Effect of Phosphate and Copper on the Fermentation of Hydroheptin

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The organism Streptomyces chartreusis IMRU 3962 produced a mixture of the antibiotics hydroheptin and chartreusin in fermentation broth. The addition of increasing levels of phosphate resulted in a corresponding increase in the production of both antibiotics, with maximum yields of 400 to 450 μg of chartreusin per ml and 80 to 100 μg of hydroheptin per ml at 0.45 to 0.55 M phosphate. Chartreusin was invariably produced at a higher ratio; however, a reversal in ratio to favor hydroheptin was attained when 0.03% copper sulfate was added to the medium, particularly at a 0.2 M KH2PO4 level, with antibiotic yields of 125 μg of hydroheptin per ml and 40 μg of chartreusin per ml.

Hydroheptin, a water-soluble polyene macrolide antifungal antibiotic, was found to be coproduced along with chartreusin in the fermentation broth of Streptomyces chartreusis IMRU 3962 (8). The initial yield was 2 μg hydroheptin per ml and 300 μg of chartreusin per ml. A medium consisting of high phosphate and copper levels was found to boost the production of hydroheptin.

The requirements for P, for the production of several polyene macrolides vary over a considerable range; low levels of phosphate are needed for the production of amphotericin A (5), while the addition of phosphate to the medium used for nystatin production decreased its yield (5). Relatively high phosphate levels were reported for candicidin (60 to 100 μg of P, per ml) (1), afyacin (330 μg/ml) (2), and mycoheptin (110 μg of P, per ml) (7). However, such levels are very low compared with the optimum level of P, (6.2 mg/ml) in the fermentation medium for hydroheptin production. This paper describes the fermentation of hydroheptin in such medium with high phosphate and copper levels.

MATERIALS AND METHODS

Culture. S. chartreusis IMRU 3962 frozen vials of vegetative mycelia (7) were used as the source of working stock cultures.

Seed medium. The seed medium consisted of 4% sucrose, 2% cotton seed meal (CSM) (Pharmamera, Traders Protein Division, Fort Worth, Tex.), 0.16% yeast extract, 0.2 M KH2PO4, and 0.1% KOH. The seed medium was placed in a 500-ml baffled shake flask (50 ml per flask).

Flask and shaker. Triple-baffled flasks (Belco Glass, Inc., Vineland, N.J.) were used with gauze closure. The flasks were shaken in model V shakers, 1-in. throw diameter (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) located in a 28°C room. The shaker speed was set at 245 rpm.

Antibiotic assay. The antibiotics hydroheptin and chartreusin were assayed spectrophotometrically (7). Except where indicated otherwise, antibiotics were assayed from 3-day fermentation broths.

Growth determinations. Microbial growths were measured by the dry weight method for fermentation in complex media and by the colorimetric method for synthetic media. For the

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HYDROHEPTIN FERMENTATION

FIG. 1. Production of hydroheptin and chartreusin at different phosphate (KH₂PO₄) levels in the presence and absence of copper (CuSO₄ · 5H₂O).

FIG. 2. Effect of minerals with positive redox potentials on the production of hydroheptin and chartreusin.

FIG. 3. Production of hydroheptin and chartreusin with the medium adjusted to different pH levels.

Hydrolyzed CSM as nitrogen source. CSM was hydrolyzed with 6 N HCl at 100°C for 18 h in sealed test tubes. After hydrolysis, the HCl was thoroughly evaporated at reduced pressure. The samples were then diluted with water and filtered through Whatman paper (no. 1). The filtrate was used as a medium constituent equivalent to 2% CSM, as in hydroheptin medium.

The following modifications were made: (i) no copper or phosphate added; (ii) with phosphate, no copper; and (iii) with phosphate and copper. With hydrolyzed CSM, the phosphate addition was sufficient for giving high hydroheptin yields with a minimal amount of chartreusin (Fig. 4). The addition of copper to the phosphate-containing medium inhibited growth as well as hydroheptin production.

Synthetic medium. The production of hydroheptin with the hydrolyzed CSM as nitrogen sources prompted an evaluation of a synthetic medium. The amino acids found in the CSM were roughly classified into two groups: acid labile (threonine, tryptophan, methionine, cystine, and serine) and acid resistant (lysine, histidine, isoleucine, valine, glycine, proline, alanine, arginine, leucine, aspartic, glutamic, tyrosine, and phenylalanine). Both groups were tested as nitrogen sources for antibiotic production. The concentrations of amino acids used simulated the levels found in the CMS as provided by the supplier (Pharmamedia). The rest of the

7nSO₄ · 7H₂O, MnSO₄ · H₂O, and CaCl₂ · H₂O were evaluated but did not significantly affect antibiotic yield.

pH and antibiotic production. The effect of different pH levels at constant phosphate concentrations was evaluated. Thus, the hydroheptin medium was adjusted to different pH levels with KOH. pH 5.8 was optimum for hydroheptin production; pH 6.0 gave slightly higher hydroheptin but also higher chartreusin (Fig. 3).

Several buffers were evaluated to determine whether pH and not phosphate is responsible for antibiotic production. The buffers tested included acetate (pH 5.0), citrate (pH 5.8), potassium acid phthalate (pH 5.8), and PIPES (1,4-piperazinebis-ethanesulfonic acid) (pH 5.8), replacing the phosphate in the hydroheptin medium. These buffers were tested at 0.2 M. Growths of the organism in these buffered media were comparable, but no detectable hydroheptin was produced in these buffered media.

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medium consisted of 4% sucrose, 0.2 M KH₂PO₄, and 0.003% of each of the following minerals: FeSO₄ · 6H₂O, CaCl₂ · 2H₂O, ZnSO₄ · 7H₂O, and MnSO₄ · H₂O. Also, growth factors (inositol, choline, niacin, and pantothenic acid) were added at concentrations simulating the CSM analyses. The inoculum was washed three times with sterile distilled water.

The acid-resistant group gave trace levels of hydroheptin, while the acid-labile group gave trace levels of chartreusin. These amino acids were subsequently tested in all possible combinations, and the following triplets produced hydroheptin: alanine-valine-glycine and proline-isoleucine-tyrosine. Further breakdown of the above combinations gave the following amino acid tandems as producers of hydroheptin: proline-isoleucine and alanine-proline (Table 1). These amino acids when used singly did not produce any antibiotic.

**DISCUSSION**

The addition of phosphate in the fermentation medium resulted in a corresponding increase in chartreusin and hydroheptin yields, and the addition of copper to the medium suppressed the production of chartreusin. The concentration of copper (0.03%) in the medium obviously is much higher than the trace mineral requirement (0.0003%) for microorganisms (9). Hence, copper must function in the media in some other capacity, possibly as an oxidant or chelator.

Iron (E₀ = 0.74), which has about the same redox potential as copper (E₀ = 0.45), did not suppress chartreusin. Cobalt (E₀ = 1.82), on the other hand, increased chartreusin yield. This indicates that the redox potential of the metals does not correlate with chartreusin suppression.

Copper in the medium could thus function as a chelating agent. Since copper readily forms biuret complexes with proteins and higher peptides, it could have formed a biuret complex with the CSM; CSM contains 36% (wt/wt) protein. The resulting copper-CSM complex apparently is less favorable for chartreusin production than for hydroheptin. In this regard, the amino acids involved in the production of chartreusin might have been more tightly bound in the complex than those responsible for hydroheptin. Indeed, an evaluation of the different amino acids found in CSM showed that certain amino acids favored either chartreusin or hydroheptin. In the synthetic medium, copper was not needed for the production of the antibiotics.

A medium consisting of acid-hydrolyzed CSM as nitrogen source did not need copper to suppress chartreusin production. The addition of copper to the hydrolyzed CSM medium suppressed microbial growth and antibiotic production. Apparently, the characteristic peptide linkages essential for the formation of biuret complexes were destroyed by acid hydrolysis. Individual amino acids, except histidine, could not form copper-amino acid complexes, thereby leaving the cation unbound and toxic to the cells.

The requirement for high levels of phosphate is of interest, particularly for this organism in the biosynthesis of hydroheptin. Biosynthesis of polypeptide macrolides (3) and chartreusin (4) both follow the polyketide route. In the biosynthetic cascade, NADPH is necessary as a reducing agent in the condensation process (6). Since the synthesis of NADPH involves phosphorylated intermediates, the organism may specifically require high phosphate levels in the media to favor or stabilize the synthesis of such intermediates.

**LITERATURE CITED**


