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Penicillic Acid Production in Submerged Culture†

L. A. LINDENFELSER AND A. CIEGLER

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

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Twenty known penicillic acid (PA)-producing *Aspergillus* and *Penicillium* cultures were grown under various conditions in shaken flasks to determine the highest yielding strains and their requirements for maximum toxin production. Abilities of the cultures to utilize eight different carbon sources in Raulin-Thom medium for mycotoxin synthesis were determined at four different incubation temperatures: 15, 20, 25, and 28°C. Of the 20 cultures, *P. cyclopium* NRRL 1888 was superior, yielding up to 4 mg of PA per ml, with mannitol as the carbon source and 25°C as the incubation temperature. Fifteen of the cultures elaborated lesser amounts of PA, whereas four strains yielded none under the test conditions. Whey from the manufacture of cottage cheese by the cultured process was also a satisfactory medium for PA production. In whey medium, yields up to 3 mg/ml were obtained with *P. cyclopium* NRRL 1888.

Mycotoxins were formed in grain as metabolic products of certain molds. One of the mycotoxins that may occur in moldy grain, particularly corn, is penicillic acid (PA) (γ-keto-β-methoxy-δ-methylene-A$^{\alpha}$-hexenoic acid). A number of *Penicillium* species causing blue-eye mold of stored corn were found to be capable of producing PA (9), and one species in particular, *P. martensii,* elaborated large quantities of the toxin on artificially inoculated corn (18). Two strains of *Aspergillus ochraceus* isolated from poultry feed were found to be capable of producing PA, along with another mycotoxin, ochratoxin A (2). In addition, Ciegler (7) observed that *A. sclerotiorum, A. alliaceus, A. ostianus, A. melleus,* and *A. sulphureus* (now *A. auricomus*) cultures were capable of concomitant production of these two toxins. Numerous other molds have also been reported to synthesize PA: *A. quercinus* (now *A. melleus*) (14); *P. baarnense* (6); *P. cyclopium* (3, 5); *P. fennelliae* (22); *P. madriti* (4); *P. palitans* (9); *P. puberulum, P. stoloniferum* (1); *P. suavolens* (now *P. roqueforti*), and *P. thomii* (17). The large number of strains capable of elaborating the mycotoxin indicates the ubiquity of PA-producing organisms in nature and the possibility for presence of the mycotoxin in stored high-moisture grain.

Although some studies have been made to determine effects of PA on small laboratory animals (1, 12, 13, 19), no published data have appeared on its toxicity to larger animals. And, contrary to current toxicity indications, a German patent (K. Schröder, German Patent 1,026,606, March 1958) advocates adding PA to animal feed as a supplement. Definitive tests are needed for obtaining toxicological data for PA in large animals.

To conduct tests with large animals, considerable quantities of PA would be required. The purpose of our experiments was to develop conditions under which optimal yields of the toxin might be obtained from select cultures in submerged fermentation.

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MATERIALS AND METHODS

Cultures and inoculum. Twenty potential PA-producing cultures were obtained from the ARS Culture Collection and maintained on malt extract or potato-dextrose agar. Three types of inocula were used in the shaken flasks. The first was actively growing vegetative cultures, which were used for inoculating flasks of Raulin-Thom medium. Inoculum was prepared by washing the spores from a 10-day-old potato-dextrose agar slant culture (25 by 150 mm) into 100 ml of Raulin-Thom medium in a 300-ml Erlenmeyer flask and incubating for 2 to 3 days on a rotary shaker at 25°C. The resultant culture was washed twice with sterile water and used at the rate of 5% (vol/vol) as inoculum. The second was vegetative inoculum for whey medium flasks, which were prepared by adding 2% of the spore suspension described below to 100 ml of whey medium in a 300-ml Erlenmeyer flask and incubating on a rotary shaker 36 h at 25°C. The resultant mycelial culture was used at the rate of 2% (vol/vol). The third was a spore suspension, which was made by washing spores from two potato-dextrose agar slant cultures (25 by 150 mm) in 50 ml of sterile

† Send reprint requests to C. W. Hesseltine at the Northern Regional Research Center.

water. The resultant suspension contained about 10^6 spores per ml and was used at the rate of 2% (vol/vol) as inoculum in Raulin-Thom shaken flasks.

Shaken-flask culture. Shaken-flask fermentations were conducted in 300-ml Erlenmeyer flasks, each containing 100 ml of medium sterilized by autoclaving. The 20 mold cultures were grown in duplicate flasks of Raulin-Thom medium (11) in which eight different compounds were tried individually as carbon sources. The compounds were glucose, fructose, sucrose, maltose, lactose, glycerol, mannitol, and soluble starch. All were used at 5% concentration, the same as that of glucose in the original Raulin-Thom formula. Flasks were incubated at 25°C on a rotary shaker (250 rpm, 5-cm strokes). Samples were aseptically withdrawn periodically for analyses.

Wheys obtained as byproducts of both the cultured and acid processes of cottage cheese manufacture were also tried as liquid substrates for PA production. Whey normally contains about 5% lactose (16). Denatured proteins resulting from autoclave sterilization were allowed to remain in the medium.

In the effort to increase PA yields, several experimental conditions were tried. In both Raulin-Thom (containing mannitol in place of glucose) and whey media, incubation temperature was varied; temperatures tested were 15, 20, 25, and 28°C. In whey medium, starting the fermentation at 28°C to promote faster initial growth was tried as a possible means for achieving earlier peak yields; flasks were incubated first at 28°C for periods varying from 1 to 3 days, then at 20 or 25°C for the remainder of the fermentation period. Both smooth and baffled flasks were tested.

PA and lactose analyses. PA content was determined during fermentations by thin-layer chromatography. Culture filtrates, appropriately diluted with water, were spotted directly onto glass plates (20 by 20 cm) layered with 0.5 ml of Silica Gel G-HR (Brinkmann Instruments Inc., Westbury, N.Y.). Plates were developed with chloroform–ethyl acetate–formic acid (60:40:1) in a lined tank (8). After the solvent fronts had traveled about 16 cm, plates were removed and air dried for about 5 min in a hood. Plates were then sprayed with 1% methanolic solution of diphenylboric acid ethanolamine complex (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and heated for 20 min at 100°C in an oven (20). This spray yields a fluorescent derivative more intense than ammonia and enables detection of smaller quantities of PA. PA appeared as a light-blue fluorescing spot under ultraviolet light. Quantitative determinations were made by visual comparisons with various amounts of pure PA standard under long-wave (366 nm) ultraviolet light. Lactose content of culture filtrates was determined with an automatic analyzer (21) by the potassium ferricyanide method (15).

RESULTS AND DISCUSSION

Considering yields without regard to carbon source of the 20 mold cultures tried in these studies, we determined that *P. cyclopium* NRRL 1888 was superior to all others for its ability to elaborate PA, producing up to 4 mg/ml (Table 1). The second highest producer was *P. puberulum* NRRL 3672, with yields up to 2 mg/ml, whereas *P. martensii* NRRL 3612 produced amounts up to 1 mg/ml. Six of the mold strains were low producers with top yields of 0.2 to 0.3 mg/ml, seven were in the trace category with highest amounts ranging from 0.01 to 0.06 mg/ml, and four produced none at all under any of the test conditions.

As expected, there was some strain variation among the various species tested; e.g., one culture of the four *P. cyclopium* strains and one of the three *P. puberulum* strains produced no detectable PA. In an early publication (17), *P. roquefortii* and *P. thomii* had been reported to synthesize PA; however, in our studies, no production was detected from either of the two strains we analyzed.

Spore suspension was the most satisfactory inoculum for shaken flasks of Raulin-Thom medium. Spore inoculum produced a fine, homogeneous mycelial growth and the highest PA yields in shaken flasks. In contrast, vegetative inoculum produced a nonhomogeneous growth of pellets of various sizes and relatively low amounts of PA. However, for whey medium in shaken flasks, the 26-h whey inoculum was most satisfactory; it promoted PA yields earlier and higher than either spore suspension or Raulin-Thom washed mycelial inoculum.

Ability to utilize specific carbon compounds for synthesizing PA varied widely among the mold strains tested, and no general pattern was apparent. For *P. cyclopium* NRRL 1888, the highest PA-producing strain, mannitol as the carbon source was superior; but maltose, lactose, and glycerol also gave good yields. For *P. puberulum* NRRL 3672, the second highest PA-producing strain, glycerol as the carbon source was superior, whereas yields from other carbon compounds were considerably lower. For *P. martensii* NRRL 3612, the third highest PA-producer, maltose and glycerol as carbon sources were best, whereas all others were inferior. Of the eight compounds, soluble starch was generally poor as a carbon source.

Of the four incubation temperatures tried, the two intermediates, 20 and 25°C, were most satisfactory (Fig. 1). In tests with the highest producing strain, *P. cyclopium* NRRL 1888 in Raulin-Thom (mannitol) medium, equivalent yields were obtained at either temperature, but fermentations conducted at the two temperature extremes, 15 and 28°C, promoted lower PA yields. At 15°C, growth was slower and not as heavy as with the higher temperatures. Incubation at the other extreme, 28°C, yielded quick, heavy mycelial growth in the flasks, but yields were lower than at either 20 or 25°C; e.g., incu-

TABLE 1. *Production of penicillic acid by 20 mold cultures grown at 25°C in shaken flasks of Raulin-Thom medium with eight different compounds as carbon sources*

Organism	NRRL no.	PA yields[a] from various carbon sources (mg/ml)							
		G[b]	F	S	M	L	Glyc	Mann	SS
A. auricomus	391	0.08[c]	0.15	0.1	0.2	0.02	0.05	0.3	0.1
A. melleus	394	0.12	0.15	0.2	0.1	0.0	0.0	0.09	0.1
A. ochraceus	3174	0.0	0.06	0.0	0.2	0.0	0.1	0.13	0.0
P. baarnense	2086	0.01	0.01	0.02	0.02	0.0	0.0	0.12	0.0
P. cyclopium	1888	1.3	1.7	1.7	2.0	2.0	2.0	4.0	0.5
P. cyclopium	6314	0.02	0.01	0.0	0.02	0.01	0.01	0.01	0.0
P. cyclopium	6315	0.0	0.0	0.01	0.01	0.01	0.0	0.0	0.06
P. cyclopium	6316	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P. fennellii	3697	0.0	0.0	0.0	0.01	0.15	0.02	0.0	0.0
P. madriti	3675A	0.0	0.1	0.3	0.02	0.02	0.2	0.0	0.0
P. martensii	3612	0.4	0.5	0.13	1.0	0.17	0.9	0.5	0.0
P. martensii	3747	0.0	0.0	0.0	0.0	0.02	0.04	0.03	0.0
P. martensii	6317	0.0	0.0	0.01	0.01	0.01	0.0	0.0	0.0
P. martensii	6318	0.2	0.1	0.3	0.03	0.0	0.0	0.0	0.0
P. puberulum	2040	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P. puberulum	3564	0.2	0.2	0.17	0.25	0.0	0.0	0.0	0.0
P. puberulum	3672	1.0	1.25	1.16	1.0	0.5	2.0	1.0	0.1
P. roquefortii	856	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P. stoloniferum	859	0.0	0.01	0.0	0.0	0.0	0.05	0.0	0.0
P. thomii	1640	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] Yields represent highest amounts detected in samples taken periodically during the fermentation.

[b] Abbreviations for carbon sources: G, glucose; F, fructose; S, sucrose; M, maltose; L, lactose; Glyc, glycerol; Mann, mannitol; and SS, soluble starch.

[c] Numbers represent averages of values from duplicate flasks. Statistically, the relative standard deviation per flask is 12%; yield changes exceeding 25% are significant at the 95% confidence level.

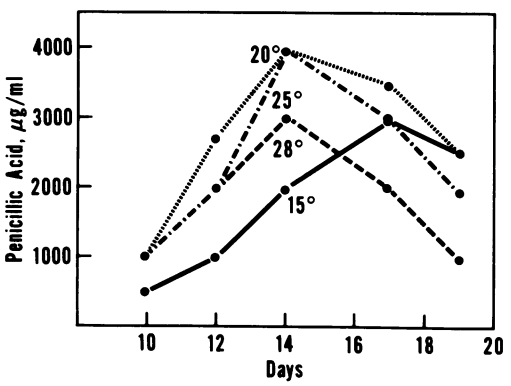

FIG. 1. *Effects of four different incubation temperatures on PA yields in a typical fermentation, with Penicillium cyclopium NRRL 1888 in Raulin-Thom medium containing mannitol as the carbon source.*

bating at 28°C for 1, 2, or 3 days before continuing at either 20 or 25°C produced lower PA amounts than continuous incubation at either 20 or 25°C.

The relatively high cost of mannitol but generally satisfactory yields by lactose suggested the use of whey. Whey from the microbial process of cottage cheese manufacture was an excellent medium for PA production. With *P. cyclopium* NRRL 1888 as a fermentation organism, yields of 2 to 3 mg/ml were regularly obtained with whey medium, compared with 3 to 4 mg/ml with the more expensive Raulin-Thom medium containing mannitol in place of glucose. For reasons not known, whey from acid-process cheese production was an unsatisfactory medium, promoting PA yields of only 0 to 10 µg/ml. Because of the normal mineral content of whey (0.6%) (16), supplementing with inorganic salts was not explored. Amending either type whey with yeast extract promoted quicker and heavier mycelial growth. However, while yeast extract stimulated cell proliferation, PA synthesis was suppressed.

When the PA peak was reached in whey medium (Fig. 2), the lactose content of the substrate simultaneously reached its lowest level. Also at that point, the total solids had been largely utilized and the pH had gradually risen from an initial 4.7 to a plateau at 7.5 to 7.8. After the PA had reached a maximum concentration, at about 10 days, the level remained constant for 1 to 2 days and then fell sharply. Also, it is known that at pH 7.5 to 7.8, the PA molecule exists in open form and might be unstable or react with lysing mycelial components (10), reducing its level in the substrate. The duration of the high PA plateau may afford an advantage, since it allows time to stop the fermentation at

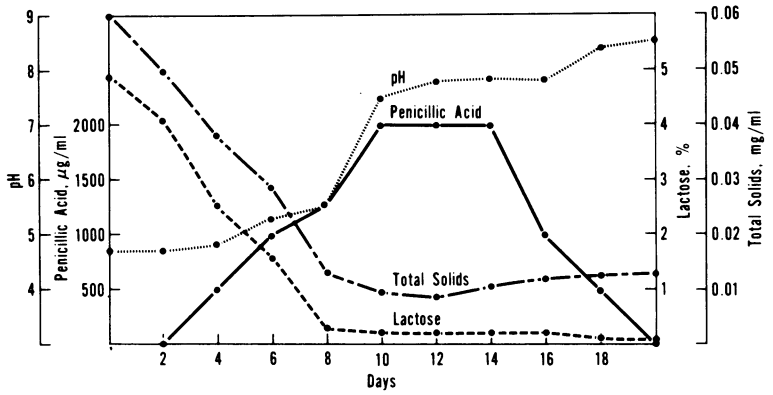

FIG. 2. *Relationship of PA production to lactose utilization, pH, and total solids in a typical fermentation, with P. cyclopium NRRL 1888 in whey medium.*

the PA optimum, before the beginning of the rapid decline.

Whey, as a by-product of the dairy industry, ordinarily is plentiful and readily available from cottage cheese manufacturers. Use of whey provides an inexpensive fermentation medium when lactose can serve as a carbon source.

Fermentations conducted in baffled 2,800-ml Fernbach flasks resulted in greatly reduced PA yields compared to those of regular, nonbaffled Fernbach flasks. Yields in the two types of flasks, using *P. cyclopium* NRRL 1888 and whey medium, were 1 mg/ml with baffled flasks and 3.5 mg/ml with regular, nonbaffled flasks. These results indicate that excess aeration could limit PA yield in this fermentation.

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