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Research Note

Potential of Two *Metschnikowia pulcherrima* (Yeast) Strains for In Vitro Biodegradation of Patulin

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ABSTRACT

Patulin contamination of apple and other fruit-based foods and beverages is an important food safety issue, as consumption of these commodities throughout the world is great. Studies are therefore necessary to reduce patulin levels to acceptable limits or undetectable levels to minimize toxicity. This study was undertaken to investigate the efficacy of two *Metschnikowia pulcherrima* strains (MACH1 and GS9) on biodegradation of patulin under in vitro conditions. These yeast strains were tested for their abilities to degrade patulin in liquid medium amended with 5, 7.5, 10, and 15 μg/ml patulin and a yeast cell concentration of 1×10^8 cells per ml at 25°C. Of the two strains tested, MACH1 completely (100%) reduced patulin levels within 48 h, and GS9 within 72 h, at all concentrations of patulin. MACH1 effectively degraded the patulin within 24 h by 83 to 87.4%, and GS9 by 73 to 75.6% at 48 h, regardless of concentration. Patulin was not detected in yeast cell walls. This indicates that yeast cell walls did not absorb patulin, and that they completely degraded the toxin. Patulin had no influence on yeast cell concentration during growth. Therefore, these yeast strains could potentially be used for the reduction of patulin in naturally contaminated fruit juices. To our knowledge, this is the first report regarding the potential of *M. pulcherrima* strains for patulin biodegradation.

Patulin (4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one) is a toxic secondary metabolite produced by *Penicillium, Aspergillus,* and *Byssochlamys* spp. *(6). Penicillium* spp., mainly *P. expansum,* is a common contaminant of fruits and fruit products. For example, it causes blue mold rot, resulting in the production of patulin *(10).* Apples and apple products are excellent substrates for *P. expansum,* a major producer of patulin *(12, 14).* It is also a common contaminant of apricots, grape, pears, and peaches, and patulin appears during the production of fruit juice concentrates, owing to its solubility in water *(17).* Patulin is classified as a Group 3 carcinogen by the International Agency for Research on Cancer, which means that there is no evidence of it being carcinogenic to humans, and that data in experimental animals on carcinogenesis are sparse *(5).*

Patulin production within fruits and their products has been investigated; it appears dependent on water activity, temperature, pH, and other chemical characteristics intrinsic to fruits *(8).* Patulin production and pH are inversely related, with patulin being unstable at high pH *(9).* In addition, temperature has been shown to affect pathogen growth and, largely, to the production of patulin *(8, 9, 12).* Patulin is stable in an acidic environment and is not destroyed during thermal processing. Therefore, it can exist in juices even after processing *(1).* Patulin contamination of fruit juices has reported worldwide *(12).*

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Several strategies have been reported for the detoxification of patulin. These include physical, chemical, and microbiological approaches *(7, 9).* However, microbiological methods are much better understood as compared with other decontamination methods *(9).* Coelho et al. *(3)* reported a greater than 90% reduction of patulin during yeast fermentation. Stinson et al. *(19)* examined and found that, of eight yeast strains tested, six reduced patulin levels to below detectable levels, while all eight strains resulted in a decrease of 99% or better in total patulin content. In our previous study, we found greater than 80% ochratoxin A degradation by using *Metschnikowia pulcherrima* strains under in vitro conditions *(11).* However, in this study, attempts have been made to evaluate the *M. pulcherrima* strains on biodegradation of patulin.

MATERIALS AND METHODS

Microorganisms and culture conditions. *M. pulcherrima* strains (MACH1 and GS9) previously isolated from apples were obtained from the Agroinnova Culture Collection Center, University of Torino, Torino, Italy, and subcultured in a potato dextrose agar slants (Merck, Darmstadt, Germany) containing 50 mg/liter streptomycin (Merck) and stored at 4°C for further studies. Cell suspensions of the individual strains were prepared by growing the yeast strains in yeast-peptone-dextrose broth and by bringing them to a final concentration of 1×10^8 cells per ml by using a hemocytometer, according to Spadaro et al. *(18).*

Yeast antagonists on biodegradation of patulin. The efficacy of yeast strains (MACH1 and GS9) on the biodegradation

TABLE 1. *Efficacy of yeast strains on in vitro biodegradation of patulin at 25°C*

Treatments	Patulin used (μg/ml)	Patulin concn and % degradation[a]					
		24 h		48 h		72 h	
		Patulin (μg/ml)	Reduction (%)	Patulin (μg/ml)	Reduction (%)	Patulin (μg/ml)	Reduction (%)
MACH1[b]	5.0	0.85 ± 0.12 G	83.0 ± 1.2 A	0.0 ± 0.0 F	100 ± 0.0 A	0.0 ± 0.0 E	100 ± 0.0 A
	7.5	1.09 ± 0.15 G	85.4 ± 1.0 A	0.0 ± 0.0 F	100 ± 0.0 A	0.0 ± 0.0 E	100 ± 0.0 A
	10.0	1.65 ± 0.21 F	83.5 ± 1.6 A	0.0 ± 0.0 F	100 ± 0.0 A	0.0 ± 0.0 E	100 ± 0.0 A
	15.0	1.89 ± 0.35 E	87.4 ± 1.3 B	0.0 ± 0.0 F	100 ± 0.0 A	0.0 ± 0.0 E	100 ± 0.0 A
GS9[b]	5.0	2.13 ± 0.14 E	57.4 ± 0.9 C	1.35 ± 0.12 E	73.0 ± 1.2 B	0.0 ± 0.0 E	100 ± 0.0 A
	7.5	2.95 ± 0.12 E	60.6 ± 1.2 D	1.98 ± 0.42 D	73.6 ± 2.3 B	0.0 ± 0.0 E	100 ± 0.0 A
	10.0	3.96 ± 0.25 D	60.4 ± 1.4 D	2.62 ± 0.25 D	73.8 ± 1.6 B	0.0 ± 0.0 E	100 ± 0.0 A
	15.0	6.21 ± 0.62 C	58.6 ± 0.8 D	3.65 ± 0.38 C	75.6 ± 2.1 B	0.0 ± 0.0 E	100 ± 0.0 A
Control	5.0	4.68 ± 0.41 D	6.4 ± 0.2 E	4.65 ± 0.62 C	7.0 ± 0.3 C	4.58 ± 0.31 D	8.4 ± 0.4 B
	7.5	7.12 ± 0.25 C	5.0 ± 0.1 E	7.05 ± 0.26 B	6.0 ± 0.2 C	6.98 ± 0.56 C	6.9 ± 0.3 C
	10.0	9.15 ± 0.36 B	8.5 ± 0.4 F	9.23 ± 0.16 B	7.7 ± 0.4 C	9.25 ± 0.25 B	7.5 ± 0.5 B
	15.0	14.32 ± 0.54 A	4.5 ± 0.3 E	14.25 ± 0.23 A	5.0 ± 0.2 D	13.99 ± 0.4 A	6.7 ± 0.2 C

[a] Values are averages of five replications. Values (±standard deviations) in the same column followed by the same letter are not statistically different by Duncan's multiple range test at $P < 0.05$.
[b] MACH1 and GS9 are different strains of *M. pulcherrima*.

of patulin in liquid media was assessed. Twenty-milliliter aliquots of peptone malt extract (PM) broth (0.5% yeast extract, 1% sucrose, 0.5% peptone, and 0.2% malt extract [pH 4.0]) in 100-ml conical flasks containing 5.0, 7.5, 10.0, and 15.0 μg/ml patulin (dissolved in 10 mg/ml water and adjusted to pH 4.0) (Sigma, St. Louis, MO) were inoculated with 0.1 ml of cell suspension of each yeast strain containing 1×10^8 cells per ml and then incubated at 25°C for 96 h in the light under shaking conditions at 200 rpm (Innova 40, New Brunswick, NJ). The following treatments were included: (i) control with only patulin, (ii) patulin plus MACH1, and (iii) patulin plus GS9. The cultures were harvested at different time intervals of 24, 48, 72, and 96 h and centrifuged at 5,000 rpm for 10 min at 4°C to obtain cell-free culture filtrate for extraction of patulin. The patulin was then extracted from the culture filtrates and prepared for high-performance liquid chromatography (HPLC) analysis. The cell pellet was used for further studies.

Extraction and determination of patulin by HPLC analysis. The patulin was extracted from each culture filtrate (15 ml) three times with ethyl acetate (30 ml each time; Sigma), cleaned by extraction with sodium carbonate, and dried with anhydrous sodium sulfate (Merck) *(12)*. Ethyl acetate was removed by rotary evaporation (Rotavapor Laborota 4000, Heidolph, Schwaback, Germany), and patulin was redissolved in 2 ml of water, adjusted to pH 4.0 with acetic acid, and determined by HPLC analysis, according to Reddy et al. *(12)*.

Method validation. The recovery was determined on a blank PM broth spiked at three concentrations of patulin (8, 30, and 50 ng/ml). Each test was performed three times, and the mean recovery values with standard deviations were 96.5% ± 1.2%, 93.5% ± 2.0%, and 94.1% ± 1.5%, respectively. The relative standard variation ranged from 4.7 to 7.7% for triplicate analyses. The detection and quantification limits were 1.04 and 1.57 ng/ml, respectively.

Studies on confirmation of either degradation or cell wall absorption of patulin. The cell wall pellet obtained from the above experiment was sonicated with saline solution (0.85% NaCl [wt/vol]) for 30 min and washed twice with saline solution by centrifugation at 4,000 rpm for 10 min at 4°C. The pellet was resuspended in 10 ml of saline solution and 10 ml of ethyl acetate. Afterward, they were thoroughly mixed and centrifuged at 4,000 rpm for 10 min at 4°C. The organic phase was separated and evaporated by flash evaporation (Rotavapor Laborota 4000, Heidolph, Schwaback, Germany), redissolved in 2 ml of water, and adjusted to pH 4.0 with acetic acid. The patulin contents were determined by HPLC analysis, according to Reddy et al. *(12)*.

Influence of patulin on yeast cell concentration. CFU were determined through serial dilution technique after 24, 48, 72, and 96 h of incubation in PM medium with patulin (5.0, 7.5, 10.0, and 15.0 μg/ml) and without patulin. Yeast cultures were spread on a surface of PM solid-medium plate and incubated at 25°C, and the number of colonies formed was counted before and after the incubation periods.

Statistical analysis. Five replications were maintained for each strain and each treatment. The design of the experiment was completely randomized with replicates. Data were subjected to analysis of variance by using SPSS (version 13.0, SPSS Institute, Inc., Chicago, IL), and statistical significance was assessed with $P < 0.05$. Duncan's multiple range test was used to separate means.

RESULTS

Two *M. pulcherrima* strains were evaluated to test their potential of patulin degradation under in vitro conditions. Patulin concentrations and percent degradation are presented in Table 1. Of the two strains tested, MACH1 completely (100%) degraded patulin at 48 h, and GS9 degraded patulin at 72 h of incubation. MACH1 effectively degraded the patulin by 83 to 87.4%, and GS9 by 73 to 75.6%, within 24 and 48 h, respectively. Both strains effectively degraded the patulin by 58.6 to 87.4% at 24 h, and by 75.6 to 100% at 48 h of incubation, even at high concentrations of patulin (15 μg/ml). We observed minor reductions of patulin in controls by 4.5 to 8.5% at all concentrations (Table 1). No detectable level of patulin was observed in yeast cell walls. This means that yeast cell walls did not absorb patulin, and

they completely degraded it. There was no influence of patulin on yeast cell concentration during growth even at higher concentrations of patulin.

DISCUSSION

Patulin contamination of fruit juices is a serious health hazard throughout the world *(12, 15)*. Our results show that the *M. pulcherrima* strains were able to degrade patulin to acceptable limits or undetectable levels during their growth in nutrient medium under in vitro conditions. Patulin degradation mediated by yeast has been studied by many authors. For example, Ricelli et al. *(13)* observed the reduction of patulin by more than 90% (initial concentration 100 μg/ml of media) by using *Gluconobacter oxydans* incubated for 32 h at 25°C. The degradation product was confirmed to be ascladiol. In another study, Coelho et al. *(3)* reported that *Saccharomyces cerevisiae* degraded more than 96% of patulin after 143 h of incubation at 25°C. In our investigation, we observed the complete degradation of patulin within 48 and 72 h by using yeast biocontrol agents, MACH1 and GS9, respectively, isolated from apples (Table 1). To our knowledge, this is the first report regarding biodegradation of patulin using *M. pulcherrima* strains.

Stinson et al. *(19)* examined and found that, of eight yeast strains tested, six reduced patulin levels to below detectable levels, while all eight strains resulted in a 99% or better decrease in total patulin contents. However, a control stored for the same period (2 weeks) had only a 10% reduction. In our study, we also found reduction of patulin in controls ranging from 4.5 to 8.5% at all concentrations of patulin (Table 1). Effective biological control with yeast is limited to products that can be fermented. Furthermore, yeast is itself sensitive to patulin, and at concentrations greater than 200 μg/ml, yeast has been completely inhibited, preventing fermentative detoxification *(20)*. The patulin concentrations used in this study had no influence on yeast cell concentration. Our results show that no detectable levels of patulin were extracted from yeast cell walls.

The patulin levels used in the present study (5 to 15 μg/ml) (Table 1), as well as the patulin contents in the controls after 72 h of incubation (4.58 to 13.99 μg/ml) (Table 1) at 25°C, were greater than the levels recommended by the European Commission *(4)* and those normally observed in fruit juices *(2, 16)*. This means that it is possible to significantly reduce higher doses of patulin to an acceptable or undetectable level by using yeast as a means of biodegradation under in vitro conditions. In terms of practical application, the two yeast strains tested seem to have the potential to detoxify patulin in fruit juices. In summary, the results of this study show complete degradation of patulin by both yeast strains during their growth under in vitro conditions. However, further studies are needed to understand the mechanism of action of yeast strains during patulin degradation.

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