CXV. A WET-CRUSHING MILL FOR MICRO-ORGANISMS

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(Received 1 April 1938)

For some years we have been engaged on the problem of extracting enzymes from micro-organisms. It is well known that most bacteria and yeasts carry out oxidations and metabolic processes at a rate incomparably higher than that of animal tissues. The peculiar virtue of micro-organisms as the source of enzymes is therefore self-evident. A certain amount of success has been attained in extracting enzymes from yeast. By drying yeast at room temperature and then resuspending in water it is possible to obtain in solution a large number of enzymes. This method, however, has one serious limitation. The fragile enzymes are destroyed by the drying procedure, and among the fragile enzymes are included the most fundamental catalysts of yeast oxidations such as the cytochrome oxidase and the coenzyme factor. This method of extracting enzymes from the dried organisms is not applicable to bacteria. In fact no generally satisfactory method is at present available for extracting enzymes from the bacterial cell. Knowledge of isolated bacterial enzymes is virtually confined to a few which can survive the autolysis of the cell.

Our approach to the problem of enzyme extraction was made by some preliminary experiments with the Barnard & Hewlett [1911] mill for grinding micro-organisms, kindly lent by the designers. Though again applicable to yeast it proved ineffective for extracting enzymes from bacteria, principally through two limitations: (a) appreciable amounts of copper were introduced into the suspension, and (b) the capacity of the mill was too small to permit the grinding of an appreciable amount of a micro-organism in a reasonable time. We proposed therefore to construct a mill which would overcome the disadvantages of the Barnard mill. The objectives of the mill were: (1) absence of copper, (2) a capacity of about 40 ml., (3) greater rapidity of crushing, (4) elimination of heating due to friction and (5) a device for continuous circulation and replacement of the suspension fluid.

The Barnard mill is essentially ball bearing, and the crushing is accomplished at the points of contact between the five balls and the phosphor-bronze pot. It occurred to us that by using a roller bearing with line contact between the rollers and the race, the efficiency of crushing might be greatly increased. The difficulty of roller bearings even of the precision type is that the clearance is never less than about 2.5μ . This means that the inaccuracies of fit would be of the same order of magnitude as the size of the average micro-organism. Dr Sears and his colleagues at the National Physical Laboratory and Mr Wise of the Hoffman Manufacturing Co., Ltd., suggested to us that zero clearance could be attained by forcing a specially tapered inner race of a roller bearing along a tapered shaft until the distance between the inner and outer races was less than the diameter of the rollers. Under these conditions the revolution of the roller is accompanied by deformation of the metal of the races at the two lines of contact.

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Description of the mill

The essential unit of the instrument is a specially made Hoffman roller bearing, the inner race of which is tapered and is made to expand by forcing it on a tapered shaft. Each roller is tightly gripped between the races which it distorts. The pressure which causes this distortion tends to smooth out small scratches or inaccuracies on the contact surfaces and any material coming between roller and race must be crushed. The whole apparatus is shown diagrammatically in Fig. 1. All parts coming into contact with the grist are made of non-corrodible steel. The outer race *O* is firmly held in a stout housing *H* which is mounted on a heavy stand. The inner race *I* is revolved by the central taper shaft *S*₁ which is connected with another shaft *S*₂ mounted in ball bearings *B*₁ and *B*₂ and carrying a driving pulley *D*. It is extremely difficult to ensure that the inner race should fit exactly on the taper shaft. In absence of a perfect fit the shaft centre will describe a small circle as it revolves. To reduce resultant strains two universal joints *U*₁ and *U*₂ are introduced into the driving shaft. If there is a slight lack of parallelism between the race faces the rollers will tend to force the inner race up or down. This tendency is checked by the ball bearings which can take a considerable thrust load. With the rollers revolving in the same direction adjacent surfaces of any pair must turn in opposite directions. To prevent the rollers grinding one another Bakelite spacers are inserted. Spacers of various shapes, sizes and materials have been tried. Thin plates of a water-resisting paper Bakelite were found to give the best results.

A suspension of the micro-organism under study is placed in the mill which is driven at 600 r.p.m. The suspension is circulated continuously through the mill by a Bayliss & Muller type of pump. The exit is in the drainage basin underneath the screw nut and the entrance is at the top of the instrument through an opening in the cover. The minimum volume of fluid required to fill the mill (including pump, etc.) to a level just above the rollers is 40 ml. When the cream of micro-organism is replaced by water the running of the mill is uneven with a tendency towards seizure due very probably to local crowding of adjacent rollers and subsequent jamming of a spacer. However in spite of the oversize fit of the rollers the mill runs smoothly when lubricated by a thick suspension of micro-organisms containing about 10% dry weight of cells. During a run of 60 min. the bulk temperature of a yeast suspension increased only 2°. To reduce the effects of possible short duration local heating the working parts are water cooled. The grist is made to serve as the cooling fluid by circulating it through a tube with cooling vanes *V* immersed in a tank containing ice-water. By suitable manipulation of clips *C*₁ and *C*₂ the pump can be made to empty the mill instead of circulating the suspension. By reversing the pump the mill can be filled with a suspension fluid. The pump is normally run to circulate 60 ml. per min., but for emptying and flushing out it is made to deliver 150 ml. per min. In the case of resistant organisms the suspension is treated for 2 hr. and

Fig. 1. Schematic diagram of the mill. For explanation see text. Overall diameter of outer race is 100 mm.

centrifuged. If a considerable amount of uncrushed cells remains, the sediment is resuspended in water and the suspension is treated in the mill for a further period. When the quantities to be treated are greater than 40 ml. in the case of an organism, which is easily crushed, e.g. baker's yeast, the mill is run continuously, the charge of 40 ml. being replaced every 10 min.

Expansion of the inner race to the desired extent is attained by screwing up the cap nut *N*. The criterion of correct adjustment is that none of the rollers can be moved in any direction relative to the races except by rolling. To minimize the very considerable force required for this adjustment the taper shaft was highly polished (finishing with a vertical motion of the polisher). The screw threads were also polished and both these and the shaft were lubricated with tallow.

Crushing of yeast

There are various methods available for estimating the extent to which a suspension of yeast has been crushed. The method of cell counts gives only a rough approximation of the percentage of cells which have been crushed. In theory a smashed yeast cell should be broken up into fine particles, and the internal liquid content should be released into the suspension fluid. In practice gradations between the intact and the completely smashed cell are found, e.g. "ghost" cells which are the shells of the original intact cells without the internal fluid. It is obviously impossible to decide by simple microscopic examination whether cells abnormal in appearance are intact. It is similarly difficult to distinguish between intact and "ghost" cells. In our opinion the more reliable measures of the extent of crushing are (1) the amount of material which passes into solution from the burst yeast cells and (2) the enzymic activity of the cell-free suspension fluid.

The suspensions of baker's yeast which we tested were prepared as follows. 100 g. of the wet cake were suspended in 50 ml. $M/10$ Na_2HPO_4 . The function of the alkaline buffer is to neutralize the acids liberated during crushing. Fig. 2

Fig. 2 Diminution in cell count with time of crushing of baker's yeast.

Fig. 3. Increase in soluble extract with time of crushing yeast.

shows the relation between the number of cells and time of crushing in the mill, with a charge of 40 ml. of the yeast cream. At the end of the first 5 min. the cell count is halved. But it is only after about 30 min. that the cell count becomes

less than 10 % of the original. Plate VIII *a* and *b* are photomicrographs of dried smears of the untreated and the crushed yeast cream. In order to obtain a satisfactory photograph of the debris in the crushed cream it was necessary to use five superimposed dry films in contrast to a single film of the normal cream.

The amount of soluble material extracted from disrupted yeast cells provides a simple and convenient measure of the extent of disruption. Our experiments show that 1 g. dry weight of a completely crushed suspension of yeast liberates about 0.43 g. of soluble material. Fig. 3 shows how the amount extracted varies with time. The total charge of yeast suspension used in this experiment was 45 ml. At the time intervals indicated in the curve a sample of 1.5 ml. was taken and diluted to 5 ml. After 20 min. centrifuging, the dry weight per ml. of the cell-free supernatant fluid was determined. Water instead of phosphate buffer was used for suspending the yeast cake. After a 5 min. run in the mill 57 % of the total soluble material had been extracted; after a 15 min. run extraction was 88 % of theory. The theoretical amount is considered to be the asymptotic value of the curve.

The untreated yeast cream diluted 1:1 with water when centrifuged for 20 min. at 3000 r.p.m. is separated sharply into a hard packed sediment of yeast and a water-clear, colourless supernatant. The cream of crushed yeast when centrifuged at the same speed and for the same length of time is separated into four layers: a brownish opaque supernatant fluid and three sharply defined layers of sediment. At the bottom of the centrifuge cup is the firm layer of uncrushed yeast; above this is a white layer of "ghost" cells and cellular debris and finally there is a loosely packed coloured layer of extremely fine insoluble particles. The relative proportions of these three layers depend upon the duration of treatment in the mill. After a run of greater than 10 min. the bottom layer is very narrow compared with the layers of disrupted material.

Most of our experiments have been concerned with the properties and characteristics of the cell-free supernatant fluid. Microscopic examination of this fluid with dark ground illumination shows the presence of large numbers of particles which are just within the limits of visibility. By prolonged centrifuging (2 hr. or more) at high centrifugal speeds (15,000 r.p.m.) these particles can be completely sedimented.

The activity of certain enzymes such as the coenzyme factor and the cytochrome oxidase is found to be associated with these particles. The topmost layer of sediment obtained by 20 min. centrifuging of the crushed suspension represents the same type of insoluble particles as are found in the supernatant except that the former are somewhat larger in size and therefore more easily sedimented.

The supernatant fluid has been found to contain the following oxidative enzymes in considerable concentration: lactic, malic, α -glycerophosphoric, triosephosphoric, hexosemonophosphoric and alcohol dehydrogenases, cytochrome oxidase, coenzyme factor and catalase. The dehydrogenases are unusually active. 1 ml. of the supernatant fluid takes up about 1000 μ l. O_2 per hour in the presence of the substrates of any of the dehydrogenases listed above. The presence of coenzyme factor in yeast has in fact been demonstrated for the first time with the aid of the "juice" of crushed suspensions. We have not attempted to make an exhaustive survey of all the oxidative and hydrolytic enzymes present in the supernatant fluid. Probably all the soluble and stable enzymes of the cell would be found.

a.

b.

a and *b.* Photomicrographs of yeast suspension before and after crushing.

c.

d.

c and *d.* Photomicrographs of *Bact. coli* before and after crushing.

Crushing of Bact. coli

Since *Bact. coli* is of average size compared with other bacteria, and is easily grown in large quantity we have carried out most of our experiments with this organism. The 24 hr. growth on broth-agar was washed off with water and filtered through glass wool. The cells were washed thrice with water, the final wash water being clear and containing no soluble material. The final suspension contained about 4 g. dry weight of cells in 40 ml. water.

Bact. coli has at most 1/300 the volume of baker's yeast. It is not surprising therefore that whereas 5 min. suffice to crush 50 % of a yeast suspension 2 hr. are necessary for a suspension of *Bact. coli*. Before treatment in the mill 1 ml. of the *coli* suspension contained 100 mg. dry weight. After a 2 hr. run in the mill, followed by sharp centrifugation, 1 ml. of the cell-free supernatant fluid was found to contain 45 mg. dry weight. The dry weight of the soluble material represents only a part of the contents of the disrupted bacteria. It does not include the dry weight of the insoluble particles and cellular debris which are sedimented. The suspension of crushed bacteria on centrifuging is resolved into four layers as in the case of yeast. The cell-free supernatant fluid is brownish in colour and opaque like yeast "juice". Plate VIII *c* and *d* are photomicrographs of dried smears of the original and crushed suspensions. In order to obtain a satisfactory photograph of the debris in the crushed suspension it was necessary to use five superimposed dry films in contrast to a single film of the normal suspension.

The difficulty experienced in extracting enzymes from bacteria has led to a certain amount of doubt as to whether the catalytic units are of a similar nature to those of animal tissues and yeast, i.e. whether discrete soluble enzymes highly specific in their catalyses exist. Examination of the extract of crushed *Bact. coli* discloses the presence of a large number of highly active enzymes. In the course of only a limited survey positive tests for some 20 enzymes were obtained. It is quite clear therefore that there is no basis for assuming that the catalytic units of bacteria are any different from those of yeast and animal tissues.

The following enzymes have been found in the *Bact. coli* extract: lactic, malic, α -glycerophosphoric, triosephosphoric, formic, succinic and triose dehydrogenases, hydrogenase, zymohehexase, triosephosphoric mutase, coenzyme factor, cytochrome oxidase, catalase, peroxidase and aspartase. The malic, triosephosphoric and triose dehydrogenases require the presence of coenzyme I for their activity. The α -glycerophosphoric, succinic and lactic dehydrogenases function independently of a coenzyme. The most active enzymes are the malic, triosephosphoric, α -glycerophosphoric and succinic dehydrogenases and hydrogenase. In presence of any of these substrates (with other components such as coenzymes, carriers or fixatives added) 1 ml. of the *Bact. coli* extract takes up 500–1000 μ l. O_2 per hour. Hexosediphosphate is "fermented" with production of CO_2 in a bicarbonate- CO_2 medium. The rate of CO_2 evolution is about 1000 μ l. per hour. It is interesting to note that with the exception of the lactic and formic dehydrogenases all the enzymes described above have been obtained for the first time in cell-free solution from bacteria. Members of this department are now engaged in a study of the properties of some of these enzymes.

Other bacteria

Some experiments have been carried out with suspensions of *Sarcina lutea*. In a run of 4 hr. 25 % of the original dry weight of the suspension was found in solution after centrifuging off the cells and insoluble material. A suspension of

Bact. subtilis was also crushed. At the end of 2 hr. 44% of the original dry weight was found in solution. Several dehydrogenases were present in the cell-free extract. The time required to crush any particular micro-organism is dependent not only on size, but also on other factors such as the presence of capsules, shape, consistency etc. It is therefore necessary to determine by experiment the time required to crush efficiently a particular micro-organism.

Applications of the mill

Our knowledge of the detailed mechanisms of bacterial oxidative and metabolic processes is comparatively meagre. This deficiency is in great part due to the difficulty experienced hitherto in resolving the bacterial cell and in analysing the component catalytic units. The analysis of the extracted juices of bacteria offers a direct approach to the study of mechanism. To take an example, the fermentation of glucose by *Bact. coli* has been assumed to go through the same type of phosphorylation cycle as that of animal tissues [v. Tikka, 1935]. Analysis of the enzymes present in the *Bact. coli* juice shows that there is a firm basis for this assumption. There are present highly active enzymes for carrying out the following reactions in the glycolytic cycle: fragmentation of hexosediphosphate into triosephosphate, dismutation of triosephosphate, oxidation of α -glycerophosphate and oxidoreduction between triosephosphate and oxaloacetate. Furthermore, the properties of these enzymes resemble very closely those of their counterparts in animal tissues and yeast. It is thus clear that the participation of coenzymes in fermentation applies as well to *Bact. coli* as to yeast. There are many other examples that could be adduced to illustrate how analysis of the expressed juice of bacteria can throw light on the mechanism of reaction. But it would be outside the scope of the present communication to develop these specialized themes further.

In recent years attention has been concentrated on the purification of the antigenic proteins of pathogenic bacteria. A difficulty is that of extracting the proteins from the intact or killed organism. If the antigenic protein is very stable and can survive the subjection of the organism to drastic chemical treatment the difficulty of extraction is easily overcome. Many of the antigenic proteins, however, are destroyed by the chemical and physical treatment necessary to disrupt the cell. The use of the mill offers another method of approach to the antigen problem and to the problem of the separation and purification of bacterial proteins. The proteins extracted from crushed bacteria are not denatured. A simple method of obtaining the undenatured proteins in dry form is to freeze the cell-free juice and dry the frozen mass in a high vacuum over sulphuric acid.

The model of the experimental mill described above was not designed to allow of complete aseptic precautions being taken. Our tests thus far have been conducted therefore with non-pathogenic bacteria. Messrs Unicam Instruments Ltd., of Cambridge, who made the present machine, are arranging to construct an improved model of the mill with provision for handling pathogens. Although the mill has been in frequent use for several months, micrometer readings show no appreciable wear in the rollers.

SUMMARY

The detailed description is given of a mill for the wet-crushing of micro-organisms. Tests with yeast and *Bact. coli* show that efficient crushing takes places in a relatively short period. The enzymes present in the expressed "juice"

are briefly described. The possibilities and applications of this mill are discussed.

Our thanks are due to Dr Sears and his colleagues at the National Physical Laboratory for their useful suggestions and Mr Wise of the Hoffman Manufacturing Co. for his advice and help. The cost of constructing the mill was defrayed by a grant from the Medical Research Council. We also wish to thank Dr Stephenson and Dr Woods of this department for supplying us with bacterial suspensions.

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