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Production of *Clostridium pasteurianum* in a Defined Medium

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A method for the growth of *Clostridium pasteurianum* in a 140-liter (total capacity) stainless-steel vessel is described. By preventing the *p*H value from falling below 5.6, the growth of cultures was prolonged. Larger amounts of the carbon source (sucrose) and the nitrogen source (ammonium ion) were supplied and consumed, and cell yields of up to 5.56 g (dry weight) per liter were obtained. The highest cell yield previously reported was 1.7 g (dry weight) per liter obtained under nitrogen-fixing conditions in 500-ml cultures. The ferredoxin content of the cells was comparable with that obtained by earlier workers.

A defined medium for the growth of the anaerobic, nitrogen-fixing bacterium *Clostridium pasteurianum* was described by Carnahan and Castle (1). It contained (per liter): sucrose, 20 g; $MgSO_4 \cdot H_2O$, 0.1 g; NaCl, 0.1 g; $NaMoO_4 \cdot 2H_2O$, 0.01 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $FeSO_4$, 0.01 g; $CaCO_3$, 10 g; biotin, 1 μg; and *p*-aminobenzoic acid, 1 μg. Cultures were grown in 500-ml lots, and nitrogen was supplied by bubbling at the rate of 10 to 15 liters/hr. In this way, cell yields of 1.5 to 1.7 g (dry weight) per liter were obtained. These authors also found that good growth was obtained when $(NH_4)_2SO_4$ (0.8 g per liter) was used instead of nitrogen gas as the nitrogen source. They did not state the yields, but optical density measurements indicate that they were much the same as under nitrogen-fixing conditions.

A similar medium was used by Carnahan et al. (2) to grow 40-liter cultures under nitrogen-fixing conditions. The only modification was that iron was added as an alcoholic $FeCl_3$ solution to give 0.1 g of $FeCl_3 \cdot 6H_2O$ per liter of medium. The final *p*H value of harvested cultures was 5.4 to 5.8, and the yield of washed, dried cells was 0.63 to 0.75 g per liter.

Lovenberg, Buchanan, and Rabinowitz (6) modified the medium of Carnahan and Castle (1), so that it contained (per liter): KH_2PO_4, 1.4 g; K_2HPO_4, 15.6 g; $(NH_4)_2SO_4$, 2.0 g; no $CaCO_3$; and the other medium constituents as in the medium of Carnahan and Castle. Lovenberg et al. did not report their cell yields, nor the scale of their operations.

C. pasteurianum is a source of the electron carrier ferredoxin, a nonheme, iron-containing protein (6). It may be isolated by the method of Mortenson (7). The aim of this work was to produce large amounts of *C. pasteurianum* for the isolation of ferredoxin.

MATERIALS AND METHODS

C. pasteurianum was strain W.5 (NCIB 9486, ATCC 6013).

Temperature. All cultures were grown at 30 C.

Determinations. Sucrose was hydrolyzed for 30 min at 100 C in 0.25 N hydrochloric acid to give glucose and fructose, which were determined by the method of Somogyi (9).

Ammonium ion was liberated as ammonia by distillation in the presence of excess sodium hydroxide. The ammonia was collected in excess boric acid and titrated against standard hydrochloric acid.

Bacterial dry weights were determined by heating a sample of wet cells at 160 C to constant weight in an infrared oven, as described by Ford (4).

The optical density of cultures was determined, after appropriate dilution, on a Hilger Biochem Absorptiometer H.627 without a filter, by comparison with water.

For 2-liter cultures, gas evolution was measured by collecting the evolved gas in a measuring cylinder via a pneumatic trough by displacement of a solution containing 22% sodium chloride and 5% sulfuric acid (carbon dioxide has a low solubility in this solution). In the case of larger cultures, gas evolution was measured with a Parkinson "P. 13" test meter. The gas volumes reported are uncorrected for pressure and temperature.

Culture maintenance was on potato broth, which contained fresh peeled potato, 25 g; glucose, 0.5 g; $CaCO_3$, 0.2 g; and water, 100 ml. The components were mixed in a power blendor and heat-sterilized (15 psi, 15 min) in half-filled 8-oz bottles. Subcultures were made weekly by use of a 10% inoculum. After 24 hr of growth, when gas evolution with considerable froth formation had occurred, the cultures were stored at 0 to 4 C.

Production of 2-liter seeds. The medium contained (per liter): sucrose, 20 g; $MgSO_4 \cdot H_2O$, 0.1 g; NaCl, 0.1 g; $NaMoO_4 \cdot 2H_2O$, 0.01 g; KH_2PO_4, 1.4 g; K_2HPO_4, 15.6 g; $FeSO_4$, 0.01 g; biotin, 50 μg; *p*-aminobenzoic acid, 50 μg; $(NH_4)_2SO_4$, 0.8 g; and silicone MS antifoam emulsion RD (Hopkin and Williams Ltd.), 0.5 ml. All the materials except $MgSO_4 \cdot H_2O$, $FeSO_4$, and antifoam were dissolved in water and sterilized together (15 psi, 15 min). The remaining materials were each sterilized separately under the same conditions [the $MgSO_4 \cdot H_2O$ and the antifoam in water; the $FeSO_4$ in 0.1 N H_2SO_4 (1 ml per 10 mg of $FeSO_4$)] and added to the main bulk, to give 2-liter lots of medium in 40-oz bottles. The final *p*H was 7.5.

Cultures were inoculated either with 50 ml from a mature potato broth culture, or with 50 to 200 ml from a previous 2-liter culture. The inoculum was either fresh, or stored at 0 to 4 C for up to 7 days.

Production of 20-liter seeds. The culture medium was the same as for the 2-liter seeds except that the $(NH_4)_2SO_4$ concentration was 2 g per liter, and that cultures were grown in 5-gal bottles. Cultures were inoculated with the whole of a 2-liter culture, which ideally had evolved about 3 liters of gas. The inoculum was either fresh or stored at 0 to 4 C for up to 7 days.

Growth of 140-liter cultures. These cultures were grown in a jacketed stainless-steel culture vessel, 140 liters in total capacity, and 21 inches (53.3 cm) in diameter. It had provision for automatic temperature and *p*H control, contained four equally spaced full-length radial wall baffles 1.5 inches (3.8 cm) wide, and was similar to the 20-liter vessel described by Elsworth, Williams, and Harris-Smith (3). The stirrer was operated at 630 rev/min, with a propeller 4⅜ inches (11 cm) in diameter.

The culture medium was the same as for 20-liter seeds, and was prepared as follows. All the materials except for $MgSO_4 \cdot H_2O$, $FeSO_4$, and antifoam were dissolved in water in the culture vessel. The weights used were based on 140 liters of medium, but the water volume was so adjusted that the additions to be made during the experiment just failed to cause the vessel to overflow. The gas space over the mixture was swept out with nitrogen until the effluent gas was oxygen-free (Orsat). The contents of the fermentor were heated to 95 C by passing steam into the jacket, raised to 15 psi by steam injection, and held at that pressure for 15 min. The vessel was cooled to 30 C and automatically maintained at that temperature, with continuous stirring. Nitrogen was passed over the charge at the rate of 1 to 5 liters per minute starting when the excess pressure was discharged during cooling. The remaining medium constituents, which had been sterilized separately as described earlier, were then added without introducing air.

A supply of 10 N NaOH was made available for *p*H control. A concentrated solution of sucrose (about 50%) and $(NH_4)_2SO_4$ (about 5%), prepared from separately sterilized solutions (15 psi, 15 min), was also made available.

Cultures were started by inoculating with a 2-liter or a 20-liter seed. The flow of nitrogen was stopped and the vessel was sealed except for an inlet, to admit antifoam (25% silicone MS emulsion RD) which was added as required, and an outlet, via the gas meter. In later cultures, inlets were provided, to supply 10 N NaOH as demanded by the automatic *p*H control, and to supply the sucrose-$(NH_4)_2SO_4$ solution via a separate pump, under manual control. Samples were taken at intervals.

The progress of cultures was followed by gas evolution, increases in optical density, and consumption of sucrose and ammonium ion.

Recovery of the cells was in a Laval type 1700 centrifuge with a flow rate of about 1 liter/min.

RESULTS

Culture maintenance. Potato broth was a very satisfactory medium for the growth of the organism. In every case, growth was apparent 24 hr after inoculation.

Production of 2-liter seeds. Growth, once started, was generally good in 2-liter bottles, but a variable, and sometimes long induction period was found. A few cultures did not grow at all. Gas evolution figures gave a good indication of the growth of a culture, and over 12 liters of gas were evolved before growth ceased, when the *p*H value had fallen to about 5.2.

The pregrowth induction period was greatest (up to 48 hr) when a potato broth culture was used for the inoculum. It was reduced to 12 to 24 hr when a portion from a previous 2-liter seed culture, which had evolved about 3 liters of gas, was used; and it was further reduced to less than 12 hr when, in addition, 0.1% yeast extract (Yeatex, light grade, English Grains Co. Ltd., Burton-on-Trent) was incorporated in the culture medium.

By cooling to 0 to 4 C and sealing, 2-liter cultures, aged from 20 to 36 hr, from which about 3 liters of gas had been evolved, could be stored for at least 7 days. No increase in the induction period for cultures initiated with such stored inocula was observed.

Production of 20-liter seeds. The growth pattern was similar to that observed for 2-liter seeds.

Description of 140-liter cultures. Five such cultures are described. In the first, which was unstirred, the *p*H was not controlled. The culture was inoculated with a 2-liter seed from which 2.2 liters of gas had been evolved. Biotin and *p*-aminobenzoic acid were included at only 5.5 μg/liter instead of the 50 μg/liter used in all other experiments.

There was an induction period before growth started. Growth was accompanied by gas evolution, and a rise in the optical density of the culture. The *p*H fell progressively, and both sucrose (carbon source) and ammonium ion (nitrogen source) were consumed. When the culture was harvested (28 hr), growth, as indicated by opti-

TABLE 1. *Culture 1: growth without pH control*[a]

Time	Total vol. of gas evolved	Optical density	*p*H value	Sucrose (g/liter)	NH_4^+ (g of N/liter)
hr	*liters*				
0	0	0	7.5	22.0	0.39
14	22	0.08	7.05	14.3	0.30
18	117	0.40	6.55	13.1	0.23
22	344	0.80	5.9	10.9	0.17
26	597	0.96	5.3	8.0	0.12
28 (harvested)	684	1.08	5.25	7.4	0.11

[a] The culture was inoculated with a 2-liter seed from which 2.2 liters of gas had been evolved. The wet weight of cells recovered was 683 g from 120 liters, equivalent to 166 g, dry weight (1.38 g/liter).

cal-density measurements, had slowed down. The yield of cells was equivalent to 1.38 g (dry weight) per liter. The final *p*H value was 5.25, and, since both the carbon source and the nitrogen source were present in excess at the end, it is surmized that growth slowed down because of the low *p*H value. The results are summarized in Table 1.

In the second culture, the *p*H was prevented from falling below 5.6 by the automatic addition of 10 N NaOH, in the hope of encouraging further growth. The stirrer was operated starting when the *p*H control was required. The culture was inoculated with a 2-liter seed from which 10.1 liters of gas had been evolved.

There was a very long induction period before growth started, but the changes then observed were similar to those in the first culture. The only difference was that, with the *p*H control, growth, as indicated by optical-density changes, continued at a high rate until the sucrose became exhausted. When the culture was harvested (88 hr), the gas evolution rate had dropped sharply. The yield of cells was equivalent to 1.57 g (dry weight) per liter (Table 2).

In the third culture automatic *p*H control at 5.6, with stirring, was afforded as in the second culture. In addition, during growth, 790 g of sucrose and 79 g of $(NH_4)_2SO_4$, were supplied over a 6-hr period. The culture was inoculated with a 20-liter seed from which 66 liters of gas had been evolved.

Following an induction period, growth occurred. The pattern of changes was similar to that observed in the earlier cultures, except that the rise in optical density was prolonged until the sucrose became exhausted. When the culture was harvested (44 hr), gas evolution was slow. The yield of cells was equivalent to 3.64 g (dry weight) per liter (Table 3).

In the fourth culture, stirring was carried out from the start, and automatic *p*H control at 5.6 was provided as before. During growth, 2,800 g of sucrose and 280 g of $(NH_4)_2SO_4$, were supplied over an 8-hr period. The culture was inoculated with a 20-liter seed from which 26 liters of gas had been evolved.

After an induction period, growth occurred with a pattern of changes similar to that in the third culture, except that the rise in optical density was greater, though it had ceased long before the sucrose became exhausted. The yield of cells was equivalent to 4.88 g (dry weight) per liter (Table 4)

TABLE 2. *Culture 2: growth with pH control*[a]

Time	Total vol. of gas evolved	Optical density	*p*H value	Sucrose (g/liter)	NH_4^+ (g of N/liter)
hr	*liters*				
0	0	0	7.5	20.0	0.42
62	62	0.33	6.7	14.4	0.34
68	193	0.92	6.1	13.1	0.30
73	412	1.46	5.65	10.2	0.24
79	772	2.08	5.6	5.3	0.14
88 (harvested)	1,113	2.80	5.6	0.8	0.06

[a] The culture was inoculated with a 2-liter seed from which 10.1 liters of gas had been evolved. The stirrer was operated from 72 hr, and thereafter 750 ml of 10 N NaOH was used in *p*H control. The wet weight of cells recovered was 742 g from 120 liters, equivalent to 189 g, dry weight (1.57 g/liter).

TABLE 3. *Culture 3: growth with pH control and added nutrients*[a]

Time	Total vol. of gas evolved	Optical density	*p*H value	Sucrose (g/liter)	NH_4^+ (g of N/liter)
hr	*liters*				
0	0	0	7.35	17.5	0.42
20	1	0.01	7.25	17.5	0.44
24	18	0.19	6.9	16.7	0.40
28	85	0.64	6.5	13.4	0.32
32	328	1.64	5.95	9.4	0.23
36	687	2.64	5.6	6.3	0.16
40	1,126	3.92	5.6	3.8	0.11
44 (harvested)	1,516	5.04	5.6	0.5	0.05

[a] The culture was inoculated with a 20-liter seed from which 66 liters of gas had been evolved. The stirrer was operated from 23 hr, and from 36 hr 900 ml of 10 N NaOH was used in *p*H control. Between 34 and 40 hr, 1,750 ml of solution containing 790 g of sucrose and 79 g of $(NH_4)_2SO_4$ was pumped into the culture at a constant rate. The wet weight of cells recovered was 1,800 g from 140 liters, equivalent to 510 g, dry weight (3.64 g/liter).

TABLE 4. *Culture 4: growth with pH control and added nutrients*[a]

Time	Total vol of gas evolved	Optical density	*p*H value	Sucrose (g/liter)	NH_4^+ (g of N/liter)
hr	*liters*				
0	0	0.05	7.25	20.0	0.49
4	1	0.11	7.05	—	—
8	27	0.39	6.8	15.4	0.42
12	217	1.16	6.25	9.7	0.35
16	520	2.16	5.7	7.7	0.20
20	1,115	4.40	5.6	12.4	0.19
24	1,643	5.46	5.6	11.0	0.39
28	1,970	6.45	5.6	6.7	0.35
32	2,241	6.60	5.6	2.3	0.31
36	2,468	6.45	5.6	0.5	0.24
38 (harvested)	2,540	6.45	5.6	0.8	0.24

[a] The culture was inoculated with a 20-liter seed from which 26 liters of gas had been evolved. The stirrer was operated from the start, and from 17 hr 2,400 ml of 10 N NaOH was used in *p*H control. Between 14 and 22 hr, 5,250 ml of solution containing 2,800 g of sucrose and 280 g of $(NH_4)_2SO_4$ was pumped into the culture at a constant rate. The wet weight of cells recovered was 1,834 g from 120 liters, equivalent to 585 g, dry weight (4.88 g/liter).

In the fifth culture, stirring was carried out from the start, and automatic *p*H control at 5.6 was provided as before. During growth, 4240 g of sucrose and 424 g of $(NH_4)_2SO_4$ were supplied over an 8-hr period. The culture was inoculated with a 20-liter seed from which at least 23 liters of gas had been evolved (a faulty gas meter prevented a precise reading).

There was almost no induction period before growth started, with the usual pattern of changes. At 20 hr, the *p*H control system was altered to cause the *p*H to rise to 5.9 over about 4 hr, and the *p*H was kept at this higher value until harvest (26 hr). The final optical density was little different from that in the fourth culture, and, despite the large excess of sucrose available, the optical density was hardly rising at harvest. Gas evolution had slowed down slightly at the end. The yield of cells was equivalent to 5.56 g (dry weight) per liter (Table 5).

Ferredoxin content of the cells. The harvested cells were used by S. G. Mayhew and J. L. Peel (ARC, Food Research Institute, Norwich, U.K.) for the isolation of ferredoxin. The yields of pure material were (per 100 g of dry cells); culture 1, 0.030 g; culture 2, 0.018 g; culture 3. 0.027 g; culture 4, 0.024 g; culture 5, 0.023 g. There is thus no evidence of an appreciable difference between the ferredoxin contents of the cells obtained in high yield from cultures 4 and 5, and of those obtained in lower yields from the earlier cultures. These yields of ferredoxin per gram of dry cells are approximately half of that reported earlier by Mortenson (7), who obtained 220.1 mg from 400 g of dry cells. (It was not clear from Mortenson's paper whether the 220.1 mg of recrystallized ferredoxin referred to in his Table 1 was derived from 400 g of dry cells or from dry cells which contained 400 g of protein. Correspondence with Mortenson has established that the former was the case.) This does not, however, necessarily reflect a lower ferredoxin content in the original cells, as the present isolations were done on approximately one-fifth of the scale described by Mortenson (7), with appropriate modifications. In these circumstances, Mayhew and Peel stated that a less efficient isolation would be expected.

TABLE 5. *Culture 5: growth with pH control and added nutrients*[a]

Time	Total vol of gas evolved	Optical density	*p*H value	Sucrose (g/liter)	NH_4^+ (g of N/liter)
hr	*liters*				
0	0	0.07	7.5	25.7	0.50
4	26	0.22	7.05	17.7	0.47
8	101	0.54	6.7	14.7	0.37
12	371	1.36	6.15	7.8	0.27
16	807	3.04	5.65	7.8	0.14
20	1,235	5.20	5.7	12.3	0.28
24	1,632	6.24	5.9	15.6	0.38
26 (harvested)	1,776	6.50	5.9	17.3	0.46

[a] The culture was inoculated with a 20-liter seed from which at least 23 liters of gas had been evolved. The stirrer was operated from the start, and from 17 hr 1,720 ml of 10 N NaOH was used in *p*H control. Between 14 and 26 hr, 6,500 ml of solution containing 4,240 g of sucrose and 424 g of $(NH_4)_2SO_4$ was pumped into the culture at a constant rate. The wet weight of cells recovered was 2,277 g from 140 liters, equivalent to 779 g, dry weight (5.56 g/liter).

DISCUSSION

In the growth of cultures, a variable and sometimes long (over 48 hr) induction period was usually observed. For this reason gas evolution data were useful in giving an immediate indication of the state of a culture. Thus, it was easy to select actively growing 2-liter and 20-liter cultures for use as seeds, or for storage at 0 to 4 C for future use as seeds. By having such a store of grown seed cultures, the main 140-liter experiments could be started at times of choice, which were independent of the induction period in the seed cultures. The reason for the induction period is not known, but it is not a basic property

of the system because in a few cases it was absent (for example, in the fifth 140-liter culture).

The medium used was the same as that of Lovenberg, Buchanan, and Rabinowitz (6) except that higher levels of biotin and *p*-aminobenzoic acid were employed. This was merely to ensure that an excess of these materials was present at all times. The iron added as $FeSO_4$ (3.7 mg of Fe per liter) was the same as that added by Carnahan and Castle (1). However, since our cultures were grown in a stainless-steel vessel, largely at *p*H 5.6, it is likely that dissolved iron, derived from the vessel, was also available.

From the work of Knight and Hardy (5), it is now known that the ferredoxin content of *C. pasteuranium* is at a very low level when the organism is grown at a very low iron concentration (0.44 mg of Fe per liter, measured). There was no significant drop in the ferredoxin content of the cells from cultures 4 and 5, in which the iron available per gram of cells was lowest, as compared with cultures 1 and 2. Therefore, it is unlikely that lack of iron limited the ferredoxin content of any of our cells. Nevertheless, in the light of the work of Knight and Hardy (5), and the finding of Carnahan and Castle (1) that added iron levels as high as 37 mg/liter were not detrimental to growth, it would be reasonable to use higher iron concentrations in future cultures of *C. pasteurianum* aimed at ferredoxin production.

It is possible to estimate from the analytical figures that in cultures 1 and 2 approximately 28 g of nitrogen, in the form of ammonium ion, were utilized for every 100 g of dry cells produced. In the later cultures, the rate of nitrogen utilization per gram of cells produced was lower, for no obvious reason. It is known, from the work of Rosenblum and Wilson (8), that considerable quantities of soluble organic nitrogen are found in cultures of *C. pasteurianum* grown in a medium containing ammonium sulfate. These authors reported that up to 50% of the nitrogen assimilated may occur outside the cells, and this probably accounts for the high ammonium ion utilization observed.

The optical density of cultures 1, 2, and 3 stopped rising when sucrose ran out, and at the same time the gas evolution rate slowed appreciably. Therefore, cell production probably continued to the end in these cultures. In culture 4, the optical density was approximately constant for the last 10 hr, although sucrose and $(NH_4)_2SO_4$ were being metabolized during this period. Similarly, in culture 5 the optical density was hardly changing at harvest, although a large excess of sucrose and $(NH_4)_2SO_4$ were still present. A possible explanation of these observations is that in cultures 4 and 5 some nutrient, other than carbon or nitrogen, essential for cell production, became exhausted. Thus, in future experiments aimed at obtaining even higher cell yields, it would be worth using the other medium components at higher concentrations and deliberately supplementing the medium with essential trace elements (e.g., manganese) in the hope of overcoming this possible deficiency.

The improvements in cell yield per milliliter of culture were obtained through *p*H control, which enabled larger amounts of sucrose and $(NH_4)_2SO_4$ to be utilized. As a result, in the best culture (no. 5) we obtained 5.56 g of dry cells per liter, compared with the highest previously reported yields of 1.7 g/liter under nitrogen-fixing conditions (1).

Whether the slightly higher cell density obtained at *p*H 5.9 (culture 5), as compared with *p*H 5.6 (culture 4), was significant is not known More work would be required to determine the optimal *p*H value at which to grow the organism. It is emphasized that by using a stirred culture vessel, measurement and control of cultural conditions were made possible. Stirring was attended by no obvious disadvantage, and, without stirring, *p*H control would have been much more difficult to achieve.

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