Influence of Carbohydrate and Nitrogen Source on Patulin Production by Penicillium patulum

W. T. STOTT AND L. B. BULLERMAN*  
Department of Food Science and Technology, University of Nebraska, Lincoln, Nebraska 68583  
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A strain of Penicillium patulum, isolated from cheddar cheese, produced patulin when grown on liquid media containing lactose and milk nitrogen sources. Patulin production was affected by the temperature of incubation, the type and amount of carbohydrate, and the type of nitrogen source present. Patulin levels generally were depressed by incubation at 5 C and low carbohydrate levels. Patulin was produced at low levels in the absence of sugars at 5 C when the mold was grown on milk nitrogen sources. No patulin was detected in cultures grown on 25% casein slurries or cheddar cheese, even though growth of the mold was extensive.

Patulin, a metabolite of several Aspergillus and Penicillium species, is quite toxic to animals (1) and has been shown to be carcinogenic to rats in sublethal doses (4). Many types of substrates have been utilized to study the production of patulin by molds and to determine the optimum conditions for production. P. patulum Bainier, grown on Raulin-Thom broth, produced up to 1.3 mg of patulin/ml of medium after 2 weeks incubation at 25 C (15). P. expansum produced up to 0.48 mg of patulin/ml of yeast extract-sucrose broth after 2 weeks incubation at 25 C (10). The largest yields of patulin have been obtained by Norstad and McCalla (11, 12) using potato-dextrose broth in which P. urticae Bainier produced up to 2.7 mg of patulin/ml after 2 weeks incubation at 25 C. Glucose and iron salts were reported to stimulate patulin production, whereas yeast extract and corn steep liquor depressed its formation (9).

Patulin production has also been limited by incubation at low temperatures. However, P. expansum and P. patulum have been reported to produce over 0.4 mg of patulin/ml of broth after 100 days incubation at 1.7 C (J. Lovett and R. G. Thompson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, E71, p. 12). P. urticae has been observed to produce up to 0.25 mg of patulin/ml of broth after 3 weeks incubation at 5 C (12), and P. expansum has been reported to produce 0.7 mg/ml after 18 weeks at 0 C (20).

Growth of patulin-producing molds may occur on foods and be accompanied by patulin production. Growth of P. expansum on apples has been reported to produce up to 125 µg of patulin/g of apple tissue (20), up to 1 mg of patulin/ml of apple sap (2), and up to 17.7 mg of patulin per apple (8). Patulin has also been found in commercial apple juices (18, 23, 24). However, growth of P. expansum on sausages and breads gave either no detectable patulin or a rapid disappearance of patulin (6, 10, 17). Lack of production of patulin or loss of patulin in these products was attributed to its reaction with sulphydryl groups present in the substrates. No nutritional limitations of the foods for patulin production were indicated.

Concern has been expressed about possible mycotoxin production on cheeses since mold growth can occur on cheese during ripening, curing, and refrigerated storage (3, 7). In one study, up to 82% of the molds isolated from cheddar cheeses were observed to be Penicillium species, and 4% of these isolates were found to produce patulin (3). The work reported here describes a study of the effects of carbohydrate and nitrogen sources derived from milk on patulin production by a strain of P. patulum isolated from cheddar cheese.

MATERIALS AND METHODS

Organism. A Penicillium isolate, obtained from cheddar cheese, was identified as P. patulum by using the keys of Raper and Thom (16). The identification was confirmed by D. I. Fennell of the Northern Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois. The organism was carried on potato-dextrose slants stored at 5 C.

Verification of patulin. Patulin production by the organism was confirmed by comparing crystalline patulin obtained from ethyl acetate extracts of potato-dextrose and Czapeks-Dox-glucose broth cultures of the organism with known patulin obtained from the laboratory of T. M. McCalla. This was done.
on the basis of melting point, infrared and ultraviolet absorption spectra, color reactions of patulin derivatives, and $R_f$ values on thin-layer chromatography plates developed in benzene-methanol-acetic acid (90:5:5, vol/vol/vol) and toluene-ethyl acetate-formic acid (60:30:10, vol/vol/vol). Derivatives of patulin were made using ammonia fumes and 4% phenylhydrazine and were compared with internal and external standards (19).

Substrates. Several basal media were employed to study growth and patulin production by 

**P. patulum.** Potato-dextrose broth, prepared by the method of Norstadt and McCalla (11), was used as a control substrate. Czapeks-Dox broth was employed as a basal salts medium. It consisted of: KCl, 0.71 g; MgSO$_4$ $\cdot$ 7H$_2$O, 0.71 g; H$_2$K$_2$PO$_4$, 1.43 g; FeSO$_4$, 0.01 g; and distilled water, 1,000 ml. By using this basal medium, carbohydrate and nitrogen sources were compared. The carbohydrates glucose and lactose were studied. Stock solutions (10%) of the sugars were prepared and sterilized by filtration. The nitrogen sources, added to the basal salts solution before heat sterilization, were as follows: NaNO$_3$, 4.29 g/liter; peptonic milk (Difco Laboratories, Detroit, Mich.), 26.8 g/liter; or acid-precipitated casein, 8.4 g/liter. After autoclaving at 121 C for 15 min, the solutions had a final pH of about 7.0. Carbohydrate solutions were then added in sufficient quantity to give levels of glucose or lactose of 3%. Filter-sterilized distilled water in the same quantity as the carbohydrate solutions was employed in the treatments having no carbohydrate. The final substrates thus obtained were: Czapeks-Dox-glucose (3% glucose-sodium nitrate, 3% glucose-peptonized milk, and 4% glucose-casein media), Czapeks-Dox-lactose (3% lactose-sodium nitrate, 3% lactose-peptonized milk, and 4% lactose-casein media), and basal salt-peptonized milk and basal salts-casein media. The peptonized milk contributed 0.7% lactose, as determined by a phenol colorimetric method (5), to media in which it was employed.

Patulin production in systems with little or no carbohydrate but with high concentrations of casein was studied using two casein slurries. One slurry was prepared from acid-precipitated casein that had been washed three times in distilled water after removal of the whey; it still contained trace amounts of salts and lactose. The second was prepared from acid-precipitated casein that was washed three more times to make it essentially free from any carbohydrate and salts. It gave no browning reaction when heated in an oven to 140 C for 4 h. Each type of casein was used to make a 25% slurry in distilled water. After heating at 121 C for 5 min to kill vegetative cells and mold spores, the pH of the medium was about 5.0.

Prior to sterilization, all of the substrates were dispensed in 50-$ml$ quantities into 250-$ml$ Erlenmeyer flasks. Each flask was inoculated with about 10$^8$ spores from a 7- to 10-day-old potato-dextrose slant culture of 

**P. patulum.** The cultures were incubated for 2 weeks at 25 C or for 8.5 weeks at 5 C. Three replicates were done in duplicate for each treatment.

The organism was also grown on cheddar cheese
ent also affected patulin production by *P. patulum*. Glucose supported toxin production better than lactose in the presence of NaNO₃ at 25 C but essentially made no difference at 5 C. Cultures containing peptonized milk or casein plus glucose generally gave better yields of patulin than when lactose was present. An exception to this was the slightly higher levels of patulin obtained by a basal salts-peptonized milk-lactose medium at 25 C. Lactose cultures in this case yielded 253 μg of patulin/ml of media. Lactose in very low concentrations (0.75%) in peptonized milk supported patulin production, but in much smaller amounts than when substantial carbohydrate was present. These results support earlier findings that glucose is an optimal carbohydrate source for patulin production (9). Cultures containing little or no carbohydrate were unaffected by temperature; growth was scant and only very low amounts of patulin were produced at either temperature.

The nitrogen source also affected the amount of patulin produced by *P. patulum*. Inorganic nitrogen in the presence of glucose supported large amounts of toxin production at 25 C, but it supported lesser amounts in the presence of lactose (Table 1). The amount of patulin obtained from lactose and inorganic nitrogen was approximately the same as that obtained when milk nitrogen sources were present at both incubation temperatures. One exception was that only 84 μg of patulin/ml of basal salts-lactose-peptonized milk was obtained at 5 C incubation.

Organic nitrogen from milk lowered patulin yields at 25 C, but it supported substantially more patulin production at 5 C than did inorganic nitrogen when used in combination with glucose. An exception was the basal salts-lactose-casein media, which actually supported more toxin production at 5 C than at 25 C incubation. Overall, casein in the basal salts medium supported more toxin production than did peptonized milk in the presence of glucose. Casein also supported some patulin production (4 μg/ml) by *P. patulum* in the absence of all carbohydrate. Mold growth on casein without carbohydrate in the basal salts medium was heavy at both 5 and 25 C, even though toxin production was very low. Mold growth on 25% slurries of unwashed and washed casein was heavy but produced no detectable patulin. Washed casein, free from any carbohydrates, did not support mold growth at 5 C.

The organism grew abundantly on the shredded cheddar cheese at both 5 and 25 C. However, thin-layer chromatography examination of extracts of the moldy cheese did not detect the presence of patulin in any of the samples.

**DISCUSSION**

The results reported here indicate that lactose and casein supported less patulin production by *P. patulum* than did glucose and inorganic salts at 25 C. At 5 C, lactose supported a similar amount of patulin production as glucose. However, peptonized milk and casein supported more patulin production at 5 C with either glucose or lactose than the inorganic nitrogen did. In the absence of added carbohydrate at 5 C, both casein and peptonized milk supported patulin production in amounts similar to inorganic nitrogen combined with either carbohydrate. In all of these cases, the total amount of patulin produced was much less than that produced under optimum conditions at 25 C with glucose and inorganic nitrogen, even though growth of the mold was quite heavy. This suggests that dairy products such as natural cheeses, which are high in casein and low in total carbohydrate, might not be good substrates for patulin production, particularly if stored at low temperatures. On the other hand, in the presence of proper nutrients and given sufficient time, *P. patulum* produced substantial levels of patulin at low temperatures. Thus, refrigeration alone would not be sufficient to

**TABLE 1. Patulin yields (micrograms/milliliter) from *P. patulum* grown at 25 and 5 C for 2 and 8.5 weeks, respectively**

| Broth substrate | Incubation (25 C) | Temp (5 C) |
|-----------------|------------------|-----------|
| Potato-dextrose | 2,771a           | 674a      |
| Czapeks-Dox-glucose | 1,369a        | 14a       |
| Basal salts-casein | 206a           | 15a       |
| Basal salts-peptonized milk | 192a | 152a |
| Basal salts-peptonized milk | 253a | 84a |
| Basal salts-peptonized milk | 16a | 13a |
| Basal salts-peptonized milk | 676b | 458b |
| Basal salts-peptonized milk | 157d | 241d |
| Casein (25% slurry) | ND<sup>a</sup> | ND<sup>a</sup> |
| Unwashed | ND<sup>a</sup> | ND<sup>a</sup> |
| Washed | ND<sup>a</sup> | NG<sup>a</sup> |

<sup>a</sup> Values reported are the average of six samples with three assays each.
<sup>b</sup> Light mycelium with >50% sporulation.
<sup>c</sup> Light mycelium with <50% sporulation.
<sup>d</sup> Heavy mycelium with <50% sporulation.
<sup>e</sup> Heavy mycelium with >50% sporulation.
<sup>f</sup> NG, No growth.
<sup>g</sup> ND, None detected.
completely prevent patulin production in a food product. These data are in agreement with those of Sommer et al. (20).

When the organism was grown on shredded cheddar cheese, the results supported these findings. Mold growth was abundant on cheese at both 5 and 25 C, but no patulin was detected in the extracts of any of the complete mold and cheese cultures.

In previous studies with meats, breads, flours, and fruit juices, it has been suggested that patulin may react with sulphydryl-containing compounds present in these foods and may be rendered nontoxic and nondetectable by physicochemical methods (10, 17, 19, 22). This could have occurred in this study in the substrates containing organic nitrogen and in the cheese. On the other hand, no patulin was detected in washed mycelia grown on the 25% casein slurries, whereas patulin was detected in mycelia grown on the basal salts media containing casein. This suggests that casein alone, the lack of carbohydrate, the lack of certain salts, and/or the presence of inhibitory factors associated with high levels of casein may have prevented patulin synthesis even though mold growth was able to occur.

Patulin production in a basal salts liquid medium by a strain of P. patulum isolated from cheddar cheese was affected by the incubation temperature, the amount and kind of carbohydrate, and the nitrogen source. An interaction between these factors apparently occurred. Growth and toxin production occurred at 5 C when provided lactose and nitrogen from milk. Thus, refrigerated storage alone would not prevent patulin production by P. patulum. The possibility exists that dairy products on which growth of P. patulum has occurred may become contaminated with patulin. However, the amount of contamination would probably be low since, in general, these products would be expected to be unfavorable substrates for patulin production. This conclusion is supported by the fact that no patulin was detected in cheese that was heavily molded by P. patulum. Additionally, the presence of sulphydryl groups in casein in products such as cheese would be expected to detoxify the small amounts of patulin that may be produced. Whether or not these reactions could be reversed to yield free patulin by such factors as low pH or the action of digestive enzymes is not known at this time.

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