Influence of Dissolved Oxygen Levels on Production of L-Asparaginase and Prodigiosin by *Serratia marcescens*

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The effect of dissolved oxygen concentrations on the behavior of *Serratia marcescens* and on yields of asparaginase and prodigiosin produced in shaken cultures and in a 55-liter stainless-steel fermentor was studied. A range of oxygen transfer rates was obtained in 500-ml Erlenmeyer flasks by using internal, stainless-steel baffles and by varying the volume of medium per flask, and in the fermentor by high speed agitation (375 rev/min) or low rates of aeration (1.5 volumes of air per volume of broth per min), or both. Dissolved oxygen levels in the fermentation medium were measured with a membrane-type electrode. Peak yields of asparaginase were obtained in unbaffled flasks (3.0 to 3.8 IU/ml) and in the fermentor (2.7 IU/ml) when the level of dissolved oxygen in the culture medium reached zero. A low rate of oxygen transfer was accomplished by limited aeration. Production of prodigiosin required a supply of dissolved oxygen that was obtainable in baffled flasks with a high rate of oxygen transfer and in the fermentor with a combination of high-speed agitation and low-rate aeration. The fermentation proceeded at a more rapid rate and changes in pH and cell populations were accelerated by maintaining high levels of dissolved oxygen in the growth medium.

The importance of oxygen supply for microbial growth and product formation by microorganisms in submerged fermentations is well documented in reviews (3, 9, 11) of the subject. Although there have been reports (1, 5, 6) of the profound effects which the amount of oxygen may have on product formation during growth, there is a paucity of information on the influence of dissolved oxygen levels, measured during the course of the fermentation, on the various metabolic activities involved in growth and product formation in submerged cultures. Since microorganisms growing in submerged culture utilize oxygen dissolved in the fermentation medium, the supply in vessels used for shaken cultures may become critical for microbial biosynthesis of specific end products. In addition, oxidation-reduction mechanisms existing in the fermentation mixture may exert a chemical influence on biosynthetic products.

The recent introduction of membrane-type electrodes provides a direct, convenient, and reliable method for routine measurement of dissolved oxygen levels as they are actually found during a submerged fermentation.

The facultative anaerobe, *Serratia marcescens*, is able to carry out metabolic functions over a wide range of aeration conditions. Heinemann and Howard (8) observed that agitation in shaken culture was essential for optimal growth of this organism and that the tumor-inhibitory enzyme, asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1), was produced during a period of zero-dissolved oxygen concentration in the fermentation medium. Prodigiosin, the characteristic red, water-insoluble, antibiotic pigment of the bacterium *S. marcescens*, was not detected in shaken-flask fermentations under conditions which yielded optimal enzyme formation. Growth in fermentors with agitation or with high rates of aeration, or both, yielded prodigiosin and reduced asparaginase titers. Harned (7) indicated that production of prodigiosin was stimulated in *S. marcescens* when fermentations were carried out in stirred-aerated fermentors.

This report is a study of the effect of dissolved oxygen concentrations on yields of asparaginase and prodigiosin by *S. marcescens* in shaken flasks and in a small fermentor.

**MATERIALS AND METHODS**

Fermentations in shaken flasks, *S. marcescens* ATCC 60, kindly supplied to us by A. W. Phillips
of Syracuse University, was used throughout this investigation. Inoculum for studies in shaken flasks was prepared as described previously (8), and 0.5% (by volume) was used for seeding. The fermentation medium in all shaken flasks consisted of 4% autolyzed yeast extract ("Maggi") Autolyzed Yeast Extract Special Light Powder, Nestle Co., White Plains, N.Y.) in distilled water adjusted to pH 5.0 with hydrochloric acid before sterilization. The flasks were closed with milk-filter discs and incubation was carried out at 26 C on a rotary shaker (250 rev/min) which described a 5.08-cm circle. Replicate fermentations were carried out and analyses were carried out at 6- to 8-hr intervals until the fermentation was complete. A flask of the fermentation beer was removed for analyses at each sampling time.

To investigate the effects of dissolved oxygen concentrations in shaken flasks on the behavior of S. marcescens and on production of asparaginase and prodigiosin, a range of oxygen transfer rates was obtained by using internal baffles (4, 10) and by varying the volume of medium (2) per flask.

To determine the effect of baffling on the S. marcescens fermentation, shaken-flask experiments were performed with 100 ml of medium per flask. “Unbaffled” flasks were standard 500-ml Erlenmeyer flasks. The “baffled” flasks were standard 500-ml Erlenmeyer flasks with stainless-steel baffles (4) clipped on to the rim of the flask. Care was taken to use flasks in which the metal baffles would fit well and maintain their original position to eliminate possible variations in oxygen-transfer rates. To investigate further the relationship between the dissolved oxygen supply and production of asparaginase and prodigiosin, fermentations were carried out with 25-, 50-, 100-, and 150-ml volumes of medium, inoculated with the same cell concentration in standard 500-ml Erlenmeyer flasks.

Experiments in fermentors. The inoculum for the fermentor was prepared in two stages starting with a quick-frozen, stock culture broth that had been stored in the frozen state at -23 C. A 2-ml amount of rapidly thawed culture was added to 100 ml of yeast extract medium (pH 7.0) in a 500-ml Erlenmeyer flask, and the culture was incubated for 24 hr at 26 C on a rotary shaker at 250 rev/min. A 40-ml amount of this flask was then added to 24 liters of yeast extract medium (pH 7.0) in a 30-liter seed tank, and the culture was incubated for 24 hr at 26 C and with aeration at 1.2 liters of air per liter of broth per min.

Fermentations were carried out in a 55-liter stainless-steel, baffled fermentor. A 37-liter amount of production medium composed of 5% yeast extract (pH 5.7) was sterilized for 30 min at 121 C and transferred into the steam-sterilized fermentor. After cooling, 200 ml of seed tank inoculum was added to the fermentor. The fermentation was incubated at 26 C with low rates of oxygen transfer (1.5 volumes of air per volume of broth per min). High rates of oxygen transfer were provided by an agitation speed of 375 rev/min from two impellers with four blades on each impeller in addition to aeration of the medium with 1.5 volumes of air per volume of broth per min.

Replicate fermentations were run in the fermentor and samples for analysis were removed at 5-hr intervals until the completion of the fermentation. Foaming was controlled with lard oil containing 2% octadecanol which was automatically added on demand.

Assay for asparaginase. Asparaginase assays of fermentation broth were carried out as described previously (8). One asparaginase unit (IU) is that amount of enzyme which liberates 1 μmole of ammonia in 1 min at 37 C.

Prodigiosin assay. Samples of whole broth culture were diluted 1:10 in 0.1 N methanolic HCl, shaken mechanically to extract all of the pigment in the cells, and centrifuged. The supernatant fluids were then diluted with methanol to contain between 0.2 and 2.5 μg of prodigiosin per ml. Readings were made at 540 nm on a Bausch & Lomb Spectronic-20 colorimeter against a known prodigiosin standard (NSC 47147), kindly supplied to us by the Cancer Chemotherapy National Service Center, Bethesda, Md.

Viable cell counts. The cell concentration in the fermentation media was determined by plating diluted samples on nutrient agar, incubating overnight at 32 C, and counting colonies.

Oxygen measurements. A Delta model 85 oxygen meter capable of detecting 0.2 mg/liter of oxygen with an accuracy of ±1% fullscale was routinely used for measurement of dissolved oxygen in the fermentation medium.

RESULTS

Table 1 summarizes changes in dissolved oxygen, yields of asparaginase and prodigiosin, pH, and cell populations during the course of typical fermentations in unbaffled and baffled shaken flasks.

In the unbaffled flask, a condition of oxygen deficiency developed which had significant effects on the course of the fermentation. No measurable dissolved oxygen in the fermentation medium from 15 to 39 hr was detected in spite of vigorous agitation of the medium. Biosynthesis of asparaginase occurred during this period of limited oxygen availability. Concurrently with the reappearance of dissolved oxygen in the fermentation medium at 48 hr, the number of viable cells and the enzyme levels decreased. Both the peak cell population and the maximal asparaginase yield occurred at 39 hr. The pH of the culture medium rose from its starting level of 5.0 to 8.5, the peak of asparaginase accumulation. A further increase in the pH of the fermentation broth to 8.9 at 54 hr was accompanied by an abrupt decrease in asparaginase level and in a reduction in the number of viable cells. Biosynthesis of asparaginase occurred at pH 6.8 to 8.5. Under these conditions, prodigiosin was not detected.

With baffled flasks, the fermentation proceeded
at a more rapid rate, increases in pH value and viable cell population were accelerated, and markedly increased levels of dissolved oxygen were attained. A low level of 4.5 mg of oxygen per liter and sharply reduced yields of asparaginase were observed at 15 hr. Under these conditions, substantial quantities of prodigiosin were produced, reaching a peak of 25 μg/ml at 24 hr.

Table 2 summarizes changes in dissolved oxygen, in asparaginase and prodigiosin yields, and in pH during the course of fermentations at 26°C with S. marcescens carried out in plain 500-ml Erlenmeyer flasks containing 25, 50, 100, and 150 ml of medium per flask. The fermentation proceeded at a more rapid rate, as indicated by accelerated increases in pH values in reduced volumes of medium. The condition of oxygen deficiency that developed in fermentations made

**Table 1. Comparison of influence of dissolved oxygen on pH, viable cell population, and asparaginase and prodigiosin production of Serratia marcescens incubated in un baffled and baffled shaken flasks at 26°C**

| Time of incubation (hr) | Unbaffled flask | Baffled flask | Unbaffled flask | Baffled flask |
|-------------------------|-----------------|---------------|-----------------|---------------|
|                         | Dissolved oxygen (mg/liter) | Asparaginase (IU/ml) | Prodigiosin (μg/ml) | pH | Viable cells (per ml) | Dissolved oxygen (mg/liter) | Asparaginase (IU/ml) | Prodigiosin (μg/ml) | pH | Viable cells (per ml) |
| 0                       | 8.5             | 0             | 5.0             | 8.5 | 0 | 0 | 5.0 | 8.0 | 0 | 0 | 5.0 |
| 8                       | 8.2             | 0             | 5.0             | 8.5 | 0 | 0 | 5.0 | 0.1 × 10^4 | 0 | 0 | 5.0 |
| 15                      | 0               | 1.1           | 6.8             | 4.5 | 0.4 | 20.0 | 8.2 | 22.0 × 10^4 | 0 | 0 | 8.2 |
| 24                      | 0               | 2.9           | 7.7             | 7.0 | 0.4 | 25.0 | 8.5 | 12.0 × 10^4 | 0 | 0 | 8.5 |
| 32                      | 0               | 2.9           | 8.0             | 6.5 | 0 | 22.5 | 8.9 | 3.5 × 10^4 | 0 | 0 | 8.9 |
| 39                      | 0               | 3.1           | 8.5             | 7.5 | 0 | 17.7 | 8.9 | 0.8 × 10^4 | 0 | 0 | 8.9 |
| 48                      | 7.0             | 1.9           | 8.8             | 7.5 | 0 | 15.0 | 9.0 | 0.007 × 10^4 | 0 | 0 | 9.0 |
| 54                      | 7.8             | 1.9           | 8.9             | 0 | 0 | 8.5 | 0.1 × 10^4 | 0 | 0 | 5.0 |

* Not measured.

**Table 2. Comparison of influence of dissolved oxygen on pH and on asparaginase and prodigiosin production of Serratia marcescens incubated in un baffled shake shake flasks with various volumes of medium**

| Time of incubation (hr) | 25 ml/flask | 50 ml/flask | 100 ml/flask | 150 ml/flask |
|-------------------------|-------------|-------------|--------------|--------------|
|                         | DO (mg/liter) | Asparaginase (IU/ml) | Prodigiosin (μg/ml) | pH | DO (mg/liter) | Asparaginase (IU/ml) | Prodigiosin (μg/ml) | pH | DO (mg/liter) | Asparaginase (IU/ml) | Prodigiosin (μg/ml) | pH | DO (mg/liter) | Asparaginase (IU/ml) | Prodigiosin (μg/ml) | pH |
| 0                       | 8.5         | 0           | 5.0           | 8.5 | 0 | 0 | 5.0 | 8.5 | 0 | 0 | 5.0 | 8.5 | 0 | 0 | 5.0 |
| 8                       | 8.2         | 0           | 5.0           | 8.5 | 0 | 0 | 5.0 | 8.0 | 0 | 0 | 5.0 | 8.0 | 0 | 0 | 5.0 |
| 15                      | 0           | 1.3         | 8.2           | 0 | 0 | 7.6 | 7.0 | 0 | 0 | 7.0 | 0 | 0 | 7.0 | 0 | 0 | 7.0 |
| 24                      | 6.0         | 0.7         | 9.1           | 0 | 0 | 8.3 | 0 | 0 | 7.0 | 0 | 0 | 7.0 | 0 | 0 | 7.0 |
| 32                      | 7.0         | 0.2         | 9.2           | 6.5 | 0 | 8.8 | 7.0 | 0 | 0 | 8.8 | 7.0 | 0 | 0 | 8.8 |
| 39                      | 7.0         | 0.1         | 9.1           | 7.5 | 0 | 9.2 | 7.0 | 0 | 0 | 9.2 | 7.0 | 0 | 0 | 9.2 |
| 48                      | 6.5         | 1.3         | 8.9           | 0 | 0 | 3.0 | 8.3 | 0 | 0 | 8.3 | 0 | 0 | 3.0 | 8.3 | 0 | 0 | 8.3 |

* Dissolved oxygen.

b Not measured.
ml of medium was a detectable yield of prodigiosin obtained due to the brief period when dissolved oxygen was absent. Sustained high levels of dissolved oxygen which exist in baffled flasks are required for production of high yields of prodigiosin.

Table 3 summarizes changes in dissolved oxygen, in asparaginase and prodigiosin yields, and in pH during the course of fermentations with S. marcescens incubated at 26 C in 5% of yeast extract (initial pH 5.7) in a 55-liter, baffled, stainless-steel fermentor with low and high oxygen transfer rates.

Oxygen deficiency that developed in the fermentation with a low oxygen transfer rate had significant effects. The dissolved oxygen level in the culture medium was not detected commencing at 15 hr, although air was supplied to the medium. Biosynthesis of asparaginase took place during this period of limited oxygen availability and reached a peak of 2.7 IU/ml at 45 hr. The pH of the culture medium rose from its starting level of 5.7 to 8.6, which was the peak of asparaginase accumulation. When the biosynthesis of asparaginase occurred at pH 7.1 to 8.6, prodigiosin was not detected.

The fermentation in the fermentor with a high oxygen transfer rate proceeded at a more rapid rate and increases in pH were accelerated. Markedly increased levels of dissolved oxygen were attained during this fermentation. Yields of asparaginase were approximately 60% less than those obtained at a low oxygen transfer rate. Substantial quantities of prodigiosin were produced and reached a peak of 240 µg/ml at 45 hr.

**DISCUSSION**

Fermentations with S. marcescens demonstrated the profound influence of dissolved oxygen levels available in the broth on biosynthesis in both shaken-flask cultures and in a small fermentor. The way in which oxygen was transferred into the fermentation directed, in a qualitative manner, the production of the desired end product. A maximum yield of asparaginase accumulated only at undetectable levels of dissolved oxygen, a condition which was achieved either in an unagitated flask or in an unagitated fermentor with a low rate of aeration (1.5 volumes of air per volume of broth per min). Production of prodigiosin, on the other hand, required a supply of dissolved oxygen that could be obtained only with the high rate of oxygen transfer in baffled flasks or in the fermentor supplied with both aeration and agitation.

In the screening and fermentation development work which utilizes shaken cultures, it is essential that an oxygen level be provided which is sufficient to meet both the growth requirements of the organism as well as the yield of desired end product. Since aeration above or below an optimal level may induce conditions unsuitable for the formation of the desired end product, precise measurement and control of this variable are essential. A number of similar situations may be recognized if control of oxygen availability is more commonly practiced during fermentation experimentation. The most direct and meaningful determination of optimal aeration during the course of a fermentation can be achieved by measurement of dissolved oxygen in the culture broth. Successful scale-up from shaken-flask fermenta-
tions to small fermentors may be more readily accomplished if optimal dissolved oxygen concentrations for formation of the desired end product are provided.

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