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# Does ultrasound improve the activity of alpha amylase? A comparative study towards a tailor-made enzymatic hydrolysis of starch

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ABSTRACT

This work reports the role of ultrasound (US) in the activity of alpha amylase through a systematic comparison of the effect of US (supplied by a US probe) in four different commercially available alpha amylases from different sources. To this end, we selected a fungal alpha amylase from *Aspergillus oryzae* (AOA), and three bacterial alpha amylases: one from *Bacillus amyloliquefaciens* (BAA), and two from *Bacillus licheniformis*, heat-stable (HSA) and Liquozyme (LQA). A Box-Behnken experimental design made possible to establish the optimum activity conditions for each enzyme. AOA and BAA were inactivated by US, in contrast with HSA and LQA that were activated. When compared to control experiments, US lead to modifications in the enzyme activity that ranged from −44% in BAA to +1657% in LQA. The magnitude of inactivation/activation and the physical and chemical mechanisms responsible for these responses were critically discussed and compared with the previously reported results, with a particular emphasis on the thermal effects of US. These findings revealed the potential of US to modulate the activity of alpha-amylase, which will impact in the development of tailor-made protocols for starch hydrolysis.



## 1. Introduction

Starch is a natural polymer with nutritional and biotechnological importance, from human and animal nutrition to paper manufacturing and biofuels. The degradation of its sub-units, amylose and amylopectin, is a key step in numerous laboratorial and industrial workflows (Robyt, 2008), and it is carried out using chemical (Wang & Copeland, 2013) or enzymatic (Blazek & Gilbert, 2010) approaches. This process is energy-intensive due to the variety and complexity of the polymeric structures originated by the different botanical sources. For these reasons, the enzymatic approach has been preferred due to the mild conditions necessary for the hydrolysis. The enzymatic hydrolysis of starch to glucose usually comprises a two-stage process: liquefaction, catalyzed by alpha amylase, and saccharification, catalyzed by glucoamylase (Olsen, 1995). Alpha amylase is able to hydrolyze the α-(1 → 4) linkage, whereas the glucoamylase is able to hydrolyze not only α-(1 → 4) but also α-(1 → 6) glycosidic linkages (Robyt, 2008). In this context, novel strategies to modify the activity of alpha amylase will have a significant impact in the different processes where this enzyme is used.

The use of ultrasound (US) energy, which produces cavitation at low frequencies caused by compression-expansion cycles of the waves, may play an important role in this hydrolysis process (Delgado-Povedano & Luque de Castro, 2015). Despite ultrasound being reported in the literature as a potential enzymatic inactivator or activator (R. Mawson, Gamage, Shiferaw, & Knoerzer, 2011), US may be predominantly considered an enzymatic inactivator due to its physical and sonochemical effects (Delgado-Povedano & Luque de Castro, 2015; Islam, Zhang, & Adhikari, 2014; R.; Mawson et al., 2011). On one hand, physical effects are essentially associated with the denaturation of proteins due to the excessive applied forces and also with the high-localized temperatures and shock-waves. On the other hand, sonochemical effects are related with the concentrations of free radicals in the interface between the bubbles and the surrounding liquid. A third effect based on mechanical and shear forces caused by microstreaming and shock can also be present (Delgado-Povedano & Luque de Castro, 2015). Furthermore,

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US can positively contribute to the activity of enzymes immobilized in polymeric resins (J. S. Alves et al., 2015; Fallavena et al., 2014), not only by increasing their original activity (J. Alves et al., 2014), but also by improving its operational stability (Martins et al., 2013) and thermodynamic control (Fallavena et al., 2014). US was recently combined with molecular sieves in order to enhance the stability and the activity of enzymes in organic reaction milieu (Fallavena et al., 2014; Paludo et al., 2015). Beyond this interaction with enzymes, US has been also successfully used in food processing (Kentish & Feng, 2014), and in the extraction of bioactive compounds (Azmir et al., 2013).

Considering our particular case of enzyme-assisted hydrolysis of starch using alpha amylase, the latest literature has described a dual role for US. For example, Yu et al (Yu, Zeng, Zhang, Liao, & Shi, 2014). found inactivation of alpha amylase from *Bacillus licheniformis* using a US bath during the starch hydrolysis. A similar behaviour was reported for an alpha amylase from *Bacillus amyloliquefaciens* submitted to a US frequency of 30 kHz by a sonotrode (Kadkhodaee & Povey, 2008). Conversely, US supplied by a US bath activated commercial alpha amylases from *Trichoderma reesei* (Souza et al., 2013) and *B. licheniformis* (Leaes, Lima, et al., 2013). Nevertheless, the experimental conditions (enzyme source, US amplitude, pH, temperature) applied in each case were different, and for this reason it is difficult to determine the optimum experimental conditions to customize alpha amylase activity using US. If we consider the different optimum ranges of pH and temperature and the multiple inactivation mechanisms that can affect each enzyme (Apar & Ozbek, 2004), the use of a US probe that provides an accurate control of the energy supplied can help in establishing comparable experimental scenarios. Hence, a simultaneous and systematic evaluation of US power, combined with other relevant parameters such as temperature and pH, will contribute to determine the factors that will tailor the enzyme activity.

The use of an experimental design strategy (Leardi, 2009) allows a simultaneous and systematic evaluation of alpha amylase working conditions and their influence in the hydrolysis process (Leaes, Lima, et al., 2013; Leaes, Zimmermann, et al., 2013). Among the available options, the use of response surface methodology based on Box-Behnken design is particularly suited, considering the estimation of the parameters of the quadratic model, the limited number of experiments necessary to obtain a complete set of information, and the possibility of applying the full range of the parameters under study (Ferreira et al., 2007; Montogomery, 2001). This latter point is particularly important to evaluate the entire range of amplitudes that can be supplied by the US probe.

Based on these elements, we aimed to evaluate the effect of US in the activity of four different commercial alpha amylases from fungal and bacterial origin commonly used in laboratory or industrial workflows. To this end, we applied a Box-Behnken design in order to find the significant effects of the factors (US amplitude, temperature, pH, and respective interaction(s)) that affected enzyme performance, influencing its application in starch hydrolysis. It was also objective of this work to identify potential strategies for tailoring the activity of the different enzymes that will improve current methods for the hydrolysis of starch in different matrices with relevance in the food industry (Awad, Moharram, Shaltout, Asker, & Youssef, 2012).

## 2. Experimental

### 2.1. Reagents and solutions

All aqueous solutions were prepared with ultra pure water (maximum conductivity of 0.055 μS $cm^{-1}$) produced by a Sartorius arium pro water purification system (Goettingen, Germany). Starch stock solution was prepared by dissolving 1.000 g of soluble starch (Sigma-Aldrich, St. Louis, MO, USA) previously dispersed in 5 mL of water (at room temperature) in ~400 mL of boiling water under continuous stirring. After 5 min, the heat was switched off and the stir was kept until the solution reached room temperature. The solution was then transferred to a 500 mL volumetric flask and the volume completed with water. This solution was stored in the refrigerator and it was used up to three consecutive days. Starch standards for the microplate measurements were prepared by dilution of the stock solution with water.

KI/$I_2$ solution was prepared by dissolving 200 mg of KI (Merck, Darmstad, Germany) in ~60 mL of ultra pure water, followed by the addition of 20 mg of $I_2$ (Sigma-Aldrich). This solution was sonicated until complete dissolution of $I_2$ and subsequently transferred to a volumetric flask of 100 mL, where the volume was completed.

Universal buffer solution was prepared by dissolving 2.91 mL of 85 g/100 g phosphoric acid (Sigma-Aldrich), 2.86 mL of acetic acid (Sigma-Aldrich) and 3.09 g of boric acid (Chem-lab, Zedelgen, Belgium) in 1000 mL of ultra pure water. The different target pH values were set using a 4 mol $L^{-1}$ NaOH solution (Fisher Scientific, Waltham, MA, USA).

Four different commercially available alpha-amylases were used (Table 1): alpha amylase from *Aspergillus oryzae* (AOA) (Sigma-Aldrich, #A8220), alpha amylase from *Bacillus amyloliquefaciens* (BAA) (Sigma-Aldrich, #A7595), and two alpha amylases isolated from *Bacillus licheniformis*, heat-stable (HSA) (Sigma-Aldrich, #A3303) and Liquozyme SC DS (LQA) (Novozymes, Bagsvaerd, Denmark). Enzyme working solutions were prepared by stepwise dilution of the commercial solutions using universal buffer at the target pH of the experiment. All solutions were firstly diluted 200 times, followed by a second dilution of 400 (AOA, BLA, HSA) or 200 times (LQA) before use in the activity assays.

For stopping alpha amylase activity, 1 mol $L^{-1}$ HCl solution was applied. This solution was obtained from the dilution of a 37 g/100 g commercial solution (Panreac, Barcelona, Spain).

### 2.2. Equipment

A block heater (Stuart SBH130D/3, Staffordshire, UK) was used to control the temperature (30–60 °C) during the assays. The starch solution was prepared using a magnetic stirrer/heater plate (MAG-H, Gerhardt, Königswinter, Germany). The tested samples were sonicated by a Branson sonifier (SLPe, Danbury, CT, USA) operating at a frequency of 40 kHz with a maximum output power of 150 W, and equipped with a 3.2 mm microtip probe (PN: 109-122-1065), using the following settings: amplitude: variable (10–70%); duty cycle: emission during 10 s followed by a pause of 5 s; and total time: 10 min. For the amplitude of 70%, the maximum instantaneous power density was 242.5 W $cm^{-2}$. Temperatures of hydrolysis milieu were measured using a digital thermometer (TM 65, Crison Instruments, Alella, Spain) equipped with a temperature-dependent resistance probe (Pt 1000, Crison Instruments).

Absorbance measurements of the starch-iodine complex were carried out at room temperature (~23 °C) under microplate format (96-well microplates, well volume ~ 340 μL, Thermo Fisher Scientific, Waltham, MA, USA), by using a Synergy HT reader (Bio-Tek Instruments, Winooski, VT, USA) controlled by Gen 5 software (Bio-Tek Instruments). The absorbance values at 580 nm were recorded 1 min after the insertion of the microplate into the reader.

### 2.3. Activity assay

The protocol sequence used to measure the activity of alpha amylase studied is illustrated in Fig. 1. First, 1500 μL of soluble starch (2000 mg $L^{-1}$) and 1500 μL of enzyme solution, comprising