



Research article

Effects of laccase and cellulase on saccharification of barley malt

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ABSTRACT

Improving saccharification of barley malt is beneficial to promoting economic benefits of beer brewers, but there are few detailed reports on the application of cellulase and laccase in barley malt. So, barley malt was pretreated by cellulase and laccase, and the malt wort and brewer's spent grains were analyzed by HPLC, FTIR and SEM in this study. The concentration of malt wort was increased significantly to 8.1 (° Bx), which increased by 28.6% after barley malt was pretreated by cellulase, but laccase could not improve saccharification of barley malt. Through analysis of sugar in malt wort and cellulose and lignin components as well as physical and chemical structures of brewer's spent grains, the increase in sugar content in malt wort was mainly due to the increase in glucose because of hydrolysis of cellulose in barley malt by cellulase. Further, laccase and cellulase should have a mutual inhibition when they are pretreated simultaneously.

1. Introduction

Beer is one of the most widely consumed drinks available among various alcoholic beverages (Rani and Bhardwaj, 2021). To improve the economic benefits of beer brewers, large-scale and collectivization have become the development tendency of brewers. When beer production is large-scale and collectivized to a certain extent, the key to improve the economic benefit of brewers is still the production technology (Bongaerts et al., 2021; Florian et al., 2006; Wu et al., 2016). Because barley malt is the main material and one of the main production costs in beer production (Rittenauer et al., 2021), the key to decrease beer production cost is to reduce the consumption of barley malt or improve the saccharification of barley malt. Replacing part of barley malt with rice, barley, cocoa pulp, wheat and other starch materials can decrease the cost of materials in beer production (Nunes et al., 2020; Steiner et al., 2012), but the replacement affects the quality of malt wort and beer (Okada et al., 2008). Improving the saccharification of barley malt is still becoming a research hotspot.

Amylase is added to the mash tun to improve the hydrolysis of starch in barley malt (Johanan et al., 2013). Brewer's spent grains can be digested by cellulase to produce bioethanol or feed, which decreases the use cost of barley malt (Feksa Frasson et al., 2018; Giacobbe et al., 2019;

White et al., 2008). In addition, barley malt also contains lignin. Laccase is used to degrade lignin from ginseng residues to increase the yield of sugars (Zhang et al., 2020). Can barley malt be pretreated by cellulase and laccase to delignify and hydrolyze cellulose to improve the saccharification? At present, there have few detailed reports.

In this study, cellulase and laccase were used to pretreat barley malt to study the effect of enzyme pretreatment on saccharification. The main sugar contents of malt wort were determined by high-pressure liquid chromatography (HPLC), and the cellulose and lignin components as well as physical and chemical structures of brewer's spent grains were also determined.

2. Materials and methods

2.1. Reagents

Barley malt was purchased from Shandong Dadi barley malt Co., Ltd, China. Laccase was purchased from Shanghai yuanye Bio-Technology Co., Ltd, China, and the activity was 120U/g. Cellulase was purchased from the Huai'an Bio-mass green bioenergy CO., Ltd, China, and the filter paper activity (FPA) was 350 U/g. Standards, such as fructose, glucose, sucrose and maltose (HPLC grade) were purchased from Sinopharm

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Table 1. Enzyme pretreatment system of barley malt.

Enzyme	Usage of enzyme (U)	Malt (g)	Pure water (mL)
Control Check (CK)	-	20	160
Cellulase (Cel)	40	20	160
Laccase (Lac)	40	20	160
Complex Enzyme (CE)	40 (Cel)+40 (Lac)	20	160

Chemical Reagent Co., Ltd, China. Acetonitrile (HPLC grade) was purchased from Sigma-Aldrich Co. LLC. Germany.

2.2. Saccharification

20 g of barley malt was moistened with 5 mL of purified water, then broken by a disintegrator (LD-400 model, Changshashi Hongjing Mechanical Equipment Co., Ltd, China). The enzyme pretreatment system was showed in Table 1, and the enzyme pretreatment was conducted at 45 °C for 2 h under natural pH. The traditional barley malt saccharification step was further used for saccharification. The solution was filtered by filter paper, then the filtrate was malt wort, and the residue was dried to constant weight to be used as brewer's spent grains.

2.3. Determination of sugars in malt wort

Reducing sugar content (RSC) and total sugar content (TSC) in the malt wort were determined by 3, 5-dinitrosalicylic acid method (DNS) and Phenol-Sulphate acid method respectively (Wu et al., 2020). Concentration of malt wort (CMW) was determined directly by hand-held saccharometer (LB20T model, Guangzhou Mingrui Electronic Technology Co., Ltd, China).

The malt wort was filtered through a 0.22 µm membrane, and the filtrate was diluted to an appropriate concentration for HPLC analysis. Waters 1525 HPLC system with a refractive index detector and an Agilent ZORBAX Eclipse XDB-C18 column (250 × 4.6mm, 5µm) were used to

determine the sugar components in the filtrate, including fructose, glucose, sucrose and maltose. The moving phase was 70% of acetonitrile and 30% of pure water, and the injection volume of the sample was 20 µL. The flow rate was held at 1.0 mL/min and the temperature of the column and the detector was 40 °C.

2.4. Determination of cellulose and lignin in brewer's spent grains

The contents of cellulose and lignin in brewer's spent grains were determined by the National Renewable Energy Laboratory method.

2.5. Fourier transform infrared spectroscopy (FTIR) of brewer's spent grains

FTIR spectra of brewer's spent grains were recorded with Nicolet iS5 FTIR spectrometer (Thermo Scientific, USA). KBr pellets for FTIR spectroscopy were prepared with 2 mg of powder of brewer's spent grains in 40 mg of KBr. Spectral measurements ranged from 400 cm⁻¹ to 4000 cm⁻¹ with 0.4 cm⁻¹ resolution.

2.6. Microscope images of brewer's spent grains

The microstructures of the fracture surface of brewer's spent grains were observed with scanning electron microscopy (SEM) (JSM-6390LV, Japan). The specimens of brewer's spent grains were first mounted on aluminium stubs, and then fracture surfaces were coated with a mixture of 60% gold particles and 40% palladium with an ion coater (Eiko IB-3, Japan) before observation.

2.7. Statistical analysis

The data were processed by ORIGIN PRO 8.0 (Origin Lab Corp., USA) or Microsoft Excel 2016 (Microsoft Corp., USA). One way ANOVA was used to analyze the experimental data. The error bars in all figures corresponded to standard errors of three replicated determinations.

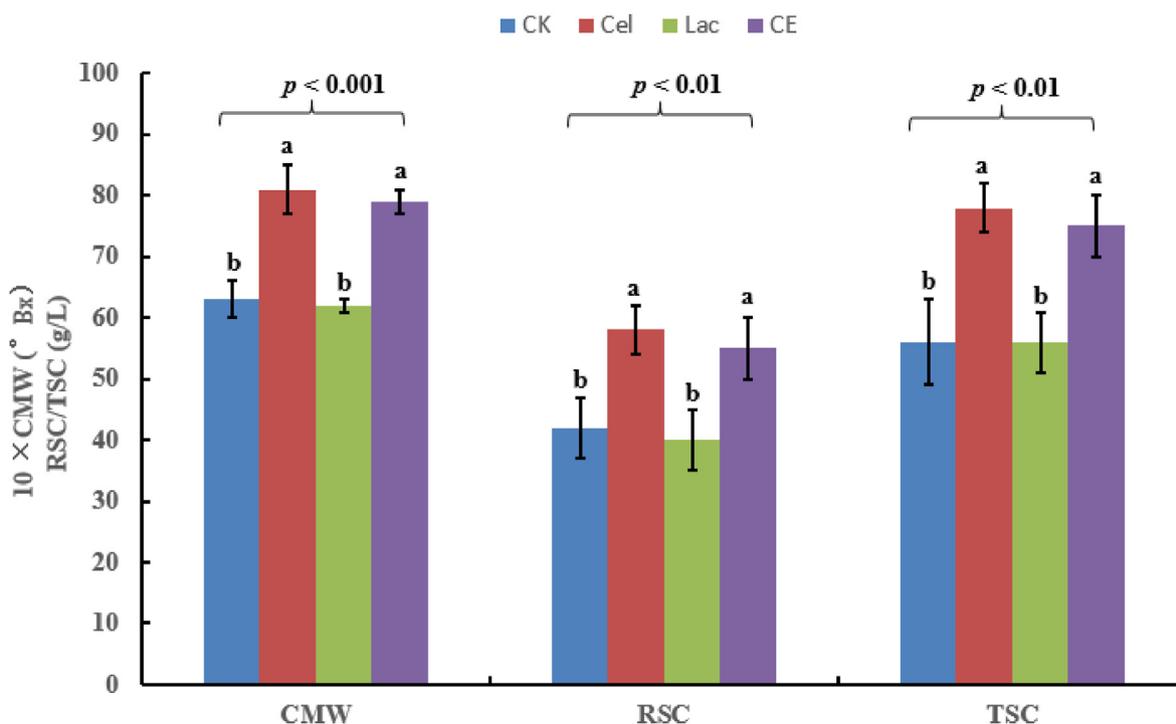


Figure 1. Sugar content (CMW, RSC, TSC) in malt wort after the barley malt was pretreated by cellulase (Cel, ■), laccase (Lac, ■) or complex enzyme (CE, ■).

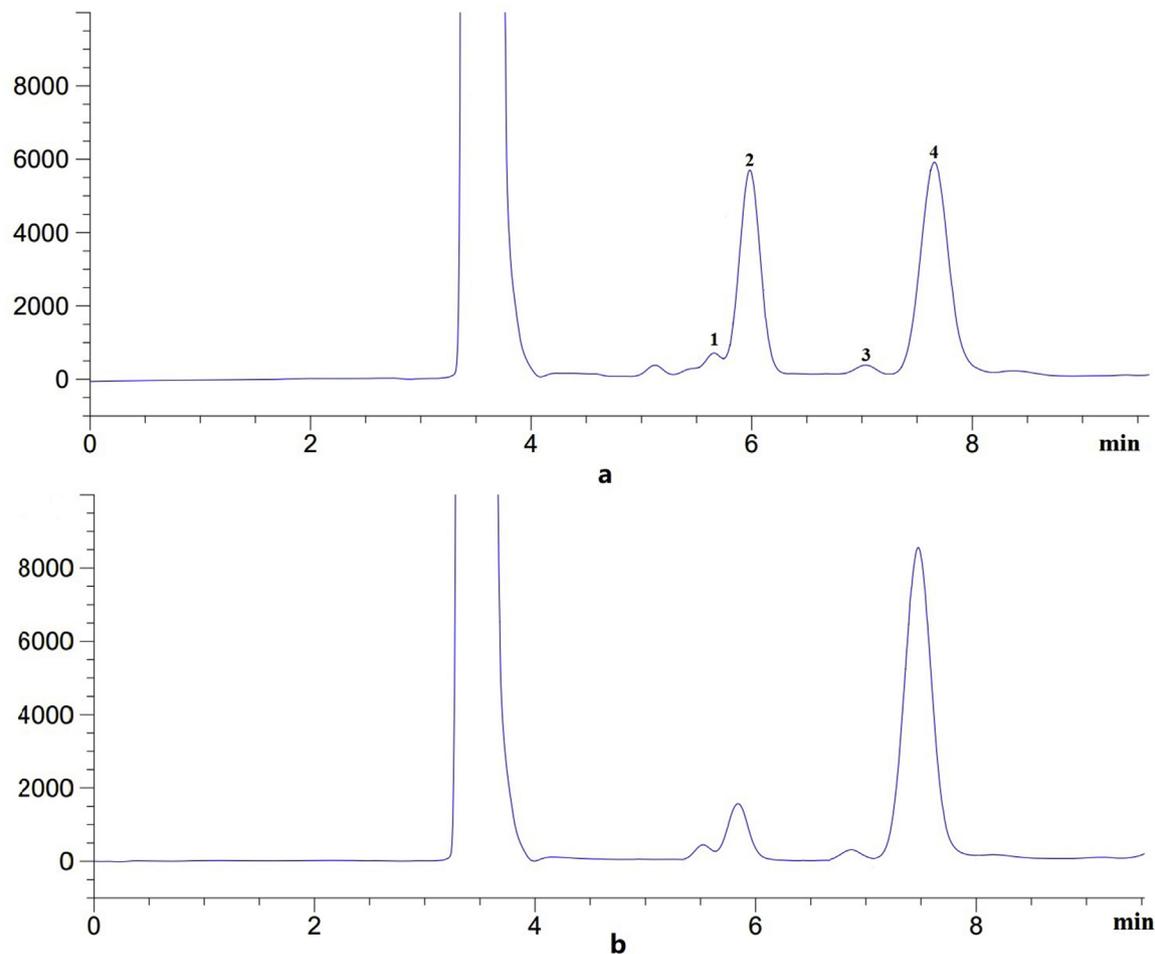


Figure 2. High pressure liquid chromatography of malt wort. (a) Barley malt was pretreated by cellulase; (b) CK. The peak 1, 2, 3 and 4 meant fructose, glucose, sucrose and maltose, respectively.

3. Results and discussion

3.1. Effect of different enzyme pretreatment on sugar content in malt wort

Brix degrees was widely used to show the concentration of malt wort in malt wort production (Savel et al., 2009), and the concentration of malt wort (CMW) was as shown in Figure 1 after barley malt was pretreated by cellulase (Cel), laccase (Lac) and complex enzyme (CE). The results of one-way ANOVA showed that the experimental data had significant statistical significance ($p < 0.01$).

The CMW was increased significantly to 8.1 ($^{\circ}$ Bx), which was 28.6% higher than that of the control check (CK) when barley malt was pretreated by Cel. While laccase pretreatment could not increase the CMW. Due to the existence of laccase in CE, the effect of pretreatment by CE was worse than that by Cel alone, but the CMW was still 20% higher than that

of CK. Further analysis of the reducing sugar content (RSC) and total sugar content (TSC) in malt wort, there was a high correlation between RSC and TSC with CMW among the four treatment systems (CK, Cel, Lac and CE). Comparing the CMW, RSC and TSC, it was found that the increase of CMW was mainly due to the increase of reducing sugar. The increase of reducing sugar by enzyme pretreatment should lead to the increase of alcohol conversion in malt wort. In other words, the same alcohol fermentation could use a smaller amount of barley malt by pretreatment with Cel.

3.2. Analysis of main sugar in malt wort

Sugars in malt wort were mainly maltose, also including glucose, fructose, sucrose, etc (Hu et al., 2014), and the main sugars in malt wort were as shown in Figure 2 and Table 2. According to the results of

Table 2. Mainly sugars content in malt wort with different pretreatments.

Mainly sugar	Retention time (min)	Content (mg/L) with different enzyme pretreatments				p
		CK	Cel	Lac	CE	
Fructose	5.66	807.3 \pm 95.32 ^b	1031.66 \pm 99.08 ^a	914.25 \pm 47.28 ^a	990.01 \pm 69.55 ^a	<0.05
Glucose	5.93	6182.33 \pm 1020.54 ^c	22154.31 \pm 1098.02 ^a	7856.35 \pm 517.62 ^c	17288.52 \pm 1197.16 ^b	<0.001
Sucrose	7.03	621.46 \pm 56.55 ^b	579.72 \pm 39.27 ^b	766.13 \pm 47.12 ^a	454.8 \pm 58.32 ^c	<0.001
Maltose	7.66	32407.52 \pm 1542.95 ^a	25134.52 \pm 2211.93 ^b	29647.73 \pm 1479.52 ^a	27767.46 \pm 2105.33 ^b	<0.01
Total		40018.61 \pm 2537.61 ^b	48900.21 \pm 3224.91 ^a	39184.46 \pm 2262.28 ^b	46500.79 \pm 3198.59 ^a	<0.01

P value came from one-way ANOVA, and superscripts in ascending order denoted significantly different (at $p < 0.05$).

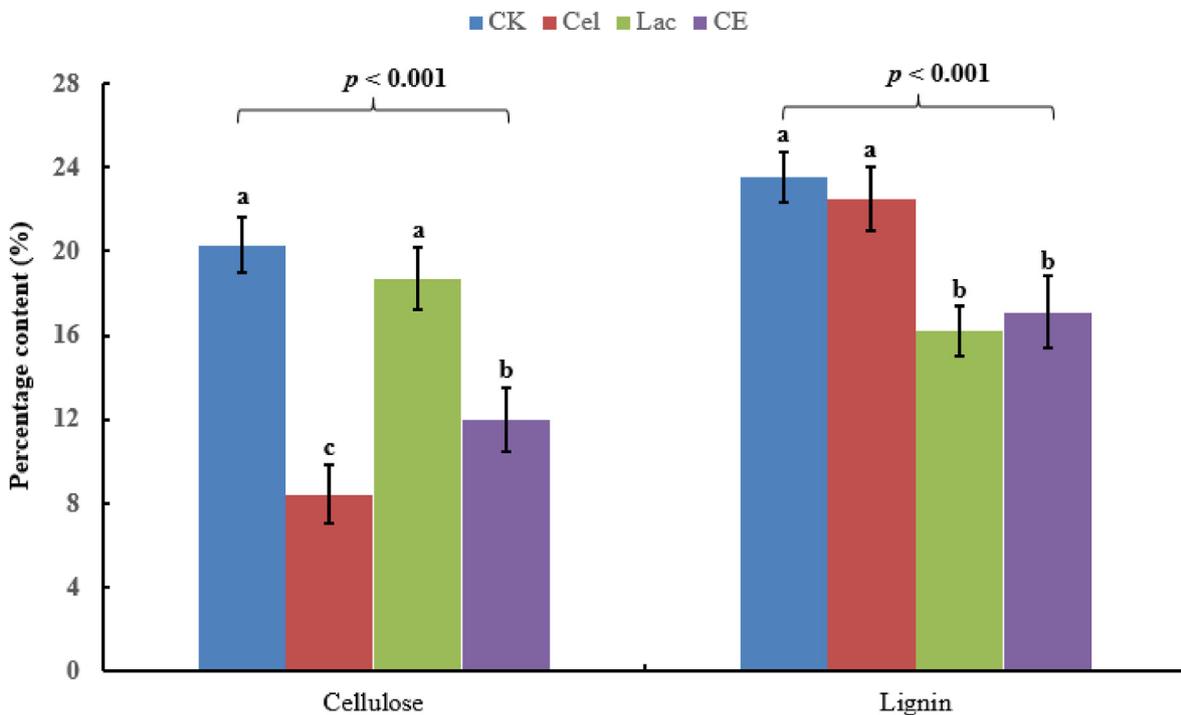


Figure 3. The content of cellulose and lignin in brewer's spent grains pretreated by cellulase (Cel, ■), laccase (Lac, ■) or complex enzyme (CE, ■).

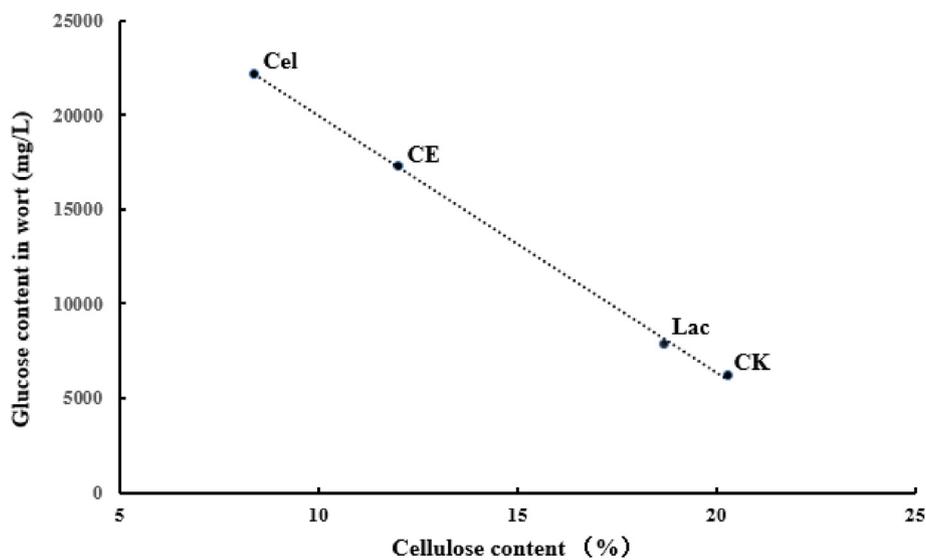


Figure 4. Relationship between the cellulose content in brewer's spent grains with the glucose content in malt wort, and the data came from the results by different enzyme pretreatments (CK, Cel, Lac and CE).

one-way ANOVA, the data of glucose, sucrose and maltose had significant statistical significance ($p < 0.01$), and the data of fructose had statistical significance ($p < 0.05$).

The total content of these four sugars was more than 85% reducing sugar, and the total content of glucose and maltose was even more than 80% of reducing sugar especially. The glucose and maltose contents in malt wort by Cel pretreatment were about 3.5 times higher and 22% lower than those of CK, respectively. It was indicated that Cel might hydrolyze cellulose in barley malt, thus greatly increasing the glucose content in malt wort. Cel also might inhibit the amylase in barley malt, which lead to the decrease of maltose content in malt wort. After barley malt was pretreated by Lac, the total content of these four sugars had no significantly different from that of CK, which indicated that Lac pretreatment could not effectively increase saccharification of barley malt.

Comparing the sugar content of malt wort by CE pretreatment with the other three pretreatment systems (CK, Cel and Lac), it was found that glucose content of malt wort by CE was much higher than that by Lac and CK, but slightly lower than that by Cel. Meanwhile, maltose content in malt wort by CE was slightly lower than that by Lac and CK, but slightly higher than that by Cel. It was indicated that Lac in CE could inhibit Cel and amylase in barley malt.

3.3. Analysis of cellulose and lignin in brewer's spent grains

The contents of cellulose and lignin in brewer's spent grains were shown in Figure 3. The results of one-way ANOVA showed that the data had extremely significant statistical significance ($p < 0.001$).

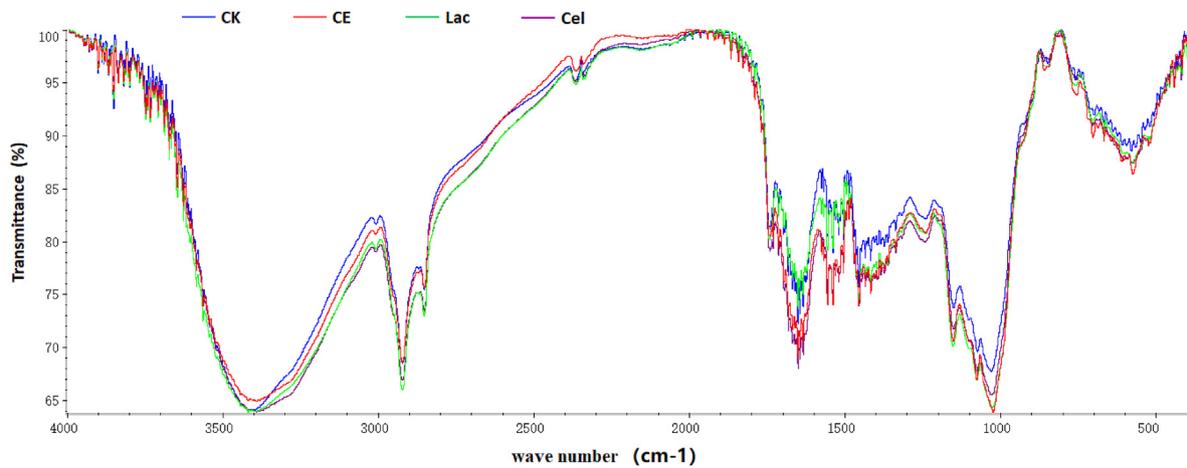


Figure 5. Fourier transform infrared spectroscopy (FTIR) of brewer's spent grains. Blue line represented control check (CK); red line represented the sample with complex enzyme pretreatment (CE); green line represented the sample with laccase pretreatment (Lac); purple line represented the sample with cellulase pretreatment (Cel).

After barley malt was pretreated by Cel, the cellulose content of brewer's spent grains decreased from 20.3% to 8.4%, and there was a little change in lignin content, which further confirmed that the hydrolysis of cellulose in barley malt by Cel resulted in the increase of glucose in malt wort. In addition, after barley malt was pretreated by Lac, the

lignin content of brewer's spent grains decreased from 23.5% to 16.2%, and there was a little change in cellulose content, which indicated that laccase could degrade lignin in brewer's spent grains, but it had little effect on the saccharification of barley malt. Further analysis of the relationship between the cellulose content in brewer's spent grains with

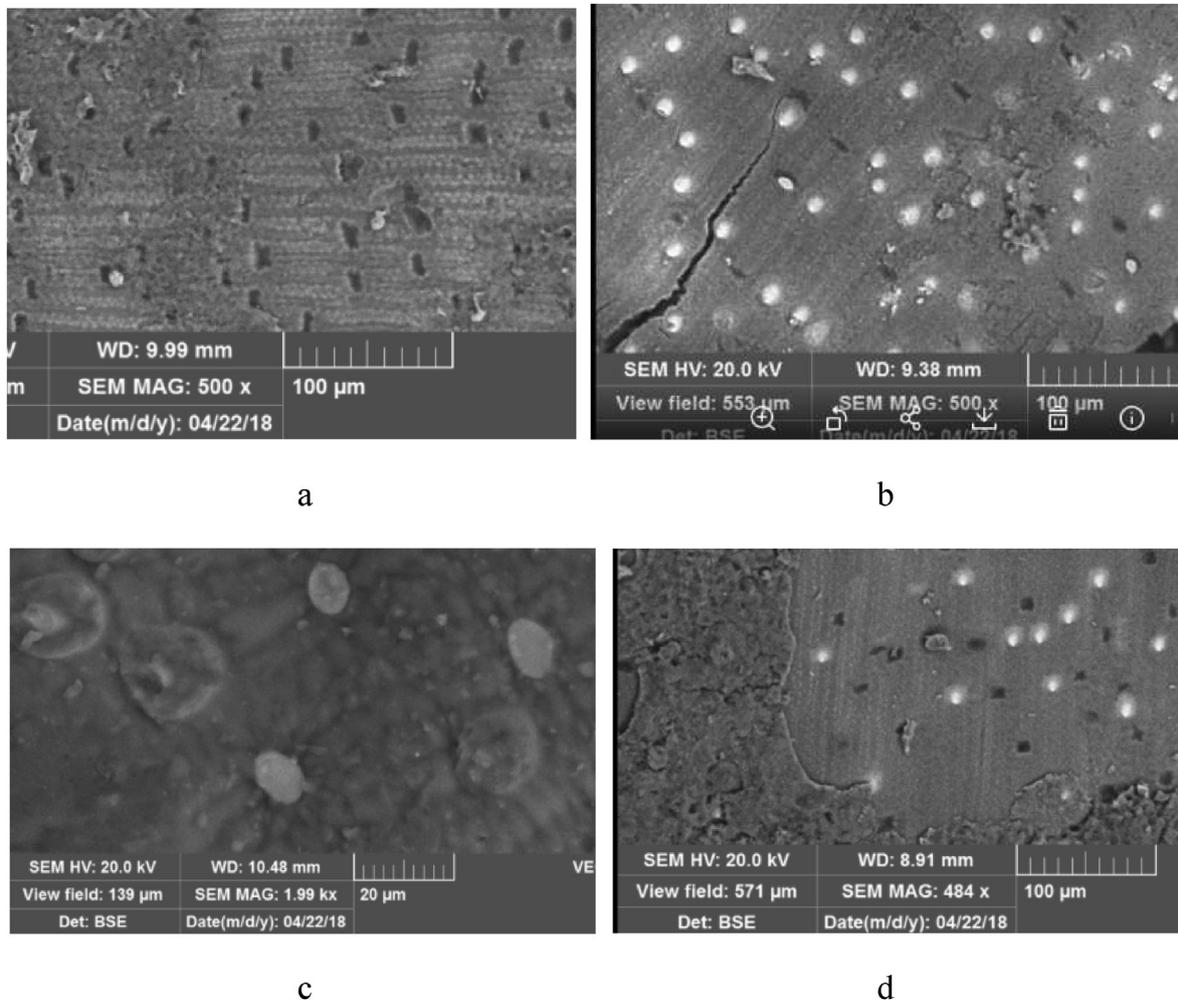


Figure 6. Microscope images of brewer's spent grains observed by scanning electron microscopy (SEM). (a) Control check (CK); (b) barley malt pretreated by cellulase (Cel); (c) barley malt pretreated by laccase (Lac); (d) barley malt pretreated by complex enzyme (CE).

the glucose content in malt wort showed that there was a strong negative correlation ($R^2 = 0.9993$), which was shown in Figure 4. The contents of cellulose in brewer's spent grains by Cel, Lac and CE pretreatment denoted significantly different, which indicated that Lac should inhibit Cel to hydrolyze the cellulose to some extent.

3.4. Analysis of physical and chemical structure in brewer's spent grains

Physical and chemical structures, especially the chemical groups and physical surface structures of lignocellulose materials should be changed after pretreatment (Li et al., 2012). The change of chemical group could be found directly by FTIR, while the physical surface structure could be seen directly by ESM. The FTIR and ESM of brewer's spent grains were as shown in Figure 5 and 6.

From Figure 5, hydroxyl group stretching vibration (3425cm^{-1} band) of the brewer's spent grains by Cel and CE pretreatment were significantly lower than that by others. Hydrocarbon bond stretching vibration (2900cm^{-1} band) of the brewer's spent grains by Laccase and CE pretreatment were higher than that of others. Moreover, the carbonyl group stretching vibration ($1600\text{--}1450\text{cm}^{-1}$ band) of the brewer's spent grains by Lac pretreatment was reduced slightly. The Cel could make the cellulose of barley malt hydrolyze into many holes (Figure 6b). Lac could loosen and destroy the barley malt structure (Figure 6c). The density and size of holes in barley malt pretreated by CE were slightly smaller than that by Cel alone, but the barley malt structure was also destroyed (Figure 6d).

Cel could hydrolyze cellulose in barley malt, while Lac could destroy lignin molecules in barley malt. However, when Lac and Cel were used together to pretreat barley malt, the destruction degree of lignin and the hydrolysis degree of cellulose of barley malt were lower than that by Lac and Cel alone. Therefore, there might be a mutual inhibition between Cel and Lac (Ruqayyah et al., 2020) in simultaneous pretreatment of barley malt.

Some researchers also found that Lac and Cel could be adsorbed on biomass and inactivate when both of them were put together at the same time, or Lac degraded lignin and produced some phenols, which would affect the hydrolysis of cellulose in biomass (Ko et al., 2015; Ladeira Azar et al., 2018; Oliva-Taravilla et al., 2016). In addition, in order to obtain a higher sugar yield of lignocellulose in bioethanol production, Lac was used to destroy the structure of biomass, which was conducive to hydrolysis of cellulose in biomass by Cel (Schroyen et al., 2015). Based on the results of this study, it was suggested that the pretreatment procedures of Lac and Cel should be carried out separately.

4. Conclusions

The concentration of malt wort was increased significantly to $8.1\text{ (}^\circ\text{Bx)}$, which increased by 28.6% after barley malt was pretreated by cellulase, but laccase pretreatment could not increase saccharification of barley malt. Through the analysis of sugar in malt wort and the cellulose and lignin components as well as the physical and chemical structures of brewer's spent grains, the increase of sugar content in malt wort was mainly due to the increase of glucose because of hydrolysis of cellulose in barley malt with cellulase. Furtherly, laccase and cellulase should have a mutual inhibition when they pretreated barley malt simultaneously.

Declarations

Author contribution statement

Jianguo Wu: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ziyi Li, Jiapei Wang, Huanwei Gan, Jiandong Wang, Cannan Yu: Performed the experiments.

Ci Jin, Guilong Yan, Wei Wang: Analyzed and interpreted the data.

Yuzhen Zhou: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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