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Effect of Medium Composition on the Growth and Asparaginase Production of *Vibrio succinogenes*

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Asparaginase synthesis by *Vibrio succinogenes* is induced by ammonium ions. Synthesis occurs throughout exponential phase, and in early stationary phase asparaginase accounts for about 5% of the total soluble protein. The organism grows best when fumarate is provided as the terminal electron acceptor of the formate-oxidizing cytochrome system. Yeast extract or enzyme-hydrolyzed proteins are effective nutrient sources. In an ammonium formate-sodium fumarate medium, where maximum growth and asparaginase synthesis occurs, the total enzyme yield (international units per liter of culture) is about one-tenth that obtainable with a good asparaginase-producing strain of *Escherichia coli*. The energetic inefficiency of *V. succinogenes* appears to cause a low yield of cells and therefore low total enzyme yield. However, the levels of asparaginase accumulated within cells raise questions about the organism's protein synthesizing system.

The rumen anaerobe *Vibrio succinogenes* has been shown to produce large amounts of the enzyme L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) (5). Because asparaginases have antileukemic activity, the vibrio enzyme was purified and characterized (3). The enzyme was found to be unique among microbial asparaginases thus far described in that glutamate is not hydrolyzed. Glutamine hydrolysis by asparaginase has been implicated as a cause of the immunosuppressive side effects of asparaginase therapy. In vitro experiments have suggested that the vibrio enzyme should be less likely to cause immunosuppression than other microbial asparaginases (J. A. Distasio and M. M. Sigel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, E174, p. 110). The vibrio enzyme has been shown to be immunologically distinct from the *Escherichia coli* enzyme now in clinical use (3). The antitumor activity of the vibrio enzyme is comparable to that of the *E. coli* enzyme when both are tested in a standard mouse tumor system (2).

The properties of the vibrio enzyme suggest that it may prove to be a clinically useful enzyme. If so, large-scale cultivation of the organism will be essential. Although cell extracts of *V. succinogenes* can contain large amounts of asparaginase, the cell yield is low (4). Anaerobic energy metabolism is inherently less efficient than aerobic metabolism, but in the case of *V. succinogenes* the problem is particularly acute. Though an anaerobe, the organism is nonfermentative and has no known catabolic pathways. The oxidation of formate (or its energetic equivalent, H_2), coupled via cytochromes, to the reduction of either fumarate or nitrate is the organism's only source of energy. The free energy change of the formate-fumarate redox couple is −20 kcal (ca.−83.72 kJ) per mol (8), and Kröger has presented growth yield data that indicate that the ATP yield may be less than 1 ATP per formate-fumarate couple metabolized (6). This yield is not only very small when compared to aerobic metabolism, but it is also small when compared to most fermentative pathways.

The low energy yield obviously limits growth and consequently presents a major obstacle to the use of the organism on an industrial scale. The present study was undertaken in an attempt to develop media and culture conditions that maximize the yield of both cells and the potentially valuable product, asparaginase.

MATERIALS AND METHODS

Organism. *V. succinogenes* was isolated by Wolin et al. (9) and has been maintained in this laboratory for a number of years. The organism is listed by the American Type Culture Collection as ATCC 29543 and by the Anaerobe Laboratory of the Virginia Polytechnic Institute and State University, Blacksburg, as VPI no. 10659.

Media components. Sodium and ammonium formate and fumaric acid were reagent grade. With the exception of the experiments described in Table 1, sodium hydroxide was used to neutralize fumaric acid and adjust media to pH 7.3 to 7.4. All other chemicals were the best available grades. Peptone and yeast extract were the products of Difco Laboratories, Detroit, Mich. Other media ingredients, products of

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Humko Sheffield, Lyndhurst, N.J., were: N-Z-Case-TT, a tryptic digest of casein; Hy-Soy, a papaic digest of soybean meal; and Hy-Cas M, a hydrochloric acid hydrolysate of casein.

Cultivation and enzyme assays. Media for all experiments, except the growth curve determinations, were prepared in 100-ml quantities, in 160-ml screw-capped bottles, adjusted to pH 7.3 to 7.4 with NaOH, autoclaved, quickly cooled to approximately 37°C, reduced by the addition of freshly prepared sterile sodium thioglycolate solution (0.05% final concentration in the medium), and inoculated (0.05% [vol/vol] or less) from a 24-h culture in the medium of Wolin et al. (9). Incubations were in air at 37°C.

Growth curve experiments were performed with 3 liters of medium in 5-liter Erlenmeyer flasks. Upon removal from the autoclave, the media were cooled and vigorously sparged with sterile N_2. Sterile sodium thioglycolate and inoculum (0.03% [vol/vol]) were added, and the flasks were sealed under positive pressure. Samples of culture (100 ml) were periodically removed by displacement with N_2. In all experiments growth was estimated turbidimetrically (and expressed as optical density [OD]) at 550 nm with a Gilford model 240 spectrophotometer using cuvettes with a 1-cm light path. An OD of 1.0 is equivalent to a cell yield of approximately 1.5 g (wet weight) or 400 mg (dry weight) per liter. Cells were harvested by centrifugation (12,000 × *g*) at 4°C and washed twice with a solution of 0.9% NaCl and 0.2% $MgCl_2 \cdot 6H_2O$. Cell pellets were stored at −70°C. Preparation of cell extracts, the asparaginase assay, and the protein determination were described previously (5). Enzyme activity is expressed as international units (IU): micromoles of NH_4^+ released per minute at 37°C. Specific activities were IU per milligram of protein.

RESULTS

Four major and several minor variables were investigated. The results obtained will be described individually.

Source of fumarate. Fumarate may be added to media or may be replaced by L-malate, L-asparagine, or L-aspartate. These compounds are converted to fumarate intracellularly (Fig. 1). When sodium fumarate was the acceptor in a medium containing 100 mM sodium formate, 0.4% yeast extract, and 0.05% sodium thioglycolate, growth was proportional to fumarate concentration up to 100 mM. When similar concentrations of L-asparate, L-asparagine, or sodium L-malate were used in place of fumarate, growth was not generally proportional to acceptor concentration. A 100 mM sodium fumarate culture yielded an asparaginase specific activity of 1.8. With the other acceptors the asparaginase level was variable and difficult to reproduce and showed no consistent correlation with the final OD of the culture.

Presence of ammonium ions. The chemical composition of the medium requires that there be a substantial concentration of cations. Table 1 presents data obtained with media prepared with solutions of the K^+, Na^+, or NH_4^+ salts of formate and fumarate. The data clearly show that NH_4^+-containing media yielded good

TABLE 1. *Growth and asparaginase production as a function of the monovalent cation salts of formate and fumarate*[a]

Formate salt	Fumarate salt	Growth	Asparaginase sp act
Na^+	K^+	0.74	1.6
K^+	K^+	0.44	2.3
K^+	Na^+	0.56	1.5
NH_4^+	K^+	0.70	11.4
NH_4^+	NH_4^+	0.60	6.8
NH_4^+	Na^+ (25 mM)	0.35	7.4
NH_4^+	Na^+ (50 mM)	0.60	5.8
NH_4^+	Na^+	0.96	9.0
NH_4^+	Na^+ (125 mM)	1.2	13.4

[a] Unless otherwise indicated, the concentrations of both formate and fumarate were 100 mM. At the pH of the medium (7.3), the concentrations of the monovalent cations were equal to the formate concentration and twice the fumarate concentration. All media contained 0.4% yeast extract and 0.05% sodium thioglycolate. Cultures were harvested in early stationary phase (24 to 30 h). Growth is expressed as OD and asparaginase specific activity as IU per milligram of protein.

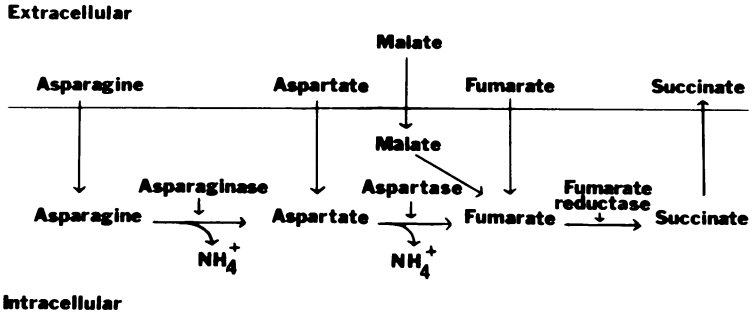

FIG. 1. *Metabolic relationships of the compounds that can provide fumarate as the terminal electron acceptor of the cytochrome-linked electron transport system.*

growth and enzyme production. A sodium formate-sodium fumarate medium (100 mM each) supplemented with 100 mM NH_4Cl grew to an OD of 1.1 and yielded an asparaginase specific activity of 6.3. When used with aspartate, asparagine, or malate, ammonium formate produced erratic and generally nonreproducible growth. The effect of ammonium ions was, however, confirmed. An ammonium formate-sodium malate culture (100 mM each) grew to an OD of only 0.49, but yielded an asparaginase specific activity of 11.2. The sodium formate-sodium malate cultures grew better but produced less enzyme. With 100 mM sodium formate, a 50 mM sodium malate culture grew to an OD of 0.61 and produced asparaginase with a specific activity of 6.6; with 125 mM sodium malate, the final OD was 1.0, but the specific activity of asparaginase was 4.5. (All of the malate cultures contained 0.4% yeast extract as well as 0.05% sodium thioglycolate.)

Media supplements. One factor that influences cell yield is the amount of ATP that must be used for the biosynthesis of small molecules. If this biosynthesis is minimized, available ATP can be used for macromolecular synthesis. Because the growth of *V. succinogenes* appears to be energy limited, attempts were made to maximize the cell yield by supplying the organism with an optimal mixture of small molecules. Experiments were performed using a basal medium of ammonium formate and sodium fumarate (100 mM each), supplemented with yeast extract and several protein hydrolysates. Cultures were harvested in early stationary phase, and the levels of growth and enzyme yield typically obtained are illustrated in Fig. 2 and 3. Yeast extract and enzyme-hydrolyzed proteins (casein or soybean meal) both supported good growth and enzyme production. However, the protein hydrolysates were required in higher concentrations (1 to 2%) than yeast extract (0.4 to 0.8%) to produce comparable growth. No growth was obtained in the absence of any supplement or when acid-hydrolyzed casein was the sole supplement. When mixed with either peptone or yeast extract, acid-hydrolyzed casein was neither stimulatory nor inhibitory. A mixture of 3% peptone and 1.2% yeast extract (as well as a number of other mixed supplements) had no

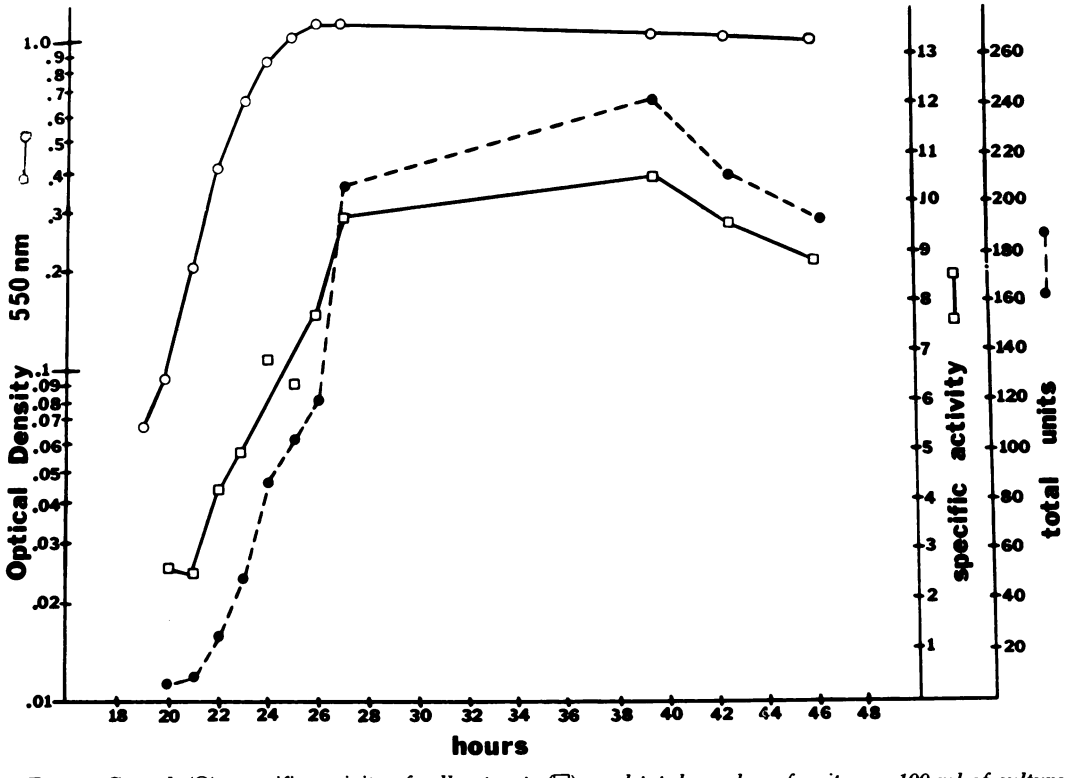

FIG. 2. *Growth* (○), *specific activity of cell extracts* (□), *and total number of units per 100 ml of culture* (●). *The medium contained ammonium formate and sodium fumarate (100 mM each), yeast extract (0.4%), and sodium thioglycolate (0.05%).*

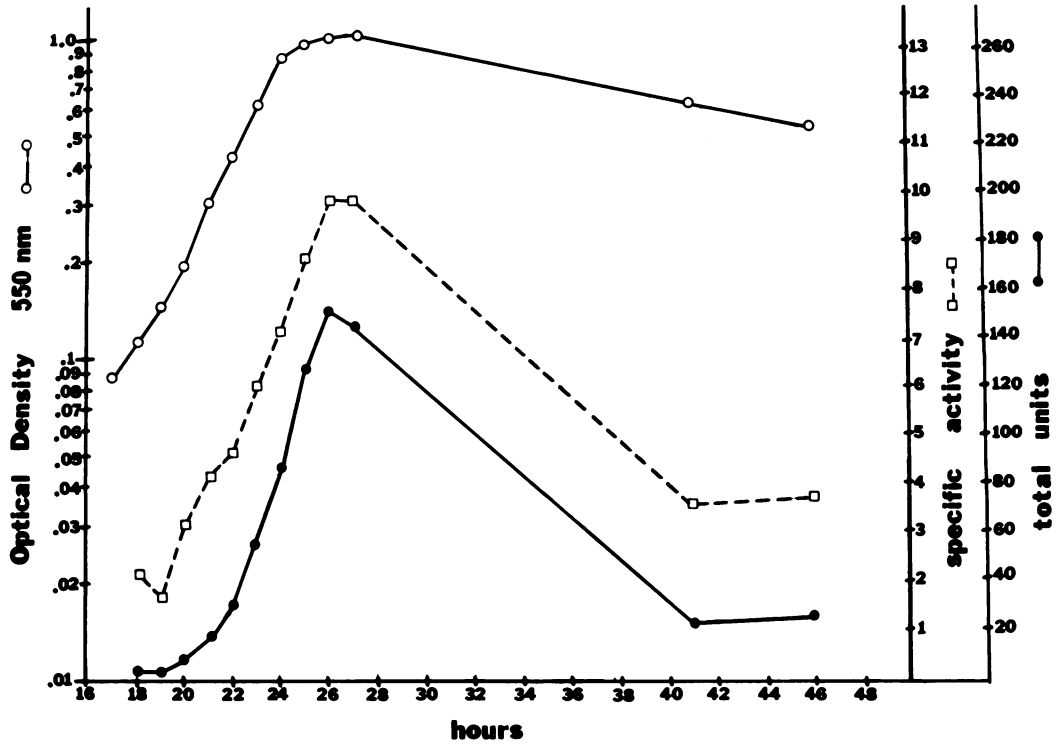

FIG. 3. *Growth* (○), *specific acitivity of cell extracts* (□), *and total number of units per 100 ml of culture* (●). *The medium contained ammonium formate and sodium fumarate (100 mM each), peptone (2%), acid-hydrolyzed casein (0.8%), and sodium thioglycolate (0.05%).*

significant advantage over 0.4% yeast extract as the sole supplement. Cells harvested from peptone-containing media were usually contaminated with a black precipitate; this was not observed with any of the other supplements tested.

Phase of growth. Growth experiments were performed to determine the optimal time to harvest the cells. Figure 2 presents the data obtained with an 0.4% yeast extract culture; Fig. 3 presents the data for a 2% peptone-0.8% acid-hydrolyzed casein culture. The basal medium contained ammonium formate-sodium fumarate (100 mM each). It is clear that the specific activity rises during exponential and early stationary phases. Figure 4 presents a differential plot of the data presented in Fig. 2 and 3. The linear increase in the proportion of the total cell mass that is asparaginase is indicative of enzyme synthesis in response to an inducer. The experiments shown in Fig. 2 and 3, as well as other growth experiments, revealed that yeast extract-containing cultures are less likely to undergo extensive lysis during stationary phase than are protein hydrolysate-containing cultures. Phase-contrast microscopy of wet mounts revealed that motility is a useful guide for harvesting the cells. The rapid motility of the organism is maintained during early stationary phase; a decline in motility indicates that the cells should be harvested.

Additional variables tested. Supplementation of media with a trace elements solution (7) appeared to increase the growth rate slightly, but was otherwise without effect. The addition of citrate at a concentration of 10 mM prevented growth from a small inoculum (0.05%). Lower concentrations of citrate were without apparent effect on growth or enzyme production. Ethylenediaminetetraacetate inhibited growth at the lowest concentration tested (0.1 mM). L-Glutamate, which has been shown to stimulate asparaginase production by *E. coli* A-1 (1), greatly decreased both growth rate and cell yield when added to media at a concentration of 1%. Rigorous anaerobic procedures in the preparation of media and incubation of cultures were without effect. Cell and enzyme yield were identical to cultures incubated in air and made anaerobic by 0.05% sodium thioglycolate and the depth of the liquid medium. Replacement of the thioglycolate by cysteine or sodium sulfide had no effect on growth or enzyme production.

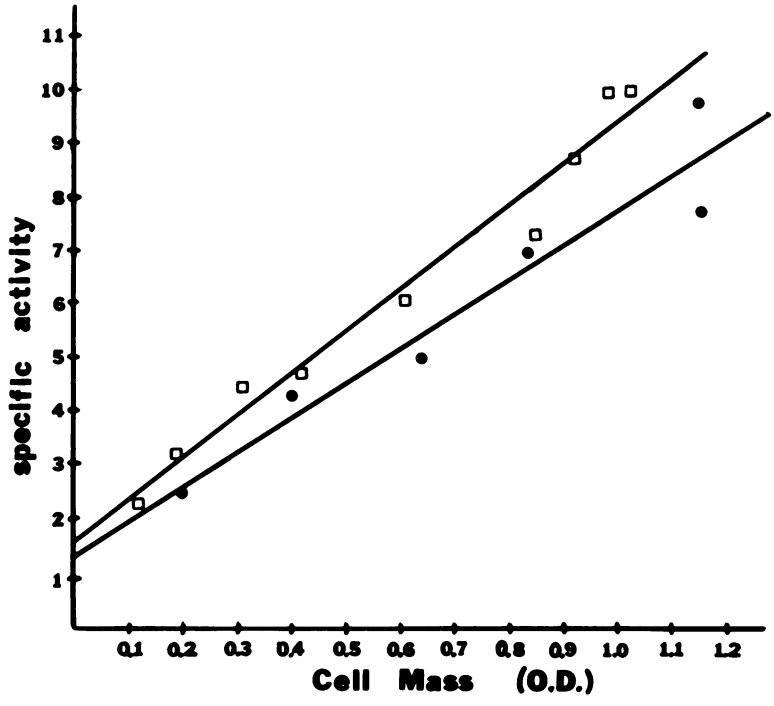

FIG. 4. *Differential plot of the growth and specific activity data of Fig. 2 and 3. Data from Fig. 2 (●); data from Fig. 3 (□).*

DISCUSSION

The experiments described in this report show that maximum production of cells and asparaginase is obtained in an ammonium formate-sodium fumarate medium supplemented with either yeast extract (0.4 to 0.8%) or an enzyme-hydrolyzed protein (1 to 2%). Initiation of growth from a small inoculum requires that the ammonium formate concentration not exceed 100 mM and the sodium fumarate concentration not exceed 125 mM. Higher concentrations of either or both frequently lead to inhibition (4). The reducing agent added to provide anaerobiosis may be 0.05% sodium thioglycolate, 0.05% sodium sulfide, or 0.05% cysteine-hydrochloride.

Fumarate, the terminal electron acceptor of the cytochrome system, is a more effective media component than the compounds that are intracellularly converted to fumarate. Experiments utilizing aspartate, asparagine, or malate yielded variable and often nonreproducible results. Nitrate, which is also a terminal electron acceptor of the cytochrome system, was not investigated. Although good enzyme production occurs in nitrate media (5), growth is poor (4), probably as a result of nitrite accumulation and a sharp rise in pH.

The requirement of ammonium ions for good enzyme production is interesting because these ions are a product of the asparaginase reaction. It is also apparent that maximum enzyme production occurs in a medium containing ammonium ions and fumarate: asparaginase has no obvious metabolic role in such an environment. It is difficult to even speculate on the regulation of asparaginase synthesis since, aside from the electron transport system (6, 8), little is known of the organism's metabolism. Figure 4 indicates that asparaginase synthesis occurs in response to an inducer present in the medium. The data suggest that ammonium ions are involved, but since these experiments were performed in complex media it is impossible to rule out the existence of a coinducer. The amount of asparaginase accumulated by the cells raises some interesting questions. The purified enzyme has a specific activity of 200 IU/mg of protein (3). Therefore, in a crude cell extract with a specific activity of 10 IU/mg of protein, asparaginase represents 5% of the total protein. That an organism should accumulate so much of a single enzyme is unusual even if the enzyme is essential for metabolism. In the case of asparaginase the activity is not only nonessential but actually destructive. The asparaginase reaction is hydrolytic and irreversible, and the enzyme is present in the soluble fraction of the cells. Assuming

that *V. succinogenes* synthesizes proteins that contain asparagine, it would appear that its protein-synthesizing system faces a formidable challenge in finding asparagine in the cytoplasm.

The majority of experiments described in this report were performed in 100 ml of medium. To test the validity of the conclusions, selected experiments were repeated in 40 liters of medium. Virtually identical data were obtained. An observation made in the course of this investigation that is of some practical importance is that none of the media described in this report was suitable for the maintenance of the organism. At 4°C viability was lost within a few days. By contrast, cultures in the medium of Wolin et al. (9) retain their viability, when stored at 4°C, for at least 3 months. It is possible that asparaginase catalysis at 4°C is responsible for the loss of viability. However, cells grown in the Wolin medium also contain significant amounts of asparaginase (5).

The data obtained in this investigation may be compared with the published data for *E. coli* A-1. Barnes et al. (1) showed that this strain, a good asparaginase producer when compared with literature reports for other organisms, yields a maximum of 27 IU/ml of culture. *V. succinogenes,* in the best medium developed in this investigation, yielded about 2.5 IU/ml of culture. This 10-fold difference must be viewed against the 30- to 40-fold energetic advantage possessed by *E. coli* growing aerobically on glucose (the conditions used by Barnes et al. [1]) compared to anaerobic growth of *V. succinogenes* on formate-fumarate. Since the *E. coli* and *V. succinogenes* enzymes have similar K_m's towards asparagine, and the vibrio enzyme is a slightly larger protein (molecular weight, 146,000 versus 135,000 for the *E. coli* enzyme), it appears that when both organisms are grown under conditions that maximize asparaginase synthesis, *V. succinogenes* is actually devoting a larger fraction of its metabolic resources to asparaginase synthesis than is *E. coli* A-1.

The study of *V. succinogenes* asparaginase was originally undertaken in an attempt to provide a new, clinically useful enzyme. The present investigation was begun in an attempt to optimize the production of the enzyme. It appears that the original goal of these studies will be achieved, but it also appears that these studies have raised a number of physiological questions about an unusual microorganism.

ACKNOWLEDGMENTS

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