

Short communication

ANTIFUNGAL AND ANTIPATULIN ACTIVITY OF *GLUCONOBACTER OXYDANS* ISOLATED FROM APPLE SURFACE*

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Fungicides are the most common agents used in postharvest treatment of fruit and are the most effective against blue mould, primarily caused by *Penicillium expansum*. Alternatively, blue mould can be treated with antagonistic microorganisms naturally occurring on fruit, such as the bacterium *Gluconobacter oxydans*. The aim of this study was to establish the antifungal potential of the *G. oxydans* 1J strain isolated from apple surface against *Penicillium expansum* in culture and apple juice and to compare it with the efficiency of a reference strain *G. oxydans* ATCC 621H. The highest antifungal activity of *G. oxydans* 1J was observed between days 3 and 9 with no colony growth, while on day 12, *P. expansum* colony diameter was reduced to 42.3 % of the control diameter. Although *G. oxydans* 1J did not fully inhibit mould growth, it showed a high level of efficiency and completely prevented patulin accumulation in apple juice.

KEY WORDS: *antagonistic microorganisms, mould growth, Penicillium expansum, toxin accumulation*

Due to the decay it leads to, blue mould can cause major losses in apple harvest, but its negative effects can be prevented by proper sanitation and efficient control. The most common cause of blue mould, the fungus *Penicillium expansum* causes fruit decay and stimulates the production of a carcinogenic mycotoxin patulin. Initially, patulin was used in pharmaceuticals as an agent, but prolonged use in human trials has revealed that it causes gastrointestinal and dermal irritation, whereas animal models have showed that patulin is genotoxic but not carcinogenic (1). The

International Agency for Research on Cancer (IARC) has classified patulin as group 3 carcinogen to humans (2). The EU was among the first to set concentration limits for several mycotoxins, including patulin, to 50 µg kg⁻¹ in fruit juices and fruit nectars, 25 µg kg⁻¹ in solid apple products, and 10 µg kg⁻¹ in apple-based products for infants and young children (3, 4). At high concentrations patulin may spoil the flavour of fruit intended for processing and its presence indicates that mouldy apples have been used in the production of juice. The most important and most common patulin-producing fungi belong to three genera of ascomycota: *Penicillium*, *Aspergillus*, and *Byssoschlamys*, representing both commensal and pathogenic species found on the surface of apples (5).

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Apples have always been among the most frequently consumed fruit, and that is why the presence of patulin may present a serious health risk for humans and affect food processing industry (6). The most common agents used to treat patulin-contaminated fruit are fungicides (7, 8). However, their harmful effects on human health and the environment are well known (6), and their overuse increases the risk of resistance in treated microorganisms. These reasons have led to an intensive search for safer biological alternatives to control fruit and vegetable spoilage and potential toxin accumulation. One such alternative is the use of microorganisms naturally occurring on fruit and vegetable surfaces (9, 10), such as *Gluconobacter oxydans*.

The genus *Gluconobacter* includes five species used in food manufacturing primarily because it is harmless to human health. This genus belongs to a group of acetic acid bacteria (11) and has the ability to partially oxidise a number of carbohydrates and alcohols. Its species can grow in highly concentrated sugar solutions and at low pH values. Their growth behaviour and response to extreme culture conditions are remarkably unique (12).

The aim of this study was to isolate naturally present *Gluconobacter oxydans* from apple surface and to determine its effectiveness in controlling the growth and patulin production of *Penicillium expansum*.

MATERIALS AND METHODS

Isolation and identification of the microbial culture

G. oxydans was isolated from old varieties of five "Jonathan" cultivars collected aseptically and washed with sterile water. Aliquots (0.1 mL) of washing samples were spread on yeast-peptone-mannitol (YPM) plates (5.0 g of yeast extract, 3.0 g of peptone, and 25.0 g mannitol per litre) and potato dextrose agar (PDA) and incubated at 25 °C for 7 days. The colonies were chosen at random and identified according to their morphology, Gram staining, cell morphology, and catalase reaction. Samples were prepared and bacterial species identified using a Microflex LT™ matrix-assisted laser desorption/ionisation time-of-flight mass spectrometer (MALDI-TOF MS) following the procedure recommended by the manufacturer (Bruker Daltonik, Bremen, Germany). A colony of

YPM agar culture was suspended in 300 µL water to obtain a homogeneous suspension of cells by vortexing for one minute. For the identification of species, the peaks from the generated mass spectra were compared with the reference spectra of the integrated database using the MALDI Biotyper Software package. All of the samples were analysed in triplicate and the final score was the mean value of three determinations.

Culture preparation

The *G. oxydans* strain, which is the usual contaminant of apples, capable of degrading patulin, was identical to the one held in the collection of microorganisms of the Zagreb University Faculty of Food Technology and Biotechnology (ZUFFTB) and recorded under serial no. 1J. The reference strain, *G. oxydans* ATCC 621H, was obtained from Valio Ltd., Helsinki, Finland. *G. oxydans* ATCC 621H was maintained at -70 °C on a medium with Ca (10 g L⁻¹ glucose, 1 g L⁻¹ yeast extract, and 3 g L⁻¹ CaCO₃) supplemented with 20 % glycerol. Prior to inoculation, the cultures were transferred twice to YPM broth. The bacterial growth was determined after 72 h of incubation at 28 °C by plate counting (CFU mL⁻¹). The cell concentration was 10⁸ CFU mL⁻¹.

The *Penicillium expansum* strain 565 was obtained from the ZUFFTB collection of microorganisms. It was stored on slants of PDA ("Biolife", Milan, Italy) at 4 °C. To obtain the inoculum, the mould was incubated on PDA slants at 25 °C for 7 days and then added 2x5 mL of sterile water solution of Triton X-100 (5 mg L⁻¹) (Sigma-Aldrich, St. Louis, MO, USA). The suspension was diluted to a concentration of 10⁶ spores mL⁻¹.

Mould growth and patulin production were determined in two ways. Fungal colony growth was determined by measuring colony diameter (mm) on YPM plates after inoculation with the reference strain *G. oxydans* ATCC 621H and with the isolated *G. oxydans* 1J to compare their effects. Aliquots (0.1 mL) of bacterial cells (10⁶ CFU mL⁻¹) were spread on YPM plates and after 2 h, 10 µL of an aqueous suspension at the conidial concentration (10⁴ spores mL⁻¹) of *P. expansum* 565 were added in the centre of each plate. Control samples did not contain *G. oxydans*. Radial colony growth was measured at three-day intervals over 15 days.

Patulin production by *P. expansum* was screened based on thin-layer chromatography (TLC) determinations from agar plugs cut from the central area of the colony. The plates were developed in the

Table 1 Mould colony growth and the presence of patulin on YPM agar

Days after inoculation	Colony diameter / mm			Patulin / agar plug		
	<i>P. expansum</i>	<i>P. expansum</i>	<i>P. expansum</i>	<i>P. expansum</i>	<i>P. expansum</i>	<i>P. expansum</i>
	565	565 + <i>G. oxydans</i> ATCC 621H	565 + <i>G. oxydans</i> 1J	565	565 + <i>G. oxydans</i> ATCC 621H	565 + <i>G. oxydans</i> 1J
3	20	-	-	nd	nd	nd
6	34	-	-	nd	nd	nd
9	44	-	-	+	nd	nd
12	52	29.5	9	+	nd	nd
15	58	33	21	+	+	nd

nd – not detected

Table 2 Effect of *Gluconobacter* strains on fungal biomass and patulin concentration in apple juice.

Days after inoculation	Fungal biomass dry weight / g mL ⁻¹			Patulin concentration / µg L ⁻¹		
	<i>P. expansum</i>	<i>P. expansum</i>	<i>P. expansum</i>	<i>P. expansum</i>	<i>P. expansum</i>	<i>P. expansum</i>
	565 (control)	565 + <i>G. oxydans</i> ATCC 621H	565 + <i>G. oxydans</i> 1J	565	565 + <i>G. oxydans</i> ATCC 621H	565 + <i>G. oxydans</i> 1J
3	0.4040±0.007	0.2092±0.039 ^a	0.0192±0.002 ^{a,b}	<3	<3	<3
6	0.5955±0.071	0.1651±0.028 ^a	0.0493±0.003 ^{a,b}	<3	<3	<3
9	0.9565±0.054	0.2055±0.028 ^a	0.0995±0.003 ^{a,b}	6.0	<3 ^a	<3 ^a
12	1.3706±0.034	0.4734±0.036 ^a	0.1912±0.029 ^{a,b}	6.2	<3 ^a	<3 ^a
15	1.3920±0.022	0.4002±0.024 ^a	0.2104±0.030 ^{a,b}	7.8	<3 ^a	<3 ^a

^aSignificantly different from control (Student's *t*-test; P<0.05).

^bSignificantly different from the ATCC 621H strain (Student's *t*-test; P<0.05).

Table 3 Comparison of *P. expansum* mould biomass inhibition by the reference *G. oxydans* strain ATCC 621H and the natural, isolated strain 1J in apple juice

Strain	Inhibition / %				
	Day 3	Day 6	Day 9	Day 12	Day 15
<i>G. oxydans</i> ATCC 621H	48.22±2.23	78.25±3.07	78.52±2.74	65.47±3.61	71.25±3.55
<i>G. oxydans</i> 1J	95.25±2.91**	91.73±3.13*	89.60±3.02*	86.05±2.73**	85.89±3.38*

*Significantly different from the reference ATCC 621H strain on days 6, 9, and 15 days (Student's *t*-test; P<0.05)

**Significantly different from the reference ATCC 621H strain on days 3 and 12 (Student's *t*-test; P<0.01)

solvent system containing toluene:ethyl acetate:formic acid (5:4:1) and after drying, patulin was detected as a fluorescent spot made visible by UV light ($\lambda=254$ nm) (13, 14). Rf values of the samples were compared with the Rf value of the patulin standard (Sigma- Aldrich, St. Louis, MO, USA) chromatographed simultaneously.

Alternatively, mould growth and patulin production were measured in clear apple juice (Maraska, Zadar, Croatia) over 15 days. Prior to the experiment, apple juice was screened for patulin. Each flask with 50 mL of apple juice was added 1 mL of spore suspension (10^6 spores mL⁻¹) alone or in combination with bacterial cells. The flasks were incubated in duplicate at 28°C for 15 days. The amount of mould biomass was determined gravimetrically by filtration through

pre-weighed Whatman no. 1 filter paper. The filter paper and mycelia were dried in a hot-air oven at 105 °C for 24 h and mycelia weight obtained by re-weighing the filter paper with dried mycelia. Patulin was determined using a Shimadzu HPLC equipped with a UV detector (Shimadzu, Kyoto, Japan). No preparation was required for the clear apple juice. Patulin was eluted with water:acetonitril:perchloric acid (95:5:0.095) and separated on a 5-µm C₁₈ chromatographic column (150 mm x 4.6 mm) at a flow rate of 1.0 mL min⁻¹. The UV detector wavelength was set at 276 nm and sample injection volume at 50 µL. Total run time was 10 min. The detection limit for patulin was 1 µg kg⁻¹ and quantification limit 3 µg kg⁻¹.

Statistical analysis

We used the statistical package Statistica v. 6.1 (SAS Institute, Cary, NC, USA) to compare the activities of the two *G. oxydans* strains in apple juice (but not in agar). The differences in mould biomass and patulin concentrations in apple juice were tested with Student's *t*-test. The differences were considered significant if the *P*-value was less than 0.05.

RESULTS AND DISCUSSION

Culture findings

G. oxydans strain 1J identified in our study had the following characteristics: negative Gram stain reaction, ellipsoidal to rod-shaped cells, single or in pairs and rarely in chains, and positive catalase test. The identity of the bacterial species was confirmed by the MALDI-TOF MS. Both the 1J and the reference ATCC 621H strains were highly inhibitory to *P. expansum* 565 growth, especially over the first 12 days (Table 1). Patulin in agar plugs cut from pure fungal dish cultures was first detected after 9 days. Rapid colony diameter growth for the reference strain was observed between days 12 and 15. Patulin production started from day 9 through day 15. The strain 1J significantly reduced the mould growth of the *P. expansum* 565 and completely inhibited patulin production.

Apple juice

Table 2 shows the effects of both *G. oxydans* strains on the growth of *P. expansum* and patulin accumulation in apple juice over 15 days. The highest increase in mould biomass growth (1.392 g mL⁻¹) was recorded on day 15. Patulin accumulation started 9 days after inoculation, and reached maximum concentration on day 15 (7.8 µg L⁻¹). Both strains significantly inhibited the growth of mould biomass over the 15 post-inoculation days compared to control (*P*<0.05), but the 1J strain was significantly more efficient (*P*<0.05). No significant differences between the strains were found in degrading patulin, as both reduced it to below the detection limit of 3 µg L⁻¹. These findings are in accordance with Ricelli et al. (6), who observed that incubation with *G. oxydans* degraded patulin in apple juices up to 96%. Ricelli et al. (6) also reported that natural microflora from apples, such as the bacterium

G. oxydans, is capable of degrading patulin to a less toxic compound called ascladiol. *G. oxydans* ATCC 621H showed partial (48%) inhibition of mould growth in apple juice at the beginning of incubation and increased inhibition (65% to 78%) from day 6 to day 15.

In turn, the inhibition of mould growth with the isolate 1J was about 92% to 95% over the first six days of inoculation. From days 12 to 15 this inhibition weakened a little to 85% and 86%, respectively, but remained significantly greater than with the ATCC 621H strain (Table 3).

It is difficult to control apple decay caused by *Penicillium* by biological means due to the high competitiveness of this pathogen (9, 15, 16). Some authors have shown that the best antagonist against fungi that cause fruit decay is the very microflora from their surfaces (17-19). Our results confirm that microflora naturally occurring on fruit surfaces could provide effective protection against fungal growth and patulin accumulation. This is a preliminary study, and our results will be validated using apples infected with *P. expansum*. Future studies should include other biocontrolling agents which naturally occur as fruit microflora, such as yeasts (20-22), and investigate their effectiveness against mould growth and patulin.

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Sažetak**ANTIFUNGALNA I ANTIPATULINSKA AKTIVNOST *GLUCONOBACTER OXYDANS* IZOLIRANE S POVRŠINE JABUKE**

Tretiranje voća fungicidima, nakon berbe, uobičajeni je način suzbijanja plave plijesni. Međutim, propadanje voća može se spriječiti i upotrebom antagonističkih mikroorganizama, kao što je bakterija *Gluconobacter oxydans*. Svrha ovoga rada bila je izolirati prirodnu mikrobnu populaciju s površine jabuka i istražiti moguće inhibitorno djelovanje *Gluconobacter oxydans* 1J na plavu plijesan, *Penicillium expansum*, najvažnijeg uzročnika kvarenja jabuka u skladištu. Najveća antifungalna aktivnost bakterije primijećena je između 3. i 9. dana, kada nije zabilježen porast kolonija, a nakon 12. dana promjer kolonije plijesni bio je manji za 42,3 %. Iako istraživana bakterija *Gluconobacter oxydans* 1J nije u potpunosti inhibirala rast plijesni u jabučnom soku pokazala je visoku razinu učinkovitosti (od 86 % do 95 %). *Gluconobacter oxydans* 1J djelomično inhibira rast plijesni i u potpunosti biosintezu patulina, ovisno o vremenu i uvjetima uzgoja.

KLJUČNE RIJEČI: *antagonistički mikroorganizmi, nakupljanje toksina, Penicillium expansum, rast plijesni*

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