APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1977, p. 257-261
Vol. 33, No. 2

Printed in U.S.A.

# Effect of Culture Conditions on Synthesis of L-Asparaginase by *Escherichia coli* A-1

W. R. BARNES, G. L. DORN, AND G. R. VELA*

*Department of Microbiology and Leland Fikes Research Institute, Wadley Institutes of Molecular Medicine, Dallas, Texas 75235; and Department of Biological Sciences, North Texas State University, Denton, Texas 76203**

Received for publication 13 August 1976

The nutritional requirements and culture conditions affecting biosynthesis of L-asparaginase in a mutant of *Escherichia coli* HAP designated strain A-1 were studied. Asparaginase activity was increased by the addition of L-glutamic acid, L-glutamine, or commercial-grade monosodium glutamate. The rate of enzyme synthesis was dependent on the interaction between the pH of the culture and the amount of oxygen dissolved in the medium. A critical oxygen transfer rate essential for asparaginase formation was identified, and a fermentation procedure is described in which enzyme synthesis is controlled by aeration rate. Enhancement of L-asparaginase activity by monosodium glutamate was inhibited by the presence of glucose, culture pH, chloramphenicol, and oxygen dissolved in the fermentation medium.

L-Asparaginase therapy, alone or in combination with other drugs, is finding increased success in the management of acute lymphocytic leukemias. In recent clinical trials, significant remissions were obtained in more than 50% of patients treated with 3,000 to 9,000 IU/kg per day (6, 10). Dosages of this magnitude require large amounts of purified enzyme, and increasing recognition of successful therapy will generate increased demand.

Recent reports (1, 5, 9) show that most organisms studied yield only 1.3 to 4.0 IU of L-asparaginase per ml of culture medium. In general, the successful production of fermentation products depends on yield per unit volume of culture fluid. Since asparaginase is extracted from whole cells, efficiency in production will be determined by cell density and the amount of enzyme each cell produces. In this report, we emphasize the amount of enzyme produced per unit of cell dry weight because it describes the yield of enzyme per cell better than the commonly used designation of enzyme per milliliter of culture fluid. The organism described here, *Escherichia coli* A-1, produces high levels of enzyme in comparison with other organisms reported in the literature (1-5, 9, 12).

The effects of culture medium, culture pH, and oxygen transfer rate on asparaginase synthesis have been shown to vary for different organisms (1-3, 5, 8, 13), and a comprehensive view of this literature reveals a complex and variable set of culture parameters that affect asparaginase in different ways. For example, *Serratia marcescens* produces greater quantities of L-asparaginase with limited aeration than it does anaerobically (5), whereas either of these conditions has little effect on *Erwinia aroideae* (8). *E. coli* A-1 shows a response to dissolved oxygen unlike that reported for other bacteria or other strains of *E. coli*. The data presented prove that a precise amount of dissolved oxygen is essential for maximal asparaginase synthesis; we assume that this critical level of oxygen is a consideration of major importance not fully recognized in the past.

## MATERIALS AND METHODS

**Organism.** *E. coli* A-1 ($Met^-$) was obtained from the stock culture collection at Wadley Institutes of Molecular Medicine. It is a mutant produced by ultraviolet irradiation of *E. coli* HAP (12). Stock cultures were maintained on slants of 3% nutrient agar (30 g of dehydrated Difco nutrient broth powder and 15 g of agar dissolved in 1 liter of distilled water) in the refrigerator. Working cultures were obtained by streaking out stock cultures and picking isolated colonies from plates showing no contamination.

**Test media.** Ten different media were tested, but only the four that gave the highest yields of asparaginase are described here. They were prepared by dissolving the commercial powder to the strength indicated in distilled water and adjusting the pH to 7.0.

**Minimal medium.** *E. coli* A-1 was grown in a medium consisting of: $NH_4Cl$, 5 g; $NH_4NO_3$, 1 g; $Na_2SO_4$, 2 g; $K_2HPO_4$, 3 g; $KH_2PO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; glucose, 10 g; methionine, 25 mg; and distilled water, 1 liter.

**Test tube cultures.** Stock cultures of *E. coli* A-1

were streaked on plates of 3% nutrient agar and checked for purity. Three isolated colonies were picked, and the cells were suspended in 3 ml of sterile 0.85% NaCl to a density of $2 \times 10^8$ to $4 \times 10^8$ cells/ml. Tubes containing 4 ml of test medium were inoculated with 0.05 ml of this suspension and incubated for 20 h at 37°C in a rotary shaker-incubator.

**Fermentor cultures.** Isolated colonies from plates of 3% nutrient agar were suspended in saline to a density of $1 \times 10^9$ to $2 \times 10^9$ cells/ml. Five milliliters of suspension was used to inoculate 500 ml of the appropriate medium in a Bioflow model C-30 fermentor (New Brunswick Scientific Co., New Brunswick, N.J.). Fermentor temperature was maintained at 37°C, air flow at 560 ml/min, and agitator (propeller) speed at 400 rpm. The pH was continually monitored and automatically adjusted, when desired, with 2 N NaOH or 2 N HCl. When used, sterile 25% glucose solution was added to the desired concentration. Foaming was automatically controlled with Corning Antifoam reagent.

**L-Asparaginase assay.** Cells were removed from 4 ml of the growth medium by centrifugation and resuspended in an equal volume of distilled water. Asparaginase activity was measured using a Technicon autoanalyzer model I (Technicon Corp., Terrytown, N.Y.) according to the procedure for ammonia determination described by Logsdon (11), but with the following modifications. Hypochlorite solution was prepared by adding 200 ml of 6% NaOCl (Purex) to 800 ml of deionized water containing 54 g of $K_2CO_3$. Alkaline phenol was prepared by adding 100 ml of liquefied phenol to 1 liter of 0.9 N NaOH. The substrate for enzyme assay was L-asparagine dissolved in a buffer solution made of 0.2 M $K_2HPO_4$ titrated with 0.1 M citric acid to pH 7.0. Assays are reported in international units; 1 U is the amount of enzyme that releases 1 μmol of ammonia per min at 37°C. The Logsdon assay was calibrated and checked periodically by measuring ammonia evolution using the Nessler reaction (12). Samples from the fermentor cultures could be held in ice for periods of less than 12 h before assay without change in asparaginase activity.

## RESULTS

**Test tube studies.** *E. coli* A-1 was grown for 20 h at 37°C in test tubes containing 4 ml of medium and assayed for asparaginase activity. The data in Table 1 show the four media that gave the highest yield of asparaginase per unit of cell dry weight.

Growth of *E. coli* A-1 in minimal medium containing 0.5% glucose was adequate ($1 \times 10^9$ cells/ml in 20 h), but no asparaginase activity was found. Addition of a mixture of L-amino acids to this medium produced cells with ample asparaginase activity. Further studies indicated that the major part of the enhancement was due to glutamic acid, although glutamic acid had no effect in the absence of the other amino acids. In the absence of glutamic acid there was very little asparaginase synthesis, and no other amino acid tested gave the incremental increase in enzyme activity seen with glutamic acid (Table 2).

The experiment described in Table 3 was performed in view of the peculiar induction of L-asparaginase activity by L-glutamic acid. Similar studies indicated that L-aspartic acid and L-asparagine do not enhance L-asparaginase activity, and a separate series of experiments has shown that L-glutaminase activity is not signif-

TABLE 1. *Yield of L-asparaginase in test tube cultures of Escherichia coli A-1 grown in different media*

| Medium | g of powder/100 ml | Final pH | IU/ml | IU/mg of cell dry wt |
|---|---|---|---|---|
| Brain heart infusion | 12.5 | 6.47 | 3.1 | 2.9 |
| | 6.25 | 7.47 | 9.5 | 6.6 |
| | 3.12 | 8.14 | 9.0 | 5.6 |
| | 1.56 | 8.31 | 0.5 | 0.5 |
| Nutrient broth | 12.5 | 6.97 | 9.8 | 4.4 |
| | 6.25 | 7.98 | 9.0 | 3.9 |
| | 3.12 | 8.46 | 3.9 | 2.7 |
| | 1.56 | 8.47 | 0[a] | 0 |
| Peptone | 12.5 | 6.79 | 6.0 | 2.8 |
| | 6.25 | 8.36 | 3.5 | 2.2 |
| | 3.12 | 8.42 | 0 | 0 |
| | 1.56 | 8.29 | 0 | 0 |
| Yeast extract | 12.5 | 6.52 | 9.0 | 3.2 |
| | 6.25 | 7.63 | 9.5 | 3.0 |
| | 3.12 | 8.19 | 8.2 | 3.5 |
| | 1.56 | 8.63 | 4.5 | 2.6 |

[a] Zero indicates amounts less than 0.1 IU.

TABLE 2. *Effect of amino acids added to minimal medium on the synthesis of L-asparaginase in E. coli A-1*

| Additions to minimal medium | IU/ml | IU/mg of cell dry wt |
|---|---|---|
| 0.5% glucose | 0 | 0 |
| AA[a] | 0.2 | 0.2 |
| 1% MSG[b] | 0 | 0 |
| AA + 0.5% MSG | 4.0 | 3.2 |
| AA + 1.0% MSG | 5.7 | 5.2 |
| AA + 1.5% MSG | 6.2 | 6.2 |
| AA + 2.0% MSG | 6.2 | 6.3 |
| AA + 2.5% MSG | 6.0 | 6.3 |
| AA + 3.0% MSG | 5.5 | 6.7 |
| AA + 3.5% MSG | 5.1 | 7.3 |
| AA + 4.0% MSG | 3.5 | 4.9 |

[a] The amino acid (AA) mixture contained 0.4% (wt/vol) asparagine, serine, and methionine; 0.3% lysine and aspartic acid, 0.2% phenylalanine, arginine, leucine, isoleucine, valine, proline, alanine, and threonine; 0.1% histidine and glycine; and 0.01% tyrosine and cysteine.

[b] Monosodium glutamate, commercial grade.

icantly enhanced by L-glutamic acid or L-glutamine.

The highest yields of asparaginase were obtained in cells pregrown in minimal medium, washed, and resuspended in the induction medium (Table 4). The data in Table 4 also show the combined effect of nutrient broth and monosodium glutamate (MSG) under these conditions.

**Fermentor studies.** A series of experiments with the Bioflow fermentor was used to confirm the effect of MSG on asparaginase activity in mass cultures of *E. coli* A-1. These studies showed that higher asparaginase activity was obtained when the culture pH was maintained at 7.5 and also that glucose caused a significant reduction in asparaginase activity (Table 5). If glucose was added to 3% nutrient broth together with MSG, asparaginase synthesis was lower than that obtained with MSG only (Table 5). In the next series of experiments, higher yields of asparaginase activity were found in cultures grown in 3% nutrient broth plus 0.5% glucose in which there was no discernible dissolved oxygen. To attain this "critical level" of oxygen, the culture pH was adjusted to 7.5 and maintained by automatic control. Adjusting the pH to 7.5 brought the level of dissolved oxygen to zero without further manipulation, although agitator velocity was maintained at 400 rpm and air flow at 560 ml/min. Figure 1 shows the kinetics of asparaginase synthesis diminished. The effect of about 0.8 mg of $O_2$ per liter of medium (10% dissolved oxygen) is seen in Fig. 2. In essence, the rate of asparaginase production could be controlled by manipulation of the culture pH and, as a consequence, the dissolved oxygen content of the medium. The effects of pH and dissolved oxygen appeared to be interdependent in that asparaginase activity diminished when the pH was lower than 7.5 or when the dissolved oxygen was greater than zero.

TABLE 3. *Effect of L-glutamic acid, L-glutamine, and MSG on asparaginase synthesis by E. coli A-1*

| Additions to 3% nutrient broth[a] | L-asparaginase | | L-glutaminase (IU/ml) |
|---|---|---|---|
| | IU/ml | IU/mg of cell dry wt | |
| | 3.4 | 2.9 | 0 |
| 0.25% glut | 9.1 | 5.0 | 0.2 |
| 1.0% | 10.0 | 5.1 | 0.2 |
| | 3.2 | 2.6 | 0.1 |
| 0.25% gln | 9.6 | 4.8 | 0.2 |
| 1.0% | 9.6 | 4.5 | 0.2 |
| | 3.2 | 2.6 | 0.1 |
| 0.25% MSG | 10.0 | 5.0 | 0.2 |
| 1.0% | 10.6 | 4.5 | 0.2 |

[a] Abbreviations: glut, Glutamic acid; gln, glutamine; MSG, monosodium glutamate, commercial grade.

TABLE 4. *Asparaginase activity in E. coli A-1 pregrown in minimal media*[a]

| Induction medium | Final pH | IU/ml | IU/mg of cell dry wt |
|---|---|---|---|
| Minimal medium | 5.25 | 0 | 0 |
| 0.5% NB[b] | 7.30 | 0 | 0 |
| 1.0% NB | 8.25 | 0.5 | 0.4 |
| 2.0% NB | 8.41 | 4.2 | 2.6 |
| 3.0% NB | 8.59 | 5.5 | 3.4 |
| 0.5% NB + 1% MSG | 8.65 | 0.5 | 0.5 |
| 1.0% NB + 1% MSG | 8.50 | 5.3 | 3.3 |
| 2.0% NB + 1% MSG | 8.40 | 19.6 | 8.0 |
| 3.0% NB + 1% MSG | 8.51 | 27.5 | 8.9 |

[a] Minimal medium with 0.5% glucose, used as growth medium. The cells were removed from the growth medium at late log phase, washed, and resuspended in an equal volume of induction medium. Growth in the induction medium was limited to approximately one cell division.

[b] NB, nutrient broth.

TABLE 5. *Effect of glucose and culture pH on L-asparaginase activity in fermentor cultures of E. coli A-1*

| Additions to 3% nutrient broth | Culture pH[a] | IU/ml | IU/mg of cell dry wt |
|---|---|---|---|
| None | 7.5 | 5.0 | 2.7 |
| None | 5.5 | 0 | 0 |
| 0.5% glucose | 7.5 | 6.8 | 2.9 |
| 0.5% glucose | 5.5 | 0 | 0 |
| 1% MSG | 7.5 | 19.8 | 8.8 |
| 1% MSG | 5.5 | 1.0 | 0 |
| 1% MSG + 0.5% glucose | 7.5 | 7.0 | 2.9 |
| 1% MSG + 0.5% glucose | 5.5 | 0 | 0 |

[a] The pH was established and maintained as indicated.

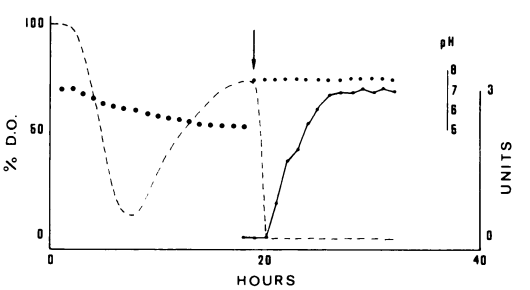


FIG. 1. *Kinetics of L-asparaginase synthesis by E. coli A-1 in the Bioflow fermentor. The broken line represents percentage of dissolved oxygen (D.O.); the solid line represents asparaginase in international units per milligram of cell dry weight; and the dots represent pH. Medium pH was adjusted (arrow) and controlled automatically thereafter.*

In contradiction to this, if the flow of air was stopped and the cultures were allowed to become anaerobic, asparaginase synthesis also ceased (Fig. 3). The synthesis of asparaginase in *E. coli* A-1 depends on proper culture pH and on oxygen uptake, but is inhibited by oxygen dissolved in the medium. This critical relationship has not been previously reported.

It is the synthesis of asparaginase that is involved, since no asparaginase activity is observed, even under optimal conditions, if chloramphenicol is added to the culture. This point is well established in previous reports (2, 13). This implies that asparaginase is formed de novo when the pH is brought to 7.5 and, consequently, dissolved oxygen to zero. To the contrary, the stimulation of asparaginase activity, i.e., synthesis, does not require extensive cellular reproduction. Cells of *E. coli* A-1 grown in minimal medium were harvested, washed, and transferred to the 3% nutrient broth-1% MSG medium and permitted to attain the critical rate of oxygen transfer in the new medium. The synthesis of asparaginase started in 8 h, although cell numbers did not increase more than twofold during that time (Fig. 4). In experiments of this type, asparaginase synthesis could be initiated at any time simply by adjusting the pH to 7.5 and allowing the dissolved oxygen to reach the critical transfer rate.

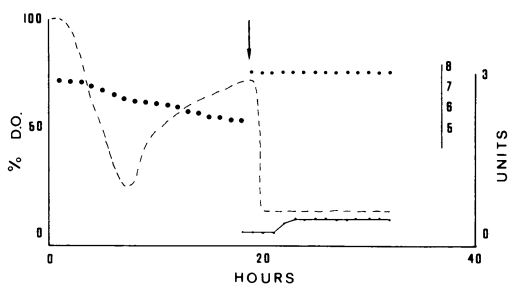


FIG. 2. *Effect of dissolved oxygen (D.O.) (10% of saturation) on asparaginase synthesis. Other details as in Fig. 1.*

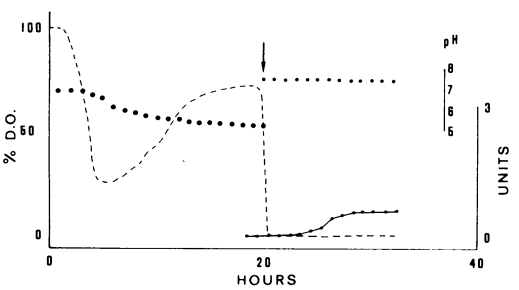


FIG. 3. *Effect of anaerobiosis on asparaginase synthesis by E. coli A-1. Symbols are as in Fig. 1. Medium pH was adjusted and air supply was turned off at the time indicated by the arrow.*

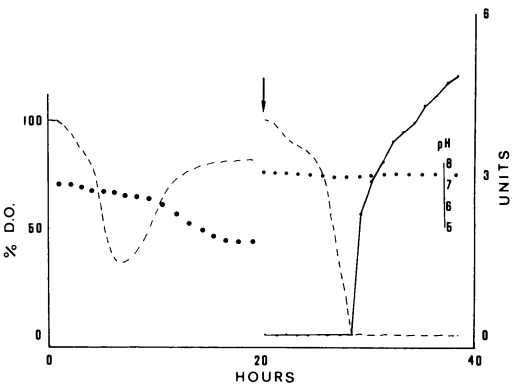


FIG. 4. *Synthesis of asparaginase by E. coli A-1 pregrown in minimal medium and transferred (arrow) to 3% nutrient broth plus 1% MSG. Symbols are as in Fig. 1.*

## DISCUSSION

Nutritional requirements for maximal synthesis of L-asparaginase vary from one microorganism to the other. Indeed, the rate of synthesis varies in the same organism as a function of culture conditions. In this report, we have shown that asparaginase activity in cells of *E. coli* A-1 depends on a complex interaction of nutrients, pH, oxygen transfer rate, and the presence of specific requirements such as glutamic acid.

The effect of MSG on synthesis is perplexing. The natural substrate for L-asparaginase is L-asparagine, but the maximal enhancement of asparaginase synthesis is obtained by adding glutamine or glutamate to the medium. Imada et al. (7) showed that many organisms that produce L-asparaginase also produce L-glutaminase, and another study (13) showed that L-asparagine induced asparaginase only under anaerobic conditions. In our experiments, MSG probably does not function as a classical enzyme inducer since L-asparagine does not affect L-asparaginase activity and other amino acids are required for the induction (Table 2). We have not ascertained the mechanism of action involved in this effect. Levels of asparaginase activity of 15 to 25 IU per ml of culture have not been reported from other experiments and can be attained in ours only by the use of MSG in a suitable fermentation broth. The synthesis of L-asparaginase represents a curious biological system. In essence, asparaginase synthesis is induced by the presence of a group of amino acids but principally by glutamic acid at a specific pH and level of respiratory activity

whether cell division occurs or not.

It should also be noted that the yield of asparaginase activity using a given culture medium should be viewed in terms of the amount of cell protein devoted to asparaginase activity. The best results in our studies (27 IU/ml) were obtained by using cells pregrown in minimal medium and transferred to 3% nutrient broth plus 1% MSG. Cells grown in minimal medium plus 3.5% MSG and the other amino acids gave only 5.0 IU/ml, but the yield of enzyme was 8.9 and 7.3 IU per mg of cell dry weight, respectively. That is, approximately the same amount of effective enzyme was produced in both cases although, if measured in terms of international units per milliliter, it appears as if the pregrown cells produce five times as much as those grown on minimal medium.

In view of this finding, it is suggested that the only valid comparison of enzyme yields is in terms of international units per milligram of cell dry weight.

The effect of oxygen transfer rate shown in these experiments is essentially in agreement with the findings of Liu and Zajic (8), but *E. aroideae* does not show the critical oxygen dependence seen in *E. coli* A-1. It is obvious that a specific rate of oxygen consumption is required for asparaginase synthesis and that dissolved free oxygen in the growth medium inhibits synthesis. The relationship between oxygen tension and the synthesis of a specific enzyme must be complex and is certainly beyond our understanding at this time.

The data presented here show that asparaginase synthesis in *E. coli* A-1 is the result of a very complex sequence of culture events. The specific nature of these events is not understood, but we have identified some of them. When these findings were applied to cultures of *E. coli* A-1 in a 1,000-liter fermentor, the yields of purified L-asparaginase were increased threefold in the production laboratories of the Wadley Institute.

## ACKNOWLEDGMENTS

This work was supported with funds provided by the Leukemia Foundation of North Central Texas and the Carl B. and Florence E. King Foundation.

We gratefully acknowledge the valuable technical assistance given by Sal Comparini.

## LITERATURE CITED

1. **Bascomb, S., G. T. Banks, M. T. Skarstedt, A. Fleming, and K. A. Bettelheim.** 1975. The properties and large-scale production of L-asparaginase from *Citrobacter*. J. Gen. Microbiol. 91:1-16.
2. **Boeck, L. D., and P. P. K. Ho.** 1973. L-asparaginase production during static incubation of aerobically grown *Escherichia coli* B. Can. J. Microbiol. 19:1251-1257.
3. **Cedar, H., and J. H. Schwartz.** 1968. Production of L-asparaginase II by *Escherichia coli*. J. Bacteriol. 96:2043-2048.
4. **Heinemann, B., and A. J. Howard.** 1969. Production of tumor-inhibitory L-asparaginase by submerged growth of *Serratia marcescens*. Appl. Microbiol. 18:550-554.
5. **Heinemann, B., A. J. Howard, and J. H. Palocz.** 1970. Influence of dissolved oxygen levels on production of L-asparaginase and prodigiosin by *Serratia marcescens*. Appl. Microbiol. 19:800-804.
6. **Hill, J. M., E. Loeb, A. MacLellan, N. Hill, A. Khan, and A. Albayrak.** 1972. Treatment of acute leukemia. Wadley Med. Bull. 2:63-76.
7. **Imada, A., S. Igarasi, K. Nakahama, and M. Isono.** 1973. Asparaginase and glutaminase activities of microorganisms. J. Gen. Microbiol. 76:85-99.
8. **Liu, F. S., and J. E. Zajic.** 1973. Effect of oxygen-transfer rate on production of L-asparaginase by *Erwinia aroideae*. Can. J. Microbiol. 19:1153-1158.
9. **Liu, F. S., and J. E. Zajic.** 1973. Fermentation kinetics and continuous process of L-asparaginase production. Appl. Microbiol. 25:92-96.
10. **Loeb, E., J. M. Hill, A. MacLellan, N. Hill, A. Khan, and R. Speer.** 1972. Five year experience with L-asparaginase in the treatment of acute leukemias. Wadley Med. Bull. 2:23-32.
11. **Logsdon, E. E.** 1960. A method for the determination of ammonia in biological materials on the autoanalyzer. Ann. N.Y. Acad. Sci. 87:801-807.
12. **Roberts, J., G. Burson, and J. M. Hill.** 1968. New procedures for purification of L-asparaginase with high yield from *Escherichia coli*. J. Bacteriol. 95:2117-2123.
13. **Svobodova, O., and S. Strbanova-Necinova.** 1973. Induction of L-asparaginase synthesis in *Escherichia coli*. Biochim. Biophys. Acta 321:643-652.