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Increasing the extraction of alpha-amylase from the malted barley by means of ultrasound

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ABSTRACT

In this study, the effects of ultrasound as emerging technology were investigated on the extraction of alpha-amylase in the mashing process. All experiments were conducted at 20 KHz on the ultrasonic generator by considering the three effective factors, temperature (20, 25 and 30°C) and ultrasonic power (13.8,27.5 and 34.5W) corresponding to ultrasonic intensities of (0.0051,0.0101 and 0.0127W/cm2)in different time intervals (10, 15 and 20 min).About determining these effects the Fuwa method assay based on the decreased staining value of blue starch–iodine complexes employed for determining an activity. The results of this assay were analyzed by Qualitek4 software using the Taguchi method to evaluate the factor's effects on enzyme activity. Consequently the results of assays showed that the yield of extraction was increased after thermosonication by comparing to the blank. At the optimum operating condition (20 min at 30 °C and ultrasonic intensity of 0.0127W/cm2 by Na-phosphate buffer (pH=8)) the process yielded 73.25U enzyme activity per ml of used malt (corresponding to extraction yield of 23.10%).

KEYWORDS

Ultrasound- extraction;
Alpha-amylase activity;
Malted barley;
Fuwa method assay;
Taguchi technique.

INTRODUCTION

Amylases are a class of hydrolases widely distributed in microbes, plants and animals. However, the activity of these enzymes is high in germinating cereals. They can specifically cleave the O-glycosidic bonds instarch, a storage polysaccharide present in seeds of various plants[1]. In regard to extensive usages of alpha-amylase in different industries and the commercial importance of diastatic power of malt in brew producing, much effort have been taken for increasing the alpha-amylase activity in the germination process of barley seeds[2-4]. In the malting and brewing industries, considering the importance of the diastatic power is very important for assessment of the activity of starch-degrading enzymes. Thus, application of high temperatures in mashing process is an economically less acceptable approach, and ultrasonication as a pretreatment may be a good technique to be tried along with conventional treatment at low temperatures. The use of ultrasound in the brewing industry is largely limited to the cleaning effects of high frequency waves, ultrasound measurements like particle sizing[5] and measurement of gravity during lautering[6]. Some researchers have demonstrated the utilization of ultrasound to control and reduce foam formation during fermentation[7]. Otherwise the use of power ultrasound, as a part of the brewing process has not been exploited till Buckow and et.al.[8,9] who studied the effects of ultrasound on the extraction and filtration of soluble solids of mash from barley malt.There is no reference about the effects of ultrasound on the extraction of alpha-amylase from grist in the literature up to now.

Recently, ultrasound-assisted extraction has been given more attention, since it has been acknowledged to play a significant role in extraction performance. A number of papers have been found in the literature dealing with the ultrasonically assisted extraction of different materials[10-29].Having high efficiency, low energy, milder conditions together with a shorter extraction time are the main positive effects of such extractions.The most common interaction mechanisms which are involved in this case are acoustically induced cavitational activity which causes heat and chemical effects. The mechanical effects of ultrasound via cavitation bubble collapse is believed to be responsible for these positive results, due to the intensification of mass transfer and easier access of the solvent to the content of cellular materials[30,31]. The release of enzymes and proteins from cells and subcellular particles is a unique and effective application of ultrasound, which causes destruction of cellular structure by a cavitation effect[32].

The present research was undertaken with the aim of enhancing the extraction amount of alpha-amylase from grist. Therefore the effects of ultrasonic power, temperature, and irradiation time on the efficiency of alpha-amylase extraction are investigated. For this purpose, the activity of alpha-amylase was kept as a means of the determination of process yield.

EXPERMENTAL

Material and Equipment

Karon in kavir barley varieties with moisture content of 9% and an average content of protein 11.5% was used in all experiments. To prevent absorption of moisture it was stored in a dry place at 20 ºC until malting. Also it must be mentioned, that for removal of dormancy, samples were stored at room temperature (25–37 ºC) for 3 months after harvest.

All chemicals with high analytical grade including iodine,KI (potassium iodide), KH2PO4 (monobasic phosphate), K2HPO4·3H2O (dibasic phosphate) and soluble starch from potato (S-2630) which used for alpha-amylase assay were obtained from Sigma-Aldrich, Fluka and Merk companies.

The sets of Gerhardt Kjeldathermand Gerhardt Vapodest 30 instrument were used for determination of the protein amount in barley seeds.

Ultrasonic irradiation was performed by means of UP 200 H ultrasonic processor horn type(20 kHz, maximum wave amplitude of 210 μm, and maximum nominal power of 460W) equipped with a radial Sonotrode S3 (3 mm diameter) designed by Hielscher GmbH (Treptow, Germany).

The set of SALD-2101 laser diffraction particle size analyzer (SHIMADZU) was used for analyzing the particle size of sample.

Methods

Planning of Experiments

In this study 3 important effective parameters namely, ultrasonic power, the time of ultrasound irradiation and temperature at 3 levels were selected for the design of experiments. 3 selected factors along with their levels were shown in TABLE 1. In this study, the activity of alpha-amylase was evaluated and Taguchi experimental method was used to determine optimum extraction conditions. As we know the Taguchi method, one of the optimization, has good reappearance of experiments concerned only with the main effects of design parameters. In principle, the Taguchi's design of experiments is used to get information such as main effects and interaction effects of design parameters from minimum number of experiments. The objectives of Taguchi method for parameter design were to find out the best combination of design parameters and reduce the variation for quality. In practice specifically27 experiments each repeated for 3 times in the 20 KHz frequency for malted flour in the solvent (sodium phosphate buffer with pH= 8) were done because of

TABLE 1: Control factors

Number	Factors	level 1	level 2	level 3
1	P^a(W)	13.8	27.6	34.5
2	t^b (min)	10	15	20
3	T^c (°C)	20	25	30

a: Ultrasonic Power; b: Irritation Time along with Thermal Treatment; c: Temperature

the sensitivity of work with enzymes and their behavior to close monitoring,evaluation and exact controlling of parameters effects. In order to analyse the results, 9 data × 3 that selected among the 27 experiments × 3 were given to software according to prediction the Taguchi technique in experiments design condition and Qualitek4 analysis using ANOVA approach was employed for finding the average effects of individual parameters on enzymatic process condition according to software output. Statistical calculations also for predicted optimum condition have shown in TABLE 3.

It must be mentioned that because of the encountering a serious problem (formation of gel) in sonication process with very small particles the planning of experiments is not included the parameter of particle size of malt flour. Moreoverwith bigger particles the amount of extraction was very low and the results would be far from the used scales in figures.

Determination thepower dissipated in a solution

In order to gain information on the actual powerinput a calorimetric method was used to calibrate theapparatus[33]. For this purpose 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. Calorimetric approach which assumes that all of the energy delivered to the system is dissipated as heat, as shown by

$$P_{diss} = \left(\frac{dT}{dt}\right)_{t=0} mC_p \qquad (1)$$

where*m* and *C*p are the mass and heat capacity of thesolvent, respectively, and (d*T*/d*t*)*t*=0 is the initial slopeof the temperature rise of the reaction mixture versustime of exposure to ultrasonic irradiation.

It is found that only about 30% of the power delivered by the transducer wasdissipated as heat, which would indicate that the other70% of the power was lost in the transfer process or byother means.

The intensity (I_{diss}, W/cm2) of ultrasound at the surface of the ultrasonic device is equal to the power dissipated (*P*diss) divided by the area of the probe tip (*A*p), as shown by

$$I_{diss} = \frac{P_{diss}}{A_p} \qquad (2)$$

TABLE 2 : Analysis of variance (ANOVA)

Number	Factors	DOF	Sums of Squares	Variance	F-Ratio	Pure Sum	Percent
1	P	2	31.725	15.862	48.853	31.076	28.97
2	t	2	12.187	6.093	18.766	11.537	10.756
3	T	2	56.86	28.43	87.557	56.211	52.402
Other/Error			20	6.493	0.324	7.872	
Total	26	107.267	100.000%				

TABLE 3 : Optimum conditions

Number	Factors	Level Description	Level	Contribution
1	P	25	3	1.282
2	t	20	3	0.691
3	T	30	3	1.421
Total Contribution from All Factors		3.394		
Current Grand Average of Performance…		70.459		
Expected Result at Optimum Condition…		73.883		

Malting stage

Barley seeds were micro-malted manually in laboratory scale according to the following procedure: samples after steeping at 16-17 ºC for 6 h in the incubator chamber were air-rested for 8 h. This process was done 3 times periodically to reach a moisture content of 45%. The subsequent germination phase was followed 96 h with keeping the 45% moisture content (with watering the samples every 4 h). Then the samples were kilned in the drying oven in gradually ramping temperature from 17 to 55 ºC over 20 h, from 55 to 65 ºC over 20 h, from 65 to 75 ºC over 6 h, and finally from 75 to 82 ºC over 4 h. The drying process was stopped with reaching the moisture content of samples to 4%. After removing the rootlets, the samples were milled and the malted flour is prepared for the next stages of the experiments. Because of the important role of the husk in forming a permeable filter bed during the extraction from the mash, it is kept as possible and milled carefully, but the endosperm is ground to particles that readily release their extract. The particle size distribution was 23.5 to 66.7 micrometer as a best range. In the present investigation mean value of particle size is 45.1 micrometer.

Sonication of the samples during the extraction of enzyme from malt

In this research commonly 50 mM Na-phosphate buffer with pH=8 was used as the best extraction medium. Approximately, 0.75 g of malt flour was weighed in duplicate into centrifuge tubes and 4 mL from extraction media was added with mixing. The sonication experiments were carried out at 20 kHz on an ultrasonic processor UP 200H. The tip of the horn was immersed into about 9 mm of 4 mL solution to be processed. All experiments were performed on solution in direct sonication at ultrasonic power of 13.8, 27.6 and 34.5 W. Ultrasonic irradiation was employed with additional agitation or shaking in order to the uniform distribution of the waves. The sonic power was controlled manually in three mentioned intensities. The ultrasonic energy was pulsed using a Duty Cycle Control in order to reduce the formation of free radicals. The cycle was set on 50% in all experiments. The solution was processed at three temperatures of 20, 25, and 30 ºC with the sonication horn for 10, 15, and 20 min. The temperature inside the solutions was intermittently checked. Extraction was terminated by centrifugation for 10 min at 5000 RPM. Thereafter the supernatants were filtered through moist filter paper into measuring cylinders and the volumes were recorded. These volumes were used in the calculation of enzyme activities. Control (0% ultrasonic vibration amplitude controller setting of nominal power) was treated similarly with the exception the elimination of the sonication stage. All experiments were performed thrice.

It must be mentioned that extraction of control under the above defined conditions was performed with regular vortexing for 5 s at 5 min intervals.

Determination of alpha-amylase activity based on decrease in starch/iodine colour intensity

Starch forms a deep blue complex with iodine and with progressive hydrolysis of the starch, it changes to red brown. Several procedures have been described for the quantitative determination of amylase based on this property. This method determines the dextrinising activity of alpha-amylase in terms of decrease in the iodine colour reaction. The dextrinising activity of alpha-amylases employs soluble starch as substrate and after terminating the reaction with dilute HCl, iodine solution is added. The decrease in absorbance at 620 nm is then measured against a substrate control. One percent decline in absorbance is considered as one unit of enzyme[34].

Enzyme assay

Starch–iodine assay according the Fuwa method was carried out as follows: assay reactions were initiated by adding 0.5mol of starch solution (20 mg/mL in 0.1 M phosphate buffer pH=7) and 0.5mol of enzyme in 0.1M phosphate buffer at pH 8.0 to reaction tube and incubated at 37°C for 30min. The reaction is then terminated by adding 1ml of 1M HCL. Following reaction termination, the mixture then diluted to nearly 10 ml with H2O, followed by the addition of 1ml of iodine reagent (0.2% iodine and 2% potassium iodide). Finally, the volume is adjusted to 10ml with distillated water and the amount of color development is determined by measuring the absorbance at 620 nm[35].

The enzyme activity was calculated according to the following equation[35]:

$$\mathbf{U/ml} = \frac{(\mathbf{OD_{620}\,control} - \mathbf{OD_{620}\,sample})}{\mathbf{OD_{620}\,mg\ starch \times t \times V}} \qquad (3)$$

The amount of extracted compound was calculated and the yield was expressed as weight ratio (%, w/w): %=

$$\frac{g\ extracted}{g\ initial}\times 100=\frac{U/g\ malt(sample\ activity-control\ activity)}{U/g\ malt(control\ activity)}\times 100 \qquad (4)$$

Where, OD control is the absorbance obtained from the starch without the addition of enzyme, OD sample is the absorbance for the starch digested with enzyme, OD /mg strach is the absorbance for 1 mg of starch as derived from the standard curve, t is the assay incubation time and V is the volume of the enzyme used in the assay.

The measured activity from the above equation for blank was 59.5 U/ml.

RESULTS AND DISCUSSION

The effects of different extraction conditions on the efficiency of process

The impacts of the various amplitude controller setting of nominal power of the device on the efficiency of extraction of alpha-amylase from malt flour was investigated at different time and temperatures. The results are shown in Figures 1 to 3, which illustrate that the activity of alpha-amylase, showed that the efficiency of extraction was increased with increasing ultrasonic power. The activity increased from 68.99 U/ml malt for a cavitation level of 10%(corresponding to extraction yield of 15.94%) to 73.25 U/ml malt (corresponding to extraction yield of 23.10%) for 25% power setting at the end of 20 min processing time. An appropriate explanation for this is that the larger the amplitude of ultrasound wave travelling through a mass medium, the more violently the bubbles collapse. As known that the enhancement in extraction efficiency obtained by the use of ultrasound is attributed to the disruption of the cell walls and the enhancement on the mass transfer of cell content to the extraction media caused by the collapse of the bubbles produced by cavitation[36].

Several factors affect the yield of the process during the extraction with ultrasound. Generally, increasing the temperature aids compound dissolution. In this research the activity obtained from sonication at any observed temperature is greater than resulting activity via thermal treatment (blank). This behavior is due to the increase in the number of cavitation bubbles formed. However the experimental results clearly indicate that the extraction efficiency of ultrasound becomes low with increasing temperature close to 30 ºC. For instance, the enzyme activity for sonication (25% of power setting) at 20 ºC was 68.57 U/ml malt (corresponding to extraction yield of 15.24%). When the temperature was increased to 25 ºC to introduce heat-induced extraction, the activity was increased to 72.48 U/ml malt (extraction yield of 21.81%) at the same employed power. This increase is higher than when temperature increased from 25 ºC to 30 ºC (proportional with enzyme activity of 73.25 U/ml malt and extraction yield of 23.10%) at the end of 20 min processing time and the constant employed power. Considering error bars it can be concluded that the enzyme extraction yield does not change significantly from 25 ºC to 30 ºC. From a practical point of view this means that in order to improve the efficiency of the extraction, the use of high temperatures may not be as useful. The explanation for reduction of the extraction efficiency with temperature may be attributed to the fact that at higher temperatures, increased vapor pressure inside the bubbles introduces a cushioning effect and hence produces less effective collapses[37].

About the effect of exposure time on the extraction of studied enzyme as one can see from figures 1 to 3, at the studied temperatures and for all the evaluated exposure times, the use of ultrasound causes an increase of alpha-amylase extracted from mash.

Quailtek4 statistical analysis of the sonication of malt flour suspension

TABLE 2 shows the detailed analysis of variance results of experiments conducted under Taguchi method. In this table the contribution of each factor was quantitatively determined by using ANOVA approach. The information summarized in TABLE 2 indicated that

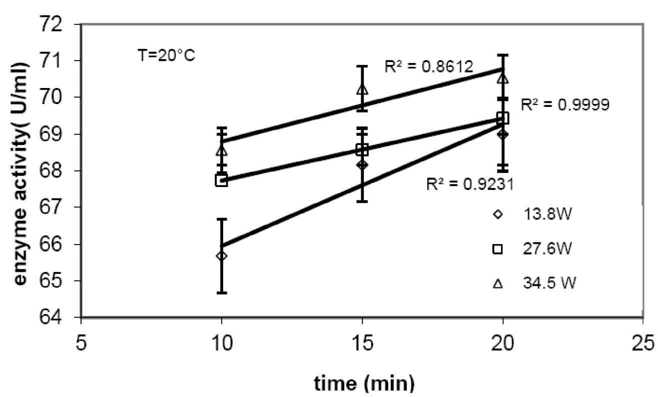

Figure 1 : Alphaamylase activity as a function of time of sonication and power level at 20°C (bars indicate± 5% standard error)

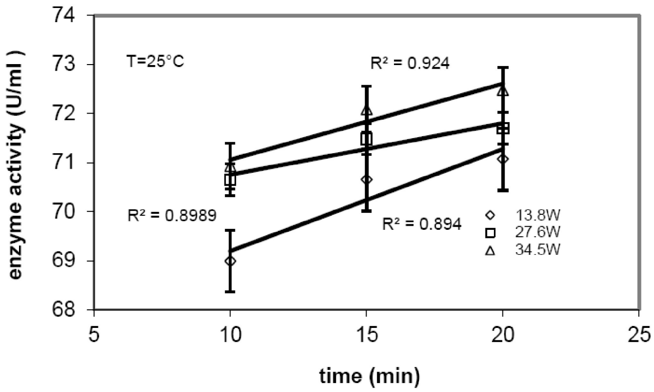

Figure 2 : Alpha amylase activity as a function of time of sonication and power levelat 25°C (bars indicate± 5% standard error)

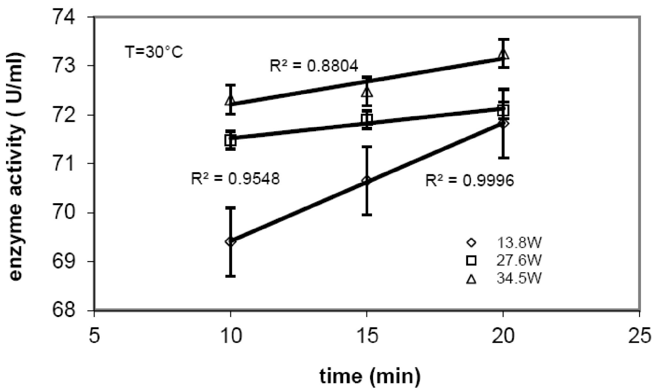

Figure 3 : Alpha amylase activity as a function of time of sonication and power levelat 30°C (bars indicate± 5% standard error)

operative temperature with a contribution percentage as high as 52.402%, had the most dominant effect on overall performance, followed by the time of exposure to ultrasonic irradiation along with thermal treatment, and power of ultrasound.

On the other hand, the degree of freedom (DOF) for each factor was 2and total DOF was 26, so the DOF for error term (which includes the interaction between introduced parameters) was 20, andfinally the variance for the error term, obtained by calculatingerror sum of squares and dividing by error degrees offreedom, is very small. Henceforth, it was difficult or impossibleto calculate the *F*-ratio, defined as the variance of eachfactor dividing by error term.If the factor is highly influencing the process response,then the F-value is large and is used to rank the factors. Fromthe obtained F-values for design parameters in the present work with 95% confidence intervalit can be concluded that each parameter's combination to total variation is significant.

Also Figure 4 depicts the main effects (average of

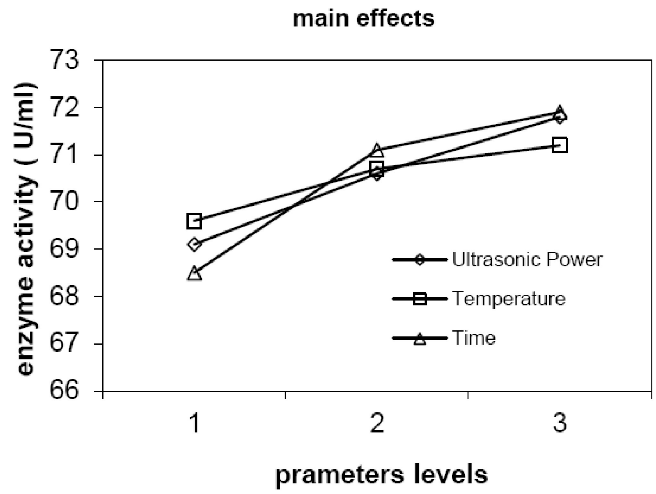

Figure 4 : Main effect of ultrasonic power, temperature and irradiation time at their selected levels on ultrasonic-assisted extraction by the Taguchi method using qualitek4 software

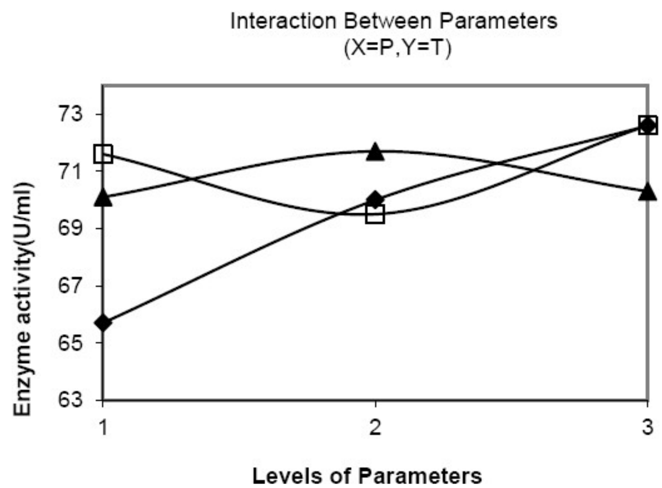

Figure 5 : Average effects of interaction between ultrasonic power and temprature by Taguchi method using Qualitek4 software.

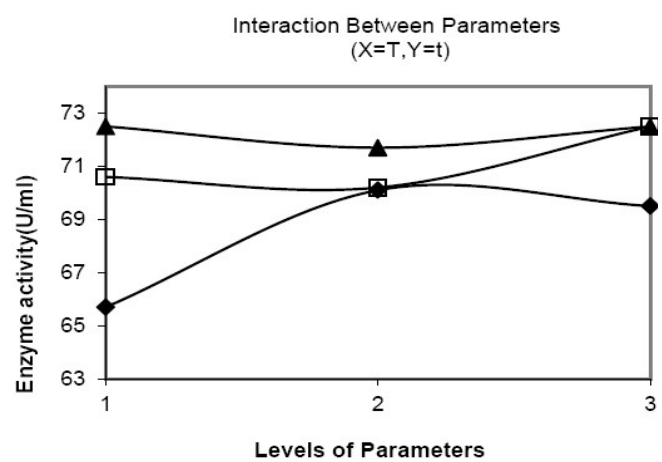

Figure 6 : Average effects of interaction between temprature and time by Taguchi method using Qualitek4 software

obtained results) of ultrasonic power, temperature and exposure time. As it is shown in this Figure the average effects of ultrasonic intensity and time of exposure to ultrasound are positive on enzyme extraction. Also the maximum effects of these parameters were in their third level.

The average effects ofinteraction between defined parameters are also presented in Figures 5 to 7.

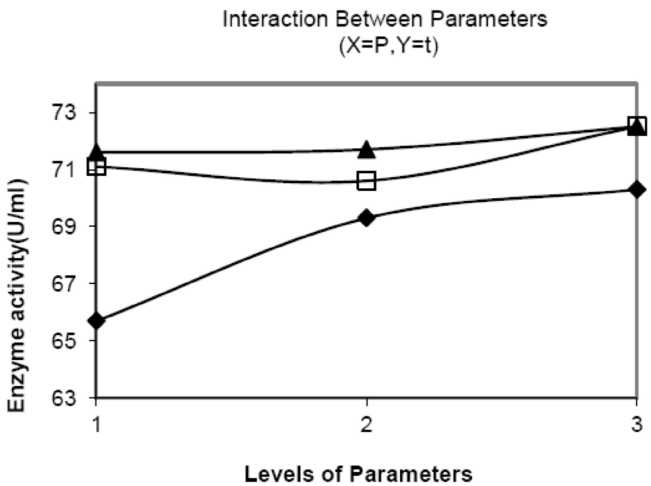

Figure 7 : Average effects of interaction between ultrasonic power and time by Taguchi method using Qualitek4 software.

CONCLUSIONS

The results obtained in this study have implications for the brewing industry. Use of ultrasonication along with thermal treatment at low temperatures was found to be useful in the case of improving the extraction efficiency of alpha-amylase from malt flour and so its activity. The results showed that the extraction yield of enzyme was increased by 23.10%in the optimum condition by comparing to blank.

Although ultrasonication during the mashing process will not eliminate the need for high temperatures enable the gelatinization of starch, it can be used to disintegrate long-chain starch molecules, and this increase in surface, together with increased temperature (within grist solution) released by bubble collapse, may enhance saccharification by amylase at low temperatures during the mashing process.

In summary the efficiency of ultrasound-assisted extraction procedure exceeded that of conventional procedure. Using ultrasound, greater yields of total extracted alpha-amylase achieved at lower extraction temperature and shorter extraction time. In addition the activity of the sonically extracted alpha-amylase was higher than that of thermally extracted preparations.

Finally for practical purpose, it must always be considered although the possibility of increasing the extraction yield of the alpha-amylase in mashing process by ultrasound waves in combination with thermal treatments has been proven by our laboratory experiments, the same may be not true for industrial applications. The reasons of the nondevelopment 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. Most results reported by researchers, 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.

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