Production of Beer with a Genetically Engineered Strain of *S. cerevisiae* with Modified Beta Glucanase Expression

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ABSTRACT

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This study used a recombinant S*accharomyces cerevisiae* strain, which expressed both β-glucanase enzyme and reduced Proteinase A expression during wort fermentations. The genetic stability and fermentation features of the strain were examined. The recombinant strain's proteinase A activity was reduced compared to the parent strain; β-glucanase was produced throughout the fermentation. The fermentation with the recombinant *S. cerevisiae* strain exhibited a larger reduction in β-glucan content than what was observed with the control strain, with β-glucan degradation above 80%. The foam stability period was reduced when the beer produced by the recombinant *S. cerevisiae* was stored for 3 months. SDS-PAGE analysis of the beer proteins indicated that lipid transfer protein 1 had disappeared. Fermentation studies indicated that based on the parameters examined, this recombinant strain was suitable for industrial beer production.

Key words: draft beer, foam, β-glucanase, proteinase A, recombinant *S. cerevisiae.*

INTRODUCTION

Draft beer is a beer that has undergone aseptic filtering and packaging but has not been pasteurized. It is increasingly favored by consumers as a mainstream beer choice because of its flavor. Due to the absence of pasteurization, a fraction of the proteinase enzyme activity, secreted by the yeast, is retained in the beer. This results in degradation of beer foam protein during storage, decreasing shelf life time in terms of beer foam. Many experiments have shown that this phenomenon is due mainly to Proteinase A degradation of beer proteins[12]. To maintain the stability of beer foam, a number of studies have been conducted on Proteinase A to identify the relationship between Proteinase A content and foam stability[1,4,20,24,26].

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In the brewing process, incomplete decomposition of β-glucan in the malt endosperm obstructs the transfer of other stored materials. As a result, some starch and proteins are retained in the cells, ultimately causing a reduction in extractive substances. Some β-glucan from the malt enters into the wort and increases the viscosity of the latter[22]. Highly viscous malt wort causes filtration difficulties, triggers the non-biological turbidity of beer, and influences beer stability and quality. Since the Millipore filters used for filtration of draft beer have an extremely small aperture, a high viscosity due to glucan molecules can obstruct the filter. Thus glucan has a greater influence during filtration of draft beer, and the content of glucanase in beer and malt during brewing has aroused great attention.

The β-glucan causes difficulty during beer filtration and affects the non-biological turbidity of the beer, influencing its quality and shelf life to a certain extent. Beer foam, especially draft beer foam, is greatly influenced by Proteinase A[6,8,23]. There have been attempts to reduce the negative influences of Proteinase A by adding Proteinase A inhibitors[5] of microbial origin and by adding β-glucanase[10], processes which doubtlessly complicate beer production and increase cost. In light of this, the industry has been exploring the possibility of constructing *S. cerevisiae* strains that express β-glucanase and exhibit a reduced expression level of Proteinase A in the hope of solving filtration problems and to obtain improved foam stability. The experiments reported in this paper were carried out to construct and then determine the genetic stability and feasibility of using a recombinant strain for brewing that addresses these issues.

MATERIAL AND METHODS

Materials

Strains. *S. cerevisiae* strain 163 and strain Shuanglu 3 are industrial production strains (sourced from Inbev Doubledeer Brewing Group, Wenzhou, PR China).

The recombinant *S. cerevisiae* strain used throughout this study was constructed by colleagues at Zhejiang University. The recombinant strain cannot grow at 37°C. Strain 163 cannot ferment the sugar melibiose and cannot grow at 37°C. The gene encoding Proteinase A was replaced by the β-glucanase gene from *B. subtilis* and strain construction was confirmed using sequencing techniques. The detailed method of strain construction is reported in the recent paper from this University by Zhang et al.[28]

Culture medium. Seed culture medium consisted of 10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl and a pH of 7.0. The fermentation medium consisted of 10 g/L whey mist, 5 g/L yeast extract, 10 g/L NaCl and a pH of 5.5.

Method of cultivation

Seed activation and cultivation: The strain was inoculated into test tubes each filled with 5 mL of LB medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L). Kanamycin (5 μL) was added and the tubes were placed in a 37°C water bath shaker for 12 h and then the yeast suspension (300 μL) was transferred into a 250 mL flask, filled with 30 mL LB medium, and incubated at 37°C for 12 h at 200 r/min until the logarithmic phase.

Initial conditions of fermentation: The yeast suspension (300 μL) was transferred into a 250 mL flask filled with 30 mL fermentation medium. This was cultivated at 37°C for 14 h at 200 r/min.

Preparation of Proteinase A crude extract and measurement of proteinase activity

Preparation of the Proteinase A crude extract. Wort samples were collected throughout the fermentation, centrifuged at 15,000 × *g* for 10 min and the resultant supernatant was analysed to determine Proteinase A activity[23].

Measurement of the Proteinase A activity. Measurement employed 2 mL of a 1% casein solution and 0.2 mL proteinase solution at 37°C for 20 min followed by the addition of 2 mL of 10% trichloroacetic acid solution. The sample was centrifuged for 15 min at 15,000 × *g*. The optical density of the supernatant was measured with the Lowry method (660 nm) about 10 min after the reaction ended. The proteinase in the blank reaction solution was deactivated with 10% trichloroacetic acid solution for 15 min.

Proteinase A activity is defined as the quantity of proteinase needed to hydrolyze 1 mg casein per min at 25°C and pH 6.0.

Measurement of β-glucanase activity

A 0.8 mL aliquot of β-glucan (30 μg/mL) solution was incubated at 40°C for 10 min followed by the addition of 0.2 mL of beer sample. After 30 min the solution was heated to 100°C for 15 min and then cooled to room temperature. A 200 μL aliquot of Congo Red (100 μg/mL) was added and the mixture was diluted with buffer to 2 mL and the optical density of the supernatant measured at 540 nm[25].

The β-glucanase activity is defined as the quantity of β-glucanase needed to hydrolyze 1μg β-glucan per min in 1 mL of culture fluid.

Measurement of foam stability during storage of draft beer

The foam stability time of draft beer samples was measured in a 20°C water bath for 30 min. The measurement of beer foam stability was performed using a NIBEM-T foam stability tester (Haffmans, VENLO Holland) and the NIBEM method[13]. According to the manufacturer's instructions, measurements were made after incubating the beer in a water bath at 20°C for 30 min.

Measurement of filterability of draft beer

The method of Esser[9] was used in the experiments and results expressed as the maximum filtrate volume.

Measurement of β-glucan

For determination of β-glucan, the sample material was suspended in phosphate buffer (pH 6.5) and mixed for 5 min at 90°C. The suspension was hydrolysed with β-glucanase (Sigma) for 60 min at 45°C. After dilution and centrifugation (10 min at 15,000 × *g*), an aliquot of the supernatant was incubated with β-glucosidase in acetate buffer at pH 4.5 and 40°C for 15 min. The glucose released was assayed with hexokinase/glucose-6-phosphate dehydrogenase[17,18].

The degradation rate of β-glucan is defined as follows: content of glucan after fermentation – content of glucan after fermentation / content of wort glucan × 100%.

Measurement of fermentation rate

A gravimetric method was used. Fermentation solutions were monitored by measuring weight loss every 24 h (expressed as g/day).

Measurement of fermentation degree

The degree of fermentation (%) was analyzed with an automatic beer analyzer (Alcolyzer Plus Beer, Anton Paar Austria)[16].

Measurement of total sugars

Total sugars were analysed according to the DNS[19,21] method. The DNS solution was prepared by dissolving 10 g of 3,5-dinitrosalicylic acid in 2 M sodium hydroxide solution. A separate solution of 300 g sodium potassium tartrate solution was prepared in 300 mL of distilled water. A hot alkaline 3,5-dinitrosalicylate solution was added to sodium potassium tartrate solution. The final volume of DNS solution was made up to 1 L with distilled water. A calibration curve was prepared with 2 g/L glucose solution.

Measurement of diacetyl

Diacetyl was measured using a gas chromatograph with a flame ionization detector and a fused capillary column coated with DB-Wax (30 m × 0.53 mm and 1.0 μm film thickness) (Auto System XL; Perkin Elmer). The carrier gas was nitrogen and the flow rate was 10 mL/min. Pretreatment involved the removal of the yeast from the broth by centrifugation (3,000 rpm, 10 min) and then heating at 60°C for 90 min under aerobic conditions to convert the α-acetolactate to diacetyl[27].

Measurement of total acids (titratable acidity)

Each beer sample (100 mL) was filtered through a No. 1 Whatman filter paper after which 10 mL of the filtrate were titrated against 0.1 N NaOH using phenolphthalein indicator until a permanent pink colour persisted for 30 sec. The titration was conducted in triplicate for each sample[11]. The total acidity was calculated as follows:

$$\% \text{ total acidity} = \text{mL of sample} \times 0.09$$

where 0.09 is the conversion factor used to convert total acids from grams to a percentage.

Flocculation analysis

Yeast flocculation was determined using a modified Helm's assay according to the method of Bendiak[2] with amendments as suggested by D'Hautcourt[7]. Flocculation is expressed as a function of the mean absorbance of the control assay ((control A – experimental A / Control A) × 100%).

Industrial production of draft beer

The recombinant *S. cerevisiae* strain was used to brew beer in a 2,000 L fermentation tank. A 12°P all-malt wort was brewed according to standard production procedures, with 10 days of fermentation at 10°C used throughout. The samples of the draft beer obtained from this test brew were measured for the various parameters.

Measurement of protein content during storage of draft beer

The method of Bradford[3] was used for this analysis.

Electrophoretic separation of beer proteins during draft beer storage

The electrophoretic separation of proteins from the draft beer samples from different storage times (1, 30, 60, 90 days) was carried out using 15% SDS-PAGE (Tris 0.6 mol/L). The gels were stained with silver-stain as described by Laemmli[14].

RESULTS AND DISCUSSION

All data presented are the average values of at least three measurements.

Proteinase A activity

Columns labeled I, II and III in Table I refer to three samples tested in parallel for Proteinase A activity at day 5 of fermentation. The recombinant *S. cerevisiae* showed differences in Proteinase A activity compared to the non recombinant strains. Proteinase A activity in the recombinant *S. cerevisiae* was 39.5% and 38.0% lower than that of the parent *S. cerevisiae* strain 163 and *S. cerevisiae* Shuanglu strain 3, respectively, indicating that the recombinant strain secreted less Proteinase A.

β-Glucanase activity

Triplicate samples from the same strain were cultivated in 10 mL YEPD medium for 24 h at 200 r/min and 30°C, then transferred to fresh YEPD medium (100 mL) at an inoculum concentration of 1/100 and cultivated under the same conditions. Samples were measured for β-glucanase activity every 8 h.

The β-glucanase activity curve of the recombinant strains is given in Fig. 1. No activity was observed until 36 h after inoculation. Activity then increased rapidly and reached a maximum value at 60 h, then dropped to a small amount, became stable after 72 h and then continued to produce β-glucanase steadily until the end of the fermentation at 120 h. The activity of β-glucanase secreted by the non recombinant *S. cerevisiae* was not determined in this experiment.

Genetic stability and brewing properties

Genetic stability of the recombinant yeast. After cultivation at 25°C, the yeast was transferred to fresh slant medium for cultivation at 25°C, then stored at 4°C and transferred for 20 generations. The 1st, 5th, 10th, 15th, and 20th generations of the recombinant strain were tested for genetic stability as follows: quantity of *S. cerevisiae* at 24 h of cultivation, maximum reduction of glucan, fermentation rate, fermentation degree, total acid, *S. cerevisiae* flocculation after the peak period, lowest reduction of diacetyl, content of glucan and wort glucan after fermentation, degradation rate of β-glucan, and Proteinase A activity. The results are presented in Table II and indicate that the *S. cerevisiae* appeared to exhibit stable inheritance of the parameters tested.

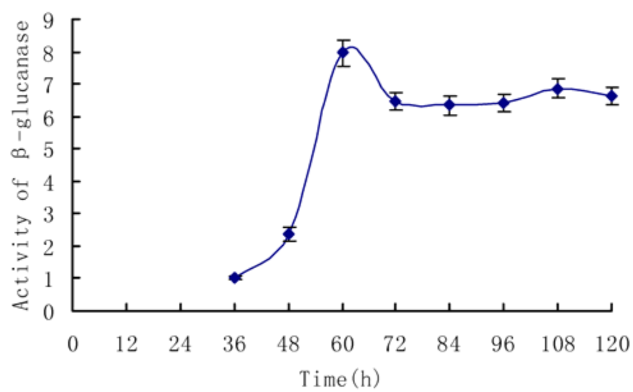

Fig. 1. The β-glucanase activity of the *S. cerevisiae* recombinant strain over time.

Table I. Proteinase A activity of recombinant *S. cerevisiae* and non-recombinant brewing yeast controls in YEPD medium.

	Proteinase A activity (U)[a]					
Strain	I	II	III	Ti	*x*i	sample/CK
S. cerevisiae 163	50.2	51.6	50.8	152.6	50.9	100
S. cerevisiae Shuanglu 3	49.1	49.3	50.6	149.0	49.7	100
S. cerevisiae Recombinant	29.4	31.2	31.7	92.3	30.8	60.5/62.0

[a] I, II and II are triplicate samples.

Table II. Genetic stability of the *S. cerevisiae* recombinant strain tested in brewery 12°P all-malt wort.

Performance	1st	5th	10th	15th	20th
The quantity of *S. cerevisiae* at a peak period after 24 h cultivation	$(3.2 \pm 0.05) \times 10^7$	$(3.8 \pm 0.09) \times 10^7$	$(3.2 \pm 0.11) \times 10^7$	$(4.0 \pm 0.03) \times 10^7$	$(4.7 \pm 0.06) \times 10^7$
Maximum reduction of sugar (g)	2.1 ± 0.1	2.6 ± 0.1	1.9 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
Fermentation rate (g/day)	1.34 ± 0.02	1.31 ± 0.02	1.44 ± 0.04	1.50 ± 0.05	1.51 ± 0.02
Fermentation degree (%)	69.97 ± 0.19	70.57 ± 0.08	70.59 ± 0.12	70.21 ± 0.16	70.35 ± 0.13
Total acid (ml/100 mL)	1.71 ± 0.01	1.81 ± 0.04	1.69 ± 0.02	1.73 ± 0.02	1.76 ± 0.03
S. cerevisiae flocculation after peak period (%)	31.00 ± 0.13	36.80 ± 0.17	50.00 ± 0.18	47.50 ± 0.12	48.70 ± 0.09
The lowest reduction of diacetyl (mg/L)	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Content of glucan after fermentation (μg/mL)	47.02 ± 0.25	46.62 ± 0.18	42.71 ± 0.22	49.37 ± 0.16	45.72 ± 0.15
Content of wort glucan (μg/mL)	156.00 ± 0.41	156.00 ± 0.41	156.00 ± 0.41	156.00 ± 0.41	156.00 ± 0.41
Degradation rate of β-glucan (%)	69.89 ± 0.48	70.12 ± 0.38	72.62 ± 0.43	68.35 ± 0.37	70.69 ± 0.35
Proteinase A activity (mg/L)	0.0188 ± 0.0009	0.0201 ± 0.0008	0.0196 ± 0.0010	0.0207 ± 0.0009	0.0201 ± 0.0009

Table III. Performance comparison between the *S. cerevisiae* recombinant strain and non-recombinant production brewing strains tested in brewery A 12°P all-malt wort.

Performance	*S. cerevisiae* recombinant strain (average)	*S. cerevisiae* 163	*S. cerevisiae* Shuanglu 3
The quantity of *S. cerevisiae* at a peak period	$(4.70 \pm 0.06) \times 10^7$	$(5.0 \pm 0.12) \times 10^7$	$(1.2 \pm 0.07) \times 10^8$
Maximum reduction of sugar (g)	2.6 ± 0.1	2.9 ± 0.1	3.1 ± 0.1
Fermentation rate (g/day)	1.50 ± 0.02	1.53 ± 0.04	1.55 ± 0.05
Fermentation degree (%)	70.21 ± 0.15	71.30 ± 0.19	71.00 ± 0.12
Total acid (mL/100mL)	1.73 ± 0.04	1.81 ± 0.01	1.89 ± 0.03
S. cerevisiae flocculation after peak period (%)	47.50 ± 0.18	50.00 ± 0.14	23.30 ± 0.22
The lowest reduction of diacetyl (mg/l)	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.01
Content of glucan after fermentation (μg/mL)	46.43 ± 0.22	124.57 ± 0.39	120.13 ± 0.31
Content of wort glucan (μg/mL)	156 ± 0.41	156 ± 0.41	156 ± 0.41
Degradation rate of β-glucan (%)	70.24 ± 0.44	20.15 ± 0.93	22.99 ± 0.80
Proteinase A activity (mg/L)	0.0201 ± 0.0011	0.0342 ± 0.0014	0.0321 ± 0.0009

Table IV. Comparison of filtration properties (12°P all-malt wort after 10 days of fermentation at 10°C).

	Filtering speed (/hL)			
sample	Filtering speed in lab (/200 mL)	Filtering speed in trial-production (/hL)	Content of glucan before the filtering	Degradation rate of β-glucan
S. cerevisiae recombinant strain	28 min	45 min	27.426	81.16%
S. cerevisiae Shuanglu 3	41 min	58 min	97.56	18.89%
S. cerevisiae 163	43 min	60 min	98.15	21.35%
Increased rate of filtering speed	31.71% 34.88%	22.42% 25.00%	-	-

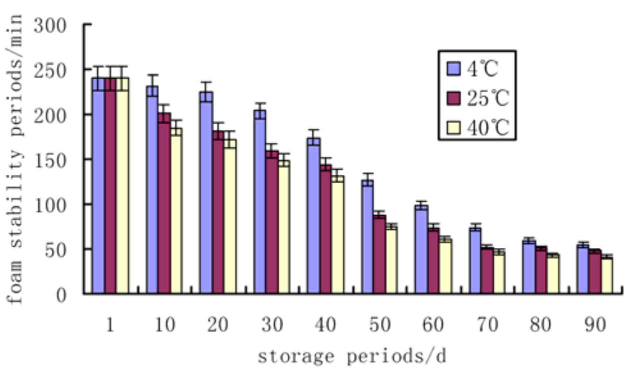

Fig. 2. Changes in foam stability during the storage of draft beer produced with *S. cerevisiae* strain 163.

Performance comparison between recombinant *S. cerevisiae* and production strains. Fermentation tests for the recombinant strain and mass production strains were conducted so that their properties could be compared. The results are shown in Table III. In addition to the stable regular properties, the recombinant strain also showed a strong degrading ability with β-glucan and a decrease in Proteinase A activity. The recombinant strain's growth and fermentation properties were similar to those of parent strain 163.

Comparison of filtering properties. The filtering speed during laboratory trials and production trials was tested. Table IV indicates that the recombinant *S. cerevisiae* had a larger reduction in β-glucan content than the parent strain 163 or strain 3. The degradation rate was above 75% with an increased filtration speed.

Changes in foam stability during the storage of draft beer

The draft beer was measured for foam stability. Foam stability declined continuously when common draft beer was stored (Fig. 2). After storage at 4°C for one month, foam stability dropped from 240 sec to 204 sec. When draft beer was stored at 25°C and 40°C, it dropped to 159 sec and 149 sec, respectively. Foam stabilities, when the beer was stored at temperatures of 12°C, 25°C, and 40°C for two months, were 98 sec, 74 sec and 61 sec, respectively. Foam stability period dropped to less than 60 sec when the beer was stored for three months. The above results indicate that good foam stability can be maintained for about one month when draft beer is stored at a low

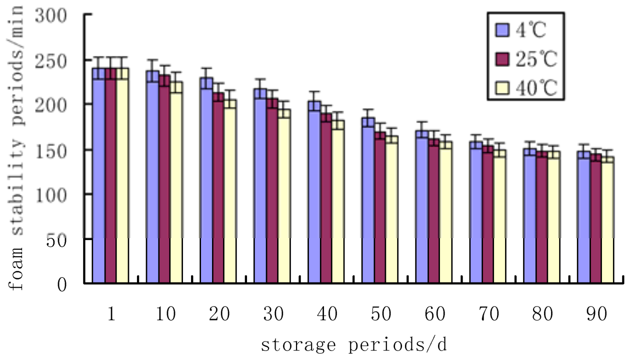

Fig. 3. Changes in foam stability during the storage of draft beer produced by the *S. cerevisiae* recombinant strain.

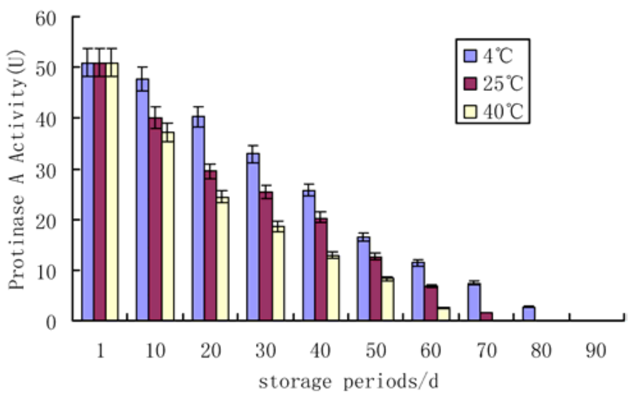

Fig. 4. Change in Proteinase A activity over time in beer produced with *S. cerevisiae* strain 163.

temperature (4°C), but stability drops when draft beer is stored at higher temperatures (25°C and 40°C) for one month, and reduces further when stored for two months at 25°C and 40°C, or three months at 4°C. Although the foam stability period was somewhat reduced when the draft beer produced by recombinant *S. cerevisiae* was stored at the same temperatures, the extent of reduction of the foam stability period, when it was stored for three months, proved to be much less than that of common draft beer (reduced by about 90 sec) (Fig. 3).

Change of Proteinase A during storage of draft beer

Proteinase A activity decreased gradually during storage of draft beer (Fig. 4). The activity decreased at different speeds at the different storage temperatures. Proteinase A still had 64.6% activity when stored for one month at 4°C. The activity levels of Proteinase A in beer stored at 25°C and 40°C were 49.9% and 36.5% of the original activity, respectively. The activity levels of Proteinase A in beer when it was stored for two months at 4°C, 25°C, and 40°C were 22.4%, 13.4%, and 4.9% of the initial activity, respectively. When stored at 40°C for 70 days, at 25°C for 80 days, or 4°C for 90 days, Proteinase A showed no signs of activity. The above mentioned results indicate that draft beer has foam decline even when stored at low temperatures. The reduction rate in the Proteinase A activity levels of draft beer produced by the re-

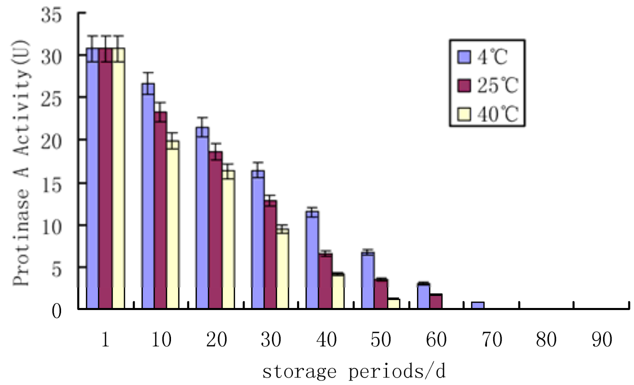

Fig. 5. Change in Proteinase A activity over time in beer produced by the *S. cerevisiae* recombinant strain.

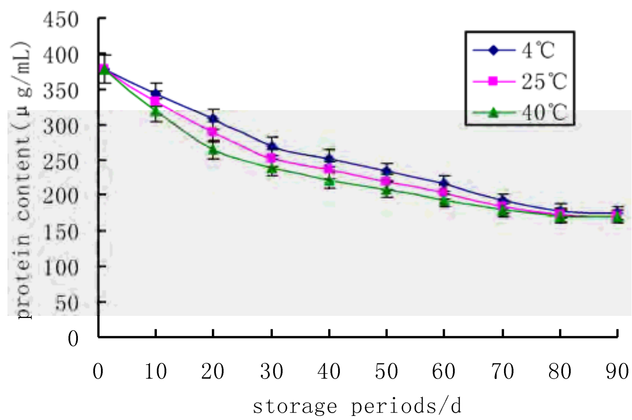

Fig. 6. Change in protein content over time in beer produced with *S. cerevisiae* strain 163.

combinant strain were similar to the rate of the draft beer produced by non recombinant yeast. When the beer was stored at 40°C for 70 days, at 25°C for 80 days, or at 4°C for 80 days, Proteinase A activity was no longer detected (Fig. 5).

Change in protein content during storage of draft beer

The content of protein during the storage of the draft beer produced with non recombinant yeast decreased continuously (Fig. 6). The content of protein decreased, when the beer was stored for one month at three different temperatures, from a starting level of 378 μg /mL to 269 μg /mL (4°C), 252 μg /mL (25°C) and 239 μg /mL (40°C). The protein content changed slightly when draft beer was stored for the second month at the three different temperatures and again when the beer was stored for the third month at 25°C and 40°C; the content of protein changed greatly under 4°C storage during the third month. This is possibly because Proteinase A still maintains a certain activity at low temperatures. The protein content of the beer produced by the recombinant strain was somewhat reduced during storage (Fig. 7). After 3 months of storage, activity was reduced by about 70 μg, whereas the protein content of the non-recombinant beer had dropped by about 200 μg during the same time period.

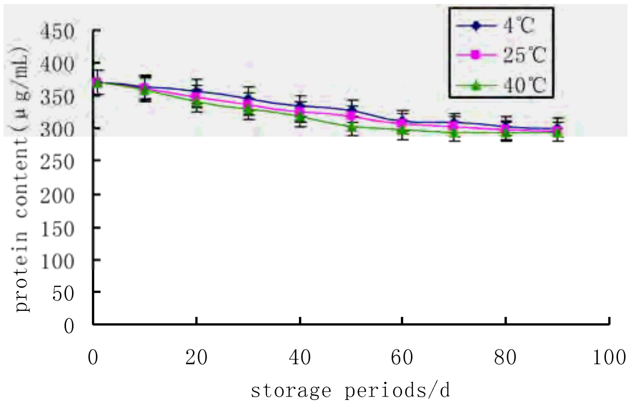

Fig. 7. Change of protein content over time in beer produced by the *S. cerevisiae* recombinant strain.

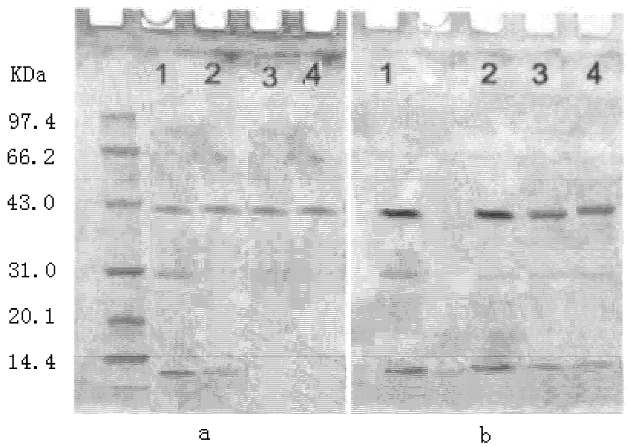

Fig. 8. Separation of proteins from beer by SDS-PAGE. Left lane is the marker lane and lanes 1, 2, 3, and 4 represent beer storage for 1, 30, 60 and 90 days respectively. a) Proteins from beer produced with *S. cerevisiae* strain 163. b) Proteins from beer produced by the *S. cerevisiae* recombinant strain.

Electrophoretic separation of proteins during draft beer storage

Electrophoretic separation of the draft beer samples (Fig. 8) stored for different time periods (1, 30, 60 and 90 days) illustrated the change in protein content. Lanes 1, 2, 3, and 4 represent storage for 1, 30, 60 and 90 days respectively. The protein band with a molecular mass of around 10 KDa became weaker when the control beer (brewed with the non-recombinant yeast) was stored for two months, indicating a low content of protein in the beer. When this beer was stored for three months, the protein band disappeared, indicating complete protein degradation (Fig. 8a). The protein band still existed when draft beer produced by recombinant yeast was stored for three months (Fig. 8b).

The molecular mass of the protein was similar to that of the lipid-transfer protein reported in the literature[15]; this protein is known as the major foam-stabilizing protein. Other proteins, with a molecular mass around 30 KDa, also disappeared from the electrophoretogram.

CONCLUSIONS

The following were tested: 24 h cultivation, maximum reduction of glucan, fermentation rate, fermentation degree, total acid, *S. cerevisiae* flocculation after peak period, lowest reduction of diacetyl, content of glucan and wort glucan after fermentation, degradation rate of β-glucan, Proteinase A activity, and foam stability during beer brewing. The physiochemical indices were normal and there were no apparent differences with the control samples. The indices tested using the recombinant *S. cerevisiae* met the quality criteria of the brewery.

The above mentioned experimental results indicate that the beer fermented with the recombinant strain had stable quality and flavor; the glucan content was reduced, and the degradation rate was above 80%. The filtration rate improved by nearly 20% and the foam stability reached 240 NIBEM units.

The pattern of SDS-PAGE of proteins in draft beer at different storage periods showed that lipid transfer protein 1(10KDa) in the draft beer after storage for 3 months had disappeared from the control, but was still present in the beer made from the recombinant yeast.

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