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The use of ultrasound in food technology I: inactivation of peroxidase by thermosonication

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Abstract

The combined effect of high power ultrasound and temperature on the activity of peroxidase type VI from horseradish suspended in water was studied. The tests were performed at 80°C using power ultrasounds having frequencies of 20, 40, and 60 kHz. Accordingly, the actual ultrasonic powers varied in the range from 0 to 120 W. Combined treatments were carried out by using a laboratory scale plant operating in continuous and in batch mode. In continuous experiments, 46 ml of suspension was circulated in the plant having a sanitation chamber of 20 ml. In the other case, two sets of experiments were carried out by using a sanitation chamber of 100 ml treating 40 or 80 ml of suspensions, respectively. It was found that the decimal reduction time of peroxidase at 80°C, D_{80}, reduces from 65 to 10 min ca. when ultrasounds are applied. In particular, D_{80} varies with ultrasonic power, sonotrode geometry and volume of suspensions submitted to the treatment. It has been demonstrated that the influence of these variables on the decimal reduction time can be grouped by considering the ultrasound power density, i.e. the ultrasound power per unit area of tip of the probe and unit volume of suspension.

Nomenclature

a	Exponential constant of Eq. (2) which describes the dependence of D_{80} on the ultrasound power, P
a_d	Exponential constant of the decay equation which describes the dependence of D_{80} on the ultrasound power density, P_d
a_i	Exponential constant of Eq. (3) which describes the dependence of D_{80} on the ultrasound power intensity, P_i
c_p	Heat capacity
D_{80}	Decimal reduction time of peroxidase inactivation at 80°C
$D_{80}(P)$	Decimal reduction time of peroxidase inactivation at 80°C as function of ultrasound power P
$D_{80}(P_i)$	Decimal reduction time of peroxidase inactivation at 80°C as function of ultrasound power intensity P_i
$D_{80}(0)$	Decimal reduction time of peroxidase inactivation at 80°C without sonication (corresponding to D_{80})
$D_{80}(\infty)$	Asymptotic value of the D_{80} decay curves
M	Mass of the sonicated solution
P	Ultrasound power
P_d	Ultrasonic density, i.e. ultrasound power P per unit of area of the horn tip and per unit of volume of sonication
P_i	Ultrasound power intensity, i.e. ultrasound power per unit of area of the horn tip

1. Introduction

The application of ultrasonic waves generating cavitation in suspensions which contain microorganisms and enzymes often has a lethal result and deactivating action (Suslik, 1988). When high power ultrasound propagates into a liquid the microbubbles, which are commonly present in it or that may form from the presence of suspended particles, will oscillate according to the pressure wave. High acoustic pressure will determine their growth and violent collapse, which is accompanied by a sudden increase of the temperature and the pressure in the surrounding area (El'piner, 1964). Many authors have reported that these phenomena are responsible for dispersion of clumps of microorganisms, modification of the cellular activity, puncturing of the cell wall and increasing sensitivity to heat. However the lethal effect of ultrasound is not the same for all microorganisms. Generally the ultrasound treatment are not much effective on small and round cells (Allinger, 1975), for example, Gram-positive bacteria, such as *Staphylococcus aureus* and enterococci (Ordonez, Sanz, Hernandez & Loper-Lorenzo, 1984) are quite resistant and even more resistant are bacterial spores (Ahmed & Russell, 1975; Boucher & Lechowich, 1979). The combined effect of ultrasonic waves and heat treatment applied

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simultaneously appears instead more effective (Ciccolini, Taillandier, Wilhem, Delmas & Strehaiano, 1997) and even more are treatments which use the combination of heat and ultrasound under pressure (Raso, Condon & Sala Trepat, 1994). The same combination of effects has been demonstrated to have a quite effective deactivating action on various enzymes of interest in food technology (Lòpez et al., 1994; Lòpez & Burgos, 1995a).

Although the possibility of deactivating enzymes or destroying microorganisms by ultrasound waves, alone or in combination with other physical treatments, has been widely used for laboratory applications in microbiology, immunology and enzymology, the same is not true for industrial applications. The reasons of the non-development on an industrial scale of this technique are numerous and in part the non-development is due to the lack of information needed for design and scale-up procedure (Mason, Lorimer & Bates, 1992). Most results reported in the scientific literature, in fact, relate deactivating and destructive action of ultrasound only to their frequency and fail to provide information about the dependence of the treatment efficiency on the actual power and power density of ultrasound. In addition, although in principle no difference is noticed on the efficiency of ultrasound in batch or in continuous applications, no definite experimental evidence has been reported.

In this work, the attention was focused on determining scale-up parameters for design application of treatment which use ultrasound in combination with conventional heat treatments. It is part of a wider research program whose aim is to verify the possibility of improving the efficiency of selected unit operations used in Food Technology by applying ultrasound in combination with traditional physical treatment.

2. Experimental procedures

Materials. Peroxidase type VI from horseradish was purchased from Sigma (St. Louis, MO). Other chemicals used were of reagent grade.

Enzyme assay. Peroxidase activity was determined by monitoring the decomposition of hydrogen peroxide by peroxidase and o-dianisidine as a hydrogen donor. The rate of color development was measured at 460 nm. Spectrophotometric measurements were made in a spectrophotometer (UV 2100, Shimadzu, Japan) equipped with a thermostated cell holder block.

Reaction medium. The reaction medium consisted of 0.1 M phosphate buffer, pH 6.5, containing 0.01% o-dianisidine, 0.005% H_2O_2 and 2% methanol (o-dianisidine was added to the assay medium dissolved in methanol). Enzyme activity was determined at 25°C using 1.99 ml of the above medium and 10 μl of enzyme sample.

Ultrasonic equipment. Four submerged ultrasonic horns (Undatim Ultrasonics S.A., Louven la Neuve, Belgium), having an exponential profile were used. Table 1 summarizes the geometrical parameters of each horn and their relative fixed frequency of operation in the range between 20 and 100 kHz. A generator (Undatim Ultrasonics S.A., Louven la Neuve, Belgium), converting 50 Hz electric energy into the desired frequency was used. The generator was equipped with a wattmeter allowing the power to be changed in the range 0–500 W. The generator was, also, equipped with an electronic device for frequency resonance search capable of maintaining constant power input throughout the entire ultrasonic treatment.

In order to gain information on the actual power input a calorimetric method was used to calibrate the apparatus (De Gennaro, 1996). According to the method proposed by Margulis and Maximenko (1990), the ultrasonic power entering the system was obtained by recording the temperature rise T against the time t, by means of a thermocouple placed in the system itself. During the calibration experiments the treated solution was kept into an adiabatic vessel to avoid thermal leak. The power, P, was calculated from the equation:

$$P = Mc_p(dT/dt), \tag{1}$$

where c_p is the heat capacity of the solution and M the mass of solution used. The sample medium was a phosphate buffer solution, pH 6.5, and in all treatments the samples were kept in a thermostated vessel where the temperature was maintained at 80°C.

Thermosonication treatment. The experimental set up consisted of three units: the frequency generator, the

Table 1
Dimensions of the ultrasonic probes

Nominal frequency (kHz)	Size	Initial diameter D (cm)	Final diameter d (cm)	D/d
20	Short	3	2	1.5
20	Long	3	1.4	2.14
40	Long	1.8	1	1.8
60	Long	1.8	0.8	2.25

probe and the sonication chamber. Two different arrangements were used. In the case of discontinuous treatments 35 or 75 ml of the reaction medium were transferred to a batch reactor having a volume equal to 100 ml, provided with an external water jacket which was connected to a thermostatic bath with external circulation (HAAKE k mod. F4, Karlsrhue, Germany) and an electronic control ±0.1°C. It was allowed to equilibrate to 80°C. Once the test temperature was reached 5 ml of the reaction medium containing the dissolved enzyme were mixed with the medium heated to the preset treatment temperature. After a short transient the temperature reached steady state and aliquots of 100 μl were taken at regular intervals to estimate the decay of the enzyme activity compared to the initial activity. During each experiment, the temperature of the reaction medium was continuously detected by means of a Chromel–Alumel thermocouple placed in the reactor and monitored by means of a digital thermometer (Roline, mod. RO 1310, Taiwan). In the case of continuous treatments 46 ml of a solution containing the enzyme were transferred into a reservoir provided with an external water jacket which was connected to a thermostatic bath with external circulation (Braun mod. Termomix 1420, Melsungen, Germany). By means of a peristaltic pump (Velp Scientifica, mod. sp311, Milan, Italy) the solution was pumped at 46 ml/min to a cup provided with an external water jacked connected to a thermostatic bath with external circulation. A Chromel–Alumel thermocouple was set in the cup in order to monitor continuously the solution temperature during the experiment. By means of another peristaltic pump the solution was transferred from the cup again to the reservoir, passing through a sampling trap. The volume in the cup containing the probe was at any time constant and equal to 20 ml. At regular time intervals (10 min) 100 μl of suspension were removed from the sampling trap and analyzed. The experiments were carried out using different sonotrodes operating at 20, 40 and 60 kHz. The results are the arithmetic mean of at least three independent runs.

Residence time distribution. Residence time distribution of the liquid phase was evaluated using the pulse method. Accordingly, the plant was filled with distilled water (pH = 7.32) and the system was allowed to reach steady conditions in the presence of ultrasound. At a given time, 1.5 ml of concentrated solution of HCl were injected at once into the reservoir of the continuous plant with the subsequent decrease of pH to values close to 2. At regular intervals (5 s) samples of liquid coming out from the sonication chamber were collected and their pH evaluated by means of a digital pH-meter (Radiometer, mod. PHM82 Standard, Copenhagen). Data analysis was performed by using the software package Table-Curve for Windows (Jandel Scientific, San Rafael, CA, USA).

3. Results and discussion

Figs. 1–3 show the decay over time of the residual activity of peroxidase normalized compared to its initial activity in experiments in which different volumes of peroxidase suspensions in water were batch treated. The treatments were performed by applying ultrasound at 20, 40 and 60 kHz, for different times. The temperature and the flow rate of water circulating in the external jacket of the batch reactor were set so that the temperature of the peroxidase suspension remained constant during the experiment. Actually, the temperature varied at ±0.4°C with respect to the average temperature of 80°C. The same is true for experiments performed in continuous mode which were discussed later on. Each curve refers to a given actual ultrasound wave power. Due to the geometrical parameters of the available 60 kHz probe (the horn of this transducer is too short to reach the bottom of the reactor) it was not possible to treat volumes less than 80 ml of suspension and then only data relative to experiments performed using 80 ml are presented. In all the experiments the peroxidase deactivation due to ultrasound treatment follows first order kinetics according to data reported into the literature (Lòpez & Burgos, 1995b).

As in other food treatment deactivation kinetics are compared by using decimal reduction times, i.e. the time required to reduce the concentration of the component under examination to 10% of its initial value, D_{80} for

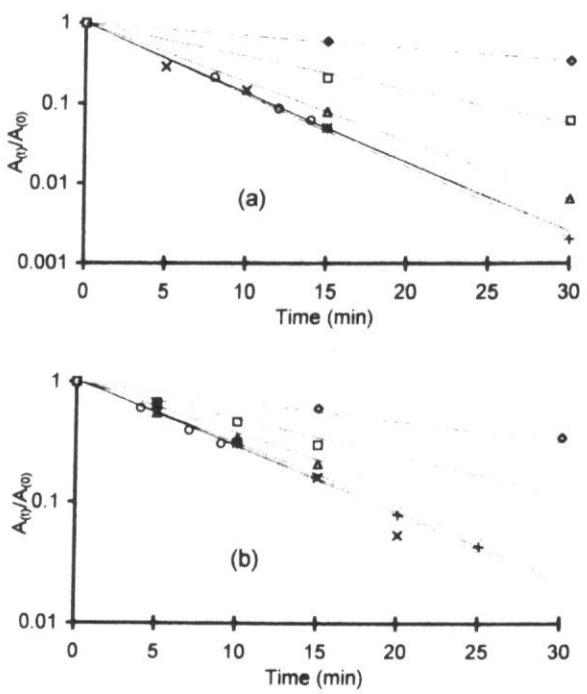

Fig. 1. Normalized residual activity, $A(t)/A(0)$, of peroxidase vs time for treatments performed in batch, (a) = 40 ml and (b) = 80 ml, applying ultrasound at 20 kHz. Power [=] W: (◇) 0; (□) 31.12; (Δ) 58.76; (×) 83.05; (+) 102.32; (○) 116.07.

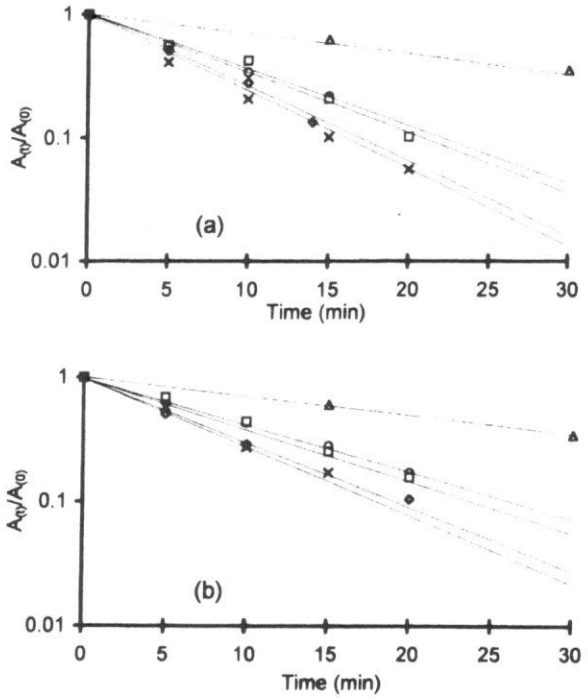

Fig. 2. Normalized residual activity, $A(t)/A(0)$, of peroxidase vs time for treatments performed in batch, (a) = 40 ml and (b) = 80 ml, applying ultrasound at 40 kHz. Power [=] W: (Δ) 0; (O) 16.53; (□) 29.18; (×) 39.01; (◇) 50.12.

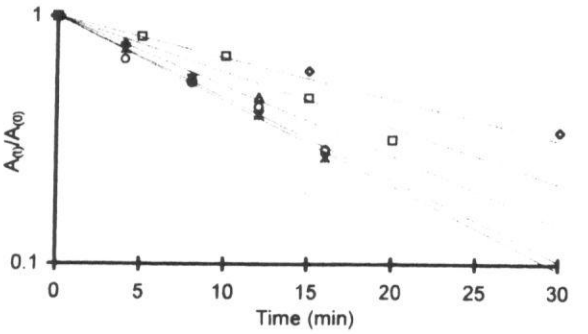

Fig. 3. Normalized residual activity, $A(t)/A(0)$, of peroxidase vs time for treatments performed in batch, 80 ml, applying ultrasound at 60 kHz. Power [=] W: (◇) 0; (□) 16.44; (Δ) 24.16; (O) 30.59; (×) 40.02.

each treatment was evaluated from the slope of the straight-lines of Figs. 1–3. The influence of the actual ultrasound power and volume on D_{80} is illustrated in Fig. 4. With increasing ultrasonic power D_{80} decreases to an asymptotic value. The experimental results clearly indicate that the deactivating efficiency of ultrasound becomes very low with increasing power of the ultrasound waves. This can be explained by considering that when ultrasonic waves with high power are used a sort of cushion of bubbles forms under the tip of the applicator separating it from the sonicated solution (Ratoarinoro, Contamine, Wilhem, Berlan & Delmas, 1995) and consequently the efficiency of the ultrasound diminishes. From a practical point of view this means that in order to improve the efficiency of the treatment the use of very high power ultrasound may be useless.

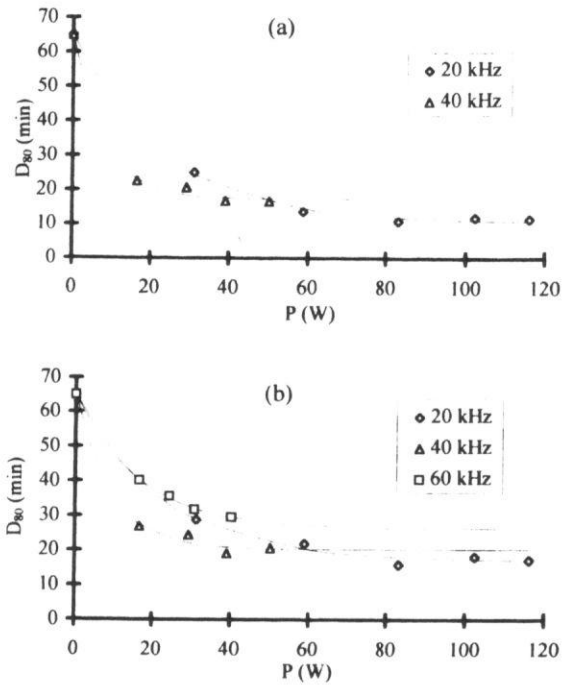

Fig. 4. Decimal reduction time, D_{80}, vs ultrasound wave actual power, P, for combined heat and sonication treatments performed in batch: (a) = 40 ml and (b) = 80 ml. Solid lines reproduce curve drawn according to Eq. (2).

The decrease in D_{80} with varying the ultrasonic wave power, P, can be described by an exponential law:

$$D_{80}(P) = D_{80}(\infty) + [D_{80}(0) - D_{80}(\infty)]\exp(-P/a), \quad (2)$$

where $D_{80}(\infty)$ is the asymptotic value of each curve; $D_{80}(0)$ the decimal reduction time corresponding to experiments performed at 80°C without sonication; a an exponential constant which provides information on the deactivating efficiency of the ultrasound wave: the larger the value of the constant a the faster the decimal reduction time decreases with increasing the actual power. Data correlate quite well with the curve described by Eq. (2).

Table 2 reports the values of the parameter a of Eq. (2) corresponding to the different experimental conditions which have been explored. It can be noticed that the parameter a does not follow any trend with varying

Table 2
Numerical values of the parameter a of Eq. (2)

Volume (ml)	Frequency (kHz)		
	20	40	60
40	22.83	7.93	n.d.
80	22.87	9.18	16.35

the batch volume and the ultrasound frequency. Therefore, this approach does not provide a good basis for design applications since it does not give any information on the influence of volume and frequency on D_{80}.

The previous results indicate that the deactivating capability of ultrasound in conjunction with heat in addition to ultrasound wave power depends on ultrasound frequency and on the amount of solution, which is treated. For practical purposes it would be useful to investigate the possibility of reducing the number of variables which govern the operation in order to facilitate scale-up procedure and plant design. For example, by considering that cavitation happens mainly near the tip of the applicator (Lorimer, 1990), it is possible that the D_{80}, which is a measure of the efficiency of the ultrasonic treatment, correlates rather with the power intensity of the ultrasound wave (the actual power transmitted to the suspension per unit area of the tip of the horn) than with the power. In Fig. 5 D_{80} was plotted versus power intensity. Data exhibits a regular behavior supporting this hypothesis. The variation of the decimal reduction time D_{80} with varying the power intensity is described by an exponential decay curve:

$$D_{80}(P_i) = D_{80}(\infty) + [D_{80}(0) - D_{80}(\infty)]\exp(-P_i/a_i), \quad (3)$$

where P_i is the power intensity given in W/cm^2 and a_i the exponential constant. Eq. (3) can be easily rearranged in the form:

$$\ln \frac{D_{80}(P_i) - D_{80}(\infty)}{D_{80}(0) - D_{80}(\infty)} = -\frac{P_i}{a_i}. \quad (4)$$

showing that $a_i/2.303$ corresponds to the increment of the power intensity which is required for a reduction to 10% of the decimal reduction time of peroxidase during treatments performed at 80°C. By using this type of representation, the influence of power intensity and ultrasound frequency on the decimal reduction time of peroxidase becomes evident. The decimal reduction time decreases exponentially by increasing the power intensity and decreases faster the lower the frequency of the ultrasound.

The influence of ultrasound frequency on the parameter a_i evaluated in the course of combined heat and ultrasonic treatments performed in batch is shown in Fig. 6. Within the range of frequency, which has been explored, a_i increases almost exponentially. Unfortunately, due to the experimental difficulties mentioned before, only two data points are available for the case in which the experiment was carried out using 40 ml of suspension and therefore a direct comparison between the two experimental situations cannot be made. However, the numerical values of a_i relative to the two set of experiments, reported in Table 3, suggest that the difference between the volumes of suspensions in the two set of experiments affects the efficiency of the treatment. The influence of the volume of the sample on the treatment efficiency may be explained by considering that when ultrasound waves of a given power intensity pass through the suspension (Lorimer, 1990) the enhancement of the pressure, and thus cavitation, becomes negligible in the portions of the solution which are located farther from the tip of the probe. In other words, data suggests that the efficiency of the ultrasonic treatment may be affected by the distribution of the ultrasonic wave within the volume of suspension, which is treated.

In order to verify this hypothesis the D_{80} experimental data of Fig. 5 were plotted against power density, P_d, i.e. the actual power per unit of area of the horn tip and per

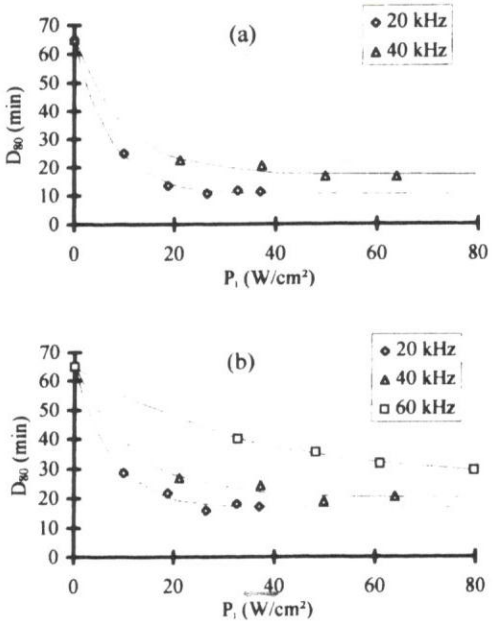

Fig. 5. Decimal reduction time, D_{80}, vs ultrasound wave power intensity, P_i, for combined heat and sonication treatments performed in batch: (a) = 40 ml and (b) = 80 ml. Solid lines reproduce curve drawn according to Eq. (3).

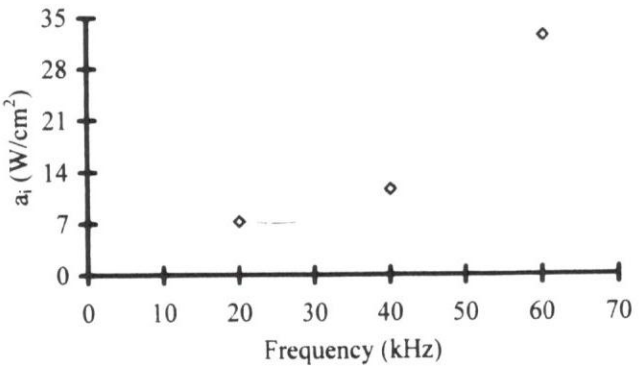

Fig. 6. Exponential constant a_i vs ultrasound wave frequency.

Table 3
Numerical values of the parameter a_i of Eq. (3)

Volume (ml)	Frequency (kHz)		
	20	40	60
40	7.26	10.10	n.d.
80	7.28	11.66	32.50

unit volume of the suspension. This procedure generated three curves each corresponding to a given ultrasound frequency (Fig. 7). Once again an exponential decay equation describes accurately the dependence of the decimal reduction time on a processing parameter that in this case is the power density P_d. The new exponential constant a_d divided by the factor 2.303 allows one to estimate the increment of the power density required for a reduction to 10% of the decimal reduction time of peroxidase during combined heat and ultrasound treatments performed at 80°C.

The influence of ultrasound frequency on the parameter $a_d/2.303$ is shown in Fig. 8. With varying ultrasound frequency, $a_d/2.303$ increases rapidly with increasing power density. If one considers that without the application of ultrasound, i.e. corresponding to 0 kHz, a_d must become equal to infinity, one can conclude that an optimal ultrasound frequency probably exists at which a_d has its minimum value. According to data which have been presented the most efficient conditions for combined heat and ultrasound treatments in batch mode seem to correspond to a frequency of 20 kHz and a power density close to 0.6.

The last experimental effort was made to verify if the conclusions derived so far could be applied also in the case of combined treatments made in continuous mode. Preliminary analysis of the fluid dynamic conditions existing in the laboratory scale continuous plant indicates that the operating conditions of the system are nearly those corresponding to an ideal CSTR (Fig. 9). This allowed us to estimate the residence time of the

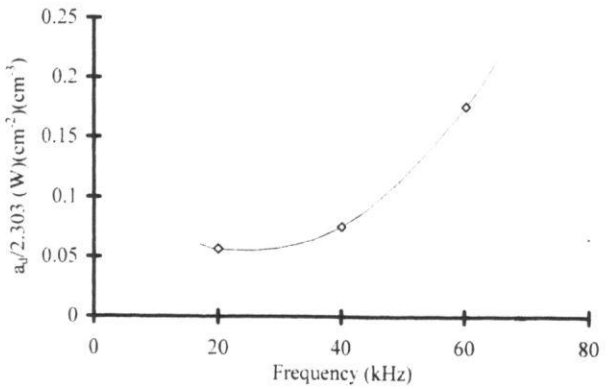

Fig. 8. Exponential constant $a_d/2.303$ vs ultrasound wave frequency.

suspension in the sonication cell simply by dividing the cell volume by the volumetric flow rate of the liquid circulating in the plant, i.e. 20 ml/(46 ml/min). In the experiments, which were performed, the total amount of liquid circulating in the plant was equal to 46 ml, therefore, each minute the liquid present in the plant made a complete circulation. The actual duration of the combined treatment was evaluated by multiplying the mean residence time of the solution in the sonication chamber (≅26 s) by the number of circulations made by the suspensions in the plant in the course of the experiment. Fig. 10 shows the decay over time of the peroxidase activity in experiments made with and without the application of ultrasound. The deactivation effect of ultrasound is quite evident. The value of D_{80} corresponding to combined heat and ultrasonic treatments was estimated from the slope of the straight line of Fig. 10 and gave a value for D_{80} equal to 9 min and 56 s. In Fig. 11 this value was compared with the prediction based on experiments performed in batch mode. The experimental data of D_{80} estimated from continuous combined treatments is quite similar to that which can be predicted by experiments run in batch mode. This confirms that power density of ultrasound may be considered a useful reduced variable to predict the behavior

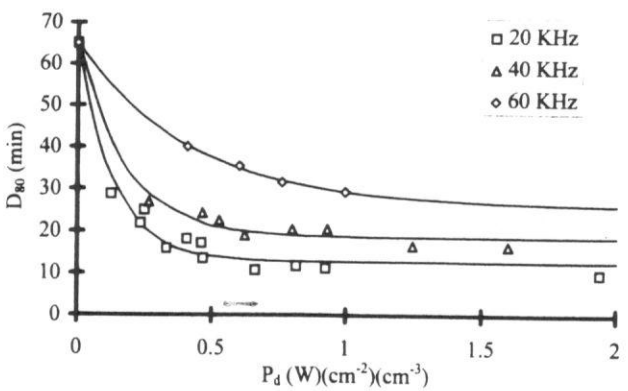

Fig. 7. Decimal reduction time, D_{80}, vs ultrasound wave power density, P_d [=] W/(cm^2)(cm^3).

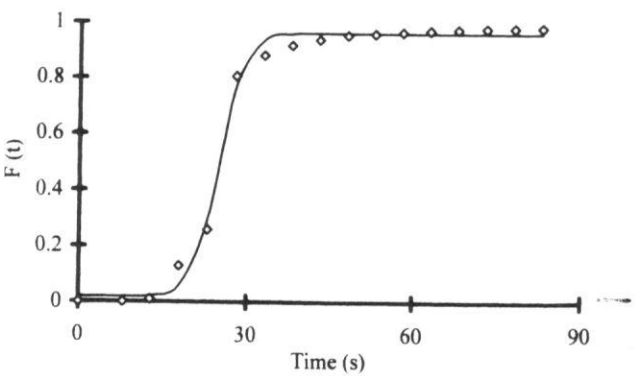

Fig. 9. Cumulative distribution function $F(t)$ vs time relative to the continuous pilot plant.

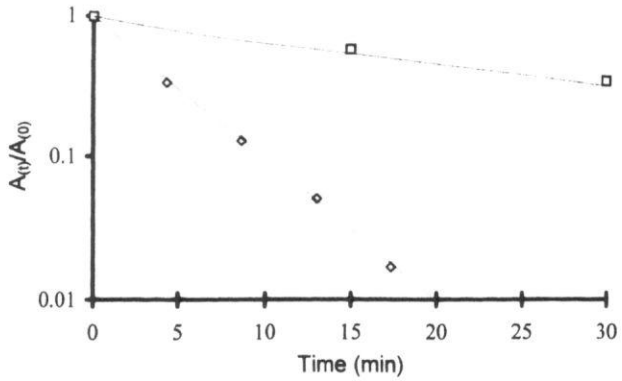

Fig. 10. Normalized residual activity, $A(t)/A(0)$, of peroxidase vs time for treatments performed by continuously by applying ultrasound at 20 kHz. Power = 59.34 W.

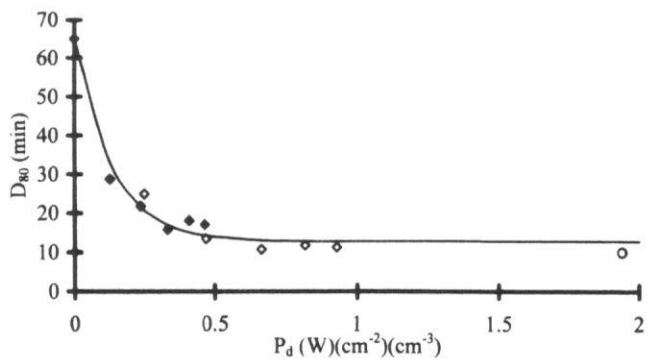

Fig. 11. Comparison between decimal reduction times evaluated during treatments performed in batch (◆) 80 ml, (◇) 40 ml, and in continuous mode (O).

of ultrasound treatments in combination with conventional heat processes.

4. Conclusions

The action of ultrasounds in combination with conventional heat treatment is quite effective in deactivating peroxidase suggesting that this technique has interesting possibilities in food technology. For design purpose it has been demonstrated that the efficiency of the combined treatment can be related to the ultrasound power density, i.e. the ultrasound power per unit area of tip of the probe and unit volume of liquid treated. In addition the experimental evidences suggest that there exists a frequency-power density combination to which corresponds to the maximum efficiency of the treatment and that the deactivation dynamics is the same whether the treatment is performed in batch or in continuous mode.

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