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Factors Affecting Patulin Production by *Penicillium expansum*†

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ABSTRACT

Patulin, a mycotoxin produced by *Penicillium* spp. during fruit spoilage, is a major concern with regard to human health because exposure can result in severe acute and chronic toxicity, including carcinogenic, mutagenic, and teratogenic effects. In this study, we investigated the effects of *Penicillium expansum* isolate, apple cultivar, storage temperature and time, and pH on the production of patulin. Patulin was analyzed by a previously developed micellar electrokinetic capillary electrophoresis method. *P. expansum* isolates originating from across Ontario produced widely differing levels of patulin, ranging from 0 to >6 mg/g by dry mycelial weight. The highest patulin levels were those for isolates displaying aggressive growth (characterized by rapidly increasing acidity) accompanied by profuse mycelial development. Distinct patterns in fungal growth rates and patulin production were evident among isolates grown in McIntosh, Empire, and Mutsu ciders. Extensive fungal growth and higher patulin levels (538 to 1,822 μg/ml on day 14) in apple ciders were associated with incubation at room temperature (25°C), although potentially toxic patulin levels (75 to 396 μg/ml on day 24) were also found in refrigerated ciders (4°C) inoculated with *P. expansum.*

Fungal spoilage of agricultural products is of great concern both because of economic losses and because of health concerns raised by the exposure of animals and humans to mycotoxins produced by spoilage. Patulin is one such mycotoxin and is produced by several species of fungi, particularly *Penicillium expansum,* commonly known as blue mold, a prevalent apple pathogen *(2, 11).* Patulin levels in apple products are of great concern because of the severe acute and chronic effects caused by the toxin. Patulin's acute toxic effects in humans include nausea, vomiting, and other gastrointestinal trauma and accompanying kidney damage, and chronic patulin exposure has been shown to induce the formation of cancerous tumors and to cause genetic mutations and embryonic developmental defects *(1, 3, 4, 6, 15).* In many countries, particularly those of the European Union, the levels of patulin in apple cider and related products are federally governed, with the most common maximum permissible level being 50 μg/liter *(5, 7, 10, 12, 14).* Apple cider producers in such countries closely monitor the levels of patulin in their cider, and the measurement of patulin levels in such products is a suggested means of evaluating the quality of fruits such as apples *(9, 10).* It is important to study the factors influencing patulin production by *P. expansum,* since this mycotoxin is generally heat stable and survives the pasteurization process, and therefore the inhibition of its production is critical in maintaining the safety of apple products *(13).*

At the onset of this investigation, we believed that considerable variation would be found in the growth patterns and patulin production capabilities of *P. expansum* isolates. We expected most of this variation to stem from genetic differences, but environmental factors such as incubation time, incubation temperature, and nutrient source (apple variety) were also expected to greatly influence the growth of *P. expansum* and its production of patulin. Characterization of the factors affecting the production of patulin by *P. expansum* may help in developing effective means of controlling patulin levels in food products.

In a previous study, we showed that micellar electrokinetic capillary electrophoresis was a rapid, economical, and effective means of measuring patulin levels in apple ciders, superior in many ways to conventional chromatographic techniques *(13).* In the present study, we used the previously developed micellar electrokinetic capillary electrophoresis method for patulin detection and analysis in determining and characterizing the factors affecting patulin production by *P. expansum,* specifically, the effects of incubation time and temperature, apple cider pH, cultivar type, and differences among *P. expansum* isolates, from which a particularly virulent strain was obtained for use in further studies.

MATERIALS AND METHODS

Microorganisms. *P. expansum* isolates were obtained from locations across Ontario, including Vineland, Woodstock, and Georgian Bay. Isolates were identified as *P. expansum* through the comparison of their colony morphology, color, growth habits, and conidia and conidiophore morphology with a certified typical *P. expansum* culture (P99418) obtained from the Fungal Culture Collection, Ottawa. All isolates were grown on potato dextrose agar (Difco Laboratories, Detroit, Mich.) plates containing penicillin and streptomycin (both at 100 μg/ml) to inhibit bacterial growth.

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Apple ciders. Healthy McIntosh, Mutsu, and Empire apples from Woodstock, Ontario, were washed and processed with a Braun MP 80 centrifugal cider extractor (Braun Inc., Woburn, Mass.). An apple was considered healthy when it did not have apparent disease spotting and its patulin level was below the detection limit of the capillary electrophoresis (CE) method used (3.8 μg/liter). The cider was centrifuged at 10,000 × *g* for 20 min to remove pulpy sediment (Sorval RC5C Plus, Mandel Scientific Co. Ltd., Guelph, Ontario, Canada). The supernatant was passed through three layers of cheesecloth, a series of paper filters (no. 54, no. 1, and no. 42, Whatman International Ltd., Maidstone, UK) with decreasing pore sizes, a 0.45-μm Gelman GHP Acrodisc GF syringe filter (Pall Canada Ltd., Mississauga, Ontario, Canada), and, finally, a disposable sterilization filter unit with a 0.22-μm membrane into a sterile glass jar (Nalgene MF75, Nalge Nunc International, Rochester, N.Y.). These processed ciders were completely sterile and free of background patulin *(13)*. The ciders were stored at −18°C until they were needed.

Chemicals and reagents. CE buffer solutions were prepared using distilled, deionized reverse-osmosis water prepared in house via a NANOpure ultrapure water system (Model D4741, Barnstead/Thermolyne, Dubuque, Iowa). The patulin standard and reagents were of the highest available quality (either 99+% or American Chemical Society grade) and were purchased from the Sigma-Aldrich Company (Oakville, Ontario, Canada), along with the antibiotics, penicillin and streptomycin. Solvents were of high-pressure liquid chromatography grade and were purchased from Caledon Laboratories (Georgetown, Ontario, Canada).

Run buffer, SDS, and standard solutions. A borate run buffer was prepared from 0.2 M sodium tetraborate ($Na_2B_4O_7$), 0.2 M boric acid (H_3BO_3), and 0.2 M sodium dodecyl sulfate (SDS), for a final solution of 25 mM borate and 50 mM SDS, adjusted to a pH of 9.0 with 1 M NaOH. The run buffer was filtered with a 0.45-μm Gelman GHP Acrodisc GF prior to its use and was replaced after every 8 to 12 injections. A stock solution of patulin standard (2,000 μg/ml) was prepared in methanol, serially diluted to 200, 100, 50, 25, 5, and 1 μg/ml, and analyzed by CE, yielding a standard curve that was linear between 1 and 100 μg/ml, with an r^2 value of 0.9990 (data not shown).

Patulin production by different isolates of *P. expansum*. Spore suspensions (6×10^6 conidia per ml) of six *P. expansum* isolates, two from Georgian Bay (P18-5B, P51-16B), two from Woodstock (PRD-1, PGD-1), one from Vineland (PM1), and one from Ottawa (Fungal Culture Collection, P99418), were prepared. Spore suspensions (100 μl) were inoculated individually into 6 ml of potato dextrose broth (PDB; Difco) for a final concentration of 1×10^5 conidia per ml and incubated in polypropylene centrifuge tubes (Corning Inc., Corning, N.Y.) at 25°C with shaking (150 rpm). Samples were removed at different time intervals (the maximum incubation time was 144 h) and centrifuged at 6,000 × *g* for 20 min. The pellet was heated at 65°C for 3 days to obtain the mycelial dry weight. The supernatant was syringe filtered with a 0.45-μm Acrodisc and frozen at −24°C for later CE analysis. There were three replicates for each isolate for each time period.

Patulin production by *P. expansum* isolate P18-5B in different apple ciders. According to the results of the experiment, *P. expansum* isolate P18-5B was the most virulent isolate with regard to growth rate, acid production, and patulin synthesis. A P18-5B spore suspension (6×10^6 conidia per ml) was prepared, and 100 μl was inoculated into each variety of sterile apple cider (6 ml) for final concentrations of 1×10^5 conidia per ml. Tubes were incubated in the dark at 4 and 25°C and were removed at various times and centrifuged at 6,000 × *g* for 20 min. The pellet was heated at 65°C for 3 days to obtain the mycelial dry weight. The supernatant was syringe filtered with a 0.45-μm Acrodisc and frozen at −24°C for later testing. Prior to CE analysis, patulin was extracted with three equivalent volumes of ethyl acetate. The combined ethyl acetate layers were dried by being passed through a pipet containing anhydrous sodium sulfate and evaporated with a gentle stream of ultrapure nitrogen. The remaining residue was resuspended in borate-SDS run buffer, vortexed, and syringe filtered immediately prior to CE analysis.

CE. A micellar electrokinetic capillary electrophoresis method was adopted from our previous work *(13)*. A Beckman P/ACE 5500 CE with a photodiode array detector, an autosampler with two rotating trays, and a cartridge interface with a cooling option was used. The CE conditions were as follows: run buffer, 25 mM borate and 50 mM SDS (pH 9.0); column, fused silica capillary (50 cm with 75-mm inside diameter); temperature, 25°C; separation voltage, 15 kV; detection, 273 nm; injection, low pressure (0.5 lb/in^2) for 5 s (30 nl); run time, 10 min.

Statistical analysis. Outlying datum points were eliminated according to a simple Q-test with a certainty level of 90%. Cubic polynomial models were used to analyze the remaining data with SAS software (SAS Institute Inc., Cary, N.C.) according to the general linear model procedure. Comparative differences were deemed significant at the 5% level. Most comparisons were made for the last datum points (since differences were most pronounced in these regions), with the exception of comparisons of patulin levels, for which concentrations were examined at peak maximums.

RESULTS AND DISCUSSION

Patulin production by different *P. expansum* isolates. The growth of each of the six *P. expansum* isolates followed a cubic model ($r^2 = 0.8886$) and showed clear variation, as was initially hypothesized (Fig. 1A). The *P. expansum* isolates fell into two groups based on their final mycelial dry weight values, a useful indicator in measuring the aggressiveness of growth. The aggressive growers (with high mycelial dry weights) included isolates PRD-1, P99418, PGD-1, and P18-5B, which showed no significant differences among each other at 144 h but were significantly different from the less aggressive growers (with low dry weights), isolates PM1 and P51-16B. Patulin production levels also differed among isolates, with some producing no patulin and others producing >6 mg/g by dry mycelial weight (Table 1). The aggressive growers also greatly reduced the pH of the growth medium (PDB) and produced detectable patulin levels, while the weaker growers (PM1 and P51-16B) produced a smaller reduction in PDB pH and no detectable patulin (Fig. 1B and Table 1). The limit of detection for patulin with the method used was 3.8 μg/liter *(13)*.

At 144 h, the dry mycelial mass of PRD-1 (an aggressive grower) was 0.0227 g, nearly two times that of PM1 (a weak grower), whose final dry weight was 0.0127 g (Fig. 1A). Fungal metabolism acidifies the PDB medium, possibly through the production of secondary metabolites and organic acids *(8)*, lowering the pH to the point at which patulin is most stable (<pH 3.5) *(13)*. The decrease in pH was found to negatively correlate with final dry mycelial

TABLE 1. *Characteristics of the patulin production of several* P. expansum *isolates grown in PDB at 25°C*

Isolate	Patulin (mg/g dry weight)[a]	Relative SD (%)[a]	Time for detectable patulin (h)[b]	Threshold pH value for patulin synthesis[c]
P99418	64.9	116	144	3.03
PM1	0	0	—[d]	—
P51-16B	0	0	—	—
P18-5B	66.6	54	96	3.43
PGD-1	31.9	34	96	3.50
PRD-1	21.3	80	96	3.27

[a] Final concentration (after 144 h); average value for three replicates.
[b] Time at which patulin was first detected.
[c] Mean for three replicates.
[d] —, isolate did not produce patulin.

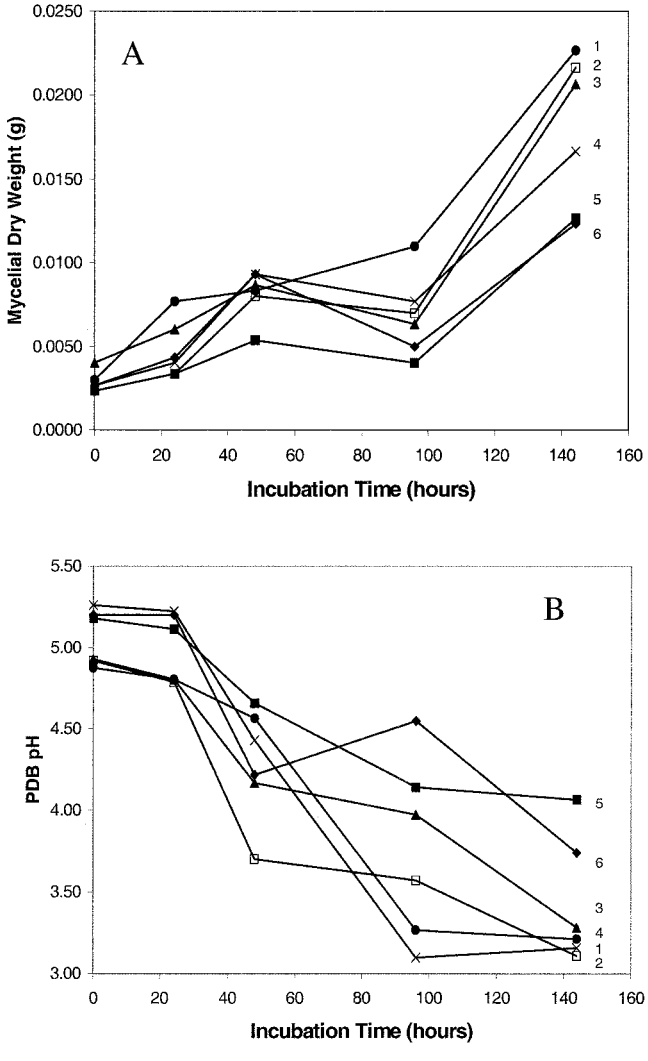

FIGURE 1. *The growth of different* P. expansum *isolates in PDB (A) and the corresponding pH change in growth media (B). 1, isolate PRD-1; 2, isolate P99418; 3, isolate PGD-1; 4, isolate P18-5B; 5, isolate PM1; 6, isolate P51-16B. Values are means for three replicates.*

weight ($r^2 = 0.7678$) according to a cubic polynomial model (Fig. 1B). We also found that each isolate displayed a unique threshold pH value above which patulin production was not detected (Table 1). Interestingly, these threshold pH values fell into the pH range of apple cider, a common natural environment for *P. expansum.* Both the mycelial

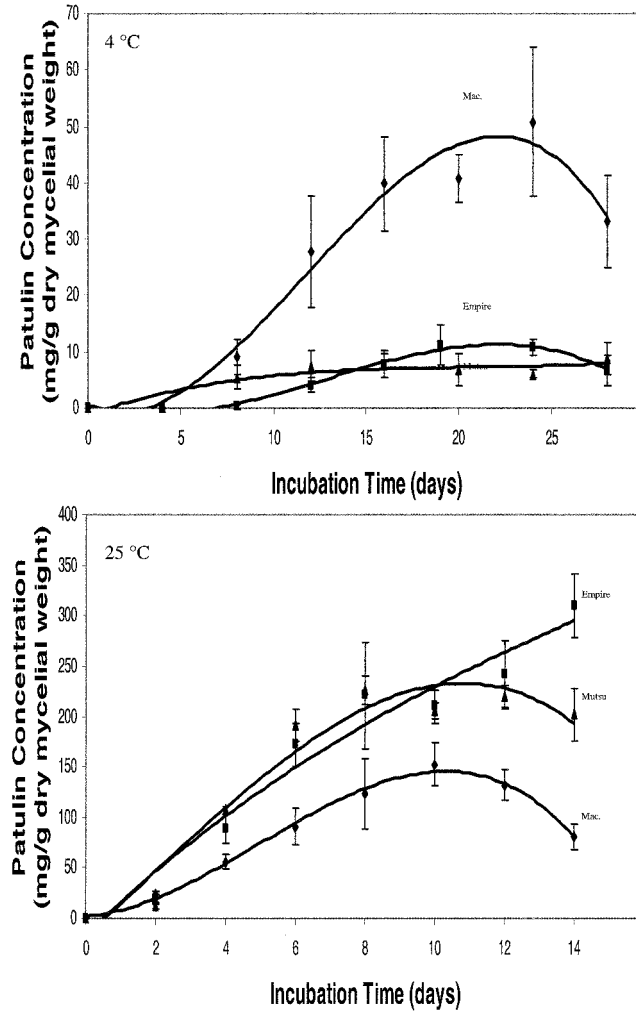

FIGURE 2. *Patulin production by isolate P18-5B in different apple ciders and at different temperatures. The patulin concentration was measured in milligrams per gram of dry mycelial weight. Values are means for four replicates.*

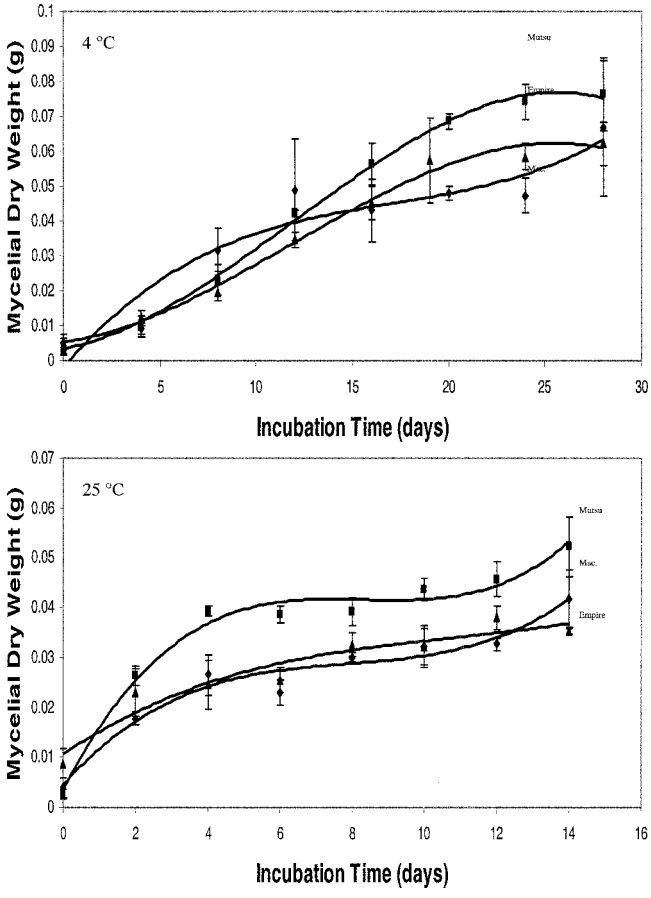

FIGURE 3. *The growth of isolate P18-5B in different apple ciders and at different temperatures. Values are means for four replicates.*

growth and the decrease in the pH value directly affected patulin production; however, a higher mycelial growth rate did not necessarily lead to a higher level of patulin production on a dry-weight basis (Table 1). Isolate P18-5B was found to be the most efficient patulin producer (Table 1) on the basis of its final patulin concentration and its strong patulin production early in the experimental period. All *P. expansum* isolates except for PM1 and P51-16B produced detectable amounts of patulin after 144 h of incubation in PDB at 25°C (Table 1). In addition, PM1 and P51-16B grew slowly and the final pH for these two isolates was the highest (Fig. 1B). Patulin is known to be unstable at pH values of >3.5 *(13).* How pH affects patulin synthesis was not specifically determined. A lower pH value stabilizes patulin chemically once it is produced. However, the fungus itself may be producing acidic metabolites to favor the production and stability of patulin, which it might use as a chemical ecological defense. It was noted that PDB is not the ideal environment for *P. expansum,* whose growth and patulin production levels were expected to be higher in apple cider (Figs. 2 and 3).

Patulin production by *P. expansum* isolate P18-5B in different apple ciders. Incubation time, incubation temperature, and apple variety were all found to strongly influence patulin production in McIntosh, Empire, and Mutsu

TABLE 2. *Initial pHs and sugar contents for sterile McIntosh, Empire, and Mutsu apple cider media*

Apple cultivar	Sugar concentration (g/liter)[a]	Initial cider pH[b]
McIntosh	35.06 ± 4.57	3.31 ± 0.03
Empire	35.03 ± 2.50	3.51 ± 0.01
Mutsu	37.25 ± 3.82	3.42 ± 0.02

[a] Mean ± standard deviation for three replicates.
[b] Mean ± standard deviation for 16 replicates.

ciders (Fig. 2). Higher temperatures (25°C) enhanced the growth of isolate P18-5B, which was the most powerful patulin producer (Table 1), although with long incubation periods under refrigerated conditions (4°C), fungal growth eventually caught up and resulted in even higher mycelial mass values (Fig. 3). Pathogen growth reached a peak after ca. 24 days at 4°C and after ca. 10 days at 25°C and followed a cubic polynomial model at both temperatures (r^2 = 0.91998 and r^2 = 0.92616, respectively) (Fig. 3). Isolate P18-5B grew best in Mutsu cider, as indicated by the high mycelial dry weights at both temperatures, possibly because of the higher sugar content of Mutsu apples (Fig. 3 and Table 2). Final mycelial weight values for P18-5B in McIntosh and Empire ciders were not found to be significantly different at either 4 or 25°C (P = 0.7245 and P = 0.0977, respectively), although the reductions in pH and the patulin production characteristics were significantly different between these two varieties (Figs. 2 through 4 and Table 1). The growth of isolate P18-5B in Mutsu cider was significantly more extensive than that in McIntosh and Empire ciders as measured by mycelial dry weight after 12 days at 4°C (P = 0.0334) and after 4 days at 25°C (P < 0.0001) (Fig. 3).

Despite its fast growth in Mutsu cider at 4°C, isolate P18-5B displayed the poorest patulin production at this temperature (Fig. 2). Isolate P18-5B's patulin production level was highest in Empire cider at 25°C (Fig. 2). At refrigeration temperature (4°C), isolate P18-5B's patulin production level was highest in McIntosh cider, a finding that give cause for concern, since McIntosh apples are a key component in most apple ciders because their widespread availability and low retail value make them attractive to cider manufacturers. Interestingly, while P18-5B's growth was poorest in McIntosh cider at a low temperature (4°C), its patulin production level was at its highest under these conditions, suggesting that the higher acidity of McIntosh cider may play an important role in patulin synthesis at this temperature, which is already stressful for the fungi (Table 2).

At both incubation temperatures, the growth of the pathogen plateaued after an initial period of rapid growth, except for P18-5B grown in Mutsu cider at 4°C (Fig. 3). During this stationary period of negligible fungal growth, the pH of the cider decreased almost linearly, while patulin levels increased (Figs. 2 through 4). The drop in the growth rate likely corresponds to a decrease in available sugars or

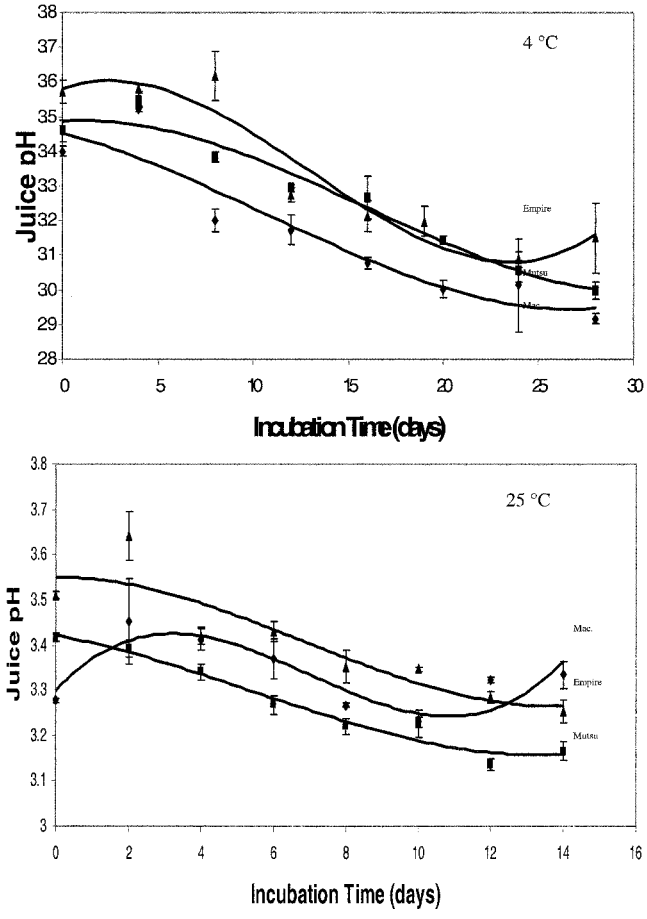

FIGURE 4. *Changes in media pH values for isolate P18-5B grown in different apple ciders and at different temperatures. Values are means for four replicates.*

other nutrients, indicating that patulin production may be linked to nutritional stress.

A secondary growth period was observed for isolate P18-5B after ca. 24 days at 4°C and after ca. 11 days at 25°C, except for Mutsu cider at 4°C, in which growth continued unabated until approximately day 24 (Fig. 2). The increasing cider acidity was seen to slow and even reverse itself at the onset of this secondary growth period, suggesting possible implications for patulin stability (Figs. 2 and 4). In most cases, patulin levels were stabilized ca. 2 days before this secondary growth stage (Fig. 3). In all cases, P18-5B produced more patulin at higher temperatures, reflective of a higher level of metabolic activity under these conditions (Fig. 2). Patulin production fitted a cubic polynomial model at both 4 and 25°C ($r^2 = 0.9193$ and $r^2 = 0.8481$, respectively). It is not clear whether the lower pH favored patulin production and fungal growth or if the higher pH caused degradation of patulin.

In general, as fungal mass increased, so did the media acidity, possibly as a result of the production of organic acids during fungal sugar metabolism. Growth initially increased rapidly and then slowed as nutrients were depleted, but it eventually increased again by the end of the study, perhaps being induced through an alternative metabolic pathway. Patulin was detected only below a certain critical threshold pH, with levels peaking shortly before the onset of secondary growth during the period of nutritional stress.

In this study, extensive differences were found among *P. expansum* isolates in terms of patulin production and fungal growth. Genetic variation may be the ultimate cause of these differences; however, environmental factors such as temperature, time, and pH could contribute greatly to the fungal growth and patulin production of *P. expansum.* Temperature affected the rate of growth of the pathogen, but not as significantly as it affected the production of patulin. Patulin concentration negatively correlated with pH value, possibly because of patulin's instability at high pH values. Mycelial growth and patulin production by *P. expansum* differed considerably among apple cultivars, with significant differences in patulin production being observed between McIntosh and Empire ciders despite the close genetic relationship between these varieties. The concentration of sugars and other chemical components unique to each apple cultivar influence the behavior of *P. expansum* (Table 2). The results of this study shed some light on the characteristics of *P. expansum,* particularly with regard to the mycotoxin patulin. Although patulin in apple juice is normally preformed, apple products such as ciders, particularly nonpasteurized ciders, may pose a serious health risk to consumers, who are increasingly interested in nonpasteurized apple ciders for better nutrition. Our findings imply a need for the regulation of patulin in apple products, particularly nonpasteurized ciders, since even slight *P. expansum* contamination can lead to high concentrations of patulin given the right isolate, temperature, storage time, and apple variety.

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