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Short Communications

The Use of Ultrasound in the Extraction of Dehydrogenases from Yeast Cells

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Because of the durable nature of yeast cell walls, mechanical disintegration of yeast is often unsuitable for the preparation of enzymes, or its use demands a very efficient apparatus and long treatments. Organic solvents, such as acetone and butanol, have been extensively used for the extraction of enzymes; however denaturation of proteins may occur even at low temperatures. By conventional autolysis, often only a small part of the enzymes can be liberated in an active state.[1] Most methods of which freezing makes a part, are inefficient in the case of yeast or cause inactivation of enzymes. However, when high pressure (2000 kg/cm²) is used at temperatures lower than −20°C, virtually 100 % breakage of yeast without noticeable inactivation of enzymes has been obtained by means of the so called x-press.[2,3]

Ultrasonic treatment has been widely used for the disintegration of bacteria, but its effect on yeast cells has been examined only in a few instances.[4,5]

Experimental. Commercial baker's yeast (Rajamäki Factories of the Finnish State Alcohol Monopoly) was exposed to ultrasonic vibration produced by the MSE (Measuring and Scientific Equipment) ultrasonic disintegrator having a nominal output of 60 W and a frequency of 18–20 kc. The instrument was equipped with a titanium probe whose tip diameter was 10 mm. Specially designed rounded bottom glass vessels, diameter 25 mm, height 65 mm and capacity *ca.* 15 ml were used to hold the yeast cells. During ultrasonic treatment, the vessels were immersed in an ice-water bath.

The viability of yeast cells was determined by staining with 0.2 % methylene blue and counting under the microscope.[6]

Protein was determined by the biuret method according to Beisenherz *et al.*[7]

Dehydrogenase enzymes, alcohol dehydrogenase (ADH) (EC 1.1.1.1.), glucose-6-phosphate dehydrogenase (ZF) (EC 1.1.1.49) and glutamic acid dehydrogenase (GDH) (EC 1.4.1.2.) were analyzed as described by Schmidt *et al.*[8] except that bicarbonate buffer (0.2 M, pH = 8.4, containing EDTA, 2.8×10^{-3} M), as proposed by Beisenherz *et al.*[7] was used instead of tris- and triethanolamine buffers. Isocitric dehydrogenase (IDH) (EC 1.1.1.42.) was assayed by the method of Ochoa.[9] Bicarbonate buffer was used in this case also. All enzymes were measured at 28°C. Samples were kept in an ice bath until they were analyzed. Material that could not be analyzed during the same day was kept overnight at −18°C.

Results and discussion. Table 1 shows the amount of enzyme activity liberated after different treatment times. The amount of protein released, and the relative number of viable cells when the maximal output of the instrument (35 W/cm²) was used are also presented. In all cases, maximal activity was obtained after 5 min. No advantage could be attained by lengthening the exposure although the amount of protein liberated increased almost linearly during the first 15 min. Either the proteins liberated after treatment for 5 min are enzymatically inactive structural proteins, or inactivation of the enzymes already in solution occurs as rapidly as active enzymes are liberated. The latter possibility was eliminated by exposing cells to the ultrasonic treatment for 5 min, centrifuging and washing the unbroken cells and cell debris, then repeating the 5 min sonic treatment. After the first sonic treatment 90 % of ADH was found in the superna-

Table 1. Effect of time on enzyme activity released, protein liberated, and viability of yeast cells. Total protein of the sample was 5.5 mg/ml. Power 35 W/cm². Enzyme activity liberated in 5 min designated as 100 %.

Length of treatment, min	Enzyme activity % ADH	ZF	IDH	GDH	Protein in solution mg/ml	Viable cells %
0	0	0	0	0	0	97
0.5	32	—	—	—	0.47	—
1.0	44	33	71	60	0.85	0
2.0	74	62	—	—	1.32	3
3.0	100	95	86	75	1.64	4
5.0	100	100	100	100	2.56	3
10.0	106	—	—	—	3.30	1
15.0	84	98	95	92	4.07	1

tant, 3 % in the washings and the rest was extracted during the second exposure.

Slight inactivation of enzymes was observed during prolonged ultrasonic treatment. This may be caused by the ultrasound itself, the free radicals formed by it, or local overheating of the solution. The temperature of the solution was lower than 15°C in all experiments, but the possibility of local overheating should be taken into account (*cf.* Hughes [10]). With the experimental conditions used, formation of free radicals is low (*cf.* Hughes [10]). The activity lost during treatment for 5 min was not more than 10 %.

The effect of output on the liberation of enzymes from yeast cells during 5 min exposure can be seen from Table 2. If

Table 2. Influence of the power used on the enzyme activity released during ultrasonic treatment. Exposure 5 min in all cases. 2 % suspension of commercial baker's yeast.

Power W/cm²	Released enzyme activity, % ADH	IDH	GDH
3.8	0.0	0.0	3
8.7	88	32	21
15.5	85	80	65
22.0	102	94	83
35.0	100	100	100

probes with a greater tip diameter are used, the power/cm² decreases and enzymes can be completely extracted from yeast cells only through longer treatment times.

Raising the salt concentration of the solution diminishes the breaking of the

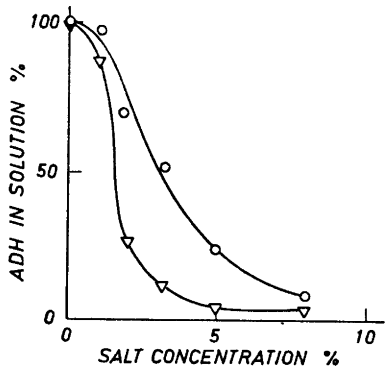

Fig. 1. The influence of inorganic salts on the effectiveness of ultrasonic treatment. The amount of ADH liberated during ultrasonic treatment of 1 % yeast suspension for 1 min in NaCl solutions (▽) or 5 min in $(NH_4)_2SO_4$ solutions (○) of different concentration. The amount of ADH liberated during treatment in pure water designated as 100 %.

yeast cells and the liberation of enzymes (Fig. 1). This phenomenon has been observed by Hughes [10] too, and is probably caused by the osmotic pressure of the solution which strengthens the yeast cell wall through dehydration.

Yeast concentration has little effect on the amount of enzymes extracted. If high yeast concentrations (20 % fresh yeast) are used, considerable quantities of the extracted enzymes may be lost with the cell debris during centrifugation.

For best results, the tip of the probe should be 1—3 mm below the surface of the liquid. If the probe is immersed deeper,

the temperature increases and local or general overheating may occur.

For the determination of the reliability of the ultrasonic method, 15 parallel samples were treated at 35 W/cm² for 5 min and analyzed for ADH and IDH. Standard deviations were 3.1 % for ADH and 7.5 % for IDH.

In comparative experiments, freezing and thawing was found to be inefficient when dilute (5—10 %) suspensions of yeast were used. Only 5 % of the ADH was liberated after 12 successive repetitions. After grinding for 10 min in a Potter-Elvehjem homogenisator, or for 7.5 min (50 000 rpm) in the Bühler homogenisator with glass beads (ø 0.2 mm), less than 5 % of ADH was extracted. Better results have been obtained by using more concentrated yeast suspensions.[11] By experience, grinding of baker's yeast with an equal volume of quartz sand in a cone shaped all-glass homogenisator results in complete breakage of cells, but only 30 % of the ADH activity can be extracted. Acetone dried yeasts have 60 % of the activity of the ultrasonically treated material.

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Synthesis of 1,3-Di-*O*-acetyl-glycerol from Glycerol and Acetic Anhydride and a Method to Determine the Ratio of 1,2-Di-*O*-acetyl-glycerol and 1,3-Di-*O*-acetyl-glycerol in a Mixture

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Several authors have reported methods for the preparation of di-*O*-acetylglycerol;[1-6] however, it seems doubtful that the product obtained by these methods was pure di-*O*-acetyl-glycerol. Furthermore, the relative occurrence of 1,2-di-*O*-acetyl- and 1,3-di-*O*-acetyl-glycerol was not determined. Langenbeck and Bollow[7] have described a method to synthesize 1,3-di-*O*-acetyl-glycerol *via* 2-amino-propan-1,3-diol but no details of the method of synthesis were given. The diester was characterized by hydrolysis and by acetylation to 1,2,3-tri-*O*-acetyl-glycerol. Smith[3] claimed that he got pure di-*O*-acetyl-glycerol by direct acetylation of glycerol with acetic anhydride but gave no experimental details. It is not clear whether 1,2-di-*O*-acetyl- and/or 1,3-di-*O*-acetyl-glycerol was obtained. The isolated product was analysed by hydrolysis and by determination of the number of acetyl groups. A mixture of mono-*O*-acetyl-glycerol, di-*O*-acetyl-glycerol and tri-*O*-acetyl-glycerol could give an apparently pure product.

We have examined the possibility of synthesizing 1,3-di-*O*-acetyl-glycerol by direct acetylation of glycerol with acetic anhydride with and without the presence of catalytic pyridine. The reactions have been carried out at different temperatures and with different proportions of acetic anhydride and glycerol. The reaction mixtures were studied by thin layer chromatography. The isolated diesters have been submitted to NMR analysis. Tri-*O*-acetyl-glycerol gives a characteristic quintet at 4.88 τ ($J \approx 5$ cps), which derives from the methine proton of the $CH\text{-}OOC\text{-}CH_3$ group. The methylene protons of the $CH_2\text{-}OOC\text{-}CH_3$ group give signals between 5.60 and 6.00 τ. The methylene protons of the $CH_2\text{-}OH$ group in 1-mono-*O*-acetyl-glycerol give a