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Unique Volatile Compounds of Sea Cucumber Beer and its Anticoagulant Activity

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HIGHLIGHTS

- The sea cucumber beer is successfully brewed by adding enzymatic hydrolysate (10%).
- The unique volatile compounds are identified in the final sea cucumber beer.
- The anticoagulant activities of sea cucumber beer are assessed.

Abstract: The sea cucumber beer was successfully brewed by the optimal dosage of sea cucumber enzymatic hydrolysate (10%) and pilot-scale brewing technology were determined, then unique volatile compounds and anticoagulant activity of sea cucumber beer were analyzed in this paper. The brewing results of sea cucumber beer showed that foam stability (244 ± 5 s), original gravity (14.03 ± 0.06 ^{o}P), alcohol level (5.90 ± 0.10 %, vol), residual sugar content (2.00 ± 0.17 g/L) and total acidity (2.40 ± 0.1 mL/100mL) were significantly lower than that of control beer, but the color (17.00 ± 0.10 EBC) and pH (4.97 ± 0.06) were obviously higher than that of the control, and 30 kinds of unique volatile compounds (nonanol, 1-decanol, nonanal, phenylethyl acetate, 6-methyl-5-hepten-2-one, etc) were identified in the final sea cucumber beer, and which were not existent in the control beer. In addition, the anticoagulant activity of sea cucumber beer was assessed by measuring the values of Activated Partial Thromboplatin Time (APTT, 127.83 ± 1.10 s), Prothrombin Time (PT, 30. 08 ± 0.40 s), and Thrombin Time (TT, 26.69 ± 1.37 s), which were higher than those of control beer and will benefit to prophylaxis and treatment of thrombosis for people.

Keywords: sea cucumber; enzymatic hydrolysate; volatile compounds; anticoagulant activity.

INTRODUCTION

Sea cucumbers, also known as holothurians, are marine invertebrates belonging to the *Phylum Echinodermata* [1]. They are low in calories and fat, but high in protein, making them a highly nutritious food source. Additionally, sea cucumbers contain a range of bioactive compounds, including antioxidants, which are beneficial for human health. For thousands of years, sea cucumber has been used as a traditional tonic food in China and other Asian countries due to its bioactive compounds and health-promoting properties [1-4]. There are over 1100 known species of sea cucumbers, with 140 of them found in China.

Scientific research has shown that sea cucumber contains numerous bioactive compounds, including collagen, glycosaminoglycans, triterpene glycosides, gangliosides, branched-chain fatty acid, and lectins [5]. Some of these compounds, such as fucosylated glycosaminoglycans [6-8] and fucan sulfate [9-10] have been shown to possess strong anticoagulant [11-12] and antithrombotic properties [13]. These compounds have advantages over other known anticoagulants, such as heparin, as they are not derived from mammals and can be obtained in high concentrations from an abundant invertebrate source [14]. Consuming foods with anticoagulant activities, such as sea cucumbers, can help to prevent and treat blood clots. The nutritional value of sea cucumber has attracted the interest of the general public, nutritionists, and pharmacologists, who have begun to use it as a nutraceutical and tonic food. Previous research has shown that sea cucumber rice wine, made by adding sea cucumber enzymatic hydrolysates, contains 30 free amino acids (1681.216 mg/L), four oligosaccharides (10999.380 mg/L), high levels of total phenols (658.850 mg/L), and nine mineral elements (1911.353 mg/kg), all of which are higher than those found in control rice wine [15].

However, there has been little research on brewing with sea cucumber. Nowadays, craft brewing is a growing industry that is characterized by the use of unique, high-quality ingredients to create a wide range of flavorful and distinct beers. At the heart of every great craft brew is a selection of high-quality, carefully chosen specialty ingredients. These ingredients can range from the traditional barley, hops, and yeast, to more exotic and unusual additions like fruit, spices, and even chocolate or coffee. One of the key elements that set craft brewing apart from mass-produced beers is the use of specialty ingredients, which can add depth and complexity to a brew. Brewing with sea cucumber has the potential to be a specialty ingredient for craft brewing, as it could not only provide a unique and novel flavor to beer, but also be beneficial to certain consumers, such as thromboembolic patients, due to its unique anticoagulant activities.

The objective of this study was to brew a novel beer with marine flavor by adding sea cucumber enzymatic hydrolysate, and to investigate its unique anticoagulant activities. The nutrient solution of sea cucumbers was obtained through proteolysis, and the optimal dosage of sea cucumber enzymatic hydrolysate was determined through laboratory-scale beer fermentation. The feasibility of brewing sea cucumber beer was then verified through pilot-scale fermentation, and the resulting beer was compared to a control in terms of physicochemical indexes, unique volatile compound levels, and anticoagulant activities.

MATERIAL AND METHODS

Materials

Top-fermenting yeast DM303 originated from Doemens (Munich, Germany), and it was preserved in China-Germany Brewing Technical Center at Qilu University of Technology. This yeast is often used to brew ale beers with ester aroma and phenolic flavor, moderate flocculability, and its optimum fermentation temperature is 20°C.

Pale ale barley malt (5.5-7.5 EBC, Harrington, cultivated in Canada) was purchased from China Yongshuntai Malt Co., Ltd. The bitter hop pellets (Qingdao Flower, α-acid 6%, harvested in 2021) were provided from Xinjiang Sapporo Agricultural Science & Development Co., Ltd (Xinjiang, China), and the aroma hop pellets (Saaz, α-acid 4.5%, harvested in 2021) were supplied from Yakima Chief (Washington, USA). Sea cucumber was produced in Yantai, Shandong, China.

Reagents

Kits for alcohol, bitterness, color, and residual sugars measurements were purchased from CDR s.r.l. (Florence, Italy). 2-Octanol was supplied from Sigma-Aldrich (Shanghai, China). The kits for APTT, PT, and TT were provided by Shanghai Sun Biotechnology Co., Ltd (China). Other reagents were all of the analytical grades.

Preparation of sea cucumber enzymatic hydrolysate

The enzymatic hydrolysate of sea cucumber was obtained by several optimum experiments, the food-grade enzyme flavorzyme from Novozymes (Beijing, China) was selected, the degree of hydrolysis was 29.00 ± 1.01% under the conditions that were hydrolysis temperature of 55°C, hydrolysis time of 4.5 h, flavorzyme dosage of 9.9%, hydrolysis pH value of 7.2, and a solid-liquid ratio of 1:25 g/mL.

Laboratory-scale fermentation trials

The 14°P brewer's wort was accomplished in 100L pilot-scale mashing vessels as follows: the ratio of pale ale barley malt was 100%, and the infusion mashing was adopted: the mashing-in temperature was 45°C (20min), the proteolytic rest was 52°C (40min), the amylolytic rest was 65°C (70min), the lautering temperature was 78°C; the boiling time was 70 min, and at 5 min before the end of boiling, the dosage of sea cucumber enzymatic hydrolysate was 0%, 5%, 10%, and 15% respectively. Yeast strain DM303 was pitched to the 14°P cooled brewer's wort in a 2L PET bottle, and the pitching rate was about 1×10^7 cells/mL. The primary fermentation was carried out at 20°C. When the apparent extract was 4°P or so, the open lid of PET bottle was closed until the diacetyl content of the green beer was reduced below 0.1mg/L. The temperature was then decreased to 0°C in a refrigerator, and the secondary fermentation continued for more than five days. Figure 1 shows the brewing diagram.

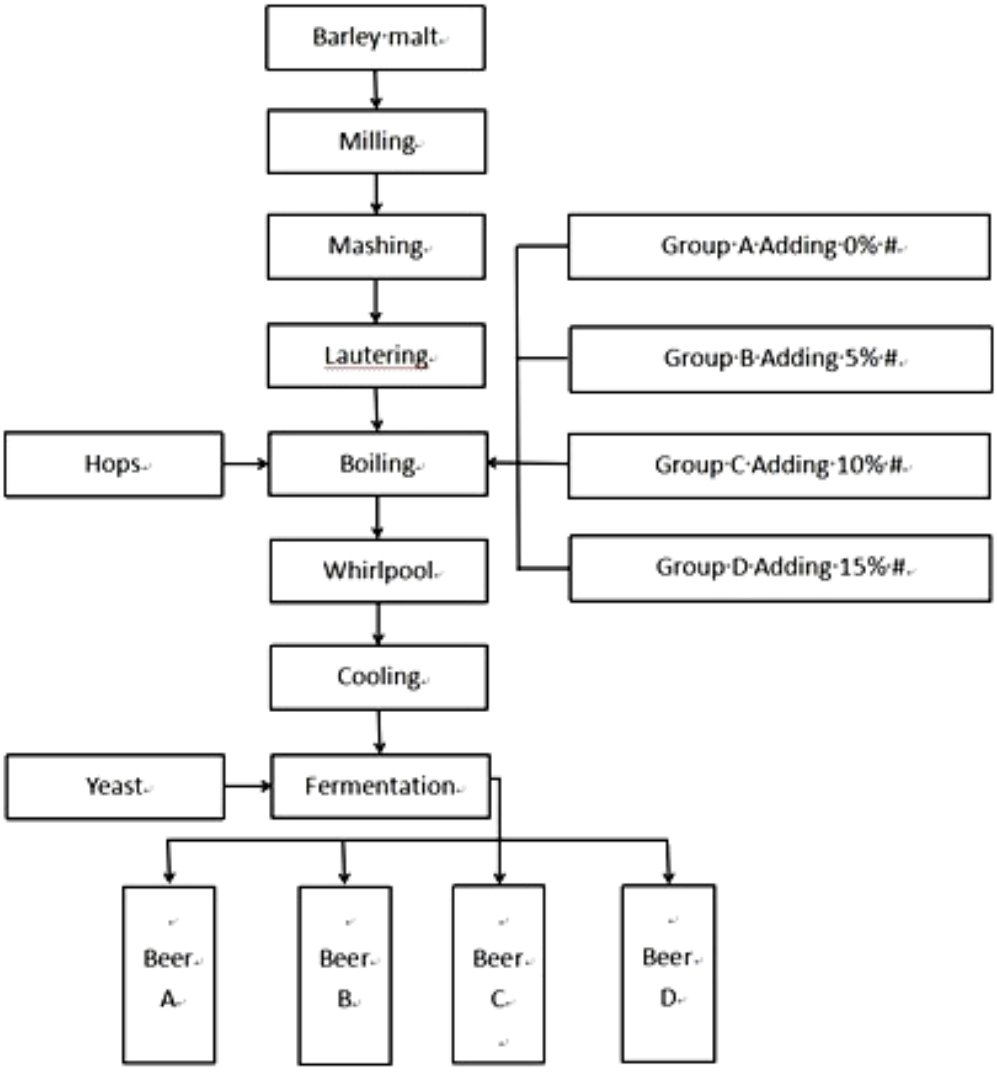

Figure 1. The flowchart of beer production process (# indicated sea cucumber enzymatic hydrolysate).

Pilot-scale fermentation experiments

Sea cucumber beers were brewed in 100L pilot-scale fermenters according to the brewing technology, which was optimized during laboratory-scale fermentation trials.

Detection of the physicochemical indexes during brewing

The yeast concentration, yeast mean diameter were detected every day using Countstar (Shanghai Ruiyu Biotech Co., Ltd, China) according to the manufacturer's protocol. The levels of diacetyl, alcohol, color, bitterness and residual sugars were detected using CDR BeerLab (CDR s.r.l, Florence, Italy), the pH was measured by a handheld pH meter, the foam stability and total acidity level were measured referring to the ASBC methods [16-17].

Determination of volatile compounds in beers

The sample was analyzed by headspace solid phase micro-extraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC-MS) from Thermo Fisher Scientific (Waltham, MA, USA). The beer sample (6 mL) and sodium chloride (3 g) were mixed well in a 15 mL headspace bottle, and the internal standard (2-octanol, 100 mg/L ethanol) of 10 μL was added. The extraction temperature was controlled at 50 °C, the sample was balanced for 5 min, extracted for 40 min by 50/30 μm DVB/CAR/PDMS extraction head (Supelco, Bellefonte, PA, USA), and the emulsifier was operated 5 min at a speed of 67×g. After extraction, the extraction head was desorbed at the GC inlet (250 °C, 5 min) and then analyzed.

The GC analysis was conducted on a TG-WAXMS column (30 m × 0.25 μm × 0.25 mm, Thermo Fisher Scientific, Waltham, MA, USA) at 250 °C. The carrier gas was high-purity helium, and the sample was injected in the splitless mode. The original temperature of the column was maintained at 40°C for 2 min, and then it was increased up to 230°C at a rate of 6°C/min.

MS analyzes were carried out by electron ionization mass (EI) in the full scan mode, using ionization energy of 70 eV and a transfer line at 250 °C. The mass acquisition range was 33-350 amu.

The contents of volatile compounds were calculated by semi-quantitative method with 2-octanol as the internal standard. The concentration of volatile compounds (mg/L) was calculated using the following formula: the concentration of analyte (mg/L) = (the peak area of the analyte/the peak area of the internal standard substance) × the concentration of the internal standard substance (mg/L).

Analysis of anticoagulant activity of pilot-scale final beers

The anticoagulant activity were measured on a SL318 coagulation analyzer (Senlan Trade, Jinan, China) using the reagent kits of APTT, PT and TT; distilled water and heparin sodium were used as negative control and positive control, respectively [12,18].

Sensory evaluation of final beers

The sensory evaluation was conducted according to Beer Judge Certificate Program (BJCP) methods. The beers were assessed by a trained panel of seven beer judges following the BJCP Beer Scoresheet [19] from five aspects: appearance, aroma, flavor, mouthfeel, and overall impression. Beer judges were asked to score the samples of each session according to their preference (where the overall score is 50 points with appearance accounting for 3 points, aroma accounting for 12 points, flavor accounting for 20 points, mouthfeel accounting for 5 points, and overall impression accounting for 10 points).

Statistical analyzes

All results in this study were expressed as the mean ± SD (standard deviation) from at least three replicates. All data were subjected to analysis of variance (ANOVA) and Multiple Range Test (Tukey's test) using Origin 2021 statistics software.

RESULTS AND DISCUSSION

The physicochemical indexes and sensory evaluation of laboratory-scale trial beers

The physicochemical indexes and sensory evaluation of beers are both of importance. Sea cucumber enzymatic hydrolysates of 0%, 5%, 10%, and 15% were added to the brewer's wort at 5 min before the end of boiling, respectively. The corresponding trial beers were marked Beer A, Beer B, Beer C, and Beer D, respectively. Table 1 showed that the dosage of sea cucumber enzymatic hydrolysate significantly affected the physicochemical indexes of beers. With the increase of enzyme hydrolysate amounts, the bitterness, alcohol level, and residual sugar content of the laboratory-scale trial beers were gradually decreased. Still, the color and pH were increased gradually. As for the sensory evaluation of laboratory-scale trial beers, hop aroma and sea cucumber flavor in Beer C were noticeable and moderate, the palate was mellow, and the

evaluated mean score by seven judges was the highest. Based on the above results, the optimized dosage of sea cucumber enzymatic hydrolysate was determined at 10%.

The physiological characteristics of yeast during pilot-scale beer fermentation

The pitching rate of yeast was linked with fermentation time, the total number of yeast cells, the stagnation period of yeast growth, residual sugar content, and volatile compounds level in beer [20]. Results in Figure 2A exhibited that the yeast pitching amount of control beer and sea cucumber beer were 1.0-1.6×10^7 cells/mL. The maximum value of control beer (8.35±0.30 cells/mL) was achieved when the fermentation on the first day and then it showed a downward trend. However, the highest yeast content of sea cucumber beer (8.64±0.10×10^7 cells/mL) was achieved when the fermentation on the second day, and then it showed a downward trend. The reason was that control beer was bunged at 2:00 on the second day of fermentation, whereas sea cucumber beer was bunged at 14:00 at noon on the second day of fermentation. Before the fermenter was bunged, yeast could absorb oxygen, and the numbers increased rapidly under aerobic conditions. After the fermenter was bunged, the anaerobic respiration of yeast produced a large amount of CO_2, absorbing some yeast to float on the surface of the fermentation broth; moreover, small amounts of yeast were sunk with the increase of fermentation pressure. Consequently, the number of yeast in the fermentation liquid was reduced.

Table 1. The physicochemical indexes and sensory evaluation points of laboratory-scale trial beers. Data are the mean ± SD of three biological replicates.

Physicochemical indexes and sensory evaluation	Beer A	Beer B	Beer C	Beer D
Color (EBC)	13.00 ± 0.00^{bcd}	14.00 ± 0.00^{acd}	15.00 ± 0.00^{abd}	16.00 ± 0.00^{abc}
Bitterness (BU)	21.67 ± 0.15^{bcd}	21.17 ± 0.06^{acd}	19.83 ± 0.06^{abd}	12.70 ± 0.10^{abc}
Alcohol (%, vol)	6.90 ± 0.10^{cd}	6.80 ± 0.10^{cd}	6.33 ± 0.06^{abd}	5.87 ± 0.06^{abc}
Diacetyl (mg/L)	0.08	0.09	0.08	0.10
pH	4.67 ± 0.06^{bcd}	4.80 ± 0.00^{acd}	5.10 ± 0.00^{ab}	5.13 ± 0.06^{ab}
Residual sugar (g/L)	3.13 ± 0.06^{bcd}	2.83 ± 0.06^{ad}	2.77 ± 0.66^{ad}	2.47 ± 0.66^{abc}
Aroma and taste	Intense aroma of hop and smooth in the mouth	Intense aroma of hop and smooth in the mouth	Intense hop aroma and moderate sea cucumber flavor, mellow in the mouth	Over intense sea cucumber flavor (tranig) and mellow in the mouth
Sensory evaluation points	42	44	48	45

[a] it was significantly different from Beer A ($p<0.05$); [b] it was significantly different from Beer B ($p<0.05$); [c] it was significantly different from Beer C ($p<0.05$); [d] it was significantly different from Beer D ($p<0.05$).

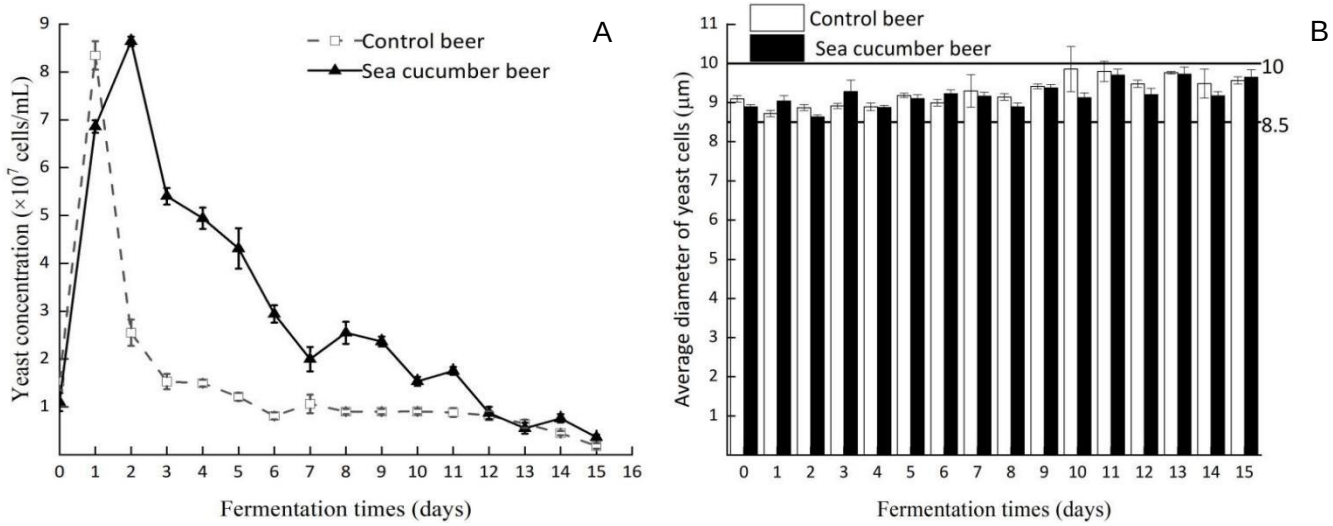

Figure 2. The variations of yeast concentration (A) and average diameter (B) during pilot-scale beer fermentation.

The yeast's size might be correlated with its physiological indexes [21-22].The acetyltransferase activity and esterase activity of yeast cells were affected by cell size. More acetate esters can be metabolized by the larger yeast cells, while more lysine was metabolized by the smaller yeast cells. From Figure 2B, it could be seen that the average diameter of yeast cells in the control beer and sea cucumber beer was in the range of 8.5-10 μm during the whole fermentation process. The results showed that the yeast diameter of the control beer and sea cucumber beer was larger over the entire fermentation process; thus, both beers had a more robust ester aroma.

Variation of the diacetyl and alcohol during pilot-scale beer fermentation

Variation of diacetyl content during pilot-scale beer fermentation can be seen in Figure 3A. There was no significant difference in the diacetyl content between the two beers during the first two days of fermentation. However, there was a significant difference in the content of diacetyl between the two beers on the fourth, sixth and eighth day of fermentation ($p<0.05$), and the content of diacetyl in control beer was significantly lower than that of sea cucumber beer. On the 8th day of fermentation, the level of diacetyl in the control beer was decreased to 0.072 ± 0.005 mg/L; while that of sea cucumber beer was only decreased to 0.254 ± 0.002 mg/L, which was reduced to 0.094 ± 0.002 mg/L till the 15th day of fermentation.

It can be seen from Figure 3B that the alcohol level trends of the two beers were similar, i.e., they increased rapidly at the early stage of fermentation and tended to stability. Lastly, in the final two beers, the alcohol level of the control beer was higher than that of the sea cucumber beer.

Compared to the control beer, the higher diacetyl content and lower alcohol level in sea cucumber beer were related to the ability of yeast's intracellular enzymes, which was inhibited by saponins from the enzymatic hydrolysates of sea cucumber, which was also consistent with the research of Kuznetsova and coauthors [23].

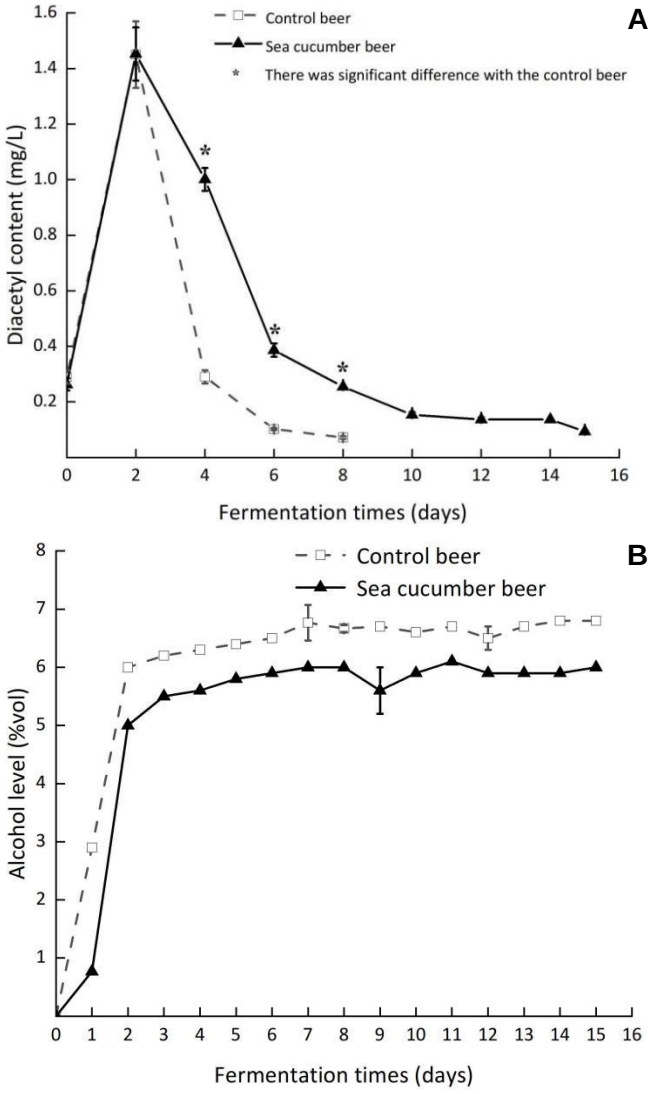

Figure 3. Variation of diacetyl content (A) and alcohol level (B)during pilot-scale beer fermentation.

The physicochemical indexes of pilot-scale finished beers

From Table 2, it could be seen that the foam stability of sea cucumber beer was reduced significantly, which might be due to the decrease of the foam-promoting polypeptide concentration or increase of the inhibitory lipid concentration by the addition of the sea cucumber enzymatic hydrolysate, which was in accordance with Goldberg and coauthors [24]. In addition, the color of sea cucumber beer was significantly increased because of the addition of brown sea cucumber enzymatic hydrolysate, but the total physicochemical indexes of two beers were within the scope of ASBC standard [16].

Volatile compounds in pilot-scale final beers

The total chromatograms of volatile compounds in the final control beer and sea cucumber beer brewed in a pilot-scale were indicated in Figure 4 (A, B). The volatile compounds identified in two beers were reported in Table 3, in which 58 and 71 volatile compounds were isolated and identified from the control and the sea cucumber beer, respectively. In contrast with the control beer, the content of volatile compounds in sea cucumber beer was significantly lower, mainly caused by the dilution of the addition enzymatic hydrolysate of sea cucumber to precursor substances in sea cucumber beer. In addition, 30 unique volatile compounds were identified in the sea cucumber beer, which were not detected in the control beer, such as nonanol, 1-decanol, nonanal, phenylethyl acetate, 6-methyl-5-hepten-2-one and so on, which was in line with previous research that the nonanol (rose fragrance and fruit aroma) and nonanal (rose fragrance) were identified in dried sea cucumber [25]. Interestingly, 19 volatile compounds were found in the control beer, while which were not analyzed in the sea cucumber beer, for example, 5-cubenol, 2-undecanol, geranyl acetate, 2-phenylethyl-amyl oxalate, azulene, α-humulene, etc. With regard to these unique volatile compounds in the sea cucumber beer were come from the sea cucumber enzymatic hydrolysate or the metabolism of yeast to sea cucumber enzymatic hydrolysate, which still needs to be further studied in the future.

Table 2. The physicochemical indexes of pilot-scale final beers. Data are the mean ± SD of three biological replicates.

Physicochemical indexes	Control beer	Sea cucumber beer
Original gravity (°P)	14.23 ± 0.06	14.03 ± 0.06
Alcohol level (%, vol)	6.73 ± 0.06	5.90 ± 0.10*
Foam stability (s)	462 ± 8	244 ± 5*
Color (EBC)	13.0 ± 0.1	17.0 ± 0.1*
Bitterness (IBU)	18.56 ± 0.15	19.47 ± 0.75
Total acidity (mL/100mL)	2.6 ± 0.3	2.4 ± 0.1
pH	4.47 ± 0.06	4.97 ± 0.06
Residual sugar (g/L)	2.37 ± 0.06	2.00 ± 0.17

* It was significantly different from control beer ($p<0.05$).

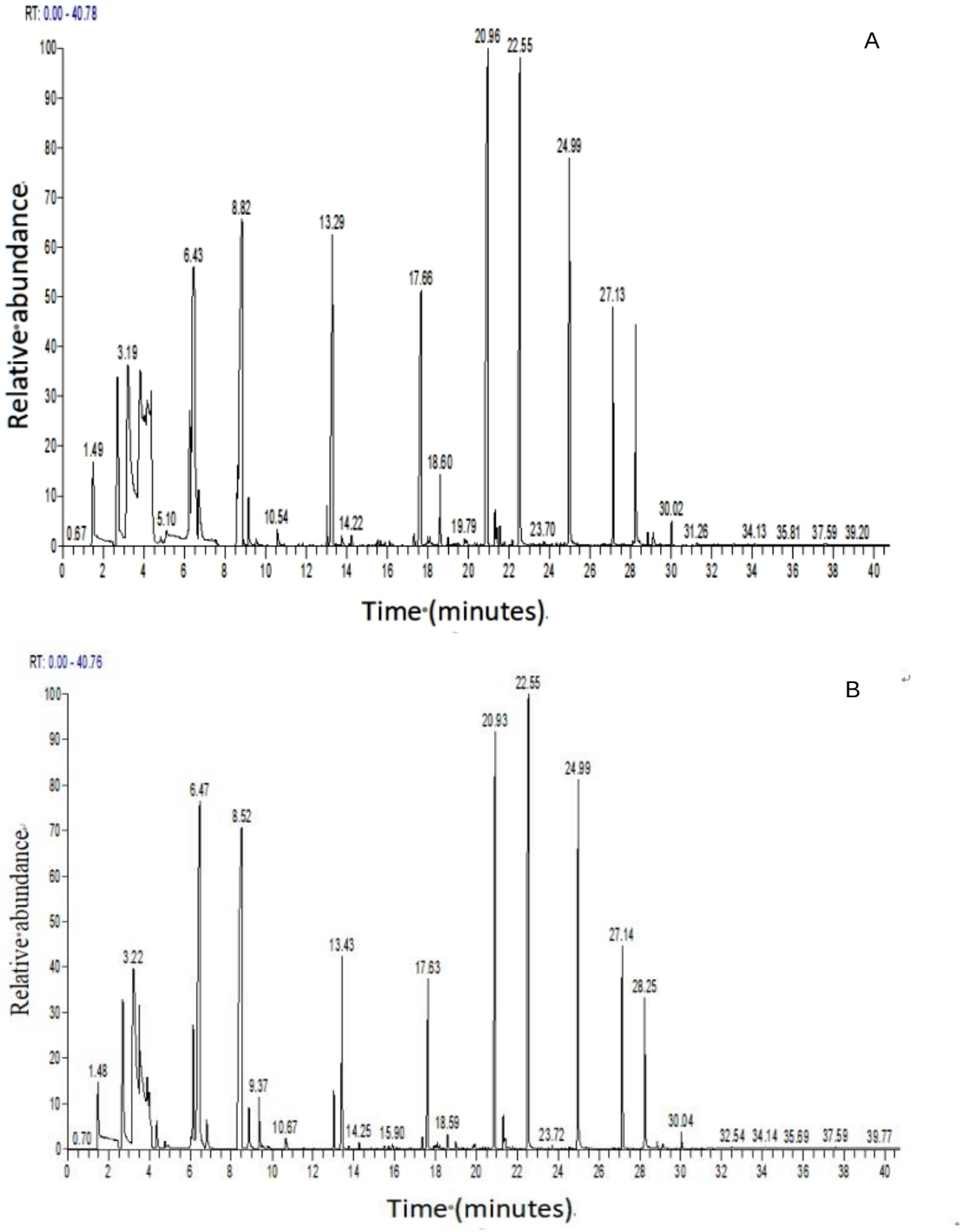

Figure 4. GC-MS chromatogram of pilot-scale beers, (A)control beer and (B) sea cucumber beer.

Anticoagulant activities of pilot-scale finished beers

Anticoagulant activities of two beers was assessed by measuring APTT, PT, and TT, comparing with heparin and distilled water. The APTT and TT assays (Table 4) of sea cucumber beer were higher than that of distilled water, control beer and heparin sodium (0.25mg/L), indicating that the sea cucumber beer had significant anticoagulant activities. The anticoagulant testing results showed that the anticoagulant activities of sea cucumber beer were significantly stronger than that of the control beer, which may be due to the addition of sea cucumber enzyme hydrolysate containing sea cucumber polysaccharide, which was in accord with the study of Luo and coauthors [26].

Table 3. The volatile compounds in pilot-scale final beers. Data are the mean ± SD of three biological replicates.

Volatile compounds	Control beer (mg/L)	Sea cucumber beer (mg/L)
1-Propanol	20.233 ± 0.001	18.020 ± 0.001
Isobutyl alcohol	40.611 ± 0.065	40.523 ± 0.066a
Isoamyl alcohol	93.663 ± 0.441	86.301 ± 0.195*
Phenethyl alcohol	4.230 ± 0.106	2.135 ± 0.009*
3-(Methylthio)-1-propanol	0.037 ± 0.003	0.023 ± 0.002*
(2S,3S)-(+)-2,3-Butanediol	—	0.008 ± 0.004
(2R,3R)-(-)-2,3-Butanediol	—	0.009 ± 0.006
Furfuryl alcohol	—	0.007 ± 0.001
Hexyl alcohol	0.010 ± 0.002	0.006 ± 0.000*
3,7-Dimethyl-3,6-dialkene-1-octanol	—	0.001 ± 0.000
Nerol	0.009 ± 0.000	0.005 ± 0.001*
2-Nonanol	—	0.003 ± 0.000
1-Decanol	—	0.023 ± 0.008*
5-Cubenol	0.004 ± 0.001	—
T-Cadinol	0.011 ± 0.002	—
8-γ-Eudesmol	0.006 ± 0.001	—
β-Eudesmol	0.010 ± 0.001	0.003 ± 0.001*
2-Undecanol	0.004 ± 0.001	—
2,6,10,10-Tetramethyl-Tricyclo[7.2.0.0(2,6)]-5-Undecanol	—	0.001 ± 0.000*
Linalool	0.010 ± 0.001	0.002 ± 0.000*
4,4-Dimethyl-Tetracyclo[6.3.2.0(2,5)0(1,8)]-9-Tridecanol	0.004 ± 0.000	—
4-Vinylguaiacol	1.113 ± 0.002	0.564 ± 0.011*
2,4-p-Tert-Butylphenol	0.055 ± 0.000	0.022±0.002*
Octyl formate	—	0.017±0.000
Ethyl acetate	28.735 ± 0.185	20.642 ± 0.004*
Isobutyl acetate	0.109 ± 0.090	0.105 ± 0.004a
Isoamyl acetate	4.876 ± 0.718	2.535 ± 0.021*
Pentyl acetate	—	0.002 ± 0.001
Heptyl acetate	0.013 ± 0.002	0.005 ± 0.000*
3-(Methylthio-2-Propanone)-Propyl acetate	—	0.037 ± 0.001
Geranyl acetate	0.035 ± 0.006	—
Phenethyl acetate	—	2.141 ± 0.026
Decyl acetate	—	0.004 ± 0.001
Ethyl butyrate	0.038 ± 0.004	0.023 ± 0.001*
Ethyl caproate	0.238 ± 0.084	0.157 ± 0.014a
Ethyl-3-hexenoate	0.002 ± 0.000	—
Ethyl heptanoate	0.005 ± 0.001	0.005 ± 0.003a
Ethyl octanoate	2.732 ± 0.105	0.889 ± 0169*
Isoamyl octanoate	0.048 ± 0.003	0.012 ± 0.003*
Decanolactone	0.014 ± 0.001	0.007 ± 0.001*
Ethyl decanoate	2.337 ± 0.003	0.784 ± 0.144*
Isoamyl isovalerate	0.022 ± 0.001	0.010 ± 0.000*
Ethyl trans-4-decenoate	0.415 ± 0.010	0.060 ± 0.009*
Ethyl dodecanoate	0.130 ± 0.007	0.043 ± 0.002*
3-Oxhydryl-Ethyl tridecanoate	0.004 ± 0.000	—
Ethyl palmitate	0.023 ± 0.004	0.005 ± 0.002*
2-Phenylethyl-Amyl	0.004 ± 0.000	—

Cont. Table 3

Dibutyl phthalate	0.011 ± 0.003	0.006 ± 0.001*
1-Hexyl formate-2-Iso-butyl formate-Phenyl	—	0.003 ± 0.001
2-(4-Methoxy-Phenyl formate)-Phenethyl benzoate	—	0.003 ± 0.001
2'-Hexyl-1,1'-Bicyclopropane-2-Octanoic acid methyl ester	—	0.001 ± 0.000
Rel-9-Octadecenoic acid [(2S)-2α-Phenyl-1,3-Dioxolane]-4β-Ylmethyl ester	0.002 ± 0.001	0.001 ± 0.000a
3,5-dimethylphenyl-Phthalic acid-2-phenylethyl ester	0.007 ± 0.003	—
rel-9-Octadecenoic acid [(2S)-2α-Phenyl-1,3-Dioxolane]-4α-Ylmethyl ester	0.003 ± 0.001	0.003 ± 0.001a
9,12,15-Octadecatrienoic acid 2-Trimethylsilyloxy-1-[(Trimethylsilyloxy) Methyl]Ethyl ester	—	0.004 ± 0.001*
Isobutyric acid	—	0.008 ± 0.000
Hexanoic acid	0.149 ± 0.002	0.088 ± 0.006*
Heptanoic acid	—	0.005 ± 0.001
Octanoic acid	2.959 ± 0.012	1.479 ± 0.076*
Nonanoic acid	0.012 ± 0.001	0.005 ± 0.000*
Methyl-Benzenepropanic acid	—	0.006 ± 0.000
Capric acid	1.146 ± 0.009	0.412 ± 0.032*
9-Decenoic acid	0.076 ± 0.002	—
(3E)-3-Decenoic acid	0.002 ± 0.001	0.018 ± 0.004*
Lauric acid	0.018 ± 0.001	0.007 ± 0.000*
Palmitic acid	—	0.004 ± 0.001
Azulene	0.016 ± 0.002	—
Octamethylcyclotetrasiloxane	1.147 ± 0.103	0.316 ± 0.028*
Hexadecamethylheptasiloxane	—	0.013 ± 0.009
Trans-2-Oxabicyclo [4.4.0] Decane	—	0.003 ± 0.000
1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-Octasiloxane	0.080 ± 0.009	—
(1R,2R)-Ethenyl-1-Methyl-2-(1-Methylethenyl)-4-(1-Methylethylidene)-Cyclohexane	—	0.006 ± 0.000
Trans-Z-à-Bisabolene Epoxide	0.010 ± 0.001	—
Phenylethylene	0.243 ± 0.019	0.200 ± 0.019
4-Ethenyl-1,2-Dimethoxy-Benzene	0.003 ± 0.000	0.001 ± 0.000*
α-Humulene	0.007 ± 0.000	—
l-Caryophyllene	—	0.009 ± 0.002
[S-(Z)]-3,7,11-Trimethyl-1,6,10-Dodecatrien-3-ol	0.019 ± 0.001	—
1,5,9,9-Tetramethyl-1,4,7,-Cycloundecatriene	—	0.017 ± 0.000
Nerolidol	—	0.003 ± 0.001
1-Methylnaphthalene	0.019 ± 0.001	—
1-Ethylnaphthalene	0.011 ± 0.001	—
(−)-Caryophyllene oxide	0.029 ± 0.001	0.002 ± 0.000*
Octaethylene glycol monododecyl ether	—	0.002 ± 0.001
6-Methyl-5-hepten-2-one	—	0.001 ± 0.000
7-Ethyl-4-tridecylene-6-one	0.007 ± 0.001	—
Nonanal	—	0.003 ± 0.001
2-Bromooctadecanal	—	0.002 ± 0.001
2,3-Dihydrobenzofuran	0.116 ± 0.001	0.048 ± 0.002*

* It was significantly different from Control beer ($p<0.05$).

Table 4. Anticoagulant activity of pilot-scale finished beer. Data are the mean ± SD of three biological replicates.

Sample	APTT (s)	PT (s)	TT (s)
Distilled water	39.38 ± 0.30[abc]	29.13 ± 0.31[abc]	11.13 ± 0.08[abc]
Control beer	46.74 ± 0.34*[bc]	28.25 ± 0.17*[bc]	14.11 ± 0.09*[b]
Sea cucumber beer	127.83 ± 1.10*[ac]	30.08 ± 0.40*[ac]	26.69 ± 1.37*[ac]
Heparin sodium (0.25 mg/L)	48.62 ± 0.20*[ab]	31.17 ± 0.40*[ab]	13.60 ± 0.05*[b]

*It was significantly different from the anticoagulant activity of distilled water ($p<0.05$).
[a] It was significantly different from the anticoagulant activity of control beer ($p<0.05$).
[b] It was significantly different from the anticoagulant activity of Sea cucumber beer ($p<0.05$).
[c] It was significantly different from the anticoagulant activity of heparin sodium (0.25 mg/L) ($p<0.05$).

Sensory evaluation of pilot-scale final beers

In Figure 5, the sensory score of the sea cucumber beer was lower than that of the control beer, in which the score was 30 and 34.5, respectively. It looks like the sea cucumber beer did carry the taste of the sea. The slight seafood flavor in sea cucumber beer led to its lower score and was not favored by most BJCP judges, but the score of 30 was also acceptable. Different people have different tastes, such as most women prefer the sweet taste to the bitter taste of beers [27]; there were still a few BJCP judges who prefer the unique seafood flavor of sea cucumber beer. It would be interesting to explore how the sea cucumber beer pairs with seafood, and what the sensory tastes like if we could have brewed it in a crisp lager style in the future research.

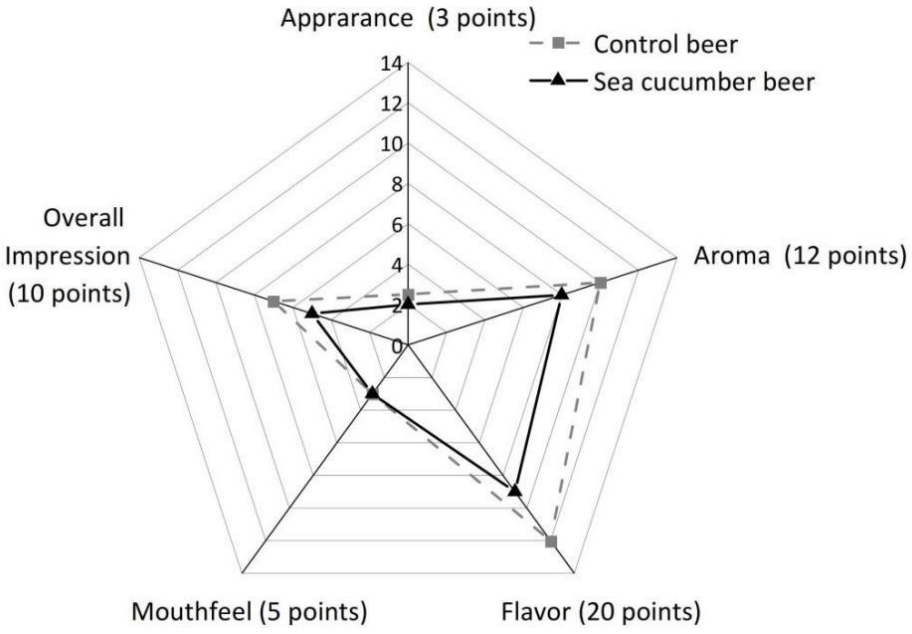

Figure 5. Sensory evaluation of pilot-scale final beer.

CONCLUSION

The sea cucumber beer was successfully brewed by adding 10% sea cucumber enzymatic hydrolysate at 5 min before the end of boiling. Its physicochemical indexes, unique volatile compounds, anticoagulant activities, and sensory evaluation were also analyzed with the control beer. Results of sea cucumber beer showed that foam stability (244 ± 5 s), original gravity (14. 03 ± 0.06 °P), alcohol level (5.90 ± 0.10 %, vol), residual sugar content (2.00 ± 0.17 g /L) and total acidity (2.40 ± 0.1 mL/100mL) were significantly lower than that of control beer, but the color (17.0 ± 0.1 EBC) and pH (4.97 ± 0.06) were obviously higher than that of the control beer. Moreover, the sea cucumber beer had a unique seafood flavor and more substantial bitterness. It was firstly found that there were some certain unique volatile compounds in the sea cucumber beer, such as nonanol, 1-decanol, nonanal, phenylethyl acetate, 6-methyl-5-hepten-2-one and so on, while there were not in the control beer. In addition, anticoagulant activities of sea cucumber were assessed by measuring the values of APTT (127.83 ± 1.10 s), PT (30.08 ± 0.40 s), and TT (20.69 ± 1.37 s), which were higher than those of control beer and will benefit to prophylaxis and treatment of thrombosis for people [26].

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