A study of the effect of calcium chloride (CaCl$_2$) and pH on the flocculation ability of *Saccharomyces cerevisiae* (NCYC 1195)

M Pienasthika, A A Brahmanti, I Purwatininrum and A K Wardani

Department of Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia

E-mail: agustinwardani@ub.ac.id

Abstract. The best fermentation ability of yeast-forming flock species is highly desirable in the bioethanol production process. *Saccharomyces cerevisiae* NCYC 1195 is known to have the ability to form flock which is influenced by the concentration of calcium chloride (CaCl$_2$) and pH. This is because calcium ions can form bonds with mannose on the surface of the cell wall of *S. cerevisiae*, while the degree of acidity will affect the charges of specific protein on the cell wall. The ability of flocculation will precipitate *S. cerevisiae* cells so that they will not be mixed with fermentation medium. Thus, the separation process of cell and fermentation media can be done quickly and efficiently, without expensive centrifugation step. The aim of this study was to determine the effect of CaCl$_2$ concentration and pH that used to determine the ability and stability of the flock of *S. cerevisiae*. Three concentration of CaCl$_2$ ($10^{-5}$, $10^{-7}$, $10^{-9}$ M) were used as well as three variations of pH (4, 5, 6). Culture was incubated at 30°C with 100 rpm agitation and analysed for flocculation ability every 4 hours during 24 hours. The stability of the flock was analysed every 24 hours during 30 days. The highest flock formation ability (73.15%) was obtained with the concentration of CaCl$_2$ concentration $10^{-9}$ M and pH 5.

1. Introduction

*Saccharomyces cerevisiae* is an Ascomycota or phylum division of fungi that able to convert sugar into ethanol, so it is widely used especially in the field of biotechnology, such as bioethanol production, fermentation of alcohol (beer, wine), bakery products, and other fermented foods [1]. The research on bioethanol continues to be carried out, given its potential to overcome the energy crisis. There have been many studies conducted using *S. cerevisiae* in the manufacture of bioethanol, for example the production of bioethanol using cassava materials [2], sugar cane ingredients [3], and lignocellulose materials [4].

Bioethanol production includes several stages, starting from material preparation, hydrolysis, liquefaction and saccharification, fermentation, and bioethanol purification. At the stage of bioethanol purification, a centrifugation process is needed which causes production costs increased. Centrifugation is the process of separating *S. cerevisiae* cells from the fermentation medium to obtain pure ethanol [5]. Therefore, to support the use of bioethanol, a method is needed that can improve the efficiency of the bioethanol production process.

The use of *S. cerevisiae* flocculent in the bioethanol production process is intended to reduce the production stage, especially the separation step by centrifugation. *S. cerevisiae* flocculent can form cell sedimentation (flock) at the bottom of the fermentation vessel. Thus, it can naturally separate the cell biomass from the media solution. There are many factors that influence the stability of *S. cerevisiae*
flocculent in forming flock [6]. The presence of calcium ions is known to trigger flocculin formation, so that the flocculent \textit{S. cerevisiae} cells can bind [7], while the pH of the medium will affect the electrostatic charges of \textit{S. cerevisiae} flocculant cell’s surface which also affects cell-to-cell contact and cell viability [8]. Therefore, this study aimed to find the best concentration of calcium chloride (CaCl$_2$) and degree of acidity (pH) that can improve the process of flocculation and improve its stability.

2. Materials and Method

2.1. Stock culture preparation

\textit{S. cerevisiae} flocculent culture was grown on PGYB media which was set at pH 5. The composition of the PGYB was 2\% of Bacteorogical peptone, 2\% glucose, 1\% flocculent \textit{S. cerevisiae} cells were incubated in the shaker water bath at 30\textdegree C, 100 rpm for 22 hours.

Preparation of stock culture in glycerol was carried out by growing 1 ml of 22-hours-\textit{S. cerevisiae} flocculent on PGYB media with a composition of 0.4 g/L Bacteorological peptone, 0.4 g/L glucose, and 0.2 g/L yeast extract, then incubated in the shaker water bath at temperature of 30\textdegree C, 100 rpm for 22 hours. The 22-hour-old incubated culture was added to the microtube by adding 40\% glycerol to a ratio of 1:1. Then stored in freezer with the temperature was set to -22\textdegree C.

2.2. \textit{S. cerevisiae} flocculent growth curve h

The growth phase of \textit{S. cerevisiae} flocculent was investigated by a total of 5\% (v/v) culture that grown onto PGYB media (3 g/L Bacteorogical peptone, 3 g/L glucose, and 1.5 g/L yeast extract), which was incubated in a shaker water bath at 30\textdegree C, 100 rpm. Then, an optical density (OD) analysis was carried out every 2 hours during 24 hours using a spectrophotometer (595 nm).

2.3. Flocculation assay

As much as 5\% (v/v) of starter culture was inoculated in 120 mL of PGYB media on 250 mL Erlenmeyer. Incubation is carried out on the shaker water bath at 30\textdegree C, 100 rpm. Flocculation ability measurements were carried out every 4 hours (two times). The first sampling (A2) was carried out by incubating the culture at room temperature for 5 minutes and the upper phase was taken for about 3 mL. The second sampling (A1) was done by homogenising the culture inside of the Erlenmeyer using vortex. Each sample was then inserted into the microtube and then washe with ddH$_2$O. Centrifugation was carried out to separate the pellets and supernatants. The pellet was then added with EDTA and buffer flocculation. Afterwards, Optical Density (OD) was measured using a spectrophotometer at 500 nm. The absorbance results of A2 and A1 are calculated using a formula as follows:

$$FA = [1-(A2/A1)] \times 100\%$$  \hspace{1cm} (1)

2.4. Stability of \textit{S. cerevisiae} flocculent

The culture was inoculated onto 50 mL PGYB media with the best treatment of pH and calcium chloride. Then it incubated in the shaker water bath at 30\textdegree C, 100 rpm for 24 hours. After 24 hours, visual flock observations were carried out manually. Furthermore, the second incubation was carried out using the first incubation culture. The test was conducted every 24 hours for 30 days.

3. Results and Discussion

3.1. Characteristics and growth profile of culture

In this study, \textit{S. cerevisiae} flocculent strain was used, specifically \textit{S. cerevisiae} flocculent NCYC (National Collection of Yeast Culture) 1195 which was commonly used in the beer industry [9]. \textit{S. cerevisiae} flocculent (NCYC 1195) was grown on PGYB media and then vortexed to homogenize cells in the medium, then left it for 5 minutes. It can be proved that the \textit{S. cerevisiae} flocculent cell which initially hovers on the medium, then formed sediments at the base of the PGYB medium.
The growth curves of *S. cerevisiae* flocculent (NCYC 1195) was determined by growing *S. cerevisiae* flocculent (NCYC 1195) on PGYB synthetic medium containing 2% (w/v) glucose, 2% (w/v) peptone, and 1% (w/v) yeast extract. The growth of *S. cerevisiae* flocculent (NCYC 1195) was observed every 1 hour in the first 6 hours, then continued to be observed every 2 hours for 20 hours by analysing the turbidity level of the medium. Figure 1 shows the growth profile of *S. cerevisiae* flocculent (NCYC 1195).

Based on Figure 1, the peak of the log phase was occurred at 12
\[\text{th}\]
hour. Therefore, the starter culture that used in this study was 12-hour-old *S. cerevisiae* flocculent, where the cell concentration was considered as maximum. This is in accordance with previous studies that also using *S. cerevisiae* in the process of bioethanol production in lignocellulose materials [9] and the production of various alcoholic beverages [10]. The use of starter culture in the log phase was expected to accelerate the phase of cell adaptation to subsequent incubation treatment.

3.2. Effect of calcium chloride addition and pH on flocculation ability

Flocculation of *S. cerevisiae* is also called social behaviour of cells when dealing with a non-conducive environment, as an effort to maintain the life of the colony [11]. This occurs due to the expression of *FLO* genes groups (FLO1, FLO5, FLO9, FLO10, and FLO11) and transcription factors such as Flo8p and Flo11p which can affect gene expression either directly or indirectly [12] for example in a condition of nutrient deficiency [13] and high ethanol concentration in the media [14]. In this study there was an increase in *S. cerevisiae* flocculation ability which indicated that the treatment that already given influenced flocculation ability. There was a significant difference between the control and the treated group. The effect of CaCl\(_2\) addition and pH is shown in Figure 2 to 4.

![Figure 1. Growth profile of *S. cerevisiae* flocculent (NCYC 1195)](image1)

![Figure 2. Effect of calcium on *S. cerevisiae* (NCYC 1195) flocculation ability at pH 4](image2)

![Figure 3. Effect of calcium on *S. cerevisiae* (NCYC 1195) flocculation ability at pH 5](image3)
Figure 4. Effect of calcium on *S. cerevisiae* (NCYC 1195) flocculation ability at pH 6

The calcium concentration which showed the highest flocculation ability was the concentration of calcium (CaCl$_2$) 10$^{-9}$ M, which was equal to 73%. Based on the resulting trend from the three variations of pH (4; 5; 6), the treatment which contain 10$^{-9}$ M concentration of calcium (CaCl$_2$) had higher flocculation ability compared to the 10$^{-5}$ and 10$^{-7}$ M calcium (CaCl$_2$) concentration. The calcium concentrations that needed to activate the flocculin are varies depending on the *S. cerevisiae* strain. In the NewFlo phenotype as used in this study, found exclusively, the strain grows as a single cell and the occurrence of flocculation does not depend on the presence of receptors, but rather depends on the presence of active lectins [15].

The cell wall of *S. cerevisiae* is composed of proteins that play an important role in the ability of adhesion, interaction and cell infection [16]. Cell topology plays an important role in the ability of cell flocculation, where the rougher the cell wall, the higher the ability of flocculation [17]. Based on the theory of *Lectin*, flocculation occurs due to interactions between flocculins, which is specific proteins that only exist in flocculant microbial cells with certain carbohydrates in the closest microbial cell wall [18].

The result of the study has shown compatibility with the theory of *Lectin Models*, which is the role of calcium as a cofactor in the process of flocculation. Calcium (Ca$^{2+}$) can activate flocculin, so that when flocculin recognizes calcium (Ca$^{2+}$) there will be cross bonds with mannose residues on the surface of the other *S. cerevisiae* cells [19]. Then, an aggregate of *S. cerevisiae* flocculent cells is formed or called flock [20]. The concentration of mannose residues on the cell wall is known to remain constant during the fermentation process [21], as well as the concentration of flocculins, while the calcium concentration will decrease at the end of the fermentation process [22], this indicates that calcium ions are taken by cells from the medium and used to activate flocculin.

Each microbe has a different optimum growth pH, this also affects the optimum flocculation pH range [23]. The degree of acidity (pH) affects the specific lectin that is on the surface of the *S. cerevisiae* flocculent cell. Specific lectin or zymolectin is a protein that only exists on the surface of *S. cerevisiae* flocculent cells. Extreme pH, which is very acidic or alkalic, can change the electrostatic charge on the cell surface, then interfere with or inhibit the interaction of the flocculin with Ca$^{2+}$ ions [24]. In addition, the electrostatic charge of the cell surface affects the distance between cells, the greater and the more negative charge on the cell surface, the further the distance between cells due to the electrostatic resisting repulsion force [10]. Some other factors that influence flocculation include genetic traits, ionic strength, the nature of the cell hydrophobicity [14], type of culture (strain), nutrient of growth media [5], and cell age [25]. In addition, it is also influenced by temperature, the presence of oxygen, cell density, agitation, sugar, and ethanol levels in the media [26].
3.3. Effect of pH and calcium addition to the appearance of the flock S. cerevisiae flocculent (NCYC 1195) during flocculation assay

The flock appearance significantly differences in the control-treated group, as shown in Figure 3, where S. cerevisiae cell growth (NCYC 1195) at pH 4 was less than pH 5 and pH 6. This was evidenced by clearer media colours. The amount of white flock that appears as fine as sand increases with the increasing of CaCl₂ concentration. This is indicated by the increasingly thick white colour formed under the medium as shown in Figure 5.

Figure 5. Appearance of S. cerevisiae flock (NCYC 1195) during flocculation assay: (a) pH 4, CaCl₂ 10⁻⁵ M; (b) pH 4, CaCl₂ 10⁻⁷ M; (c) pH 4, CaCl₂ 10⁻⁹ M; (d) pH 5, CaCl₂ 10⁻⁵ M; (e) pH 5, CaCl₂ 10⁻⁷ M; (f) pH 5, CaCl₂ 10⁻⁹ M; (g) pH 6, CaCl₂ 10⁻⁵ M; (h) pH 6, CaCl₂ 10⁻⁷ M; (i) pH 6, CaCl₂ 10⁻⁹ M; (j) Without Treatment

At pH 5, the formed flock form clumps under the growth medium. Whereas at pH 6, it appears that cells grow more than in pH 4 and pH 5. This is characterised by a more turbid colour change in the medium. Turbidity that appears on the medium also indicates that not all cells from clots (floc) under the growth medium. It is also seen in the formed flock, which is more diffuse and less.
Based on previous studies [27], the optimal pH that capable to producing the highest *S. cerevisiae* flocculation ability is pH 5. The optimal pH condition for flocculation is needed to maintain cell viability, maintaining enzymes that are on the cell surface that can support the occurrence of *S. cerevisiae* flocculation.

### 3.4. Stability test of flocculent *S. cerevisiae* (NCYC 1195)

The stability test of flock in this study was carried out 30 times to determine the flock that is still formed up to 24 hours. Considering that the longer the flock can be formed, the better the result. It is because the cell mass can be reused in the next fermentation batch. In this study, it was found that flock remained in shape for 30 days of testing. However, the appearance of the formed flock cannot be uniform (Figure 6). This is consistent with previous studies [28] which found that *S. cerevisiae* cells can be reused up to 20 batches of fermentation.

![Figure 6. Flock formed on: (a) Day 1; (b) Day 10; (c) Day 20; (d) Day 30](image)

### 4. Conclusions

*Saccharomyces cerevisiae* (NCYC 1195) with the best treatment, specifically the addition of calcium chloride (CaCl$_2$) with the concentration up to $10^{-9}$ M at pH 5 can form the highest flock with the percentage up to 73.155%. The flock was also known to be stable for 30 days, although with different appearances. Further research is needed to find out other factors that influence flocculation and stability. Thus, it can be said that *S. cerevisiae* (NCYC 1195) has the potential to be used as a culture in the production process of bioethanol in the industry, because it can streamline the process of bioethanol purification.

### References

[1] Mortier A, Soares E V 2007 Separation of yeast by addition of flocculent cells of *Saccharomycess cerevisiae* World J. Microbiol. Biotechnol. **23** 1401-1407.

[2] Choi G W, Kang H W, Moon S K 2009 Repeated-batch fermentation using flocculant hybrid, *Saccharomyces cerevisiae* CHFY0321 for efficient production of bioethanol Biotechnol. Prod. Process Eng. **84** 261-269.

[3] Ramos C L, Duarte W F, Freire A L, Dias D R, Eleutherio E C A, Schwan R F 2013 Evaluation of stress tolerance and fermentative behavior of indigenous *Saccharomyces cerevisiae* Braz. J. Microbiol. **44** 3 935-944.

[4] Idi A, Mohamad S E 2011 Bioethanol from second generation feedstock (lignoselulose biomass) Interdis. J. Contem. Res. Bus. **3** 8 919-935.

[5] Wang D, Wang Z, Liu N, He X, Zhang, B 2008 Genetic modification of industrial yeast strain to obtain controllable newfloo flocculation property and lower diacetyl production Biotechnol. Lett. **30** 2013-2018.

[6] Jia B J, Yu J M 2012 The research status and development trend of microbial flocculant *Phys. Procedia* **24** 425-428.

[7] Stewart G G 2018 Yeast flocculation—sedimentation and flotation Ferment. **42** 28 1-32.

[8] Halina S, Nathan L 2007 Lectins Springer

[9] Wood J A, Orr V C A, Luque L, Nagendra V, Berruti F, Rehmann, L 2015 High-Throughput
screening of inhibitory compounds on growth and ethanol production of *Saccharomyces cerevisiae* Bioenergy Res. 8 423–430.

[10] Nayyar A, Walker G, Canetta E, Wardrop F, Adya A K 2014 Cell surface properties and flocculation behaviour of industrial strains of *Saccharomyces cerevisiae* Int. J. Appl. Microbiol. Biotechnol. Res. 2 64-72.

[11] Herker E 2004 Chronological aging leads to apoptosis in yeast J. Cell Biol. 164 501–507.

[12] Singh V, Azad G K, Sariki S K, Tomar R S 2015 Flocculation in *Saccharomyces cerevisiae* is regulated by RNA/DNA Helicase Sen1p FEBS Lett. 589 3165–3174.

[13] Dranginis A M, Rauceo J M, Juan C E Peter L N 2007 A Biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions Microbiol. Mol. Biol. Rev. 71 282-294.

[14] Claro F B, Rijsbrack K, Soares E V 2007 Flocculation onset in *Saccharomyces cerevisiae*: effect of ethanol, heat and osmotic stress J. Appl. Microbiol. 102 693–700.

[15] Sampermasn M, Soares E V 2005 Flocculation onset in *Saccharomyces cerevisiae*: the role of nutrients J. Appl. Microbiol. 98 525-531.

[16] Jendretzki A, Wittland J, Wilk S, Straede A, Heinisch J J 2011 How do I begin? sensing extracellular stress to maintain yeast cell wall integrity Eur. J. Cell. Biol. 90 740-744.

[17] Nayyar A, Walker G, Canetta E, Wardrop F, Adya A K 2017 Influence of cell surface and nanomechanical properties on the flocculation ability of industrial *Saccharomyces cerevisiae* Strains J. Food. Res. 6 5 1-10.

[18] Miki B L, Poon N H, James A P, Seligy V L 1982 Possible mechanism for flocculation interactions governed by the Gene FLO1 in *Saccharomyces cerevisiae* J.Bacteriol. 150 878-889.

[19] Vallejo P, Martiner J, Villa 2013 Cell aggregations in yeast and their applications Appl. Microbiol. Biotechnol. 97 2305-2318.

[20] Speers R A, Wan Y Q, Jin Y L, Stewart R 2006 Effect of fermentation parameters and cell wall properties on yeast flocculation J. Inst. Brew. 112 246-254.

[21] Nayyar A, Walker G, Canetta E, Wardrop F, Adya A K 2017 Flocculation in industrial strains of *Saccharomyces cerevisiae*: role of cell wall polysaccharides and lectin-like receptors J. Inst. Brew. 123 211–218.

[22] Vidgren V, Londeborough J 2011 125th Anniversary review: yeast flocculation and sedimentation in brewing J. Inst. Brew. 117 4 475–487.

[23] Soares E V 2010 Flocculation in *Saccharomyces cerevisiae*: a review. J. Appl. Microbiol. 110 1–18.

[24] Kang Y, Heui S K, Yong S J H, Ryu Y W 2006 Flocculation of an isolated flocculent yeast, *Candida tropicalis* HY200, and its application for efficient xylitol production using repeated-batch cultivation J. Microbiol. Bioethanol. 16 12 1874-1881.

[25] Mulders S V, Ghequire, M, Daenen Luk, Verbelen P J, Verstrepen K, Delvaux, F R 2010 Flocculation gene variability in industrial brewer’s yeast strains Appl. Gen. Mol. Biotechnol. 88 1321-1331.

[26] Bruckner S, Mosch H U 2012 Choosing the right lifestyle: adhesion and development in *Saccharomyces cerevisiae* Fed. Eur. Microbiol. Soc. 36 25-58.

[27] Li E, Yue F, Chang Q, Guo X, He, X, Zhang B 2013 Deletion of intragenic tandem repeats in Unit C of FLO1 of *Saccharomyces cerevisiae* increase conformational stability of flocculin under acidic and alkaline conditions PLOS ONE 8 1 53428 1-10.

[28] Wang F Z 2009 Construction of flocculent industrial yeast by the yeast flocculation Gene Flo1 Appl. Biochem. Microbiol 45 5523-5530.