NUTRITIONAL REQUIREMENTS FOR HYDROGENOMONAS EUTROPHA

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Received for publication August 30, 1961

ABSTRACT

REPASKE, ROY (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Nutritional requirements for Hydrogenomonas eutropha. J. Bacteriol. 83: 418-422. 1962.—A simple apparatus for the autotrophic cultivation of Hydrogenomonas eutropha in 100-ml shake cultures is described. Nitrogen, in the form of ammonium, nitrate, or urea, was used for growth; nitrite could not be utilized. Optimal growth occurred at pH 6.4 to 6.8 at 30 C. H. eutropha grew best in an atmosphere containing 15 to 25% oxygen and 10% carbon dioxide. Below these concentrations each of the gases was limiting. Growth was shown to be dependent on iron, and the rate of growth was a function of iron concentration and its state of oxidation.

Although hydrogenomonads have remarkable synthetic capabilities, they have not been studied as extensively as might be expected. Investigations with hydrogenomonads have usually involved hydrogenase, carbon dioxide fixation, or the oxyhydrogen reaction, and in these studies, growth characteristics of the organisms have received only incidental attention. Perhaps the limited interest is not surprising because of the restrictions imposed by growth on solid medium, by the protracted incubation time, and by the hazardous gas mixture required. As a result of the work of Cohen and Burris (1955), who demonstrated that high cell yields of Hydrogenomonas facilis could be obtained in mass culture in liquid medium, the first two considerations no longer apply. The present paper describes some of the growth characteristics and nutritional requirements of autotrophically grown H. eutropha in liquid culture. [The culture was obtained from A. G. Marr. It was isolated and described as Hydrogenomonas sp. by Bovell (1957), who subsequently termed the organism H. eutropha.]

MATERIALS AND METHODS

The medium used for autotrophic culture of H. eutropha was essentially that of Cohen and Burris (1955), modified in respect to the trace mineral content. Since variations in pH and nutrients were to be tested, it was convenient to sterilize various stock solutions separately and to combine them aseptically when constituting the medium. The culture medium was prepared in 500-ml heavy-walled Erlenmeyer flasks containing 94 ml of sterile 0.025 M potassium phosphate buffer (pH 6.3) to which was added 1 ml each of solutions A, C, D, E, and 2 ml of solution B. Solution A contained CaCl2·2H2O, 1 g; NH4Cl, 10 g; and NaCl, 1 g per 100 ml; solution B contained NaHCO3, 5 g per 100 ml; solution C contained MgSO4·7H2O, 1 g per 100 ml; solution D contained Fe(NH4)2(SO4)2, 80 mg per 100 ml; and solution E contained the following trace elements per liter: CoCl2·6H2O, 0.2 mg; MnCl2·4H2O, 400 mg; CuSO4·5H2O, 2 mg; Na2MoO4·2H2O, 10 mg; and ZnSO4·7H2O, 10 mg. All solutions were sterilized by autoclaving except ferrous ammonium sulfate, which was sterilized by Seitz filtration. In early studies an equivalent quantity of ferric iron (FeCl3) was used in place of ferrous iron. Iron supplied as the ethylenediaminetetraacetic acid chelate was not as effective as the free salt.

The organisms were grown in a gas mixture containing 70% hydrogen, 20% oxygen, and 10% CO2, with exceptions as noted. Cohen and Burris (1955) found that growth of H. facilis, in a closed system, was limited when gas consumption resulted in a partial vacuum; this observation was confirmed with H. eutropha.

Best growth was obtained when the gas was maintained at atmospheric pressure by a water-displacing system (Fig. 1). A simple and effective water-displacing system was made with two 5-gal carboys; one served as a water reservoir (A) and the other (B) as a gas reservoir. The carboys were connected by two water bridges (C and D), each with an alternate long and short...
stem in opposite carboys, to make repeated transfer of water between bottles convenient. The gas mixture was prepared by filling the gas bottle with acidified water and displacing (through D) appropriate volumes of water into the water reservoir with each of the individual gases (gases added through E). As the organisms consumed gas, water was displaced back into the gas reservoir through the second bridge (C). The gas reservoir was connected to a glass manifold (F), which was supported above a New Brunswick gyrotry (water bath) shaker by rods fastened to the platform.

After the medium was inoculated with a 1 to 3% inoculum, the flask was fitted with a sterile rubber stopper assembly, containing a cotton-filled glass bulb used to filter the incoming gas and a culture-sampling tube. The sampling tube, which extended to the bottom of the flask, was designed to accommodate a syringe for withdrawing the culture. Between samplings, the external end of this tube was covered to maintain sterility. Inoculated culture flasks were connected to the gas manifold by rubber tubing and evacuated through the manifold with a water aspirator, and the gas mixture was admitted to the flask. A continuous supply of gas mixture was then available while the cultures were being incubated in the water bath maintained at 30°C. Under optimal growth conditions, 100 ml of culture consumed more than 1 liter of gas mixture in 24 hr.

Solid medium, for slants and streak plates, was prepared by the addition of 2% Noble agar (Difco) to the mineral salts medium. Iron salt was added after the agar had been sterilized, since autoclaved iron forms hydroxides not utilized by the organism (Ruhland, 1924). These cultures were placed in a Brewer jar from which the heating element had been removed. The jar was evacuated, connected to the gas reservoir, and the cultures were incubated at room temperature.

RESULTS

H. eutropha grows rapidly in liquid medium, reaching the stationary phase of growth in less than 24 hr. Figure 2 shows a typical growth curve based upon turbidity measured at 660 nm and total protein, determined by the biuret method of Stickland (1951). The average generation time, calculated from protein and optical density values, was 3.5 to 4 hr, and the final cell count usually obtained was $2 \times 10^9$ cells per ml. It was recently found that, when aeration was increased by making four baffles in the wall of the flasks, growth also increased; however, results reported in this paper were obtained with regular flasks.

Effect of temperature and pH. Figure 3 shows the effect of temperature on the rate and extent of growth. The maximal rate of growth occurred between 30 and 35°C. At 25°C the rate was somewhat less, and at 40°C the growth rate was reduced by more than 50%. Temperatures within this range had a pronounced influence on the extent of growth. Although the rate of growth at 30°C and 35°C was essentially the same, total growth in cultures at 35°C was approximately 25% less.
The effect of pH on the rate of growth is shown in Fig. 4. These represent maximal rates before the buffering capacity was overcome in the media at low pH. Even at pH 6.7, the pH began falling after 12 hr as a result of chloride accumulation after the bulk of the ammonium ion had been metabolized. Since the extent of growth paralleled the growth rate, pH 6.4 to 6.9 was considered optimal.

Nitrogen sources. Ammonium chloride, potassium nitrate, potassium nitrite, and urea were tested as nitrogen sources for autotrophic growth; of these, only nitrite did not support growth of H. eutropha. Atkinson (1955) reported that best growth of H. facilis occurred with ammonium salts between 0.01 and 0.03 M. In the mineral medium described in Methods, the final ammonium chloride concentration was 0.019 M, equivalent to 26 mg of total nitrogen per 100 ml of medium. A fully grown culture of H. eutropha contained 125 mg of total nitrogen, representing approximately 20 mg protein nitrogen or 77% of the initial ammonium nitrogen (Fig. 2). These data agreed with the finding that growth was limited if the ammonium chloride concentration was reduced 50%. Higher concentrations of ammonium chloride (0.038 M) were inhibitory for growth. A similar concentration of urea nitrogen also suppressed growth, but inhibition was caused at least in part by the pH which rose to 8.5.

Requirement for oxygen and carbon dioxide. H. eutropha was tolerant to as much as 25% oxygen.
In fact, equivalent growth (rate and extent) occurred in an atmosphere of 15 to 25% O₂; higher concentrations of oxygen were not tested. Some limitation on growth was observed when the oxygen was decreased to 10%, and with 5% oxygen, growth was poor. H. facilis (Schatz and Bovell, 1952), H. pantotropha (Kaserer, 1906), and H. carboxydovorans (Kistner, 1953) also were reported to be insensitive to high partial pressures of oxygen. This feature distinguishes these hydrogenomonads from H. vitrea, H. flava (Niklewski, 1910), and H. ruhlândii (Packer and Vishnia, 1955), which grew only in concentrations of oxygen below 10%.

Autotrophic growth of H. eutropha in 20% oxygen was not affected when the carbon dioxide was increased to 15%, but a 40 to 50% reduction in growth occurred when the carbon dioxide atmosphere was decreased to 5%. Restricted growth, imposed by 5% CO₂, was also evident with limiting (10%) oxygen.

Effect of iron. Autotrophically grown H. eutropha showed a dramatic response to milligram concentrations of added iron. With subsaturating amounts of iron, growth was a function of the concentration and the state of oxidation of iron. Carry-over of iron with the inoculum was minimized by using an inoculum prepared from a culture without added iron. A series of flasks containing the usual autotrophic medium, minus iron, were supplemented with appropriate dilutions of ferrous [Fe(NH₄)₂(SO₄)₂] or ferric [FeCl₃] iron to give final concentrations of 0.001, 0.005, 0.01, 0.05, and 0.1 μg of added iron per ml. After 24 hr incubation and again after 48 hr, samples were removed for optical density measurements (Fig. 5). Growth was considerably better with ferrous than with ferric iron at the concentrations shown. This difference disappeared when the iron content exceeded 1 μg per ml. Additional experiments of this type gave qualitatively similar results, although quantitative differences in growth were observed between experiments. These differences probably resulted from varying trace quantities of iron in each series of media. It should be possible to use this organism for milligram iron assay, if appropriate precautions are taken to prepare an iron-free medium or one of standardized low-iron content.

**DISCUSSION**

H. eutropha grows rapidly in shake culture, when provided with the proper gas mixture near atmospheric pressure. Nevertheless, growth was still limited by the solubility of one of the gases, since increased agitation increased growth. This limitation was particularly apparent in 10-liter mass cultures, where yields had been increased from 1.5 to 3.5 g per liter (wet weight) by increasing the rate at which gas was passed through the culture.

The requirement for at least 15% oxygen for maximal growth is significant in grouping this organism with the other oxygen-tolerant hydrogenomonads: H. facilis, H. agilis (Niklewski, 1914), H. pantotropha, and H. carboxydovorans. It is curious, however, that Schatz and Bovell (1955) reported that H. facilis can grow "autotrophically in traces up to 30 per cent but not 40 per cent oxygen" although their prescribed gas mixture contained 6% oxygen, which is definitely limiting for H. eutropha. Most other investigators who have cultivated this organism on solid medium have also used less than 10% oxygen. Cohen and Burris (1955) used 22% oxygen in their experiments, but they did not comment on the effect of the increased oxygen. Schlegel (1953), who isolated a hydrogenomonad which he identified
as *H. facilis*, reported that optimal growth occurred with 20 to 30% oxygen.

The unequivocal growth response of *H. eutropha* to traces of added iron (Fig. 5) indicates that this organism should be of interest for studies of iron metabolism. Since ferrous iron was much more effective than ferric iron at the same concentration, it is possible that growth with ferric iron was dependent upon reduction of iron to the ferrous state, and growth was governed by the rate of reduction. Ferric hydroxide (autoclaved iron) would not permit growth even when 5 μg of iron were added per ml of medium. Iron has been implicated as a cofactor for hydrogenase from a variety of organisms (Peck, San Pietro, and Gest, 1956). The clearly demonstrable iron requirement for *H. eutropha* was facilitated by the use of a simple chemically defined medium. In addition, the iron requirement may have been exaggerated by an iron demand by the hydrogenase of these cells. However, iron has not yet been shown to be associated with hydrogenase of *H. eutropha*.

**LITERATURE CITED**


