Novel System for Improved Control of Filamentous Microorganisms in Continuous Culture

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We describe a system devised to overcome the tendency of fungal mycelium grown in continuous cultures to adhere to the walls and hamper the outflow of the fermentation vessel, with resulting difficulties in attainment of true steady-state conditions.

The culturing of mycelium-forming fungi necessitates a series of precautions to prevent accumulation of the organism on the walls and probes of the fermentation vessel. These accretions of organism result in erratic measurements of steady-state concentrations of biomass and make it impossible to attain a true steady state. The accumulations in the overflow system act as a filter, retaining the organisms while allowing medium to flow through, with resulting discrepancies in residence times of the organism and medium (4).

A device for an improved control of culture volume and organism concentration was described by Righelato and Pirt (5) which consists of a pinch valve that periodically closes and opens the air-culture effluent tube which clears the system.

In the present communication, a different and simpler system is described which enables prolonged, continuous fermentation in a wide range of dilution rates with minimal interference with the steady state system, as shown by the data on the formation of an intracellular proteinase by a strain of Penicillium chrysogenum.

The fermentation vessel used was an all-glass fermentor (Stölzle, Vienna, Austria) with a working volume of 3 liters. Air was supplied through the stirring shaft of the impeller at a rate of 1.5 liters of air per minute per liter of culture medium. The outflow system consisted of a glass standpipe inserted through a stuffing box in the bottom of the vessel. The standpipe was ground off obliquely to form a weir lip and allow an efficient overflow of culture broth. A glass, pH electrode and an oxygen electrode were inserted through the glass cover of the vessel. Nutrient medium, an antifoam agent, and KOH for the pH-stat operation were metered through inlets in the cover. To reduce the occurrence of accretions of mycelium on the walls of the vessel near the surface of culture medium, the glass vessel and the standpipe were coated with a solution of silicone oil (Bayer) in trichloroethylene (Merck) followed by heating at 200°C for several hours.

The test organism, strain D6/341/13 of P. chrysogenum, was cultured at 25°C and pH 5.8 in a synthetic medium containing: 22.2 g of glucose, 1.22 g of (NH₄)₂HPO₄, 1.00 g of yeast
Table 1. Continuous culture experiments: mycelial dry weight, concentration of nutrient components, and proteinase activity in steady state at five dilution rates

| Dilution rate (hr⁻¹) | Mycelial dry weight | Glucose (g/liter) | Ammonia-nitrogen (g/liter) | Proteinase (P) activity (P units/g, dry wt) | Period of culture in steady state (hr) |
|---------------------|---------------------|-------------------|-----------------------------|--------------------------------------------|--------------------------------------|
|                     |                     | Standard deviation (%) of mean |                        |                                            |                                      |
| 0.030               | 10.98               | 0.97              | 3.7                        | 0.37                                       | 11.0                                  | 12                                   |
|                     | 11.16               |                   | 3.9                        | 0.34                                       | 11.3                                  | 24                                   |
|                     | 11.24               |                   | 4.1                        | 0.35                                       | 9.2                                   | 36                                   |
|                     | 11.21               |                   | 3.9                        | 0.35                                       | 10.5                                  | 48                                   |
|                     | 11.02               |                   | 3.9                        | 0.34                                       | 11.7                                  | 60                                   |
|                     | 11.12               |                   | 3.9                        | 0.35                                       | 11.0                                  | 72                                   |
| 0.048               | 11.09               | 2.64              | 3.8                        | 0.59                                       | 22.9                                  | 12                                   |
|                     | 10.87               |                   | 4.1                        | 0.60                                       | 28.3                                  | 24                                   |
|                     | 10.51               |                   | 4.2                        | 0.68                                       | 36.6                                  | 36                                   |
|                     | 10.32               |                   | 4.1                        | 0.60                                       | 25.4                                  | 48                                   |
|                     | 10.42               |                   | 3.8                        | 0.66                                       | 26.5                                  | 60                                   |
|                     | 10.42               |                   | 3.9                        | 0.65                                       | 27.8                                  | 72                                   |
|                     | 10.29               |                   | 3.9                        | 0.62                                       | 29.1                                  | 84                                   |
| 0.062               | 9.65                | 2.71              | 4.0                        | 0.74                                       | 37.5                                  | 12                                   |
|                     | 10.24               |                   | 4.4                        | 0.73                                       | 40.0                                  | 24                                   |
|                     | 10.21               |                   | 4.3                        | 0.74                                       | 38.2                                  | 36                                   |
|                     | 9.93                |                   | 4.1                        | 0.72                                       | 45.7                                  | 48                                   |
|                     | 10.51               |                   | 4.4                        | 0.75                                       | 42.1                                  | 60                                   |
|                     | 10.24               |                   | 4.3                        | 0.72                                       | 38.0                                  | 72                                   |
|                     | 10.21               |                   | 4.4                        | 0.72                                       | 39.1                                  | 84                                   |
| 0.086               | 8.08                | 1.13              | 7.6                        | 0.84                                       | 51.6                                  | 24                                   |
|                     | 8.23                |                   | 7.2                        | 0.84                                       | 49.2                                  | 36                                   |
|                     | 8.06                |                   | 7.4                        | 0.84                                       | 52.0                                  | 48                                   |
|                     | 8.10                |                   | 7.7                        | 0.86                                       | 53.6                                  | 60                                   |
|                     | 8.26                |                   | 7.5                        | 0.83                                       | 53.8                                  | 70                                   |
| 0.097               | 5.41                | 0.24              | 17.4                       | 0.91                                       | 36.2                                  | 12                                   |
|                     | 5.39                |                   | 17.2                       | 0.93                                       | 38.0                                  | 18                                   |
|                     | 5.42                |                   | 17.3                       | 0.92                                       | 37.3                                  | 30                                   |
|                     | 5.41                |                   | 17.2                       | 0.92                                       | 38.0                                  | 36                                   |

Fig. 2. Influence of operating control device on partial pressure of oxygen in the culture medium during mycelial growth in a batch process. Stirring speed of the impeller was increased every 30 min for 30 sec.
extract, 1.00 g of $\text{KH}_2\text{PO}_4$, 0.50 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g of $\text{FeSO}_4 \cdot 2\text{H}_2\text{O}$, 0.01 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.01 g of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.50 g of NaCl.

Mycelial dry weight was determined according to a method which allowed the measurement of dry weight and enzyme activity in the same sample (3).

Glucose concentration was determined iodometrically (1) and the ammonia-nitrogen was determined colorimetrically by using the phenol-hypochlorite reaction. Proteinase activity of the mycelium was measured by a casein method at 37 C, pH 7.8 (2), and expressed as proteinase units. Partial pressure of oxygen was measured with a galvantic-type oxygen electrode (Radiometer, Copenhagen) previously sterilized chemically.

Our system is based on the following observation. Stirring a liquid in a cylindrical vessel causes the surface of medium to form a rotational paraboloid, which becomes steeper when the stirring is accelerated. The liquid creeps up the walls of the vessel, whereby the height of elevation is a function of stirring speed. A periodic rise in stirring speed results in a periodic elevation of the surface of medium near the walls of the vessel. With the standpipe inserted near the walls as a constant-level device, a periodic rise in stirring speed would result in a periodic drainage of a certain amount of liquid through the overflow. This loss of liquid is gradually compensated for by the continuous feeding of fresh medium. To carry out periodic alteration of stirring frequency automatically, a rheostat was built into the power circuit of the stirring motor. The rheostat was cut out periodically by a constant-cycle electric motor (type 220 B, 1 rev/hr, Crouzet, Haase, Vienna). The circuit diagram is shown in Fig. 1. Every 30 min the programming disc operated the microswitch, thereby cutting out the rheostat for 30 sec and effecting a rise in stirring speed which caused an overflow of 30 to 60 ml. This 2% variation in working volume was neglected. The device was applied to a study on the formation of an intracellular proteinase of $P. \text{chrysogenum}$ in continuous culture (cf. H. Brunner and M. Röhr, Abstr. Comm. Meet. Eur. Biochem. Soc. 7th, p. 262, 1971).

Continuous fermentation experiments were performed at dilution rates in the range of 0.030 to 0.097 hr$^{-1}$. The steady state was easily maintained for up to 3 weeks without any disturbance by accretions of mycelium and was only limited by the emergence of foreign organisms penetrating through the stuffing box of the stirring shaft. Without the device, serious disturbances due to mycelial accretions at the weir lip occurred within 12 to 36 hr, depending on the dilution rate, i.e., mycelial concentration in the vessel. Table 1 gives steady-state values of the mycelial dry weight and glucose and ammonia-nitrogen concentrations, as well as proteinase activity present in the mycelium at several dilution rates gained in an "en suite," continuous-fermentation campaign started at a dilution rate of 0.030 and proceeding stepwise up to a dilution rate of 0.097. The steady levels in the concentrations of nutrient components and in the values of proteinase activity in the mycelium confirmed the effectiveness of the device.

As an increase in stirring speed is concomitant with an increase of dissolved oxygen in the culture medium, the influence of the control device on oxygen partial pressure was studied. To determine this effect significantly at varied mycelial concentrations, the partial pressure of oxygen was recorded continuously in a culture experiment performed in the same vessel under the same conditions at a dilution rate of zero, i.e., a batch experiment (Fig. 2). Each operational step (30 sec) of the control device caused a small, but fully reversible and thus negligible, rise in the partial pressure of oxygen in the culture medium in the whole range of biomass concentration without any effect on the steady state in continuous cultivation.

Simplicity of construction, low costs, and an excellent control of steady-state conditions with minimal interference with the system recommend the use of the device for accurate studies on continuous culture of filamentous organisms.

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