Solid-Substrate Fermentor for Ochratoxin A Production

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Received for publication 4 November 1974

A laboratory-scale fermentor designed for solid-substrate fermentation was constructed and tested. Its capacity to produce ochratoxin under varied conditions was determined with wheat as substrate. Ochratoxin yields of 2,000 to 2,500 µg/g of wheat were regularly obtained, and occasionally yields as high as 4,000 µg/g were obtained. The most critical factor in the fermentation was initial substrate moisture content; wheat tempered at 30 to 31% moisture produced the highest yields. Other variables tested were agitation and aeration rates, initial static culture time, and inoculum types and volumes.

The demand for gram quantities of mycotoxins for testing purposes has brought into focus the need for larger, special production equipment and technology for its use. Heretofore, mycotoxins have been produced on solid substrate only in the laboratory, mainly in shaken-flask culture (1, 3, 5). In contrast to conventional liquid fermentations, many mycotoxins are produced on solid substrates, usually high-moisture grain, inoculated with a specific mold culture. Although grain fermentations may be conducted in laboratory equipment, no large-scale, solid-substrate equipment is currently available or in common usage in the United States.

In Japan solid-substrate fermentations are being conducted commercially in automated equipment (2). The main products are fermented foods and enzymes used in manufacture of soy foods. The substrate is cereal grain, usually wheat or rice. Apparently these fermentations do not require continuous agitation, and only occasional turning of the solid medium is involved.

In the United States, the fermentation industry virtually centers around liquid culture in deep tanks. Many aspects of the process with liquid substrates are less involved than with solid media; e.g., effectual agitation may be accomplished with impellers, or sometimes the usual aeration will provide the necessary motion. A completely different approach is required for the mechanical movement of grain or other solid substrates to achieve the required agitation in a fermentation process.

In this investigation, a laboratory-scale, solid-substrate fermentor was constructed and the production of ochratoxin (OT) was studied as a model system. OT was the mycotoxin of choice because of its relative ease of assay and extraction from fermented substrate. Experimental variables tested with the fermentor were initial substrate moisture levels, agitation and aeration rates, initial static culture time, and inoculum types and volumes. In addition, analyses were made periodically during the fermentation for time of OT optimal yield and for moisture and volume increases of the substrate.

MATERIALS AND METHODS

Fermentor. Originally we considered construction of a small, baffled, rotating drum fermentor similar to the laboratory-scale units developed for gluconic acid production (6) and later tried at the Northern Regional Research Laboratory in the early penicillin work. However, to explore the anticipated number of variables in a minimum of time, we constructed two fermentor units, each consisting of four simulated cross sections of a 13-in. (33-cm) diameter, baffled, stainless-steel drum (Fig. 1). Each section was 3 in. (7.6 cm) wide and had tight-fitting Plexiglas sides to permit observation during operation. The four drum sections were spaced on a common shaft of stainless-steel pipe, and each was secured to the shaft with an Allen set screw in an aluminum hub. The shaft rotated in two half-bearings located near the ends. Power source was a Zero-Max variable speed drive (1 to 40 rpm) equipped with chain and sprocket drive. Fermentors were protected from heat generated by the power units with styrofoam insulation board shields.

The individual drum sections were equipped with four baffles each to facilitate agitation. For the most effective agitation, it was considered desirable to have baffles of a size and shape that would permit all of the substrate to be elevated and subsequently dropped at some point during each revolution. Through experimentation, it was found that baffles 3 in. (7.6 cm) long and bent upward at a 30° angle in the direction of
rotation 1.5 in. (3.8 cm) from the ends effected the desired agitation with wheat substrate loads of up to 1 kg.

A sampling port for each fermentor consisted of a 1.25-in. (3.7-cm) round hole in the drum shell midway between two baffles. This port was used for charging the fermentor, inoculating, making water additions for moisture control, and withdrawing samples for analysis during the fermentation period. Tight-fitting rubber stoppers served as simple but effective closures for the ports.

To test the effect of aeration on the fermentation by-product yields, the four-sectional fermentors were equipped to allow continuous passage of sterile air through each section. The shaft of stainless-steel pipe had four 3/16-in. (0.48-cm) holes drilled through the pipe walls to allow passage of air forced through the hollow shaft. A rotary joint was installed on each end of the shaft for connecting of air hoses.

The fermentor units could be readily dismantled for cleaning and resterilizing. The power block assembly, mounted with bolts through a slotted base plate, could be moved backward so that the drive chain was easily removed from the sprockets. After removal of the rotary joints from the shaft ends, the assembly of four drum sections could be lifted as a unit off the half bearings. Loosening the Allen set screws allowed the individual sections to be slid from the shaft for cleaning and preparation for reuse. Sterilization was accomplished by reassembling the fermentor sections on their shaft and treating as a unit with ethylene oxide gas.

Preparation of substrate. Since OT was the desired fermentation product, wheat was used as the substrate (3). Hard, red winter wheat (Parker) was used throughout these studies. To permit ready infection by mold hyphae, the wheat kernels were lightly cracked. Wheat fines were removed by screening. For each fermentor 1 kg of cracked wheat was tempered and sterilized in a 2,800-ml Fernbach flask. Tempering was accomplished by adding sufficient tap water to bring the wheat moisture content to 30 to 31%. The flasks were covered with metal foil to prevent evaporation and shaken occasionally to facilitate moisture distribution. After standing overnight at room temperature, any wheat masses were thoroughly broken up by shaking. For sterilization, the flasks were autoclaved 45 min at 121.5 C. The wheat was cooled overnight and transferred to the fermentors by pouring from the flasks through a wide-mouthed, sterile, stainless-steel funnel inserted in the sampling ports.

Culture and inoculum. The culture used for OT production in these studies was Aspergillus ochraceus NRRL 3174, obtained from the Agricultural Research Service Culture Collection.

Spores for inocula were produced either on malt extract agar in 6-oz (178 ml) culture bottles or on bread cubes by the method of Sansing and Ciegler (4). Spore suspensions from 2-week-old cultures in the culture bottles were prepared by adding 30 ml of sterile H2O and dislodging the spores with a wire loop; 5 ml of the suspension was added to each fermentor as inoculum. Sporulated bread cubes were used for inoculum either as dry cubes added directly to the fermentors or as an aqueous spore suspension made from the cubes. For the dry inoculum, four cubes were added to each fermentor. The aqueous bread cube spore suspension was prepared by adding 60 ml of sterile H2O to a flask containing 20 of the cubes and agitating vigorously to dislodge spores; 5 ml of the resultant suspension containing about 10^6 spores/ml was added to each fermentor as inoculum. Fermentations were conducted in a 25 C constant temperature room.

Sampling and analyses. Approximately 20 g of fermented wheat was removed daily from each fermentor for analysis. Samples were obtained by opening the sampling port while the fermentor was stopped, with the port situated near its lowest position of the cycle. Samples were tested for volume, moisture, and OT content. Temperature checks were also made with a sterile thermometer inserted into the wheat. Volume was ascertained by measuring a 10-g sample after lightly tamping in a graduated cylinder. Moisture content was determined by calculating the weight loss of a 10-g sample dried 24 h in a 110 C drying oven.

Wheat samples were extracted and assayed for OT by the methods of Hesseltine et al. (3). Final quantitative determinations were made by visual comparisons, under ultraviolet light (wavelength, 366 nm), with varying amounts of pure OT-A standard.

Starting fermentations in static culture. Theoretically, beginning the fermentation in static culture before starting regular agitation would allow initiation of mycelial penetration into individual wheat kernels without interruption from motion and friction created by agitation in a freshly inoculated fermentor. Static fermentation was accomplished by loosening the Allen set screw in the hub of one or more fermentors to allow the shaft to turn within the hub and the individual unit or units to remain stationary. Static culture for varying periods of time was tested by beginning the fermentation with three of the fermentors stationary and one revolving as a control. After 4, 6, and 8 days, agitation was started by tightening the Allen set screw of a particular fermentor.
RESULTS AND DISCUSSION

OT yields of 2,000 to 2,500 µg/g of wheat were regularly obtained and occasionally yields as high as 3,000 to 4,000 µg/g were achieved with the four-sectional solid-substrate fermentor. These yields are well above those previously reported from shaken-flask culture. The highest reported yield from shaken flasks was 1,497 µg/g of wheat (3).

The OT peak was usually reached in 10 to 12 days (Fig. 2). A lag phase of 4 days was followed by a very rapid OT increase during the next 5 to 7 days. After the peak had been reached, there was usually a brief leveling off for 1 to 3 days, followed by a gradual diminution of yield.

From Fig. 2, it will be noted also that during the OT production cycle there was a corresponding increase in both the wheat moisture content and volume. The moisture increase was the result of carbohydrate metabolism yielding H₂O as one of the by-products. A slight moisture decrease occurred during the first 2 days, or early lag phase, since little H₂O was produced before active metabolism had begun, and at the same time some substrate moisture was given up to the fermentor atmosphere.

The wheat volume increase during the OT production phase closely paralleled the moisture increase. The volume usually increased 60 to 70% during the normal OT production cycle, and in prolonged experiments it increased by 100%. Individual wheat kernels swelled as the moisture content rose, taking on a "puffed wheat" appearance. The wheat expansion is a factor that must be considered in calculating initial substrate loads in relation to total fermentor capacity. Baffled fermentors can lose their agitation efficiency with initial overload-

ing and subsequent substrate expansion during fermentation.

The initial wheat moisture level is the most critical of all fermentation conditions. Highest yields were obtained with a starting moisture level of 30 to 31% (Fig. 3). Either higher or lower initial moisture content resulted in inferior OT production. Lower moisture resulted in a longer lag phase, a longer period to reach production peak, and a reduced yield. Higher moisture resulted in substrate adhering to fermentor walls and also rendered the substrate more vulnerable to bacterial contamination; yields were also inferior. If after the fermentation had begun it was found that the wheat moisture content was too low, it was both possible and practical to correct the deficit. Sterile water was added in increments of 5 ml or less to obviate sticking of wet substrate to the fermentor walls. Good OT yields were obtained when the substrate moisture was adjusted even though belatedly.

It is apparent that the highest agitation rate, 16 rpm, resulted in the highest OT yields (Table 1). However, considerably more time was re-

![Fig. 3. Effect of initial moisture content on OT yields. Fermentation conditions: initial wheat moisture, varied; inoculum, 10⁶ spores in 5 ml of H₂O; agitation rate, 10 rpm; aeration, none.](image-url)

![Fig. 2. Relationship of OT production to increases in wheat moisture and volume. Fermentation conditions: initial wheat moisture, 31%; inoculum, 10⁶ spores in 5 ml of H₂O; agitation rate, 10 rpm; aeration, none.](image-url)

| Agitation (rpm) | No. of runs | Fermentation time (days) | Ochratoxin yields |
|----------------|-------------|--------------------------|------------------|
|                |             |                          | Range (µg/g)     | Avg (µg/g) |
| 0              | 4           | 7-10                     | 100-200          | 150       |
| 1              | 2           | 8-9                      | 2,300-2,500      | 2,400     |
| 6              | 2           | 8-11                     | 2,000-2,500      | 2,250     |
| 8              | 2           | 12                       | 2,000            | 2,000     |
| 10             | 2           | 11-13                    | 1,700-2,500      | 2,080     |
| 16             | 5           | 12-19                    | 2,000-4,000      | 2,860     |
quired to reach the production peak than at lower agitation rates. Yields as high as 4,000 µg/g were obtained at 16 rpm, but 12 to 19 days were required at that speed. With agitation of 1 rpm, yields of 2,300 to 2,500 µg/g were obtained in only 8 to 9 days. Agitation rates of 6, 8, or 10 rpm demonstrated no advantage over 1 rpm, but shorter fermentation times were required to reach peak yields with all speeds under 16 rpm. At the low extreme, 0 rpm, the advantages of agitation are very dramatic; the yield was only 100 to 200 µg/g with no agitation. Brief periods of agitation once or twice daily did not improve yields in the otherwise static fermentations.

Likewise, starting the fermentation with static culture had no advantage. As initial static time was increased, progressively lower yields resulted, and the fermentation with no initial static time produced highest OT yields (Fig. 4). Agitation is obviously necessary and should be started as soon as inoculation is completed.

Extensive aeration studies were not conducted because introduced air even at low rates was apparently deleterious to the fermentation. Yields with forced aeration were always inferior to those with no introduced external air.

Of the several types of inocula used in the four-sectional fermentors, either of the two aqueous suspensions, whether from agar or from bread cubes, produced higher amounts of OT than the dry, sporulated bread cube inoculum. Of the two aqueous suspensions, that from sporulated bread cubes was preferred, because producing and harvesting the spores was less laborious than with the agar method.

Typical of most mold fermentations, heat was generated during the OT fermentation. After a 4-day lag phase, there was a gradual rise of 1 to 2 C per day until the temperature leveled off at 33 to 33.5 C concomitant with peak OT production. The temperature might have risen considerably more, except that the narrow-width construction of the four-sectional fermentors allowed some heat dissipation. In larger vessels a greater heat buildup would be expected. High temperatures could greatly reduce product yields, and means for cooling would be necessary for conducting solid-substrate fermentations in larger units.

Agitation serves a number of useful purposes in the OT fermentation. It effectively distributes spore inoculum. It maintains homogeneity throughout the fermentation period, preventing mycelial mass formation characteristic of static mold culture, and restricts growth to individual kernels. Agitation promotes aeration by exposing individual wheat kernels to the fermentor atmosphere momentarily during each revolution. One very important function of agitation is that of facilitating heat exchange and preventing localized overheating of substrate.

The superior OT yields obtained with the four-sectional fermentor compared with those from shaken flasks might be attributed to the more efficient substrate agitation achieved in the baffled fermentor. In the shaken flask, wheat substrate seems only to slide around, and individual kernels probably surface only randomly for exposure to fermentor atmosphere compared with the regularity of the baffled fermentor. Also, the agitation rate can be more precisely controlled in the fermentor than in shaken flasks.

The results obtained with OT-A as a model system in the four-sectional fermentor demonstrate the potential of solid-substrate fermentations in larger vessels for production of mycotoxins generally and possibly of other secondary metabolites. The concepts for fermentor design and operation resulting from these studies should be useful for scaling up equipment and conducting other solid-substrate fermentations.

ACKNOWLEDGMENTS

We thank S. P. Rogovin and R. R. Montgomery for valuable suggestions, Elsie E. Vanegraft for OT-A standard used in this work, and C. M. Each for help in designing and constructing the fermentors.

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**Fig. 4.** Effect of varying initial static fermentation time on OT yields. Fermentation conditions: initial wheat moisture, 31%; inoculum, 10⁶ spores in 5 ml of H₂O; agitation after static period, 10 rpm; aeration, none.
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