Effect of Heavy Water on Three High Producing Strains of *Penicillium chrysogenum*

KASHEED MOHAMMED, DANIEL A. NONA, and MARTIN I. BLAKE

Abstract □ Three high antibiotic-producing strains of *Penicillium chrysogenum* (Wisconsin Q176, 51-20F3, and 49-133) were examined for their ability to grow and produce penicillin in nutrient solutions containing pure heavy water. Penicillin titer values and pH changes were observed over the culture study period. Wisconsin Q176 responded most favorably. Continuous feeding of dextrose and potassium phenylacetate gave better antibiotic titers than a single addition of the two components. With either feeding regimen, potassium phenylacetate was more effective as a precursor than *N*-(β-hydroxyethyl)phenylacetamide or α-phenylacetamide. L-Leucine added together with potassium phenylacetate further enhanced penicillin production. The production of the yellow pigment associated with Wisconsin Q176 in protio nutrient cultures was suppressed in deuterium oxide nutrient cultures.

Keyphrases □ Deuterium oxide effect—*Penicillium chrysogenum* penicillin production □ Penicillin production, *P. chrysogenum*—deuterium oxide effect □ Media additives, effect—penicillin production □ Additives, single, multiple feedings—penicillin production, *P. chrysogenum*

Extensive studies have been reported in the literature pertaining to the effects of deuterium oxide on various organisms. In each case, deuterium oxide was substituted for water either partially or *in toto* in the nutrient medium. These investigations have been summarized by Flaumenhaft *et al.* (1) and by Katz (2), who thoroughly surveyed deuterium oxide effects on organisms.

Nona *et al.* (3) studied the effect of deuterium oxide on the culturing of *Penicillium janczewskii* and the antifungal activity of fully deuteriated griseofulvin. In an earlier report, Mohammed *et al.* (4) investigated the effect on the growth and antibiotic production of a number of low producing strains of *Penicillium*. Mycelial dry weights, pH variations, and penicillin potency over a 90-day study period were included in that study.

In the present study three high producing strains of *Penicillium*, the parent strain and two mutants, were cultured in water and deuterium oxide by a continuous feeding technique and by a single addition of dextrose and a precursor.

EXPERIMENTAL

Strains of *Penicillium chrysogenum*.—Three high producing strains of *Penicillium* were used in the present study: Wisconsin Q176[1] and two of its mutants, Wisconsin 51-20F3[2] and Wisconsin 49-133.[2] Two methods for preserving the stock cultures were utilized: on agar slants (4) at 5° and by lyophilization of healthy spores. The spores were suspended in 1.0 ml. of calf serum and the suspension was freeze-dried. The lyophil cultures were stored at 5°. Propagation of the spores from the lyophil tube was effected by first suspending the freeze-dried material in Sabouraud liquid medium. Agar slants were then prepared from this enriched medium. Fungal spores obtained from these slants were used for inoculation of the seed inoculum medium.

Preparation of the Seed Inoculum.—A standard salt mixture was used throughout this study. The composition of the mixture was (g./100 ml. of solvent): $NaNO_3$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.025; KH_2PO_4, 0.05; $ZnSO_4 \cdot 7H_2O$, 0.005; $CuSO_4 \cdot 5H_2O$, 0.0003; $FeSO_4 \cdot 7H_2O$, 0.005. To prepare the seed inoculum 7.0 g. of dextrose was added to the basic salt mixture. The pH of the solution was adjusted to 6.5 with 25% potassium hydroxide solution. Finally calcium carbonate, 1.2 g., was added. Ninety milliliters of this medium was placed in a 1-l. conical flask; the flask was stoppered with nonabsorbent cotton, capped with aluminum foil, and then sterilized. One loopful of spores obtained from an agar slant prepared as described under *Strains of Penicillium chrysogenum* was used to inoculate the flask. A thick vegetative growth was obtained within 48–54 hr. which was then used for inoculating the fermentation nutrient medium.

Protio and Deuterio Nutrient Media.—The protio and the deuterio fermentation media both had the same nutrient composition: the basic salt mixture as indicated under *Preparation of Seed Inoculum*, together with dextrose and a precursor, or a combination of precursors, as listed under *Procedures*. The protio nutrient medium and a deuterio nutrient medium contained distilled water and pure deuterium oxide (99.6%) as the solvents, respectively. However, in preparing the deuterio nutrient medium, the exchangeable hydrogen of the components was replaced with deuterium by dissolving the nutrient mixture in deuterium oxide and lyophilizing. This procedure was repeated to ensure complete exchange. Finally the pH was adjusted to 6.5 with 25% (w/v) potassium hydroxide in deuterium oxide, and 1.0 g. of calcium carbonate/100 ml. of solution was added. After sterilization, 10 ml. of inoculum was added to each flask/100 ml. of nutrient medium. In the case of the deuterio inoculation, the seed inoculum was filtered through a Seitz pressure filter, and the vegetative mycelial growth was washed with deuterium oxide under aseptic conditions prior to inoculation of the sterilized deuterio fermentation nutrient medium.

Procedures.—The sterilization techniques, the measurement of pH, and penicillin bioassay determinations have been described in an earlier paper (4). Preparation of the seed inoculum and all fermentations were conducted on an Eberbach rotary shaker at 210 r.p.m. Incubation temperature was kept at 26–28°, and the relative humidity was between 20 and 30%. In all cases, the pH of the fermentation medium was adjusted to 6.5 with 25% potassium hydroxide solution, and 1.0 g. of calcium carbonate was added/100 ml. of medium. One hundred milliliters of nutrient medium was added to a 3-l. conical flask; the flask was plugged with nonabsorbent cotton, capped with aluminum foil, and sterilized.

One series of experiments involved a single addition of dextrose and a precursor or precursors to the basic salt mixture prior to sterilization. The concentration of dextrose was 3.0%. The following precursors were studied (g./100 ml. of nutrient solution): potassium phenylacetate, 0.3; α-phenylacetamide, 0.3; *N*-(β-hydroxyethyl)phenylacetamide, 0.3; and L-leucine, 0.06.

In a second study, dextrose, 2.0 g., and potassium phenylacetate, 0.3 g., or *N*-(β-hydroxyethyl)phenylacetamide, 0.3 g., dissolved in 8.0 ml. of solvent were fed continuously to the basic salt mixture which contained 1.0% dextrose.

In a third experiment, a solution of dextrose containing 2.0 g. in 8.0 ml. of solvent was fed continuously to the basic salt mixture which contained, in addition to 1.0% dextrose, 0.3% α-phenylacetamide.

Following sterilization, the nutrient medium in each flask was inoculated with 10 ml. of seed inoculum. After fermentation had

[1] Obtained from Northern Utilization Research and Development Div., Peoria, IL 61604

[2] Supplied by Dr. J. F. Stauffer and Dr. M. P. Backus, Professors of Botany, University of Wisconsin, Madison, WI 53706

Table I—Penicillin Production, pH Plateau, and Time for Maximum Penicillin Production by *Penicillium chrysogenum* Strain, Wisconsin Q176

Additive	Peak Penicillin Production, u./ml.	Time for Maximum Production, days	pH Plateau
Potassium phenylacetate (H_2O)[a]	849	6.5	7.81–7.84
Potassium phenylacetate (D_2O)[a]	601	6.5	7.00–7.10
Potassium phenylacetate (H_2O)[b]	755	6.5	7.81–7.90
Potassium phenylacetate (D_2O)[b]	511	6.5	6.91–6.92
L-Leucine + Potassium phenylacetate (H_2O)[b]	896	7.5	7.82–7.91
L-Leucine + Potassium phenylacetate (D_2O)[b]	742	6.5	7.10–7.11
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[a]	812	7.5	7.79–7.84
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[a]	348	8.5	7.02–7.08
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[b]	751	7.5	7.89–7.95
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[b]	375	8.5	7.01–7.05
α-Phenylacetamide (H_2O)[a]	785	6.5	7.81–7.91
α-Phenylacetamide (D_2O)[a]	306	7.5	7.12–7.22
α-Phenylacetamide (H_2O)[b]	780	6.5	7.78–7.93
α-Phenylacetamide (D_2O)[b]	284	5.5	7.12–7.21

[a] Continuous feeding. [b] Single addition, prior to sterilization.

proceeded for 20–30 hr., the flasks containing 1.0% dextrose in the basic salt formula were fed continuously as indicated in the second and third experiments above. The pH of the continuous feed mixture was adjusted to 6.5 with 25% potassium hydroxide in water or deuterium oxide and sterilized separately in a 250-ml. conical flask. A 240-mm. length of rubber tubing was connected to the tip of a 10-ml. microburet equipped with a Teflon stopcock, and the top was plugged with nonabsorbent cotton. The apparatus was wrapped in aluminum foil and sterilized in a dry heat oven at 180° for 4–6 hr. A sterile transfer chamber was used for transferring the feed mixture to the buret. The rubber tubing from the buret was wrapped with the flask's sterile cotton plug and inserted into the flask in such a way as to have at least 100–120 mm. of the tubing extending inside the flask below the cotton plug. The culture nutrient flask was placed on the shaker with the buret fitted securely with a vinylized three-finger jaws clamp attached to a heavy-based buret stand. The solution was introduced at the rate of 0.08 ml. per hour and required 100 ± 4 hr. for the entire solution to be added. Triplicate flasks were prepared for each single addition and continuous feeding experiment.

RESULTS AND DISCUSSION

Growth Observation—The three strains of *Penicillium* studied grew well in the protio seed nutrient medium. Profuse growth was evident within 24 hr. The culture appeared very thick and gelatinous after 48 hr. Fermentation of the protio or deuterio culture medium which contained all the additives introduced at one time produced greater mycelial growth than the corresponding continuous slow feeding culture. Numerous globular pellets were produced in the protio fermentation cultures that had the nutrients added in a single addition. These pellets were tan in color and varied in size from 1 to 5 mm. in diameter. When deuterium oxide was used as the solvent, the organisms manifested morphological changes. Small spherical pellets appeared after 60–70 hr. of fermentation. These pellets were white to gray in color and varied in size from 1 to 3 mm. in diameter.

After 24 hr. fermentation, both the protio and deuterio cultures which contained an initial 1.0% dextrose in the basic salt mixture showed sparse pellet formation. These pellets varied in size from 1 to 4 mm. in diameter in the protio culture and 1 to 2 mm. in diameter in the deuterio culture. Continuous slow addition of the feed solution to either the protio or deuterio fermentation medium resulted in an enlargement rather than an increase in the number of pellets. After 150–160 hr., the increase in size of the pellets ranged from 4 to 8 mm. in diameter in the protio culture and 2 to 6 mm. in diameter in the deuterio culture.

A yellow pigment was secreted in all protio cultures fermented by Wisconsin Q176. This phenomenon characteristic of the strain was not observed in any of the deuterio fermentation cultures.

Penicillin Production—The three strains of *Penicillium* under study responded differently in protio and deuterio fermentation culture. Overall, penicillin production and the pH plateau were higher for the protio fermentation culture than for the corresponding deuterio fermentation culture.

Tables I, II, and III show penicillin production, pH plateau, and time for maximum penicillin production for strains Q176, 51-20F3, and 49–133. Continuous feeding of dextrose and potassium phenylacetate gave a higher penicillin assay than single addition. Soltero and Johnson (5) obtained increased penicillin titers by the continuous feeding of glucose or sucrose to the protio fermentation media. These workers used a single addition of lactose in the fermentation media as the control. In the present study, a repressive effect in penicillin yield was noted in all deuterio fermentation cultures. Similar observations were reported in an earlier paper (4). However, at that time, low antibiotic-producing strains of *Penicillium* were examined, and the additives were introduced in a single addi-

Table II—Penicillin Production, pH Plateau, and Time for Maximum Penicillin Production by *Penicillium chrysogenum* Strain, Wisconsin 51-20F3

Additive	Peak Penicillin Production, u./ml.	Time for Maximum Production, days	pH Plateau
Potassium phenylacetate (H_2O)[a]	1952	7.5	7.76–7.80
Potassium phenylacetate (D_2O)[a]	79	6.5	7.11–7.13
Potassium phenylacetate (H_2O)[b]	1352	6.5	7.63–7.65
Potassium phenylacetate (D_2O)[b]	21	6.5	7.11–7.15
L-Leucine + Potassium phenylacetate (H_2O)[b]	2173	7.5	7.87–7.91
L-Leucine + Potassium phenylacetate (D_2O)[b]	40	6.5	6.96–6.98
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[a]	1701	7.5	7.86–7.90
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[a]	79	6.5	6.94–6.97
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[b]	1607	6.5	7.89–7.91
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[b]	68	6.5	6.99–7.00
α-Phenylacetamide (H_2O)[a]	944	7.5	7.68–7.71
α-Phenylacetamide (D_2O)[a]	69	6.5	6.96–6.98
α-Phenylacetamide (H_2O)[b]	898	7.5	7.80–7.81
α-Phenylacetamide (D_2O)[b]	56	6.5	6.80–7.01

[a] Continuous feeding. [b] Single addition, prior to sterilization.

Table III—Penicillin Production, pH Plateau, and Time for Maximum Penicillin Production by *Penicillium chrysogenum* Strain, Wisconsin 49-133

Additive	Peak Penicillin Production, u./ml.	Time for Maximum Production, days	pH Plateau
Potassium phenylacetate (H_2O)[a]	1993	6.5	7.40–7.80
Potassium phenylacetate (D_2O)[a]	120	6.5	6.97–6.99
Potassium phenylacetate (H_2O)[b]	1372	6.5	7.19–7.32
Potassium phenylacetate (D_2O)[b]	20	6.5	7.00–7.02
L-Leucine + Potassium phenylacetate (H_2O)[b]	2397	6.5	7.86–7.91
L-Leucine + Potassium phenylacetate (D_2O)[b]	50	7.5	7.02–7.04
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[a]	1731	7.5	7.28–7.80
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[a]	80	7.5	6.98–7.00
N-(β-Hydroxyethyl)phenylacetamide (H_2O)[b]	1545	7.5	7.76–7.80
N-(β-Hydroxyethyl)phenylacetamide (D_2O)[b]	71	6.5	6.98–6.99
α-Phenylacetamide (H_2O)[a]	1164	7.5	7.69–7.74
α-Phenylacetamide (D_2O)[a]	112	7.5	7.07–7.10
α-Phenylacetamide (H_2O)[b]	1101	7.5	7.68–7.73
α-Phenylacetamide (D_2O)[b]	39	6.5	6.93–7.48

[a] Continuous feeding. [b] Single addition, prior to sterilization.

tion. Table I shows that continuous feeding of dextrose and potassium phenylacetate to the deuterio fermentation medium gave higher penicillin production than a single addition, 601 u./ml. *versus* 511 u./ml. In the corresponding deuterio experiments with mutant strains 51-20F3 and 49–133, the penicillin titers were drastically reduced. The two mutants gave higher penicillin titers than the parent strain (Q176) in protio fermentation culture. In deuterio cultures, the parent strain yielded more penicillin than the mutants. Christensen (6) indicated that the virulence of mutants placed in a different environment may equal, exceed, or be less than the parent strain. Changes in the original characteristics of the mutants usually result in altered production of enzymes and organic acids.

L-Leucine significantly stimulated penicillin production in protio fermentation culture (Tables I, II, and III). In deuterio fermentation culture enriched with the amino acid, the stimulating effect in penicillin production did not occur with strains 51-20F3 and 49–133. Strain Q176 produced 742 u./ml. with L-leucine and 511 u./ml. without L-leucine. This represents an increase of 45.2%. Foster (7) explained that leucine served as a precursor for the azolactone moiety of the penicillin molecule.

Continuous feeding or a single addition of dextrose and *N*-(β-hydroxyethyl)phenylacetamide in protio culture showed relatively high penicillin titers when fermented with either of the three strains of *Penicillium* (Tables I, II, and III). Penicillin production in the related deuterio fermentation culture with strains 51-20F3 and 49–133 (Tables II and III) showed about a twentyfold reduction in antibiotic production and about a twofold reduction with strain Q176 (Table I).

Dextrose administered by either feeding regime to α-phenylacetamide-enriched protio culture did not greatly affect antibiotic production. In deuterio fermentation culture, Wisconsin Q176 (Table I) gave higher penicillin titers than the other two strains.

Tables II and III show that strains 51–20F3 and 49–133 were limited in their capacity to produce antibiotic in a D_2O environment. This severe reduction in antibiotic production in each case is apparently related to a low pH plateau. With these two mutants, it appears higher pH plateaus are necessary to activate the enzyme system required for penicillin biosynthesis. The parent strain Q176 consistently maintained a higher pH plateau and higher penicillin titers in each deuterio fermentation culture studied.

Table I indicates that in deuterio fermentation culture, addition of the precursor potassium phenylacetate gave the highest penicillin titers among the precursors added singly. A combination of L-leucine and potassium phenylacetate further enhanced penicillin production. In deuterio fermentation culture (Tables II and III), severe repression in antibiotic production was evident with each precursor investigated. However, no significant differences were observed in the quantities of penicillin produced. Apparently the amount of penicillin produced in deuterio fermentation culture is not as dependent on the type of precursor used as on the strain of *Penicillium*.

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