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Conditions for the Production of L-Asparaginase 2 by Coliform Bacteria

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Of 28 coliforms, five strains of *Escherichia coli* were particularly active in elaborating L-asparaginase 2, the form of the enzyme useful in the treatment of some forms of cancer. Since it is advantageous to start purification of the enzyme from highly active cells, cultural conditions necessary for good growth and high enzyme yield have been studied. Gentle aeration proved suitable for good growth as well as high enzyme content. Stationary cultures gave poor growth, whereas vigorous aeration gave good growth but resulted in a marked decrease in the enzyme content of the cells. L-Asparaginase 2 has been purified about 40-fold by a combination of ammonium sulfate and ethyl alcohol precipitations.

There has been renewed interest in L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) since Kidd (10, 11) observed that guinea pig serum inhibits the growth of Gardner lymphosarcoma in mice, and Broome (1, 2, 3) provided evidence that L-asparaginase in the serum is the antitumor factor. Since then many workers have confirmed these observations, and the study of Yellin and Wriston (20) has provided conclusive evidence that a highly purified guinea pig serum L-asparaginase, homogeneous by ultracentrifugation and immunoelectrophoresis, does, in fact, inhibit Gardner lymphosarcoma in C3H mice. However, commercial production of L-asparaginase appeared desirable only after Mashburn and Wriston (12) showed that L-asparaginase from *Escherichia coli* also inhibits tumors in mice.

The discovery that *E. coli* produces two distinct enzymes, L-asparaginase 1 and L-asparaginase 2 (6, 7, 18), and the availability of a simple method for calculating amounts of either enzyme in a mixture of the two (6) induced us to study available *E. coli* and other microbial cultures for the elaboration of either of the two L-asparaginases under different growth conditions. Selection of a highly active culture with respect to L-asparaginase 2 would be an asset in producing large quantities of the enzyme. Study of the literature revealed little information on yields of the enzyme from bacterial cultures and on cultural conditions for optimal enzyme production. While we were investigating this problem, Roberts et al. (16) reported on the production of L-asparaginase by coliform and other bacteria in a variety of culture media but made no attempt to show how much of the total activity at *p*H 8.0 was due to L-asparaginase 2, the enzyme useful in the treatment of some forms of cancer in animals and man (5, 14, 15). Although Roberts et al. (16) claimed to obtain optimal enzyme yields under aerobic growth conditions, Schwartz et al. (18) reported that maximal yields of L-asparaginase 2 are produced under "briefly anaerobic" conditions. The work reported here evaluates different *E. coli* cultures with respect to their ability to produce the two L-asparaginases under different growth conditions by using the procedure described by Campbell et al. (6).

MATERIALS AND METHODS

Bacterial cultures. The following cultures were obtained through the courtesy of R. W. Reed, Microbiology Department, McGill University, Montreal: *E. coli* B, B/r, K-12(Y mel), K-12(B-1), *mutabile*, 0/55, 0/86, 0/125, K-12(T-3), K-12(T-26), W677, 9001, 9002, 58-161; 10 other cultures of *E. coli* isolated in the clinical laboratory of the department; and a culture of *E. freundii*. The National Research Council of Canada was the source of two cultures, *E. coli* K-12 (NRC 2006; ATCC 11303) and *E. coli* B (NRC 2005). A culture of *E. coli* was also obtained through the courtesy of S. Sonea, Microbiology Department, University of Montreal. The cultures were maintained on slopes of nutrient agar. They were allowed to grow for 2 to 3 days at 22 C and then were stored at 4 C.

Media. Medium A contained 1.0% peptone, 0.6% beef extract, 0.33% KH_2PO_4, and 0.1% L-asparagine. Medium B was corn steep water medium prepared essentially as described by Roberts et al. (16). A 10% medium was also used. Medium C was 2%

Hycase S.F. (Sheffield Chemical Co., Norwich, N.Y.) containing 0.02% yeast extract solids. Medium D was 2% Soytone (Difco) containing 0.02% yeast extract solids. Medium E contained 0.5% peptone, 0.5% beef extract, 0.5% yeast extract, and 0.1% L-asparagine. Medium F was medium A, but containing 0.5% L-asparagine instead of 0.1%. Medium G contained 0.2% peptone, 0.2% beef extract, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.6 mg of $FeSO_4 \cdot 7H_2O$ per 100 ml, 0.5 mg of $MnSO_4 \cdot H_2O$ per 100 ml, 0.01% $CaCl_2$, 0.05% NaCl, 1.0% $(NH_4)_2SO_4$, and 0.5% L-asparagine. All of the above media were adjusted to *p*H 6.9 to 7.0 before sterilization at 121 C of pressure for 20 min.

Harvesting of cells. A 2- to 3-day-old growth on nutrient agar slants was used to inoculate the inoculum medium. Growth for 24 hr in the appropriate liquid medium provided the final inoculum. A 1:9 (v/v) ratio of final inoculum to growth medium was used throughout.

In the case of stationary cultures, 500 ml of the medium was placed in 1-liter conical flasks which were inoculated and held at 22 C for 48 hr. The vigorously aerated cultures, in which sterile air was bubbled through the medium, were grown for 18 hr at 22 C in 2 liters of medium contained in 3-liter flasks. Frothing was prevented by the addition of Dow Antifoam A. Most of the testing for the production of L-asparaginases, however, was done by using shaken cultures (Dubnoff water bath shaker or an Eberbach rotary shaker, 100 rev/min) at 22 C; 100 ml of the medium was placed in 250-ml conical flasks when the Eberbach shaker was used, and 20 ml of the medium was placed in 50-ml conical flasks when the Dubnoff shaker was used. At the end of the growth period, the cells were centrifuged at 4 C, washed twice with deionized water, and resuspended in deionized water for determinations of dry weight and L-asparaginase activity or for further purification.

Assay of L-asparaginase. L-Asparaginase activities were measured at 37 C in a Dubnoff water bath shaker at *p*H 8.4 and 5.0, by use of borate and acetate buffers, respectively, by the procedure described by Campbell et al. (6). The reaction mixtures contained 0.2 millimole of borate or acetate buffer (*p*H 8.4 and 5.0), respectively, 20 μmoles of L-asparagine, and an appropriate volume and dilution of cell suspension (or enzyme extract) in a total volume of 2.0 ml. After incubation with shaking for the desired periods of time, the reaction was stopped by the addition of 0.5 ml of 15% trichloroacetic acid. The ammonia liberated in the reaction was measured by direct nesslerization (6). The yellow color was read at 450 nm in a Beckman DB-G spectrophotometer, and the amount of ammonia liberated was calculated from a standard curve prepared from NH_4Cl solution. It was established that under the conditions of the assay the enzyme reaction was linear with time and also that the NH_3 liberated was due to L-asparaginase activity alone.

The determination of the two forms of L-asparaginase in the presence of one another was carried out by employing the equations given by Campbell et al. (6). The activities are expressed in terms of international units (IU) of L-asparaginase, which has been defined as that amount of enzyme which will release 1 μmole of ammonia in 1 min at the initial maximal rate.

Determination of protein was carried out by the spectrophotometric method of Groves et al. (8).

Partial purification of the enzyme. All of the steps described below were carried out, as far as possible, in the cold (4 C). The washed cells were freeze-dried in a VirTis freeze-dryer. The enzyme was extracted from this lyophilized material either by grinding to a paste with an equal weight of alumina (Fisher, 80 to 200 mesh), by using a mortar and pestle (12), or by sonically treating a heavy cell suspension in tris-(hydroxymethyl)aminomethane (Tris)-PO_4 buffer (*p*H 8.6) for 20 min with a Blackstone sonicator (13). The extract was centrifuged at 20,000 × *g*, and the extraction was repeated on the residue. The pooled supernatant fluids were treated to remove nucleic acids by adding 0.05 volume of 1 M $MnCl_2$ dropwise and with stirring. After stirring for 1 hr, the solution was centrifuged at 23,000 × *g* for 45 min, and the residue was discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant fluid to a concentration of 50% saturation, keeping the *p*H 8.0 constant by the addition of alkali. After stirring for 30 min, the precipitate was removed by centrifugation at 13,000 × *g* for 20 min. The $(NH_4)_2SO_4$ concentration was raised to saturation concentration, and the precipitate obtained was centrifuged as before. Most of the L-asparaginase 2 activity was present in this precipitate (Table 7), which was dissolved in water and dialyzed against repeated changes of water until salt-free and finally dialyzed against physiological saline.

This fraction was then dialyzed against 0.02 M Tris-PO_4 buffer (*p*H 8.0) and fractionated with ethyl alcohol. A 1.5-volume of cold ethyl alcohol was added dropwise with stirring. After 30 min of continuous stirring, the solution was allowed to stand at 4 C for 1 hr. The precipitate was removed by centrifugation, and the supernatant fluid was retained for further processing. Similarly, precipitates obtained with 1.8-, 2.5-, 3.0-, and 3.5-volumes of ethyl alcohol were collected by centrifugation and dissolved in physiological saline. The 2.5-volume precipitate, which contained most of the L-asparaginase 2 activity, was retained.

RESULTS

Growth and enzyme production under stationary conditions. Since it had been reported that *E. coli* K-12 produces more L-asparaginase under "briefly anaerobic" conditions (18), the available bacterial cultures were examined for the production of L-asparaginases under stationary conditions in a variety of media. When these cultures were grown in medium A they gave the results presented in Table 1. Four other media, modified with respect to their peptone, L-asparagine, and inorganic salt concentrations, were then tested for the elaboration of L-asparaginases by a culture of *E. coli* U.M. (Table 2).

TABLE 1. *L-Asparaginases produced by some bacterial cultures under stationary growth conditions*

Bacterial culture	Cell yield (mg/ml of culture)	L-Asparaginases produced (IU/g of dried cells)	
		L-Asparaginase 1	L-Asparaginase 2
Escherichia coli U.M.	0.23	30.2	263.4
E. coli B (NRC 2005)	0.16	19.0	141.5
E. coli K-12 (NRC 2006; ATCC 11303)	0.18	41.1	66.5
Aerobacter aerogenes	0.30	54.5	Nil
Pseudomonas species	0.33	138.1	Nil
E. coli U.M.[a]	0.30	33.0	238.6

[a] This culture was grown differently from the others. It was a 3-day growth at 22 C in 2,500 ml of medium taken in a 3-liter round flask.

TABLE 2. *Effect of medium composition on cell yield and production of L-asparaginases by E. coli U.M.*

Medium	Cell yield (mg/ml of culture)	L-Asparaginases produced (IU/g of dried cells)	
		L-Asparaginase 1	L-Asparaginase 2
A	0.27	30.5	260.7
E	0.47	44.6	152.4
F	0.30	42.7	287.7
G	0.20	29.3	98.0

TABLE 3. *Growth and production of L-asparaginases by E. coli U.M. under conditions of vigorous aeration*

Medium	Cell yield (mg/ml of culture)	L-Asparaginases produced (IU/g of dried cells)	
		L-Asparaginase 1	L-Asparaginase 2
Medium A	1.38	31.5	138.2
Medium A plus 0.2% Casitone	1.60	24.5	138.5

Growth and enzyme production under conditions of vigorous aeration. Although stationary cultures gave good enzyme yields, as compared with values given by Campbell et al. (6), the growth obtained was poor. However, when *E. coli* U.M. was grown by blowing sterile air through the growth medium, there was a marked increase in growth, but the amount of L-asparaginase 2 produced by this culture decreased to about half of that produced in the same medium under stationary conditions (Table 3, compare with results in Tables 1 and 2).

Growth and enzyme yields in shaken cultures. Because of the decrease in enzyme yield as a result of vigorous aeration, the effect of milder aeration on enzyme yield was investigated. It was found that shaking on a rotary or Dubnoff shaker not only increased cell yields to the levels obtained under vigorous aeration, but also gave the high yields of L-asparaginase 2 obtained in stationary cultures. Consequently, all subsequent testing and culturing of cells for preparing L-asparaginase 2 were done in shake cultures. Three *E. coli* cultures were examined for their growth and ability to elaborate L-asparaginases in medium A at 37 C in the Dubnoff shaker (Table 4). All subsequent studies were carried out at 22 C, the ambient temperature of the laboratory.

Table 5 presents the results of screening of available coliform bacteria for the production of L-asparaginases in medium A. When the six best enzyme producers were examined in this medium after 48 hr of growth, a slight increase in cell yield was accompanied by a decrease in the enzyme content of the cells.

While this work was in progress, a paper by Roberts et al. (16) appeared which reported very high yields of total L-asparaginase activity in several coliform and other bacteria. In view of these high yields, some of the cultures tested in our laboratory in the media used by these workers were examined. Indeed, 2% Hycase-0.02% yeast extract produced the highest yields and was superior to our media (Table 6). The 5% corn steep water medium gave higher yields of L-asparaginase 2 than the 10% medium (Table 6). The addition of 0.1% L-asparagine to 2% Hycase-0.02% yeast extract did not in any way affect the production of L-asparaginases or the cell yield of *E. coli* U.M., *E. coli* K-12 (NRC 2006), and *E. coli* B (NRC 2005).

TABLE 4. *Production of L-asparaginases by shake cultures of E. coli at 37 C*

Culture	Cell yield (mg/ml of culture)	L-Asparaginases produced (IU/g of dried cells)	
		L-Asparaginase 1	L-Asparaginase 2
Escherichia coli U.M.	1.5	Nil	359.5
E. coli B (NRC 2005)	1.4	Nil	174.8
E. coli K-12 (NRC 2006)	1.5	6.9	101.8

TABLE 5. *L-Asparaginases produced by shake cultures of coliform bacteria in medium A*

Culture identity	Cell yield (mg/ml of culture)	L-Asparaginases produced (IU/g of dried cells)		Culture identity	Cell yield (mg/ml of culture)	L-Asparaginases produced (IU/g of dried cells)	
		L-Asparaginase 1	L-Asparaginase 2			L-Asparaginase 1	L-Asparaginase 2
Escherichia coli B	0.90	38.0	682.4	*E. coli* 58-161	0.89	48.6	75.5
E. coli B/r	0.83	46.3	131.6	*E. freundii*	1.15	113.6	194.9
E. coli K-12 (Y-mel)	0.94	47.7	56.9	*E. coli*	1.21	11.3	113.2
E. coli K-12 (B-1)	0.95	10.9	62.5	*E. coli*	1.14	—[a]	—[a]
E. coli mutabile	1.31	44.3	92.5	*E. coli*	1.24	44.6	172.9
E. coli 0/55	1.20	34.4	189.9	*E. coli*	1.00	30.1	177.9
E. coli 0/86	1.04	6.8	283.3	*E. coli*	1.01	19.2	226.3
E. coli 0/125	0.93	46.8	385.5	*E. coli*	1.06	Nil	216.9
E. coli K-12 (T-3)	0.98	17.9	69.9	*E. coli*	1.23	24.6	145.2
E. coli K-12 (T-26)	0.94	61.4	56.1	*E. coli*	1.13	6.2	204.1
E. coli W677	0.81	44.7	65.9	*E. coli*	0.73	18.6	240.3
E. coli 9001	0.91	66.9	540.6	*E. coli*	1.21	29.7	104.9
E. coli 9002	1.25	43.3	123.1	*E. coli* U.M.	1.10	83.2	313.9

[a] No activity at concentration tested.

TABLE 6. *Production of L-asparaginases by shake cultures of some coliform bacteria in different media*

Culture identity	Cell yield mg. ml.$^{-1}$ culture		L-Asparaginases produced (IU/g of dried cells)			
			24 hr		36 hr	
	24 hr	36 hr	L-Asparaginase 1	L-Asparaginase 2	L-Asparaginase 1	L-Asparaginase 2
Medium C						
E. coli B	1.16	1.50	84.0	848.1	60.8	748.2
E. coli 0/86	1.23	1.70	74.7	422.6	55.9	353.4
E. coli 0/125	1.31	1.78	135.2	723.9	29.1	618.4
E. coli 9001	1.04	1.37	126.3	811.8	16.4	655.8
E. coli U.M.	1.39	1.89	108.0	446.7	65.0	454.2
Medium D						
E. coli B	1.05	1.51	76.1	592.2	50.3	551.8
E. coli 0/86	1.20	1.56	1.5	222.6	41.3	184.3
E. coli 0/125	1.76	1.98	93.9	332.6	53.9	345.7
E. coli 9001	1.04	1.35	47.7	504.1	75.9	442.5
E. coli U.M.	1.29	1.71	144.3	453.1	126.1	365.2
Medium B						
E. coli B	1.26	1.65	40.5	576.9	64.5	595.1
E. coli 0/86	1.38	1.70	17.5	272.2	16.8	240.0
E. coli 0/125	1.33	1.81	60.5	358.2	58.5	302.1
E. coli 9001	1.27	1.49	45.0	245.2	52.3	259.7
E. coli U.M.	1.39	1.77	36.4	240.5	55.9	179.8
Medium B[a]						
E. coli B	1.25	1.81	89.6	223.6	80.9	270.6
E. coli 0/86	1.39	1.74	30.8	157.9	8.9	153.3
E. coli 0/125	1.35	1.90	52.8	196.1	54.6	220.2
E. coli 9001	1.18	1.61	32.4	92.0	37.4	87.0
E. coli U.M.	1.39	2.11	42.8	51.2	45.9	56.9

[a] With 10% corn steep water.

Purification of L-asparaginase 2. Attempts at partial purification of L-asparaginase 2 by using a combination of $(NH_4)_2SO_4$ and ethyl alcohol precipitation, after removal of nucleic acids by $MnCl_2$ precipitation, resulted in a 40-fold purification of this enzyme (Table 7).

DISCUSSION

From the results presented in this paper, it is apparent that cultural conditions markedly influence L-asparaginase 2 production by coliform bacteria. Of the 28 coliforms tested, *E. coli* B, *E. coli* 0/86, *E. coli* 0/125, *E. coli* 9001, and *E. coli* U.M. were found to be good producers of this enzyme, when they were grown in nitrogen-rich media and under conditions of gentle aeration. That vigorous aeration is detrimental to the profuse elaboration of this enzyme is not surprising in view of the extreme lability of this enzyme under conditions promoting surface denaturation, as reported by Yellin and Wriston (21) with guinea pig serum L-asparaginase, and in view of my experience with purified commercial preparations from *E. coli*.

There is a great deal of interest in finding microorganisms which are good sources of this enzyme, particularly those producing relatively little toxin. Thus far, only *E. coli* is being commercially exploited, although a wealth of microorganisms await to be examined for the production of L-asparaginase 2, as is evident from reports of antitumour activity of L-asparaginases from *Serratia marcescens* (17), *Mycobacterium tuberculosis* (9), and, very recently, *Erwinia carotovora* (19). An asparaginase from compressed bakers'

TABLE 7. *Partial purification of E. coli* L-*asparaginase 2*

Fraction	L-Asparaginase activity at *p*H 8.4 (IU/ml)	Proteins (mg/ml)	Specific activity	Percentage recovery
First sonic-treated material	3.45	11.4	0.3	
Second sonic-treated material	2.67	11.8	0.2	
Supernatant fluid after nucleic acid removal	2.37	6.3	0.4	65
Precipitate at 50% saturated $(NH_4)_2SO_4$	1.55	23.25	0.1	5
Precipitate at 100% saturated $(NH_4)_2SO_4$	14.63	10.60	1.4	60
1.5-Volume ethyl alcohol precipitate	0.72	0.788	0.9	3
1.8-Volume ethyl alcohol precipitate	0.92	0.238	3.9	4
2.5-Volume ethyl alcohol precipitate	6.53	0.570	11.5	27
3.0-Volume ethyl alcohol precipitate	1.14	0.187	6.1	6
3.5-Volume ethyl alcohol precipitate	0.33	0.125	2.6	2

yeast (4) has no antitumour activity, being cleared very rapidly from the blood. A strain of *Saccharomyces cerevisiae* examined in this laboratory was found to produce only L-asparaginase 1. Thus, if it can be shown that only L-asparaginase 2 has antitumour activity, as appears to be the case, then it is clear that Campbell et al. (6) have made useful contribution by describing a method of calculating how much of either L-asparaginase is present in a mixture of both. By employing this method, one has a reasonable chance of picking out useful sources of L-asparaginase 2 with potential antitumour activity without resorting to animal testing in the preliminary stages.

While this work was in progress, Roberts et al. (16) reported high yields of L-asparaginase from *E. coli* but did not determine how much of these activities at *p*H 8.0 are due to L-asparaginase 2. They concentrated mainly on purification of the enzyme, presumably so that enough pure enzyme might be available for clinical work. Prior to the report of Roberts et al. (16), the yields obtained in this laboratory appeared to be higher than those reported in the literature (6). However, it is pertinent to point out here that, although Roberts et al. (16) obtained high yields in their initial screening of cultures, they failed to record the same high yield when they grew *E. coli* H.A.P. in bulk for purification of the enzyme. This is evident from Table 4 of their paper, wherein a specific activity of 0.5 is recorded for the "ruptured cell suspension." If the protein content of *E. coli* cells is taken to be around 50% of their cell weight, then this would work out to an activity of 0.25 IU/mg of dried cells. This value, as compared to the extremely high 0.95 to 1.0 IU/mg of dried cells recorded in Table 2 of Roberts et al. (16), is understandable in the light of the results obtained in this laboratory. Thus, if vigorous aeration was employed by Roberts et al. in harvesting cells on a larger scale for enzyme purification, the drop in activity can be expected. Apparently, only gentle aeration appears to enable one to obtain both good growth and high enzyme yield.

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