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Effect of Glucose and Low Oxygen Tension on L-Asparaginase Production by a Strain of *Escherichia coli* B

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Cultural and nutritional requirements for maximum L-asparaginase synthesis were determined. Conventional aerobic and anaerobic fermentations were not satisfactory. The former yielded larger quantities of cells containing minimal amounts of L-asparaginase, whereas the latter supplied only minute amounts of bacteria that contained an abundance of enzyme. However, the combination of these classical methods, i.e., allowing growth to proceed aerobically until the mid to late exponential phase and then forcing the facultative microbial cells toward anaerobic metabolism by static incubation, produced 2.6 international units of enzyme per ml of fermentation broth when glucose was present. Enzyme synthesis was not induced by terminating aeration-agitation in the absence of glucose, nor was it induced in the presence of glucose when aeration was continued. Use of 0.2 M phosphate buffer resulted in a constant *p*H near the optimum value of 7.5 during L-asparaginase formation. Addition of 0.05% L-asparagine prior to induction was also beneficial, but other amino acids or their catabolites failed to increase biosynthesis of L-asparaginase.

Kidd (16) observed that certain lymphomas of the mouse and rat could be suppressed effectively by treatment with normal guinea pig serum. Broome (4, 5) later determined that the factor present in serum responsible for this effect was the enzyme L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1). Mashburn and Wriston (18) found the enzyme from *Escherichia coli* effective against mouse leukemia. L-Asparaginase has since been detected in numerous other microbial genera by a number of investigators (20, 23, 24), although gram-negative organisms are generally most productive.

This study was undertaken to facilitate, by optimal fermentation conditions, procurement of the large quantity of material required for clinical trial. Crystallization of L-asparaginase by Ho et al. (14) was accomplished from fermentation broth prepared under the improved fermentation conditions.

MATERIALS AND METHODS

Bacterial cultures. All cultures were obtained from the Lilly collection of lyophilized microorganisms. The strain of *E. coli* B used throughout this study was Lilly culture C532.5, a natural phage-resistant variant obtained by classical methods of single-clone isolation from ATCC 13706.

Media and growth conditions. Slant cultures of C532.5 were incubated overnight at 37 C on Trypticase Soy Agar (BBL) and then were lyophilized. Fermentor inoculum was grown in wide-mouth, 250-ml Erlenmeyer flasks containing 50 ml of Trypticase Soy Broth (TSB). Flasks inoculated with lyophilized pellets were incubated overnight at 37 C on a G53 New Brunswick shaker rotating at 250 rev/min. Fermentors were aerated (0.4 v/v/m) and stirred (400 rev/min) 40-liter vessels of conventional design containing 25 liters of medium. They were seeded with approximately 2.72×10^6 cells per ml of broth volume. The fermentation medium was a modified TSB (MTSB) prepared according to the TSB formulation with two exceptions; Hy-Case amino (acid hydrolyzed casein, Sheffield Chemical Co.) was substituted for Trypticase and glucose was omitted.

Semianaerobic aging for enzyme induction. After cells were grown aerobically, incubation was continued for 60 min, without agitation or aeration, at 37 C in a closed vessel. The head space was one-fourth to one-half of the total fermentor volume. There was no exchange or replacement of the entrapped gases. Unless otherwise indicated, 0.5% glucose, 0.2 M potassium phosphate (*p*H 7.5), and 0.05% L-asparagine were added when aging began.

L-Asparaginase assays. Bacteria were removed from the growth medium by centrifugation at 4 C. The cell pellet was resuspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.5 and sonically treated. The sonically-treated material was centrifuged at high speed, and the resulting supernatant fluid was assayed at pH 5.0 by the method of Campbell et al. (7) for the antilymphoma asparaginase, EC-2. Enzyme activity was recorded as international units (IU) per milliliter of culture broth, one unit being equal to that quantity of enzyme which released one μmole of ammonia per minute from L-asparagine at the maximum rate.

Oxygen measurement. A modification of the galvanometric membrane electrode described by Johnson et al. (15) was used to observe oxygen activity.

RESULTS

Growth cycle and enzyme production. C532.5 grew rapidly in the MTSB medium (Fig. 1). Exponential cell replication ended after 7 hr of incubation, after which growth slowed progressively until the maximum stationary phase was reached at the 9th hr. In contrast, dry weight continued to increase through the 11th hr of incubation. The L-asparaginase activity per unit of fermentation broth was low throughout the cycle, being no greater than 0.04 IU/ml at any time. The greatest activity per unit of biomass was found during the earlier portion of exponential growth. Highest total enzymatic activity per unit of broth volume was realized later, just before the stationary phase, when the decrease in enzyme per cell was more than counterbalanced by the increase in cell mass. Enzyme activity is therefore reported as IU/ml of broth, since our goal was maximum enzyme production per unit of fermentation volume.

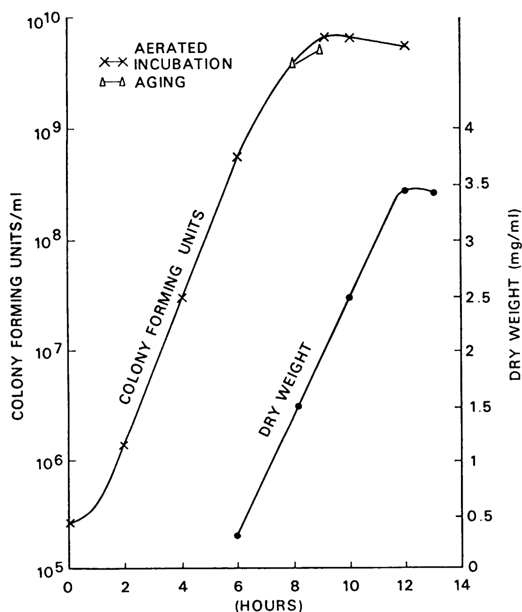

FIG. 1. *Growth of C532.5 in MTSB medium.*

Effects of glucose and aging. Although growth in the MTSB fermentation medium was similar to that in the TSB vegetative medium, it was noted that the glucose content of the latter was rapidly depleted. Since the catabolism of a readily oxidizable carbohydrate frequently results in growth stimulation, glucose was added to the MTSB medium before autoclaving. Only a slight increase in growth resulted, and no significant change in the asparaginase level of aerated broths was detected. Schwartz et al. (22) observed higher asparaginase yields when previously aerated *E. coli* cultures were allowed to stand without agitation or aeration. This procedure elicited no response from *E. coli* C532.5 unless at least 0.75% of glucose was added to the fermentation medium. This quantity was not exhausted during the period of aerated growth. The 20-fold increase in asparaginase observed was found to be directly related to the presence of glucose during the static incubation period succeeding aeration (Table 1), an interval which we define as aging. Aging without glucose did not afford high titers of L-asparaginase; neither did aerated growth in the presence of glucose unless succeeded by aging.

Effect of pH during aging. An increase in acidity was observed during aging in the presence of glucose; the pH value frequently dropped from about 6.8 to 5.2. To establish the relationship between pH and L-asparaginase induction during aging, pH was controlled through the addition of NaOH, HCl, or both. Table 2 illustrates the critical role of pH. Maximum enzyme production was obtained at pH 7.5, with a gradual decrease at lower pH values, as well as a sharp decline at higher values. Phosphate

TABLE 1. *Effect of glucose and aging on L-asparaginase biosynthesis*

Cell growth	Glucose in growth medium	Cells aged after growth	Glucose present during aging	L-Asparaginase (IU/ml)
Aerobic	No	No		0.04
Aerobic	0.75%	No		0.05
Aerobic	No	Yes	No	0.04
Aerobic	No	Yes	Yes	0.81[a]
Aerobic	0.75%	Yes	Yes	0.79[a]
Anaerobic	0.75%			0.33

[a] Results obtained prior to pH control during aging.

TABLE 2. *Effect of pH on biosynthesis of L-asparaginase during aging*

*p*H	Enzyme activity (IU/ml)
5.0	0.81
6.0	1.70
6.5	1.91
7.0	2.29
7.5	2.62
8.0	1.74
9.0	0.1

TABLE 3. *Effect of buffers during aging*

Buffer addition	*p*H after buffer addition	*p*H after aging	Enzyme activity (IU/ml)
None	6.8	5.6	1.14
$NaHCO_3$(0.2 M)	7.3	7.0	2.41
$NaHCO_3$(0.4 M)	7.4	7.3	2.03
Tris base (0.05 M)	7.5	6.2	1.61
Potassium phosphate (0.1 M)	7.5	7.1	2.51
Potassium phosphate (0.2 M)	7.5	7.4	2.67

buffer was more effective than carbonate or Tris for producing high enzyme levels (Table 3).

Aging time. The length of the aging period was critical. Enzyme synthesis began almost immediately upon cessation of aeration-agitation and continued at a constant rate for 40 min (Fig. 2). Synthesis then declined sharply. The enzyme activity reached the maximum at 60 min and remained at a constant level for the 2nd hr. A 15% loss occurred during the 3rd hr. Even when aeration-agitation was resumed after aging, the enzyme concentration remained unchanged at the maximum level for several hours.

Effects of glucose concentration on enzyme synthesis during aging. Production of L-asparaginase was proportional to glucose concentrations between 0.1 and 1.8 mg/ml. It was maximal at 2 to 5 mg/ml (Fig. 3). Normal induction was obtained whether the glucose was added immediately before, or as much as 30 min after, aeration was stopped. When available, 3.2 mg/ml was catabolized during the aging period, although only about 2 mg/ml was required for maximum enzyme synthesis (Table 4).

Inductive response as affected by aerated growth time. When the aging procedure was applied to aerobically grown cells, enzyme synthesis per unit of broth volume was maximum by 8 hr of aerobic incubation (Fig. 4), representing 1.7 IU/mg of dry cells. Maximum synthesis per

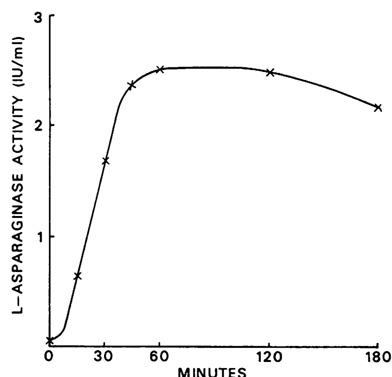

FIG. 2. *Effect of aging on L-asparaginase production.*

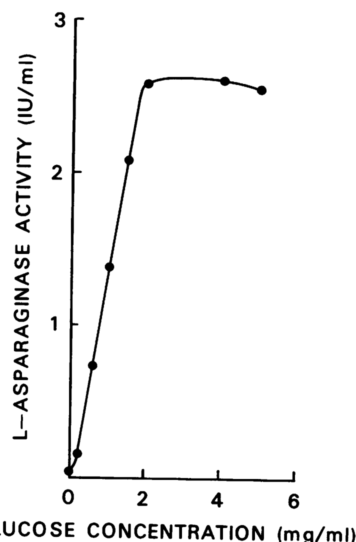

FIG. 3. *Effect of glucose concentration on L-asparaginase production during aging.*

TABLE 4. *Glucose utilization and effect on L-asparaginase production*

Glucose level (mg/ml)				Enzyme activity after aging (IU/ml)
Remaining after aerated incubation	Added after aerated incubation	Present at beginning of aging	Remaining after aging	
0	0	0	0	0.04
0	5.0	5.0	1.83	2.59
1.72	0	1.72	0	2.31
1.72	5.0	6.72	3.42	2.45
4.07	0	4.07	0.93	2.6
4.07	5.0	9.07	6.23	2.1

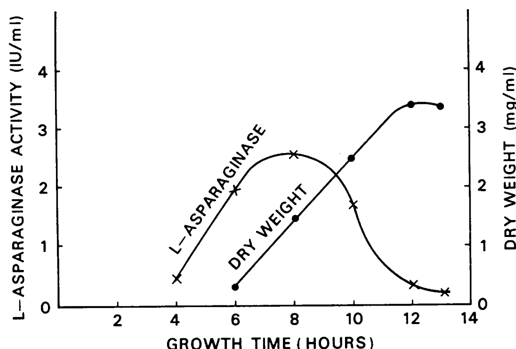

FIG. 4. *Time course of enzyme production versus dry weight of C532.5.*

unit of dry weight, 6.6 IU/mg, was achieved by 6 hr, although cell weight was still only 0.3 mg/ml. Only 0.1 IU/mg was obtainable when dried cell weight reached its peak at 12 hr. Asparaginase synthesis is demonstrably most efficient during logarithmic growth and declines sharply thereafter. This suggests that the pattern and capacity of enzyme biosynthesis depend on the rate and type of metabolism and are governed by changes of metabolic conditions inherent in the growth cycle, as Beck and von Meyenburg (1) have reported for yeast.

Oxygen tension of medium. Schwartz et al. (22) observed that when an aerated culture of *E. coli* K12 was allowed to incubate for an additional 20 min in the absence of aeration (briefly anaerobic culture), larger amounts of L-asparaginase were obtained than when the culture was harvested immediately after aerated growth. Cedar and Schwartz reported the enzyme was produced only in cells grown anaerobically (8) and later suggested that the supply of oxygen is likely to be limiting to cultures having high cell densities, even when shaken in air (9). Heinemann et al. (13) confirmed that the dissolved oxygen level is very low under certain conditions of aerated incubation and demonstrated the enhancement of L-asparaginase yields by oxygen limitation. We showed initially that C532.5 produced little asparaginase during aerated growth. Anaerobically grown cells were 30-fold richer in enzyme content when activity was based on dry cell weight, but activity per unit of culture medium was only eightfold greater because dry weight per unit of broth was reduced by 73%.

By monitoring dissolved oxygen in the fermentor, we found that the oxygen activity of the medium dropped to its lowest point between the 7 and 8 hr of aerated incubation. This reflected maximum oxygen demand near the terminal portion of logarithmic growth (at high cell density, as Cedar and Schwartz had suggested), but the minimum oxygen level was still 45% of saturation (Fig. 5), whereas the asparaginase level was <0.04 IU/ml. When aeration-agitation was held constant, the rapidly increasing oxygen demand was abruptly reversed, declined sharply for 30 min, and then gradually stabilized and again began to increase slowly. The presence of glucose during aeration increased the oxygen demand slightly and delayed the reversal for 30 min; otherwise, the tracings were identical to those of Fig. 5.

When aeration-agitation was terminated after 7 hr and glucose, phosphate, and L-asparagine were added, oxygen activity plummeted to zero saturation within 15 min. Enzyme synthesis began 6 to 7 min after aeration was terminated (Fig. 2), while oxygen activity was still about 20% saturation.

Carbon dioxide can be fixed by many heterotrophs. It has been shown by Gaffney (11) to reduce the lag period in aerobic catabolism of glucose and by Caldwell et al. (6) to be incorporated into the carboxyl function of the succinate produced by *Bacteroides ruminicola* during glucose fermentation. To determine whether the asparaginase synthesis associated with reduced oxygen tension might actually be a consequence of carbon dioxide accumulation, we sparged carbon dioxide through the culture medium during the aging period, but observed no effect. Sparging nitrogen to sweep out residual oxygen and hasten the drop in oxygen activity likewise did not produce a detectable effect.

Modification of growth medium. Attempts to increase asparaginase production by altering the levels of each substrate in the medium or by substituting other components for Hy-Case

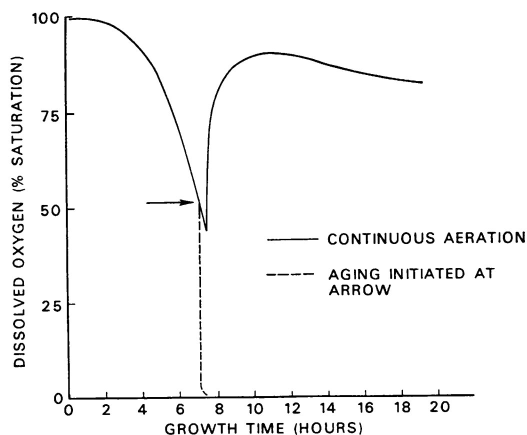

FIG. 5. *Typical fermentor-dissolved oxygen level.*

TABLE 5. *Effect of various medium modifications on asparaginase production by C532.5*

Substrate change	Substrate level (%, w/v)	L-Asparaginase (IU/ml)
Control[a]		2.6
Hy-Case amino	0	0.26
Hy-Case amino	0.85	0.83
Hy-Case amino	3.4	1.67
Soy peptone	0	0.29
Soy peptone	0.6	2.68
Phosphate	0	1.14
NaCl	0	0.53
Substitutes for Hy-Case amino		
N-Z Amine type A	1.7	1.82
Amber EHC	1.7	1.24
Purified casein	1.7	0.91
Corn steep liquor	1.7	0.36
Wilson's peptone 159	1.7	0.23

[a] Contained Hy-Case amino, 1.7%; soy peptone, 0.3%; K_2HPO_4, 0.25%; and NaCl, 0.25%.

amino were unsuccessful (Table 5). Growth remained essentially unchanged except when the substitutes for hydrolyzed casein were unhydrolyzed substrates which did not contain predominantly free amino acids. Then both growth and enzyme levels were much reduced. Enzymatic casein hydrolysates were inferior to the more completely degraded acid hydrolysates. Higher levels of soy peptone produced no effect, but omission of this supplement reduced enzyme synthesis Phosphate was also required, although it was not determined whether it supplied a need for inorganic phosphate or functioned primarily through its buffering action. Even though Hy-Case amino contains 40% NaCl, separate addition of the salt could not be omitted. When a corn steep medium was prepared by the method of Roberts et al. (21), various results were obtained from different lots of corn steep. The best of three lots tested gave results, without aging, almost equal to the MTSB medium with aging. However, cells from the corn steep medium would not respond to induction by glucose and aging.

Addition of individual amino acids, either included when the medium was prepared or added just prior to aging, generally elicited little or no response. The enzyme substrate, L-asparagine, did stimulate production as much as 50% when the optimum level of 0.05% was added before aging (Table 6). Repression by aspartic acid was virtually absent.

Screening of gram-negative microorganisms. Eighteen strains of gram-negative organisms, including eleven *E. coli* strains, were screened in the MTSB medium for asparaginase production. The enzyme was present in most strains (Table 7), but the quantity varied greatly among strains of the same species, in agreement with earlier reports (20, 21). Twelve strains produced at least twice the activity of C532.5 during aerated growth, with one strain of *E. coli* producing 14 times that amount; but, of the group tested, only C532.5 contained higher levels of asparaginase after aging in the presence of glucose.

TABLE 6. *Effect of amino acid additions prior to aging*

Amino acid	Addition level (g/liter)	L-Asparaginase (IU/ml)
None		1.7
L-Asparagine	0.1	2.0
L-Asparagine	0.25	2.2
L-Asparagine	0.5	2.65
L-Asparagine	1.0	2.58
L-Asparagine	5.0	2.45
D-Asparagine	0.5	1.28
L-Aspartic acid	5.0	1.73
Glycine	0.5	1.36

TABLE 7. *Effect of aging on biosynthesis of L-asparaginase by various gram-negative microorganisms*

Culture	L-Asparaginase activity (IU/ml) after	
	Aerated growth	Aging
Escherichia coli B, C532.5	0.04	2.6
E. coli B, ATCC 11303	0.05	0.05
E. coli B, ATCC 15597	0.08	0.07
E. coli B/1	0.1	0.12
E. coli B/1-7	0.05	0.05
E. coli Q13	0.05	0.05
E. coli K12	0.05	0.05
E. coli Crooks	0.16	0.14
E. coli T_6Host, ATCC 12435	0.22	0.21
E. coli, T_6-resistant	0.14	0.13
E. coli, cephaloridine-resistant	0.57	0.63
E. coli, patient isolate	0.05	0.07
Erwinia amylovora, ATCC 7398	0.1	0.1
E. atroseptica	0.08	0.08
E. carotovora	0.11	0.13
E. carotovora	0.32	0.19
Pseudomonas aeruginosa, ATCC 9027	0.33	0.28
Serratia marcescens	0.09	0.11
S. marcescens, NRRL B284	0.07	0.07

DISCUSSION

This study reports a novel method for the production of L-asparaginase. Glucose is known

to repress a number of other inducible degradative enzymes, such as β-galactosidase (3), nicotinamide adenine dinucleotide-independent glycerophosphate dehydrogenase (19), tryptophanase (2), and arginase (17). Some workers have specifically reported that glucose inhibits synthesis of L-asparaginase (9, 12, 21). We have, nevertheless, demonstrated the ability of glucose to increase the level of asparaginase in our strain of *E. coli*, which is unique in this respect among the limited number of microbial cultures we have tested. Low oxygen tension is a coordinate requirement. Oxygen limitation has previously been reported as a requirement for the formation of another enzyme. Downey et al. (10) found that nitrate reductase was induced in *Bacillus stearothermophilus* by nitrate only when cells were grown anaerobically. They concluded that oxygen functioned as a repressor of enzyme formation. Involvement of a similar mechanism is suggested in biosynthesis of L-asparaginase by C532.5, aerobically grown cells being repressed while anaerobically grown cells are derepressed, although requiring glucose to trigger biosynthesis. Further, aerobic repression is reversible by static incubation; strict anaerobiosis is not required.

Our method for production of L-asparaginase, employing high aeration for cell proliferation and reduced oxygen tension for enzyme synthesis, has several advantages over conventional aerobic or anaerobic methods. In addition to allowing rapid production of maximum cell volume with excellent enzyme synthesis per cellular unit, it permits subdivision of the fermentation procedure into two distinct phases, growth and biosynthesis. Effects on cellular reproduction and physiology may be studied independently of, or in conjunction with, biosynthesis. The biosynthetic phase may then be studied as an independent system, medium and incubation conditions being modified as desired before triggering enzyme synthesis by the addition of glucose when the level of dissolved oxygen is subinhibitory.

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