

Full Length Research Paper

Influence of ultrasonic stimulation on the germination of barley seed and its alpha-amylase activity

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In this study, the influence of ultrasonic stimulation was investigated on the germination of barley and alpha-amylase activity grains in the dry state before steeping. All experiments have been performed using an ultrasonic horn operating at a fixed frequency of 20 KHz in 3 different ultrasonic power (20, 60 and 100% setting from total electrical power of device (460W) and time (5, 10 and 15 min) at constant temperature (30°C). For determining the effects of these parameters, the enzymatic activity was assayed by measuring the reducing sugars released as a result of the alpha-amylase action on soluble starch using 3,5-dinitrosalicylate reagent (DNS). The results of these assays were analyzed by Qualitek4 software using Taguchi statistical method to evaluate the factor's effects on the amount of enzyme activity. The results of assays showed that the activity of this enzyme was increased as a result of the increasing germination rate in the sonicated seeds. Also the findings indicated that both ultrasonic power and treatment time with a contribution percentage as high as 48.456% and 45.273% respectively, had the dominant effects on overall performance.

Key words: ultrasonic stimulation, alpha-amylase activity, germinated barley, Taguchi statistical method.

INTRODUCTION

The application of ultrasound to biotechnological processes has recently attracted the attention of some research groups. In biotechnological processes, ultrasonication method is widely used for laboratory scale and it does not require sophisticated equipment or extensive technical training. The structure and function of biological molecules can be changed by the ultrasound irradiation. The most common interaction mechanisms which involved in this case are either heat or chemical effects and acoustically induced cavitation activity. In addition to these, changing the function of biomolecules by ultrasonication can also be caused by mechanical effect that is shear stress developed by eddies arising from shock waves.

Ultrasound exerts its effects mainly through a phenomenon called cavitation. Cavitation is the formation, growth and, sometimes, the implosion of microbubbles created in a liquid when ultrasound waves propagate through it. The collapse of the bubbles leads to energy accumulation in hot spots where temperatures of above 1000 K and pressures of approximately 500 MPa have been measured (Suslick, 1990). It is well known that the ultrasonic waves have the potential to influence the living cells. Ultrasound acts as an alternative stress on cells or tissues. A number of papers have been published dealing with the ultrasonically assisted stimulation of different cells and tissues. Research in this field of ultrasonic was expanded not only in the clinical medicine, biomedical engineering (Gavrilov et al., 1996) and biotechnology process (Vollet et al., 1998; Schläfer et al., 2002; Lanchun et al., 2003) but also on the other field of industry such as physics (Lin et al., 1995), chemical (Liu et al., 2007) and mechanical engineering (Zhu et al., 1999). High efficiency, saving energy, improved biological activity, mass transfer enhancement and shortening process time are the main positive effects of such treatments.

In biotechnology process and food industry ultrasoni-

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Abbreviations: OD_t = optical density of test sample at 540 nm; OD_s = optical density of the maltose standard; EV = extraction volume (mL); DF = dilution factor; t = incubation time (min); V = used volume of diluted enzyme extract (mL); m = weight of malt flour (g); Mw = molecular weight of maltose.

cally stimulated seed germination offers the possibility of increased productivity for large scale farm crops and in more general horticulture. Agricultural crop yields are dependent on the quality of the plant variety and on the percentage seed germination and growth. The effect of the suspension media on the ultrasonically stimulated germination of seeds from a temperate *Cymbidium* species has been studied. Pretreatment with ultrasonic waves resulted in the best germination but the ultrasonic treatment induced quicker germination and faster rhizome growth (Chio and Chung, 1991). Extensive efforts have applied sonication under dry conditions which may be carried out up to several months before actual sowing (Abramov, 1994). Examples of the use of this process include ultrasonic treatment leading to a three fold enhancement in sunflower seed germination in soil, 2 to 3 days faster germination of corn seeds (Hebling and Silva, 1995; Shors et al., 1999) and a ten-day reduction in the ripening time of tomatoes (Abramov, 1994) or shorter period to form a gel as a result in a faster release of starch during subsequent cooking in a sonicated rice grain in water (Paniwnyk, 1993). Detailed discussions on the results of these investigations can be found in various reviews (Gordon, 1963), articles (Aladjadjiyan, 2002; Shimomura 1990; Weinberger and Measures, 1968) and books (Povey and Mason, 1998). Reports and the intriguing possibility that the use of ultrasound may enhance the stimulation of some seeds have led us to examine the feasibility of ultrasound-induced effective germination of barley seeds in order to increase its alpha-amylase activity.

Alpha-amylases (endo-1,4- α -D-glucan glucohydrolase EC 3.2.1.1) are a class of hydrolases widely distributed in nature, that is in the higher plants, animals, and microorganisms (bacteria, Fungi origin). They can specifically cleave the O-glycosidic bonds in starch, a principal storage polysaccharide present in seeds of various plants and other related oligo and polysaccharides. These enzymes have a great significance with extensive biotechnological applications in food, brew, textile and paper industries. Alpha-amylase, one of the most valuable enzymes is important in the metabolism of maltose and maltodextrins. As well as being used as an additive in detergent, it can be used for such things as the removal of starch sizing from textiles, the liquefaction of starch, preparation of digestive aids and the proper formation of dextrin in baking. Starch depolymerization by amylases is the basis for several industrial processes such as the preparation of glucose syrups and brewing. Cereal alpha-amylases play a very important role in the starch metabolism in developing as well as germinating cereals (Muralikrishna and Nirmala, 2005).

Conventionally, this industrially very important enzyme is not always satisfactory in respect of its activity and cost. Extensive research activities are performed to solve these problems. For instance as a means for solving this problem, an increase in productivity of the enzyme have

been attempted by genetic altering (DNA recombination) particularly for barley embryo's (Rikiishi et al., 2001, Wong et al., 2004, Wu and Rodinguez, 2000) and protein engineering technology for altering the properties of alpha-amylase to obtain an enzyme with properties tailored for specific application (Svendersen et al., 2002, 2004) and a method of improving the reaction rate of the enzyme in the enzyme reaction systems. In this regard, in an article by Tull and et.al, 2003 transfection the aleurone protoplasts of barley to express synthetic genes encoding cytosolic and secreted forms of the alkalophilic *Bacillus* alpha-amylase, alkBA disclosed. The alpha-amylase activity in the cytosol of transfected protoplasts was increased 4 fold compared to the controls (Tull et al., 2003). with respect to the method of activating the enzyme, there are reports on activation by adding oxidizing agent (Ishii et al., 2006) to an enzyme reaction system, activation by adding carbonate ions (Antrim, 1993) to specific amylase or enhancing the reaction by sonication the mixture of the enzyme-substrate (Barton et al., 1996) and any other method like that. In the malting and brewing industries the diastatic power as it is popularly known is very important for assessing the activity of starch-degrading enzymes and the brewing industry depends on the activity of alpha-amylase, improved yield and/or rate of barley seed germination is valuable in the area of commercial brew producing. For this purpose the action of botanical gibberlic acid growth hormones (Taiz and Starks, 1977) and ethylene (Eastwell and Spencer, 1982a, b) were investigated in the release of amylase from barley aleurone layer. The aleurone cells of barley secrete substantial quantities of protein in response to GA3 (Melcher and Varner, 1971). GA increases the synthesis of poly (A) RNA, as well as the level of translatable alpha-amylase messenger RNA. However, the massive cell walls of the aleurone layer pose a formidable barrier to the release of protein into the surrounding medium. Extensive degradation of barley aleurone cell walls in response to GA3 was detected (Ashford and Jacobsen, 1974; Pomeranz, 1972). Taiz and Starks have found that the hormone simultaneously stimulates both DNA synthesis and DNA degradation in aleurone cells, resulting in enhanced rates of DNA turn-over and subsequently increase alpha-amylase release (Taiz and Starks, 1977). A similar result has been recorded by Eastwell and Spencer as a result of ethylene treatment. They found that ethylene promotes the release of amylase from isolated aleurone layers by enhancing the production of the cell wall-degrading enzyme. Ethylene affects the production of only those enzymes that require GA3 for their synthesis (Eastwell and Spencer, 1982a, b). However, today, the brewing and distilling industries are cautious in using grain which has been treated with added GA and/or ethylene, because of the risk of excessive proteolysis occurring in the endosperm of the grain. Moreover the malt having been germinated by the addition of gibberlic acid is liable to dissolve to greater

extent than is necessary which can result in enhanced coloring during drying and in an increased malting loss. In order to avoid these unfavorable results developing the physical methods to increase the germination rate and the alpha-amylase activity are indispensable. In this regard Ress et al., have examined the influence of ionized radiation on germination of barley. They found ionizing radiation prior to the malting process causes a stimulating effect on barley seeds and increase the enzyme activity (Ress et al., 1987).

As mentioned above because of the commercial importance of diastatic power of malt in brewing and industrial application of alpha-amylase, in this work, the attention was focused on the investigation of the ultrasound effects to treat barley seeds before steeping for greater and faster germination as well as the influence of quicker germination on the alpha-amylase activity. For this purpose the day of germination and alpha-amylase activity were kept as a means of determining rate and yield of process respectively. As far as we know no reference about the effects of ultrasound on the stimulation of barley seeds and its alpha-amylase activity have been found in the literature.

MATERIALS AND METHODS

Chemicals, reagent, solution and samples

The chemicals include soluble starch obtained from potato, $(C_6H_{10}O_5)_n$ (S-2630), sodium potassium tartarate tetrahydrate $[KOCOCH(OH)CH(OH)COONa \cdot 4H_2O]$ (S-2377), 3,5-dinitrosalicylic acid $[(O_2N)_2C_6H_2-2-(OH)CO_2H]$ (D-0550), sodium phosphate mono-basic anhydrous $[NaH_2PO_4]$ (S-0751), maltose monohydrate $[C_{12}H_{22}O_{11} \cdot H_2O]$ (M-5885), sodium metabisulfite $[Na_2S_2O_5]$ (71928), sodium phosphate $[NaH_2PO_4]$ (S0751) and maleic acid (disodium salt) $[C_4H_2Na_2O_4 \cdot xH_2O]$ (M-9009). All of these materials with high analytical grade were obtained from Sigma-Aldrich and Fluka companies and used in alpha-amylase assay.

3, 5-Dinitrosalicylic acid solution is used for measuring the reducing sugar. For preparation of this solution first 10 g of 3, 5 dinitrosalicylic acid and 10 g of NaOH were dissolved in almost 600 ml distilled water. Then 192 g of tartaric acid sodium potassium with constant mixing is added to the solution. Afterward with adding 2 g of melted phenol and 0.5 g of metabisulfite, the total volume of solution was adjusted to 1000 ml with distilled water.

Soluble starch solution is used for liquefaction purpose as substrate. For preparation of this solution, 10 mg of soluble potato starch was mixed in 1 mL of 0.1 M malate buffer with pH = 5.5. After that the solubilization was facilitated by heating the starch solution in a glass beaker directly on a heating/stirring plate using constant stirring. Then the solution were brought to boil and maintained at this temperature for 15 min. Finally it was cooled to room temperature with stirring.

Karon and kavar 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°C until malting. Also it must be mentioned, that for removal of dormancy, samples were stored at room temperature (25 – 37°C) for 3 months after harvest.

Equipments

The sets of Gerhardt Kjeldatherm and Gerhardt Vapodest 30 instru-

ment were used for determination the amount of protein in barley seeds. Ultrasonic irradiation was given by means of UP 200 H horn type (20 kHz, maximum wave amplitude of 210µm and maximum nominal power output 460W) equipped with a radial Sonotrode S3 (3 mm diameter) designed by Dr. Hielscher GmbH (Treprow, Germany).

Experiment design

In this study 4 important effective parameters namely, ultrasonic power, the time of ultrasonic irradiation, temperature and frequency were selected. Since in our design problem the operating temperature and frequency were fixed, only 2 variables were remained for the design of experiments. Taguchi method was employed to design experiments condition and evaluate the factor's effect on stimulation and activity of enzyme. The experimental trials were arranged in an L9 orthogonal array matrix. Accordingly, 9 experiments, repeated for 3 times, considering the 2 parameters that defined above were done in the 20 KHz frequency for barley seeds. The Qualitek-4 software, which is designed for Taguchi experiments, was used for the optimization, analysis of result and determination the main effect or average effects of individual parameters on enzymatic process conditions and interactions between factors as shown in the Figures 2 to 4. The term interaction is used when the change in operational level of one factor influences the performance of other factor/s. The standard option from the analysis menu was selected for analysis of the transformed data from the experiments to software. The optical density was chosen as a yield of process. As there is need of maximization of the yield so "bigger is better" was performed as the optimization criteria. At last with determination of the optimum condition (Table 2) by software the conformation experiments were done with 90 and 95% confidence interval.

Sonication of the samples

The ultrasonication experiments were carried out at 20 kHz on the ultrasonic generator. The tip of the horn was immersed about 9 mm into solution to be processed. All experiments were performed on samples (10 g barley seeds) dispersed in 80 ml of tap water in direct sonication at ultrasonic intensity of 20, 60 and 100% of 460 W (maximum output power of device) with additional agitation or shaking that was employed, to avoid standing waves or the formation of solid free regions for the uniform distribution of the ultrasonic waves. 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 constant temperatures of 30°C with the sonication horn for 5, 10 and 15 min. The temperature of water circulating in the water batch was set and the temperature inside the solution was intermittently checked so that the temperature of the solution remained constant during the experiments.

Malting stage

Barley seeds were micro-malted manually in laboratory scale according to the following procedure: samples after steeping at 16 - 17°C for 6 h in the incubator chamber, air-rested for 8 h and it was done 3 times alternately to reach a moisture content of 45% and the subsequent germination phase followed 96 h with keeping the 45% moisture content (with watering the samples every four hours). Following on from germination the malted grain was dried. Drying of the green malt was done in kiln. The samples were kilned in the drying oven in gradually ramping temperature from 17 to 55°C over 20 h, from 55 to 65°C over 20 h, from 65 to 75°C over 6 h and final-

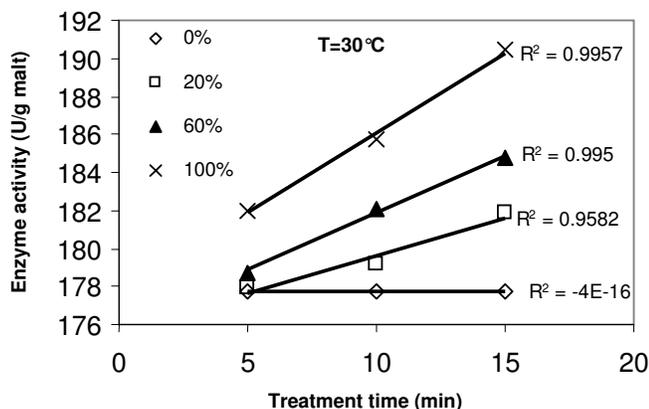


Figure 1. Alpha-amylase activity versus time, barley sonication before steeping at 30°C.

ly from 75 to 82°C over 4 h. Once kilning was finished (with reaching the moisture content of samples to 4%) the malt is allowed to cool, rootlets were removed and the cleaned and milled malt was prepared for the next stages of the experiments (Osman, 2002).

Extraction of enzymes from malt

In this research commonly 50 mM sodium phosphate buffer with pH = 8 were used as the best extraction media. This buffer enhances the release of more enzymes rather than another media such as the mixture of NaCl in water owing to the fact either the high pH or added phosphate ions (higher concentration) as pointed out by Osman. Approximately, 0.75 g malt flour was weighed in duplicate into centrifuge tubes and 4 mL extraction media was added with mixing. Extraction was performed for 30 min at 30°C with regular vortexing for 5 s at 5 min intervals and was terminated by centrifugation for 10 min at 2826 g. The supernatants were filtered through wet filter paper into measuring cylinders and the volumes were recorded. These volumes were used in the calculation of enzyme activities (Osman, 2002).

Alpha-amylase assay according to method of reducing sugars

This method determines the increase in reducing sugars as a result of amylase action on starch. The major defect in this assay is a slow loss in produced colour and destruction of glucose by constituents of the DNSA reagent. To overcome these limitations, a modified method for the estimation of reducing sugars was developed. Sodium sulphate was added in order to prevent the oxidation of the reagent (Gupta et al., 2003). The reagent is composed of dinitrosalicylic acid, potassium sodium tartrate (Rochelle salt), phenol, sodium bisulfite (or sodium sulfite), and sodium hydroxide. During the reaction, color development is stabilized by Rochelle salt and enhanced by phenol, and sulfite protects the reagent and reducing sugar from oxidation (Decker et al., 2003; Wang et al., 1997).

To assay alpha-amylase by measuring the reducing sugars produced by its action on soluble starch, beta-amylase was inactivated by heat treatment for 7 min at 70°C, in the presence of 20 mM CaCl₂. Exactly 0.1 mL of heated and appropriately diluted (20 in this study) extract was added with mixing to 0.1 mL thermal pre-equilibrated soluble starch solution. The reaction was continued for 10 min and terminated by adding 2 mL 2 M NaOH. The reducing sugars were measured by adding 1.0 mL 3,5-dinitrosalicylic acid

solution freshly prepared then mixing and boiling for 5 min. Standards and controls were treated similarly with the exception that the enzyme extract was added to the controls after NaOH. The samples, controls and the standard were read at 540 nm after cooling to room temperature and making up the contents of solution to 10 ml with distilled water. Reducing equivalents were calculated from calibration graphs obtained using absorbance data for standard solutions of maltose reacted with DNS as above (Osman, 2002).

One unit of alpha-amylase activity was defined as the quantity of enzyme that released the amount of reducing sugars per minute equivalent to one micromole of maltose, under the above defined assay conditions.

The enzyme activity was calculated according to the following equation (Osman, 2002).

$$U/g\ malt = OD_t / OD_s * \mu g_{realised\ maltose} * EV * DF / t / V / m / M_{w_{maltose}}$$

It has been established that 4 mL used to extract 0.75 g malt flour, yielded on average 2.95 mL.

RESULTS AND DISCUSSION

The efficacy of ultrasonic stimulation on the germination of barley and alpha-amylase activity was investigated at 30°C and cavitation levels between 20 and 100% settings of output power of device. Resulting activation are shown in Figure 1.

Analysis of variance, ANOVA approach and average effects of individual parameters (P, t) on the enzymatic process conditions and interactions between factors are shown in the Table 1 and Figures 2, 3 and 4. The results of the ANOVA reveal that the value of treatment time and the cavitation level (ultrasonic power) with minor difference in the relative percentage contribution, which reached 45.273% and 48.456% respectively made the major contribution to overall performance. The percentage errors are only 6.27%, which indicates that all the major contributing factors related to the objective of study have been considered. It can be clearly seen that all the major effects of two parameters are significant. Further, plots 2 and 3 also indicate that the relative contributions of ultrasonic intensity and treatment time increase with their increasing levels. The average effects of ultrasonic power and the time of exposure to ultrasound were positive on stimulation of barley seeds germination, therefore enzyme activity and the maximum effects of these parameters were in the their third level. Based on the data listed in Table 1 there was no doubt the activity of alpha-amylase was increased in the sonicated grains.

As can be seen from the Figure 1, it is obvious that increasing ultrasonic power increases alpha-amylase activity. The activity increased from 177.74 for 0% to 190.431 (U/g malt) for a cavitation intensity of 100% at the end of the 15 min processing time. This can be explained by the work of Suslick (1990) on the cavitation on liquid-solid systems: according to his statements when cavitation occurs in a liquid close to a barley seeds boundary, cavity collapse generates high-speed jets of liquid. The potential energy of the expanded bubble is

Table 1. Analysis of variance (ANOVA) showing the effect of ultrasonic power (P) and the variable of time (t) as significance of the main effects.

Number	Factors	DOF	Sums of squares	Variance	F-Ratio	Pure sum	Percent
1	P	2	0.009	0.004	101.468	0.009	48.456
2	t	2	0.008	0.004	94.868	0.008	45.273
Other/Error		22	0.000	0.000			6.271
Total		26	0.019				100.000%

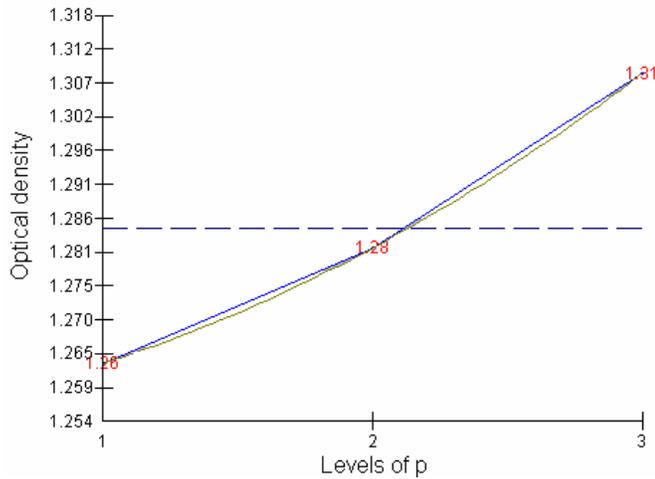


Figure 2. Average effect of ultrasonic power by Taguchi method using qualitek4 Software.

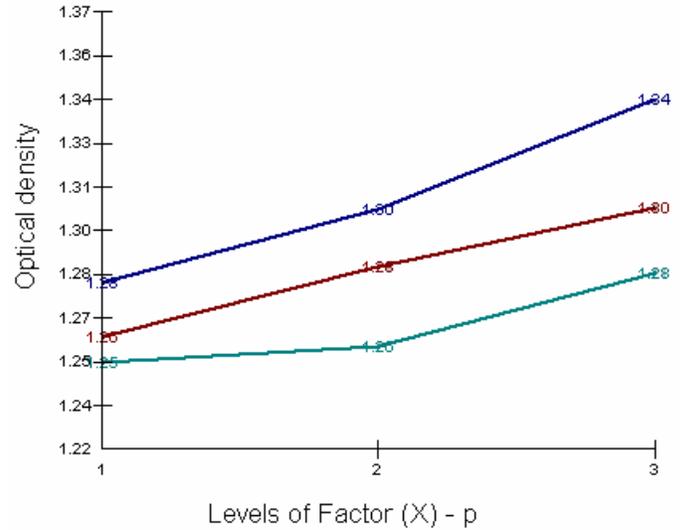


Figure 4. Average effects of interaction between time and ultrasonic power by Taguchi method using qualitek4 software.

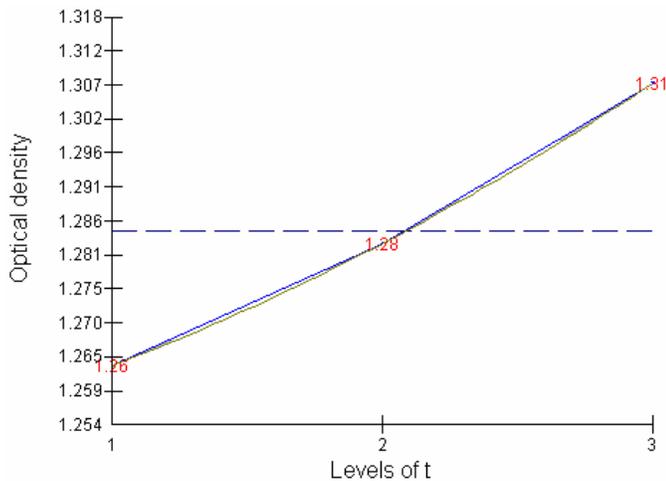


Figure 3. Average effect of irradiation time by Taguchi method using qualitek4 software.

converted into kinetic energy of a liquid jet that moves through the bubble's interior and penetrates the opposite bubble wall. These jets hit the surface with tremendous force (Suslick, 1994). This process can cause severe damage at the point of impact and can fragment the seed shell and produce larger porosity on the surface of barley

grains. Shell fragmentation dramatically decreases the resistance of the seeds shell against the water diffusion and facilitates the passage of the water molecules across the cell wall. Therefore as a result of increasing the mass transfer rate of the target components, the sonicated tissue absorbs an extra volume of water so that the absorbed water is given to embryo most freely and simply. This could be inducing the more alpha-amylase release and lead to quicker germination and faster embryo growth, such that pretreatment with ultrasonic waves resulted in better germination after sonication and therefore the activity of alpha-amylase in the sonicated grains was much more, compared by nonsonicated seeds at constant interval time.

Also by using the barley grains as treated above, the germination period is shortened to 4 to 5 days depending upon the cavitation level and exposure time to ultrasonic waves, from the usual 7 days. A number of factors have been proposed to account for both the observed enhancement alpha-amylase activity and reduction the time of the germination phase. The mechanism by which ultrasound induced enhancement in alpha-amylase activity as a result of better germination or reduction in the time of malting period in the barley seeds is unknown, but there are several possible explanations.

Table 2. Optimum condition and performance

Number	Factors	level Description	Level	Contribution
1	P	100	3	0.024
2	t	15	3	0.023
Total Contribution from All Factors ...				0.047
Current Grand Average of Performance...				1.284
Expected Result at Optimum Condition...				1.331

The enhancement of cell wall fluidity as a result of the mobilization of endosperm nutrients might be one factor promoting the germination in ultrasonic stimulation and increasing the alpha-amylase activity. It is possible that endosperm modification, including starch degradation by means of ultrasound, subsequently may lead to increase in the rate of enzyme-catalyzed hydrolysis reactions within the barley grain itself and also increase efficiency in the action of alpha-amylase. Nevertheless, we believe the reason for the increased barley alpha-amylase activity lies in the ultrasound stimulation, which caused more water retention before steeping and better germination after sonication in barley grains. Obviously, at high cavitation intensity levels there will be more damage to the barley's seed shells, resulting in a higher stimulation and more alpha-amylase release, no matter what mechanisms might be involved.

Commercial perspectives

Subjecting the barley seeds to direct sonication substantially reduced the time for germination and increased the alpha-amylase activity but it may be questionable as to whether or not the present study is to provide a method of promoting the germination of seeds by processing a large quantity of seeds at one time thereby providing a commercially viable scale of operation. In answer it must always be considered although the possibility of increasing the rate of barley germination and alpha-amylase activity by ultrasonic waves, has been proved to laboratory scale, the same may be not true for industrial applications. Moreover the difficulty in this method is in obtaining equal ultrasonic waves exposure in all areas of treated seed lots. The reasons of the non-development on an industrial scale-up 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 et al., 1992).

Conclusion

Two main conclusions can be drawn from the information presented in this paper. Ultrasound waves used in treating the barley seeds before soaking results in better and faster germination. This is due to shell fragmentation leading to more water retention capacity in dry grains.

Secondly, the stimulation of the germination process of barley caused by ultrasound is as a result of enlargement in the pores size of the seed's shell for better hydration. All these increase alpha-amylase activity causing faster germination of barley seeds and two-day reduction in the malting period in sonicated barley grains in water.

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