NOVEL IMMOBILIZATION METHOD OF SACCHAROMYCES CEREVISIAE THROUGH FLOCCULATION PROCESS USING POLYACRYLAMIDE-GRAFTED-STARCH

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Abstract

Cells immobilization has been interesting for many researchers to explore and investigate some materials as an immobilization agent. This effort aims to enhance cells productivity during fermentation. The objective of this research was to immobilize cells by developing a novel immobilization through the cells trapping in flocs using the grafted flocculants, i.e., Polyacrylamide Grafted Starch (St-g-PAM). This method was applied to trap the cell of Saccharomyces cerevisiae in a certain concentration of molasses solution (substrate). The ability of cells to survive in the flocs after flocculation and the presence of impurities (kaolin) in the substrate were also investigated. The incubation of yeast 10% (w/v) in the substrate was controlled at 30 °C and 125 RPM, for 24 hours. Grafted flocculant solution was added to the substrate, i.e., 10, 20, 30 and 40% (v/v). At 40% (v/v) and without impurities, the turbidity was recorded at 184 NTU equivalent to 8.96×10^{11} trapped cells/L. The presence impurities to increase the turbidity. It was monitored at 288 NTU equivalent to 5.12×10^{11} trapped cell/L. It means that the impurities in the medium inhibit the physical localization of yeast. Generally, this work had succeeded in trapping yeast into flocs, and the cell's metabolism was proven not interfered.

Keywords: Bioethanol, Flocculation, Immobilization, Modified polyacrylamide, Saccharomyces cerevisiae.

1. Introduction

Recently, the bioenergy field has been becoming an interesting research theme due to the rapid decreasing of petroleum-based oil resources [1]. The government of Indonesia ruled in substituting petroleum using a blending method such as bioethanol blended to a gasoline engine. The composition of the blend is about 5-25% (v/v) bioethanol so that the engine performance is still working normally. Bioethanol, a potential renewable bioenergies source, can be produced through a biological route that is fermentation [2]. This fermentation converts the sugar or starchy substrate by utilizing microbial metabolism of free or immobilized cells [3].

Many researchers have been interested in developing immobilized cells due to its performance (such as yield, productivity, and easily of product separation) is better than that of free cells [4, 5]. Some advantages of the yeast immobilization include high cell densities, lowered risk of microbial contamination, better control of the processes, as well as reuse of the immobilization for batch fermentation and continuous fermentation [5, 6]. There are four major categories according to the physical mechanism employed, namely: (i) attachment or adsorption onto solid carrier surfaces, (ii) natural or artificial flocculation, (iii) entrapment within a porous matrix and (iv) encapsulation [7-12].

Some materials have been reported successfully to be developed as an immobilizing agent/support. Most of these materials were employed to attach, encapsulate or entangle cells, i.e., gel matrix material of polyvinyl alcohol (PVA) and Ca-alginate[13], lyophilized cellulose gel [14], corncob piece [15], combined sponge-alginate [16], sweet sorghum stalks [5], sweet sorghum stalk juice [17], thin shell silk cocoons [18], dried spongy fruit of luffa [19], acrylamide gel [20], a new alginate-maize stem ground tissue matrix [21], sodium alginate gel [4] and sugarcane bagasse [22].

According to Walsh and Malone [23], there are some obstacles dominated by mass transfer problems when we use the immobilized cells used in the fermentation. The mass diffusion of substrate and product pass through the matrix significantly affected bioethanol productivity.

Flocculation is one of the most commonly used methods for water purification. Commercial flocculants can be classified into two categories, namely inorganic flocculant agent and polymeric flocculant. From these two flocculants, polymeric flocculants are more effective [24]. These flocculants can be natural and synthetic polymers. Polyacrylamide is one of the most effective synthetic polymers as a flocculant because it has a strong binding force against suspended particles. However, it is not resistant to mechanical shear and has low degradability. Starch is a natural, cheap, abundantly available and easily degraded. Thus, integration of those advantage properties enables to enhance the flocculation effectivity.

Grafting, one type of copolymerization, is to modify polymer through inserting one polymer chains to another polymer. One polymer act as a backbone and the others act as the grafted polymer. Some researcher reported this copolymerization between the organic polymer and inorganic one. Huang et al. [25] investigated starch-graft-poly (2methacryloyloxyethyl) trimethyl ammonium chloride (St-g-PDMC). This grafted flocculant exhibited two excellent properties, i.e. high flocculation effectivity and antibacterial property [25]. The others investigated various types of starchy materials such as taro, cassava, taro modification, modified cassava. These natural polymers act

as a backbone of polyacrylamide. The flocculation performance could be compared with inorganic flocculants without altering the acidity (pH) [24]. The flocculation mechanism can be explained using both network theory and bridging theory. The network mechanism is addressed by forming a network that connects some particles through hydrogen bonds, van der walls, or other bonds. While the bridging mechanism, it is preceded with neutralizing the solid particle charge by the active group of the flocculant. The active group will bind/attack one or more sides of the particles so that a chemical bridge is formed. Finally, the compacted flocs are formed and settled down rapidly [26].

Based on the description above, this study aims to investigate the immobilization of S. cerevisiae using flocculation method. Grafted flocculant of St-g-PAM is used due to not altering the acidity during flocculation. Thus, the floc formation may be expected not interfering the cell metabolism. At the same time, the diffusivity barriers in the solid matrix could be overcome. In this investigation, the presence of impurities in the molasses substrate was also studied to know the cells trapping sensitivity of St-g-PAM during flocculation.

2. Experimental Part

2.1. Material

Fresh molasses ($C_{12}H_{22}O_{11}$) and starch were supplied from Colomadu Sugar Factory (PG. Colomadu) and from the local market in Solo (Indonesia), respectively. Saccharomyces cerevisiae nᵒDY 7221 brand Fermiol from DSM Food Specialties B.V., the Netherlands. Yeast extract, Glucose, Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), Ammonium chloride (NH_4Cl), Calcium chloride Dihydrate ($CaCl_2.2H_2O$), Phenol, Sulfuric acid (H_2SO_4), Methanol, Tetramethylethylenediamine (TMEDA), Hydroquinone, Acrylamide and Potassium persulfate ($K_2S_2O_8$) were purchased from Merck, Germany. All chemicals were used without purification.

2.2. Preparation

The preparation of grafted flocculant followed our previous research by Kaavessina et al. [24]. The inoculation of S. cerevisiae 10% (w/v) was prepared in sterile medium containing: yeast extract 2.5 g/L, glucose 20 g/L, $MgSO_4.7H_2O$ 25 g/L, NH_4Cl 1.3 g/L, and $CaCl_2.2H_2O$ 0.06 g/L. The incubation of S. cerevisiae 10% (w/v) in the sterile substrate (molasses) was carried out in an incubator controlled at 30 ºC and 125 RPM, for 24 hours. There are two types of substrate in this investigation, i.e., pure substrate dan contaminated substrate. The contaminated substrate was prepared by adding the suspension of kaolin with a concentration 1 g/L into the substrate. Grafted flocculant solution was added to the molasses medium with the varied concentration, i.e., 10, 20, 30 and 40% (v/v). During the flocculation of S. cerevisiae, the temperature was controlled at 30 ºC for 24 hours. In this study, each variable was repeated three times, and the data was taken from the mean value of the repetition data.

2.3. Analysis

Turbidity is a parameter to determine the level of turbidity of the medium. The sample measured by using portable turbidity meter type ECTN100IR. The

images of cells existence were captured by using two types of digital microscope: Dino-lite edge 5MP type AM7515 for magnification above 250x and Dino-lite basic type AM2111 for magnification below 250x.

Void fraction or porosity of flocs was also analysed first to ensure a place that would trap the cells. The void fraction of flocs was determined using the analogy of the general equation as defined by Eqs. (1) and (2) [27]:

$$void\ fraction\ = \frac{total\ volume\ -volume\ of\ solids}{volume\ volume} \tag{1}$$

thus,

$$void\ fraction\ of\ flocs\ = \frac{volume\ of\ flocs\ -volume\ of\ solids}{volume\ of\ flocs} \tag{2}$$

or,

$$void\ fraction\ of\ flocs = \frac{volume\ of\ porous}{volume\ of\ flocs} \tag{3}$$

The effect of flocculant addition on trapped cells is expressed using cell trapping efficiency (Ƞ) and defined by Eqs. (4) and (5) :

$$Ƞ = \frac{\sum cells\ before\ flocculation - \sum cells\ after\ flocculation}{\sum cells\ before\ flocculation} \tag{4}$$

or,

$$Ƞ = \frac{trapped\ cells}{cells\ before\ flocculation} \tag{5}$$

3. Results and Discussion

The grafted flocculant was used to the physical localization of the cells. In our previous research [24], the flocculation of kaolin suspension using this flocculant showed that kaolin particles were trapped in the mesh formed by grafted flocculant. Thus, we proposed the hypothesis that cells will be trapped in the same phenomenon. As depicted schematically in Fig. 1, the cells are trapped in the polymeric mesh, i.e., polyacrylamide, which was grafted on starch. The cells are still moving freely and contacted directly with the substrate without any mass transfer obstacles. The performance of grafted flocculant solution in yeast immobilized was studied using the varied doses, i.e., 10, 20, 30 and 40% (v/v).
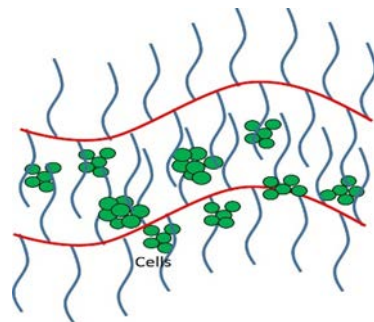

Fig. 1. Schematic of trapped cells in grafted flocculant (polyacrylamide grafted starch).

Kaolin as an impurity in the substrate

The main parameter of flocculant performance is its capability on flocculating and settling suspended particles in a liquid medium. It is measured quantitatively in decreasing turbidity of the test liquid. Grafted flocculant was tested its performance to flocculate the suspended cells of S. cerevisiae. The sensitivity of flocculant was also investigated by testing in the different substrate, i.e., (i) pure molasses medium and (ii) molasses medium, which is added by kaolin (as impurities). Further, the last substrate is called contaminated substrates. For example, the sensitivity when the flocculant dose is about 40% (v/v) as presented in Fig. 2. Figure 2 can be divided into two zones to explained this sensitivity that is (i) zone I (0-6 hours) and zone II (6-12 hours). Zone I shows that the turbidity tends to decrease during flocculation. This phenomenon has occurred in both pure and contaminated substrate. In this zone, the turbidity of the contaminated substrate is always higher than that of a pure one. At 0 hours, the turbidity was recorded at 472 and 406 NTU for both the contaminated and the pure substrate, respectively. At the end of the zone I (6 hours). It was recorded at 276 NTU and 263 NTU for the same substrate sequence. This phenomenon might be explained that flocculation in pure substrate shows the networking mechanism. The formed floc was restraining yeast from settling in the bottom. The other mechanism (bridging) occurred in the presence of kaolin. Flocculant will trap both yeast and kaolin simultaneously. The competition will be occurred both of them to be trapped in flocs. In this research, it was identified that kaolin was more dominating in the flocs than the yeast. Further, this conclusion has a consistency by void fraction analysis that the presence of untrapped yeast in the pure substrate is less than that of the contaminated one.

The rate of decreasing turbidity was depicted in Fig. 2 and could be calculated quantitatively from the depletion gradient at a certain time interval. This rate indirectly indicates the settling velocity. The average settling velocity in the zone I was recorded at 22.61 NTU/h and 35.8 NTU/h for the contaminated and pure substrate, respectively. In other word, the settling velocity of flocculation for the contaminated substrate is 1.6 times faster than that of a pure one. This phenomenon could be explained that kaolin is more easily bound in flocculants. The presence of kaolin causes the flocs to be more easily sedimented.

The value of turbidity in zone II has different trends compared to the zone I, where the presence of impurities tended to increase slightly. At 8 hours, the flocs started damage due to cell metabolism has broken the binding between flocculant and kaolin. The activity of cells metabolism produces carbon dioxide (CO_2) gas that cleaving the flocs caused kaolin and yeast are detached back into the medium and increase the turbidity. Without impurities, the turbidity of the substrate was decreasing due to the binding of flocculant and yeast is still strong and no disturbance. This phenomenon might be explained that the impurities (kaolin) are more dominant to be trapped in flocs than the yeast. The flocculation for 24 hours was also monitored to determine the maximum time of flocculation. The turbidity of the contaminated substrate was still increasing from 288 NTU (12 hours) to 295 NTU (24 hours). While, for the pure substrate, the turbidity was only slightly decreasing from 184 NTU (12 hours) to 174 NTU (24 hours). It may be highlighted that formed flocs are more stable in the pure substrate than that of the contaminated one. Further, this flocculation time (24 hours) was used in some other analysis. This result was also supporting the conclusion that the bridging mechanism occurred in substrate contaminated substrate (contaminated substrate).
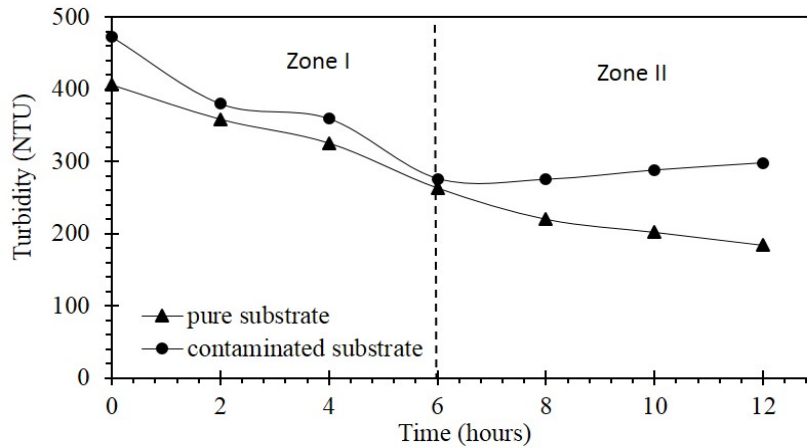
Fig. 2. Turbidity measurement during flocculation of grafted flocculant (40% (v/v)) for pure substrate and contaminated substrate.

Flocculant dose

The flocculant dose was also investigated to know the performance of grafted flocculant as depicted in Fig. 3. This figure shows that an increase of flocculant dose in the range of 0-20% (v/v) is accompanied by decreasing sharply of turbidity in both substrates. For dose between 20-40% (v/v), especially for the pure substrate, the decreasing of turbidity is still sharp, relatively. Another side, the contaminated substrate showed a different trend. There is an increase of turbidity slightly when raised its dose. This phenomenon could be recorded that the best dose of flocculant is 20% (v/v) for the contaminated substrate. For pure substrate, we have not yet to notice the optimum flocculant dose due to the depletion has still occurred in the highest range of the studied flocculant dose.
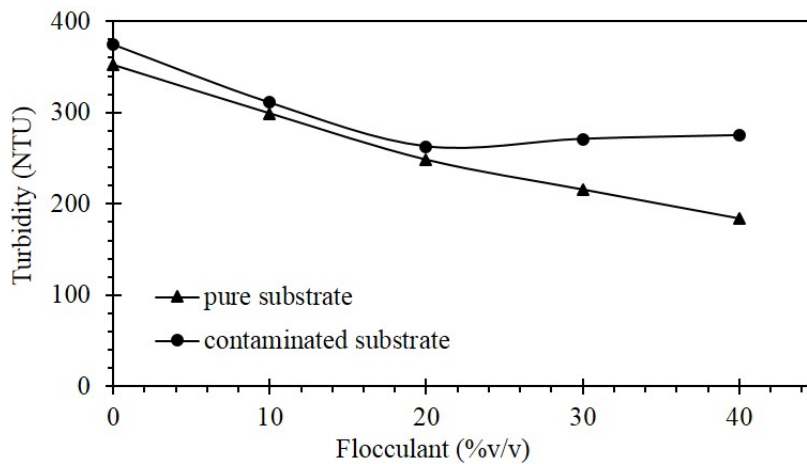
Fig. 3. Impurities effect on settling with different dose of flocculant (24 hours).

The presence of kaolin began to appear its influence when the dose of flocculant is 20% (v/v). At this dose, the void fraction was about 0.73 and 0.83 for pure substrate and the contaminated substrate, respectively. Above 20% (v/v), the different trends are clearly apparent. For pure substrate, the depletion of the void fraction has still occurred in this investigation range.

This depletion is almost 1.5 times when compared to the flocculant dose between 10% to 40% (v/v). At the same time, the increasing flocculant dose almost does not affect the void fraction when the substrate contains impurities (kaolin) as seen in Fig. 4. It might be proved that the presence of kaolin inhibits the entrapment process of the yeast. This statement is appropriate with the statement before that the impurities (kaolin) are more dominant to be trapped in flocs than the yeast.

The flocculant dose was also influencing the void fraction of formed flocs. Void fraction is one of the parameters used to determine the pore size of the sedimentation. This parameter shows the pore capacity filled by the yeast and the other particles. Thus, the value of void fraction related to the number of trapped cells in the flocs.

The relation between the void fraction and flocculant is depicted in Fig. 4. This figure is also showing the effect of kaolin as impurities in the substrate. At dose 10% (v/v), the void fraction is almost same 0.9. It means that in this dose, the presence of kaolin was not influencing the flocculation of yeast.

Relation of the flocculant dose and the trapped cells was also studied. The trapped cells indicate the number of cells successfully immobilized through flocculation. At a glance from Fig. 5, the trapped cells in the pure substrate are always higher than that of substrate contaminated substrate.

It may be explained that the particles size of kaolin is smaller than the yeast. This condition causes the kaolin particles to be easily trapped in the flocs. Thus, the yeast was restrained to trapped in the flocs. For example, at flocculant dose 10% (v/v), the trapped cells show at 5.76×10^{11} cells/L and 4.05×10^{11} cells/L for the pure and contaminated substrate, respectively.
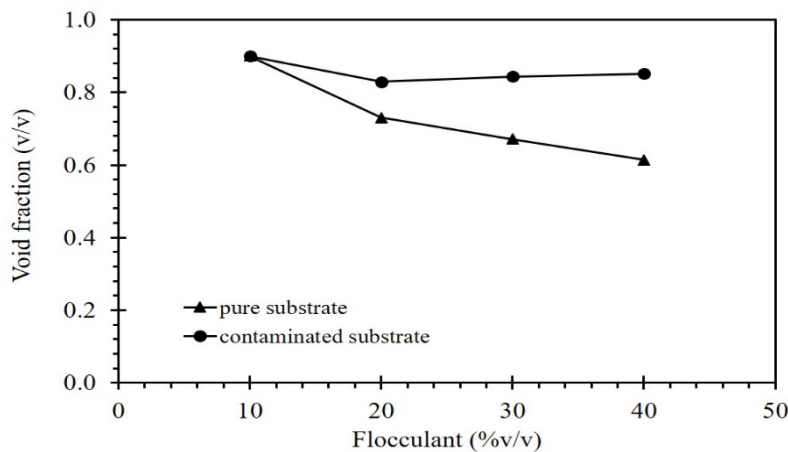

Fig. 4. Effect of flocculant dose towards void fraction.
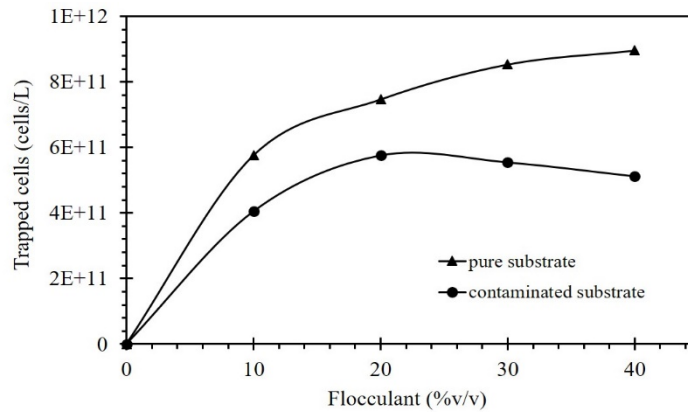
**Fig. 5. Effect of flocculant dose to number of trapped cells.**

The efficiency of flocculant to trapped cells is showing in Fig. 6. The efficiency was studied in the different flocculant dose, i.e., 10, 20, 30 and 40% (v/ v). In this range, the highest efficiency of flocculant in the pure substrate is about 93% with flocculant dose at 40% (v/v). It may be explained that the flocculant dose at 40% is forming more flocs and also trapping more cells. However, in the contaminated substrate, the efficiency of flocculant tends to lower than the pure one. For example, the efficiency becomes 49% from 60% at flocculant dose of 10% (v/v) if we added kaolin (1 g/L) in the substrate. Again, this result supported the previous statement that the presence of impurities (kaolin) inhibited to the trapping of cells in the formed flocs.

The free cells and immobilized cells in both substrates were observed to know the yeast resistance, especially the yeast in the flocs. This observation is displayed in Figs. 7 and 8. Figure 7 is showing the condition of the free cells. It can be seen that each cell are free to move and their metabolism is normal. While Figs. 8(a) and (b) are showing the dense of yeast in the flocs. This condition indicates that there is no obstacle significantly affecting the metabolism of yeast. Yeast could contact to the substrate bulk without any diffusivity problems.
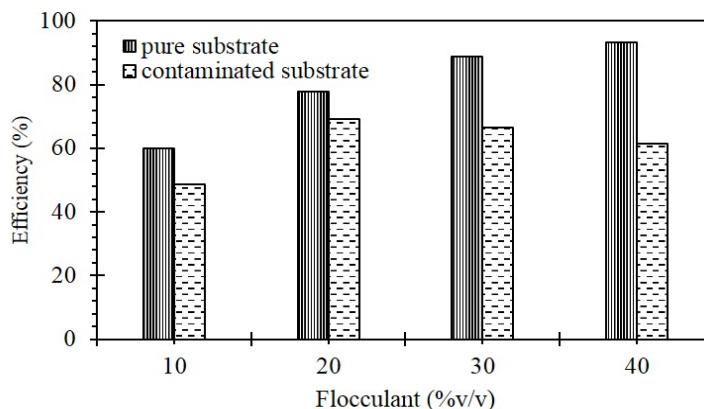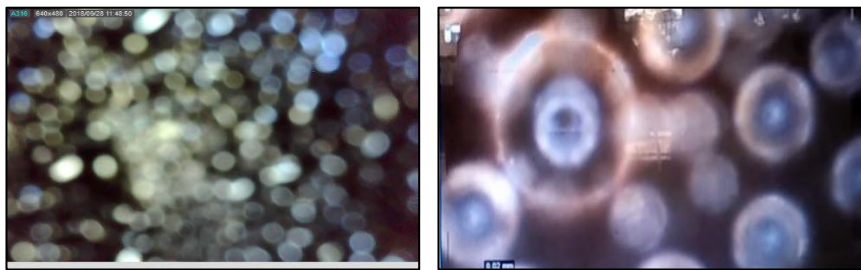

Fig. 6. Cell trapping efficiency in the different dose of flocculant for both pure and contaminated substrate.
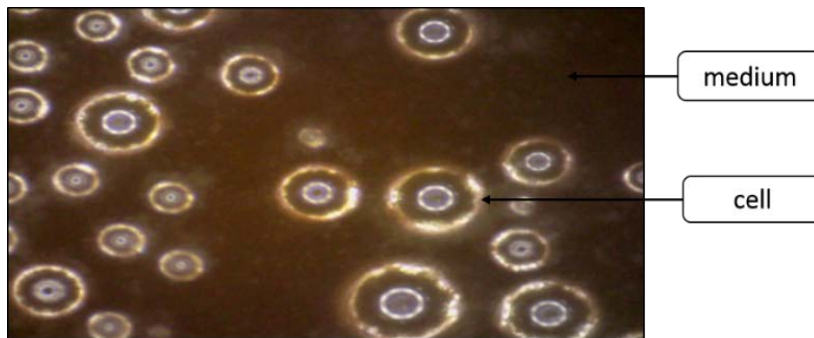
Fig. 7. Image of free cells (200x magnification).

(a)   (b)

Fig. 8. Images of immobilized cells in flocs:
(a) 200x magnification, and (b) 800x magnification.

4. Conclusions

The cell immobilization using the flocculation method is successfully developed. The impurity in the substrate is inhibiting the trapping cells into the formed flocs. Thus, it is recommended that this immobilization required the initial treatment of the substrate, i.e., removal of impurities in the substrate, etc. The presence of cell (especially yeast) in the flocs does not interfere with its metabolic life. Yeast is still growing and doing a cleavage of its cells to breed.

Acknowledgement

Greek Symbols	
η	Cell trapping efficiency

Abbreviations	
NTU	Nephelometric Turbidity Units
PG	Pabrik Gula
RPM	Rotation per minute
St-g-PAM	Polyacrylamide Grafted Starch

References

1. Bai, F.W.; Anderson, W.A.; and Moo, Y.M. (2008). Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances*, 26(1), 89-105.

2. Eiadpum, A.; Limtong, S.; and Phisalaphong, M. (2012). High-temperature ethanol fermentation by immobilized coculture of kluyveromyces marxianus and saccharomyces cerevisiae. *Journal of Bioscience and Bioengineering*, 114(3), 325-329.

3. Bhadana, B.; and Chauhan, M. (2016). Bioethanol production using saccharomyces cerevisiae with different perspectives: substrates, growth variables, inhibitor reduction and immobilization. *Fermentation Technology*, 5(2), Art. No 1000131, 1-4

4. Inal, M.; and Yiğitoğlu, M. (2011). Production of bioethanol by immobilized saccharomyces cerevisiae onto modified sodium alginate gel. *Journal of Chemical Technology & Biotechnology*. 86(12), 1548-1554.

5. Ariyajaroenwong, P.; Laopaibon, P.; Jaisil, P.; and Laopaibon, L. (2012). Repeated-batch ethanol production from sweet sorghum juice by saccharomyces cerevisiae immobilized on sweet sorghum stalks. *Energies*, 5(4), 1215-1228.

6. Moreno-García, J.; García-Martínez, T.; Mauricio, J.C.; and Moreno J. (2018). Yeast immobilization systems for alcoholic wine fermentations: Actual trends and future perspectives. *Frontiers in Microbiology,* 9, 241.

7. Bickerstaff, G.J. (1997). Immobilization of enzymes and cells. *Humana Press*, 1, 1-11.

8. Fraser, J.; and Bickerstaff, G. (1997). Entrapment in calcium alginate. *Humana Press,* 1, 61-66.

9. O'Reilly, A.M.; and J.A. Scott. (1995). Defined coimmobilization of mixed microorganism cultures. *Enzyme and Microbial Technology*, 17(7), 636-646.

10. Kourkoutas, Y.; Bekatorou, A.; Banat, I.M.; Marchant, R.; and Koutinas, A.A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production: A review. *Food Microbiology*, 21(4), 377-397.

11. Park, J.K.; and Chang, H.N. (2000). Microencapsulation of microbial cells. Biotechnology *Advances*, 18(4), 303-319.

12. Nussinovitch, A. (2010). *Bead formation, strengthening, and modification, in polymer macro- and micro-gel beads: Fundamentals and applications*. New York, United States of America: S*pringer Science + Business Media*.

13. Rakin, M.; Mojovic, L.; Nikolić, S.; Vukasinovic, M.; and Nedovic, V. (2009). Bioethanol production by immobilized saccharomyces cerevisiae var. ellipsoidal cells. *African Journal of Biotechnology*, 8(3), 464-471.

14. Winkelhausen, E.; Velickova, E.; Amartey, S.A.; and Kuzmanova, S. (2010). Ethanol production using immobilized saccharomyces cerevisiae in lyophilized cellulose gel. *Applied Biochemistry and Biotechnology,* 162(8), 2214-2220.

15. Laopaiboon, L.; and Laopaiboon, P. (2012). Ethanol production from sweet sorghum juice in repeated-batch fermentation by saccharomyces cerevisiae

immobilized on corncob. *World Journal of Microbiology and Biotechnology*, 28(2), 559-566.

16. Bangrak, P.; Limtong, S.; and Phisalaphong, M. (2011). Continuous ethanol production using immobilized yeast cells entrapped in loofa-reinforced alginate carriers. *Brazilian Journal of Microbiology,* 42(2), 676-684.

17. Jin, H.; Liu, R.; and He, Y. (2012). Kinetics of batch fermentations for ethanol production with immobilized saccharomyces cerevisiae growing on sweet sorghum stalk juice. *Procedia Environmental Sciences*, 12, 137-145.

18. Rattanapan, A.; Limtong, S.; and Phisalaphong, M. (2011). Ethanol production by repeated batch and continuous fermentations of blackstrap molasses using immobilized yeast cells on thin-shell silk cocoons. *Applied Energy*, 88(12), 4400-4404.

19. Behera, S.; Mohanty, R.C.; and Ray, R.C. (2011). Ethanol production from mahula (Madhuca latifolia L.) flowers with immobilized cells of Saccharomyces cerevisiae in Luffa cylindrica L. sponge discs. *Applied Energy*, 88(1), 212-215.

20. Aykut, G.; Hasirci, V.N.; and Alaeddinoglu, G. (1988). *I*mmobilization of yeast cells in acrylamide gel matrix. *Biomaterials*, 9(2), 168-172.

21. Razmovski, R.; and Vučurović, V. (2011). Ethanol production from sugar beet molasses by S. cerevisiae entrapped in an alginate–maize stem ground tissue matrix. *Enzyme and Microbial Technology*, 48(4), 378-385.

22. Singh, A.; Sharma, P.; Saran, A.K.; Singh, N.; and Bishnoi, N.R. (2013). Comparative study on ethanol production from pretreated sugarcane bagasse using immobilized Saccharomyces cerevisiae on various matrices. *Renewable Energy*, 50, 488-493.

23. Walsh, P.K.; and Malone, D.M. (1995). Cell growth patterns in immobilization matrices. *Biotechnology Advances*, 13(1), 13-43.

24. Kaavessina, M.; Distantina, S.; and Fadilah. (2017). Synthesis of grafted flocculants based on several kinds of starch and its performance in water turbidity removal. *MATEC Web Conference*, 101.

25. Huang, M.; Liu, Z.; Li, A.; and Yang, H. (2017). Dual functionality of a graft starch flocculant: Flocculation and antibacterial performance. *Journal of Environmental Management,* 196, 63-71.

26. Yang, Z.; Yang, H.; Jiang, Z.; Cai, T.; Li, H.; Li, H.; Li, A.; and Cheng, R. (2013). Flocculation of both anionic and cationic dyes in aqueous solutions by the amphoteric grafting flocculant carboxymethyl chitosan-graft-polyacrylamide. *Journal of Hazardous Materials,* 254-255, 36-45.

27. Darby, R. (2001). *Chemical engineering fluid mechanics* (2nd ed.). New York, United States of America: Marcel Dekker Inc.