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Influence of *p*H on the Growth Characteristics of *Neisseria gonorrhoeae* in Continuous Culture

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A series of three continuous cultures of *Neisseria gonorrhoeae* was performed at *p*H values ranging from 6.4 to 7.2, with an average duration of 3 weeks. The maximal yield obtained was 1.12 mg/ml at a *p*H of 6.75 and a dilution rate of 0.26 per hr. Some morphological variations were observed at lower dilution rates, but complement-fixation titers remained stable for the duration of the cultures.

In recent years, there has been a steadily growing interest in the use of continuous culture, both for the study of the biological properties of microorganisms and for the large-scale production of cell mass. The techniques, theory, and applications of the method were recently summarized by Málek and Fencl (8).

From studies on the growth of *Neisseria gonorrhoeae* in batch cultures (1), it was found that the cell yield varied considerably, depending on the *p*H at which the culture was controlled. At the same time, morphological variations were observed, and some dependence on *p*H was again noted. The continuous culture technique made possible an examination of these phenomena under well-defined steady-state conditions over a long period of time.

MATERIALS AND METHODS

Organism. A freshly isolated strain of *N. gonorrhoeae*, designated no. 80408, obtained from the National Bacteriological Laboratory, Stockholm, Sweden, and preserved by storage in liquid nitrogen (2), was used in all experiments. The colonial appearance was of the type 4 described by Kellogg et al. (7).

Medium. The bacteria were grown in the following medium: Proteose Peptone No. 3 (Difco), 30.0 g; K_2HPO_4, 3.0 g; KCl, 6.5 g; glucose, 10.0 g; polyglycol antifoam (Dow Chemical Corp., Midland, Mich.), 0.1 ml; distilled water, 1,000 ml. The glucose was sterilized separately and added to the medium prior to inoculation. The *p*H was controlled automatically during the course of the experiments by the addition of 2 N NaOH.

Cultivation technique. The culture vessel (Biotec, Stockholm, Sweden) and ancillary equipment used in the experiments were as described by Holmström and Hedén (5), with the exception of the *p*H control which was effected by a modified system (1) with the use of a peristaltic tube pump via an electromagnetic relay. The working volume chosen was 500 ml.

The inoculum was prepared as for batch cultures (1) with aeration provided by a 5% CO_2 in air mixture during the initial stage of batch growth, 0.2 liter of normal air per min being used after the commencement of continuous culture. The temperature was maintained at 37 C, and the stirrer speed was approximately 1,000 rev/min.

Values of measured parameters were recorded after a minimal turnover of 10 volumes of the culture fluid to ensure steady-state conditions. The purity of the cultures was checked both by Gram staining and by examination of the colonial appearance after incubation on peptone-agar plates for 24 hr at 37 C.

Analysis. Bacterial growth was estimated during the course of cultivation by means of optical-density measurements made on an Eel Spectra absorptiometer (Evans Electro-selenium Ltd., Harlow, Essex, England) at a wavelength of 580 mμ. However, on attaining steady-state conditions, dry-weight determinations of the cell density were made.

Glucose was determined according to the method of Redéi and Nagy (11). Acetic acid was analyzed by gas chromatography with a Perkin Elmer Model F6 Fractometer having a 90-cm column containing 12.5% Carbowax 20M and 3% H_3PO_4 on a powdered Teflon packing (9). Preliminary experiments with a column of 20% Tween 80 on acid-washed 60-mesh Chromosorb W (3) showed that no other C_3–C_6 fatty acids were present in more than trace quantities (0.01%).

The amino acid pattern of the medium was elaborated by means of paper chromatography (12).

Complement-fixation titrations were kindly performed by B. Gästrin at the National Bacteriological Laboratory, Stockholm, Sweden. The values were then related to the dry-weight measurements to correlate the value obtained with the quantity of cellular material.

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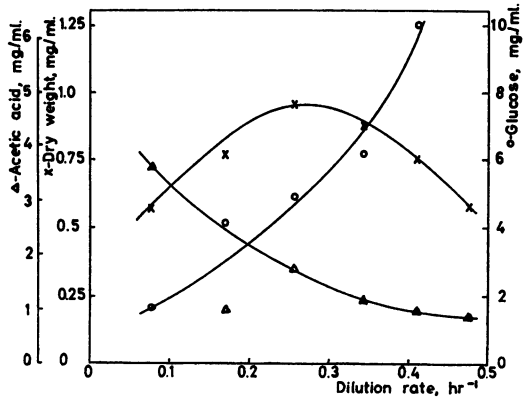

FIG. 1. *Continuous culture of Neisseria gonorrhoeae at pH 6.4, showing dry weight, glucose concentration, and acetic acid production.*

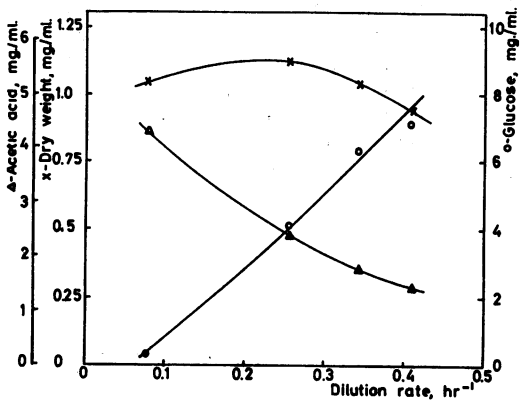

FIG. 2. *Continuous culture of Neisseria gonorrhoeae at pH 6.75, showing dry weight, glucose concentration, and acetic acid production.*

RESULTS AND DISCUSSION

Because of the earlier observed dependence of growth characteristics of the organism on the *p*H level, a series of three independent experiments was made. In the light of this experience, the values chosen were 6.4, 6.75, and 7.2. The duration of each cultivation was approximately 3 weeks with dilution rates in the range 0.08 and 0.46/hr. The highest dilution rate was selected after calculation of the maximal specific growth rate obtained under batch culture conditions.

The results obtained can be seen in Fig. 1, 2, and 3. At a *p*H of 6.75, the relationship most closely followed that obtained with organisms grown on simple media under limiting growth conditions (4). In the latter type of example, there was a known limiting substrate, but in the present work with a complex medium of unknown composition it was very difficult to determine which particular substance or substances limited the bacterial concentration. At *p*H values above or below 6.8, there was a marked decrease in cell concentration when the dilution rate fell outside the range from 0.2 to 0.25/hr. This decrease at lower dilution rates can be explained because there is considerable lysis, which could be observed both microscopically and by an increase in foaming. At *p*H 7.2, this was most noticeable. The reduced yield at higher dilution rates was very probably due to a reduction in the specific growth rate at those particular *p*H values, as compared with the optimal *p*H.

Preliminary batch experiments showed glucose to be by no means essential for growth in this medium, but both the specific growth rate and total yield of bacteria were considerably increased by its inclusion. It was, therefore, incorporated

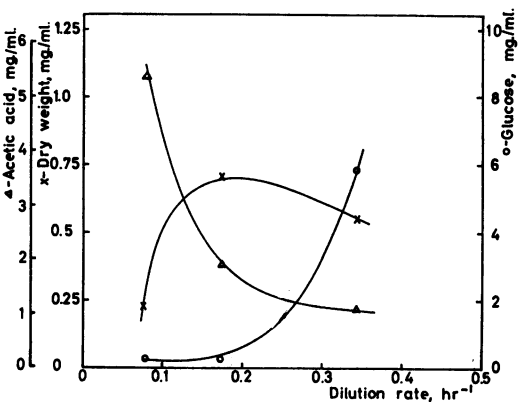

FIG. 3. *Continuous culture of Neisseria gonorrhoeae at pH 7.2, showing dry weight, glucose concentration, and acetic acid production.*

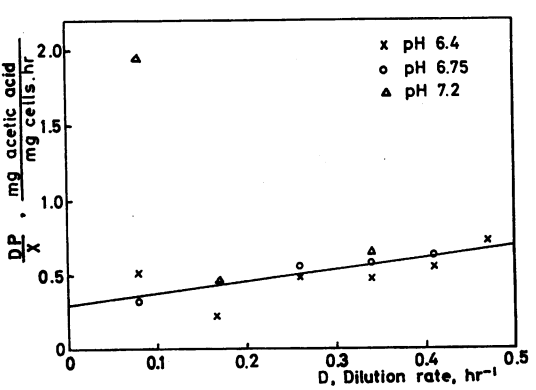

FIG. 4. *Kinetic representation of the production of acetic acid by Neisseria gonorrhoeae in continuous culture.*

into the medium in such quantities that it should not be the growth-limiting substrate. In all three experiments, the quantity of glucose consumed at lower dilution rates was relatively higher than would be expected from the cell density. This means that products other than cells must be formed. Gas chromatographic analysis of the culture fluid for fatty acid content showed that acetic acid was the only low molecular weight product, the concentrations produced by the bacteria being relatively high in relation to cell density and glucose consumption. In all experiments, there was an increase in acetic acid concentration with decreasing dilution rate. This corresponded with an increase in the consumption of glucose. At *p*H 7.2, the glucose was completely exhausted at dilution rates below 0.2/hr and the acetic acid concentration was exceptionally high.

This result contrasts sharply with the findings of Pirt (10), who reported that, under aerobic conditions with *Aerobacter cloacae*, there was no production of acetic acid except at very high dilution rates, and under conditions of partial oxygen deficiency.

If, however, the production of acetic acid is examined kinetically (6), as shown in Fig. 4, it can be seen that acetic acid formation is independent of *p*H, in spite of the different cell yields obtained at each *p*H level.

The straight-line relationship was drawn after an analysis of the data by the method of least squares. The result can be interpreted by the use of the following equation:

$$\frac{DP}{X} = \alpha D + \beta$$

where D = dilution rate per hour; P = product concentration, i.e., acetic acid (mg/ml); X = cell concentration (mg/ml); α = growth-associated yield constant (mg/mg); and β = cell productivity constant (mg/mg hr).

It can be seen that the growth-associated yield constant, obtained from the slope of the line, 0.78 mg of acetic acid per mg of cells, is a relatively small factor in the formation of acetic acid compared with the cell productivity constant of 0.3 mg of acetic acid per mg of cells per hr. This means that, in the presence of excess glucose, there is a form of overflow metabolism which proceeds independently of the growth rate, but that the acetic acid production is also partially growth-associated.

Since the medium was complex and contained compounds of high molecular weight, it was impossible to perform a complete chemical analysis. Free amino acids were analyzed, however, and it was observed that lysine was present in the medium supply but was exhausted at all dilution rates. Threonine and methionine were exhausted at a dilution rate of 0.08/hr, the concentration increasing with increasing dilution rate.

The bacterial suspension was examined regularly for morphological variations, and it was found that, in the culture maintained at a *p*H of 6.75, large cells, originally observed in batch cultures, were only observed at a dilution rate of 0.08/hr. Generally, the bacteria retained the characteristic diplococcal form. At the other *p*H levels, large cells were present with dilution rates as high as 0.26/hr. It is quite possible that these variations are connected with changes in the amino acid composition of the medium.

Complement-fixation titers were made on culture samples taken at the same time as those for other determinations. When corrections were applied for the different bacterial concentrations, the titers were remarkably constant at a sample dilution of 1:64, with the particular serum used in the tests (Positive 1214, National Bacteriological Laboratory, Stockholm, Sweden), when the dry weight was of the order of 1 mg/ml. Since the cultures were made over a period as long as 3 weeks, this factor is of considerable importance, as it indicates the possibility of producing large quantities of stable antigens.

The experiments have shown that it is feasible and practical to grow *N. gonorrhoeae* at relatively large yields in relatively simple equipment with low capital investment. The conditions giving the greatest yield of cells (*p*H 6.75; dilution rate, 0.26/hr) also gave bacteria of normal morphology and most economical use of medium.

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