

Fermentation Kinetics and Continuous Process of L-Asparaginase Production

F. S. LIU AND J. E. ZAJIC

Chemical and Biochemical Engineering, Faculty of Engineering Science, The University of Western Ontario, London, Ontario, Canada

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For the purpose of obtaining L-asparaginase in quantities from *Erwinia aroideae*, cell growth and enzyme formation were investigated in both batch and continuous fermentation. Using yeast extract as a growth-limiting substrate, the relationship between specific growth rate and substrate concentration was found to fit the Monod equation. The optimum temperature for enzyme production was 24 C, although cell growth was higher at 28 C. The enzyme yield reached its maximum of 4 IU/ml during the negative acceleration growth phase which occurs just prior to stationary growth. Compared to batch fermentations, the continuous fermentation process gave a lower enzyme yield except when the fermentation was conducted at a dilution rate of 0.1 hr⁻¹. The graphical method frequently used for prediction of continuous fermentation does not apply to L-asparaginase production by *E. aroideae*. The optimum temperature for enzyme production in continuous process was 24 C, which was the same as in batch process. Increasing the temperature from 24 to 28 C resulted in a 20% loss of enzyme yield.

A large amount of research has been conducted upon the biosynthesis of L-asparaginase since Mashburn and Wriston (6) demonstrated antitumor activity. Thus far most publications on L-asparaginase production have dealt with the nutrient requirements of microorganisms for optimal enzyme production. Far less research has been completed upon fermentation kinetics and continuous production processes. Kinetic studies would allow the prediction of fermentation rate, product yield, and the control of the fermentation process. A continuous fermentation has advantages of productivity, ease of control, uniformity, and low labor costs. However, the difficulties of maintenance of sterility and stability in continuous process must be carefully considered. If these problems are resolved, a continuous process is favored.

Several species of *Erwinia* have been reported to provide promising sources of L-asparaginase and have proved to be effective against solid tumors (8, 10). In the present study, the fermentation kinetics and the continuous production of L-asparaginase by *Erwinia aroideae* are described.

MATERIALS AND METHODS

Batch fermentation. *Erwinia aroideae* NRRL B-138, which produces high yields of L-asparaginase with antitumor activity (8), was employed through-

out this investigation. This culture was obtained from Northern Regional Research Laboratory, U.S. Department of Agriculture. The stock culture was maintained on agar slants containing 0.1% K₂HPO₄, 0.5% tryptone, 0.5% yeast extract, and 2% agar. The same medium (without agar) was used to prepare inoculum. Inoculum was obtained by transferring a loopful of *E. aroideae* from a slant culture to 100 ml of medium in a 500-ml Erlenmeyer flask and incubating it for 12 hr at 24 C on a rotary shaker (New Brunswick Scientific Co., model G53) running at 200 rev/min. The fermentations were carried out in a 1-liter microfermenter (VirTis Company, model MF-1000-CA) with 1 liter of medium. The culture medium consisted of 1.0% lactose, 1.5% yeast extract, and 0.1% K₂HPO₄. The pH was adjusted to 7.5 prior to sterilization. A 0.02% concentration (v/v) of antifoam (Nalco 127, Alchem Ltd.) was added to control foaming. The fermenter was inoculated with 1% of vegetative inoculum and was incubated for 16 hr. Temperature was maintained at 24 C by a water bath around the fermenter. Aeration was at the rate of 1.0 liter/min (1.0 vol of air per vol of medium per min), which was found to be optimal for enzyme production (the fermenter was not equipped with baffle). Agitation rate was 550 rev/min, which was provided by a magnetic stirrer.

Continuous fermentation. The continuous fermentations were conducted in a single-stage system. The 1-liter fermenter described above was used in the continuous runs. All experiments were carried out at a 1-liter operating volume. The fermenter was first

run batchwise for 16 hr and then was shifted to continuous operation. A Sigmamotor pump was used to maintain constant feed rate. To maintain constant volume in the fermenter, the cells and products were removed at the same continuous rate. For continuous feeding, 4 liters of medium (containing 0.02% antifoam) were prepared in a 6-liter Erlenmeyer flask and sterilized for 40 min at 121 C.

Harvesting of cells. Cells were separated from the broth by centrifugation (Sorvall, model RC2-B refrigerated centrifuge) at $10,000 \times g$ for 15 min and were washed twice with 1/30 M phosphate buffer (pH 7.0). Samples of washed cells were resuspended to their original volume in 0.1 M sodium borate buffer (pH 8.5).

L-Asparaginase assay. L-Asparaginase activity was determined by measuring the amount of ammonia formed by nesslerization. A 0.5-ml sample of cell suspension, 1.0 ml of 0.1 M sodium borate buffer (pH 8.5), and 0.5 ml of 0.04 M L-asparagine solution were mixed and incubated for 10 min at 37 C. The reaction was then stopped by the addition of 0.5 ml of 15% trichloroacetic acid. The precipitated protein was removed by centrifugation, and the liberated ammonia was determined by direct nesslerization. Suitable blanks of substrate and enzyme-containing sample were included in all assays. The yellow color was read in a Beckman DB-G spectrophotometer at 500 nm. One international unit (IU) of L-asparaginase is that amount of enzyme which liberates 1 μ mole of ammonia in 1 min at 37 C.

RESULTS

Effect of yeast extract on growth rate. The studies on nutrient requirements of *E. aroideae* (5) conducted in our laboratory have shown that yeast extract is essential for cell growth as well as L-asparaginase production. Figure 1 shows the time course of cell growth and enzyme production at different levels of initial yeast extract concentrations. No yield of L-asparaginase was observed when the cells were grown in the medium containing 0.05% yeast extract. A level of 3% yeast extract is inhibitory to both cell growth and enzyme synthesis as compared to 1.5% yeast extract. However, 3% yeast extract did give yields of cells and enzyme, respectively, of 3.55 g/liter and 3.41 IU/ml, and hence yeast extract was used in a Lineweaver-Burk plot, i.e., $1/\mu$ versus $1/S$ (Fig. 2). Here μ represents the specific growth rate in the exponential growth phase, and S is the concentration of yeast extract which is close to the initial concentration because of the low cell concentration. As shown in Fig. 2, a linear relation was observed. The following growth rate was then obtained by using the Monod equation (7): $\mu = (0.526 S)/(1.18 + S)$. Therefore, $\mu_{\max} = 0.526 \text{ hr}^{-1}$. K_s , the saturation constant, is 1.18 g/liter.

Effect of temperature on growth rate. The effect of temperature on growth and en-

zyme formation in batch fermentation was studied. The optimum temperature for growth and L-asparaginase production were 28 C and 24 C, respectively (Fig. 3). At 20 C, the total enzyme activity was close to that synthesized at 24 C although growth was poor (only 2.5 g/liter). Thus, a product with high specific activity (1,850 IU/g dry weight) can be obtained at 20 C if the productivity is not of direct concern. The activation energy of cell growth was found to be 8,500 cal/mole from an Arrhenius plot of exponential specific growth rate as a function of temperature.

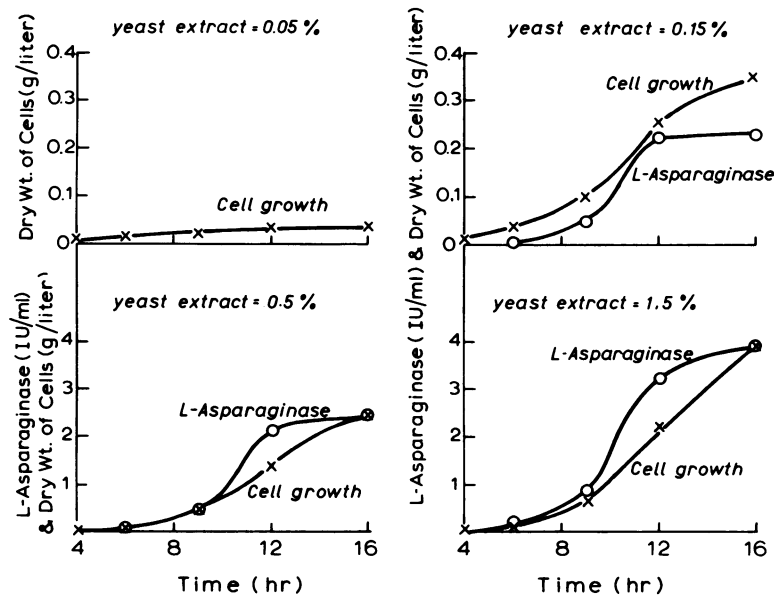
Relation of growth rate to enzyme synthesis. Luedeking and Piret (4) observed that the specific production rate of lactic acid was proportional to the specific growth rate for *Lactobacillus delbrueckii*. However, the batch production of L-asparaginase by *E. aroideae* (Fig. 4) does not show this association (Fig. 5), i.e., there is an absence of a linear relationship between the specific production rate and the specific growth rate.

Prediction of continuous fermentation behavior from batch fermentation data. From the material balance of a single-stage continuous fermentation, the following equation can be obtained at the steady-state condition: $D = (dC_x/dt)/C_x$, where D is the dilution rate. The value of C_x can be predicted graphically from a plot of (dC_x/dt) against C_x which is obtained from batch fermentation data. In the same manner, C_p can be estimated by plotting (dC_p/dt) against C_p . Figure 6 illustrates the graphical solution for a continuous fermentation. The data were obtained from Fig. 4. From these data, six continuous runs were planned. These included dilution rates in the range of 0.05 to 0.5 hr^{-1} . As shown in the right-hand portion of Fig. 6, enzyme concentrations increased with decreasing dilution rate. It was expected that 0.05 hr^{-1} run would give the highest enzyme yield of 3.7 IU/ml and cell mass of 5.9 g/liter.

Continuous fermentation. Of six continuous runs, all achieved steady state within 2 to 3 days. The cell concentrations were higher than expected (Fig. 7). Only at a dilution rate of 0.1 hr^{-1} was the enzyme yield as expected. It was also this dilution rate that gave the highest enzyme yield. "Washout" was observed at the dilution rate of 0.5 hr^{-1} .

Contrary to the batch fermentation data, a linear relationship between specific production rate and specific growth rate was observed in continuous fermentation (Fig. 5).

As shown in Fig. 3, the fermentation temperature affected both enzyme yield and cell growth in batch fermentation. An effect of



Time course of cell growth and L-Asparaginase production.

FIG. 1. Time course of cell growth and L-asparaginase production by *Erwinia arordeae* at various concentrations of yeast extract. The medium contained 1.0% lactose, 0.1% K_2HPO_4 , and 0.05 to 1.5% yeast extract. Fermentations were conducted at 24 C.

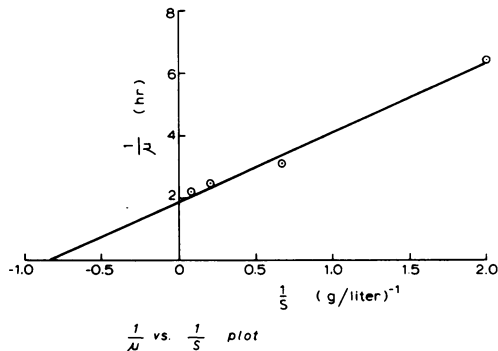
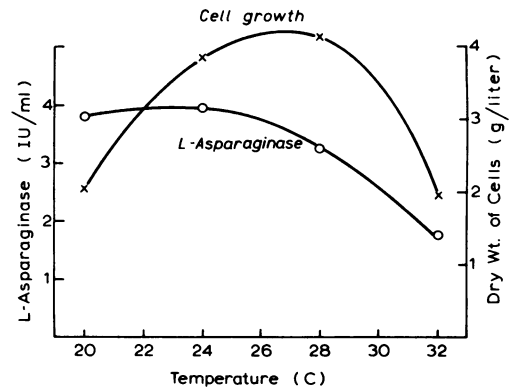


FIG. 2. Reciprocal plots of specific growth rate versus reciprocal of limiting substrate for *Erwinia arordeae*.

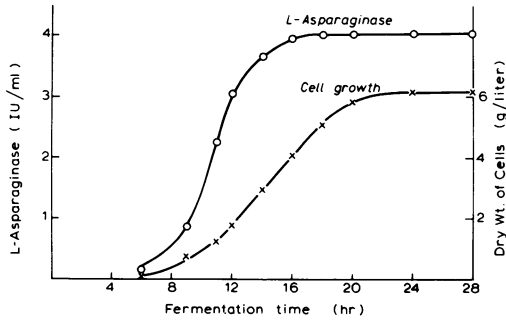
temperature in the continuous process was also investigated. Fermentation was first carried out at 24 C for 7 days. Steady state was achieved in 3 days (Fig. 8). As the temperature was shifted to 28 C, enzyme yield started to fall, and a new steady state was achieved in 24 hr. A loss of 20% enzyme yield was observed due to temperature shift. When the temperature was readjusted to 24 C, the enzyme yield



Effect of temperature on cell growth and L-Asparaginase formation.

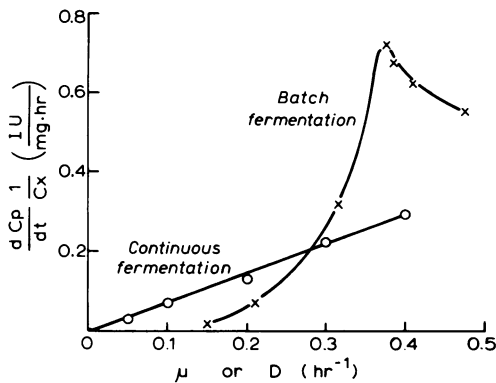
FIG. 3. Effect of temperature on cell growth and L-asparaginase formation in batch fermentations using *Erwinia arordeae*.

increased and reached its original steady state in 24 hr. No significant change in cell concentration was observed during the temperature shift.



Time course of L-Asparaginase fermentation.

FIG. 4. L-Asparaginase fermentation by *Erwinia aroideae* at 24 C. The medium contained 1.0% lactose, 1.5% yeast extract, and 0.1% K_2HPO_4 .

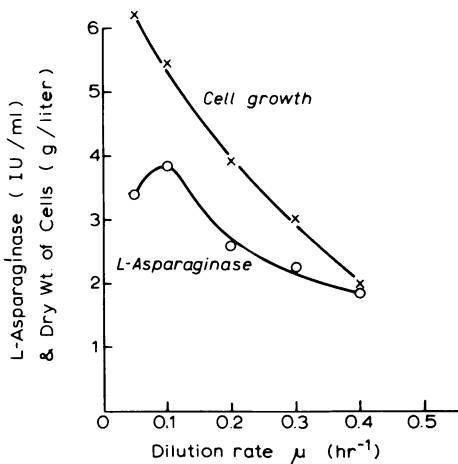


Plot of $\frac{dC_p}{dt} \frac{1}{C_x}$, vs μ or D .

FIG. 5. Plot of $(dC_p/dt)/C_x$, specific production rate, versus μ specific growth rate; or D , dilution rate for *Erwinia aroideae*.

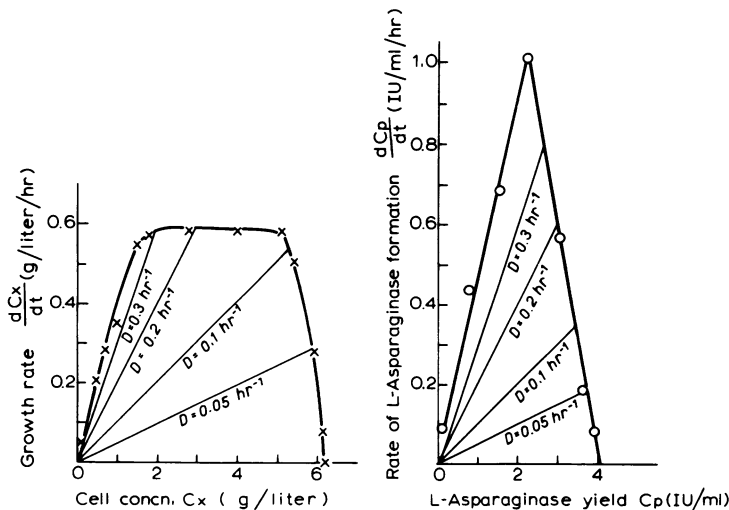
DISCUSSION

Cell growth and L-asparaginase production are usually stimulated by supplying complex forms of nitrogen such as peptone, yeast extract, and casein hydrolysate (2, 9). *E. aroideae*, however, gave satisfactory cell mass and enzyme yield only when yeast extract was present. From Fig. 1, yeast extract is considered to be the growth-limiting substrate, thus making it possible to apply the Monod equation to analysis of batch fermentation data for



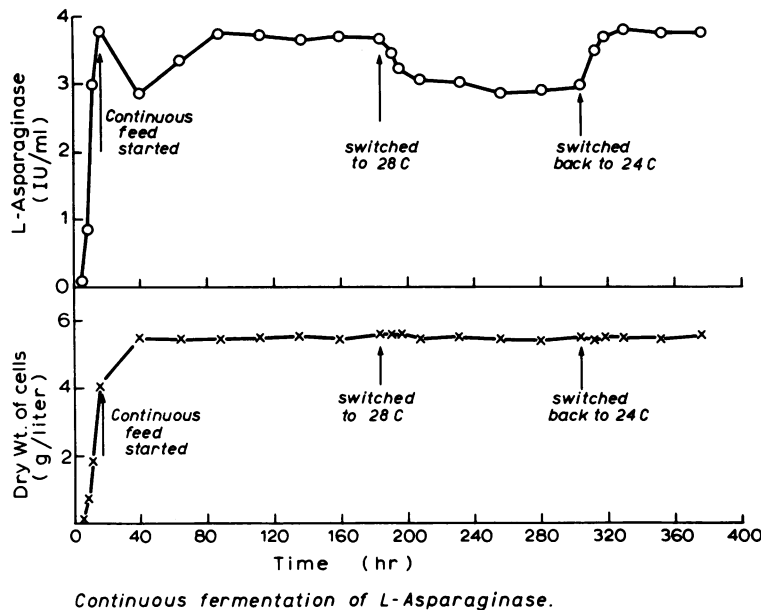
Effect of dilution rate on L-Asparaginase production.

FIG. 7. Effect of dilution rate on cell growth and L-asparaginase production in a single vessel using *Erwinia aroideae*.



Graphical solution for continuous fermentation.

FIG. 6. Graphical solution for a single-stage continuous fermentation of L-asparaginase using *Erwinia aroideae*.



Continuous fermentation of *L*-Asparaginase.

FIG. 8. Time course of a single-stage continuous fermentation of *L*-asparaginase by *Erwinia aroideae*. The dilution rate is 0.1 hr^{-1} .

L-asparaginase synthesis by *E. aroideae*.

Enzyme formation in either batch or continuous process was found to be greater when *E. aroideae* was grown at 24 C than at 28 C. The latter temperature is optimal for growth. The activation energy for cell growth is 8,500 cal/mole, which is close to that for the fungus *Penicillium chrysogenum* (1).

The growth rate equation derived by Kono and Asai (3), which can be applied to entire fermentation processes, can be used to analyze asparaginase production. The calculated values of product concentration, although not shown here, did not fit well with the observed values in the constant growth and declining growth phase. Nevertheless, the equation for product concentration in the additional phase, a term used by Kono and Asai, can be used in these two phases since the production rate was inversely proportional to the concentration of product present.

The observed values of cell and enzyme yield data of continuous fermentation process did not agree with the predicted values obtained from batch fermentation data. The cell concentrations in the continuous fermentation were higher than expected. On the contrary, the enzyme yields were lower than expected except at a dilution rate of 0.1 hr^{-1} . A comparison batch and continuous processes show they both differ in kinetic behavior (Fig. 5). This suggests a significant accumulation of intermediate metabolites between substrate and product

formation in the batch fermentation. This is probably due to the difference in physiological state between the organism in continuous fermentation and in batch fermentation.

ACKNOWLEDGMENTS

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