Full Length Research Paper

Flocculation phenomenon of a mutant flocculent *Saccharomyces cerevisiae* strain: Effects of metal ions, sugars, temperature, pH, protein-denaturants and enzyme treatments

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The flocculation mechanism of a stable mutant flocculent yeast strain *Saccharomyces cerevisiae* KRM-1 was quantitatively investigated for potential industrial interest. It was found that the mutant flocculent strain was NewFlo phenotype by means of sugar inhibition test. The flocculation was completely inhibited by treatment with proteinase K, protein-denaturants and carbohydrate modifier. The absence of calcium ions significantly inhibited the flocculation, indicating that Ca^{2+} was specifically required for flocculation. The flocculation was stable when temperature below 70°C and pH was in the range of 3.0 - 6.0. The flocculation onset of the mutant flocculent strain was in the early stationary growth phase, which coincided with glucose depletion in the batch fermentation for the production of ethanol from kitchen refuse medium. The results are expected to help develop better strategies for the control of mutant flocculent yeast for future large-scale industrial ethanol fermentation.

Key words: Kitchen refuse, *Saccharomyces cerevisiae*, flocculation, lectin-like, newflo phenotype.

INTRODUCTION

Flocculation of yeasts is usually described as a phenolmenon wherein cells could aggregate spontaneously and form flocculation which sediment rapidly in culture medium (Miki et al., 1982a). This capacity of yeast cells has been traditionally utilized by the brewing and wine industries and more recently, in continuous ethanol fermentation processes (Ge et al., 2006; Kida et al., 1992; Teunissen and Steensma, 1995; Seong et al., 2006; Nonklang et al., 2009). The self-flocculating yeast strains produce higher cell concentration under proper condition in the bioreactor and provide efficient separation process of yeast cells from fermenting mash at the end of fermentation. Thus, it is possible that flocculent yeast could be advantageous in industrial fermentation as part of the process in bioethanol production.

The mechanism of yeast flocculation was intensively explored in the past two decades. Several hypotheses have been proposed, among them, lectin-like model was most widely accepted, based on the observations that flocculation was reversibly inhibited by free sugars in the medium. In this model, a specific lectin-like component present in the cell wall of the flocculent strain will recognize and adhere to α-mannans carbohydrate on an adjoining cell, with Ca^{2+} ions acting as cofactors activating the binding capacity (Miki et al., 1982a, b). Therefore, for the flocculation of cells, the presence of Ca^{2+} in culture was required. In addition, other fermentation process parameters in terms of temperature, pH, sugar, ethanol concentration, etc, giving impact on flocculation were reported as well. However, due to yeast cell flocculation variability, the understanding and monitoring of cell flocculation is a concern in industrial application (Verstrepen et al., 2003).

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Saccharomyces cerevisiae is the preferred yeast for most ethanol fermentation due to their high ethanol yield, high productivity and high ethanol tolerance (Lin and Tanaka, 2006). In previous study, the non-flocculating strain of *S. cerevisiae* ATCC 24860 was selected for ethanol production using kitchen refuse medium, a new potential biomass in Japan (Praneetrattananon et al., 2007). During successive cultivation, the strain steadily flocculated in kitchen refuse medium, but not in artificial medium under the same fermentation condition. The experiment was repeated three times and the same result was obtained, indicating that the specific association of yeast flocculation with kitchen refuse medium. The isolated mutant flocculent strain of *S. cerevisiae* KRM-1 kept steady flocculating ability in kitchen medium and giving high ethanol production performance in batch and repeated-batch fermentation (Ma et al., 2009). Therefore, *S. cerevisiae* KRM-1 could be considered as a good candidate for bioethanol production using kitchen refuse medium. However, it appears no research has been done to address the flocculation characteristics of any mutant generated from this strain so far. Hence, for a better understanding of the flocculating ability of this strain, and seeking the optimum conditions for controlling flocculation formation in future practical industrial production, this work investigated the effect of fermentation process parameters, such as temperature, pH, sugar and calcium ions, on flocculation of *S. cerevisiae* KRM-1.

MATERIALS AND METHODS

Chemicals and enzyme

Culture media components and all the mineral salts were purchased from Wako Pure Chemical Industries, Limited. Sugars (mannose, glucose, maltose, sucrose and galactose) were purchased from Sigma Chemical Company, Louis, Mo, USA. Proteinase K (30 U/mg) were obtained from Sigma. All other reagents were of analytical grade or biochemical grade and were used without further purification.

Preparation and saccharification of the kitchen refuse

Food waste was provided by the local supermarket (Wakamatsu, Kitakyushu and Fukuoka, Japan). About 70 kg of kitchen refuse were collected in spring in the Kyushu area and was separated into 3 groups by hand: the carbohydrate group (that is, rice, bread, pasta, noodles, etc), the protein group (that is, fish, beef, pork, chicken, etc) and the vegetable/fruit group. Thereafter, the kitchen refuse was again mixed in the following proportions: 43% (w/w) carbohydrates, 19% (w/w) protein and 38% (w/w) vegetables and fruits. The subsequent saccharification and pre-treatment for medium preparation were done as previously described (Praneetrattananon et al., 2007). The saccharified liquid was called 'kitchen refuse medium,' and was adjusted to various concentrations for inoculum preparation and fermentation.

Strains, media and pre-cultivation

The mutant flocculent strain *Saccharomyces cerevisiae* KRM-1

used in this study was obtained from wild type strain *S. cerevisiae* ATTCC 24860. The yeast cells were maintained at 4°C on YM agar slants containing 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose and 20 g/L agar, with fortnightly sub-culturing until usage.

For pre-cultivation of strain, one loop of *S. cerevisiae* KRM-1 from YM agar slants was added to a 300 ml Erlenmeyer flask, which contained 50 ml sterilized kitchen refuse medium with 30 to 50 g/L of glucose concentration or YM medium with 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L glucose under natural pH condition. In order to stimulate the growth of yeast strain, 2 g/L yeast extract, 1 g/L peptone were also added to kitchen refuse medium. The culture was incubated at 30°C on an orbital incubator shaking at 100 rpm. After 24 h of growth, the cells were harvested by centrifugation (3000×g, 10 min) and used for latter investigation.

Measurement of flocculation

Flocculation was measured using the method provided by Bony et al., (1998) and modified as follows: for flocculation measurement yeast cells were deflocculated by two washes in 50 mM sodium acetate, pH 4.5, 5 mM EDTA (ethylene diamine tetraacetic acid) buffer and twice with distilled water. The cells were resuspended in flocculation buffer (50 mM sodium acetate, 5 mM $CaCl_2$, pH 4.5) while the OD at 600 nm was adjusted to about 2. Flocculation induced by Ca^{2+} in flask was shaken at 100 rpm for 30 min. Then 5 ml of cell suspension was put into 10 ml tubes and left vertically and undisturbed for 6 min to allow for settling. 0.3 ml of samples was taken from just below the meniscus and the optical density was determined using spectrophotometer. Flocculation ability (F) was determined by the following equation: $F = (1 - B/A) \times 100\%$. Where A is the absorbance at 600 nm (A_{600}) immediately before cells were shaken in flocculation buffer and B is the absorbance at 600 nm (A_{600}) after the flocculation settled for 6 min.

Effect of sugars and pH on flocculation

Stationary phase cells grown on YM medium or kitchen refuse fermentation medium were harvested by centrifugation, washed twice with distilled water and resuspended. Influences of sugars (mannose, glucose, maltose, sucrose and galactose) on flocculation were determined by addition to the resuspended cells in flocculation buffer (50 mM sodium acetate, 5 mM $CaCl_2$, pH 4.5). After incubation at 30°C for 30 min with constant gentle mixing, samples were subjected to the flocculation assay. The influence of pH on flocculation was examined by the method reported before (Smit et al., 1992). Yeast cells were harvested and washed with double-distilled water and resuspended in the pH range of 2.0 - 6.0, a 50 mM Na acetate-5 mM $CaCl_2$ buffer was used and a 10 mM Tris-HCl-5 mM $CaCl_2$ buffer was used for pH 7.0 to 9.0. After shaking at 30°C for 30 min, the flocculation ability was measured immediately.

Treatment of cells with proteolytic enzymes and chemical modification of cell surface protein and carbohydrate components

For enzyme treatments, cells were harvested and washed twice, then resuspended in 50 mM sodium acetate buffer (pH 6.5), to a final concentration equivalent to an A_{600} of 2.0. Then about 0.1 mg/ml Proteinase K concentrations was added and incubated at 37°C for 1 h with constant gentle mixing. For protein-denaturants treatments, 8 M urea or 5 M guanidine·HCl was added to a cell suspension of flocculent cells in the 50 mM sodium acetate buffer (pH 4.5) and incubated at 30°C for 30 min with constant gentle

mixing. For carbohydrate modification, flocculent cells was treated with 20 mM of periodate oxidation at 0°C for 30 min in the dark.

After appropriate treatments described above, samples were washed three times with distilled water and subjected to the flocculation assay as described above.

Effect of glucose concentration on the flocculation onset of the mutant flocculent strain in kitchen refuse medium

The batch fermentation were conducted in 1 L jar-bioreactor, 10% (v/v) inoculum broth were added with final working volume of 800-ml. Reactor conditions were maintained at 30°C with agitation at 100 rpm for 24 h. Flow rate of 0.1 vvm air was supplied at first 8 h to stimulate the growth of yeast. The pH of culture was recorded constantly but not regulated.

RESULTS AND DISCUSSION

Flocculation characteristics of *S. cerevisiae* KRM-1

The mutant flocculent yeast strain *S. cerevisiae* KRM-1 was recently isolated during the repeated-batch fermentation using kitchen refuse as medium. Large cell aggregates or flocculation occurred very quickly and sedimented within 5 min in the jar bioreactor at the end of cultivation in the formulated ethanol-producing medium. The cells of parent and mutant strain from the stationary phase were harvested, after washing three times with distilled water and suspending in flocculation buffer, subjected to light microscope observation. As shown in Figure 1b, large flocculation morphology was clearly observed indicating strong cells interaction of flocculent strain. In contrast, Figure 1a illustrated that cells of the non-flocculent strain interacted too weakly to form aggregates.

Figure 2 showed the possible model of conversion of non-flocculent to flocculent cultures. During the repeated-batch fermentation, some cells of parent strain gained flocculent characteristic by the stimulation from environmental factors such as pH, temperature and the composition of medium (Soares and Seynaeve, 2000). In this study, pH was considered as the possible factor which contributed to the mutant flocculation of the yeast strain, because the significant difference between the two kinds of media is that the pH value of kitchen refuse medium is lower than that of artificial medium, comprising an amount of lactic acid and acetic acid produced after saccrification which varied from 3 to 5. This hypothesis will be examined in future study. The mutant flocculent cells could spontaneously sediment to the bottom of fermentor; however, the non-flocculent cells were removed by the operation of medium change gradually. Therefore, the proportions of flocculent cells continue to increase and finally became dominant in the culture. For obtaining the flocculent strain, the flocculation was isolated from fermentor, after removing the liquid fraction which included the free cells. The organism was cultured on YM medium for several generations and only single colony was picked up to test its flocculation ability in culture

medium. When the colony showed stable flocculent phenomena, the flocculation were isolated and cultured on the YM medium again. The experiment was repeated for three times to ensure the purity of the isolated strain.

Flocculation phenotype of the mutant flocculent strain

Yeast flocculation was thought to be caused by calcium-dependent bonding between the flocculent cell's surface proteins (lectin-like) and specific sugar residues (Miki et al., 1982a,b). Two flocculation phenotypes could be distinguished by their sugar inhibition pattern: the NewFlo type, which was inhibited by mannose, glucose, maltose and sucrose but not by galactose; and the Flo1 type, which was inhibited by mannose but not by glucose, maltose, sucrose and galactose (Stratford and Assinder, 1991). To confirm the flocculation phenotype of the mutant flocculent yeast in this study, the yeast pattern of inhibition by sugar was tested. As shown in Figure 3, inhibition of flocculation was observed after the addition of 300 to 400 mM mannose, sucrose, maltose and glucose, but not inhibited by galactose indicating a typical NewFlo phenotype for *S. cerevisiae* KRM-1.

Effect of calcium ions on flocculation

The lectin hypothesis developed by Miki et al. (1982a,b) suggests that zymolectin (lectin-like) on the flocculent cell surfaces bind specifically to mannose residues of wall mannan on adjacent cells. The role of calcium ions has been interpreted as necessary to maintain the correct conformation of the zymolectins (Miki et al., 1982a; Taylor and Orton, 1978). Therefore, the influence of calcium ions on flocculation of mutant strain was investigated in this study. From Figure 4, it can be seen that flocculation was completely inhibited in the absence of Ca^{2+}, the flocculating rate increased significantly with the addition of Ca^{2+} and reach the maximum of around 85% when Ca^{2+} concentration in buffer was 0.1 mM. And further addition of Ca^{2+} did not facilitate the flocculation rate indicating the role of Ca^{2+} on flocculation was saturated. Furthermore, flocculation of the mutant flocculent strain could be inhibited in the presence of EDTA (date not show), which was consistent with the previous report that removal of calcium by EDTA chelation causes inhibition of flocculation (Straver et al., 1994). In addition, the influence of Mg^{2+}, Fe^{3+}, K^+, and Na^+ on flocculation were also tested while similar results were not obtained (data not shown), indicating that Ca^{2+} was specifically required for flocculation of the mutant flocculent yeast and fitted well with the calcium-bridging theory.

Effect of treatment with enzyme, protein-denaturants and carbohydrate modifier on flocculation

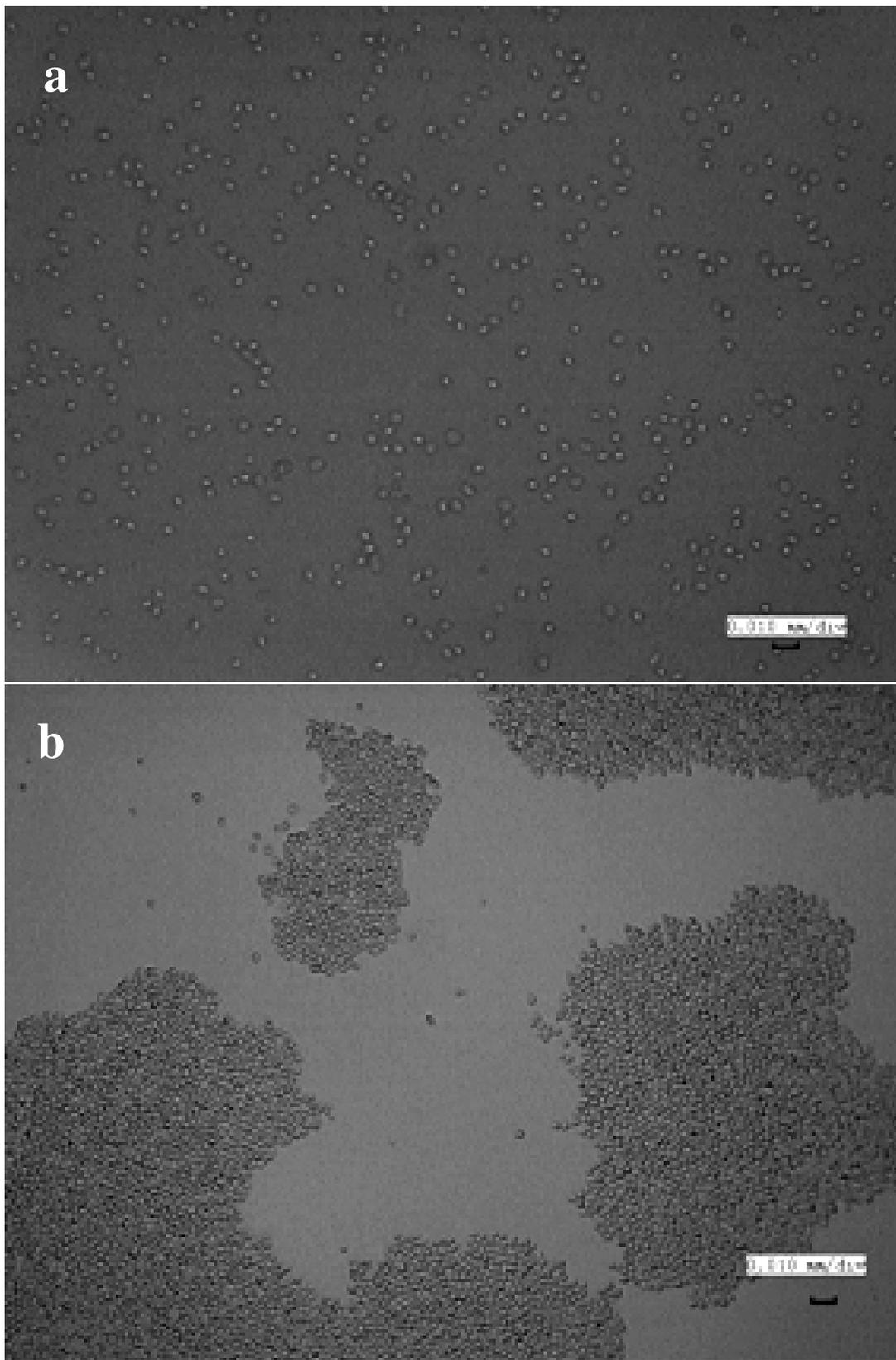Flocculation mediated by cell surfaces specific protein

Figure 1. Optical micrograph illustrating **(a)** the parent nonflocculent strain and **(b)** the mutant flocculent strain *S. cerevisiae* KRM-1. Both yeasts from the stationary phase were harvested, washed three times with distilled water and suspended in flocculation buffer. Scale bars represent a scale of 10 µm.
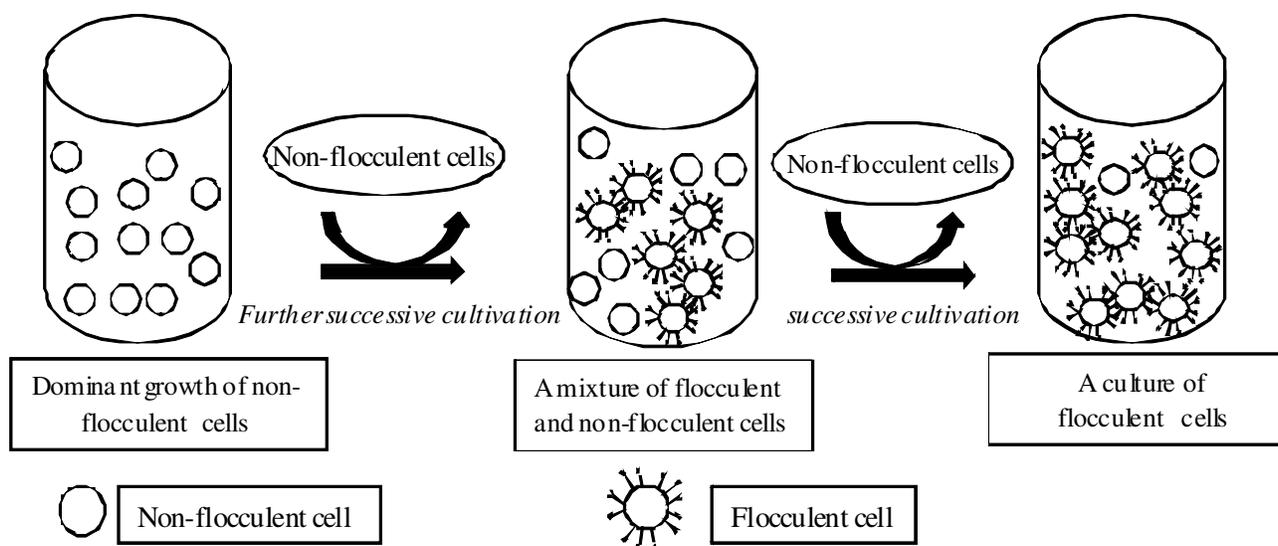
Figure 2. The possible Models of conversion of non-flocculent to flocculent cultures.
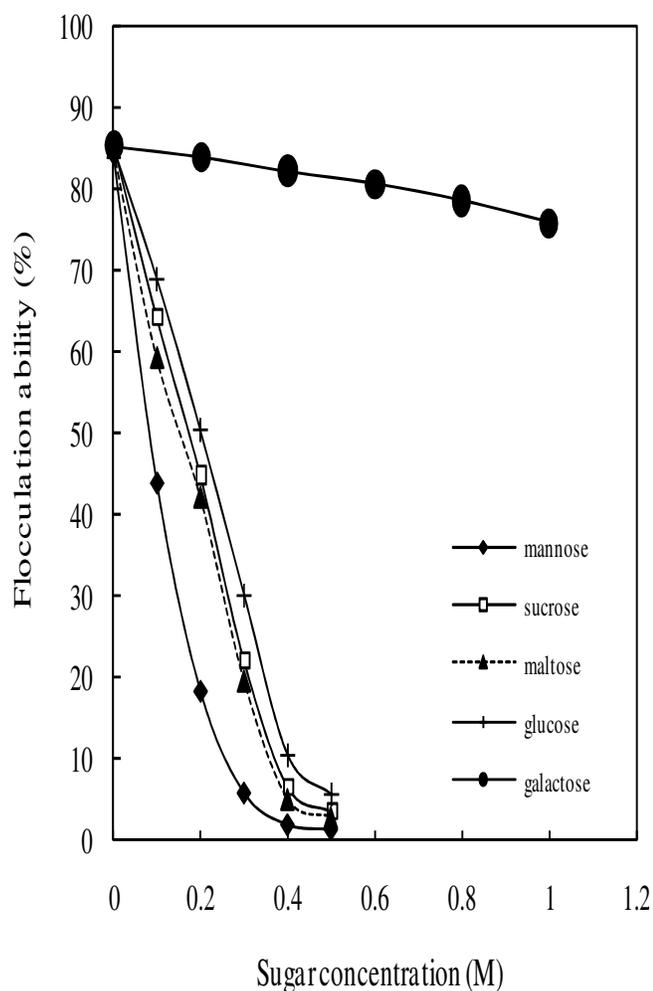

Figure 3. Effect of sugars on flocculation of the mutant flocculent strain *S.cerevisiae* KRM-1. Values are mean of three independent experiments.

has been known to be irreversibly abolished by protease treatments in other yeast (Nishihara et al., 1982; Straford, 1992, 1993). In order to check whether the flocculation of the mutant flocculent strain was also mediated by specific interaction between cell surfaces specific protein and carbohydrates, the stationary phase of yeast strain was tested for flocculation after treatments with enzymes, protein-denaturants and carbohydrate modifier. Figures 5a and b illustrate the effect of treatments with protein-denaturants on flocculation ability of mutant cells. Treatments of cells with the 8 and 5 M guanidine·HCl at 30°C for 30 min caused a complete and irreversible loss of flocculation-forming ability of the mutant flocculent strain. Figures 5c and d depicted the effect of proteinase K and periodate oxidation on flocculation of cells. Large flocculation disappeared and free cells suspended in the buffer indicating the interaction of cells was disrupted and chemicals caused failure of flocculation-forming ability of mutant flocculent strain. Therefore, it was evident that both protein and carbohydrate modifier on the cell surface play essential roles in the flocculation of the mutant flocculent strain.

Effect of pH and temperature on flocculation of the mutant flocculent strain

Figure 6 showed the effect of pH and temperature on flocculation of the mutant flocculent strain. The mutant strain from the stationary phase was examined over the various pHs in the range of 2.0-9.0. Flocculation occurred optimally across a pH range of 3.0 - 6.0, with significant decrease at lower pH (pH < 2) (Figure 6a). Yeast flocculation was optimal in slightly acidic conditions, pH 3.5 to 5.8 (Jin and Speers, 2000; Soares et al., 1994). Low pH inhibition of flocculation was also
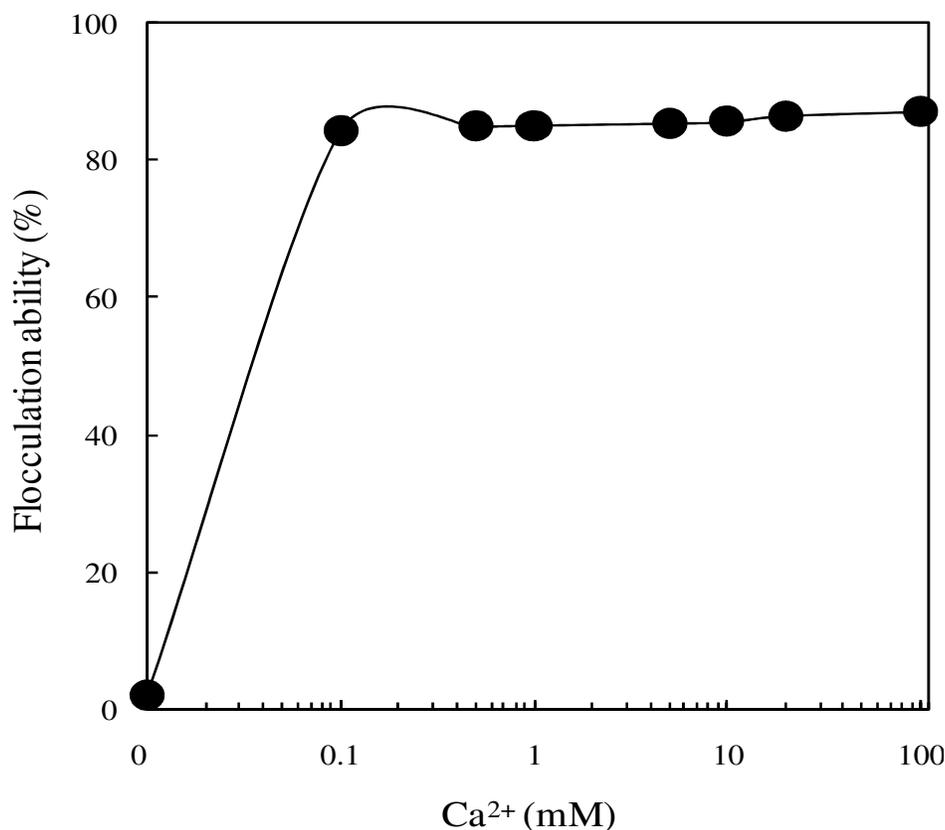
Figure 4. Effect of Ca^{2+} ions on flocculation of the mutant flocculent strain *S. cerevisiae* KRM-1. Cells were suspended in sodium acetate buffer (pH 4.5, 50 mM) containing different Ca^{2+} concentrations. Suspensions were agitated at 100 rpm, during 30 min, before flocculation was measured. Values are mean of three independent experiments.

more effective against NewFlo type strain due to conformational changes which occur when the electrostatic charge of surface proteins changes (Jin and Speers, 2000; Soares et al., 1994).

Temperature is one of the most important factors affecting growth and metabolism of yeasts. The exposition of yeast cells to supra-optimum temperature increases protein aggregation and denaturation, changing the structure of membranes and affecting different cellular processes (Estruch, 2000). To understand the effect of temperature on flocculation ability of the mutant flocculent strain, the cells were incubated at different temperatures and the flocculation ability of resulting samples was determined. The temperature dependence of flocculation ability of the mutant flocculent strain is depicted in Figure 6b. It can be seen that the cells' complete loss of their ability to flocculate with increase in temperature and almost complete deflocculation occurred at 80°C. The optimal temperature was 30°C, giving the best flocculation ability of 85%.

Taking together, the yeast flocculation can be easily deflocculated by pH change and higher temperature provides industry with a very simple technique to separate cells from media at any stage during fermentation.

Effect of glucose and ethanol concentration on the flocculation onset of the mutant flocculent strain in kitchen refuse medium

For characterization of the flocculation profile under laboratory conditions, batch fermentations with the mutant flocculent strain were carried out using the kitchen refuse medium. The effect of glucose and ethanol concentration in the medium on the flocculation mutant flocculent strain in this paper was studied as well. As Figures 7a and b showed that the flocculation of the cells was triggered when the early stationary growth phase was reached. It was consistent with the previous report that the Newflo phenotype cells progressively loss their flocculation ability in the early period of growth and become only flocculent towards the end of the active phase of growth, which could be partly due to increased transcription of flocculin genes at the post-diauxic shift (Stratford and Assinder, 1991). According to the result, the cells reach stationary phase under glucose concentration of 100 g/L for 12 h, however, under the high glucose concentration condition (210 g/L), it was prolonged to around 21 h. Therefore, it seems like high concentration of glucose can retard the cells reaching
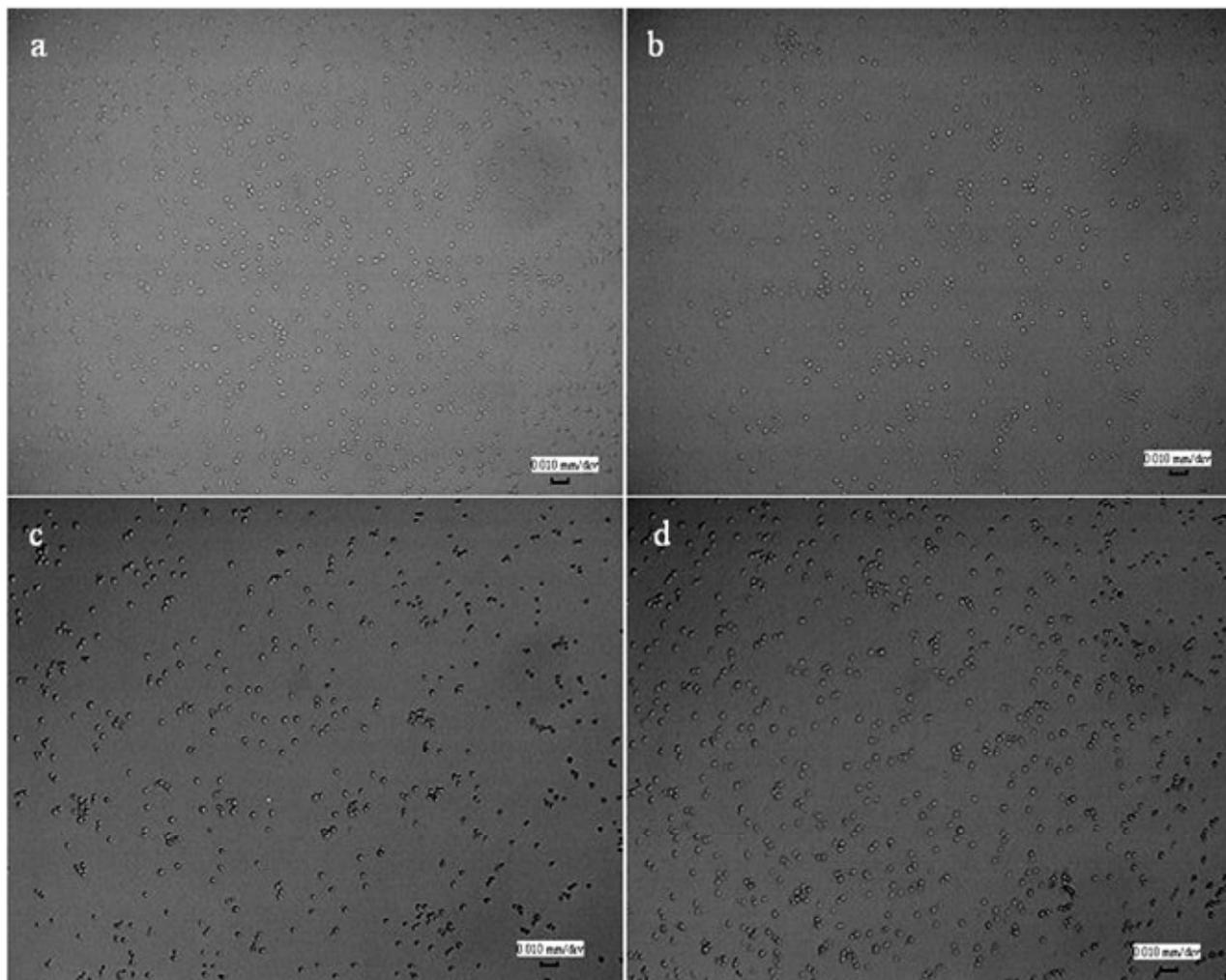
Figure 5. Yeast cells of the mutant flocculent strain *S. cerevisiae* KRM-1 observed by optical microscopy after treatments with **(a)** urea **(b)** guanidine·HCl, **(c)** proteinase K and **(d)** periodate oxidation. Scale bars represent a scale of 10 μm. Each result of experiments repeated three times.

stationary growth stage while no direct influence on cells flocculation. According to Soares and Mota (1996), in all situations flocculation is triggered after glucose depletion in the medium, which clearly shows a causal link between glucose depletion and the induction of yeast flocculation (Soares and Mota, 1996). Moreover, it was also found that the ethanol concentration as high as 99.87 g/L which was produced at the late fermentation stage did not give any influence on cells flocculation (Figure 7b). It was possible that stress factors such as high ethanol concentrations may induce the Flo genes through the numerous stress-responsive heat-shock elements that are found in the promoter region of these genes (Verstrepen et al., 2003; Teunissen et al., 1993). It is generally accepted that the onset of flocculation, in brewing yeasts of NewFlo phenotype, is triggered at the end of respire-fermentative growth phase, which coincides with the carbon source exhaustion and the attain-

ment of the higher ethanol concentration (Sampermans et al., 2005).

It is well known that high ethanol productivity could be achieved by using flocculent yeast. Therefore, the flocculating control is crucial in ethanol fermentation. Taking into account the data obtained in this work, Ca^{2+} concentration and pH range in kitchen refuse medium is suitable for the flocculation of the mutant strain. In order to achieve a high ethanol concentration and stable flocculation ability, the initial glucose concentration should be controlled at around 200 g/L in kitchen refuse medium.

Conclusion

The effect of sugars, calcium ions, enzymes, protein-denaturants, carbohydrate components, pH and temperature on the mutant flocculent strain was quantitatively
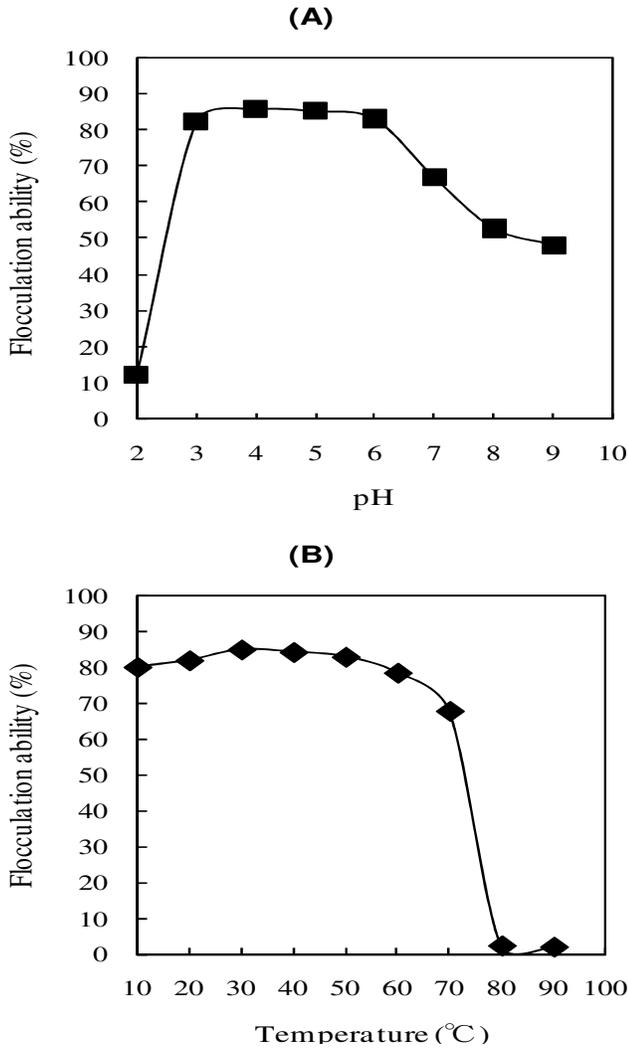
(A)

(B)

Figure 6. Effect of the pH (A) and temperature (B) on flocculation of the mutant flocculent strain *S. cerevisiae* KRM-1. Values are mean of three independent experiments.

investigated in this study. It was found that the mutant flocculent strain was NewFlo phenotype, which was inhibited by mannose, glucose, maltose and sucrose but not by galactose. The flocculation of mutant flocculent strain could be inhibited in the absence of Ca^{2+} or in the presence of EDTA, indicating that Ca^{2+} was specifically required for flocculation of the mutant flocculent yeast. The yeast flocculation deflocculated when enzyme, protein-denaturants and carbohydrate components were treated. The flocculating activity was stable when temperature was below 70°C and pH was in the range of 3.0 - 6.0. Flocculation onset of the mutant flocculent strain was in the early stationary growth phase, which coincides with the glucose depletion in the culture medium. The obtained results will help to develop strategies to monitor the flocculation of mutant strain, and consequently improve its ethanol fermentation performance in practical industrial production.
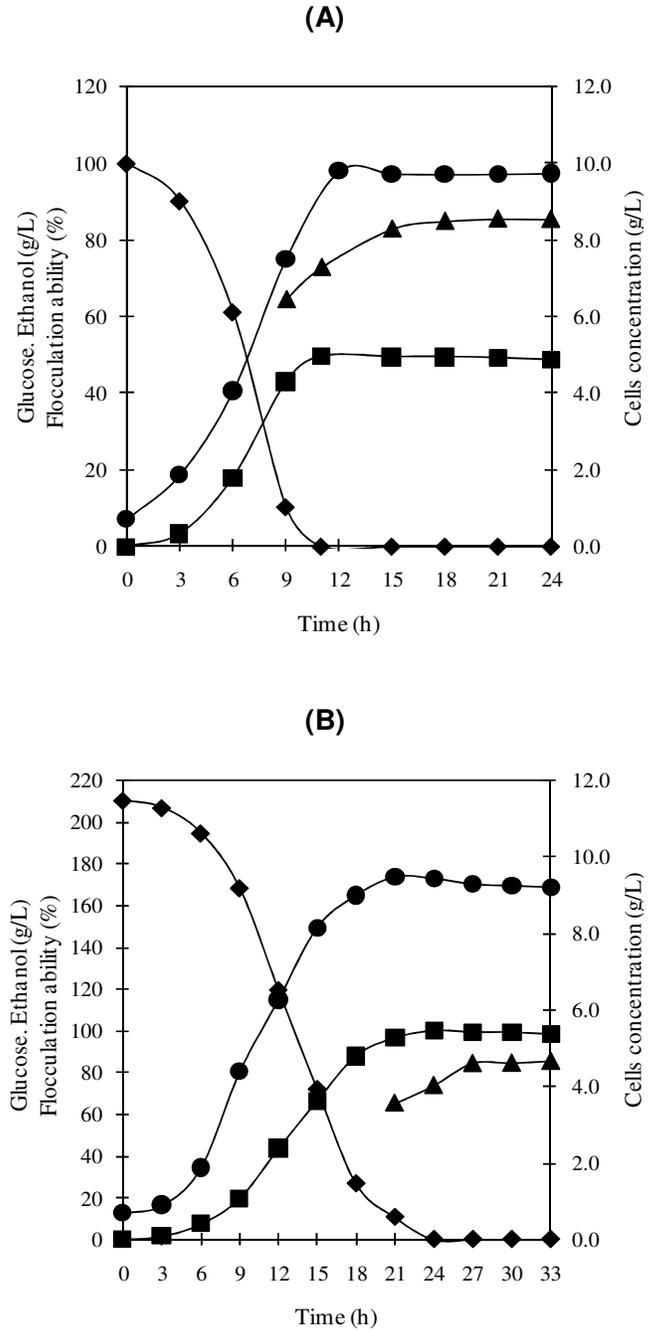
(A)

(B)

Figure 7. Relationship between growth, glucose and flocculation of the mutant flocculent strain *S.cerevisiae* KRM-1 in the kitchen refuse medium. The initial glucose concentration was 100 g/L **(A)** and 210 g/L **(B)**, respectively. (◆) Glucose concentration, (■) Ethanol concentration, (●) Cell concentration, (▲) flocculation ability. Values plotted represent the average of 3 assays of the same sample.

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