APPLIED MICROBIOLOGY, Mar. 1969, p. 462-466

Vol. 17, No. 3
Printed in U.S.A.

Disintegration of Microorganisms and Preparation of Yeast Cell Walls in a New Type of Disintegrator

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Received for publication 19 November 1968

Microbial cells were disintegrated in a new type of rotary disintegrator with a disc stirrer by a combination of shear force layers, collisions, and rolling of glass beads which were brought into motion by the stirrer. The rate of disintegration at a given dry bed volume of Ballotini beads and a given volume of cell suspension is proportional to the peripheral velocity of the stirrer up to 18 m/sec. Horizontal arrangement of the stirrer increases the effectiveness about five times; 100% disintegration of yeast cells was achieved under optimal conditions within 72 sec at a concentration of 3.5 g (dry weight)/100 ml of suspension, and within 96 sec at a concentration of 10.5 g (dry weight)/100 ml. At 17.5 g (dry weight)/100 ml, the stirrer began to slip. The cell walls of yeast were obtained at the desired degree of crushing and the course of purification was determined by infrared spectral analysis.

Considerable attention has been devoted to methods of disintegration of microorganisms. Most common methods of cell crushing employ glass beads. For this type of cell disintegration, one can use high-speed stirrer homogenizers (4, 5, 12, 13), particularly with an adapted stirrer (8) or with the working vessel moving reciprocally (7). D. Freedman and J. W. Ross (U.S. Patent 3,190,568) described a continuous disintegrator based on the principle of shaking with plastic beads. Phillips and co-workers (9) devised an apparatus based on the peristaltic-pump principle. A review article on the various disintegration methods has been published by Wimpenny (14).

The isolation of the cell wall from intact cells and from protoplasm is usually achieved by differential centrifugation in solutions of high densities (6). The purity of the isolated cell walls is determined microscopically (2), chemically, and radiobiologically (3). Some authors used infrared (IR) spectral analysis for following the changes in cell components of whole or partially extracted microbial cells (1, 10). In the present work, IR spectral analysis has been applied to determining the purity of the cell walls.

MATERIALS AND METHODS

Cultures. For this work, we used baker's yeast from the Kolín distillery, *Escherichia coli*, *Aspergillus niger*, and *Basidiomyces* sp. from the Collection of the Institute of Microbiology, Czechoslovak Academy of Sciences, Prague. The three last-named organisms were grown in 1.5-liter fermentors in synthetic media under aerobic conditions.

Apparatus. Disintegrators of 1,000 to 1,450 ml capacity were designed and made at the Research Institute for Organic Syntheses at Pardubice; the 300-ml disintegrator was made in the workshop of the Institute of Microbiology in Prague. Large-volume disintegrators were tested in two arrangements, vertical (Fig. 1), with a glass vessel and glass stirrer, and horizontal (Fig. 2), with a metal vessel and stirrer, either smooth or provided with curved grooves radiating from the center. The vertical disintegrator consists of a glass disintegrating vessel and a motor-driven disc stirrer, both provided with a cooling mantle.

Experimental procedure. The cell material was washed three times with physiological saline and twice with distilled water in the centrifuge and then suspended in distilled water to 3.5, 10.5, and 17.5 g (dry weight)/100 ml. Disintegration was carried out with glass beads (0.45 to 0.5 mm), the ratio of the dry bed volume of beads to that of the microbial suspension being 1.7. This ratio had been found to be optimal in preliminary experiments. The total volume completely fills the working space of the disintegrator.

The rate of cell disintegration was followed by determining the supernatant fluid extinction at 280 nm (bacteria and or fungi). With yeast, the cells were first heat-killed and then stained with methylene blue. In addition, the degree of disintegration was

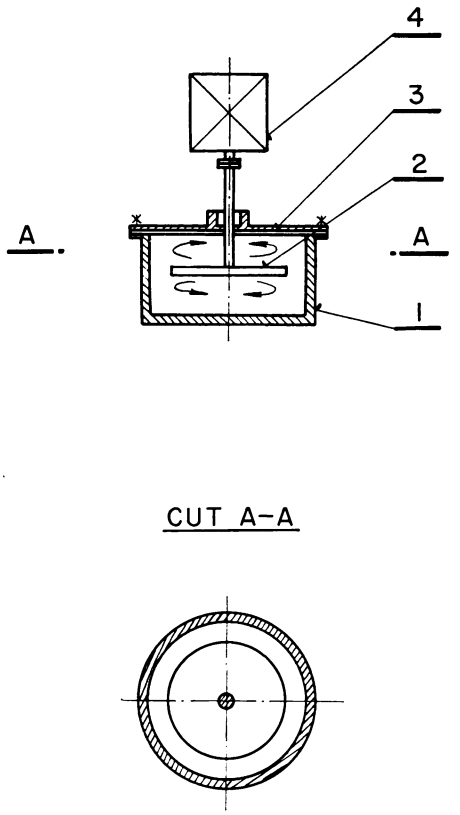

FIG. 1. *Scheme of vertical disintegrator. (1) Disintegrator vessel. (2) Disc stirrer. (3) Disintegrator lid. (4) Electric motor.*

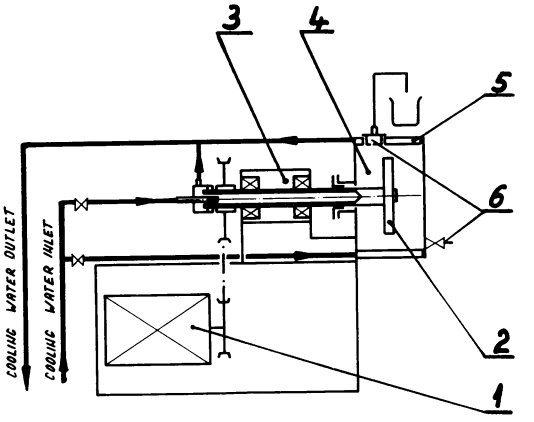

FIG. 2. *Scheme of horizontal disintegrator. (1) Electric motor. (2) Disc stirrer. (3) Shaft bearing. (4) Disintegrator vessel. (5) Cooling jacket. (6) Inlet and outlet of-suspension.*

TABLE 1. *Disintegration of different types of microorganisms in a vertical disintegrator at a peripheral speed of 18 m/sec*

Organism	Concn	Time required for 100% disintegration
	%	*min*
Escherichia coli.....	3	3
Aspergillus niger.....	3.5	2
Basidiomyces sp.....	3	2

determined from alcohol dehydrogenase (ADH) activity as described by Racker (11).

During disintegration experiments, the samples were withdrawn at short intervals (determined by preliminary experiments) and the degree of disintegration was determined. The average experimental time for 100% disintegration was corrected by a series of further experiments in which the sample was withdrawn only after disintegration was complete. Disintegration rates are expressed as the arithmetic means of 15 to 20 determinations.

For preparation of yeast cell walls, a 300-ml disintegrator was employed. After 100% disintegration of the yeast cells, the cell wall material was isolated by washing five to seven times with physiological saline. (Washing was carried out by agitating a very dilute suspension with a vibration-stirrer.) After the third washing, the material was again briefly disintegrated so that the collisions of the beads with ruptured cell walls removed any adhering protoplasm. Finally, the cell walls were washed twice with distilled water, centrifuged, partially concentrated in a rotary evaporator, and freeze-dried under reduced pressure. The purity of the cell walls was determined by the staining method of Bianchi (2) and by IR spectral analysis; a KBr cell in a Unicam 200 spectrophotometer was used.

RESULTS AND DISCUSSION

The principle of disintegration in this new type of disintegrator is based on intense stirring of a cell suspension with added solid bodies. The vertical device is shown schematically in Fig. 1. The ratios of diameter to height of the disintegration vessel, of vessel diameter to disc diameter, and of vessel height to the distance of the stirrer disc from vessel bottom are 1.9, 1.25, and 2.6, respectively. In the horizontal disintegrator, the relations of the individual dimensions of the vessel are identical, but the stirrer is placed in the center. The stirrer disc imparts a rotary movement to the mixture, the individual bodies moving along stream lines. Due to a velocity gradient between the individual stream lines, shear forces proportional to the apparent viscosity of the medium are created in the mixture. Collisions of the individual dense bodies also occur. In addition to these effects, one should also consider the effect of rolling of beads with different speeds of rotation. This implies that in the apparatus the

TABLE 2. *Efficiency of different types of disintegrators for crushing baker's yeast*

Concn of suspension	Disintegrator	Peripheral speed of stirrer	Time required for 100% disintegration	Amt of 100% disintegrated material (g/liter of working volume)	Avg productivity (g per liter per min)
%		*m/sec*	*min*		
3.5	Vertical, open 1 liter, smooth stirrer	9	9	18	2
		18	5	18	3.6
	Horizontal, closed, 1.2 liters, smooth stirrer	9	2	18	9
		18	1.2	18	15
	Horizontal, closed, 1.45 liters, grooved stirrer	18	1.2	18	15
10.5	Vertical, open, 1 liter, smooth stirrer	9	18	53	2.9
		18	12	53	4.4
	Horizontal, closed, 1.2 liters, smooth stirrer	18	2.5	53	21.2 (17.5)
	Horizontal, closed, 1.45 liters, grooved stirrer	18	1.6	53	33
		22	1.6	53	33
17.5	Vertical, open, 1 liter, smooth stirrer	9	14	87	6.2
		18	>30	87	2.9
	Horizontal, closed, 1.2 liter, smooth stirrer	9	4	87	22.0 (16.1)
		18	8	87	10.9
	Horizontal, closed, 1.45 liter, grooved stirrer	18	5	87	17.5

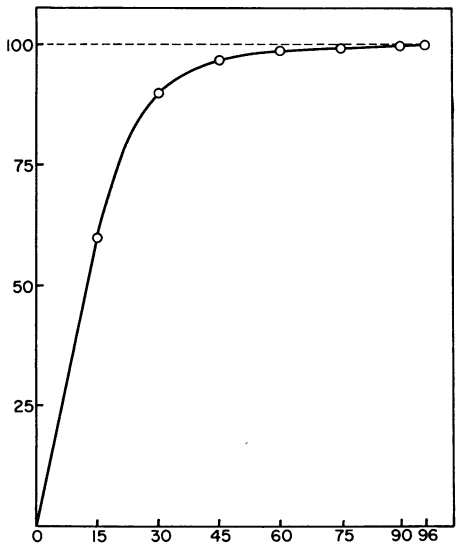

FIG. 3. *Time course of disintegration of yeast suspension (10.5%) in a horizontal disintegrator at a peripheral speed of stirrer of 18 m/sec. Abscissa: time (minutes); ordinate: percentage of disintegrated cells.*

disintegrating effect is achieved through a combined kinetic action of the beads. The apparatus permits the operator to select any desired temperature.

Table 1 shows the results obtained in the disintegration of different types of microorganisms on a vertical disintegrator at a peripheral speed of the stirrer equal to 18 m/sec. Under these conditions *E. coli* differs from the other microorganisms in the time required for complete disintegration. This may be due to the size of the beads relative to the cell size.

We used yeast cells for carrying out a more detailed comparison of the rate of disintegration with regard to: the suspension concentration used, the peripheral speed of the stirrer, the stirrer shape, and the position of the disintegrator (horizontal or vertical). The results are shown in Table 2 and the time course under the conditions of most rapid disintegration in Fig. 3. The increase in ADH activity in the disintegrated material is in agreement with the progress of morphological disintegration. From Table 2, we conclude that, at a peripheral speed of the disintegrator stirrer equal to 18 m/sec, complete disintegration is achieved in roughly half the time required at a speed of 8 m/sec. With a suspension of 17.5% (dry weight), at a speed of 18 m/sec, the stirrer begins to slip owing to the high viscosity of the suspension.

By employment of a horizontally placed stirrer, it is possible to reduce the time required for complete disintegration to about one-fifth the time required with a vertical arrangement. At the same time, one can use higher concentrations of suspension (10.5 g/100 ml), whereas the time

required for disintegration remains practically the same. A further increase in the peripheral speed to 22 m/sec with this type of disintegrator has no effect on the rate of disintegration. As with the vertical device, the stirrer begins to slip at a concentration of 17.5 g (dry weight)/100 ml. A grooved stirrer reduces slipping. For rapid preparation of a smaller amount of material, it is advisable to employ a suspension of 10.5 g (dry weight)/100 ml in a horizontal apparatus. To obtain larger amounts of material in a single run, it is desirable to employ a suspension of 17.5 g/100 ml in the same apparatus. Since it is assumed that the time lost by filling and emptying the device is the same for both types (not included in the calculation), the use of 10.5

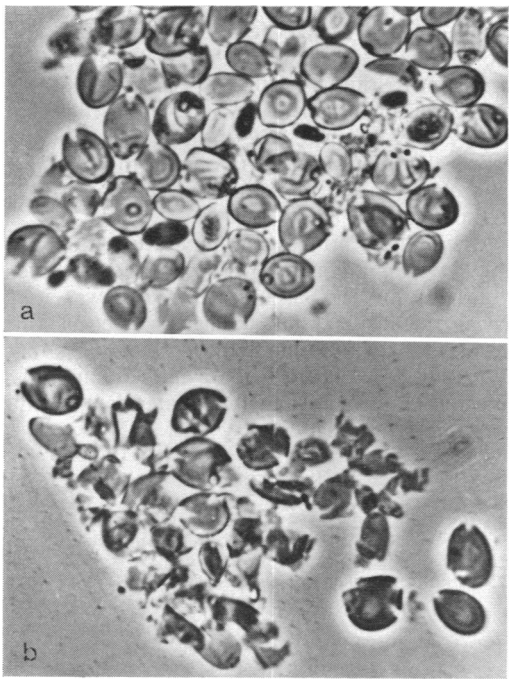

FIG. 4. *Yeast cell walls at different degrees of disintegration in a horizontal disintegrator. (a) Disintegrated for 1 min; (b) disintegrated for 6 min.*

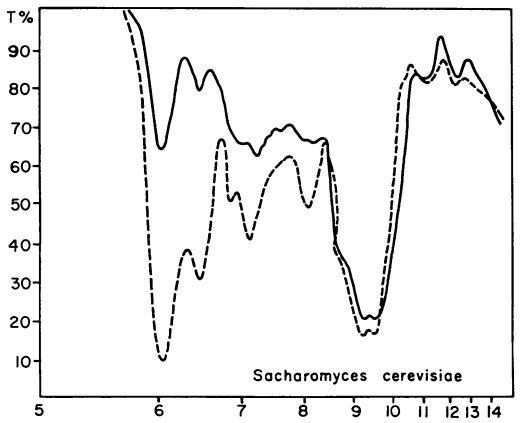

FIG. 5. *IR spectrum of whole yeast cells (broken line) and of isolated cell walls (solid line).*

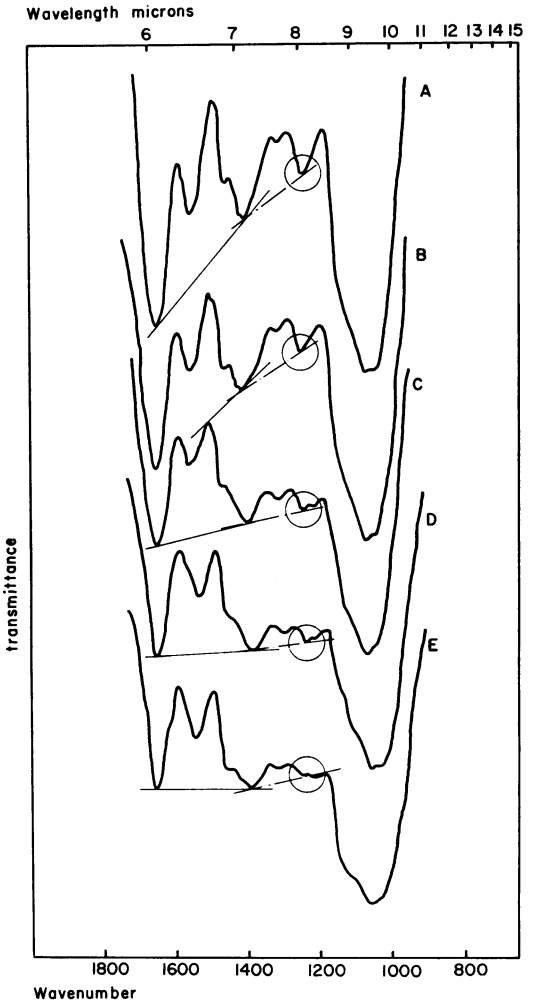

FIG. 6. *IR spectra of yeast cell walls, indicating the presence of whole cells and the removal of protoplasmic remnants. (A) Cell wall preparation containing 10% intact cells (compared with original suspension). (B) Cell wall preparation containing 5% intact cells. (C) Material disintegrated to 99.5%, washed twice with physiological saline and water, and differentially centrifuged. (D) Material treated as in C, purified by an additional disintegration (for 6 min) and washed once with water after centrifugation. (E) Pure cell walls.*

g/100 ml in a horizontal disintegrator is recommended. The cell walls are mostly intact after the disintegration times shown above and are perforated in one place only (Fig. 4a). By prolonging the time of disintegration, one can prepare cell walls of the desired degree of fragmentation (Fig. 4b).

The rate of disintegration is dependent upon a number of factors. The optimal size of glass beads used and the optimal ratio of their dry bed volume to the volume of the cell suspension was determined in preliminary tests. The rate also depends on the peripheral speed of the stirrer, on its vertical or horizontal placement, and on the concentration of the cell suspension. The apparatus permits one to select the most suitable conditions for disintegration of a given microorganism.

The purity of the isolated cell walls can be followed by IR spectral analysis. Figure 5 shows the spectrum of intact cells and isolated yeast cell walls. The spectrum of the cell walls displays a markedly decreased intensity of most absorption bands as compared with that of intact cells. The resulting spectrum of the whole cell represents a sum of the spectra of the individual components forming the cells. According to a simplified interpretation, the spectrum of *Saccharomyces cerevisiae* shows principal absorption bands at 6.05 μm, due to the C=0 group of proteins, polysaccharides, and, to a lesser extent, nucleic acids. The peak at 6.45 μm corresponds to the NH group of proteins. The absorption bands at 6.9 and 7.2 μm are caused by the linear chains, CH_2–CH_2, and the absorption band at 8.1 μm indicates the P=0 group of nucleic acids and polyphosphates. Polysaccharides are characterized by the broad absorption band formed by several of the polysaccharide groups in the region from 8.6 to 10 μm.

The spectrum of the isolated cell wall exhibits the absorption band at 8.6 to 10 μm, which is characteristic of polysaccharides, but the band representing P= 0 practically disappears. The other bands are reduced in proportion to the content of the corresponding groups outside the cell wall. During isolation of the cell walls, the lines connecting the peak of the absorption band at 7.1 μm, with that at 8.1 and 6.05 μm, are characteristically changed as the protoplasmic components are removed in the course of purification. As shown by Fig. 6, the slope of these connecting lines thus indicates the purity of the cell wall in the course of its isolation or purification, this being true particularly of the connection between the peaks at 7.1 and 8.1 μm. The effect of whole cells on the character of the spectrum is shown in Fig. 6 by curves A and B which, for the sake of illustration, were obtained from a mixture of pure walls and intact cells (10% cells in the original suspension for curve A and 5% cells for curve B). Curves C, D, and E represent the course of cell wall purification from a suspension disintegrated to the extent of 99.5%. The original absorption peak representing P=0 (8.1 μm) is markedly reduced in the cell wall spectrum, and a new peak appears at 8.3 μm, the P=0 absorption peak predominating. The situation is similar with the spectrum of the further purified wall (D); however, in the completely pure cell wall (E), the two peaks exhibit practically identical absorption. Further washing of such walls has no effect on the character of the spectrum.

The 5-liter horizontal apparatus described above (manufactured by Willy A. Bachofen Maschinenfabrik, Basel, Switzerland) was used as a model for constructing production-scale apparatus. A size-series of disintegrators has been developed, the 5-, 50-, and 200-liter disintegrators, to be described at a later date, permit continuous disintegration.

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