Title: Retrogradation behavior of corn starch treated with 1,4- α -glucan branching enzyme

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Abstract

The retrogradation behavior of corn starch treated with 1,4- α -glucan branching enzyme (GBE) was investigated using rheometry, pulsed nuclear magnetic resonance (PNMR), differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR). Dynamic time sweep analysis confirmed that the storage modulus (G') of corn starch stored at 4 °C decreased with increasing GBE treatment time. PNMR analysis demonstrated that the transverse relaxation times (T_2) of corn starches treated with GBE were higher than that of control during the storage at 4 °C. DSC results demonstrated that the retrogradation enthalpy (ΔH_r) of corn starch was reduced by 22.3% after GBE treatment for 10 h. Avrami equation analysis showed that GBE treatment reduced the rate of starch retrogradation. FTIR analysis revealed that GBE treatment led to a decrease in hydrogen bonds within the starch. Overall, these results demonstrate that both short- and long-term retrogradation of corn starch were retarded by GBE treatment.

Key words: Corn starch, 1,4- α -Glucan branching enzyme, Short-term retrogradation, Long-term retrogradation

Chemical compounds studied in this article

Sodium hydroxide (PubChem CID: 14798); Potassium bromide (PubChem CID: 253877).

1. Introduction

Starch retrogradation is a process in which starch molecules in an amorphous state undergo transformation to a more ordered crystalline state (Farhat, Blanshard, & Mitchell, 2000; Kohyama & Nishinari, 1991; Xie, Hu, Jin, Xu, & Chen, 2014). The transformation can be divided into two stages: short-term and long-term retrogradation. Short-term retrogradation is mainly caused by the aggregation of amylose, which occurs during the early stage of storage. Long-term retrogradation is more closely related to the recrystallization of amylopectin, which usually requires a longer storage time (Banchathanakij & Supphantharika, 2009; Yao, Zhang, & Ding, 2003). Starch retrogradation usually results in a decline in the quality of starch-based foods. Consequently, many methods have been used to inhibit starch retrogradation, including physical treatment (Khunae, Tran, & Sirivongpaisal, 2007), chemical treatment (Adebowale & Lawal, 2003) and enzymatic treatment (Cai & Shi, 2010). Among these methods, enzymatic treatment is receiving greater attention because it can make retrogradation resistance a property inherent to starch, so that the resistance is retained through processing.

One strategy to produce an enzyme-modified, retrogradation resistant starch is to increase the ratio of amylopectin to amylose. This leads to slower retrogradation because amylopectin has a slower recrystallization rate than amylose. The enzyme that forms the basis of this strategy is 1,4- α -glucan branching enzyme (GBE, EC 2.4.1.18), which belongs to the α -amylase family. GBE is known to cleave the α -1,4 glucosidic linkage of an existing glucan chain and transfer the cut end to the

6-position of a glucose residue within the cleaved chain or within another glucan chain, creating an α -1,6 glucosidic linkage (Devillers, Piper, Ballicora, & Preiss, 2003; Kajiura et al., 2011). GBE has been widely used in previous studies to modify a variety of polysaccharides. The modified products include highly branched starch (Le et al., 2009; Takata et al., 2010), highly branched dextrin (Lee, Yoo, Ryu, Kim, & Yoo, 2008) and cyclic glucans (Takata, Takaha, Okada, Hizukuri, Takagi, & Imanaka, 1996; Takata, Takaha, Okada, Takagi, & Imanaka, 1996). Furthermore, GBE has substantial potential in the food baking industry, since the addition of GBE has an antistaling effect and decreases crumb firmness (Wu, Liu, Yan, & Jiang, 2014). However, few studies have concentrated on the retrogradation behavior of starch modified by GBE. Systematic and deep research on the retrogradation properties of corn starch treated with GBE is still needed.

In this study, multiple analytical methods, including rheology, pulsed nuclear magnetic resonance (PNMR), differential scanning calorimetry (DSC), and Fourier transform infrared spectroscopy (FTIR) were used to investigate the retrogradation behavior of corn starch treated with GBE. The information provided by these methods may allow us to better guide the production of starch with intrinsic retrogradation resistance and expand the application of GBE.

2. Materials and methods

2.1. Materials

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67 Native corn starch was purchased from Zhucheng Xingmao Corn Developing
68 Co., Ltd (Shandong, China). The *gbe* gene encoding GBE was from *Geobacillus*
69 *thermoglucosidans* STB02 (GenBank accession no. KJ 660983); GBE was produced
70 from *Escherichia coli* BL21 (DE3) harboring plasmid pET-20b (+)/*gbe*. The
71 recombinant enzyme was purified by immobilized metal affinity chromatography
72 using a HisTrap chelating column (5 mL, GE Healthcare).

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74 2.2. Preparation of corn starch treated with GBE

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76 Corn starch was suspended in deionized water (30%, w/v), and adjusted to pH
77 7.5. This corn starch milk was incubated with GBE (300 U/g dry weight of starch) at
78 50 °C for 4, 6, 8, and 10 h, with shaking (150 r/min), to produce GBE-treated corn
79 starch (Li et al., 2016). The starch samples were washed with 10 volumes of
80 deionized water to remove the GBE and small oligosaccharides. The solid was
81 collected and vacuum dried at 45 °C for 12 h. Finally, the dried samples were ground
82 to a powder fine enough to pass through a 100-mesh sieve. The control was the corn
83 starch treated at 50 °C without the presence of GBE.

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85 2.3. Dynamic viscoelastic measurements

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87 Dynamic viscoelasticity was measured using a rheometer (AR G2, TA

Instrument Inc., USA) equipped with a parallel plate system (20 mm diameter) at a gap of 1.00 mm. Each starch sample (6%, w/v) was prepared in deionized water, and boiled in a water bath for 30 min with agitation provided by a magnetic stirrer. At the end of the heating period, the slurry was immediately transferred to the 4 °C rheometer plate. Excess material was wiped off with a spatula and silicon oil was applied to the exposed sample edge to prevent water evaporation during the measurements. The sample was allowed to equilibrate for 2 min prior to testing. The dynamic time sweep test was performed for 2 h at 1.0 Hz and 2% strain (Hong, Wu, Liu, & Gu, 2015). The 2% strain was in the linear viscoelastic region.

2.4. Pulsed nuclear magnetic resonance (PNMR) relaxation analysis

The proton relaxation time was determined using a low resolution NMR spectrometer (NMI20, Niumai Instruments Ltd., China). Each starch sample was dispersed in deionized water to obtain a concentration of 6% (w/v). These samples were boiled in a water bath for 30 min with continuous stirring to obtain completely gelatinized starch pastes. Samples of each paste (1-2 g per sample) were transferred to glass NMR tubes, which were then sealed with plastic wrap. The tubes were stored at 4 °C for 0, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, or 8.0 h. At the conclusion of the storage time, each sample was subjected to PNMR analysis using the Carr-Purcel-Meiboom-Gill (CPMG) pulse sequence to analyze the relaxation time. The number of echoes for each CPMG sequence was 17,000. The number of acquired data points was 1,363,416.

Eight scans were carried out for each measurement (Tang, Hong, Gu, Zhang, & Cai, 2013).

The data of the intensity (A) versus time (t) was obtained. The transverse relaxation time (T_2) was calculated by using an exponential function (Eq. 1), which fit the data well.

$$A = A_0 \exp(-t/T_2) \quad (1)$$

where A_0 is a relative measure of the amount of water fraction corresponding to T_2 , and T_2 represents the more mobile fractions of water in the system.

2.5. Differential scanning calorimetry (DSC)

The thermal properties of starch samples were analyzed using a differential scanning calorimeter (DSC8000, Perkin-Elmer Co., USA). Each starch sample (2 mg per sample) was placed in an aluminum pan, and then deionized water was added to each with a microsyringe to achieve a solid-water ratio of 1:2 (w/w) (Chen, Ren, Zhang, Tong, & Rashed, 2015; Morikawa & Nishinari, 2000). The sample pans were hermetically sealed, equilibrated at room temperature for 24 h, and then heated from 30 to 100 °C at a heating rate of 10 °C/min. An empty pan was used as a reference. After gelatinization, the samples were stored at 4 °C for 1, 3, 5, 7, 14, 21, or 28 days, and then rescanned from 30 to 100 °C at the rate of 10 °C/min (Banchathanakij et al., 2009). The onset temperature (T_o), peak temperature (T_p) and conclusion temperature (T_c) were determined from each curve. The gelatinization enthalpy (ΔH_g) and

retrogradation enthalpy (ΔH_r) were calculated from the area of the main endothermic peak.

The DSC data were further analyzed using the Avrami equation (Mua & Jackson, 1998; Xu et al., 2013) which is displayed as Eq.(2):

$$X(t) = (\Delta H_t - \Delta H_o) / (\Delta H_{\infty} - \Delta H_o) = 1 - \exp(-kt^n) \quad (2)$$

where $X(t)$ is the fraction of crystallized starch, ΔH_t is the retrogradation enthalpy of the t day-retrograded sample, ΔH_o is the enthalpy of the 0 day-retrograded sample, ΔH_{∞} is the retrogradation enthalpy of the 28 day-retrograded sample, k is the rate constant and n is the Avrami exponent.

2.6. Fourier transform infrared spectroscopy (FTIR) analysis

Starch samples were suspended in deionized water (25 g) to a final concentration of 6% (w/w). The mixtures were pasted using a rapid viscosity analyzer (RVA) (RVA-Tecmaster, Newport Scientific Pty. Ltd., Australia). After cooling to room temperature, the samples were stored at 4 °C for 14 days, and then freeze-dried. The freeze-dried samples were passed through a 100-mesh sieve. These samples were mixed with potassium bromide (KBr) at the rate of 1:60 (w/w) and ground until each mixture was homogeneous. Each mixture was compressed into a slice and fixed on a slide holder for Fourier transform infrared spectroscopy (FTIR) (Nicolet iS10, Thermo Nicolet, America) at 27 °C. The FTIR instrument was equipped with a deuterated triglycine sulphate detector and a temperature-controlled,

single-reflectance diamond attenuated total reflectance accessory with a sealed sapphire anvil. Each sample was scanned 32 times with a resolution of 4 cm⁻¹ (Lian, Zhu, Wen, Li, & Zhao, 2013).

2.7. Statistical analysis

All tests were performed with at least three independent replicates. Data reported in all tables are expressed as the mean $\pm$ standard deviation, which were calculated using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences among the data were analyzed for statistical significance using the Duncan multiple-range test. Differences resulting in values of $p < 0.05$ are considered significant.

3. Results and discussion

3.1 Short-term retrogradation behavior of corn starch treated with GBE

3.1.1 Dynamic viscoelastic properties

The dynamic viscoelastic properties of corn starch treated with GBE were investigated using a time sweep measurement. In the dynamic rheological analysis of gelatinized starch, the storage modulus (G') reflects the elastic property resulting from the junction zones of the three-dimensional network structure of the amylose and

swollen granule composite system. The higher G' value indicates that the formation of three-dimensional networks and crosslinking of the starch samples are enhanced (Cho, Choi, & You, 2009). The development of the initial gel network structure during storage is governed by the aggregation of amylose (Nagano, Tamaki, & Funami, 2008). Therefore, G' is usually selected to evaluate the short-term retrogradation of starch.

The G' value of the control showed a behavior with a swift rise (Figure 1a). After a long ageing period (2 h), the control showed a continuous increase in G' without a plateau region. Similar phenomenon was found by Tang et al. (2013) who studied the rheological properties of the rice starch-xanthan gum system. These results indicate that the aggregation of amylose took place and the gel network structure was formed during storage. However, the G' values of corn starches treated with GBE increased slower than that of the control during the whole ageing period. Also, the G' values of corn starches treated with GBE were all lower than that of the control. The G' decrease became more pronounced with increased treatment time. The longest treatment time, 10 h, led to the greatest reduction of G' . These results demonstrate that GBE treatment decreases the structure development rate and retards the short-term retrogradation behavior with increasing treatment time.

Previous studies have shown that the changes in the amylose content of starch during GBE treatment. It was observed that the amylose content was reduced from 18.90% to 0.75% when tapioca starch was modified by GBE (Le et al., 2009). Furthermore, the amylose content of normal maize starch decreased from 32.8% to

12.8% after partial GBE treatment (Li et al., 2014). According to these previous studies, a proposed mechanism is shown in Figure 2 (adapted from Le et al., 2009; Lee et al., 2013). It was speculated that the amylose was partially cleaved during GBE treatment, resulting in lowered amylose content in the starch treated with GBE. Consequently, the phenomenon above might be interpreted as a decrease in amylose content caused by GBE treatment, which led to a decrease of the gel network structure development rate. Moreover, the longer the treatment time, the higher the degree of modification, which results in a reduction of the amylose content of the starch.

In the dynamic rheological analysis of gelatinized starch, another important parameter, the loss modulus (G'') represents the viscous property of the temporary molecular network structure (Cho et al., 2009). The ratio of G'' to G' is defined as the loss tangent ($\tan \delta$), which is known to reflect the viscoelastic behavior of samples (Abd Karim, Norziah, & Seow, 2000). Consequently, the loss tangent ($\tan \delta$) can be used to study the changes in amylose aggregation of corn starch treated with GBE. As shown in Figure 1b, $\tan \delta$ values decreased with increasing ageing time. This suggests that retrogradation took place in all samples. Similar result has been reported previously (Choi & Yoo, 2009). The $\tan \delta$ values of corn starch treated with GBE were higher than that of the control, and increased with increasing GBE treatment time. This means that there was more viscous component and less elastic component in corn starch treated with GBE than in the control. These observations confirm that the aggregation of amylose or the short-term retrogradation of corn starch was inhibited by GBE treatment.

3.1.2 PNMR analysis

Low-resolution NMR is one of the most important techniques for studying physical and chemical structure in complex systems. In PNMR analysis, the transverse relaxation time (T_2) is sensitive to the relaxation behavior of the proton signal, and it reflects the relaxation rate of water molecules. T_2 is usually used as an important parameter to study the water mobility in food systems. In the starch paste system, the lower T_2 value means that the water molecules and starch molecules combine closely, which led to lower water mobility. In contrast, the higher T_2 value reflects higher water mobility. In other words, the water molecules were less bounded to starch molecules (Ablett, 1992).

As shown in Figure 3, the T_2 values of all samples at 4 °C decreased with increasing storage time, indicating an overall decrease in water mobility. Similar result was found in the bread system (Chen, Long, Ruan, & Labuza, 1997). Starch retrogradation is a process in which the gelatinized starch is transformed from the amorphous state to a more stable crystalline state. The starch molecules rearrange during the storage period. This limits the movement of water molecules, which leads to a decrease in water mobility (Leung., Agnuson., & Bruinsma., 1983).

The T_2 values of the corn starch samples treated with GBE were all higher than that of the control at the appropriate storage time. Moreover, the T_2 value of the corn starch treated with GBE tended to increase with increasing of GBE treatment time,

suggesting that the water mobility of corn starch was higher. The short-term retrogradation of starch is a process of amylose recrystallization. The recrystallization of amylose involves the movement of water molecules. The water molecules move into the crystal layer during the recrystallization process (Tang et al., 2013). The increase in water mobility indicated that fewer water molecules were incorporated into the crystalline structure of the starch. Fewer amorphous areas were transformed into recrystallization areas. In other words, GBE treatment restrained the recrystallization of amylose or the short-term retrogradation, just proved the conclusion obtained from the time sweep experiment above.

3.2 Long-term retrogradation behavior of corn starch treated with GBE

3.2.1 Thermal properties

The gelatinization properties of starch samples determined by DSC are summarized in Table 1. The gelatinization temperature (onset temperature, T_o ; peak temperature, T_p ; and conclusion temperature, T_c) of corn starch treated with GBE slightly decreased compared to that of the control. The gelatinization enthalpy (ΔH_g) of the control was 11.42 J/g (Table 1), which was consistent with a previous study (Fu, Wang, Li, Zhou, & Adhikari, 2013). The corn starch treated with GBE exhibited a slight decrease in ΔH_g , in the range of 0.87-1.65 J/g, compared with the control. As GBE treatment may change the molecular structure of the starch, especially the

amylose content mentioned above, the gelatinization properties of starch were changed. The corn starch treated with GBE was more prone to being gelatinized and less thermal energy was needed for melting.

DSC analysis of the retrograded samples exhibited a lower retrogradation temperature (55 °C) than the gelatinization temperature (data not shown). The retrogradation enthalpy (ΔH_r) value indicates the melting of crystals formed by amylopectin rather than amylose during the storage period (Hu, Xu, Jin, Tian, Bai, & Xie, 2011). The changes in ΔH_r of starch samples during storage at 4 °C are shown in Table 2. The ΔH_r of all samples increased strongly with increasing storage time, and the ΔH_r of the control increased from 2.62 J/g to 6.64 J/g after 28 days of storage. These results indicate that recrystallization occurred during storage and more energy is required to destroy the crystals, leading to an increase in the ΔH_r . The ΔH_r of corn starch treated with GBE decreased compared with that of the control, and the longer the GBE treatment time, the lower the ΔH_r became. The ΔH_r of the starch treated for 10 h declined by 22.3% when stored for 28 days. These results demonstrate that the long-term retrogradation behavior of corn starch was retarded by GBE treatment.

In previous literature, it was found that the weight and number-average degree of polymerization (DP_w and DP_n) of waxy corn starch were considerably decreased from 21.6 to 16.9 and from 15.4 to 13.0, respectively, after GBE treatment (Lee et al., 2013). Also, Kittisuban, Lee, Supphantharika, & Hamaker (2014) used GBE to modify seven types of starch and found that the enzymatic treatments of all starches resulted in a decrease in the proportion of long amylopectin chains and an increase in the

proportion of short amylopectin chains. From the above information, it is speculated that portions of the long amylopectin chains are transformed into short amylopectin chains when corn starch is incubated with GBE (Figure 2). Generally, starch with short amylopectin chains is not liable to retrograde during the long-time storage (Kim, Ryu, Bae, Huong, & Lee, 2008). As a result, the long-time retrogradation behavior of the corn starch treated with GBE is retarded.

The Avrami equation has been widely used to study the kinetics of starch retrogradation. The Avrami exponent (n) and the crystallization rate constant (k) are significant parameters in the Avrami model. The Avrami exponent (n) is associated with the mode of crystal nucleation, while the crystallization rate constant (k) reflects crystals growth and the speed of crystallization (Mua et al., 1998; Zhang et al., 2014). Table 2 shows the changes in Avrami exponent (n) and crystallization rate constant (k) that result from GBE treatment. The experimental data were fitted well with the Avrami model, as the determination coefficients (0.9801-0.9910) were quite close to 1. The values of the Avrami exponent of all samples were below 1, suggesting that the crystal nucleus appeared at once during recrystallization process. The values of the Avrami exponent of corn starches treated with GBE were higher than that of the control, and the longer the GBE treatment time, the higher the Avrami exponent. Meanwhile, the corn starches treated with GBE had crystallization rate constants lower than that of the control. Particularly, the crystallization rate constant of corn starch treated with GBE for 10 h was 27.6% lower than that of the control. Taken together, these results indicate that GBE treatment slowed down the recrystallization

process, and then retarded the long-term retrogradation behavior.

3.2.2 FTIR analysis

To further study the long-term retrogradation of corn starch, samples were analyzed using FTIR spectrometry. The FTIR spectra of starch samples are displayed in Figure 4. All samples exhibited a wide and strong infrared absorption peak from 3600 cm^{-1} to 3000 cm^{-1} that corresponds to the O-H stretching vibration of starch (Lian, Liu, Guo, Li, & Wu, 2013; Wu, Lin, Chen, Wu, & Xiao, 2012). Comparing the shapes of the FTIR peaks around 3400 cm^{-1} from all samples, those from corn starches treated with GBE were less flat than that from the control. Moreover, as the GBE treatment time lengthened, this tendency became more prominent, indicating that GBE treatment lowers the number of hydrogen bonds formed during the aging period. Because fewer hydrogen bonds formed indicate a lower retrogradation rate, the retrogradation rate is indeed slowed by GBE treatment.

In addition, it is noted that the infrared absorption peaks around 3400 cm^{-1} of the corn starches treated with GBE shifted to lower wavenumbers, compared with that of the control. The wavenumber decreased from 3406.64 cm^{-1} to 3384.46 cm^{-1} when the treatment time increased from 0 to 10 h, demonstrating that the strength of the hydrogen bonds decreased with GBE treatment. These results indicate that the long-term retrogradation of corn starch was retarded by GBE treatment, which is consistent with the conclusion drawn from the DSC experiment above.

4. Conclusions

The retrogradation behaviors of corn starch treated with GBE that depended on the treatment time were investigated using a series of methods. All the results demonstrate that both short-term retrogradation and long-term retrogradation of corn starch were retarded after GBE treatment. This inhibition of starch retrogradation may be attributed to the transglycosylation activity of GBE, which leads to a reduction in the amylose content and an increase in the amylopectin content of the starch. Therefore, it may provide a new method for preparing corn starch products resistant to retrogradation, which might be applied to the development of starchy foods with longer shelf lives.

The effect of GBE treatment on the retrogradation of corn starch depended on the treatment time of GBE. However, it is important to consider production efficiency when this technology is applied in industry. So the reasonable GBE treatment time should be considered between the anti-retrogradation effect and the production efficiency. Furthermore, the deep-seated mechanism on the molecular level involved the molecular structure of corn starch treated with GBE should be investigated further to explain the phenomenon above.

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485

Figure Captions

Fig. 1. G' values (a) and $\tan \delta$ values (b) of corn starch treated with GBE during storage at 4 °C for 2 h.

Fig. 2. Schematic diagram of the main enzymatic reaction of corn starch with GBE.

Fig. 3. Relaxation time (T_2) of corn starch treated with GBE during storage at 4 °C for 8 h.

Fig. 4. Fourier transform infrared spectra of corn starch treated with GBE after storage at 4 °C for 14 days.

Tables

Table 1

Gelatinization temperatures and enthalpy values of corn starch treated with GBE ¹.

Sample	T_o (°C)	T_p (°C)	T_c (°C)	ΔH_g (J/g)
Control	67.95±0.23 ^a	71.80±0.30 ^a	75.70±0.27 ^a	11.42±0.34 ^a
4 h	67.80±0.27 ^a	71.64±0.30 ^a	75.59±0.21 ^a	10.55±0.18 ^b
6 h	67.69±0.13 ^a	71.45±0.25 ^{ab}	75.30±0.31 ^{ab}	10.33±0.13 ^{bc}
8 h	67.38±0.28 ^{ab}	71.17±0.27 ^{ab}	75.18±0.21 ^{ab}	10.02±0.18 ^{bc}
10 h	67.04±0.25 ^b	70.88±0.20 ^b	74.66±0.35 ^b	9.77±0.23 ^c

T_o : onset temperature; T_p : peak temperature; T_c : conclusion temperature.

¹ All tests were performed with at least three independent replicates. Mean ± standard deviation values in the same column followed by different letters are significantly different ($p < 0.05$) by Duncan's test.

505 **Table 2**

506 Retrogradation enthalpy and Avrami kinetic parameters of corn starch treated with GBE after 4 °C storage ¹.

Sample	ΔH_r (J/g)							Avrami kinetic parameters		
	1 d	3 d	5 d	7 d	14 d	21 d	28 d	<i>n</i>	<i>k</i>	<i>R</i> ²
Control	2.62±0.24 ^a	3.79±0.28 ^a	4.55±0.28 ^a	5.37±0.16 ^a	6.03±0.14 ^a	6.31±0.23 ^a	6.64±0.12 ^a	0.60	0.47	0.9897
4 h	2.44±0.20 ^a	3.36±0.39 ^{ab}	4.22±0.17 ^{ab}	4.81±0.23 ^{ab}	5.47±0.13 ^{ab}	5.85±0.32 ^{ab}	6.01±0.36 ^b	0.64	0.46	0.9801
6 h	2.12±0.16 ^{ab}	3.11±0.16 ^b	3.94±0.18 ^{abc}	4.53±0.30 ^b	5.18±0.23 ^{bc}	5.47±0.15 ^{bc}	5.68±0.21 ^{bc}	0.65	0.43	0.9910
8 h	1.87±0.37 ^{ab}	2.76±0.17 ^{bc}	3.58±0.34 ^{bc}	4.24±0.21 ^{bc}	4.87±0.37 ^{bc}	5.23±0.31 ^{bc}	5.42±0.18 ^{bc}	0.69	0.38	0.9856
10 h	1.60±0.38 ^b	2.41±0.13 ^c	3.38±0.31 ^c	3.85±0.20 ^c	4.63±0.23 ^c	4.94±0.26 ^c	5.16±0.21 ^c	0.72	0.34	0.9876

507 ¹ All tests were performed with at least three independent replicates. Mean ± standard deviation values in the same column followed by different
508 letters are significantly different ($p < 0.05$) by Duncan's test.

Fig. 1

Fig. 2

Fig. 3

Fig. 4