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Continuous Culture Used for Media Optimization

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A technique is described in which continuous culture is used for the optimization of media in terms of growth-supporting ability. The technique consists of identifying growth-limiting nutrients by observing the reaction of the continuous system to injection of suspected growth-limiting nutrients into the growth vessel.

The selection of media for growth of microorganisms is usually based on a combination of experimentation and logic, relying to a large extent on previous media that have been successful. When the amount of growth or growth rate obtained with a particular organism on a particular medium is believed to be less than optimal, recourse to experimentation is required.

The experiments are normally carried out by changing the concentration or nature of medium constituents, and comparing the growth rate or amount of growth obtained as a consequence of the change with that obtained on the basal medium. By a sequence of experiments using shake flasks, an improved medium, i.e., one that supports a greater cell concentration or a higher growth rate, can usually be obtained. Although in principle the procedure is simple, considerable experimental work is required because generally the experimenter does not know which nutrient has been growth limiting, either with respect to growth rate or cell crop obtained. Therefore, in the initial experimental series, all the possible growth-limiting nutrients must be increased in concentration, each in its own flask or flasks. Having established which nutrient is the growth-limiting one, the concentration is then varied in a series of flasks to establish the relationship between its concentration and the cell concentration or growth rate it supports. Then the cycle is repeated in a search for a second and subsequent growth-limiting factor.

The development of continuous culture techniques, based upon the theoretical and experimental formulations of Monod (6) and Herbert et al. (5), have been used widely for approaching problems in microbial physiology (see ref. 3). In this paper we would like to suggest that the use of continuous culture under nutrient-limited conditions provides an efficient means for optimizing culture media in terms of increasing the cell concentration the media are capable of supporting. As a consequence of this procedure, it is also possible to estimate the quantitative needs of the cells for the different nutrients.

MATERIALS AND METHODS

Microorganism and media. The microorganism used in this study was *Pseudomonas* C which has been previously described (1, 7).

MSM medium is a minimal salts medium to which 1% methanol was added, as described by Foster and Davis (4). MSM-M medium was a modification, as described by Chalfan and Mateles (1). M-3 medium was a further modification, as developed using the procedure described in this paper. The compositions of the three media are given in Table 1.

Cultures of the bacteria were maintained on agar slants of MSM-M medium containing 1% methanol. Inocula for continuous culture were grown batchwise in MSM-M medium containing 1% methanol, and after good growth several milliliters of the inoculum was added to the continuous culture vessel.

Continuous culture technique. The experiments were carried out in a New Brunswick BioFlo model C-30 continuous fermentor operated at 35 C with an aeration rate of 100 ml/min and an agitation rate of 600 rpm unless otherwise noted. The volume of medium in the fermentor varied with the agitation rate, and was in the range of 336 to 340 ml. Medium withdrawal was via the standard overflow tube. The pH was maintained at pH 7 by means of a Radiometer model 28 and model 11 Automatic Titration system which added a 5 N solution of sodium hydroxide as required. The fermentor vessel was fitted with an Ingold pH electrode model 401-MH.

The growth vessel was inoculated with several milliliters of inoculum, and the medium pump was started after good growth was observed to be taking place. The dilution rate used for these experiments was 0.32/h, corresponding to a mean residence time of about 3.1 h. Steady state was generally achieved within 10 to 20 h of making a change in the composition of medium in the medium reservoir and was confirmed by several measurements of cell concentration (optical density [OD]) or concentration of metha-

TABLE 1. *Composition of the media used in this work*

Medium	Reference	Composition														
		Methanol	$(NH_4)_2SO_4$ (g)	$NaNO_3$ (g)	$MgSO_4 \cdot 7H_2O$ (g)	Na_2HPO_4 (g)	$NaH_2PO_4 \cdot H_2O$ (g)	KCl (mg)	$CaCl_2 \cdot H_2O$ (mg)	$FeSO_4 \cdot 7H_2O$ (mg)	$CuSO_4 \cdot 5H_2O$ (μg)	$ZnSO_4 \cdot 7H_2O$ (μg)	H_3BO_3 (μg)	$MnSO_4 \cdot 5H_2O$ (μg)	Na_2MoO_4 (μg)	MoO_3 (μg)
MSM	4	V[a]		2.0	0.2	0.21	0.09	40	15	1	5	70	10	10		10
MSM-M	1	V	1.0		0.1	2.1	0.9	40	15	1	5	70	10	10		10
M-3	This paper	V	2.5		0.3	0.3	0.15	60	20	2	80	80	20	20	20	

[a] V, Various.

nol, as measured by gas chromatography on samples of medium taken from the growth vessel (1).

After a steady state was achieved, the process of seeking the growth-limiting nutrient began. As the aim was to increase the ability of the medium to support a high cell concentration, the carbon source, methanol, was intentionally chosen as the growth-limiting nutrient, and its concentration (S_0) in the medium reservoir was set initially at 1.0 g/liter. So long as methanol remains the limiting nutrient, an increase in its concentration in the medium reservoir should yield a proportional increase in cell concentration in the growth vessel, and furthermore the steady-state concentration (S) of methanol in the growth vessel should not change from its previous value. If these conditions were not fulfilled (if the cell concentration failed to increase proportionally, or if S increased substantially), it indicated that a nutrient other than methanol had become growth limiting as a result of the increase in the value of S_0, and a search was carried out as follows.

Concentrated solutions of the various components of the nutrient medium were injected individually into the growth vessel, so as to provide approximately double the concentration that was present in the medium contained in the reservoir and being fed into the growth vessel. After a single injection, observations on the cell and methanol concentrations were made for a period of 1 to 3 h. In the event that no significant change was observed, it was concluded that the particular nutrient injected had not been growth limiting and the next one was injected. A significant response, i.e., an increase in cell concentration coupled with a decrease in methanol concentration, indicated that the nutrient injected had been growth limiting, and when this was observed the concentration of the nutrient in the reservoir was increased appropriately. Then, 10 to 20 h were allowed for a new steady state to be achieved, and the process of increasing the concentration of methanol in the medium was continued until once again a less-than-proportional increase in cell concentration together with an increase in S was observed, when the search procedure was commenced anew.

Assays. Cell concentration was measured on a Gilford model 240 spectrophotometer by means of the OD at 650 nm. Dry weight of cells was linear with optical density up to 0.8 OD, and 1 OD was found to be equal to a cell concentration of 0.73 mg (dry weight)/ml.

Methanol concentration was determined by gas chromatography as previously described (1).

RESULTS AND DISCUSSION

Figure 1 presents results from the initial phase of the investigation designed to improve the MSM-M medium so that it would support a cell concentration higher than the 2 to 3 g/liter previously reported (1). Only after the copper content of the medium was increased, by injecting a copper sulfate solution into the growth vessel, was there a significant change either in the cell concentration or in the residual methanol concentration (S). Thus, it was concluded that copper had become the growth-limiting nutrient when the concentration of methanol in the reservoir medium was raised from 1 g/liter to 2 g/liter. Following this observation, $CuSO_4 \cdot 5H_2O$ was added to the reservoir medium to a final concentration of 80 μg/liter, and a new steady state was attained in which the cell concentration had approximately doubled while the residual methanol, S, remained the same. Then, the concentration of methanol in the reservoir, S_0, was raised to 3 g/liter, and the process was continued. The results of these experiments are summarized in Table 2.

As may be seen in Table 2, in some cases an increase in S_0 resulted in a decrease in cell concentration. This decrease in cell yield was generally associated with polysaccharide production but this was not always the case, as can be seen when the methanol was raised to 7 g/liter. Invariably, however, a failure of the cell concentration to respond more or less linearly to an increase in methanol concentration was reflected in a large increase in residual methanol. This is fully in accordance with the results

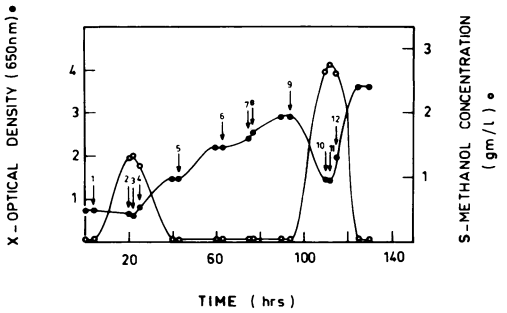

FIG. 1. *Changes in cell and methanol concentrations during growth of Pseudomonas C cells in a chemostat. Pseudomonas C cells were grown for 20 h in a chemostat with MSM medium containing methanol (1 g/liter), $(NH_4)_2SO_4$ (1 g/liter), as nitrogen source, and $CuSO_4 \cdot 5H_2O$ (10 μg/liter) at 35 C. Dilution rate was 0.35/h, with stirring of 600 rpm and aeration of 0.5 volume/volume per min. OD of cell suspension was measured at 650 nm, and the methanol concentration was that in the growth vessel (S). Numbers indicate additions as follows: 1, Methanol (1 g/liter) added to the medium in the reservoir (S_0 methanol = 2 g/liter); 2, $MnSO_4 \cdot 5H_2O$ (10 μg/liter) added to the growth vessel; 3, $CuSO_4 \cdot 5H_2O$ (10 μg/liter) added to the growth vessel; 4, $CuSO_4 \cdot 5H_2O$ (70 μg/liter) added to the medium in the reservoir; 5, methanol (1 g/liter) added to the medium in the reservoir (S_0 methanol = 3 g/liter); 6, methanol (1 g/liter) added to the medium in the reservoir (S_0 methanol = 4 g/liter); 7, $(NH_4)_2SO_4$ (0.5 g/liter) added to the growth vessel; 8, $(NH_4)_2SO_4$ (1.5 g/liter) added to the medium in the reservoir; 9, methanol (1 g/liter) added to the medium in the reservoir (S_0 methanol = 5 g/liter); 10, $FeSO_4 \cdot 7H_2O$ (1 mg/liter) added to the growth vessel; 11, $MgSO_4 \cdot 7H_2O$ (0.1 g/liter) added to the growth vessel; 12, $MgSO_4 \cdot 7H_2O$ (0.1 g/liter) added to the medium in the reservoir. The same procedure was continued until S_0 was 10 g/liter.*

expected from application of the basic theory, according to which $X = Y(S_0 - S)$, where X is cell concentration, Y is the yield coefficient relating consumption of limiting nutrient to production of cell mass, and S_0 and S are as previously defined. Since $S \ll S_0$ and $X \approx Y \cdot S_0$, the concentration in the growth vessel of limiting nutrient, S, depends only on the dilution rate (D) and cell-substrate parameters and is independent of S_0, the concentration of the limiting nutrient in the inflowing medium (5).

A further result of identifying a succession of limiting nutrients is that the stoichiometric requirement for the nutrients can be readily calculated from the cell concentration and the concentration of limiting nutrient. Table 3 pre-

TABLE 2. *Yield and concentration of Pseudomonas C cells in a steady state with methanol as a sole carbon source*[a]

S_0[b]—CH_3OH (g/liter)	X[c]-OD (650 nm)	S[d]—CH_3OH (mg/liter)	Yield[e] (%)	Additions to the medium[f]	Polysaccharide formation
1	0.72	48	53.7		–
2	0.66	1,280			+
	1.46	47	54.6	$CuSO_4$ (70 μg)	–
3	2.20	48	55.5		–
4	2.39	46			–
	2.92	58	54.5	$(NH_4)_2SO_4$ (1.5 g/liter)	–
5	1.45	2,670			+
	3.61	45	54.0	$MgSO_4$ (0.2 g/liter)	–
6	3.97	48		KCl (0.02 g/liter)	–
	4.09	46		Na_2HPO_4 (0.15 g/liter)	–
	4.20	43	52.0		–
7	1.93	7,200			–
	4.35	520		$FeSO_4$ (1 mg/liter)	+
	4.95	44	52.5	$MnSO_4$ (10 μg/liter)	+

[a] Cells were grown in the chemostat in MSM medium with ammonium sulfate (1 g/liter) and $CuSO_4$ (10 μg/liter). Growth was at 35 C, with constant stirring of 800 rpm and aeration of 1 volume of air/volume of growth medium per min. The dilution rate (D) was 0.32/h, and the pH was controlled at 7.0 ± 0.1. The influence of the different substances on cell density was measured by the addition of the substances to the growth vessel, not in the steady state. Only when there was an increase in cell density were these compounds added to the medium reservoir and cell density was measured at the new steady state.

[b] S_0, concentration of methanol in the medium added to the growth vessel.

[c] X, Cell concentration in a steady state.

[d] S, Steady-state concentration of methanol in the growth medium.

[e] Yield is expressed as grams of dry weight of cells per gram of methanol utilized.

[f] The concentration given is the final concentration in the medium.

TABLE 3. *Nutrient requirements for growth of Pseudomonas C as determined by continuous culture*

Nutrient	Mg of nutrient/g of cell (dry wt)
N	110.0
Mg	7.8
K	6.2
Ca	1.2
P	36.0
Fe	0.59
Zn	0.0036
Cu	0.0041
Mo	0.0018
B	0.0006
Mn	0.0013

sents such data for the bacterium used in this investigation, grown with 1% methanol.

The technique is time and effort saving because it is not necessary to run many shake flasks in parallel or to deal with several cultures at once. A very important factor, contributing to the efficiency of the method, is that it is not necessary to make measurements in the steady state to determine which nutrient is growth limiting. Usually, the methanol concentration was raised in the reservoir at the end of the work day. The next morning samples of broth were analyzed, and if it appeared that a nutrient other than methanol was growth limiting, as indicated by an increase in S, the injection program was started at once. As it was necessary to wait only an hour or two to observe a response, it was possible in a single day to test four to eight nutrients suspected of being growth limiting and to obtain a definite answer on each one. The fact that a steady state could not be achieved as a result of the injection was not important, as a significant response to the injection was interpreted qualitatively as a sign that the nutrient giving rise to the disturbance was the growth-limiting one, and then this nutrient was added to the medium in the reservoir at a concentration adequate to ensure that it would not be growth limiting during the further experimentation.

Based upon a series of experiments, the improved medium (see Table 1) was developed. Figure 2 shows the relationship obtained between S_0 and cell concentration using the improved medium M-3 at three times the regular concentration and at a dilution rate of 0.35/h. It is clear that a linear relationship was obtained until the oxygen transfer capacity of the apparatus became a limiting factor.

Figure 3 summarizes a series of experiments showing the relationship between dilution rate and cell concentration, residual methanol concentration, and yield for continuous cultures carried out with S_0 = 2.0 g/liter. A maximal yield of 0.55 to 0.56 g of cell/g of methanol was obtained when the dilution rate was 0.32 to 0.38/h. This result compares favorably with the yield of 0.3 to 0.4 g of cell/g of methanol found in many bacterial systems (see ref. 2) and 0.38 to 0.41 obtained by continuous culture of *Pseudomonas methylotropha* (Imperial Chemical Industries Ltd., French patent 2,117,530, 1972). Not only did M-3 medium support a high cell concentration in continuous culture, but it also did so in batch culture. Furthermore, the long lag reported when log-phase batch inocula were transferred to fresh media (1) was not seen.

Figure 4 compares growth curves obtained in batch culture on M-3 medium with those obtained on MSM-M medium.

The technique described here would be useful not only for improving media for organisms capable of growth in relatively simple defined media, but also for highly fastidious organisms. For instance, we are presently studying the growth requirements of certain very fastidious lactic acid bacteria which are grown in continu-

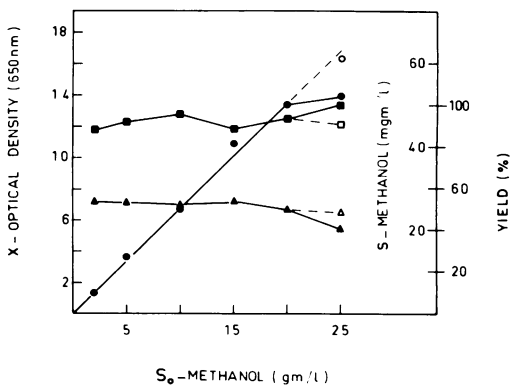

FIG. 2. *Effect of different concentrations of methanol (S_0) on the steady-state concentration of Pseudomonas C cells (X) in a chemostat culture. Cells were grown in a threefold concentrated M-3 medium, containing $(NH_4)_2SO_4$ as a nitrogen source, at 35 C. The stirring was 1,000 rpm with aeration of 4 volumes of air/volume of growth medium per min and dilution rate (D) of 0.35/h. The pH was controlled in the range of 7.0 ± 0.2. Symbols: ●, Cell concentration in the steady state (X); ■, methanol concentration in the growth vessel in the steady state (S); ▲, yield value (gram of cell [dry weight] per gram of methanol utilized). Broken lines, aeration was with 5 volumes of air/volume of medium per min.*

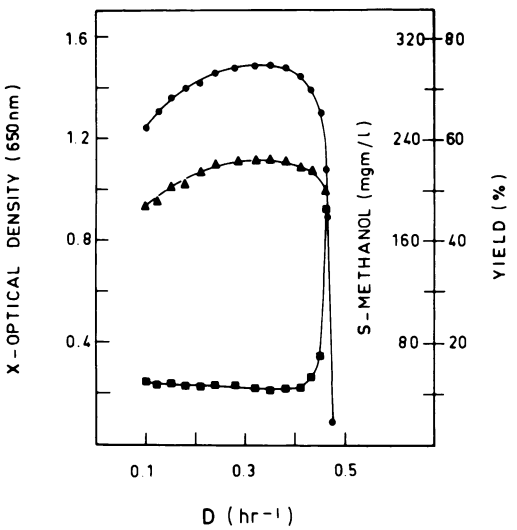

FIG. 3. *Influence of dilution rate (D) on the steady-state concentration of Pseudomonas C cells (X) and methanol (S) in a chemostat culture. Cells were grown in MSM medium containing $(NH_4)_2SO_4$ (1 g/liter) and supplemented with $CuSO_4$ (10 μg/liter) at 35 C. The stirring was 600 rpm with aeration of 0.5 volumes of air/volume of growth medium per min. The pH was controlled in the range of 7.0 ± 0.1. Methanol concentration was 2 g/liter. Symbols: ●, Cell concentration in a steady state (X); ■, methanol concentration in the growth vessel in a steady state (S); ▲, yield value (grams of cell [dry weight] per gram of methanol expended).*

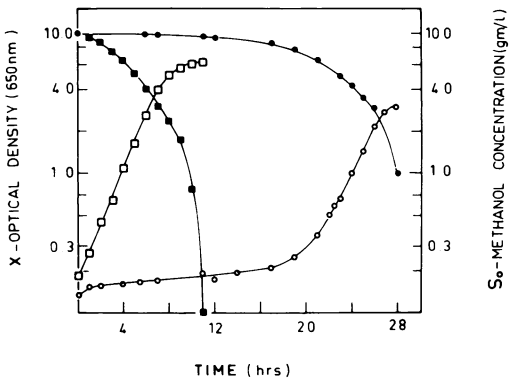

FIG. 4. *Growth of Pseudomonas C cells in batch cultures on MSM-M and M-3 medium, with 1% methanol. Symbols: ○, ●, Growth on MSM-M medium; □, ■, growth on M-3 medium. Open symbols represent values for cell concentration and closed symbols are values for methanol concentration in the medium. A log-phase inoculum grown on the same medium was used.*

ous culture on medium composed of several complex components, including yeast extract and peptone. The addition of certain other complex materials, such as tomato juice, is known to give a great stimulation to growth rate and to the amount of growth obtained. The continuous culture technique can be used as a rapid reliable bioassay in the fractionation of the tomato juice to identify the fraction responsible for the growth stimulation, and four to six fractions can be assayed per day in parallel with the fractionation procedure.

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