EFFECTS OF ULTRASONIC RADIATION ON GROWTH AND FERMENTATION IN THE YEAST, *SACCHAROMYCES CEREVISIAE**

by

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INTRODUCTION

In developing and testing apparatus and techniques for a study of the biological action of ultrasonic waves, experiments have been performed to evaluate the effects of ultrasonic radiation on suspensions of the yeast, *Saccharomyces cerevisiae*. Much of the previously-reported work involving irradiation of microorganisms with ultrasound has been concerned with the utility of such treatment in killing pathogens, sterilizing fluids, and releasing from cells certain of their morphological and chemical components, using high-intensity ultrasound at relatively low frequencies in the kilocycle range. In general, the results have shown that low-frequency, high-intensity ultrasonic waves exert their action by mechanically shattering cells (BECKWITH AND OLSON[1], BECKWITH AND WEAVER[2]; see also GREGG[3]). VON EULER AND SKARZYNSKI[4], however, found that with higher frequencies (800 kc) and low power (3 watts), growth and fermenting ability of yeast could be markedly inhibited without other evidence of damage to the cells. KATSNELSON AND KENOKH[5] reported no effects on growth, but an actual stimulation of 12% in fermentation rate as a result of irradiation of yeast at 425 kc (intensity not stated).

The results of the present studies should be of interest, in that ultrasonic waves of relatively high frequency (1 megacycle/second) have been used at carefully controlled power levels approximating 10 watts. The effects of such radiation on yeast have been estimated by tests of growth and fermentation in comparison with unirradiated control samples from the same cultures. Comparisons have also been made of the effects of continuous-wave and of pulsed radiation, in which the material is subjected to rapidly repeated (600/sec) bursts of ultrasonic energy. In addition, the relative susceptibility to irradiation of younger and of older cultures has been studied. The physical characteristics of the ultrasonic beam have been carefully evaluated, and the actual power and intensity levels to which samples were subjected have been determined as accurately as possible. The procedures used are outlined below in some detail, dealing with the culture methods for the yeast, the irradiation routine, and the techniques of estimating effects.

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MATERIAL AND METHODS

The yeast used in these experiments was *S. cerevisiae*, Strain 15.56, from a culture originally obtained from Dr FREDERICK G. SHERMAN of this laboratory. Stock cultures on agar slants prepared with READER's medium (READER[6]), as modified by SHERMAN AND CHASE[7], were stored at 4° C. For each experiment, a fresh slant was planted and incubated 48 hours at 30° C. Twelve hours before irradiation, a tube of sterile $M/15$ KH_2PO_4 was inoculated from this slant to an optical density of about 0.3. Depending upon the population density required for the experiment, a varying amount of this suspension (0.5 to 1.0 ml) was used to inoculate 40 ml of modified READER's medium in a special culture flask-absorption tube (SHERMAN AND GRANT[8]), the photometric center-point of which had previously been noted. This culture was then incubated for approximately 12 hours, with rocking, at 30° C.

Cultures were harvested at optical densities of approximately 0.163, corresponding to a population of about $2.2 \cdot 10^7$ cells/ml; or of approximately 0.3, representing a population of about $3.3 \cdot 10^7$ cells/ml. These figures were determined by repeated cell counts of cultures at different points in their growth cycles. All experiments reported here were performed on yeast from cultures harvested at one or the other of these 2 points in their growth.

The cells were separated from the culture fluid as sterilely as possible by centrifugation, washed 3 times with sterile $M/15$ phosphate buffer, and finally resuspended in sterile buffer to an optical density of 0.3. Samples from this suspension were placed into plastic vessels for irradiation (see below), and a portion reserved for a control suspension.

Suspension of cells in the non-nutritive buffer solution insures that subsequent tests for effects of irradiation will be made on irradiated cells themselves, and not on their unirradiated progeny. The use of suspensions of standard optical density permits irradiation of cells from cultures in different stages of population growth, without introducing as an added variable differences in the density of the suspensions being irradiated. Hemacytometer counts showed that throughout the range used here, a linear relationship exists between optical density and numbers of cells.

The vessels used for irradiation (see Fig. 1) were made of thin-walled cellulose acetate-butyrate tubing 1 cm in diameter. The ends of the body tube, which has a volume of about 2.5 ml, were cut at an angle of approximately 65° in different planes, to prevent the occurrence of standing waves set up by reflections between parallel end walls. In preparing the vessels for use, membranes of thin latex were stretched over the ends, held in place by rubber bands. The vessels were washed with sterile distilled water, plugged with cotton, and placed on their sides about 6 inches below an ultraviolet lamp for 12 hours' sterilization. Before irradiation, the body tube of each vessel was filled with standard yeast suspension, the plug replaced, and the vessel mounted in the center of the focal region of the ultrasonic beam.

The ultrasonic generator, a 2-stage transmitter employing an electron-coupled oscillator and a push-pull power amplifier, was constructed in the Metals Research Laboratory, Graduate Division

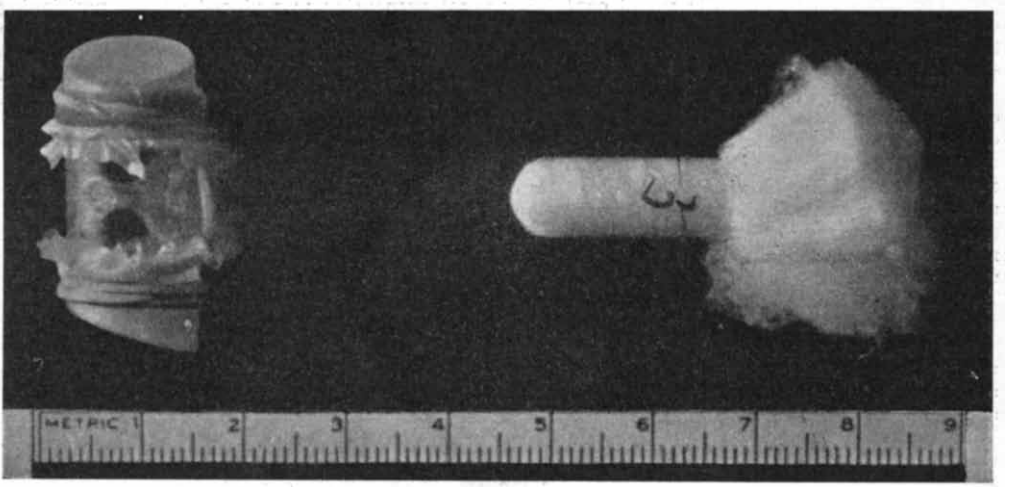

Fig. 1. Plastic vessel used to contain yeast under irradiation. Only the body-tube (left) is filled with yeast suspension.

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of Applied Mathematics. A switch is provided for operating the unit either in a continuous-wave (CW) fashion, or as a pulsed CW transmitter. Four switch positions change the duty cycle (DC) as follows: a. 100% (CW); b. 75%; c. 50%; d. 25%; these figures are approximations. Fig. 2 shows photographs of oscilloscope tracings of a. 25% and b. 50% duty cycle pulses. In any of the pulsed-operation settings, the repetition-rate is 600 cycles/sec; the duty-cycle percentages represent the portion of the repetition-period (1/600 sec) occupied by the burst of ultrasonic energy, which is of a continuous-wave nature during the "on" time.

The crystal is an X-cut quartz plate 1.5 inches in diameter, mounted in a specially designed holder inserted at the end of a modified 10-gallon aquarium. The aquarium is filled with degassed distilled water serving as the coupling agent between crystal and material being irradiated. Opposite the crystal a rubber-covered absorption cell, filled with steel wool and castor oil, prevents the reflection of sound waves which have passed through the irradiated material and thus provides a travelling-wave system for irradiation.

To increase the intensity (W/cm^2) to which small samples may be exposed in the ultrasonic field, without increasing the voltage applied to the crystal, a planoconcave spherical lens of polystyrene is mounted in front of the crystal. The lens has a focal length of 2.5 inches. It operates reliably at incident power levels below about 25 watts. Scanning measurements with a thermocouple probe have shown that approximately 85% of the ultrasonic energy reaching the focal region of the lens is focussed into a field 1 cm in diameter. (For technical details, see BRONZO AND ANDERSON[9]).

A Siemens Sonotest meter placed in front of the crystal in the water bath was used to determine the output levels required to produce the desired total power in the beam, at each of the oscillator switch positions. The Sonotest power readings, made with the lens in place, were used to calibrate a vacuum-tube voltmeter across the output circuit. Before each experiment, the region of maximum intensity in the focussed beam was located by the use of a thermocouple probe mounted on a co ordinate positioning device and connected to a recording potentiometer. The thermocouple junction

Fig. 2. Photographs of oscilloscope tracings of pulsed radiation; a. 25% duty cycle; b. 50% duty cycle

s imbedded in a bead of thermosetting plastic (Castolite) approximately 2 mm in diameter; absorption of heat by the plastic causes deflection of the potentiometer and permits measurements of relative intensity in small areas of the beam.

For irradiation, a plastic vessel containing yeast suspension was placed on the positioning device so that its center lay in the precise location indicated by the probe as the region of maximum intensity. Power was then applied at the desired level by duplicating the previously determined voltmeter reading. Control samples were placed in a corner of the water bath shielded from radiation.

With average power set at 10 watts, the maximum intensity in the focal region of the beam, calculated from thermocouple measurements, is approximately 32 W/cm^2 over a central area of 0.03 cm^2. The values fall off peripherally in such a way that the average intensity, over the entire area of 0.78 cm^2 intercepted by the end of an irradiation vessel, radius 0.5 cm, is approximately 10.5 W/cm^2. Keeping this intensity constant, samples were exposed, in various experimental combinations, to CW radiation for 10 minutes, to 50% DC pulsed radiation for 20 minutes, and to 25% DC pulsed radiation for 40 minutes. Preliminary experiments showed that these times were approximately minimal for consistent effects. The necessity of a 40-minute irradiation period using 25% DC pulsed radiation required that control cells remain in the buffer suspension for unfavourable lengths of time. Therefore, only a small number of experiments included this type of radiation, to establish its relative effectiveness.

Following irradiation, 0.5 ml portions of each sample, including the control, were added to modified Warburg vessels containing 3.5 ml of modified Reader's medium. To each vessel (see Fig. 3) a 10 × 75 mm Pyrex absorption tube had been joined, at a predetermined position and angle, forming a vessel similar, on a small scale, to the culture flask described by SHERMAN AND GRANT[8]. The modified Warburg vessels, charged in duplicate as sterilely as possible, were mounted on Warburg manometers, using a silicone sealant. The contents of each vessel were then tipped down into its absorption tube and the initial optical density read in a Coleman Junior Spectrophotometer at 4200 A, using as a standard a matched absorption tube containing medium alone. The manometers were placed on the shaking device of a water bath at 30° C and the vessels flushed for 10 minutes, with shaking,

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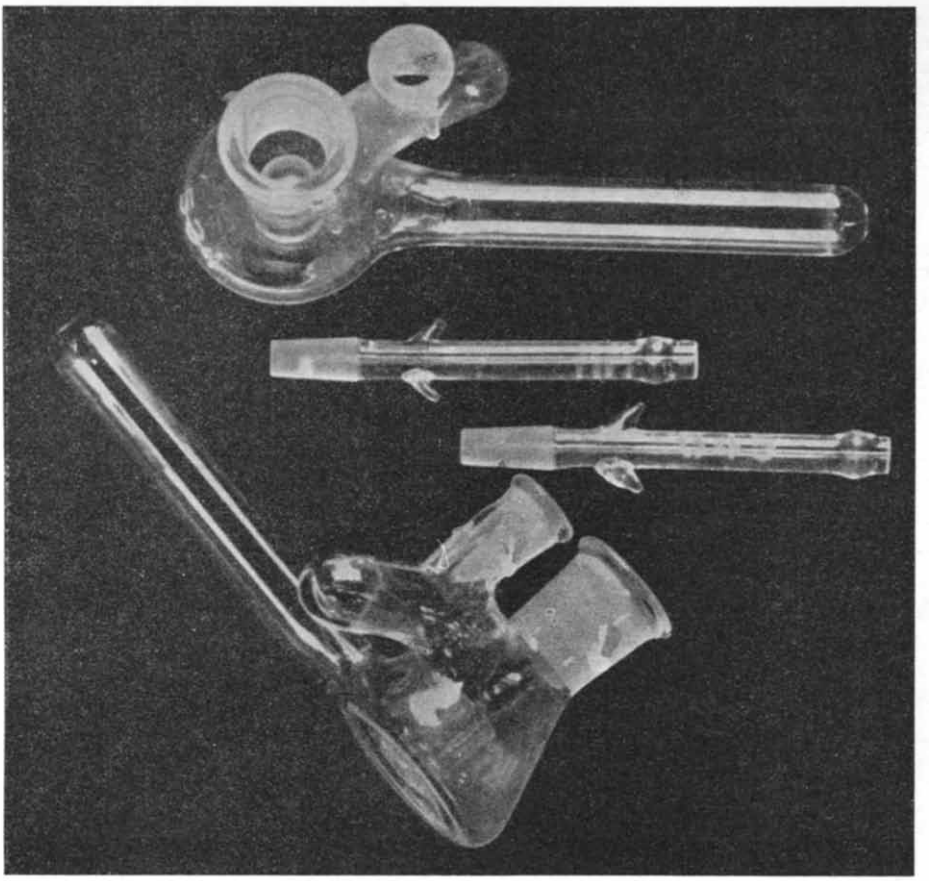

Fig. 3. Modified Warburg reaction vessels used for fermentation and growth studies of normal and irradiated yeast. Total volume of culture = 4 ml.

using a gas mixture containing 95 % N_2 and 5 % CO_2. A 60-minute determination of CO_2 production was then carried out, with readings at 10-minute intervals.

At the close of the determination, the cocks were opened, the vessels removed from the water bath and dried, and a second reading of optical density performed. It should be understood that all optical density readings were made without removing the vessels from the manometers, by tipping the entire assembly so that the contents of the vessel ran down into the absorption tube. The vessels were returned to the water bath for incubation, with shaking, for a period of 7 to 8 hours. Occasionally, experiments were continued for 10 hours; it was thought inadvisable to incubate for longer periods, as sterile conditions could not be insured during the centrifugation of the yeast suspensions and the setting up of the Warburg vessels. However, during this series of experiments no evidence of contamination of the cultures has been noted. During incubation, optical density readings of the cultures were made at approximately 1–1.5 hour intervals.

In the manner just described, it was possible to determine fermentation rate during the first hour, and growth behavior during the first 8 hours after irradiation, of the same samples of yeast in comparison with their controls.

In most of the experiments, 0.5 ml aliquots of each of the control and irradiated samples were removed from the material remaining in the plastic vessels after the Warburg vessels had been inoculated. These aliquots were diluted 1:8 with a 1:10,000 solution of eosin-Y in $M/15$ KH_2PO_4. Hemacytometer counts (26 squares for each of 2 preparations from each sample, at 430 ×) of stained and unstained cells were made to determine the proportion of dead (stained) cells in each of the samples (see PATT *et al.*[10]). Numbers of dead cells in each were expressed as percentages of the total numbers of cells counted. Figures for irradiated samples were corrected by subtracting from them

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the uniformly small percentages of dead cells found in the control samples. Using an ocular micrometer at a magnification of 430×, the dimensions of stained and unstained cells were measured. During these microscopical operations, the samples were searched for evidences of the physical destruction of cells by irradiation.

Where statistical comparisons of results were made, values for t were calculated using the small sample grouped method ("Student"):

$$t = \frac{(\bar{v}_1 - \bar{v}_2)\sqrt{n_1 + n_2 - 2}}{\sqrt{n_1\sigma_1^2 + n_2\sigma_2^2}} \sqrt{\frac{\overline{n_1 n_2}}{n_1 + n_2}}$$

where $\bar{v}_1$ = mean$_1$; $\bar{v}_2$ = mean$_2$; σ_1 = standard deviation of $\bar{v}_1$; σ_2 = standard deviation of $\bar{v}_2$ In computing values for P, $(n_1 + n_2 - 2)$ degrees of freedom were used.

RESULTS

Effects on fermentation rate

The effect of both continuous-wave (CW) and pulsed radiation under the conditions of these experiments was a marked reduction in the rate of anaerobic CO_2 production during a one-hour period shortly after irradiation. This is true, however, only of irradiated cells from "younger" cultures, exposed shortly after their entry into the log phase of growth; it is not true of older cultures. Using "young" cells, the magnitude of the reduction varied with the mode of application of the ultrasonic energy.

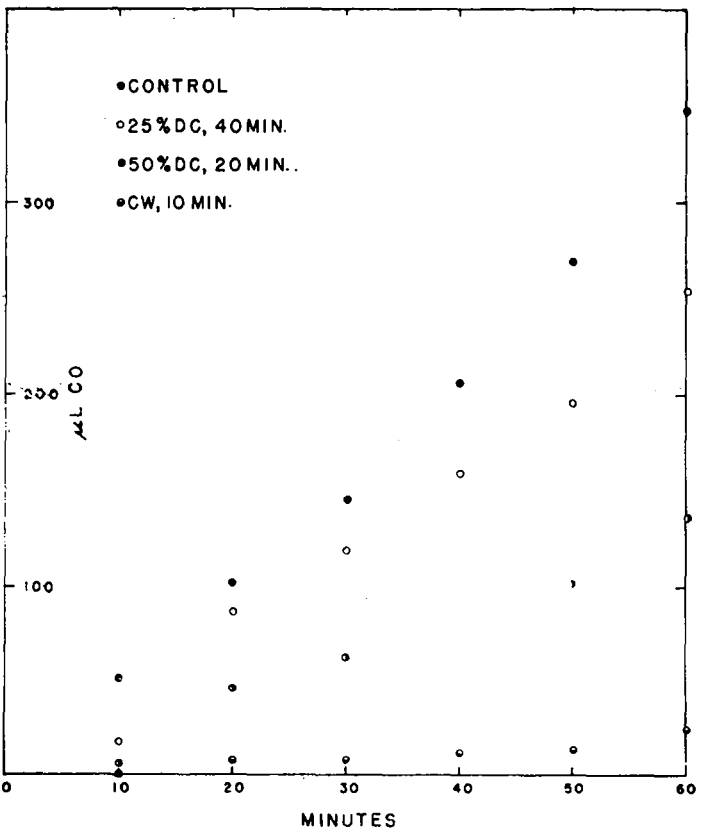

Fig. 4. Fermentation rates of normal and irradiated "young" yeast, Experiment 1. Abbreviations: DC = Duty Cycle; CW = Continuous Wave.

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Fig. 4 presents the results of Experiment 1, in which samples from the same culture were exposed as indicated. The effect of 10 minutes' exposure to CW radiation was a 93% reduction of fermentation rate; 20 minutes of 50% DC pulsed radiation reduced the fermentation rate 60%; 40 minutes of 25% DC pulsed radiation reduced it only 27%. Thus, with the same average power in all cases, a short exposure to CW radiation caused much more severe inhibition than longer exposures to pulsed radiation.

Fig. 5 illustrates the effect on fermentation rate of 20 minutes' exposure to 50% DC pulsed radiation in another experiment (#4). In this case the reduction in fermentation rate was 55% of the rate of the control sample; CW and 25% DC pulsed radiation were not used. The difference between the fermentation rates of the controls in experiments 1 and 4 is not a reflection of differences between the yeast samples, but resulted from reduction in the amplitude of oscillation of the shaker, for mechanical reasons.

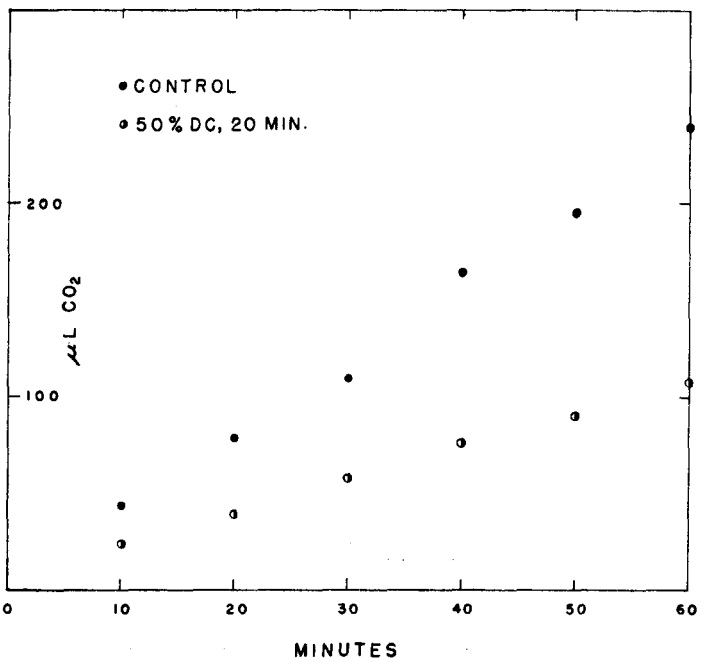

Fig. 5. Fermentation rates of normal and irradiated "young" yeast, Experiment 4. DC = Duty Cycle.

The results shown in Figs. 4 and 5 are representative of those obtained in 7 similar experiments on yeast from "young" cultures. Table I summarizes the results of these experiments, comparing the per cent inhibition of CO_2 production rate caused by exposure to 50% DC pulsed radiation and to CW radiation for the indicated times. Although between different experiments there is variation in the effectiveness of both kinds of radiation, and although the number of experiments using CW radiation was small, the differences in effect are significant.

Table II shows comparisons between the per cent inhibition of CO_2 production and the percentage of cells from samples of the same cultures found, using the eosin-staining technique, to have been killed by irradiation. In the majority of cases a marked correspondence between these values is evident.

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TABLE I

COMPARISON OF PER CENT. INHIBITION IN RATE OF CO_2 PRODUCTION CAUSED BY PULSED AND CW IRRADIATION

Exp. #	*Pulsed, 50% DC, 20 minutes*	*CW, 10 minutes*
1	60 %	93 %
2	31 %	72 %
3	45 %	82 %
4	55 %	—
5	29 %	60 %
6	44 %	—
7	20 %	—
Mean	40 %	77 %
Standard deviation	14.5	13.9

Significance of difference: $t = 3.64$; $P = 0.0058$

TABLE II

COMPARISON OF PER CENT. INHIBITION IN RATE OF CO_2 PRODUCTION AND PER CENT. DEAD CELLS IN SAMPLE AT BEGINNING OF CO_2 DETERMINATION

Exp. #	*Pulsed, 50% DC, 20 minutes*	*CW, 10 minutes*
7	I 20 %	—
	D 22 %	
5	I 29 %	I 60 %
	D 28 %	D 47 %
2	I 31 %	I 72 %
	D 37 %	D 68 %
6	I 44 %	—
	D 42 %	
3	I 45 %	I 82 %
	D 55 %	D 72 %
4	I 55 %	—
	D 52 %	

I – % inhibition
D – % dead cells

Without presenting figures for comparison, irradiation of suspensions from older cultures, which had attained an optical density of 0.3 (about $3.3 \cdot 10^7$ cells/ml), produced much smaller effects than irradiation of the younger cultures. In 5 experiments out of the 6 performed, in which irradiation was identical with that given younger cultures, the rates of fermentation of irradiated cells were not different from those of the control samples.

Effects on growth

For estimation of the effects of irradiation on growth, semi-logarithmic plots of values for optical density against time were prepared. Figs. 6 and 7 show the data from experiments 1 and 4 treated in this manner. Each point represents the mean of duplicate cultures. From these figures it is clear that the 8- to 10-hour incubation period was insufficient to permit maximum growth of the cultures; therefore, no conclusions may be drawn as to the effects of irradiation on total growth.

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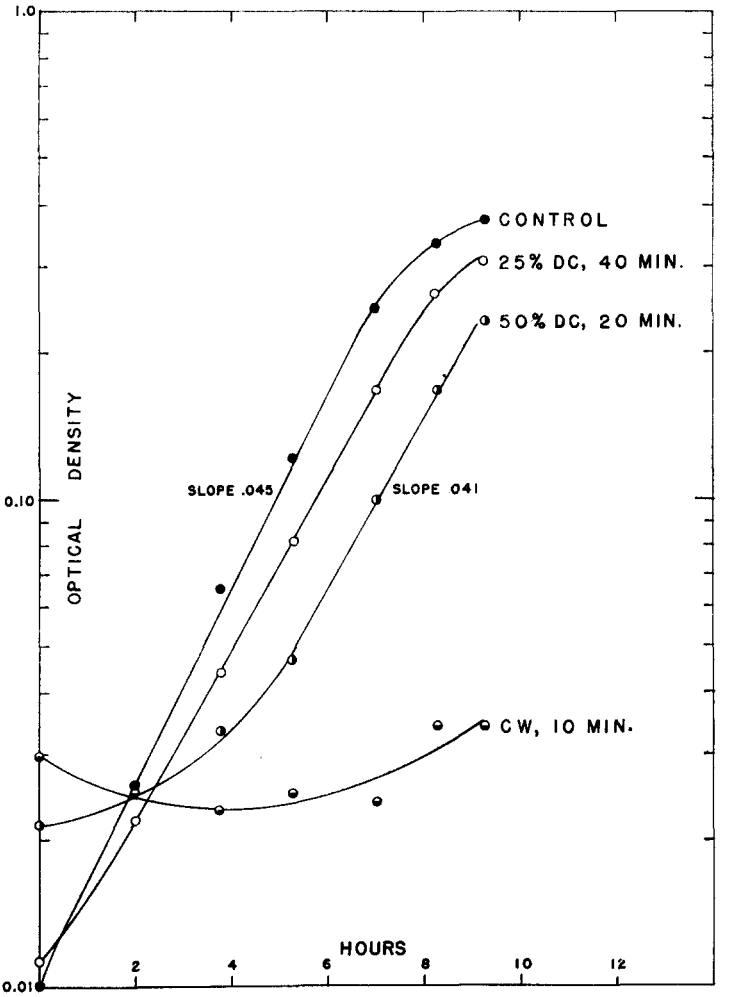

Fig. 6. Growth curves of normal and irradiated "young" yeast, Experiment 1. Fermentation data shown in Fig. 4 were obtained during the 0–2 hour growth period of these cultures. Abbreviations as in Fig. 4.

The irradiated cultures show a prolonged lag phase in comparison with the controls. As a consequence, the optical densities of irradiated cultures soon fall below those of the controls and remain in this relationship throughout the incubation period.

A marked peculiarity is the fact that the initial optical densities of cultures inoculated with irradiated cells are greater than those of control cultures, although the samples were identical before irradiation. This anomaly is accounted for by the observation that, while control cells become evenly dispersed throughout the culture, cells from irradiated suspensions form large, flocculent clumps when introduced into the culture medium following irradiation. The masses of irradiated cells cause artificially high initial optical density readings. In cultures whose cells had been, from other evidence (per cent. inhibition of fermentation rate, percentage of cells staining with eosin), most severely affected by irradiation, the initial high density reading is followed

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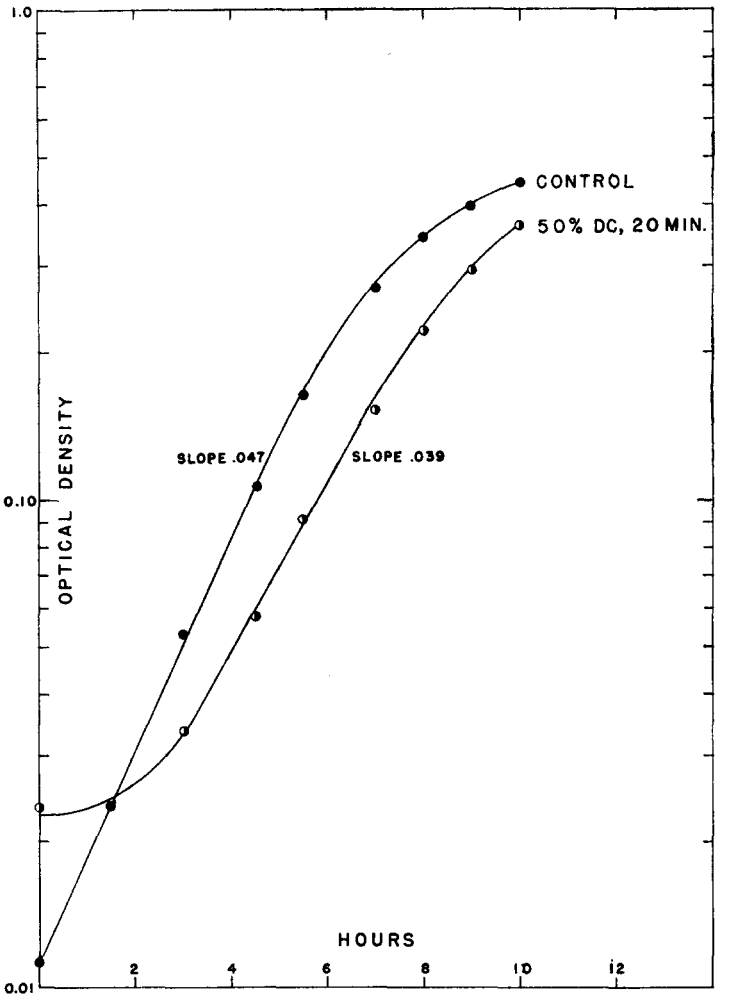

Fig. 7. Growth curves of normal and irradiated "young" yeast, Experiment 4. Fermentation data shown in Fig. 5 were obtained during the 0–2 hour growth period of these cultures. Abbreviation as in Fig. 5.

by a period of declining density. This temporary increase in per cent. transmission may be accounted for by assuming either that large clumps are breaking up, or that lysis of heavily-damaged cells is occurring. The clumping of cells is the only phenomenon observed in these experiments which, without eosin staining, gives any visible indication of damage to the cells.

At any point in its growth cycle, the relative growth rate of a culture is determined by its increase in mass, or in numbers of cells, during a given time interval. During the period in the growth cycles of interest here, a linear relationship exists between cell numbers and optical density; therefore, the increase in optical density of a culture may be used to determine its relative growth rate. The growth curves on a semilogarithmic plot are straight lines during the logarithmic phase of growth, throughout which the rate of increase is constant. Relative growth rates were calculated for all cultures,

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in the series of 7 experiments on yeast from "young" cultures, by selecting a standard portion of the logarithmic scale of optical densities lying within the log phase of all cultures (0.06 to 0.15). The increase represented (0.09), divided by the time in hours required for the culture to realize this amount of growth, yields the slope of the growth curve during this period, or the relative growth rate.

In Table III are listed the slopes of the growth curves of control and irradiated cultures, determined in this manner. For the difference between the mean slope of all control cultures, and the mean slope of all cultures from cells irradiated 20 minutes with 50% DC pulsed ultrasound, the P value indicates a high degree of significance. The small number of cultures from cells irradiated 10 minutes with CW ultrasound makes it less certain that their slopes are different from those of the controls.

TABLE III

COMPARISON OF RELATIVE GROWTH RATES (SLOPES OF GROWTH CURVES DURING LOG PHASE) OF CONTROL AND IRRADIATED CULTURES ("YOUNG" CULTURES)

Exp. #	*Slope, control*	*Slope, irradiated 50% DC 20 minutes*	*Slope, irradiated CW 10 minutes*
1	0.045	0.041	—
2	0.043	0.041	0.037
3	0.045	0.039	0.045
4	0.047	0.039	—
5	0.045	0.041	0.036
6	0.045	0.039	—
7	0.045	0.041	—
Mean	0.045	0.040	0.039
Standard deviation	0.00115	0.00108	0.00495
Significance of differences		Control and 50% $t = 7.76$ $P =$ less than 0.0001	Control and CW $t = 2.7$ $P = 0.027$

In summary, the effects on growth produced by ultrasonic irradiation of cells from "young" cultures are a prolongation of the lag phase and a depression of the relative growth rate during the log phase of growth. The effects on the prolongation of the lag phase are greatest with 10 minutes' irradiation with CW ultrasound, less with 20 minutes' irradiation with 50% DC pulsed ultrasound, and (so far as can be concluded from the present data) least with 40 minutes' irradiation with 25% DC pulsed ultrasound.

Fig. 8 is representative of the results obtained in a series of experiments in which cells from "older" cultures were irradiated in the same way. It is evident that the effects on the growth of these cells are so slight as to make it impossible to differentiate between the control cultures and the cultures from irradiated cells. Table IV lists the relative growth rates of control and irradiated cultures from this series. The sample is admittedly small for statistical comparisons; the slight apparent differences between the mean slope of the controls and the mean slopes of the two irradiated series are not significant. The results of hemacytometer counts of stained cells in these experiments also show no differences between control and irradiated cultures in the numbers of cells staining with eosin.

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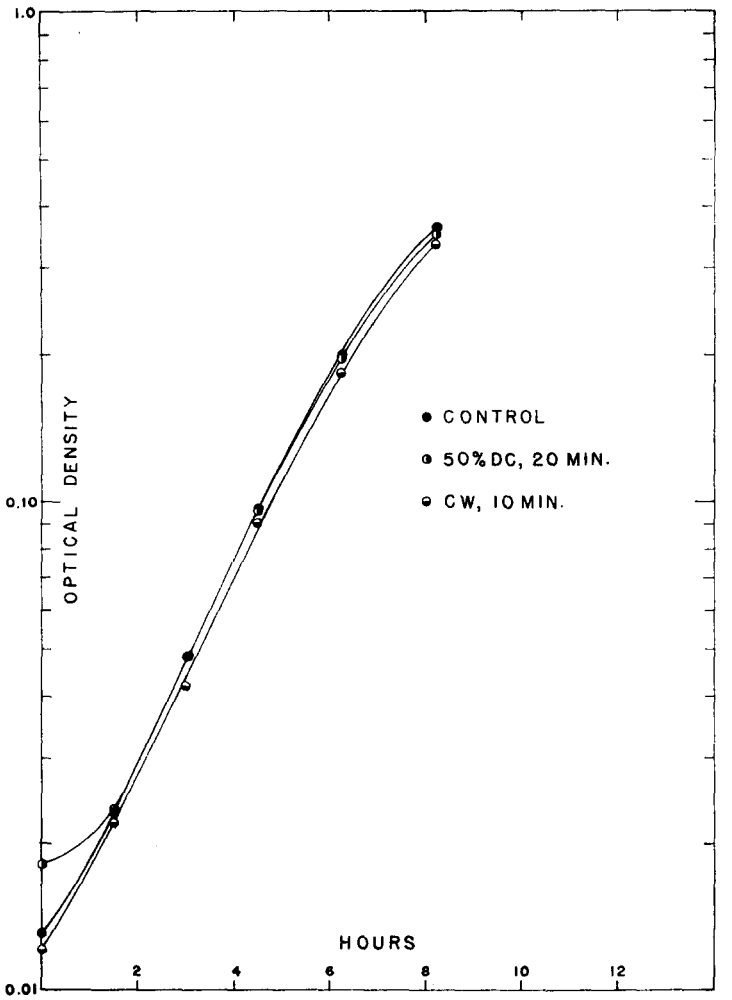

Fig. 8. Growth curves of normal and irradiated yeast from older culture. Abbreviations as in Fig. 4.

TABLE IV

COMPARISON OF RELATIVE GROWTH RATES (SLOPES OF GROWTH CURVES DURING LOG PHASE) OF CONTROL AND IRRADIATED CULTURES ("OLDER" CULTURES)

Exp. #	*Slope, control*	*Slope, irradiated, 50% DC 20 minutes*	*Slope, irradiated, CW 10 minutes*
I	0.056	0.053	0.056
II	0.047	0.047	0.045
III	0.050	0.047	0.047
IV	0.039	0.036	0.033
Mean	0.048	0.046	0.045
Standard deviation	0.0071	0.0071	0.0095
Significance of differences		Control and 50% $t = 0.283$ $P = 0.78$	Control and CW $t = 0.355$ $P = 0.73$

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DISCUSSION

Effects on fermentation

It is clear from the results presented above that exposure of yeast to ultrasonic radiation under the conditions of these experiments does not stimulate fermentation (*cf.* KATSNELSON AND KENOKH[5]).

VON EULER AND SKARZYNSKI[4] showed that 60 minutes' irradiation of fresh yeast at 800 kc, 3 watts, brought about a 44% reduction in fermentation rate. Since 40 minutes' exposure caused an almost complete loss of growth capacity, the conclusion was that the effect of radiation in depressing the fermentation rate was not a specific inhibition of the zymase system, but reflected generalized damage to the cells indicated by their loss of growth capacity as well.

The close agreement between the figures for per cent. inhibition of fermentation rate and for per cent. of cells killed by irradiation justifies a similar conclusion from the present experiments. Effects on fermentation rate evidently cannot be considered apart from effects on viability. The results suggest that if a cell is not killed by irradiation, its capacity to ferment glucose is not significantly altered.

Effects on growth

Prolongation of the lag phase in cultures inoculated from irradiated suspensions is the result to be expected if exposure to ultrasound merely kills a certain proportion of the cells and thus reduces the effective inoculum. However, it is doubtful that this reduction of inoculum, alone, would also produce the significant depression of growth rates noted. RICHARDS[11] reported that the only changes noted in the growth curves of yeast cultures prepared with reduced inocula consisted of prolongation of the lag phase, delayed entry into the log phase, and extension of the log phase until the final populations were equal. CLARK[12] showed that the growth rates of a series of yeast cultures remained the same, even though the amount of seed yeast in a constant volume of inoculum was varied from 5 cells/ml to $8 \cdot 10^6$ cells/ml. In the present experiments on "young" cultures irradiated with 50% DC pulsed ultrasound, the maximum reduction of effective inoculum (indicated by the stained-cell counts) amounted to about 55%, or a reduction from $3.3 \cdot 10^7$ to about $1.5 \cdot 10^7$ cells/ml. Evidently ultrasonic radiation causes some unfavourable condition in addition to the death of a certain proportion of the cells in the inoculum, resulting in a retardation of the growth rates of surviving cells.

In speculative terms, three possibilities seem to present themselves. It is conceivable that irradiation brings about conditions unfavourable for growth either indirectly, by producing a toxic substance in the suspending medium, or directly, by causing sublethal damage to surviving cells. Alternatively, the lysis of large numbers of cells heavily damaged by irradiation might release into the medium toxic factors in quantities sufficient to depress the growth rates of surviving cells.

VIRTANEN AND ELLFOLK[13] demonstrated the formation of appreciable quantities of oxidation products (peroxide, nitrites, nitrates) in aqueous media irradiated with ultrasound at 300 kc, intensity about 10 W/cm^2. It is possible that similar reactions occur during irradiation at higher frequencies. The efficacy of any such mechanism in affecting growth in the present experiments would be reduced by the fact that the buffer solution exposed to radiation was diluted 3:1 in the final culture medium.

Direct effects on surviving cells could take many forms; any change in the intra-

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cellular systems involved in protein synthesis, for example, would interfere with growth as well as with reproductive activities. No direct evidence is available, however, to indicate the real nature of any such possible alteration.

The other possibility, that of release of toxic substances by injured cells, remains to be demonstrated. It is significant that no evidence of the production of proliferation-promoting substances has been noted (see LOOFBOUROW *et al.*[14]). This may indicate a fundamental difference between the effects of ultrasound and those of ultraviolet radiation on yeast. Possibly the lethal action of ultrasonic radiation brings about death and dissolution too rapidly to permit the production of growth-enhancing factors by injured cells.

Comparative effectiveness of CW and pulsed radiation

With average power maintained at a constant level in all applications, exposure of yeast to CW ultrasonic radiation is always most effective, even at exposures of short duration. The peak power required to produce an average power of ten watts is much greater with pulsed radiation than that necessary to produce the same average power with CW radiation. This is true because the measuring devices indicate, in the pulsed beam, the average power between the peak value in the "on" time and the minimum value in the "off" time. Therefore, peak amplitudes are greater with lower duty cycles, and least with CW radiation. Nevertheless, in pulsed applications the effectiveness of radiation decreases with decrease in duty cycle, even though the exposure times be prolonged. Thus, the effect of radiation is not, as might be conceivable, enhanced by subjecting cells to rapidly repeated bursts of high-amplitude ultrasonic vibration.

Comparisons of the relative effectiveness of pulsed and CW radiation also demonstrate that the severity of the effects on yeast does not depend on the total energy impinging on the irradiated material. With the average power or intensity constant in all cases, the total energy per unit time (averaged over many cycles of pulsed radiation) to which the samples are exposed in different applications remains the same. Therefore, in these experiments, in 20 minutes at 50% duty cycle, the sample receives twice as much energy as in 10 minutes of CW radiation. However, cells irradiated for 10 minutes with CW ultrasound are affected much more severely than those exposed twice as long to 50% DC pulsed ultrasound.

The experimental results indicate that the lighter effects of heavier applications of energy in the pulsed techniques result from the recovery period provided during the "off" time between pulses. At a 25% duty cycle, a burst of high-amplitude vibration strikes the sample every 600th of a second, lasts 1/2400 sec, and is followed by an "off" period lasting 1/800 sec. As the duty cycle increases, the amplitude of the vibrations necessary to produce equivalent average power decreases; the length of the "on" time increases; the length of the available recovery period between pulses decreases; *and the damage increases.* Thus, decreased effectiveness seems to accompany increased recovery-time between pulses.

Mode of action of ultrasound on yeast

According to WULFF, FRY *et al.*[15]: "The propagation of ultrasonic vibrations through living tissues is accompanied by a variety of physical factors such as: 1. heating caused by absorption of acoustic energy; 2. periodic pressure changes; 3. radiation pressure; 4. streaming or flow in viscous media; and, 5. high temperatures and pressures associated

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with cavitation, defined as the formation of holes in liquid media. Any or all of these factors may produce significant and measurable changes in the state of a living system." These factors would seem to apply, with minor modifications, to a system composed of individual cells suspended in a fluid medium.

The results of the present experiments demonstrate that heating, at least in terms of the suspension as a whole, is not a significant factor in causing the effects of ultrasonic radiation on yeast suspensions. Measurements taken with a small, naked thermocouple junction, placed in the center of a yeast suspension and subjected to irradiation in the focal region of the beam, showed an average temperature increase in the neighbourhood of 10° to 15° C. The initial temperature of the suspension was 24.5° C; therefore, the equilibrium temperature under irradiation hardly exceeded a maximum of 40° C, which is well below the thermal death point of yeast.

The non-critical nature of increased equilibrium temperatures in these experiments is further indicated by the fact that the yeast suspensions subjected to 50% DC pulsed radiation were exposed twice as long as those irradiated with CW ultrasound. With the average power maintained at 10 watts, changes in the duty cycle of the radiation do not appreciably affect the equilibrium temperature in the suspension. If exposure to elevated temperature were a significant factor, then the pulsed radiation, acting over 20 minutes, should have been more effective than the CW application acting over 10 minutes; actually, the opposite result was obtained.

These considerations do not elucidate the possible role of intrinsic heat-absorption of the cells themselves, in relation to the recovery periods afforded by pulsed ultrasound. Estimation of the remaining factors listed above as contributing to ultrasound damage to yeast must await further research.

Relative susceptibility of "young" and "older" cultures

As described above, the present experiments show that suspensions of cells from yeast cultures harvested late in the log phase are more resistant to damage by ultrasound than cells harvested early in the log phase. The older cultures, although still in the phase of their growth cycle characterized by a constant rate of increase, are undoubtedly of greater physiological age. They are approaching the point at which reproduction progressively slows, and where exhaustion of nutrients and accumulation of metabolic wastes impose limits to growth. Thus, nearing this point, their over-all rate of metabolism is probably lower than that of the younger cultures which have just entered the log phase. Unfavourable conditions, including those postulated as effects of ultrasonic irradiation, would be expected to affect rapidly metabolizing cells more severely than cells at a lower level of activity.

Data from micrometric measurements of cells indicate that this is a promising avenue of approach. Measurements showed that in all cases dead cells, stained with eosin after irradiation, were considerably smaller than unstained cells. Their volumes, calculated from the two-dimensional measurements using the formula for a prolate spheroid, were on the average about 30% of the mean volume of unstained cells. These stained cells, as mentioned earlier, were most numerous in heavily damaged irradiated cultures. It appears possible that young cultures might differ from older cultures in the proportion of young, small cells to older, larger cells. If this is true, and if ultrasonic radiation selectively kills small, young cells, an explanation might be at hand for the different susceptibilities of young and old cultures. However, it remains to be established

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whether the small, stained cells in irradiated cultures were selectively killed because of their small size and presumed youth and vigorous metabolism. The possibility remains that lethal effects are random, and that killed cells subsequently shrink to a volume smaller than that of undamaged, unstained cells. Additional experimentation is needed to clarify these points; further speculation at this time is unwarranted.

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SUMMARY

Samples from young and older cultures of *S. cerevisiae*, Strain 15.56, suspended to standard optical density in buffer solution, were exposed to CW or pulsed ultrasonic radiation for different periods. Frequency was 1 Mc; a plastic lens focussed the beam into a region in which average intensity, over an area of 0.78 cm^2, was about 10.5 W/cm^2. Pulsed radiation, repetition-rate 600/sec, was applied in 25% and 50% duty cycles. Effects were estimated by determination of fermentation rate and growth behaviour, in special flasks.

Ten minutes' irradiation of young cells with CW ultrasound produced greater effects than longer exposure to pulsed radiation. Fermentation was markedly inhibited; the lag phase of cultures from irradiated cells was prolonged, and the relative growth rate depressed. Counts of killed cells (stained with eosin) showed that inhibition of fermentation could be accounted for on the basis of population reduction. This decrease in effective inoculum explains also the prolonged lag phase, but not the depressed growth rates. Growth inhibition may be caused by direct, sublethal damage to surviving cells, or by the production of toxic substances in the medium during irradiation. Cells from older cultures were essentially unaffected by radiation.

Heating of the suspension is not the factor causing damage; other physical effects were not evaluated. The ineffectiveness of pulsed radiation is attributed to the provision of recovery periods between pulses.

RÉSUMÉ

Des échantillons de cultures jeunes ou vieilles de *S. cerevisiae* souche 15.56, sous forme de suspensions de densité optique standard dans des solutions tampons, sont exposées à un rayonnement ultrasonique continu ou discontinu pendant des temps variables. La fréquence est de 1 Mc; une lentille de matière plastique concentre le faisceau dans une région où l'intensité moyenne, sur une aire de 0.78 cm^2, est d'environ 10.5 W/cm^2. Les radiations discontinues ont une période de 1/600. L'irradiation dure 25 ou 75% de la période. La vitesse de fermentation et le comportement en cours de croissance, dans des flacons spéciaux, servent à apprécier les résultats obtenus. L'irradiation de cellules jeunes pendant 10 min, avec des ultra-sons continus, a des effets plus importants que l'exposition prolongée à des radiations discontinues. La fermentation est notablement inhibée; le temps de latence de cultures de cellules irradiées est prolongé, et la vitesse relative de croissance diminuée. La numération des cellules tuées (colorées à l'éosine) montre que l'inhibition de la fermentation s'explique par la réduction de la population. Cette diminution de l'inoculum effectif explique aussi le prolongement de la phase de latence, mais non la diminution de la vitesse de croissance. L'inhibition de la croissance peut être le résultat d'une lésion directe, subléthale, des cellules survivantes, ou de la production de substances toxiques dans le milieu au cours de l'irradiation.

L'élévation de température de la suspension n'est pas responsable des lésions; les autres effets physiques n'ont pas été déterminés. L'inefficacité des radiations discontinues peut être attribuée à l'existence de périodes de repos entre les irradiations.

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ZUSAMMENFASSUNG

Proben junger und alter Kulturen von *S. cerevisiae*, Stamm 15.56, welche in Pufferlösungen zu normaler optischer Dichte suspendiert waren, wurden während verschieden langer Perioden kontinuierlichen Ultraschallwellen oder Ultraschallimpulsen ausgesetzt. Die Frequenz war 1 Mc; eine Polystyrolinese lenkte den Strahl in ein Gebiet, in dem die mittlere Intensität auf einer Fläche von 0.78 cm^2 ungefähr 10.5 W/cm^2 betrug. Ultraschallimpulse mit einer Wiederholungszahel von 600/sec wurden mit Wirkungsbreiten von 25 % und 50 % angewendet. Die Wirkungen wurden durch Bestimmung der Gärgeschwindigkeit und der Wachstumseigenschaften in Spezialflaschen bestimmt.

Eine Bestrahlung junger Zellen während 10 Minuten mit kontinuierlichen Ultraschallwellen rief eine grössere Wirkung hervor als ein längeres Aussetzen an Ultraschallimpulse. Die Gärung wurde bemerkenswert gehemmt, das Anfangsstadium der Hefevermehrung wurde in Kulturen bestrahlter Zellen verlängert und die relative Wachstumsgeschwindigkeit herabgesetzt. Zahlen getöteter und mit Eosin gefärbter Zellen zeigten, dass die Gärungshemmung auf Grund der Populationsreduktion erklärt werden konnte. Dieses Abnehmen wirksamen Inokulums erklärt ebenso das verlängerte Anfangsstadium der Hefevermehrung, aber nicht die herabgesetzte Wachstumsgeschwindigkeit. Die Wachstumshemmung kann durch direkte, sublethale Schädigung der überlebenden Zellen oder durch die Produktion toxischer Substanzen im Medium während der Bestrahlung verursacht werden. Zellen älterer Kulturen bleiben im wesentlichen bei der Bestrahlung unangegriffen.

Das Erhitzen der Suspension ist nicht der Faktor der die Schädigung verursacht; andere physikalische Effekte wurden nicht ausgewertet. Der Tatsache, dass zwischen den einzelnen Ultraschallimpulsen Erholungspausen auftreten, ist die Wirklosigkeit der Bestrahlung mit Ultraschallimpulsen zu zuschreiben.

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