates was performed as previously described (Castoria et al. 2001), with slight modifications: on the final day of incubation, when no more patulin was expected to be detected in the cultures, a TLC analysis was carried out. The TLC plate was dried completely to evaporate all solvent residues, placed in an empty Nunc™ plate (24.5 × 24.5 cm) and covered with an LB soft (0.7 %) agar overlay seeded with *E. coli*, which had been prepared with the volumes described above for the Nunc plate assay. After solidification, the plate was incubated overnight at 37 °C. Bacterial growth rendered the agar overlay red, due to the pigmentation of bacterial cell walls by tetrazolium red. The location of antibacterial compound(s) (i.e. patulin) on the TLC plate was indicated by the presence of a cleared area in the red *E. coli* lawn.

## Experiment 2: Optimization of patulin degradation in liquid culture

A microtiter plate assay was described in Castoria et al. (2005). The pre-inoculum was prepared by inoculating cells of *R. kratochvilovae* LS11 in 50 mL of LiBa medium in flasks, which were incubated on a shaker at 23 °C for 24–36 h. Subsequently, the cultures were centrifuged for 20 min at 4,000 rpm, the cells were resuspended in 200 μL of LiBa, and the concentration of the pre-inoculum adjusted to $1.0 \times 10^5$ CFU/mL, corresponding to values of 0.01 optical density (OD) at 595 nm. Two hundred microlitres of these cell suspensions were supplemented with 100–500 μg/mL of patulin and distributed over the 96 wells of sterile microtiter plates. The plates were then incubated for 10 days at 23 °C under non-shaking conditions.

In the present work, we tested shaking the microtiter plate cultures (200 rpm) in an attempt to speed up the bioconversion of patulin. The progress of biodegradation was monitored on a daily basis for 6 days in order to establish the first time point when no patulin remained.

The preparation of pre-inoculum was done essentially as described above, according to Castoria et al. (2005). The resuspended yeast cells in fresh LiBa were then distributed over the wells of the microtiter plate. Finally, patulin was added separately to the wells. Several factors were evaluated for assay optimization. The first was the assay volume of LiBa in each well (Table 1, part B). The second factor to optimize was patulin concentration of the growth medium (Table 1, part B). The goal was to obtain the lowest concentration possible that allowed for the detection of patulin and its degradation products. The plates were placed on a rotary shaker and growth was monitored on a daily basis over 6 days by determining the OD at 595 nm in a Microplate Reader (Bio-Rad Laboratories, Hercules, CA,

1392

Int. J. Environ. Sci. Technol. (2014) 11:1387–1398

USA). At specific time points (Table 1, part B), the contents of two replicate wells per treatment were harvested, centrifuged at 9,000 rpm for 5 min and filter-sterilized with 3-mm-diameter cellulose acetate filters (Iwaki Italia s.r.l.). In order to cut down the number of steps, we tested to omit the filtering step after centrifugation. Forty microlitre aliquots of cultures from each patulin concentration were placed on *E. coli* soft agar lawns and on TLC plates in parallel, to compare the two methods of detection.

Experiment 3: Development of a patulin biodegradation assay in semi-solid culture conditions

A patulin degradation assay in semi-solid growth medium was elaborated as an alternative method for a high-throughput, quick screening of patulin-degrading microorganisms. Before arriving at the final assay, several parameters and conditions were tested and adjusted (Table 1, part C).

Different filter materials were tested. Round filter or membrane discs of different sizes (Table 1, part C) were excised and distributed in empty, square Petri dishes (10 × 10 cm). Different volumes of patulin at a concentration of 2,000 μg/mL in acidified water were placed onto the filters (Table 1, part C). Aliquots of LiBa soft (0.7 % w/v) agar were placed onto the patulin-containing filters with a Distriman pipette (Gilson, Middleton, WI, USA). Many different volumes of semi-solid LiBa were tested for the three different filter disc sizes. Cells of LS11 were transferred from 24- to 48-h-old plate cultures by means of sterile toothpicks onto the solidified LiBa semi-solid media on the filters. The square Petri dishes were sealed with Parafilm® and placed in double plastic bags, with a moist paper towel in the outermost bag to avoid desiccation of the filters, and incubated at 23 °C. Patulin degradation in the filters with semi-solid medium was monitored daily. Every day, a fresh layer of LB soft agar seeded with *E. coli* and supplemented with tetrazolium red (Sigma-Aldrich) was prepared and poured into a large Nunc™ plate (24.5 × 24.5 cm). Filters were transferred onto the soft agar lawn and the plates were incubated at 37 °C for 24 h. In parallel, tests were carried out to establish the approximate quantities and concentrations of patulin to be used in filters in order to inhibit the growth of *E. coli* and to obtain small but clear inhibition zones.

Experiment 4: Patulin biodegradation products in semi-solid medium on a filter support

The assay was carried out in large scale to extract and purify sufficient amounts of biodegradation product(s), in order to test by HPLC whether the semi-solid conditions permitted LS11 to carry out biodegradation of patulin in a manner similar to that already described for liquid culture (Castoria et al. 2011). For the biodegradation in large-scale liquid culture, strain LS11 was grown in 200 mL of LiBa supplemented with 150 μg/mL of patulin in an Erlenmeyer flask of 500 mL. The pre-inoculum had been prepared in the manner described previously. After 4 days of incubation on a shaker at 150 rpm, at 23 °C, the suspension was centrifuged, and the spent medium was first acidified to pH 2, extracted twice with ethyl acetate, and the solvent completely evaporated. The purified DPA was quantified, and the ratio of initial patulin to resulting DPA could be determined. In the present assay in semi-solid conditions, each of the three square Petri dishes (10 × 10 cm) (each considered as a replicate within each experiment) were filled with 36 cellulose (6 mm Ø) filters, which were aligned inside the dishes. An aliquot of patulin stock solution (4 mg/mL) was dried by nitrogen stream, re-dissolved in high purity water (pH 4.0), and 2.5 μL corresponding to 10 μg of patulin was placed on each filter. Once dried, 30 μL of LiBa soft agar was placed on each filter and allowed to solidify. Finally, cells of LS11 were scraped from an overnight culture grown on YPD agar and added by means of sterile toothpicks. Plates were incubated at 23 °C in sealed plastic bags. Patulin degradation was monitored on a daily basis by removing 1–2 filters and analysing them by TLC and HPLC. After 3 days, patulin had been completely converted into biodegradation products, and all remaining filters were harvested. For patulin and DPA extraction, the filters were placed in a glass tube, and 1 mL of glacial acetic acid and 15 mL of ethyl acetate were added. The glass tube was shaken by vortexing at the highest settings for 1 min. The two phases, the organic and aqueous ones, were allowed to separate, and the upper phase was transferred to a clean glass tube. The extraction procedure was performed twice. The extracts were dried by rotary evaporation and re-dissolved in 1 mL of ethyl acetate. TLC analyses were performed as described previously, by assaying aliquots of 20 μL on the chromatography plates. Aliquots of 100 μL of the extracts were dried by nitrogen stream and re-dissolved in high purity water (pH 4.0) before HPLC analyses. The experiment was performed three times.

## Results and discussion

### Sensitivity of *E. coli* to patulin, but not to its biodegradation products

In experiment 1, patulin was toxic to *E. coli* at each concentration present in culture supernatants of LS11, that is, 50, 100, 250 and 500 μg/mL. The 40 μL aliquots that were placed in each lane of the TLC plate represent the effective amounts of 2, 4, 10 and 20 μg of patulin. In Fig. 2b, only the inhibition zone resulting from the smallest dose of added patulin (2 μg) is presented, since higher quantities resulted in enormous zones of inhibition, which practically covered the TLC plate
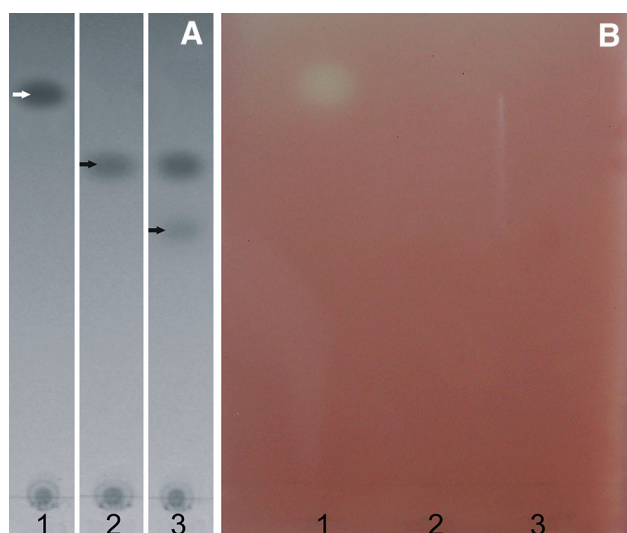
**Fig. 2** TLC analysis of patulin (*white arrow*, $R_f = 0.58$) and its degradation products (*black arrows*, $R_f = 0.46$ and $0.38$) in the absence (**a**) and in the presence (**b**) of *E. coli*



**Fig. 3** TLC detection of patulin (**a**) corresponds to its detection in an inhibition assay of *E. coli* (**b**). The same samples are assayed in lanes 1–3 (**a**) as in wells 1–3 (**b**)

(data not shown). In Fig. 2a, lane 1 has been loaded with 2 µg of patulin in $H_2O$ (pH 4), and lanes 2 and 3 with filtrates of 3-day LiBa cultures of *R. kratochvilovae* strain LS11 grown in the presence of 50 µg/mL of patulin (lane 2) or in the presence of 100 µg/mL of patulin (lane 3). The toxicity of patulin, the spot with $R_f$ 0.58 (white arrow in Fig. 2a; lane 1), was seen as a halo of inhibition in the *E. coli* lawn (Fig. 2b). No inhibition was observed in lanes 2 and 3, where samples had undergone complete biodegradation of patulin and instead two degradation products (black arrows in Fig 2a), with $R_f$ 0.46 (desoxypatulinic acid) and $R_f$ 0.38 (unknown compound) appeared. Forty microlitre aliquots removed from LS11 cultures grown in microtiter plates in LiBa supplemented with 100 µg/mL and 200 µg/mL of patulin were assayed in parallel on TLC plates and on LB soft agar seeded with *E. coli* to examine how the two assays of detection compared. The lower concentration of patulin gave rise to smaller zones of inhibition, and the biodegradation proceeded more rapidly than at the higher concentration (see next section). Figure 3 shows an example of the situation after 6 days of biodegradation: all the patulin in the 200 µg/mL LiBa culture had been biodegraded (lane 3 and well 3; Fig. 3a, b, respectively). Aliquots of the same controls and of LiBa medium were added in parallel to the TLC sheet (Fig. 3a) and to the *E. coli* lawn in the 6-well plate (Fig. 3b): lane 1 and well 1 contain 8 µg of patulin in $H_2O$ (pH 4); lane 2 and well 2 contain 8 µg of patulin in LiBa medium; lane 3 and well 3 contain the culture filtrate of *R. kratochvilovae* strain LS11 grown for 6 days in LiBa medium in the presence of 200 µg/mL of patulin; well 4 contains $H_2O$ (pH 4); well 5 contains LiBa medium, and well 6 is an empty control (only *E. coli*). The presence of a patulin spot ($R_f$ 0.58) on the TLC plate (Fig. 3a) matched perfectly the appearance of an inhibition
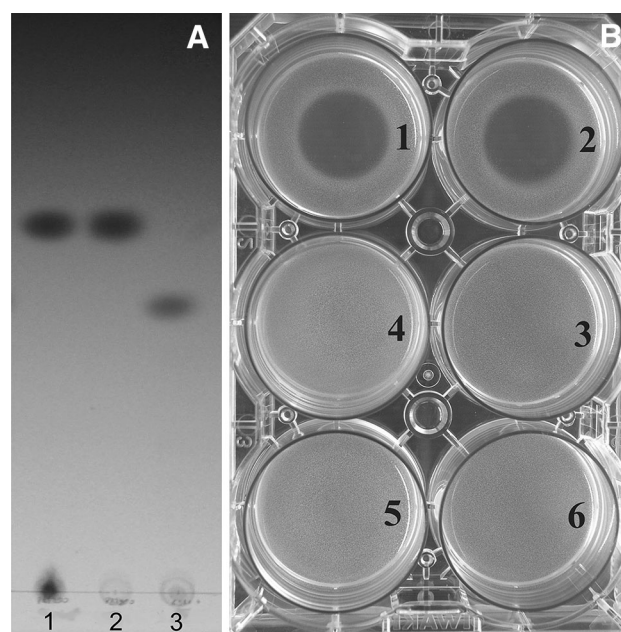
zone in the 6-well plate (Fig. 3b). For all time points and both concentrations of patulin, there was a complete correlation over 108 cases of the disappearance of the inhibition of *E. coli* growth and the complete bioconversion of patulin into DPA as visualized by TLC (data not shown). The Pearson's product moment correlation between patulin concentration and *E. coli* growth inhibition was $r = 0.94$, with an associated *p* value of $<2.2 \times 10^{-16}$. Patulin is a broad-spectrum antibiotic, and *E. coli* is one of the most sensitive microorganisms (Yates 1986). In contrast to patulin, DPA is not inhibitory to several species of *Bacillus*, *Staphylococcus*, *Sarcina lutea*, *Micrococcus clavus* and *Saccharomyces cerevisiae* when applied at a concentration of 46 µg/mL (Scott et al. 1972). The probable reason for the lowered toxicity of DPA as compared to patulin is that the toxic lactone moiety of patulin (Ciegler et al. 1971) is absent in DPA. The broad-spectrum toxicity of patulin is attributed to its propensity to react with sulfhydryl groups in all living cells, whether these are attached to enzymes or to important cellular components, such as cysteine and glutathione (Moake et al. 2005). When patulin reacts with –SH groups, it does not only exert its toxicity, but at the same time, it becomes inactivated by being conjugated and disrupted (Fliege and Metzler 2000). DPA has lost the ability of patulin to react with glutathione and is much less toxic to human lymphocytes than patulin (Castoria et al. 2011). The insensitivity of *E. coli* to desoxypatulinic acid and to the second metabolite (with $R_f$ 0.38) together with its extreme sensitivity to patulin was utilized to develop a fast and reliable assay for patulin detection. The analytical TLC method, previously the only method used for quick detection

1394

Int. J. Environ. Sci. Technol. (2014) 11:1387–1398

of the presence or absence of patulin in biodegradation experiments, can be replaced by the *E. coli* sensitivity assay developed in this study, as a screening tool.

Experiment 2: Optimization of patulin biodegradation in liquid culture

The conclusion of this modified assay is that the assay time for the microtiter 96-well plate was cut down from 10 days (Castoria et al. 2005) to 3 or 6 days, respectively (see below), and that less patulin could be used. The assay worked well with 200 or 100 μL volumes of LiBa medium per well, but in order to economize on patulin, the 100 μL volume was preferred. The minimum concentration of patulin required for consistent detection of the emergence of the two biodegradation products was 100 μg/mL in LiBa (Fig. 2; lane 3). The time-course biodegradation experiment in microtiter plates revealed that the higher concentration of patulin (200 μg/mL) had undergone complete conversion into desoxypatulinic acid by day 6 (Fig. 3). In contrast, 100 μg of patulin per mL was completely converted by day 3 (Fig. 2). The drop volume of supernatant placed onto the soft agar lawn was optimized. Smaller volumes of supernatant (5, 10, 20 μL) gave no or unclear inhibition zones, whereas larger drops (50 μL) resulted in too large zones of inhibition in the *E. coli* soft agar lawn. We found that 40 μL drops were optimal for clear and consistent zones of inhibition (Fig. 3b). Patulin detection by TLC correlated with its detection by growth inhibition of *E. coli* (Fig. 3). Zones of inhibition in the *E. coli* soft agar lawns of the 6-well plates were formed only in wells to which drops of patulin standard (200 μg/mL) or culture filtrates of *R. kratochvilovae* LS11 in which patulin was still present had been added (Fig. 3b).

In summary, the new 96-well microtiter plate assay for rapid screening of biodegradation of patulin is as follows: Grow *R. kratochvilovae* strain LS11 in YPD broth culture overnight or on YPD agar for 2 days at 23 °C. Centrifuge the liquid culture and prepare a $5 \times 10^5$ CFU/mL yeast cell suspension in fresh LiBa medium, supplemented with 100 μg/mL of patulin. Transfer 100 μL aliquots to the wells of the 96-well plate. Alternatively, transfer 24-h-old yeast cell growth from YPD agar plates to the 96-well plate. Each well contains 100 μL of LiBa medium, supplemented with 100 μg/mL of patulin. Use a minimum of six wells per treatment. Incubate the plates on a rotary shaker at 200 rpm for 3 days and monitor the growth every day by reading the $OD_{595}$ in a Microplate Reader (Bio-Rad Laboratories). At the end of each day of incubation, pool the contents of 2 wells and centrifuge it at 9,000 rpm for 5 min. Place a 40 μL drop of supernatant on the *E. coli* soft agar (0.7 % agar) lawn made in a Nunc™ Plate. Incubate the plate for 1 day at 37 °C and observe zones of inhibition.

Validation of the filter/semi-solid agar assay

In experiment 3, many variables were tested and optimized. The result of experiment 3 is the final, optimized assay (see description below), which is rapid and uses a minimum of patulin, in accordance with the initial aim. Solid LiBa medium (2 % agar) had been tested as a support for biodegradation but turned out to be too dense to allow rapid diffusion of patulin for the reaction to proceed. In contrast, 0.7 % agar did allow patulin to become biodegraded by the yeast. Therefore, all subsequent tests utilized LiBa medium with 0.7 % (w/v) agar. First, patulin dissolved in acidified water (pH 4) was added to the filters. The filters were subsequently covered with semi-solid LiBa medium. After biodegradation, any patulin remaining on the filters diffused into the LB soft agar that had been seeded with *E. coli*. The sizes of inhibition haloes produced were larger with increasing diameter of filters and with increasing quantities of patulin used. The optimal volumes of LiBa soft agar for the different filter sizes were determined to be 250 μL for discs of 11 mm in diameter, 30 μL for discs of 6 mm in diameter and 5 μL for discs of 3 mm in diameter. Using filters of 11 mm in diameter, the smallest quantity of patulin that produced an inhibition halo was 5 μL of a stock solution of 2,000 μg/mL, whereas using filters of 3 and 6 mm in diameter, 1 μL of patulin was sufficient to provoke reproducible and clear haloes of 8–9 mm in diameter. In patulin controls, no differences in the sizes of inhibition zones were observed over the 4 days of incubation. *R. kratochvilovae*, strain LS11, required only 24 h to degrade 1 and 2 μL of patulin of a concentration of 2 mg/mL (corresponding to 2 and 4 μg of patulin, respectively), whereas it required an additional 24 h (i.e. a total of 48 h) to degrade 3 μL of the same concentration of patulin.

The final patulin biodegradation bioassay in semi-solid medium is therefore as follows: filter paper discs (Albet®LabScience) of 6 mm in diameter are placed in a square Petri plate (10 × 10 cm), and 1 μL of patulin at a concentration of 2 mg/mL in acidified water (pH 4) and 30 μL of LiBa soft agar are placed on the filter, in that order. LS11 cells are then transferred to the solidified LiBa soft agar with a toothpick or by pipetting a small volume of cells in suspension. The plate is incubated in a moist chamber, created by sealing with Parafilm, placing it in double plastic bags, with damp paper towels in the outermost bag. After 24 h (or 48 h) of incubation at 23 °C, the filters containing yeast cells which have grown in the LiBa medium (see pink growth in centres of filters in Fig. 4a) are individually transferred onto a layer of LB soft agar seeded with *E. coli* and incubated at 37 °C for 24 h. The formation of inhibition haloes in the bacterial growth in the proximity of filters is indicative of the presence of patulin. *E. coli* growth appears red in Fig. 4b, since tetrazolium red was

**Fig. 4** The filter assay for patulin biodegradation. **a** After 24 or 48 h, profuse growth of *R. kratochvilovae* LS11 is visible on filters. **b** Filters still containing patulin are surrounded by haloes of inhibited bacterial growth
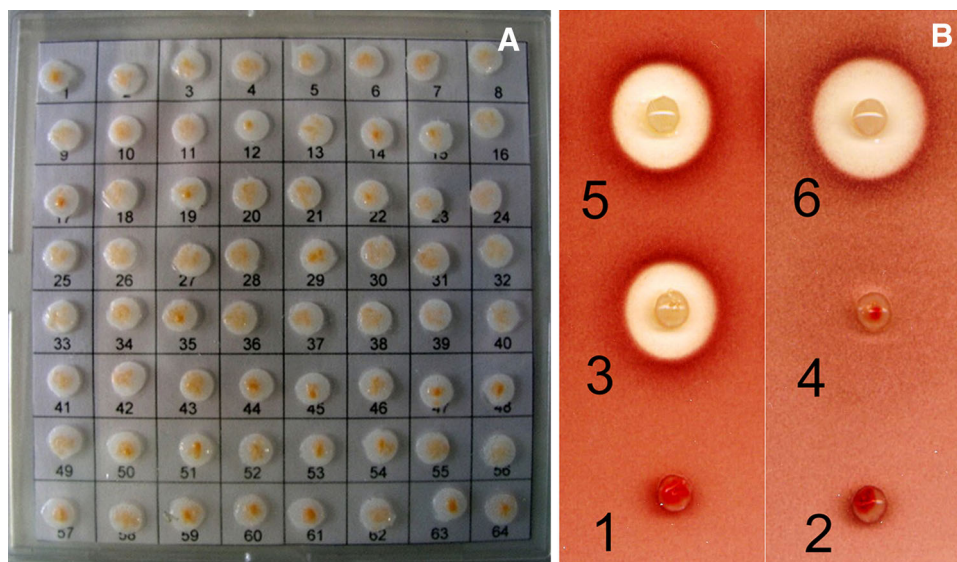


added to the LB soft agar (Fig. 4b). Filters 1 and 2 were negative controls, that is, no patulin was added (24 and 48 h incubation, respectively). To each of the filters, numbered 3, 4, 5 and 6 (Fig. 4b), aliquots of 3 μL of 2 mg/mL (corresponding to 6 μg) of patulin had been added. In filter 3, strain LS11 had grown for only 24 h, so not all the patulin had been biodegraded. In contrast, when LS11 had grown for 48 h, all the patulin in the filter had been biodegraded (filter 4). Filters 5 and 6 were patulin (positive) controls (24 and 48 h) in the absence of the yeast.

The filter extracts from patulin biodegradation in semi-solid medium were also analysed by TLC and HPLC to investigate the formation of biodegradation compounds. Figure 6 reports the quantitative results of HPLC analyses of filter extracts (experiment 4). After 3 days of incubation at 23 °C, LS11 was able to reduce up to 97.6 % of the amount of patulin initially placed on filters (333 μg of patulin/mL LiBa soft agar) (Fig. 5a; lanes 4, 5 and 6), as compared to the non-inoculated control. The other lanes of the TLC plate in Fig. 5a were as follows: 40 μg of patulin standard (lane 1), 10 μg of patulin in LiBa (lane 2) and strain LS11 in LiBa grown in the absence of patulin (lane 3). Figure 5b shows an HPLC chromatogram of the pooled filter extracts of lanes 4, 5 and 6 in Fig. 5a. Peak 1 has the retention time (Rt) of 10.58 min and corresponds to patulin. Peak 2 has the Rt of 11.43 min and corresponds to DPA. Thus, the observed decrease in patulin over the 3 days of incubation corresponded to the formation of DPA. Its identity was confirmed by comparing its retention time with the one of a desoxypatulinic acid standard, as described by Castoria et al. (2011). The quantification of patulin and desoxypatulinic acid (Fig. 6) was calculated from the HPLC results as shown in Fig. 5b. Patulin (grey bars) was converted into DPA (black bar) in a ratio almost identical to that observed in 50 mL LiBa liquid culture biodegradation experiments (Castoria et al. 2011). In liquid culture, 150 μg/

mL of patulin was converted into 87 ± 11.7 μg/mL of DPA. In the present study, the initial 333 μg/mL of patulin present in filters (calculated by dividing the amount of patulin, 10 μg, with the volume of soft agar; 30 μL, which was placed on each 6-mm-diameter filter) was converted into 193 ± 24 μg/mL of DPA (Fig. 6). For both types of assay, an initial amount of 100 mg of patulin is converted over 3 days to approximately 58 mg of DPA. Experiment 4 confirmed that the conversion of patulin to DPA proceeded in a similar way on filters as that already described for liquid culture.

During the course of biodegradation of patulin, two metabolites were formed, but with time only desoxypatulinic acid remained. At higher patulin concentrations, the biodegradation reaction was always slower, regardless of the type of assay used. The filter assay, which was developed in the present study, was possible to perform by using less patulin than in the liquid medium assay. The smallest amount required in order to detect biodegradation products was 2 μg patulin per filter (1 μL of 2,000 μg/mL), whereas the smallest amount required per sample for the microtiter plate assay was 4 μg patulin (40 μL of 100 μg/mL). The notion of employing a filter as a support came about as a consequence of the observation that patulin in suspension diffused so readily in the semi-solid medium that it was impossible to confine it to the specific position of the agar where the suspension had been placed. Small agar slices containing patulin were easier to handle when a membrane or filter was attached to them. The filters did not interfere with the inhibition of *E. coli* growth or with the chemical extraction procedure. As clearly demonstrated in this work, the two assays monitor the same biodegradation pathway; both lead to the conversion of patulin into DPA (Fig. 5) in the same ratios (Fig. 6).

Microbial degradation of mycotoxins has been extensively studied. It can be carried out by a large array of

**Fig. 5 a** TLC analysis and **b** HPLC analysis of extracts of a 3-day culture of strain LS11 on filters in LiBa soft agar supplemented with patulin
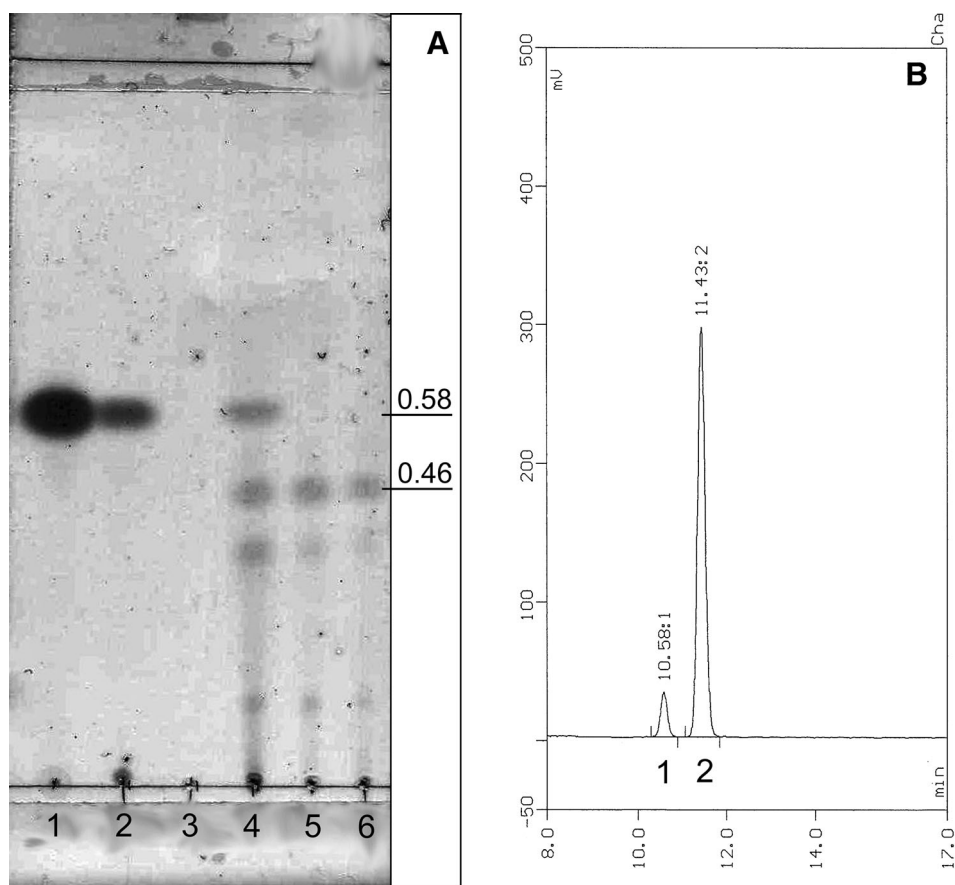


organisms, including yeasts, bacteria and filamentous fungi (Karlovsky 1999). The products of biodegradation of individual mycotoxins differ, depending on the chemical bonds within each mycotoxin that are targeted by the respective biodegradation pathway. For the most part, the products of patulin biodegradation had not been identified (Anderson et al. 1979; Harwig et al. 1973; Stinson et al. 1978; Sumbu et al. 1983) until recently, when ascladiol (Moss and Long 2002; Ricelli et al. 2007) and desoxypatulinic acid (DPA) (Castoria et al. 2011) were characterized. The two compounds probably use alternative pathways, since one process is anaerobic (leading to ascladiol) and the other is aerobic (leading to DPA). They derive from the breakage of bonds that are located in separate ends of the patulin molecule. Ascladiol is the result of the opening of the pyran (six-membered) ring of patulin. It thus keeps the $\alpha$, $\beta$-unsaturated lactone portion of patulin and the chromophore characteristics of patulin (the furan ring and the double-bonds),which explains the similarities in their UV spectra. Instead, DPA is formed through the hydrolysis of the lactone ring, into a $\alpha$, $\beta$-unsaturated carbonyl system and a carboxyl group.

The structure of the second biodegradation product (with $R_f$ 0.38) is still unknown. In liquid culture biodegradation experiments, this compound is fairly rapidly converted further, and at the end, DPA is the only

biodegradation compound present (unpublished data). Its transient nature has precluded large-scale purification and structural elucidation. However, in the filter bioassay, this compound is typically more stably maintained, and further conversion proceeds more slowly. This feature of the filter assay has allowed us to produce, purify and attempt to isolate the second metabolite (work in progress). Thus, besides its utility in the screening of multiple samples, the filter assay, which is described in this work for the first time, holds promise to become a useful tool for the stable production of intermediates of patulin biodegradation. The structural elucidation of intermediates is important for the complete characterization of the biodegradation pathway.

The choice of a liquid or solid support-based system for the production of secondary microbial metabolites, whether they are antibiotics or products of biodegradation, has long been an issue in industrial microbiology, where augmenting the quantities of commercially valuable metabolites can enhance the returns. Examples of antibiotics and mycotoxins, whose production has been greatly improved on a solid support, include aflatoxin, ergot alkaloids, zearalenone, surfactin, cephamycin C, penicillin, cyclosporine A, tetracyclines and iturin, as reviewed by Robinson et al. (2001). Solid-state fermentation has also been used for microbial biodegradation of noxious substances on an

Int. J. Environ. Sci. Technol. (2014) 11:1387–1398

1397
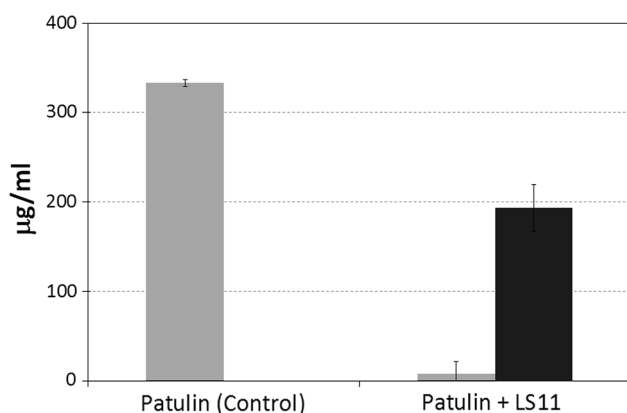


**Fig. 6** Patulin (*grey bars*) is converted into desoxypatulinic acid (DPA; *black bar*) by strain LS11 over 3 days of incubation on filters covered with LiBa

industrial scale. The advantages with these systems and the filter assay presented in this work as compared to liquid culture systems are many. In general, bioconversion on solid matrices does not require energy input for the agitation of cultures. In addition, ample oxygen is readily available and the products are more stably maintained (Pérez-Guerra et al. 2003). The filter assay has promise to be useful in other microbial biodegradation or secondary metabolite production systems, in particular, when stability is an issue or when extraction and purification is required from a solid support, due to the higher metabolite production on a solid medium. It is also inexpensive and does not require sophisticated equipment or facilities.

The presence of mycotoxins in food and foodstuffs represent a constant challenge. *P. expansum* and patulin are mostly associated with processed pome fruit products that derive from mouldy apples. However, as demonstrated by Gonçalves et al. (2006), there are other potential environmental sources of patulin. *P. expansum* in tap water could constitute yet a health hazard to consumers, and this is an area which has received little attention. One source of decontaminating microbial agents is the fruits where the patulin-producing fungi have their habitat. Further studies on the various biodegradation and detoxification pathways are needed in order to understand how ascladiol, DPA and the unknown biodegradation product might be connected. The new assays will provide the necessary tools for the advancement of this area.

## Conclusion

The present study described the development of two assays suitable for high-throughput screening of patulin biodegradation in multiple samples. One was based on filter paper coated with semi-solid (0.7 % w/v) agar Lilly–Barnett medium, an entirely new assay, and the other was a modification of an existing assay, accommodating for smaller volumes of

medium and multiple samples in a microtiter plate. We demonstrated in this work by quantifying the major biodegradation product, desoxypatulinic acid that the same patulin biodegradation pathway was operating in the two assays. The conversion of patulin into biodegradation products, normally monitored through TLC, could equally be monitored in an assay based on the growth of *E. coli*, thus allowing multiple samples to be processed simultaneously. These biodegradation assays will be useful, for example, when screening members of a genomic library or biodegradation-altered mutants of *R. kratochvilovae*. In addition, the availability of the alternative bioassays—in liquid and in solid media—would facilitate the chemical isolation and characterization of biodegradation products, which is the prerequisite for determining the pathway for patulin biodegradation. More thorough knowledge of the biodegradation pathway can also provide specific information for the advancement of novel methods for the detection of patulin in foodstuffs and drinking water, for example, by using bioprobes that derive from the gene that encodes the first enzyme of the biodegradation pathway.

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