Food Control 64 (2016) 142–150

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Biodegradation of patulin by a *Byssochlamys nivea* strain

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A R T I C L E I N F O

Article history:
Received 26 August 2015
Received in revised form
25 November 2015
Accepted 12 December 2015
Available online 18 December 2015

Keywords:
Biodegradation
Patulin
Filamentous fungi
Apple puree
Byssochlamys nivea

Chemical compounds studied in this article:
Patulin (PubChem CID: 4696)
Ethyl acetate (PubChem CID: 8857)
Acetonitrile (PubChem CID: 6342)

A B S T R A C T

This paper describes the discovery and isolation of a new wild-type microorganism, FF1-2 strain, for biodegradation of high concentration of patulin. The filamentous fungus FF1-2, identified to belong to *Byssochlamys nivea* (CCTCC M2013547), was enriched and isolated from the fermentation liquor pit mud. The patulin degradation by the FF1-2 strain was studied under such different conditions as pH, temperature and culture media. The strain grew and degraded patulin at 20 ~ 46 °C and pH 3.0–5.0. The change of pH studied had little effect on the patulin degradation capability in contrast with the significant temperature effects. The optimum temperature for patulin degradation was 37 °C, at which patulin at 500 μg/mL level in the culture media was degraded to a virtually undetectable level in 5 days. 97.1% of patulin was degraded in the apple puree at 30 °C in 9 days. This study for the first time found that filamentous fungi have the ability to degrade patulin. The experimental results manifest the potential of the FF1-2 strain in dealing with patulin contamination in such food industries as fruit processing.

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1. Introduction

Mycotoxin contamination is a severely wide-spread problem in the world, particularly in developing countries (Egmond, Schothorst, & Jonker, 2007; Topcu, Bulat, Wishah, & Boyaci, 2010). Patulin (PAT) is one of the most frequently found mycotoxins in human foods, such as apples and the related products (Janotová, Čížková, Pivoňka, & Voldřich, 2011; Sant'Ana, Rosenthal, & Massaguer, 2008). Many studies in the past five decades have demonstrated that patulin could cause serious health problems for humans due to its toxicity (Moake, Padilla-Zakour, & Worobo, 2005; Puel, Galtier, & Oswald, 2010). The provisional maximum tolerable daily intake for patulin set by the Joint FAO/WHO Expert Committee on Food Additives is 0.4 mg/kg body weight/day (Janotová et al., 2011; JECFA., 1995); and the maximum permitted levels of patulin established by the European Union (2006) are 50 μg/mL in apple juices, 25 μg/mL in purees and 10 μg/mL in young children's foods (Dalié, Deschamps, & Richard-Forget, 2010; Marín et al., 2011; Sanzani, Reverberi, Punelli, Ippolito, & Fanelli, 2012).

Over the past thirty years the interests have been steadily increasing on removal or degradation of patulin through applications of adsorption filters, chemical modification, electromagnetic irradiation, or biological processes. Though the first three approaches have exhibit some practical or potential application, they both suffer from their limitations. For adsorption filters, among its various disadvantages (Fuchs et al., 2008; Gökmen, Artık, Acar, Kahraman, & Poyrazoğlu, 2001; Moake et al., 2005), the most challenging question confronted is the patulin is just removed from the samples instead of the environment. In cases of chemical detoxification, ambiguous degradation mechanism and hazardous concerns hampers its application in food industry (Moake et al., 2005). Biological methods for patulin removal are less expensive, more effective, more metabolic ways for patulin removal. Moreover, many microorganisms can be safely applied to food processing.

The biological methods so far mainly focus on yeast and lactic acid bacteria (LAB) approaches. For the former, in 1978 Stinson et al. successfully removed patulin in alcoholic fermentation with 8 yeast strains. However, the identical treatment didn't seem to apply to quality control of juice (Stinson, Osman, Huhtanen, & Bills, 1978).

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http://dx.doi.org/10.1016/j.foodcont.2015.12.016
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Then in 1983, Sumbu et al. concluded most yeasts exhibit degradation function of patulin only when the concentration of patulin is below 200 μg/mL, over which fermentative detoxification of the yeasts is inhibited (Sumbu, Thomart, & Bechet, 1983). In recent years, *Rhodosporidium kratochvilovae* was found to present patulin degradation ability (Castoria et al., 2011; Zhu et al., 2015) without violating Sumbu's concentration limitation.

As for the latter approach of the afore-mentioned biological methods (LAB appoach), in 2008 Fuchs et al. found, among 30 different LAB strains, *Bifidobacterium animalis* VM 12 could reduce patulin levels by 80% (Fuchs et al., 2008). In 2010 with two *Enterococcus faecium* strains Topcu et al. reduced patulin levels by 41.6% and 45.3%, respectively. For the mechanism of patulin reduction with LAB, it is assumed that LAB could bind toxins to form mycotoxin–bacteria complex (Topcu et al., 2010), which, accordingly, implies there are two drawbacks in the removal of patulin with LAB, one is the approach is only applicable to liquid samples, the other is the “apparent” reduction of mycotoxin is more of being hidden than degraded.

As above described, most biological methods screening valid microbial strains for patulin degradation are somewhat trial-and-error ones, which depends largely on luck and is time-consuming. So, the establishment of more efficient microbial strain screening methods for high concentration patulin degradation in various state samples appears imperative. Fortunately, a careful look at the picture of the screening microbial strain methods for degrading environmental pollutants (Hormisch et al., 2004; Liu, Yu, Wang, Ye, & Cong, 2011; Santos, Monteiro, Braga, & Santoro, 2009; Zheng, Levin, Pinkham, & Shetty, 1999) finds an inspiring practice, which is based on the notion that when hazardous materials are used as the sole carbon source in culture media (SCSCM), the microbial strains that can survive and grow promise to be able to degrade the very carbon source specifically and efficiently. The microbial strains obtained in those practice exhibited tremendous capacity to degrade hazardous materials of high concentrations. To the best of our knowledge, the application under such notion has not extended to screening microbial strains for patulin degradation.

We herein report an approach of screening a new wild-type microbial strain based on the notion of SCSCM, which can degrade high concentration patulin. The optimal degradation conditions were also discussed, and the screened microbial strain reduced patulin to a virtually undetectable level in the culture media and to a very low level in apple puree.

2. Materials and methods

2.1. Chemicals and media

Ethyl acetate, dichloromethane, acetone, and petroleum ether (analytical grade) were purchased from Tianli Chemical Reagent Co., Ltd. (Tianjin, China). Acetonitrile (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, USA). Trifluoroacetic acid was obtained from Shanghai Kefeng Chemical Reagent Co., Ltd. (Shanghai, China). Solutions were prepared with deionized water. All the solutions were filtered through 0.45 μm membranes (Fisher Scientific) prior to HPLC analysis.

2.2. Preparation of patulin solutions

2.2.1. Standard solution of patulin

Patulin (4-hydroxyl-4H-furo (3, 2c)-pyran-2(6H)-one) was purchased from Sigma Aldrich (Sigma–Aldrich, Israel). Solid patulin was dissolved in methanol-deionized water (1:1, v/v) to obtain 100 μg/mL standard patulin solution.

2.2.2. Working solution A

Solid patulin (Sigma–Aldrich, Israel) was dissolved in deionized water (adjusted to pH 4.0 with acetic acid) with a final concentration of 5 mg/mL.

2.2.3. Working solution B

Penicillium expansum strain FP2 (CCTCC NO: M2014040), a strong patulin producer, was provided by College of Food Engineering and Nutritional Science of the Shaanxi Normal University. FP2 strain was incubated into sterilized apple puree (121 °C, 20 min) at 28 °C in dark for 10 days. The patulin extract from the apple puree culture was prepared according to MacDonald's procedure (MacDonald, Long, Gilbert, & Felgueiras, 2000) with slight modifications: the mixture of the fungus and the apple puree was fully ground, followed by three times extraction with ethyl acetate. The extracted solution was cleaned by liquid–liquid extraction with a 1.5% sodium carbonate solution and dried with anhydrous sodium sulfate. The solvent was removed by evaporation in rotary vacuum evaporator at 40 °C. The remaining material was then dissolved in acidified water (pH 4.0 adjusted with acetic acid) and was then lyophilized to obtain a syrupy mixture. The mixture was purified by silica gel column chromatography using dichloromethane-acetone (30:1, v/v) as the eluting solvent. The eluted fractions with an R_f value virtually the same to that of the standard patulin solution were collected and evaporated under reduced pressure, and the resultant product was re-purified by silica gel column chromatography using petroleum ether–ethyl acetate (1:1, v/v) as the eluting solvent. After solvent removal, a white solid material was obtained and its structure was determined by ^{1}H NMR, ^{13}CNMR and ESI-MS. The ^{1}H NMR spectra were recorded using Bruker Avance 300 at 300 MHz in presence of $CDCl_3$: δ 6.07–5.77 (m, 2H), 4.66 (d, $J = 17.2$ Hz, 1H), 4.36 (dd, $J = 17.2$, 3.9 Hz, 1H), 3.23 (s, 1H). Residual solvent peaks were used as internal references for ^{1}H NMR spectra: chloroform (δ 7.26 ppm). ^{13}CNMR spectra were recorded using Bruker Avance 300 at 75 MHz in presence of $CDCl_3$ and the chemical shifts (δ) were reported in parts per million (ppm) relative to the residue solvent chloroform peaks (77.00): δ 168.60, 149.83, 146.26, 111.19, 107.48, 88.87, 59.56. ESI-MS experiments were performed on Bruker Esquire 3000plus (Bremen, Germany) in positive ionization mode. Calcd for $C_7H_6NaO_4(M + Na)^+$: 177.0158, ESI-MS found: 177.0160. According to the analysis of the spectra data, the white solid was identified as patulin. The patulin prepared according to the above mention procedure was dissolved in deionized water (adjusted to pH 4.0 with acetic acid) to obtain 7 mg/mL working solution B.

2.3. Isolation of microbial strains

The samples of fermentation pit mud for liquor production were obtained from Chang' an liquor industry Co., Ltd., in Shaanxi Province, China. These samples were collected from the surface down to 5 cm in depth in March, 2013.

The mineral salt medium (MSM) used for enrichment in the experiment is a modified one with reference to Ikunaga et al. (2011), and the detailed compostion of MSM is provided in Supplementary Information (SI 1).

Each 1.0 g pit mud sample was mixed with 100 mL of sterile physiological saline. The mixture was activated for 12 h at 37 °C and 200 rpm. Then, 5.0 mL of the mixture was added to 75 mL of patulin MSM solution (3.5 mL working solution B was added to 71.5 mL of sterilized MSM with a final concentaration at 300 μg/mL). It was followed by incubation for 5 days at 37 °C and 200 rpm in dark. 5.0 mL of aliquot of the incubation solution was then further cultured under the same procedure with fresh patulin MSM solution, except that the concentration of patulin was increased to

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