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Strategy for Prevention of Weakly Flocculating Characters in Bottom Brewing Yeast Strains

Chul Cheong, Karl Wackerbauer[1], and Soon Ah Kang*

Department of Fermented Food Science, Seoul University of Venture & Information, Seoul 137-070, Korea
[1]Department of Biotechnology, Technical University of Berlin, Berlin, Germany

Abstract To prevent weakly flocculating characters of bottom brewing yeast during first fermentation, various technical investigations were carried out using strain of *Saccharomyces cerevisiae*. It appeared that the propagation at 10°C promoted the molecular structure and biochemical composition of cell wall in favor of flocculation. The yeast grown at 20°C by addition of zinc ion also had a stimulating effect on flocculation behavior during first fermentation cycle. The zinc ion did not influence directly on the changes of cell wall in favor of stronger flocculence. The increased fermentation activity of yeast due to addition zinc ion was rather responsible for the intensified flocculation capacity. It was concluded that the weakly flocculating characters of bottom brewing yeast during first fermentation can be solved by using yeast propagated at 10°C or by means of yeast by addition of zinc ion during propagation.

Key words: flocculation, pure culture, fermentation ability, mineral ion

Introduction

Flocculation of brewing yeast cells occurs at the end of fermentation of beer. The yeast cells flocculate spontaneously and the process is reversible (1,2). Flocculation is governed by the competition between electrostatic repulsion and polysaccharide-protein bond (3-5). Furthermore, the phenomenon of flocculation is well known to be associated with a calcium-dependent lecitin activity on the cell surface (6,7). Control of flocculation behavior of yeast is an important aspect with regard to yeast management and final product quality (8). Strongly flocculating yeasts is prematurely sedimented out of beer fermentation, thereby causing fermentation and beer quality problem. In contrast, weak flocculation will lead to problems during largering and subsequent filtration of the beer (9). The mechanism of flocculation is not yet completely understood and is still the subject of much controversy. It is a very complex progress which depends on various factors such as the yeast strain (genetics, physiological state, and metabolism) (10-12), the composition of the culture medium, and the propagation conditions (pH, temperature, agitation, and aeration) (13,14). The cell concentration influences flocculation, a reduction of 50% in cell concentration leads to a decrease of about 25% in floc size (15,16). Garsoux *et al.* (17) and Lund (18) reported that cells cultivated at 10°C exhibited flocs during the late exponential phase. The growth temperature did not inhibit cell-cell interactions but probably induced or repressed the synthesis of a cell wall component involved in flocculation. Low growth temperatures increased flocculation capacity approximately 4-fold, compared to growth at high temperatures (19). Van Hamersveld *et al.* (20) noted that above a temperature of 15°C, the bond strength, floc size, and settling rate of the flocs decrease considerably. Top strains were generally more hydrophobic than bottom strains, due to higher surface protein concentrations (21). The increase of flocculence as a function of culture time correlates also with an increase of the cell hydrophobicity (21-25). Patel and Ingledew (26) observed that a relationship between yeast flocculation and intracellular acid-soluble glycogen has been established. In the present work, various technical investigations were studied for prevention of weakly flocculating characters of bottom brewing yeast during first fermentation using strain of *Saccharomyces cerevisiae*.

*Corresponding author: Tel: +82-2-3470-5273; Fax: +82-2-523-6767
E-mail: sakang@suv.ac.kr
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Materials and Methods

Strain of yeast A strains Rh, *S. cerevisiae*, was used in this study which is a bottom strong flocculent fermentation brewing yeast. This strain was obtained from the Brewing and Research and Teaching Institute in Berlin.

Preculture All yeasts were inoculated into of 200-mL Erlenmeyer flask containing 50 mL of wort and incubated statically at 25°C for 48 hr.

Propagation of yeast Fifty mL of preculture were inoculated in a 10-L stirred conical glass flask (Schott Duran) that contained 5 L of sterile wort, and propagated with agitation at 100 rpm under continuous aeration at different temperatures. The sterile wort contained (mg/L) total nitrogen 1.018, and free amino nitrogen 186, zinc 0.10, and the content of fermentable extract was 12%.

Fermentation Yeast cells were harvested after growth to stationary phase and then were pitched 5-L glass tube fermentor containing 2.5 L of 12% wort for fermentation tests and then fermented at 20°C for 6 days. After fermentation, the yeast was harvested and used again for the next fermentation test.

Viability assay Viable cells were measured with a microscope after staining with Mg-1-aniline-8-naphthalene-sulphuric acid (ANS, Merck Pharma, Darmstadt, Germany). Viability was calculated by dividing the number of viable cells by the total number of cells, with results given as percentages.

Glycogen assay Glycogen content was determined by the modification described by Quain (27). For the determination of the glycogen, the alkali- and acid-soluble fractions were treated with amyloglucosidase (EC.3.2.1.3.; Asp. Niger, Boehringer Mannheim, Germany) and the released glucose estimated using an enzymatic colorimatic kit (Boehringer Mannheim).

Hydrophobic interaction chromatography for flocculation (HICF) assay The test was based on modified method of Jibiki *et al.* (28). Yeast cells were harvested by centrifugation and washed twice with 100 mM sodium acetate buffer (pH 4.2). Subsequently, the cells were resuspended in the same buffer to give a suspension with about 5% wet weight per volume. The concentration of the yeast corresponded to approximately 2.5×10^7 cells/mL. The hydrophobic colume was prepared in the following way: the Bio-Spin disposable chromatography colume obtained from Bio-Rad Laboratories was used; the gel was phenyl sepharose CL-4B of Pharmacia; it was packed in the colume to a volume of 0.25 mL and equilibrated with 100 mM sodium acetate buffer containing 1.0 M NaCl. Subsequently 0.1 mL of the cell suspension was applied to the colume and 3 mL of the buffer containing NaCl was then added. The eluent was collected, and the absorbance was measured at 660 nm in a spectrophotometer. All the preparation steps for yeast suspension were carried out at 4°C to suppress the metabolic activity of yeast cells. The absorbance of 0.1 mL of the cell suspension diluted with 3 mL of the same buffer containing NaCl was also measured. The HICF value was defined by the proportion of retained cells and was calculated by using the following equation: HICF value (%) = 100 (A applied - A eluent)/A applied, where A applied is the absorbance of 0.1 mL of the cell suspension diluted with 3 mL of the same buffer containing NaCl, and A eluent is the absorbance of the eluent.

Results and Discussion

Influence of propagation step Figure 1 shows the changes of fermentation ability and cell in suspension during first cycle dependent on the propagation step. The second cycle was involved regarding fermentation activity and flocculation for direct comparison. The 3 samples showed a similar fermentation activity during first cycle. The onset of flocculation was among the samples almost the same in the first cycle. The 3 samples of first cycle showed a delayed fermentation activity and a weak flocculation intensity compared to second cycle. Hence the increase of propagation step had no influence on flocculation behavior and fermentation activity in the first cycle. It was suggested that the yeast cell might be adapted to environmental condition during propagation and improved flocculation intensity according to frequent propagation step.

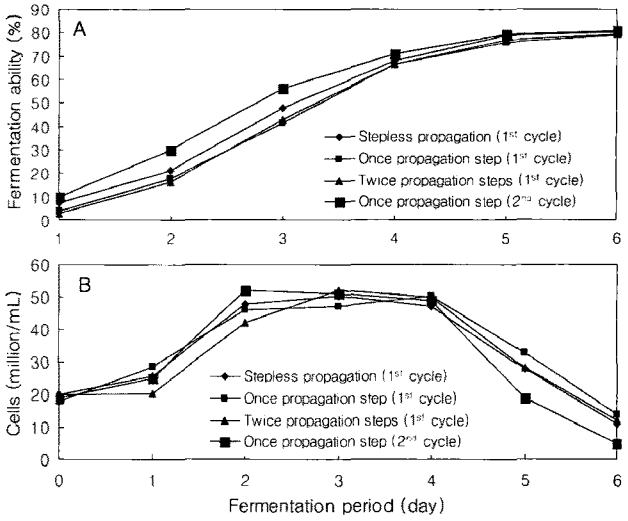

Fig. 1. Changes of fermentation ability (A) and number of cells in suspension (B) grown at different propagation step during propagation.

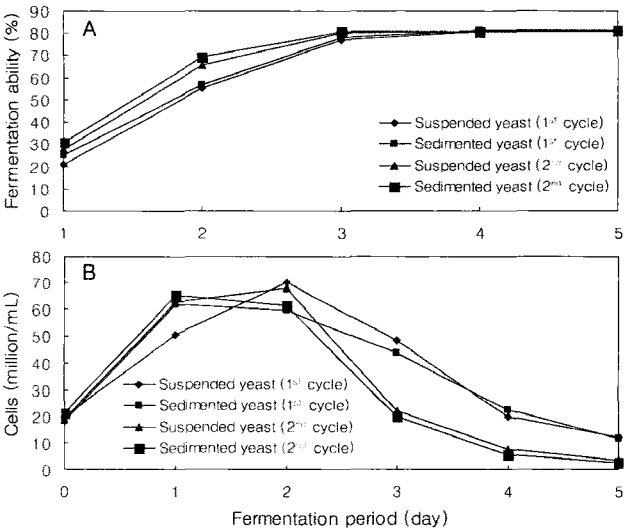

Fig. 2. Changes of fermentation ability (A) and number of cells in suspension (B) grown at different yeast crop during propagation.

Influence of yeast crop The results of this study that the samples which were pitched from suspended and sedimented yeast after propagation respectively indicated a similar fermentation activity and change of cell in suspension during first and second cycle are shown in Fig. 2. The samples during second cycle showed a stronger flocculation behavior considerably compared to first cycle independent on variation of yeast crop. So these data showed increasing flocculation capacity with increasing generations and consequently the variation of propagation crop had no effect on flocculation capacity and fermentation activity in the first cycle. The reason why the flocculation capacity was increased with increasing generations can be explained because the enhanced collision among the cells due to intensified fermentation activity occurred. In general, all yeast suspension just after propagation was used for the first fermentation in the brewing industry. For the next fermentation, it is used only the yeast which was harvested

Table 1. Yeast growth at different incubation ratio

Incubation ratio	Extract (%)	Maximum cells (million/mL)	Maximum cells after incubation (hr)
1:250	4.18±0.2[1)]	187±7.4	50±2.5
1:500	4.96±0.30	178±10.7	60±4.7
1:1,000	5.02±0.31	180±14.4	68±3.6

[1)]Means±SD (n=3).

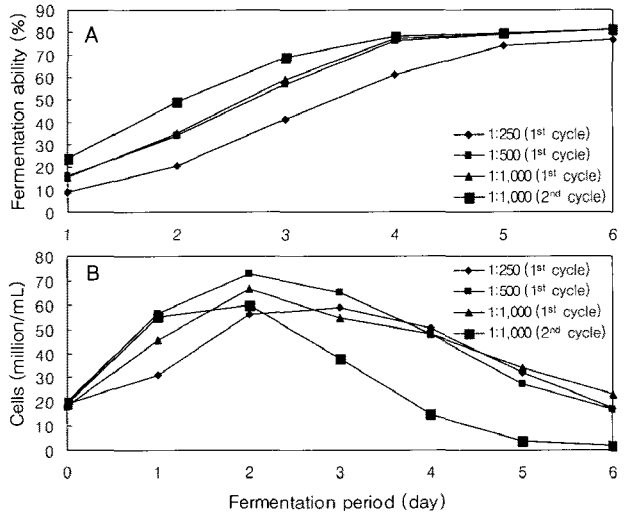

Fig. 3. Changes of fermentation ability (A) and number of cells in suspension (B) grown at different incubation ratio during propagation.

and repitched after first fermentation from cylindroconical tanks. Thereby it is occurred a selection. So it is enriched with strong flocculation yeast in cylindroconical tanks like Van der Aar (8) reported.

Influence of incubation ratio The results of the influence of incubation ration are shown in Table 1 and Fig. 3. The samples which were grown under incubation ratio of 1:250 showed that the maximum cell number was reached after 50 hr. In contrast, the samples which were grown under incubation ratio of 1:500 and 1:1,000 indicated that the maximum cell number was reached after 60 and 68 hr, respectively. Hereby the all samples showed a similar yeast yield at the end of propagation.

The sample of 1:250 indicated the lowest attenuation degree during first cycle. In addition, the samples of 1:500 and 1:1,000 began to sediment at second fermentation day, whereas this was occurred at the sample of 1:250 at third fermentation day. However, the all samples showed a similar flocculation intensify at the end of fermentation so that the same cell number in suspension was observed. The sample of second cycle showed a higher fermentation activity and flocculation intensity considerably compared to those of first cycle. Hereby it was conclude that the weakly flocculating characters of bottom brewing yeast in first cycle was not influenced by different generation frequency.

Influence of temperature Effect of the cultivation

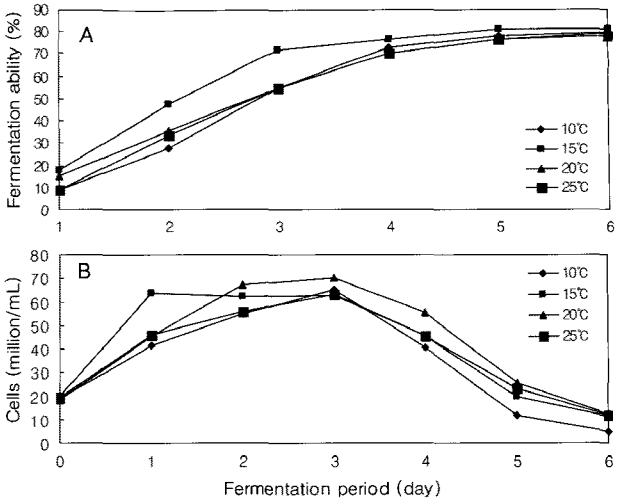

Fig. 4. Changes of fermentation ability (A) and number of cells in suspension (B) grown at different temperature during propagation.

temperature was studied, using 4 temperatures: 10, 15, 20, and 25°C. The results of the samples using pitching yeasts grown at different temperature are observed in Fig. 4. It was showed that the temperature of propagation had no effect on flocculation behavior between 15, 20, and 25°C which was confirmed by Fontana *et al.* (4). It also was found that the sample using pitching yeast propagated at 15°C led to the highest fermentation activity. All samples flocculated at the third fermentation day. In addition, it was noticeable that the sample using pitching yeast grown at 10°C flocculated more intensive than those of samples from other temperatures, so that the clarification of young beer was reached earlier. So the cell number in suspension was 4.7 million/mL from propagation at 10°C at the end of fermentation whereas this was above 11 million/mL at the samples from the propagation of 15, 20, and 25°C, respectively. Hence the yeast grown at 10°C positively influenced on the flocculation behavior during first cycle, while the propagation above 15°C had a negative effect on flocculation ability. It is assumed that 10°C-cultivation leads to accumulation of mannoprotein on the cell surface which is connected with flocculation capacity. In literature, various statements have been made concerning temperature of propagation with regard to flocculation capacity (17,19).

The pronounced difference regarding flocculation ability between propagation at 10 and 20°C, respectively, can be obtained in Fig. 5. The yeasts grown 10 and 20°C showed, as predicted, intensive fermentation activity at the first cycle than those of second cycle. Hereby it also was obtained that the sample using pitching yeast grown at 10°C indicated strong flocculation ability at the first cycle. This can be observed at the second cycle from the sample using pitching yeast propagated at 20°C. Therefore it was achieved a constant flocculation character during first cycle using propagation at 10°C and the weakly flocculating character could avoided during first cycle.

Van Hamersveld *et al.* (20) showed a good correlation between temperature and flocculation capacity. Patel and Ingledew (26) demonstrated a relationship between acid-soluble glycogen and flocculation capacity. However, it

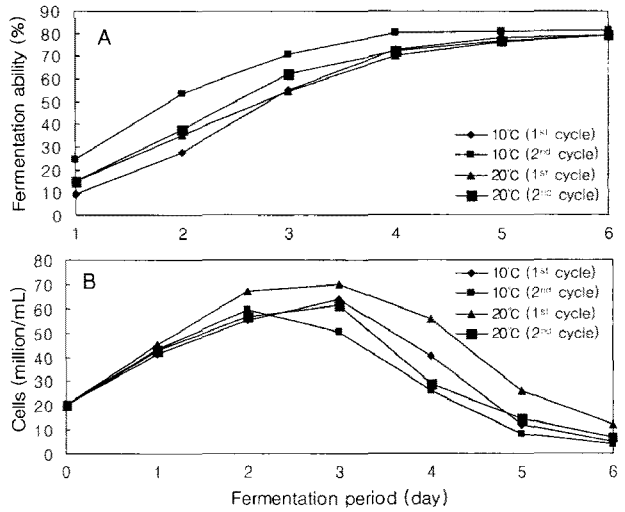

Fig. 5. Comparison of fermentation ability (A) and number of cells in suspension (B) between 1st and 2nd cycle grown at different propagation temperature.

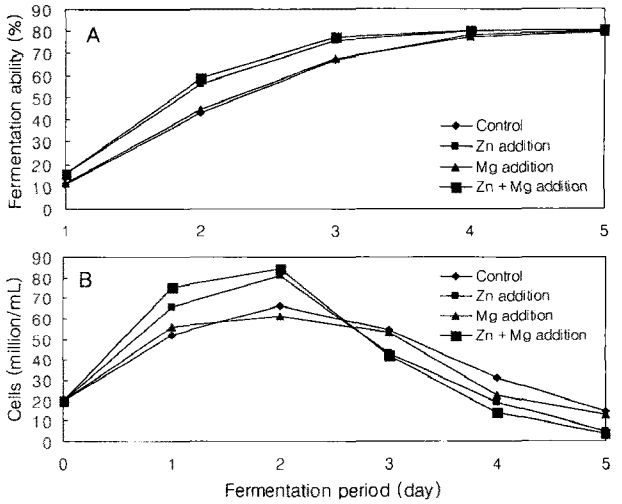

Fig. 6. Changes of fermentation ability (A) and number of cells in suspension (B) grown at different addition of metal ion during propagation.

Table 2. Hydrophobicity and glycogen content dependent on the propagation temperature

Propagation temperature (°C)	Hydrophobicity (%)	Glycogen (% d.w.)
10	77±4.6[1)]	19.6±0.9
15	72±5.0	25.9±1.8
20	71±4.3	26.8±1.3
25	76±3.4	24.0±0.9

[1)]Means±SD (n=3).

was not fully explained the reason why the yeast grown at low temperature during propagation led to a stronger flocculation behavior. To study the reason of improved flocculation behavior during first cycle using propagation at 10°C, hydrophobicity and glycogen content were investigated. As shown in Table 2, the yeast grown at 10 and 25°C, respectively, showed a slightly higher value of hydrophobicity compared to those of grown at 15 and 20°C. The yeast grown at 10°C indicated a lower content than those of yeasts cultivated at above 15°C. Van der Aar *et al.* (25) have been found a positive relationship between temperature and flocculation capacity. However, the difference among the temperatures with regard to hydrophobicity and glycogen content was insignificant in this investigation. From the view of results, no correlation between propagation at 10°C and hydrophobicity or glycogen content regarding flocculation behavior was obtained. The propagation at 10°C may result in a modification of the molecular structure and biochemical composition of yeast cell wall in favor of flocculation and consequently a modification of flocculating ability. However, the regulation of temperature with respect to flocculation will require investigation. It is suggested that the low temperature of propagation may be lead to a stronger activation or accumulation of factors in a cell wall, which affect flocculation capacity, like substances (such as mannan protein) described by Miki *et al.* (29).

Influence of metal ions Influence of the metal ions was studied, using zinc and magnesium ions. As shown in Fig. 6, the results showed the changes of fermentation and cell number during first cycle dependent on addition of metal ions. The samples using pitching yeasts grown at 20°C with zinc ion during the propagation indicated enhanced fermentation activity and a higher cell growth compared to control sample. The onset of flocculence was observed at second fermentation day in all samples. The samples using grown by addition of zinc ion sedimented compared to control sample considerably. These results could also confirmed by the samples that were grown at 15 and 25°C. Many workers have been published concerning the action of metal ions on the flocculation of brewing yeasts (30-33). The phenomenon of flocculation is well known to be associated with a calcium-dependent lecitin activity on the cell surface (7).

As shown in Fig. 7, the sample using pitching yeast grown by addition of zinc ion showed a stronger fermentation activity and a higher cell growth during first cycle compared to control sample during second cycle. The sample using pitching yeast grown with addition of zinc ion also indicated that cell number in suspension such as control sample showed during second cycle. So the problem of weak flocculation behavior above propagation at 15°C during first cycle could solve using addition of zinc ion. A physiological state of yeast (formation and activity of mannan protein on the cell wall) and a corresponding composition of wort (reduction of sugar concentration) as well as a mechanical energy (CO_2-convection flow) are a basic conditions for the formation of flocculation like Masy *et al.* (5) and Dengis *et al.* (1) suggested. In this study, the following flocculation mechanism of zinc ion can be proposed. The zinc ion accelerates the absorption of maltose by yeast. Hereby a repressive effect of sugar on the formation of flocculation abolished like Stratford and Assinder (34) described. There is basically repulsion between cell surfaces. The repulsion force between yeast cells was overcome by means of CO_2-convection flow.

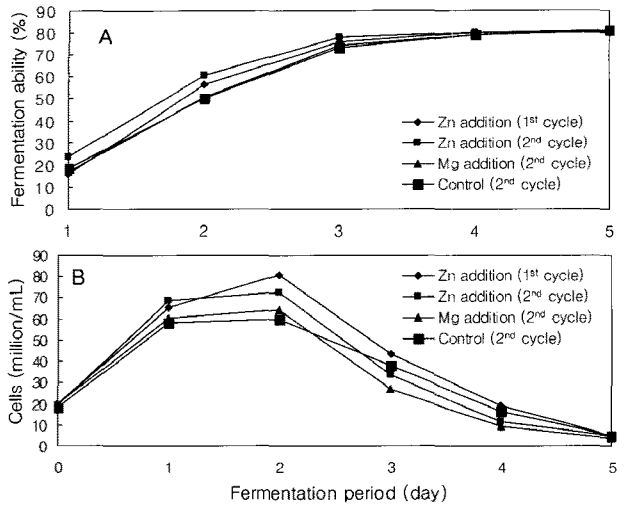

Fig. 7. Comparison of fermentation ability (A) and number of cells in suspension (B) between 1st and 2nd cycle grown at different addition of metal ion during propagation.

This results from to an increased frequency of collision between the cell-cell, cell-flocs as well as the flocs-flcos, which lead to bigger flocs. Consequently, the yeast flocculated intensive and sedimented faster during first fermentation. From the results of this study, it was assumed that the deficiency of zinc ion can regarded as a reason of weak flocculation capacity during first fermentation in bottom brewing yeast. It seemed that the zinc ion did not influence on the changes of cell wall in favor of increased flocculation behavior like suggested by Russel and Stewart (31). The increased fermentation activity by means of addition of zinc ion is responsible for the enforced flocculation capacity. Nischihara *et al.* (35,36) and Dengis and Rouxhet (6) have been found a inducing effect on flocculence of magnesium ion. The results indicated that the fermentation activity and flocculation capacity were not improved by addition of magnesium ion during propagation. It is clear that the rate of flocculation depends upon a complex relationship between cell concentration, mechanical energy input and pH of the suspending medium. From these experiments, it was concluded that low temperature and addition of zinc ion during propagation are a very significant factors for flocculation capacity.

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