Ballistic Disruption of *Penicillium chrysogenum* Cells

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**ABSTRACT**

Hamilton, Pat B. (University of Wisconsin, Madison) and S. G. Knight. Ballistic disruption of *Penicillium chrysogenum* cells. Appl. Microbiol. 10:577–579. 1962.—The use of glass beads in a high-speed mixing device to rupture organisms was applied to molds. The use of a mixer in which the propeller shaft enters from the top into a metal mixing chamber made it possible to immerse the whole device in a salt water and ice mixture so that the temperature of the glass-bead slurry could be kept below 5 C without difficulty. Mycelia, glass beads, and buffer in a 1:2:3 (w/w) ratio gave above 95% breakage in 15 min with *Penicillium chrysogenum* cells and in 4 min with *Rhizopus nigricans*. Some of the factors influencing breakage are discussed.

Many methods have been described for the extraction of enzymes from mold mycelia. These methods usually were developed for bacterial cells and have not proved as efficient with mold mycelia, which are more resistant to disruption. Acetone-treated mycelia have been extracted with various buffers to yield active preparations of soluble enzymes (Bentley, 1953). Even extraction with dilute alkali has proved valuable (Jagannathan and Singh, 1953; Neilands, 1952). However, these extractive procedures have the fault of giving rather dilute preparations. A more serious fault is the failure to extract the particulate oxidases.

The oxidases are usually extracted by a method which ruptures the cell wall as well as the cell membrane and therefore permits the exit of the entire contents of the cell. Grinding with sand in a mortar (Casida and Knight, 1954) is the simplest method, but it is laborious and gives incomplete breakage and a dilute extract. Disruption by agitation with alumina in a 10-ke sonic oscillator (Sih, Hamilton, and Knight, 1958) is easier but retains the other faults. The Hughes Press (Hughes, 1951) provides excellent breakage in a short time but is limited by the small volume that can be handled. Cheng (1954) reported the presence of oxidases in extracts prepared by milling overnight in a ball mill, a method recommended by its simplicity despite its obvious limitations. The use of a colloid mill with glass beads is especially adapted to the rupture of large quantities of mycelia (Garver and Epstein, 1959). This method, in our hands, was limited by the requirement for a thin slurry of mycelia and beads, the necessity of extensive and complicated cooling equipment, and by the fact that it cannot be readily adapted to the preparation of small amounts of enzymes that are especially needed in preliminary studies. Lamanna and Mallette (1954) used common laboratory mixers such as the Waring Blender and Virtis homogenizer to agitate mixtures of glass beads and unicellular organisms. The biggest fault of this simple method is the difficulty in dissipating the heat generated during the 2-hr period required to achieve reasonably complete breakage of a resistant mold such as *Penicillium chrysogenum*. To provide a more suitable method for the breakage of mold mycelia, an attempt has been made to extend the use of a mixing device to quickly rupture mycelia in a glass-bead slurry under controlled temperature conditions.

**Materials and Methods**

The extensive heat production, which is the main limiting factor in the rupture of molds with agitated glass beads, requires complicated refrigeration devices or an unacceptable decrease in the agitation rate. It was felt that this heat might better be dissipated if the mixing chamber were made of metal rather than glass and if the drive shaft and blades entered the chamber from the top, so that the whole device might be immersed in a cooling liquid, thus helping to eliminate the need for internal heat-exchange devices. An apparatus with these design characteristics which is commercially available is the Servall Omnimixer, which was used in these experiments. This apparatus is rated at 16,000 rev/min and was used with the 400-ml stainless-steel chamber which contains mixing blades with a radius of revolution of 28 mm.

*P. chrysogenum*, which during several years study in this laboratory has been found to be exceptionally resistant to the usual breaking methods, was grown submerged in the synthetic medium of Sih and Knight (1956) for 48 hr at 25 C from a spore inoculum. After forced aeration, cultures in 20-liter heavy-wall Pyrex bottles were harvested by passing through cheesecloth. The hyphae were washed three times in cold distilled water and finally collected on a Büchner funnel. The moist hyphal cake was stored at 2 C until used.

When desired, the required amount of hyphal cake was weighed out and mixed in the agitation chamber with measured amounts of beads (120 to 130 μ in diam; Minne-
The loaded mixing chamber was immersed in a bucket of ice water at 0°C or a salt water and ice mixture at −15°C. The simple ice-water coolant was sufficient to keep the slurry temperature at 15 to 20°C, depending on the breaking conditions. The temperature of the slurry was easily kept below 5°C when the chamber was immersed in the −15°C salt water and ice mixture. The rapid flow and shearing action inside the chamber prevented the formation of an ice sheath on the interior surfaces of the chamber when the chamber was immersed in the brine.

When the temperature of the slurry had cooled to 2°C, the mixing chamber was connected to the shaft from the motor and the agitation was started. At appropriate time intervals, the motor was stopped, the chamber opened, the temperature of the slurry was taken, and samples removed for the determination of breakage and enzyme activity. The samples were placed in tubes and centrifuged at 700 × g for 10 min to sediment the glass beads and hyphae. The supernatant fluid was stored at 5°C until it was assayed. Total nitrogen was determined by a semimicro-Kjeldahl technique (Wilson and Knight, 1952). The cytochrome c oxidase assay (Smith, 1955) and peroxidase assay (Cheng, 1954) were modified for use on a Beckman DK-2 recording spectrophotometer with 1-ml cuvettes.

RESULTS AND DISCUSSION

Figure 1 shows the kinetics of release of cellular contents into the supernatant fluid when the hyphae: bead: buffer ratio is 1:1:2 (w/w). There is an obvious differential release of the two enzymes from the mycelia. Peroxidase reaches its maximal level in almost half the time required for the release of cytochrome oxidase. This observation has theoretical value as well as practical importance. If inactivation is assumed to play no major role, this clearly indicates a structural differentiation between the two enzymes. In fact, these two enzymes may be separated by centrifugation; the peroxidase is a soluble enzyme and the oxidase is particulate (Sih et al., 1958). The further increase of nitrogen in the supernatant after the two enzymes reach their maximum is probably explained by the differential release of another component not bearing either of the two enzymes. This differential release of enzymes indicates that the rupture of hyphae may be a progressive multi-hit process. The first stage would be minor damage to the cell envelope, with the result that only relatively small, soluble molecules could escape from the cell. The next step would involve more serious damage to the cell envelope, with the opening of larger fissures so that larger particles might escape. Another possible explanation is the rupture of the cell, releasing the soluble enzymes, followed by a differential release of the cytochrome oxidase particle from the cell “hull” (Marr and Cota-Robles, 1957). This is probably not true because safranine-stained smears of the sediment from centrifugation at 700 × g show nonstaining husks with fractured edges of the same over-all dimensions and form as the intact cells which are stainable. Therefore, it is likely that the cytochrome oxidase particle is free in the cytoplasm.

Increasing the amount of liquid in the agitation mixture so that the cell: bead: buffer ratio becomes 1:1:3 (w/w) had no effect on the release of the mycelial components except, of course, that the concentrations in the supernatant were lower, as would be expected when the mixture was diluted. The only other noticeable difference was that the temperature rise in the agitation chamber was smaller than with the more concentrated mixtures. This probably results from the better heat-transfer properties of the less viscous fluids. Failure of the breakage rates to respond to dilution was at variance with the results of Lamanna and Mallette (1954), who, with Escherichia coli and bakers’
yeast, found that with a fixed number of cells, the smaller the volume of the suspending liquid, the greater the rate of disruption. This nondependence on dilution, which is expected only of noncollision mediated processes, is probably explained by the high viscosity of the suspension which, at the start of agitation, was thick enough to be transferred with a spatula, thus preventing free flow of the liquid.

The mechanism of killing of bacteria by sonic treatment is considered to be a mechanical disruption of the cell (Marr and Cota-Robles, 1957), and the release of cellular components from bacteria under such treatment is exponential. The release of material from <i>P. chrysogenum</i>, however, was arithmetical (Fig. 1), despite the fact that the mechanism of breakage is undoubtedly mechanical disruption. An explanation is that the disruption in this system occurs only in a restricted area around the propellor blades where shearing action is imparted to the beads, as opposed to a sonic oscillator where an organism may receive energy sufficient for rupture, regardless of its position.

The effect of increasing the quantity of beads was to increase the rate of breakage. Figure 2 shows that doubling the amount of beads over that in Fig. 1 almost halves the time required to achieve maximal breakage. This result agrees with that of Lamanna and Mallette (1954), who found that for a given volume of suspension greater breakage was obtained by increasing the quantity of beads. The ratio of beads to suspension was not further increased in these experiments, as the 1:2:3 ratio was felt to be a suitable compromise between breakage time, dilution, and the time required to clean the beads. The fact that the disruption rate increased with the increase of bead to suspension ratio shows that the energizing system was not saturated at the lower concentrations of beads.

Breakage of better than 95% of the hyphae of <i>P. chrysogenum</i> in 15 min is unparalleled by any other method in our hands. Another advantage of this method, which is readily appreciated, is that it yields extracts more concentrated than those obtained by other methods. Figure 1 shows that extracts containing 5 mg of N/ml are easily obtained, while grinding with sand gives extracts containing 2 mg of N/ml (Sih and Knight, 1956). The ready control of temperature by the simple means of a bucket of ice water and salt removes the heat factor which heretofore limited the application of agitated beads to the rupture of molds. The use of a stirring device in the coolant vat aids in controlling the slurry temperature, but such a device was not routinely used because, for these experiments, the temperature could be adequately controlled without it.

This method was also tested on <i>Rhizopus nigricans</i> (mycelia supplied by Marvin Knop of this laboratory), a nonseptate mold, which is rather susceptible to the older methods of breaking. With a hyphae:beads:buffer ratio of 1:2:3, maximal release of nitrogen into the supernatant fluid occurred in 4 min. Although bacteria were not tested, there would seem to be no reason why they would not be advantageously broken by this method.

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**LITERATURE CITED**


