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Ultrasound processing of amyloglucosidase: impact on enzyme activity, stability and possible industrial applications

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ABSTRACT. This study evaluated the effect of ultrasound processing as a pre-treatment of amyloglucosidase on the enzymatic activity and stability. The activity was evaluated under optimal (65°C/ pH = 4.5) and non-optimal conditions of temperature and pH and its stability was evaluated during storage at 8°C. The enzyme solution was processed at 9.5 W L^{-1}, 40 kHz, 23°C and at pH 3.5, 4.5, and 5.5, for up to 120 min. The activity was measured at 35, 65 and 80°C. The US process was able to increase, reduce or not alter the enzymatic activity, depending on the conditions applied. These modifications depended on the pH of the enzyme solution, the ultrasound processing time and the activity temperature. In different ultrasound conditions, mainly at 35 and 65°C, the enzyme activity did not change, demonstrating that this technology can be used for other purposes, such as microbial inactivation, without affecting the enzyme. The activity increase (up to 15%) occurred under non-optimal pH and temperature conditions (pH 3.5 or 5.5/ 80°C), suggesting that ultrasound promoted stabilization and enzymatic protection. This result is interesting in the starch saccharification, which requires the enzymatic reaction at high temperatures. Therefore, such results can increase the application range of this enzyme in different industrial applications.

Keywords: ultrasonics; pre-treatment; enzymatic solutions; enzymatic activity; activation.

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Introduction

A*myloglucosidase/* glucoamylase (AMG) (EC 3.2.1.3) is an enzyme that hydrolyzes α-1,4 and α-1,6 glycosidic bonds from the non-reducing ends of starch and dextrins, producing glucose (Lin, Felberg, & Clark, 1993). Its main application is the starch saccharification, for the production of glucose for different industries: from the production of food glucose to obtaining raw material for ethanol production, which can be used in the production of perfumes, medicines, alcoholic beverages and others (Tribst & Cristianini, 2012). In addition, the AMG use in the juice industry is increasing, in order to reduce the starch content in certain fruits (Ribeiro, Henrique, Oliveira, Macedo, & Fleuri, 2010).

However, the use of enzymes for large-scale industries presents two obstacles: high production cost and low stability under process conditions that use for example, high temperature, diverse pH values, affecting enzymatic activity. (Patel, Singhania, & Pandey, 2016). Thus, several alternatives are being developed to minimize these limitations, such as enzyme immobilization, genetic engineering (Patel et al., 2016) and emerging technologies, such as high pressure, microwaves and ultrasound, which began to be studied to promote enzymatic activation and stabilization (Tribst & Cristianini, 2012; Mazinani & Yan, 2016; Dalagnol, Silveira, Silva, Manfroi, & Rodrigues, 2017).

The ultrasound (US) is a technology which consists of using acoustic energy, with frequencies higher than 20 kHz. US has been proposed in food processing and preservation, as well as improving operations involving heat and mass transfer. (Chemat, Huma, & Khan, 2011). A new focus on the ultrasound application is its effect on enzyme structure and activity, with several studies and different approaches (Islam, Zhang, & Adhikari, 2014; Huang et al., 2017). However, there is still a need for studies of US processing of AMG.

In fact, most of the studies in the literature use ultrasound to promote the inactivation of undesirable food enzymes (Sulaiman, Soo, Farid, & Silva, 2015; Arroyo, Kennedy, Lyng, & O'Sullivan, 2017). On the

other hand, ultrasound technology can not only inactivate, but also activate certain enzymes, depending on the process conditions (Nadar & Rathod, 2017). Since various enzymes catalyze desirable reactions, it is of interest to improve or increase enzymatic efficiency. Moreover, depending on the process conditions, ultrasound technology may not affect the enzymatic activity. In this case, the application of ultrasound aims to ensure the microbiological quality of the enzymatic solution, without interfering with the speed of the reaction (Soares et al., 2019), since several studies have demonstrated its ability to inactivate microorganisms (Betts, Williams, & Oakley, 2014; Huang et al., 2017).

Furthermore, ultrasound can promote enzymatic reactions on different approaches: assisting the reaction, as a pre-treatment to the enzyme or as a pre-treatment to the substrate (Wang et al., 2018). In the literature, most studies evaluate the effect of ultrasound during the enzymatic reaction. For instance, the works of Leaes et al. (2013), Wang et al. (2017), and Oliveira, Pinheiro, Fonseca, Cabrita, and Maia (2018) studied the amyloglucosidase reaction assisted by ultrasound, but the effect of this technology as a pre-treatment of enzymatic solution was not evaluated. This can be an interesting approach for the enzyme production to enhance the further application. Moreover, it is important to evaluate the effect of ultrasound on the enzyme isolated, since the application of this technology could lead to an increase in enzymatic activity or to ensure microbiological quality of commercial enzyme solutions.

Therefore, the need to better understand the effect of ultrasound technology on the activity of enzymes of commercial interest, under different conditions, is highlighted. Consequently, the objective of this work was to evaluate, for the first time, the effect of ultrasound processing as pre-treatment of AMG solution on the further enzyme activity under optimal and non-optimal conditions of pH and temperature, as well as on the stability of the enzymatic solution under activation conditions during storage at 8°C.

Material and methods

Ultrasound reactor

During the experiments, an ultrasonic bath (Unique, model USC 2800 A, Indaiatuba, Brazil) with a frequency of 40 kHz and a volumetric power of 9.5 W/L (determined following the method described by Tiwari, Muthukumarappan, O'Donnell, and Cullen, (2008) was used. The temperature was controlled using a stainless-steel heat exchanger coupled to the ultrasonic bath and to an external bath, to maintain the temperature at 23 during the entire process.

Determination of amyloglucosidase activity and optimum pH and temperature conditions

Amyloglucosidase produced by Aspergillus *niger* was obtained from Prozyn Biosolution (Butantã, Brazil).

To determine the optimum pH and temperature of the enzyme, the AMG activity was performed at pH values of 3.5, 4.0, 4.5, 5.0 and 5.5, and at temperatures of 50, 55, 60, 65 and 70°C (in a water bath). Amyloglucosidase activity was determined according to Soares et al. (2019) with some modifications. The reducing groups released by the enzyme action were determined using the 3,5-dinitrosalicyclic acid (DNS) method proposed by Miller (1959).

To this end, 0.5 mL of the enzymatic solution (0.1 g L^{-1} in 0.05 mol L^{-1} sodium acetate buffer at different pHs) was added to 0.5 mL of the soluble potato starch solution (Êxodo, state São Paulo, Brazil at 10 g L^{-1}) in 0.05 mol L^{-1} sodium acetate buffer at respective pHs. The mixture was incubated for 10 min. at different temperatures and pHs. Then, all steps such as stop the reaction, spectrophotometer reading, standard curve construction were performed in the same way as Soares et al. (2019).

The condition with the highest activity (pH and temperature) was established as optimum with 100% of the enzymatic activity. The relative enzymatic activity was calculated based on the optimum condition.

Effect of ultrasonic processing on enzyme activity

The AMG solution was processed using the ultrasound technology, in different conditions. Then, after ultrasound processing, the enzyme activity and stability were evaluated. This approach simulated a possible procedure for the enzyme producer, similarly to those proposed by Tribst, Augusto, and Cristianini (2013): the enzyme solution pre-treatment would simulate a possible procedure to obtain a different product by enhancing its properties (for example: increase of the enzyme activity, thermal resistance, stability or activity at specific conditions, or even guarantee microbial stability without using additives). For instance,

this approach has been studied for other technologies, such as the high pressure homogenization (Tribst et al., 2013) or high hydrostatic pressure (Leite Júnior, Tribst, & Cristianini, 2017). However, the use of ultrasound with this objective is rare in the literature. Moreover, this approach is different to the proposal of reaction assisted by ultrasound, such as those described by Leaes et al. (2013), Wang et al. (2017), and Oliveira et al. (2018).

The ultrasonic bath was filled with 6.5 L of the enzyme solution (0.1 g L^{-1}, in 0.05 mol L^{-1} sodium acetate buffer) at different pHs (3.5, 4.5 and 5.5) and processed at temperature of 23°C. After 5, 15, 30, 60, 90 and 120 min. of process, 10 mL of the enzymatic solution were collected, and the enzymatic activity was performed. The activity was determined at temperatures of 35, 65 and 80°C (in a water bath). As a control, 10 mL of an unprocessed aliquot was collected, and its activity was analyzed under the same conditions as the processed samples.

After ultrasound processing the enzymatic activity analysis was performed according to the procedures described in section 2.2 and the relative enzymatic activity after processing was calculated considering the non-processed samples under the same conditions of pH and temperature.

Evaluation of enzymatic stability

The evaluation of enzymatic stability was performed in the conditions that ultrasound resulted in activation. The enzymatic activity was measured after storing the enzyme solutions under refrigeration (8°C) for 24 hours, according to Tribst and Cristianini (2012), in order to assess whether the activations are reversible. The relative enzymatic activity after storage was calculated considering the activity after the storage processing and right after ultrasonic processing, at the same pH and temperature.

Experimental design and statistical analysis

Experimental design and statistical analyzes were performed as Soares et al. (2019).

Results and discussion

Determination of amyloglucosidase activity and optimum pH and temperature conditions

The AMG activity at different temperature and pH is shown in Figure 1. The optimal condition of the enzyme (higher activity) was determined at a temperature of 65°C and pH 4.5. At this condition the activity was 32688.8 U g^{-1} of enzyme, which was considered as 100%. These results are similar to those found by Tribst and Cristianini (2012) .

Temperature and pH variation resulted in significant changes in enzyme activity, promoting a reduction of 43.3% at 50°C (pH 3.5) and 68.6% at 70°C (pH 5.5). In general, the AMG activity was almost constant in the evaluated pH range, when at temperatures below 65°C. It indicates that the high temperature associated with extreme pH (in relation to the optimum conditions) promotes the loss of enzymatic activity (Vandersall, Cameron, Nairn, Yelenosky, & Wodzinski, 1995; Liu et al., 2015). Liu et al. (2015) demonstrated that heating at temperatures ⩾ 70°C promotes unfolding of the α-helix, which is often correlated with the loss of activity. In addition, at high temperatures, tryptophan residues were partially exposed, indicating changes in the tertiary structure of the enzyme.

After defining the optimum conditions of AMG activity (pH 4.5 and 65°C, respectively), three conditions of pH were chosen for processing: 4.5, 3.5 and 5.5. In each one, three conditions of temperature were selected for analysis: 65, 35 and 80°C.

Table 1 shows the results of the unprocessed AMG activity, performed under different pH and temperature conditions. It was found that temperature promoted significant changes in the activity. In temperatures higher than the optimum condition (increase from 65 to 80°C), a lower activity was observed in the different pH values [reduction of 79.7 (pH 4.5) to 90.3% (pH 5.5)]. Regarding pH, the greatest reductions on the AMG activity were observed by increasing the pH from 4.5 (optimum) to 5.5 [reduction from 27.7 (at 65°C) to 65.6% (at 80°C)], with more drastic structural changes.

Different capital letters indicate a significant difference ($p < 0.05$) between the different temperature of the samples and different lowercase letters indicate a significant difference ($p < 0.05$) between the different pH of the sample. Mean ± standard deviation of nine replicates.

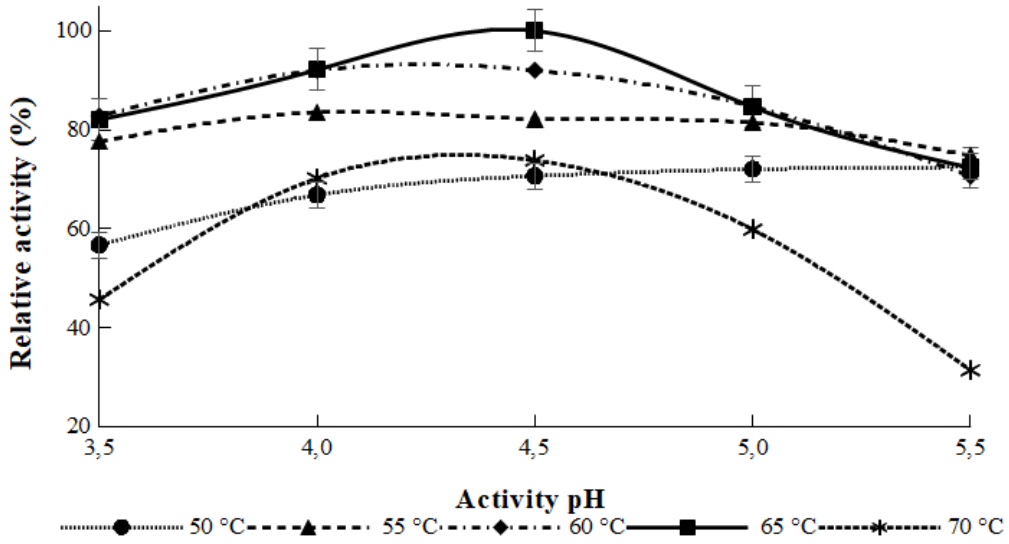

Figure 1. Effect of pH and temperature on AMG activity. Vertical bars represent standard deviation.

Table 1. Enzymatic activity (U g^{-1}) of unprocessed amyloglucosidase under different pH and temperature conditions.

Process and analysis pH	Analysis Temperature (°C)		
	35	65	80
3.5	10759.7 ± 299.5 b,B	26804.8 ± 662.0 b,A	5363.7 ± 18.1 b,C
4.5	12474.5 ± 295.6 a,B	32668.8 ± 772.0 a,A	6648.3 ± 78.4 a,C
5.5	11289.7 ± 356.8 b,B	23634.1 ± 158.9 c,A	2288.2 ± 37.2 c,C

Effect of ultrasonic processing on enzyme activity

In order to evaluate the effect of ultrasound on AMG activity, the enzymatic solutions were processed with ultrasound under the same pH conditions as the control samples (pH 3.5, 4.5, 5.5, 9.5 W L^{-1}, 40 kHz, 23ºC for up to 120 min.). Subsequently, the AMG activity of processed and unprocessed was measured at the temperatures of 35, 65 and 80°C.

Figure 2 shows the relative enzymatic activity after AMG processing, demonstrating that the US process promoted changes in amyloglucosidase activity: ultrasound increased, reduced or did not change the enzyme activity. These results were dependent on the pH of the enzymatic solution, the ultrasound time applied and the temperature of the enzymatic activity.

Ultrasound can be used throughout three targets concerning the enzymatic reactions: mixed reaction system, enzyme and substrate (Wang et al., 2018). Therefore, different factors can explain the changes on enzyme activity due to ultrasound.

Firstly, ultrasound can change the enzyme molecule. The physical (high temperature and pressure) and chemical (free radical formation) effects of the ultrasonic process promote changes in the enzyme's conformational structure, leading to modification in enzymatic activity (Rojas, Trevilin, Funcia, Gut, & Augusto, 2017). The physical (high temperature and pressure) and chemical (free radical formation) effects of the ultrasonic process promote changes in the enzyme's conformational structure, leading to modification in enzymatic activity. Therefore, the energy added to the system by US process can result in a change in the interactions responsible for the native conformation of the enzyme, as electrostatic forces, Hydrogen bonging, Van der Waals interactions (Chemat et al., 2011). Each conformational change can increase or decrease the enzymatic activity.

In fact, Yu, Zeng, and Lu (2013) applied ultrasound as a pre-treatment and reported a change in the secondary structure of tyrosinase, with increased β-sheet content, which resulted in an increase in the enzyme activity by a better structural stabilization, activation of isoenzymes or exposure of new active sites. In another study, also applying the ultrasound as a pre-treatment, the pepsin activation was correlated with changes in the secondary structure showing the lower β-sheet content and higher β-turn, as well as changes in the tertiary structure showing an increase in fluorescence intensity of tryptophan (Yu, Zeng, Zhang, Liao, & Shi, 2014). However, in the same study, a reduction was observed in the activity of α-amylase and papain notwithstanding the same process conditions. Therefore, it is not possible to establish a rule about the effect of ultrasound process on enzymes, since the enzymes present different characteristics and resistances due to several factors such as differences in the amino acid sequence and in the enzymatic structure (Islam

et al., 2014), as well as the various ultrasonic processing conditions. Consequently, it is important to evaluate the effect of ultrasonic processing on different enzymes of interest. No study applied the ultrasound as a pre-treatment in the AMG enzyme.

Secondly, ultrasound can change the substrate. In fact, ultrasound can promote the depolymerization of starch with the release of glucose (Chemat et al., 2011), which can facilitate the interaction enzyme-substrate.

Finally, ultrasound can enhance the reaction, reducing the limiting barrier of diffusion between enzyme and substrate, which increases the mass transfer in the reaction. Acoustic cavitation, which is the main effect of ultrasound, generates a large amount of energy, which results in acoustic streaming transmitted to the system (Nadar & Rathod, 2017; Wang et al., 2018).

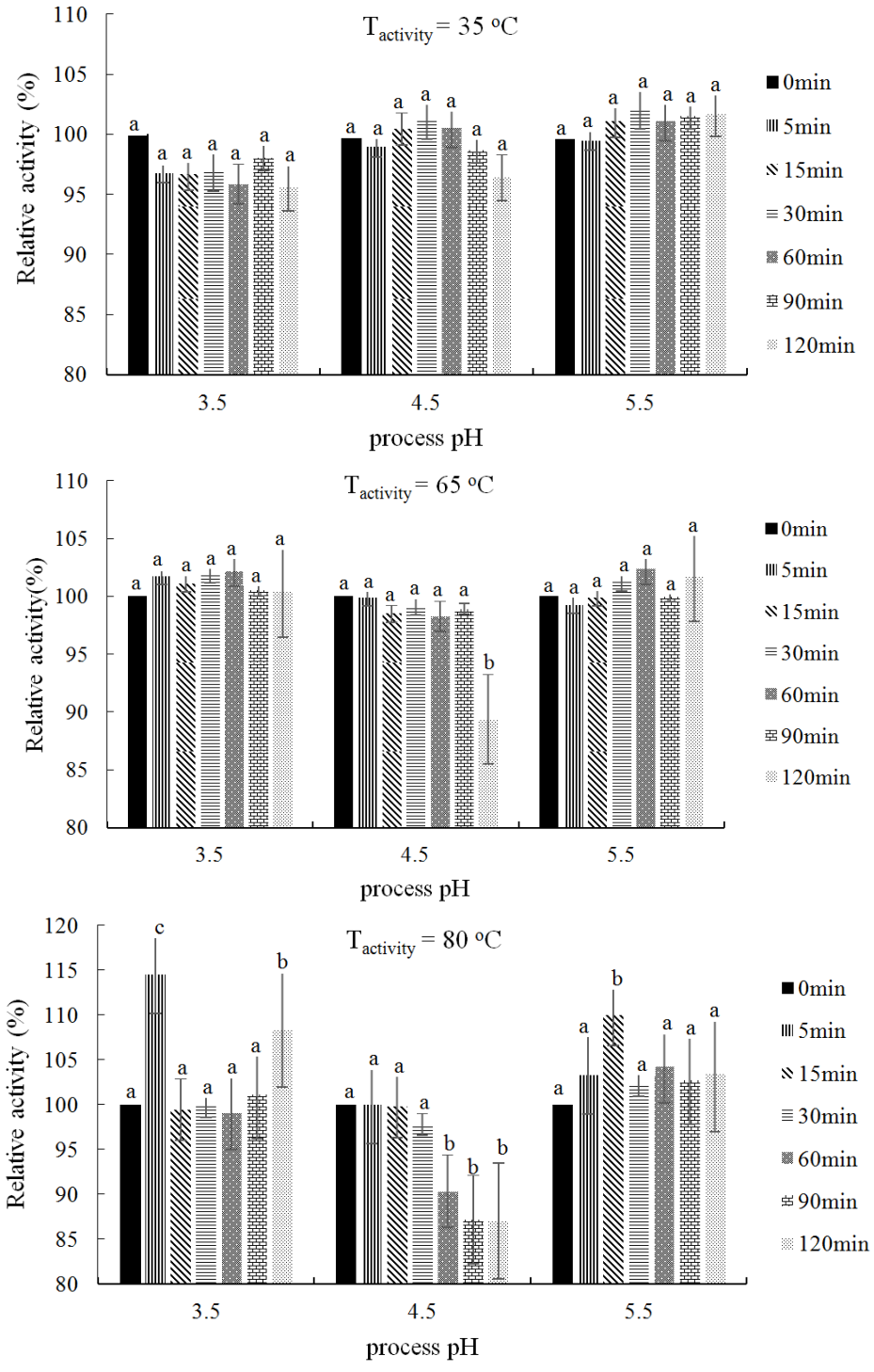

Figure 2. Relative enzymatic activity after AMG processing (REAP) at different temperatures immediately after the ultrasonic process at 23°C at different pH values and processing time. Equal letters between the unprocessed sample and processed samples at different times at the same pH and temperature did not differ statistically at 5% probability by the Tukey test.

For instance, Leaes et al. (2013), Wang et al. (2017), and Oliveira et al. (2018 studied the amyloglucosidase reaction assisted by ultrasound, but not the effect of this technology as a pre-treatment of enzymatic solution. However, as the reaction and modification of both enzyme and substrate happens simultaneously, it is difficult to describe how ultrasound enhances the enzymatic reaction - highlighting the importance of the present work.

Furthermore, there are other important differences between the results obtained in the present work and those of the study of Leaes et al. (2013), and Oliveira et al. (2018).

Leaes et al. (2013) verified higher products formation during hydrolysis in the ultrasonic bath up to 70°C, whereas, we verified an increase in the enzymatic activity when measured at 80°C after the ultrasonic bath processing at 23°C. These results show that the effects of ultrasound on the AMG are different when the enzyme solution is processed or when the reaction is performed under ultrasound.

In this context, the study of Oliveira was conducted using a probe ultrasound. Despite the positive result, the scale up of probe applications at the industrial level is difficult, limiting its relevance for this purpose (Gogate & Kabadi, 2009), in special due to the intense wear and acoustic field distribution across the reactor. In addition, the authors observed that the US increased products formation when combined with lower temperatures (40-60°C), indicating that the effect of ultrasound on the AMG is dependent of the equipment type, the conditions applied and the form applied (only in the enzymatic solution or in the reactions carried out under ultrasound). In fact, even the same amount of added ultrasonic energy, though considering different systems, can result in opposite behaviour (Rojas, Trevilin, & Augusto, 2016), due to differences on the acoustic field distribution.

Complementarily, from the industrial point of view, it is difficult to perform the reaction under ultrasound, which would be expensive due to the processed volume, limiting the process viability. In this way, the pre-treatment by ultrasound only in AMG can be an alternative to increase the economic viability of the process aiming at higher compound production with less economic and technological cost.

The main application of amyloglucosidase is the starch saccharification, in which the starch needs to be preheated at higher temperatures (95-105°C) and the pH needs to be adjusted to 5.8-6.5 to promote the liquefaction by α-amylase activity (Crabb & Mitchinson, 1997). Consequently, it would be interesting to activate the enzyme at high temperatures and pHs, resulting in time and energy savings. In addition, another possible application of amylases is together with acidic pectinolytic enzymes, in order to promote juice clarification (Lee, Yusof, Hamid, & Baharin, 2006; Kothari, Kulkarni, Maid, & Baig, 2013). In this context, the activation of amyloglucosidase at lower temperatures is also important, since pectinases perform better at temperatures of 30-50°C (Kashyap, Vohra, Chopra, & Tewari, 2001).

When the AMG activity was evaluated at 35 and 65°C, the ultrasound processing did not cause any changes in its activity, regardless of the pH ($p > 0.05$, Figure 2), except when the enzyme was processed at pH 4.5 for 120 min., and the activity was evaluated at 65°C. In this case, a reduction of 10% ($p < 0.05$) in its activity was observed (Figure 2). On the other hand, when the activity was evaluated at 80°C, there were changes in the enzyme activity after applying the ultrasound in the three evaluated pHs ($p < 0.05$, Figure 2). Samples processed at pH 3.5 showed a 15 and 8% increase after 5 and 120 min. of ultrasound application, respectively. At pH 4.5, there was a decrease in the relative activity, with a maximum reduction of 13%. Finally, at pH 5.5, there was a 15% increase in activity after 15 minutes of pre-treatment ($p < 0.05$, Figure 2).

In this context, US process at 23°C may have promoted stabilization/enzymatic protection, reducing the loss of activity at high temperatures (80°C). This activation may be interesting when the enzyme is applied to the saccharification of starch. It allows the enzymatic process to be carried out at a temperature higher than the optimal temperature, resulting in energy and time savings and, thus, meeting the industrial demand.

Moreover, according to Svensson, Svendsen, Svendsen, Sakai, and Ottesen (1982), *Aspergillus niger* produces two forms of amyloglucosidase (isoenzymes), both found in commercial enzymatic solutions, in different proportions. These forms are designated as amyloglucosidase I and II, and they have different characteristics, such as amino acid composition, molecular weight, carbohydrate content and amide grouping, resulting in different properties (Svensson et al., 1982). Therefore, the possible presence of two isoenzymes in the commercial solution used in this study may also explain the difference found in AMG behavior, such as activations at different times of ultrasound (5 and 120 min.) in the same process/ activity condition (pH = 3.5 temperature 80ºC).

Finally, the maintenance of AMG activity in several conditions after ultrasonic processing is also an interesting result. Several studies have already demonstrated the microbial inactivation capacity of ultrasound (Betts et al., 2014; Huang et al., 2017). Therefore, ultrasound could be used to ensure the microbiological quality of the commercial enzymatic solution during storage, without interfering in the efficiency of the reaction performed later (Soares et al., 2019). In fact, amyloglucosidase can be commercialized as solution, which facilitates its application but results in stability problems. Microbiological control is often guaranteed with the use of preservatives, such as potassium sorbate and sodium benzoate. Although these preservatives have been accepted as safe, they have been avoided by the consumer, resulting in the need to develop new approaches to ensure the enzyme solution microbiological stability without using additives. Among several emerging technologies studied for this purpose, ultrasound technology can be an alternative (Soares et al., 2019).

It is important to determine the enzymatic stability in order to assess whether the activations caused by the US processing are transient or permanent to identify possible ways to maintain the enzymatic activation after the process. However, most studies have not addressed this feature. The enzymatic stability of the AMG activated by the US process, under different processing conditions, was evaluated after one day of storage at 8°C. The REAS results are shown in Figure 3.

The results obtained in Figure 3 show that the unprocessed enzyme presented no significant difference in activity during storage at pHs 3.5 and 5.5, indicating that AMG was stable when kept in solution at 8°C for 24 hours. However, the processed enzymes show a decrease in activity after storage, returning to close to 100% activity.

Therefore, we can conclude that although ultrasound provides an increase in AMG activity, these changes were reversible. Equal activity for the processed and unprocessed enzymes indicates that the processed AMG returned to its native configuration after a rest period. Consequently, if ultrasound is proposed as a method to increase AMG activity, this should be performed just before the application.

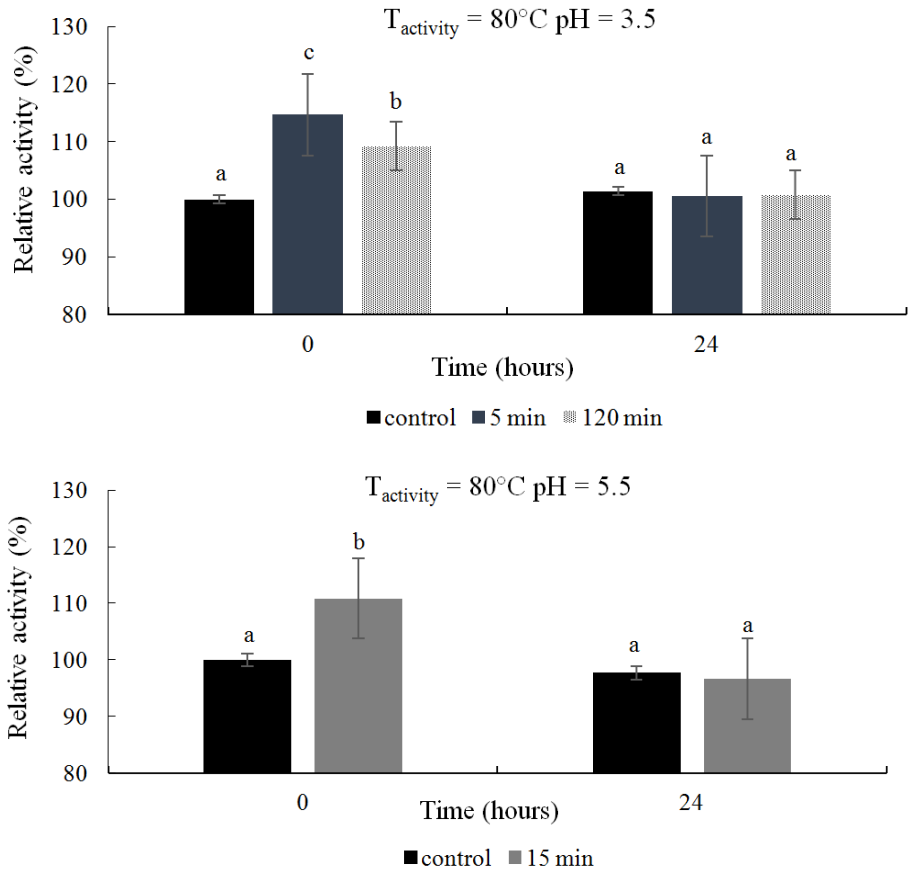

Figure 3. Amyloglucosidase activity activated by the US process after 24h of storage at 8°C (REAS). Equal letters between unprocessed and processed samples at different analysis times at the same pH and temperature did not differ statistically at 5% probability by the Tukey test.

Conclusion

Ultrasound was able to increase, maintain or decrease amyloglucosidase activity after processing. The changes depended on the pH of the enzyme solution, processing time and activity temperature. Activations occurred at 80ºC, suggesting that US promoted stabilization. This result is interesting in the application of starch saccharification, which requires the active enzyme at high temperatures. Therefore, the results show that US can increase AMG activity, especially in non-optimal pH and temperature conditions, increasing the range of applications of this enzyme in the industry. However, further studies need to be performed to increase enzymatic stability of the activated enzymes during storage.

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