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Bioethanol Production Using Alginate from Sargassum binderi as an Immobilization Matrix for Saccharomyces cerevisiae D.01 cells in a Batch Reactor with Circulation.

Yumechris Amekan*, and Guntoro.

Faculty of Biotechnology, Universitas Kristen Duta Wacana, Yogyakarta 55224, Indonesia.

ABSTRACT

Bioethanol is a renewable energy source that can be used as a mixture with fossil fuels which are developed and applied in various countries, including Indonesia. Bioethanol fermentation process optimization can be performed using immobilized microbial cells. Sargassum binderi is potential to be developed and used as a source of alginate. The aim of this study is to determine the effectiveness of alginate from algae S. binderi as the matrix in the process of yeast S. cerevisiae D.01 cells immobilization for bioethanol production process in a batch reactor with circulation as well as its optimization which includes the effect of sucrose levels, repeated use of immobilized cells and beads storage time. Saccharomyces cerevisiae D.01 cells immobilized with alginate 3% derived from S. binderi produces the highest ethanol with 3.72% (w/v) or 76.65% of the maximum theoretical yield. S. cerevisiae D.01 cells immobilized with alginate stored in media without nutrients at a temperature of 4-10 °C for 10, 20 and 30 days showed decreased ethanol production ability of 3.2%, 5.9% and 9.1% compared with immobilized cells that are immediately used. S. cerevisiae D.01 immobilized on a matrix of alginate 3% were able to tolerate the substrate sucrose to a concentration of 30% and can be used in up to three subsequent fermentation processes.

Keywords: Alginate, Sargassum binderi, Immobilization cell, Saccharomyces cerevisiae, Bioethanol, Fermentation

Corresponding author

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INTRODUCTION

The Indonesian government consideres energy security a serious matter because Indonesia is highly dependent on fossil fuels as the main energy source for industry, transportation and even electricity generation [1]. Indonesia's energy consumption continues to rise whilst energy production growth slows, particularly oil production. Indonesia's energy consumption increased by 3.9% in 2015 (having nearly doubled over the last 15 years led by growth in fossil fuels), its energy production grew by just 1.4% (its lowest growth rate since 1988) and oil production continued to decline (falling to its lowest level since 1969) [2]. The government's response to reduce dependence on fossil fuels was issuing Presidential Regulation No. 5/2006 on the National Energy Policy to develop alternative energy sources as a substitute to oil fuel and issuing Presidential Instruction No. 1/2006 concerning the provision of biofuels as a fuel source.

Bioethanol is a renewable energy source that can be used as a mixture with fossil fuels which are developed and applied in various countries, including Indonesia. Bioethanol fermentation process optimization can be performed using immobilized microbial cells. Cell immobilization reduces the risk of washout that can occur because the microbial cell is very small and has a density close to that of water meaning that the microbes can easily enter the product stream [3]. Other advantages of the use of immobilized cells compared with free cells is the ease of separation of product and process control, the ability to be used repeatedly and the low risk of contamination, so that production costs can be lowered [4]. Cell immobilization with Ca-alginate developed by Goksungur and Zorlu [4], in principle, is a process of trapping the cell suspension in porous space/calcium alginate beads which are hardened. The trapping method is very easily applied to various types of cells such as bacteria, cynobacteria, algae, fungi and yeast. Alginate is selected as the supporting matrices for cell immobilization because the enzyme activity in the alginate matrix is more stable than that which is trapped in a matrix of another polymer type. Additionally the alginate does not have toxic effects [5].

Alginate is a natural polysaccharide that comprises 30 to 60% of brown algae (on dry weight basis). Until now, Indonesia still imports alginate from several countries such as France, Britain, China, Philippines, Germany and Japan. It is quite ironic given the fact that Indonesia is an archipelagic country with a long coastline of 81,000 km and has about 28 species of brown algae from 6 genus (Dyctyota, Padine, Hormophysa, Sargassum, Turbinaria and Hydroclathrus) [6]. Sargassum grows abundantly in tropical countries such as Indonesia and has a high population density, so that in times of blooming on sub-littoral areas it will form a very wide field of macroalgae [7]. In this regard, Sargassum is one type of algae with potential to be developed and used as a source of alginate.

Bioethanol production both in batch and continuous reactor, using Zymomonas mobilis immobilized cells with Na-alginate as a matrix, has shown that the productivity of bioethanol production is influenced by microbial strains, glucose concentration, the rate of intake of substrate and pH [8]. The aim of this study is to determine the effectiveness of alginate from algae S. binderi as the matrix in the process of yeast S. cerevisiae D.01 cells immobilization for bioethanol production process in a batch reactor with circulation as well as its optimization which includes the effect of sucrose levels, repeated use of immobilized cells and beads storage time.

MATERIALS AND METHODS

Microorganism

The D.01 strain of Saccharomyces cerevisiae was obtained from the Microbiology Laboratory, Faculty of Biotechnology, Duta Wacana Christian University. This strain isused by Madukismo Sugar Factory to produce alcohol from molasses.

Alginate Extraction

Brown seaweed S. binderi was collected in August 2015 from the south coast of the province of Yogyakarta, Indonesia. Harvested seaweed was washed with tap water, shade-dried and then processed with a blender, so that the powder could be stored until used.

Extraction was performed in accordance with Calumpong et al. [9] with some small modifications. Samples were dried to constant weight in an oven, soaked for one night in a 2% formaldehyde solution to eliminate pigments, then washed with distilled water and added to a 0.2 M HCl solution before being set aside for 24 h. After this period, the samples were washed once again with distilled water before being extracted under agitation for 5 h with a 2% sodium carbonate solution. Sodium alginate was precipitated with ethanol then purified twice with ethanol before been dried at room temperature.

Cultivation of Saccharomyces cerevisiae D.01

The yeast cells were grown in a sterile solution (121°C, 15 min.) containing 100 g/l glucose, 10 g/l peptone, and 5 g/l yeast extract. After one day, the mixture was centrifuged (2000 rpm, 20 min.) and suspended in sterile water (0.15 l), following a method described elsewhere [10].

Fermentation medium with glucose

A sterile solution (121°C, 20 min.) was prepared with 100 g/l glucose, 10 g/l peptone, and 5 g/l yeast extract. This solution was used in the batch fermentation experiments.

Fermentation medium with sucrose

A sterile solution (121°C, 20 min.) was prepared with 100, 200 and 300 g/l sucrose, 20 g/l peptone, and 10 g/l yeast extract. This solution was also used in the batch fermentation experiments.

Calcium alginate beads

For immobilization in beads, 2, 3 and 4% (w/v) sodium alginate was dissolved in 0.10 l water and added to a 0.15 l suspension of S. cerevisiae D.01 in a beaker. The solution was shaken gently. A $CaCl_2$ solution with a final concentration of 0.2 M was prepared in a separate beaker. The mixture containing the cells and the sodium alginate was added dropwise to 0.150 L of the $CaCl_2$ solution using a 0.010 l syringe. The beads were hardened in this solution for 1 h [10]. After hardening in the $CaCl_2$ solution, the beads were rinsed with sterile water to be used in the subsequent fermentation experiments. The beads had a diameter of 3 to 4 mm.

Batch fermentation experiments using the S. cereviciae D.01 immobilized cells

Batch fermentation was performed in accordance with Duarte et al. [10]. Approximately 100 g of calcium alginate beads was added to a 1.5 l batch reactor with circulation containing 1.0 l of the fermentation medium with glucose or sucrose as carbon source. All steps prior to fermentation were carried out under sterile conditions. Samples were collected during the fermentation period. The beads were filtered and rinsed with sterile water and added to a fresh fermentation medium as described above. This procedure was repeated in the subsequent fermentation experiments.

Analytical methods

Reducing sugar was determined according to the 3,5-dinitrosalicylic acid method [8]. Ethanol was assayed in a GC-14B-Shimadzu gas chromatograph with a flame ionization detector. The column was CBP-10 medially polar 230W x 140D x 360H mm. The oven, column, and injector temperature were 180 °C, 180 °C and 250 °C respectively. Theoretical yield was calculated as the actual ethanol produced x 100 divided by the theoretical maximum [4].

RESULTS AND DISCUSSSION

Effect of Alginate Concentration as Immobilized Matrix of S. Cerevisiae D.01 on Ethanol Yield and Productivity

The results of ethanol yield and productivity by S. cerevisiae D.01 immobilized cells with a cell density of 3.1×10^8 CFU/ml, which was immobilized with alginate 2, 3 and 4% using glucose 100 g/l as a substrate for 36 hours fermentation is presented in Table 1. In general it seems that the ethanol production continues to

increase for up to 18 hours of fermentation, then ethanol production is stagnant and subsequently decreases slightly due to stagnant consumption of glucose.

As shown in Table 1, S. cerevisiae D.01 cells immobilized with alginate 3% are able to consume up to 97.5 g/l substrate and produce ethanol 37.2 g/l, equivalent to 92.11% of the maximum theoretical yield, after 18 h of fermentation. S. cerevisiae D.01 cells immobilized with alginate 2% consume 98.8 g/l substrate and produce ethanol 36.1 g/l (71.49% of the max theoretical yield), while cell immobilized with alginate 4% consume 99.1 g/l substrate and ethanol yield 35.2 g/l (67.72% of the max theoretical yield). These results indicate that, the differences of alginate concentrations that used to immobilize affect the ability of substrate and product diffusion into and out of the matrix. For S. cerevisiae D.01, beads immobilized with alginate 3% have the most appropriate pore density for the substrate entry and product release, as well as provide appropriate space for the cell activity in the fermentation process so that more ethanol is produced. Beads with 4% alginate have a dense, hard physical appearance and are resistant to rupture when pressed manually. The stiff matrix (rigid) causes smaller substrate diffusion efficiency resulting in lower production of ethanol. Beads with alginate 2% are too soft and disintegrate easily due to the release of carbon dioxide gas from the fermentation in the matrix which can increase the diameter of beads due to increasing cell density of S. cerevisiae D.01 [11]. Added diameter reduces ethanol production due to the reduction in surface area for mass transfer of substrates into and through the beads, as reported by Gilson and Thomas [12].

Table 1: Ethanol yield, productivity and glucose consumption by Saccharomyces cerevisiae D.01 immobilized using alginat 2, 3 and 4 % in batch reactor with circulation (30 °C, 36h fermentation time, glucose 100 g/l as substrate)

Alginate concentration	Ferm. time (h)	Ethanol (g/l)	Glucose cons. (g/l)	Ethanol Yield (% of theoretical)	Productivity (g/l.h)
2 %	6	13.2	35.2	73.37	2.20
	12	33.7	93.9	70.22	2.81
	18	36.1	98.8	71.49	2.00
	24	36.0	99.0	71.15	1.50
	30	35.8	99.0	70.75	1.19
	36	35.6	99.0	70.35	0.98
3 %	6	17.0	42.4	78.44	2.83
	12	34.6	85.3	79.36	2.88
	18	37.2	97.5	76.65	2.12
	24	36.9	99.0	72.92	1.54
	30	36.2	99.0	71.54	1.21
	36	36.0	99.1	71.07	1.00
4 %	6	15.2	47.1	63.14	2.53
	12	31.7	91.1	68.08	2.64
	18	34.2	98.8	67.72	1.90
	24	35.1	99.1	69.29	1.46
	30	35.1	99.1	69.29	1.17
	36	35.2	99.1	69.49	0.98

The level of substrate consumption by S. cerevisiae D.01 immobilized with alginate 2, 3 and 4% is relatively similar, yet different amounts of ethanol are produced. This may be caused by the difference in concentration of dissolved oxygen present in the fermentation media due to the mixing process at the time of dissolution of the glucose and the use of a pump for circulating medium. The concentration of dissolved oxygen in the fermentation medium can reduce the ethanol product because oxygen can serve as a final

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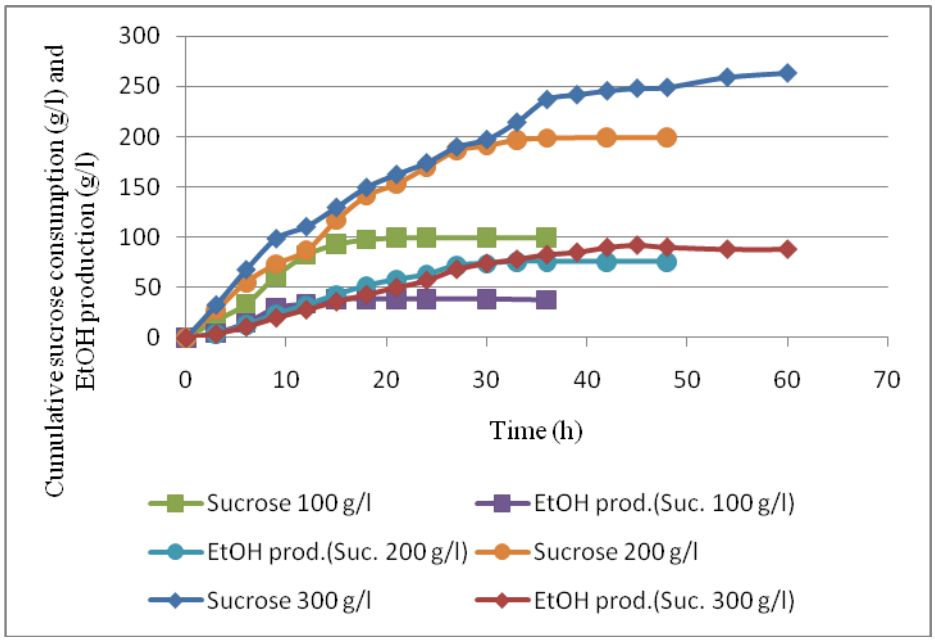
electron acceptor replace acetaldehyd. As a consequence, the substrate is not converted to ethanol but converted into cell biomass.

Effect of Sugar-Sucrose Concentration on Ethanol Yield and Productivity by S. cerevisiae D.01 Immobilized With Alginate 3%

To test the ability of immobilized cells to produce ethanol using cheaper substrate, performed with the fermentation using sucrose as a substrate. The sucrose consumption and ethanol production profile by S. cerevisiae D.01 immobilized cell with alginate 3% (cell density 4.0×10^8 CFU/ml) using sucrose as substrate in a batch fermentor with circulation is presented in Figure 1. The fermentation was performed with various sugar concentration to increase product concentrations. The initial sugar concentrations were 100, 200 and 300 g/l.



Sugar concentration is critical to this fermentation and influencing the rate of production and the final yield in addition to physiological growth of yeast. Initial sugar concentration has also been found to determine the amount of alcohol. In the present study, maximum ethanol production was obtained in the medium containing 300 g/l sucrose yielding ethanol 92 g/l, while medium containing 100 g/l and 200 g/l sucrose yielded ethanol 39 g/l and 76 g/l, respectively. This result shows the conversion of sucrose to ethanol during fermentation. In anaerobic conditions or in the absence of the final electron acceptor from the outside of the cell, yeast is forced to use acetaldehid, which is a compound products of decarboxylationof pyruvic acid, to be played as a final electron acceptor in order to regenerate NAD^+ needed in the glycolysis to generate the energy for yeast cells survival.

The increase in the ethanol production was observed with an increase in sugar concentration. This provides evidence that the alginate matrix appears to be effective in protecting cells against high osmotic pressure because of high sugar concentration. According to Barros et al. [13], high sugar concentrations will be act as inhibitors due to the high osmotic pressure causing plasma cell membrane disintegration and resulting in cell death, and therefore reduction in ethanol production. In addition, the process of circulation appears to also improve the contact between substrate and the beads and process of CO_2 discharge which is produced during the ethanol fermentation process, so that the reaction equilibrium will always favour the formation of ethanol.

Effect of Repeated Use of S. cerevisiae D.01 Immobilized with Alginate 3% on Ethanol Yield and Productivity using Sucrose as a Substrate

To test the stability of the immobilized cells in producing ethanol, the fermentation process was carried out repeatedly 3 times in a row. The ethanol production by S. cerevisiae D.01 immobilized with alginate 3%

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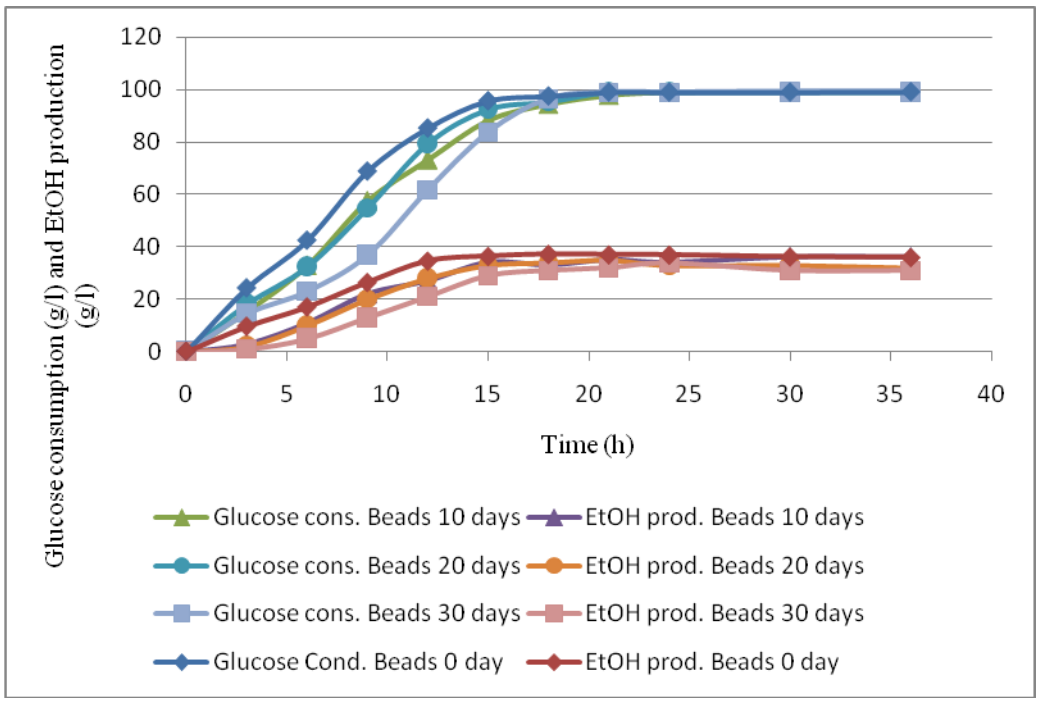
(cell density 4.0 × 10^8 CFU/ml) using sucrose as substrate (concentration 100, 200 and 300 g/l) for consecutive three batches is shown in Table 2. Immobilized S. cerevisiae D.01 cells in the medium containing 100 g/l sucrose gave similar ethanol yields on the first and second running (39 g/l and 41 g/l, respectively) then decreased in the third batch (36 g/l). Solutions containing 200 g/l and 300 g/l sucrose gave increasing ethanol yield over three batches. The results show that the immobilized S. cerevisiae D.01 in 3% alginate matrix is stable enough to be used repeatedly in batch fermentation with circulation even to 300 g/l sucrose concentration. This proves that the alginate matrix may protect cells from the osmotic pressure of the substrate used. A slight drop of ethanol in the third batch with 100 g/l sucrose as substrate, was probably caused by the pasteur effect on the presence of oxygen given sucrose consumption was still increasing. Repeated batch fermentation has the advantage of improving ethanol productivity, reducing the time of inoculum preparation [14-16].

Tabel 2: Ethanol yield and sucrose utilization during batch fermentation using immobilized S. cerevisiae D.01 (pH 6, temp. 30 °C, change of medium after 36 h (Sucrose 100 g/l), 48 h (Sucrose 200 g/l), 60 h (Sucrose 300 g/l)).

Run	sucrose (100 g/l)			Sucrose (200 g/l)			Sucrose (300 g/l)		
	Ethanol (g/l)	% of theoretical yield	Suc. used (%)	Ethanol (g/l)	% of theoretical yield	Suc. used (%)	Ethanol (g/l)	% of theoretical yield	Suc. used (%)
1	39.0	82.30	92.70	76.0	75.60	98.30	92.0	72.50	82.75
2	41.0	84.70	94.25	79.0	78.30	98.65	92.0	70.90	84.67
3	36.0	73.40	96.00	81.0	79.60	99.50	111.0	77.0	93.97

The Effect of S. cerevisiae D.01 Cell Age at Immobilization on Ethanol Yield and Productivity using Glucose as a substrate

The results of stability tests performed on immobilized S. cerevisiae D.01 (cell density 4 × 10^8 CFU/ml) aged 10, 20 and 30 days using 100 g/l glucose as substrate is shown in Figure 2.



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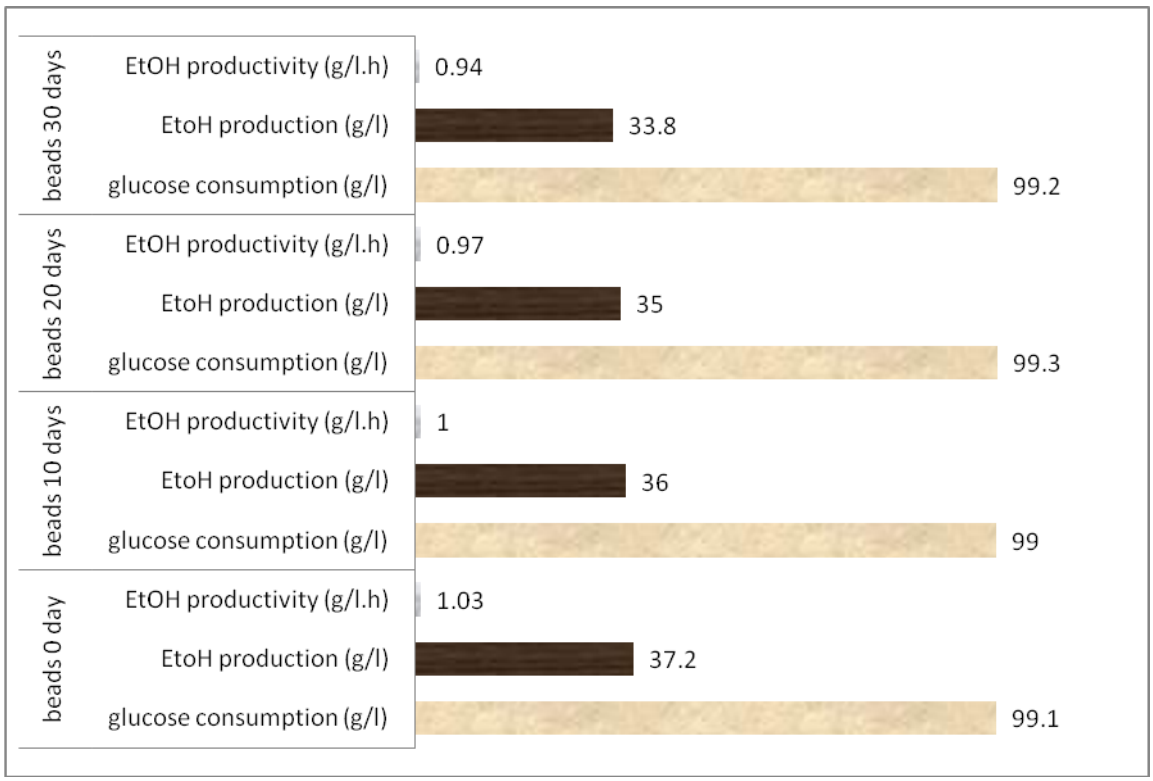
In the early stages of fermentation substrate consumption rate period was low, 15.3, 18.1 and 14.5 g/l respectively, for the immobilized cells aged 10, 20 and 30 days. Initial consumption rate in newly immobilized cells was 24.2 g/l. The slow consumption of the substrate by the immobilized cell aged 10, 20 and 30 days can be explained by cell adaptation from being stored in media without nutrition. The highest substrate consumption rate (24.2 g/l) in newly immobilized cells was observed at 0–3 hours of fermentation, whilst immobilized cell aged 10 and 20 days had maximum consumption (both 24.6 g/l) at 6-9 and 9-12 hours respectively. Cells aged 30 days had maximum consumption (22 g/l) at 12-15 hours of fermentation. This result indicates that the length of storage time of the beads affects the time needed by immobilized cells S. cerevisiae D.01 for adaptation in the fermentation medium.

In the present study, the highest ethanol production was observed in freshly immobilized cells after 18 hours of fermentation (37.2 g/l). The highest ethanol production for immobilized cells aged 10 and 20 days was observed after 21 hours of fermentation with 36 and 35 g/l respectively. Immobilized cells aged 30 days yielded maximum ethanol after 24 hours at 33.8 g/l. Ethanol yield reduction due was attributed to decreased fermentation activity, even S.cerevisiae D.01 cell death, due to the absence of a source of carbon, nitrogen and other vital elements during the storage process. Figure 3 shows that the yield of the ethanol decreased with the length of immobilized cell storage in the medium without nutrients compared with newly immobilized cells, ethanol yield in immobilized cells aged 10, 20 and 30 days decreased by 3.2%, 5.9% and 9.1% respectively. Thus, it can be said that the longer the beads are stored at conditions without nutrient, the lower the yield and productivity.



CONCLUSION

Saccharomyces cerevisiae D.01 cells immobilized on a matrix of alginate from Sargassum binderi with a concentration of 3% produces the highest ethanol with 37.2 g/l or 76.65% of the maximum theoretical yield. Saccharomyces cerevisiae D.01 cells immobilized with alginate stored in media without nutrients at a temperature of 4-10 °C for 10, 20 and 30 days showed decreased ethanol production ability of 3.2%, 5.9% and 9.1% compared with immobilized cells that are immediately used.

Saccharomyces cerevisiae D.01 immobilized on a matrix of alginate 3% were able to tolerate the substrate sucrose to a concentration of 300 g/l and can be used in up to three subsequent fermentation processes.

List of Abbreviations

GC: Gas chromatography; S.cerevisiae: Saccharomyces cerevisiae; S. binderi: Sargassum binderi; Sucrose cons: Sucrose consumption; EtOH prod.: Ethanol production.

Declarations

Authors' contributions

Yumechris Amekan carried out the lab scale alginate extraction, S. cerevisiae D.01 immobilization, bioethanol fermentation and the determination of ethanol content using glucose and sucrose as substrates and drafted the manuscript. Guntoro coordinated the study, contributed to the analysis of the results and in the improvement of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Data made available to all interested researchers upon request.

Consent for publication

All authors approved the manuscript.

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