A Continuous Culture Study of Growth of *Bacillus cereus* T

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Growth of an oligosporogenic strain of *Bacillus cereus* T was studied in continuous culture. The maximum specific growth rate obtained was 0.47 h^{-1} . RNA content increased linearly with increasing growth rate. Protein content decreased at high growth rates, but the DNA content of the culture was not affected by the growth rate. Activities of enzymes involved in glutamate utilization and energy production increased with increasing growth rate. The maximum specific activity for all enzymes assayed was obtained at a dilution rate of about 0.30 h^{-1} . Changing the concentration of zinc or manganese in the medium by as little as 10^{-7} M from the concentration optimum for growth caused a significant reduction in the steady-state bacterial population. Zinc appeared to affect the efficiency of conversion of substrate to biomass. Manganese was very toxic at concentrations slightly above the concentration optimum for a specific growth rate.

INTRODUCTION

The majority of studies on the control of spore formation have been performed in batch culture. These studies used media which were designed to give efficient sporulation; they were poorly optimized with respect to requirements necessary for vegetative growth. Investigations conducted under constant conditions which are optimum for either growth or sporulation may give better insight into the processes of growth and sporulation. One, and possibly the best, method by which these parameters can be achieved is by continuous culture. Dawes & Thornley (1970) demonstrated that differentiation could be studied under the conditions imposed by continuous culture. The main shortcomings of continuous culture studies of differentiating organisms have been (i) the inability to grow the organisms at rates approaching the maximum specific growth rate and (ii) the selection of sporulation phase mutants. These problems are interrelated because the selection of sporulation mutants is most pronounced at slow growth rates. At slow growth rates a portion of a sporogenic culture will initiate the sporulation process. These sporulating cells do not divide and therefore do not contribute to the maintenance of the population. Sporulation mutants with lesions in late function spore genes (*spoIV*) similarly do not divide or contribute to population maintenance. Sporulation mutants altered early in the sporulation cycle (*spoO*) are enriched for because they can reverse early sporulation processes and resume vegetative growth. At fast growth rates (approaching the maximum specific growth rate) selection of mutants in sporulation-specific genes is minimum because any cell which can no longer divide is quickly removed from the culture. Development of a medium which will allow growth of the culture at rates approaching the maximum specific growth rate may go a long way towards solving the problem of mutant selection.

We have developed a medium for the growth of an oligosporogenic strain of *Bacillus cereus* T. This medium allows growth under conditions of glucose limitation to a maximum

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specific growth rate of 0.47 h^{-1} . In batch culture the oligosporogenic strains and the sporogenic parent exhibit the same growth rates and enzyme activities. During the development of this medium we observed extreme sensitivity to the concentration of various trace metals. The importance of trace metals for vegetative growth has previously been dismissed as not critical (Weinberg, 1970). The results presented in this report show that alterations in medium trace metal content greatly affect the yield of the culture.

METHODS

Organism. A variant strain of *Bacillus cereus* T was isolated by the method of Holliday (1956). This strain was designated Sp⁺. An oligosporogenic mutant of this strain was isolated from a chemostat culture of the sporogenic strain and designated OSp.

Medium. A defined medium (GG) was designed from experiments conducted in continuous culture (Summers *et al.*, 1979). The medium contained $1.1 \times 10^{-2} \text{ M}$ -glucose, $1.5 \times 10^{-2} \text{ M}$ -glutamate, $4.5 \times 10^{-3} \text{ M}$ -(NH_4)₂SO₄, $1.0 \times 10^{-2} \text{ M}$ -K₂HPO₄, $4.0 \times 10^{-4} \text{ M}$ -NaH₂PO₄, $4.92 \times 10^{-4} \text{ M}$ -MgCl₂, $2.04 \times 10^{-5} \text{ M}$ -CaCl₂, $4.0 \times 10^{-10} \text{ M}$ -CuSO₄, $1.69 \times 10^{-8} \text{ M}$ -CoCl₂, $5.10 \times 10^{-7} \text{ M}$ -MnCl₂, $3.3 \times 10^{-6} \text{ M}$ -ZnSO₄ and $1.8 \times 10^{-6} \text{ M}$ -FeSO₄. This medium allowed cultivation of the OSp strain under conditions of glucose limitation to a critical dilution rate of 0.47 h^{-1} . Strains were maintained on plates of either 0.8% (w/v) nutrient agar supplemented with 0.2% (w/v) yeast extract or GG agar.

Unless otherwise noted, dilutions were prepared in a buffer of 0.15 M-NaCl, 0.05 M-Tris and $5 \times 10^{-3} \text{ M}$ -EDTA at pH 7.5 (STE buffer).

Cultivation. Batch cultures were prepared by inoculating 25 ml GG medium with either heat-shocked spores (80 °C, 30 min) or vegetative cells obtained from 24 to 36 h GG agar plates. Following 16 h incubation at 23 °C the cultures were incubated at 34 °C until the A_{600} reached 1.5. [When spores were used 0.05% (w/v) yeast extract was added to aid germination: no yeast extract was added in any subsequent transfers.] This culture was diluted 1 in 10 with fresh medium and incubated at 34 °C until the A_{600} reached 1.5 and then used to inoculate the chemostat or diluted 1 in 10 into side-arm flasks for studies on batch growth.

The chemostat consisted of a 1 l flask with a 500 ml working volume. The temperature was maintained at 34 °C by a constant temperature bath. The culture was agitated with a magnetic stirrer and aerated by sparging with filtered air at 750 ml min^{-1} . The pH was maintained at 7.0 by automatic addition of 0.5 M-NaOH.

Care was taken to avoid precipitation during the preparation of the medium. The medium was prepared twofold concentrated in two 40 l carboys containing sterile water and sufficient concentrated HCl to give a final pH of 2.0. To one carboy was added sterile carbon, nitrogen and phosphorus sources. The other carboy contained the complement of trace metals. The medium was delivered from the two carboys to the growth vessel in equal volumes by a peristaltic pump (Harvard Apparatus, Millis, Mass., U.S.A.). A detailed description of the design and operation of the chemostat has been presented elsewhere (Summers *et al.*, 1979).

After any change in the system, either due to sampling or change in medium composition, the culture was allowed a minimum of 10 residence times to re-establish a steady state.

Methods of sampling and assay. Samples of approximately 100 ml were taken as a single portion into a chilled flask and used for all analytical determinations.

A 20 ml portion was immediately added to 20 ml cold 10% (w/v) trichloroacetic acid and held at 0 °C for 15 min. The cells were harvested by centrifugation, resuspended in 2 ml 0.5 M-HClO₄ and stored at -20 °C. This sample was used for determination of cellular RNA, DNA and protein.

The cells from three further 10 ml portions of the chilled sample were harvested by centrifugation (8000 g, 10 min, 4 °C). The supernatants were stored at -20 °C and used for determination of residual nutrients. The cells were washed twice with distilled water and dried to constant weight at 80 °C for determination of dry weight.

For enzyme determinations, the cells from 40 ml of the chilled sample were harvested by centrifugation (8000 g, 10 min, 4 °C). The cells were washed twice with cold STE buffer, resuspended in 10 ml STE buffer and broken by ultrasonic disruption. Cell debris was removed by centrifuging (8000 g, 10 min, 4 °C). The supernatant was kept in an ice bath until used for determination of enzyme activities.

Culture turbidity was determined using a Klett-Summerson colorimeter with a no. 66 red filter. Samples were diluted to give a reading of between 25 and 100 Klett units to ensure linearity.

Glucose, phosphate and ammonia concentrations were determined by the methods of Miller (1959), Fisk & Subbarow (1925) and Weatherburn (1967), respectively. Prior to determination of residual glutamate, unused ammonia was removed by evaporating 5 ml of sample to dryness under alkaline conditions (pH 8.0). The residue was resuspended to the original volume in distilled water and the glutamate was assayed by the dinitrophenylation

reaction (Ghuysen *et al.*, 1966). The amount of a given substrate used was determined by subtracting the amount present in the spent medium from the amount assayed to be present in the complete medium.

The DNA and RNA contents of the cells were determined by the diphenylamine and orcinol reactions, respectively, as described by Schneider (1956). The protein concentration was determined by the Lowry method. Fumarase (EC 4.2.1.2) activity was determined by the method of Kanarek & Hill (1964). Malic dehydrogenase (EC 1.1.1.37) and alkaline phosphatase (EC 3.1.3.1) were determined as described by Yoshida (1965) and Garen & Levinthal (1960), respectively. Glutamic-oxaloacetic transaminase (GOT; L-aspartate:2-oxoglutarate aminotransferase; EC 2.6.1.1) and glutamic-pyruvic transaminase (GPT; L-alanine:2-oxoglutarate aminotransferase; EC 2.6.1.2) activities were assayed as described in Sigma technical bulletin no. 55-UV.

RESULTS

Isolation of strain OSp

Routine plating of chemostat cultures of the Sp^+ strain on GG plates demonstrated two colony types. Strain Sp^+ produced small, convex, mucoid colonies. Phase contrast microscopy showed that these colonies contained more than 95% free spores after 48 h at 34 °C. The second colony type (OSp) was larger, non-mucoid and essentially free of spores after similar incubation. The OSp strain comprised 50% of the population of a chemostat culture ($D = 0.29 \text{ h}^{-1}$) after 8 d when the culture was inoculated with the Sp^+ strain. Conversely, when the chemostat was inoculated with strain OSp, no Sp^+ cells could be detected after 10 d ($D = 0.29 \text{ h}^{-1}$) by plate counts on GG agar. The growth rates of the two strains in batch culture on GG medium supplemented with 0.02% yeast extract were identical throughout the vegetative phase and diverged only slightly during differentiation (Fig. 1). The Sp^+ strain sporulated completely after 24 h. Strain OSp produced only 0.1%

Fig. 1

Fig. 2

Fig. 1. Growth (●, ○) and percentage of spores (▲, △) for the sporogenic (●, ▲) and oligosporogenic (○, △) strains grown in batch culture in GG medium supplemented with 0.02% yeast extract. Viable and spore titres were determined by plating on GG agar plates; heat-resistant spores were detected by heating dilution tubes to 80 °C for 30 min before plating.

Fig. 2. Macromolecular composition of strain OSp in a continuous culture at various growth rates. Samples were taken after 10 residence times at the indicated dilution rate. Glucose was the limiting nutrient at all dilution rates except $D = 0.48 \text{ h}^{-1}$; at this dilution rate no steady state could be achieved ($\mu_{\text{max}} = 0.47 \text{ h}^{-1}$). The macromolecular content is expressed as a percentage of the cellular dry weight [% dry wt = $100 \times (\text{mg macromolecule ml}^{-1}) / (\text{mg cell dry wt ml}^{-1})$]. Dry weight (●), protein (○), RNA (▲) and DNA (△).

Table 1. *Carbon and nitrogen consumed by strain OSp in a chemostat at different dilution rates*

Dilution rate (h ⁻¹)	Dry weight (g l ⁻¹)	Carbon consumed (g l ⁻¹)‡			Nitrogen consumed (g l ⁻¹)		
		From glucose	From glutamate	Total	From glutamate	From (NH ₄) ₂ SO ₄	Total
0.11*	1.898	0.792	0.816	1.608	0.191	-0.008	0.183
0.23*	2.140	0.792	0.819	1.611	0.192	0.027	0.219
0.28*	2.140	0.792	0.821	1.613	0.192	0.040	0.232
0.38*	2.088	0.792	0.816	1.608	0.191	0.064	0.255
0.46*	1.723	0.792	0.683	1.475	0.160	0.064	0.224
0.51†	0.131	0.206	0.115	0.321	0.027	0.000	0.027

* Glucose-limited at steady state.

† Sample taken after 24 residence times at this growth rate.

‡ When no glucose could be detected in the spent medium it was assumed that glucose consumption was complete.

spores after 24 h and a maximum of 6% after 36 h. Both strains were sensitive to the phage ϕ CL, a phage lytic to *B. cereus* T. Continuous culture studies were conducted with strain OSp.

Effect of growth rate on the physiology of strain OSp in continuous culture

Strain OSp was grown under conditions of glucose limitation in GG medium over a wide range of dilution rates. The maximum growth rate obtained with glucose limitation at steady state was 0.47 h⁻¹. This agrees well with the maximum growth rate obtained in batch culture with the same medium ($\mu_{\max} = 0.46$ h⁻¹). At growth rates slightly above this value a slow wash-out began and a glucose-limited steady state could not be re-established.

The RNA content of the culture increased linearly with growth rate (Fig. 2). DNA content was constant over all growth rates examined. The protein content of the cells decreased as the growth rate increased.

The carbon and nitrogen required for growth could each be derived from two sources. Table 1 presents results for the utilization of these substrates expressed as carbon or nitrogen used per litre of medium. Glucose was the limiting nutrient at all dilution rates examined up to the critical dilution rate. Glutamate utilization was constant at most dilution rates, but decreased somewhat as the critical dilution rate was approached. The total nitrogen removed from the medium increased as the growth rate increased to a maximum at $D = 0.38$ h⁻¹. This could occur by increasing the use of nitrogen from ammonium sulphate at faster growth rates or by more efficient scavenging of the nitrogen derived from glutamate metabolism. The culture did not attain glucose limitation at a growth rate of 0.10 h⁻¹ if ammonium sulphate was omitted from the medium (not shown). Additionally, in the complete medium at a dilution rate of 0.11 h⁻¹ there was a slight increase in the ammonium ion concentration in the medium (Table 1). These observations suggest that variable glutamate-nitrogen use is more likely. Two transaminases involved in glutamate utilization were monitored at various growth rates in order to investigate changes in glutamate utilization. Both GOT and GPT activities increased as the growth rate increased from $D = 0.08$ h⁻¹ to $D = 0.30$ h⁻¹ (Table 2). The total transaminase activity (GOT plus GPT) was maximum near a dilution rate of 0.30 h⁻¹. At higher dilution rates there was a decrease in transaminase activity. These results, together with those for nitrogen utilization presented above, suggest that the nitrogen derived from glutamate metabolism at slow growth rates is not efficiently used and is lost by the cell as ammonium ions.

Batch culture studies suggest that various citric acid cycle enzymes are not active in

Table 2. *Specific activity of selected enzymes of strain OSp at various dilution rates*

Enzyme	Specific activity [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]				
	Dilution rate (h^{-1})				
	0.08*	0.20*	0.30*	0.39*	0.52
Glutamic-oxaloacetic transaminase	62.4	203.8	256.3	195.1	181.4
Glutamic-pyruvic transaminase	24.24	23.86	42.53	48.86	48.48
Fumarase	0.038	0.338	0.495	0.445	0.378
Malic dehydrogenase	0.172	0.322	0.332	0.270	0.260

* Glucose-limited at steady state.

Table 3. *Comparison of selected enzyme activities of Sp⁺ and OSp cells grown in batch culture*

Cells were grown to a turbidity of 100 Klett units. Cell extracts were prepared as described in Methods.

Enzyme	Specific activity [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$]	
	Sp ⁺	OSp
Fumarase	0.179	0.087
Malic dehydrogenase	0.167	0.117
Glutamic-oxaloacetic transaminase	122.9	182.1
Glutamic-pyruvic transaminase	11.57	13.15
Alkaline phosphatase	0.039	0.046

vegetative cells and are induced only after depletion of readily fermentable carbohydrates and/or the induction of sporulation (Srinivasan, 1965; Freese & Fujita, 1976). If this assumption were correct, one would expect a decrease in the specific activities of fumarase and malic dehydrogenase with increasing growth rates. In batch culture both of these enzyme activities were shown to be higher during sporulation than during vegetative growth (Charba & Nakata, 1977). The activities of both fumarase and malic dehydrogenase increased with increased growth rate to a maximum value at a dilution rate of 0.30 h^{-1} (Table 2). No alkaline phosphatase activity was observed over the range of dilution rates examined.

Since the frequency of conversion of Sp⁺ to OSp was relatively high, enzyme assays could not be carried out with Sp⁺ cells under conditions of continuous culture. The enzyme activities of the Sp⁺ and OSp strains were therefore compared in extracts of cells grown in batch culture. As seen in Table 3, the enzyme activities in the two strains were similar. Alkaline phosphatase, not detected in chemostat cultures of strain OSp, was detected in extracts of both strains grown in batch culture. This is probably due to the physiological heterogeneity of cell types found in batch culture.

Effect of trace metal concentration on growth

Early in these studies we observed that a significant fluctuation in the population was caused by a slight alteration in the trace metal composition of the medium. We therefore examined the effect of trace metals on the growth of strain OSp. The chemostat was operated at a constant dilution rate of 0.40 h^{-1} as the concentration of the trace metal was varied, and fluctuations in turbidity, dry weight and nutrient utilization at steady state were monitored; the results of experiments with zinc and manganese are presented.

The dry weight of cells was dependent on the zinc concentration (Fig. 3). Maximum dry weight was obtained at a zinc concentration 10-fold lower than that necessary for glucose-limited growth at the maximum specific growth rate. The RNA content increased linearly with zinc concentration. Cellular DNA content was constant. The protein

Fig. 3. Effect of zinc concentration on growth and macromolecular composition of strain OSp grown in a chemostat at a dilution rate of 0.40 h^{-1} . Macromolecular content is expressed as in Fig. 2. Dry weight (●), protein (○), RNA (▲) and DNA (△).

Table 4. *Effect of zinc concentration on dry weight and nutrient consumption of strain OSp in a continuous culture at a growth rate of 0.40 h^{-1}*

Zinc concn (μM)	Klett units	Dry weight (g l^{-1})	Carbon consumed (g l^{-1})	Nitrogen consumed (g l^{-1})	Phosphorus consumed (g l^{-1})
0.167	530	2.183	1.297*	0.139	0.060
0.248	570	2.338	1.495*	0.148	0.062
0.281	590	2.445	1.505*	0.141	0.063
0.330	690	2.915	1.539*	0.179	0.072
0.495	670	2.838	1.573*	0.192	0.078
1.100	550	2.150	1.566*	0.199	0.082
2.200	525	2.069	1.544*	0.197	0.081
3.300	510	2.033	1.529*	0.181	0.080
6.600	550	2.028	1.498*	0.174	0.080
16.500	550	2.043	1.474*	0.174	0.071

* Glucose-limited steady state.

concentration varied over the range of zinc concentrations tested with a pattern opposite to that of the dry weight. The observation that the maximum dry weight was not obtained at the zinc concentration needed for the optimum growth rate was interesting. This could be caused either by increased substrate utilization or by more efficient conversion of substrate to biomass at lower zinc concentrations. The amount of glucose used was constant at all zinc concentrations assayed (no residual glucose was detected in the spent medium). The increase in the utilization of the other substrates over the range of zinc concentrations monitored was not sufficient to account for the increased dry weight (Table 4). The enhanced yield must therefore be due to the increased efficiency with which substrate was metabolized.

Examination of the effect of manganese concentration on growth was conducted with zinc at the concentration which gave the maximum dry weight in the zinc studies (Table 4, $0.33 \mu\text{M}$). Again, the dilution rate was maintained at 0.40 h^{-1} . Slight alterations in the manganese concentration produced a significant variation in dry weight. In this case, however, substrate utilization closely followed the dry weight (Table 5). For manganese it appears that at concentrations below the optimum for growth the culture was limited by manganese availability and at concentrations slightly above the optimum the toxicity of manganese inhibited growth. The RNA content decreased linearly as the manganese

Table 5. *Effect of manganese concentration on dry weight and nutrient consumption of strain OSp in continuous culture at a growth rate of 0.40 h⁻¹*

Manganese concn (μM)	Klett units	Dry weight (g l^{-1})	Carbon consumed (g l^{-1})	Nitrogen consumed (g l^{-1})	Phosphorus consumed (g l^{-1})
0.051	103	0.41	0.650	0.177	0.007
0.153	210	0.82	0.839	0.171	0.023
0.255	780	3.24	1.625*	0.287	0.065
0.383	740	3.09	1.654*	0.308	0.056
0.510	680	3.09	1.618*	0.302	0.056
1.020	565	2.59	1.540*	0.273	0.057

* Glucose-limited steady state.

concentration was raised above the optimum concentration. DNA and protein concentrations were constant at all manganese concentrations tested (results not shown).

DISCUSSION

The results presented here demonstrate that the growth rate of a culture alters the physiological state of that culture. One other unexpected observation was that a slight alteration in the trace metal complement of the medium significantly altered primary metabolism.

At slow growth rates ($D \leq 0.11 \text{ h}^{-1}$) strain OSp could not use glutamate-derived nitrogen as the sole nitrogen source. The amount of nitrogen available from the metabolism of glutamate was sufficient to satisfy the requirements of the culture if it were efficiently assimilated. The enzymes needed for efficient glutamate-nitrogen utilization were not fully induced at slow growth rates. The decrease in nitrogen consumption observed near the critical dilution rate ($D = 0.46 \text{ h}^{-1}$, Table 1) paralleled the decrease in glutamate-carbon utilization at this growth rate. This is probably because at this fast growth rate the cell was unable to sustain a sufficient level of glutamate metabolism to supply its energy demand.

Previous reports (Hanson *et al.*, 1963; Charba & Nakata, 1977) have shown that in batch cultures of bacilli fumarase and malic dehydrogenase activities were very low at the end of vegetative growth and only during differentiation did the activities of these enzymes rise. These enzyme activities can be induced in continuous culture by increasing the growth rate. The similarity in the enzyme activities in the two strains grown in batch culture is an important observation and indicates that the regulation of these enzymes in the OSp strain is the same as that found in the Sp⁺ strain and that they are not regulated by a sporulation-specific gene(s). It is more likely that their regulation is directly related to cellular energy demand rather than the availability of nutrients or the growth phase.

Weinberg (1970) has suggested that trace metal concentrations required for secondary metabolism may be three orders of magnitude higher than needed for growth. He reported that these elevated concentrations of trace metals do not interfere with vegetative growth. This concept is in contradiction to our findings. Slight ($\pm 10^{-7} \text{ M}$) fluctuations of either zinc or manganese concentration altered the vegetative metabolism of the cell. The trace metal effect can be observed at growth rates lower than those presented, but the effect is not as striking. We have shown that by varying the trace metal content of the medium the efficiency of substrate utilization can be altered. This conversion of carbon and/or nitrogen to biomass is very efficient under optimum conditions (optimum ratio of trace metals for a particular growth rate).

The selection of mutants in chemostat culture has hampered the use of this technique in the study of sporulation. We chose to study the OSp strain due to its similarity to the parental

strain with respect to growth and enzyme activities (Fig. 1, Table 3). The results for macromolecular content and nutrient utilization are typical of a glucose-limited steady-state culture. The system is in agreement with the model of Dawes & Mandelstam (1969) for a differentiating organism in continuous culture. The problem of selection of sporulation mutants in continuous culture might be circumvented by operating at fast growth rates and by not operating the chemostat for extended periods. The potential of the chemostat in the study of differentiation should not be overlooked. The observations presented in this report should be useful in understanding the metabolism of bacilli in continuous culture and form a basis on which to build so that continuous culture techniques may be better used to study the technically more difficult problem of differentiation.

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