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

To cite this article: M Pienasthika et al 2020 IOP Conf. Ser.: Earth Environ. Sci. 475 012082

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A study of the effect of calcium chloride (CaCl₂) and pH on the flocculation ability of Saccharomyces cerevisiae (NCYC 1195)

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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 (CaCl₂) 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 CaCl₂ concentration and pH that used to determine the ability and stability of the flock of S. cerevisiae. Three concentration of CaCl₂ (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 CaCl₂ 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
floculent in forming flock [6]. The presence of calcium ions is known to trigger flocculin formation, so that the floculent \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} floculent culture was grown on PGYB media which was set at pH 5. The composition of the PGYB was 2% of Bacteriological peptone, 2% glucose, 1% floculent \textit{S. cerevisiae} cells were incubated in the shaker water bath at 30°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} floculent on PGYB media with a composition of 0.4 g/L Bacteriological peptone, 0.4 g/L glucose, and 0.2 g/L yeast extract, then incubated in the shaker water bath at temperature of 30°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°C.

2.2. \textit{S. cerevisiae} floculent growth curve h
The growth phase of \textit{S. cerevisiae} floculent was investigated by a total of 5% (v/v) culture that grown onto PGYB media (3 g/L Bacteriological peptone, 3 g/L glucose, and 1.5 g/L yeast extract), which was incubated in a shaker water bath at 30°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°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 was washed 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:

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

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°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).

![Figure 1](image1.png)

**Figure 1.** Growth profile of *S. cerevisiae* flocculent (NCYC 1195)

Based on Figure 1, the peak of the log phase was occurred at 12\textsuperscript{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\textsubscript{2} addition and pH is shown in Figure 2 to 4.

![Figure 2](image2.png)  ![Figure 3](image3.png)

**Figure 2.** Effect of calcium on *S. cerevisiae* (NCYC 1195) flocculation ability at pH 4  **Figure 3.** Effect of calcium on *S. cerevisiae* (NCYC 1195) flocculation ability at pH 5
The calcium concentration which showed the highest flocculation ability was the concentration of calcium (CaCl₂) $10^{-9}$ 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 (CaCl₂) had higher flocculation ability compared to the $10^{-5}$ and $10^{-7}$ M calcium (CaCl₂) 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.](image)

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 \textit{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 \textit{S. cerevisiae} flocculation.

3.4. Stability test of flocculent \textit{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 \textit{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
\textit{Saccharomyces cerevisiae} (NCYC 1195) with the best treatment, specifically the addition of calcium chloride (CaCl\textsubscript{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 \textit{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.

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