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The intensification of amyloglucosidase-based saccharification by ultrasound

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ABSTRACT

The present report studied the role of ultrasound (US) energy in the amyloglucosidase-based starch hydrolysis using two complementary approaches: (i) in the activity of six commercially-available amyloglucosidases (using soluble starch as substrate), and (ii) in the hydrolysis of four pure starches from different botanical sources. This corresponds to the first systematic evaluation of the role of US in starch hydrolysis mediated by amyloglucosidase, being a consequence of our previous report that assessed the effect of US in the activity of alpha-amylase (LWT – Food Science and Technology 84 (2017) 674–685). Regarding amlyloglucosidases, three enzymes obtained from *Aspergillus niger* (AN1-AN3), and Spirizyme Achieve (SPA), Spirizyme Fuel (SPF) and Spirizyme Ultra (SPU) were submitted to a Box-Behnken experimental design in order to establish the optimum conditions for their maximum activity. In the presence of US, we found both inactivation and activation, ranging from -88% (AN3) to 699% (SPA). The US promoted the enzyme activity when combined with lower temperatures (40–60 °C), with a marked effect in Spirizyme enzymes. Based on the optimum conditions established by the experimental design, we also evaluated the role of US in the glucose yield resulting from the hydrolysis of pure starches (corn, rice, potato, wheat). In this case, US led to higher glucose yields in all conditions tested. The enhancement factors observed ranged from 1.2 (AN1, rice starch) to 65 (SPA, potato starch) times. We compared these findings with previous reports, which highlighted the role of US in intensifying amyloglucosidase-based saccharification in mild conditions, by simultaneously influencing both enzyme and substrate. Hence, US power has to be fine-tuned for each particular enzyme in order to maximize process intensification.

1. Introduction

Starch is a natural polymeric carbohydrate produced by green plants. Beyond its energetic storage role for plants, starch is also a ubiquitous raw material with multiple and distinct applications [1]. From the food and feed sectors, to the pharmaceutical and paper industries, starch is a key raw material with economical relevance, being responsible for a turnover of 7.6 billion euros in 2016 in the European Union [2].

The partial or total hydrolysis of starch in its sub-units (glucose) is a common process to prepare starch-based products, as well to control the starch content in different vegetable matrices [3]. Although different strategies could be used for processing starch [4], this hydrolytic process commonly resorts in the use of enzymes capable to disrupt the glycosidic bonds of the polymer in mild conditions [5]. This process is based on the complementary action of two enzymes: alpha-amylase and amyloglucosidase, responsible for the cleavage of glycosidic bonds (α-1,4 for alpha-amylases and α-1,4/ α-1,6 for amyloglucosidase, respectively) [6].

In this context, the application of ultrasound (US) energy can be an additional tool to control enzyme activity, and consequently, the overall process. The cavitation phenomena originated by US transducers can play a key role in the modulation of enzyme activity due to its potential influence in the physical and chemical parameters of both enzyme and substrate [7,8]. The effect of US in enzyme-based systems may lead to either activation or inactivation of the enzymes due to its physical, chemical and mechanical effects [9–11]. Regarding starch hydrolysis, the study of the effects of US on the enzyme activity is described for both alpha-amylase and amyloglucosidase. For alpha-amylase, US energy is described as a potential activator or inactivator [12]. Indeed, this behavior can be a result of the combination of multiple parameters such as the heat tolerance of the enzyme or the potential formation of chemical species with high oxidant potential [13,14]. In the case of amyloglucosidase, the knowledge about the influence of US on its activity is scarce.

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To date, only a few reports assessed the potential influence of US in the enhancement of the saccharification process. The results reported by Leaes et al. [15] for the influence of US (using a US bath) in the commercial amyloglucosidase from Spirizyme Fuel showed a consistent activation pattern along all the experimental conditions tested, with particular impact at low temperatures (< 40 °C). Using mixtures of alpha-amylase and amyloglucosidase, the presence of US was associated with a process intensification, with an average increase of 40% in the total reducing sugars yield during the hydrolysis of cassava waste samples [16]. Khanal et al. [17] also reported a nearly 20-fold reduction in particle size of corn starch when sonicated at different levels of US power in the presence or absence of a commercial mixture of amylolytic enzymes (Stargen). The same enzyme mixture was also applied to the US assisted hydrolysis of yam [18] and yields (content of fermentable sugars) increased 25% due to the sonication of the mixture when the process was conducted at 60 °C. The recent contribution of Wang et al. [19] also highlighted the role of US in assisting the amyloglucosidase mediated hydrolysis of potato starch. US acted in both substrate and enzyme, by improving solubilization and swelling of starch, and also by accelerating mass transfer to the active site due to structural modifications of the enzyme.

Regarding the current state of the art, where information about the influence of US in the activity of amyloglucosidase is limited to some enzymes and substrates, commonly associated with experimental conditions insufficiently detailed and incomparable, we aimed a systematic assessment of US effect in the activity of amyloglucosidase enzymes. To this end, we selected six different commercially-available enzymes used in laboratory and industrial environments, which were submitted to different experimental conditions in a systematic fashion through a Box-Behnken design [20]. Furthermore, we complemented this evaluation with a second study of the effect of US in the glucose yield of US-assisted amyloglucosidase-based starch hydrolysis of four pure starches from different botanical sources (rice, potato, corn, wheat).

2. Experimental

2.1. Reagents and solutions

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 (2.000 g L^{-1}) resulted from the dissolution of 1.00 g of soluble starch (PN: S9765; Sigma-Aldrich, St. Louis, MO, USA) in 500 mL of water. To ensure a complete dissolution, soluble starch was dispersed in 5 mL of water and added to a portion of the final volume (~400 mL) of boiling water under continuous stirring until complete dissolution (a clear solution was obtained). Starch standards were prepared by dilution from the stock solution.

KI/I_2 solution was prepared by dissolving 200.0 mg of KI (Merck, Darmstadt, Germany) in ~60 mL of ultra-pure water, followed by the addition of 20.0 mg of I_2 (Sigma-Aldrich). The final volume of this solution was 100 mL. Sonication by an ultrasound bath accelerated the complete dissolution of I_2.

The universal buffer solution was composed of 2.91 mL of 85% (w/w) *o*-phosphoric acid (Sigma-Aldrich), 2.86 mL of acetic acid (Sigma-Aldrich) and 3.09 g of boric acid (Chem-lab, Zedelgen, Belgium) in a final volume of 1000 mL. The desired pH values were adjusted to the corresponding target values by adding the suitable volumes of a 4.0 mol L^{-1} NaOH solution (Fisher Scientific, Waltham, MA, USA).

Four pure starches from rice (PN: S7260), corn (PN: S4126), potato (PN: S4251) and wheat (PN: S5127) were obtained from Sigma-Aldrich. A stock solution of 1000 mg L^{-1} of D-Glucose (Sigma-Aldrich) was prepared by dissolving the solid in the respective volume of water. A working solution of 200 mg L^{-1} prepared by diluting the stock solution, and then used to prepare the microplate standard solutions. Glucose concentrations were determined using the Glucose Oxidase/Peroxidase (GOPOD) assay supplied as single reagent kit by Megazyme (Wicklow, Ireland).

Table 1
Estimated regression coefficients of the BBD model for the activity of amyloglucosidase from *Aspergillus niger* (AN1).

	Coef	SE Coef	T[a]	P
pH	0.331103	0.729596	0.454	0.681
Temperature	0.334103	0.268312	1.245	0.301
US	−0.02612	0.009694	−2.694	0.074
pH * pH	0.103761	0.035158	2.951	0.060
Temperature * Temperature	−0.027281	0.027174	−1.004	0.389
US * US	0.000169	0.000068	2.485	0.089
pH * Temperature	−0.008007	0.001032	−7.755	0.004
pH * US	−0.000537	0.001121	−0.479	0.664
Temperature * US	−0.018718	0.005614	−3.334	0.045

S[b] = 0.003789, R^2 = 99.1%, R^2 (adj) = 97.6%.
[b]Standard error of the noise.
[a]Normalized regression coeffcient.

Six commercially-available amyloglucosidases were studied throughout this work (Table S1): three amyloglucosidases from *Aspergillus niger* supplied by Sigma-Aldrich: AN1 (PN: A9913), AN2 (PN: A7095), AN3 (PN: 10115); and other three amyloglucosidases supplied by Novozymes (Bagsvaerd, Denmark): Spirizyme Achieve (SPA), Spirizyme Fuel HS (SPF), Spirizyme Ultra (SPU). Enzyme working solutions were obtained by stepwise dilution of the commercial solution. Details about the enzymes and dilutions used for each enzyme were compiled in Table S1.

For stopping the enzyme hydrolysis, a 1.0 mol L^{-1} HCl solution obtained from the dilution of a 37% (w/w) commercial solution (Panreac, Barcelona, Spain) was used.

2.2. Apparatus

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 1/8″ 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 sonication time: 10 min. For the amplitude of 70%, the maximum instantaneous power density was 242.5 W cm^{-2} (the individual power values applied are described in the experimental Tables S2–S7). The temperature (30–80 °C) of the activity assays was controlled through a block heater (Stuart SBH130D/3, Staffordshire, UK). Samples resulting from the hydrolysis of insoluble starches were centrifuged by an Astor 8 centrifuge (Astori Tecnica, Poncarale, Italy) at 350*g* during 5 min.

Absorbance measurements were performed at the desired wavelength under microplate format (96-well flat bottom 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). Wavelengths were set according to the assay performed: amyloglucosidase activity or glucose yield (more details at the description of each assay).

2.3. Amyloglucosidase activity assay

The protocol for the determination of the activity of amyloglucosidase was based on our previous work with alpha-amylase [13] (Fig. S1). In brief, 1500 μL of soluble starch stock solution (2.00 g L^{-1}) were mixed with the same volume of enzyme solution into a 4 mL amber glass vial (e.g. Sigma-Aldrich, PN: 27217, 15 mm × 45 mm × 8 mm). The composition of the 1500 μL of enzyme solution added to each vial was defined by a variable volume of enzyme working solution (1500 μL for AN3 and 500 μL for all other enzymes) and the remaining volume of universal buffer solution at the same pH (0 μL for AN3 and 1000 μL for all other enzymes). This mixture was homogenized and sonicated during 10 min