THE FERMENTATION OF GREEN ALGAE (*Spirogyra majuscule* Kuetz) USING IMMOBILIZATION TECHNIQUE OF Ca-ALGINATE FOR *Saccharomyces cerevisiae* ENTRAPMENT

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ABSTRACT

A study of batch fermentation of green algae (Spirogyra majuscula Kuetz) from Pengging Lake, Boyolali, Central Java for bioethanol source using immobilization technique of Ca-alginate for Saccaromyces cerevisiae entrapment has been done. The scope of the study emphasized on the best condition for the processes of hydrolysis and fermentation. Concentration of sulfuric acid and hydrolysis time were varied with 0.1, 0.2, 0.3, 0.4 and 0.5 M for 30, 90, 150, 210, 270, 330, 360, 390, 420, and 450 min to obtain the maximum glucose content of UV analysis. Na-alginate : yeast ratio and fermentation time were varied with 1:5, 2:4, 3:3, 4:2 and 5:1 (w/w) for 1, 2, 3, 4, 5, 6 and 7 days. Distillation at 70–80 °C was deployed to purify the fermentation product. The ethanol content in the product was analyzed using gas chromatography-flame ionization detector (GC-FID). The result of study showed that maximum glucose content was obtained 2.1% from 0.2 M sulfuric acid for 6 h of hydrolysis. Maximum ethanol content was obtained 54.1% from 2:4 ratio of Na-alginate : yeast (w/w) for 4 days of fermentation. The study also concludes that immobilization technique of Ca-alginate increase alcohol content compared to without immobilization of green-algae fermentation.

Keywords: *Ca-alginate; immobilization; green algae; bioethanol*

ABSTRAK

Telah dilakukan penelitian fermentasi dari alga hijau (Spirogyra majuscula Kuetz) dari danau Pengging, Boyolali, Jawa Tengah sebagai sumber bioetanol dengan teknik imobilisasi Ca-alginat untuk penjebakan Saccaromyces cerevisiae. Lingkup dari penelitian ini menekankan pada kondisi terbaik pada proses-proses hidrolisis dan fermentasinya. Konsentrasi asam sulfat dan waktu hidrolisis divariasikan 0,1, 0,2, 0,3, 0,4, dan 0,5 M selama 30, 90, 150, 210, 270, 330, 360, 390, 420, dan 450 menit untuk mendapatkan kandungan glukosa maksimum dari analisis UV. Rasio dari Na-alginat : ragi dan waktu fermentasi divariasikan dengan perbandingan 1:5, 2:4, 3:3, 4:2 dan 5:1 (b/b) selama 1, 2, 3, 4, 5, 6 dan 7 hari. Distilasi pada 70–80 °C dilakukan untuk memurnikan produk fermentasi. Kandungan etanol dalam produk dianalisis dengan menggunakan kromatografi gas detektor ionisasi nyala (GC-FID). Hasil dari penelitian ini menunjukkan bahwa kandungan glukosa maksimum diperoleh sebanyak 2,1%, yang diperoleh dari hidrolisis 0,2 M asam sulfat selama 6 jam. Kandungan etanol maksimum sebanyak 54,1%, yang diperoleh dari fermentasi dengan perbandingan 2:4 dari Na-alginat : ragi (b/b) selama 4 hari. Penelitian ini juga menyimpulkan bahwa teknik imobilisasi Ca-alginat meningkatkan kandungan alkohol dibandingkan dengan tanpa imobilisasi dari fermentasi alga hijau.

Kata Kunci: *Ca-alginat; imobillisasi; alga hijau; bioetanol*

INTRODUCTION

The depletion of fossil fuel is a problem related to the energy source. On the other hand, fossil fuel as energy source has caused accumulation of carbon dioxide in the atmosphere or environment [1]. These problems should be solved by seeking alternative sources that is environmentally friendly. Biofuel is one of the alternative sources. Production of biofuel from renewable sources is one of the most sustainable alternatives to petroleum fuels and a viable means for environmental and economic sustainability [2].

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Generally, biofuel can be yielded from the conversion of biomass. Bioethanol is one of biofuels that releases lower emission of carbon monoxide. Sucrose, starch and lignocelluloses are kinds of biomass that are able to be converted for bioethanol production. The main problem of the bioethanol production from biomass is that huge competion with food production yielded from agricultural lands is possible. The source of sugar and starch for bioethanol production should be selected from plants or grasses that are not cultivated for food production.

Almost 300,000 algae species are distributed worldwide in seawater, freshwater, and wastewater [3-5]. Algae can be cultivated and used as feedstock for the production of biodiesel and bioethanol [6]. Green algae of chlorella consist of 29–31% carbohydrate that has capability of producing 20–30% bioethanol [7]. Biofuel provides advantages if deployed in an engine. One of the biggest advantages of biodiesel as alternative fuel is that it can be used in existing diesel engines without modification, and its suitability for blending in at any ratio with petroleum diesel [8]. Bioethanol also mixes easily with gasoline and also can be deployed in an engine without a modification. One of the advantages of green algae for bioethanol production is that green algae has chlorophyll that undergoes photosynthesis and produces carbohydrate. Beside, it needs no special cultivation, control and has continuity of cropping because of the short growth [6]. This high yield is due to the fact that algae require less energy for the production of supporting tissue than land plants, and they have the capability to take up nutrients over their entire surface [9].

Some algae have been already known as sources for food or bioactive compounds, such as *Griffthsia, Ulva, Enteromorpa, Gracilaria, Euchema* and *Kappaphycus* [6]. Until today, macroalgae has a commercial market as a food product, mainly associated with the Asian market, account for 83 to 90% of the total value of macroalgae [10] The utilization of macroalgae for biofuel has been intensively reported. The attractive alternative of macroalgae for bioethanol production lies in the high carbohydrate content (e.g. polysaccharide) [11]. The high carbohydrate content can be converted to various gas and liquid fuels [12]. The efficiency of ethanol production largely depends on the availability of suitable substrate, yeast strain and method employed [13]. *Spirogyra sp* consists of 64 % carbohydrate that is relatively high for starting material of bioethanol [6].

Saccharomyces cerevisiae is ideal for ethanol production because of fast growth rates, efficient glucose repression, efficient ethanol production and a tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels [14]. Fermentation process with the strain using immobilization technique is possibly able to obtain good yield compared to conventional one. The ease of separation product and longer yeast life-time is offered by the technique [10,15]. Ca-alginate is one of immobilization matrixes that are able to be used for fermentation process. The yeast as biocatalyst is dopped into the gel matrix of Ca-alginate in order to improve the ethanol product during the fermentation process [10]. In this study, some parameters that influence the fermentation *Spirogyra majuscula* Kuetz to bioethanol will be studied.

EXPERIMENTAL SECTION

Materials

Green algae (*Spirogyra majuscula* Kuetz) was obtained from Pengging lake, Boyolali, Central Java. Local yeast was from local market, Na-alginate was obtained from local chemical store in Solo, Central Java. Calcium hydroxide (p.a), phenol (p.a), sulfuric acid 98%, urea, ethyl acetate, sodium chloride, acetic acid (p.a), propanol (p.a), acetaldehyde and ethanol (p.a) were obtained from E. Merck, Germany.

Instrumentation

Spectrophotometer UV-Vis used was Shimadzu UV-1601PC. Gas chromatography-flame ionization detector (GC-FID) was Perkin-Elmer AutoSystem XL with 30 m DB5 column from J&W scientific.

Procedure

Algae determination and preparation of Spirogyra majuscula Kuetz

Algae determination was done based on physical form and morphology of green algae in *Laboratorium Taksonomi Tumbuhan*, Universitas Gadjah Mada, Yogyakarta. For the preparation, *Spirogyra majuscula* Kuetz was cleaned with many times aquadest after with flowing tap water. The exposure of sunlight was done for 3 days before grinding with electric blender up to powder form. The powder form of sample was sieved about 150 mesh.

Algae hydrolysis and analysis of glucose content

Each sulfuric acid used was gently poured about 100 mL and mixed with 5 g algae powder. Sulfuric acid and hydrolysis time were varied 0.1, 0.2, 0.3, 0.4, 0.5 M for 30, 60, 90, 120 min at about 90–100 °C with constant stirring. Filtration of mixture solution was done to obtain hydrolysate of glucose after heating accomplished. Further hydrolysis with concentration of H_2SO_4 optimized was done to obtain data of glucose content for 30, 90, 150, 210, 270, 300, 330, 360, 390,

420, 450 min. In the glucose analysis, a standard curve of glucose concentration (0, 50, 100, 150 and 200 ppm) versus absorbance of UV/Vis spectrophotometer was made for glucose determination. About 1 mL glucose solution was added aquadest up to 25 mL. 5 mL H_2SO_4 98% and 1 mL 5% phenol solution was added in the solution. The sample was diluted from 1 mL up to 10 mL. Blank solution was made from 2 mL aquadest, 5 mL H_2SO_4 98%, and 1 mL5% phenol solution. Glucose content in the sample was measured using UV/Vis spectrophotometer with linear regression method. Curve of sulfuric acid concentration vs glucose content and hydrolysis time vs glucose content were also made. Determination of glucose content in the sample was by standards plotting.

Immobilization of Ca-alginate

A yeast and nutrition media was made about 50 mL by mixing each 0.5 g urea and yeast variation of 0.5 g (1% w/v), 1 g (2% w/v), 1.5 g (3% w/v), 2 g (4% w/v), 2.5 g (5% w/v). Solution of Na-alginate was made by addition of cold aquadest up to 50 mL with concentration of 1; 2; 3; 4; 5% (w/v). About 50 mL nutrition media and 50 mL Na-alginate solution was stirred until homogen solution obtained. Solid gel of Ca-alginate was formed by addition of 5 mL 2 % $CaCl_2$ solution with an injection syringe in each 100 mL solution. After about 30 min, gel solid was washed with aquadest to remove excess of sodium ions. To develop bacterial growth, granules were incubated overnight in the oven at 30 °C. Granules were stored in the 2% (w/v) yeast at 4 °C before used.

Fermentation process

Glass flasks for media holder were firstly sterilized in the autoclave at 121 °C for an hour. Hydrolysate of glucose solution from detoxification with $Ca(OH)_2$ was adjusted at pH 4-5 by addition of citrate buffer. About 20 mL glucose solution was neutralized and 6 g immobilation granules with yeast and nutrition formed of Ca-alginate was mixed in the 100 mL glass flask.

Determination of Na-alginate : yeast ratio and optimization of fermentation time

Fermentation was done for 3 days with ratio of matrix, yeast and urea (see Table 1) to obtain the ratio of Na-alginate : yeast. Fermentation was varied for 1, 2, 3, 4, 5, 6, 7 days based on the best composition of Na-alginate : yeast (w/w). The comparation between Ca-alginate and urea and without urea with the same optimized condition was also investigated (see Table 2).

Separation of fermentation product and glucose determination

Distillation of raw fermentation product to separate ethanol and impurities was carried out. Distillation was done at 70–80 °C using water condensers. Distillate was then weighed. Analysis of product was done for fine product after separation using GC-FID with column temperature (40–150 °C), heating rate 10 deg/min and nitrogen carrier.

Table 1. Ratio composition of matrix, yeast and urea

Sample	Na-alginate:yeast (w/w)	Urea (g)
A	1 : 5	0.5
B	2 : 4	0.5
C	3 : 3	0.5
D	4 : 2	0.5
E	5 : 1	0.5

Table 2. The ratio of Ca-alginate and urea and without urea

Sample	Yeast content	Na-alginate content	Urea (g)
F	Optimum weight	-	-
G	Optimum ratio with Na-alginate	Optimum ratio with yeast	0.5

RESULT AND DISCUSSION

Sample Determination and Preparation of Green Algae

Based on the investigation of the morphology and physically appearance, the algae used for the fermentation was *Spirogyra majuscule* Kuetz. The taxonomy of the algae was Chlorophyta (division), Chlorophyceae (class), Zygnematales (ordo), Zygnemataceae (family), *Spirogyra* (Genus), *Spirogyra majuscula* Kuetz (species). After preparation treatment, the colour change from light to dark green algae occurred. It was also seen that the texture of the *Spirogyra majuscula* Kuetz changed from soft and glat form to rude after drying. The change of the color was influenced by adaptic chromatic of algae pigment for responding quality of the light received. The texture change was influenced by the lost of water content of algae (see Fig. 1).

Determination of Glucose Content

The glucose content of *Spirogyra majuscula* Kuetz after hydrolysis was determined using UV/Vis spectrophotometer. The mineral acid of sulfuric acid was used for hydrolysis because this acid was strong enough to crack carbon bonding of algae. The present of glucose was indicated by orange color of solution when tested with Benedict reagent. The curve of absorbance and glucose content was shown on Fig. 2. The graphic of glucose standards using spectrophotometer showed that linear equation of the curve was $y = 0.004178x + 0.0666$ with coefficient

Fig 1. *Spirogyra majuscula* Kuetz before and after drying treatment

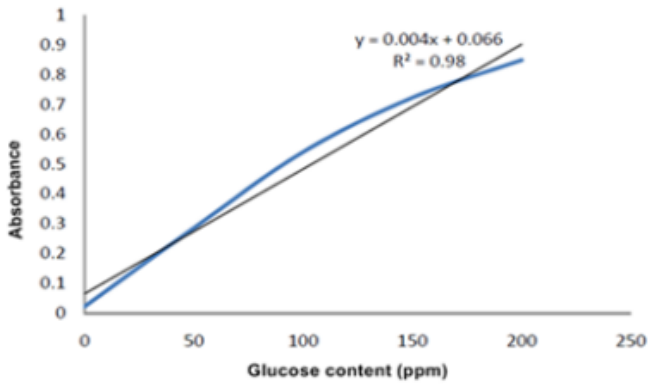

Fig 2. Curve of absorbance vs glucose standards measured with UV/Vis Spectrophotometer

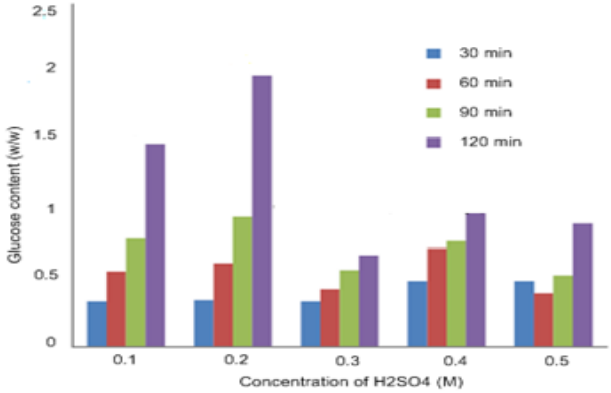

Fig 3. Diagram of glucose content of *Spirogyra majuscule* Kuetz after hydrolysis with H_2SO_4

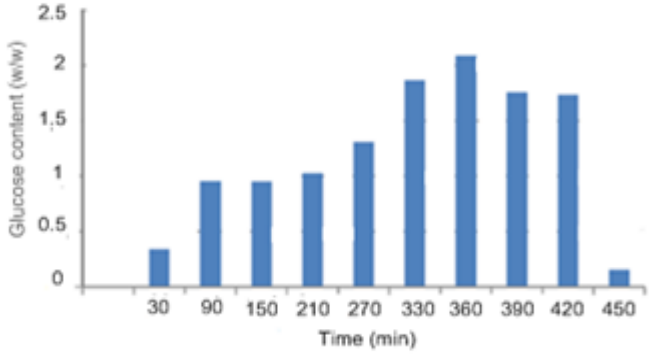

Fig 4. Diagram of the glucose content vs time variations from the range 30-450 min

Fig 5. Granules of Ca-alginate with yeast content

linearity about 99%. The glucose content of hydrolysates after measurement with spectrophotometer was shown on Fig. 3. The measurement showed that the highest glucose content about 1.98% (w/w) was obtained from the solution of 0.2 M H_2SO_4 for 120 min of hydrolysis time. Hydrolysis with 0.1 M H_2SO_4 showed that less than 0.2 M acids used was still insufficient for contributing H^+ for hydrolysis for maximum cracking. The increase of concentration from 0.3 M up to 0.5 M caused reducing glucose content of hydrolysis. This was possibly affected by decrease of effectivity of protons to crack the bonding, because of imbalancing of the proton contents and water content in the solution. The less water with increasing protons reduced hydroxyl ions that are important for bonding agent with carbonium.

Hydrolysis time of 30–120 min showed that increasing glucose content was not maximally reached. The extension of hydrolysis time with 0.2 M H_2SO_4 was then done until maximum glucose reached. The further hydrolysis was done up to 450 min (see Fig. 4). The

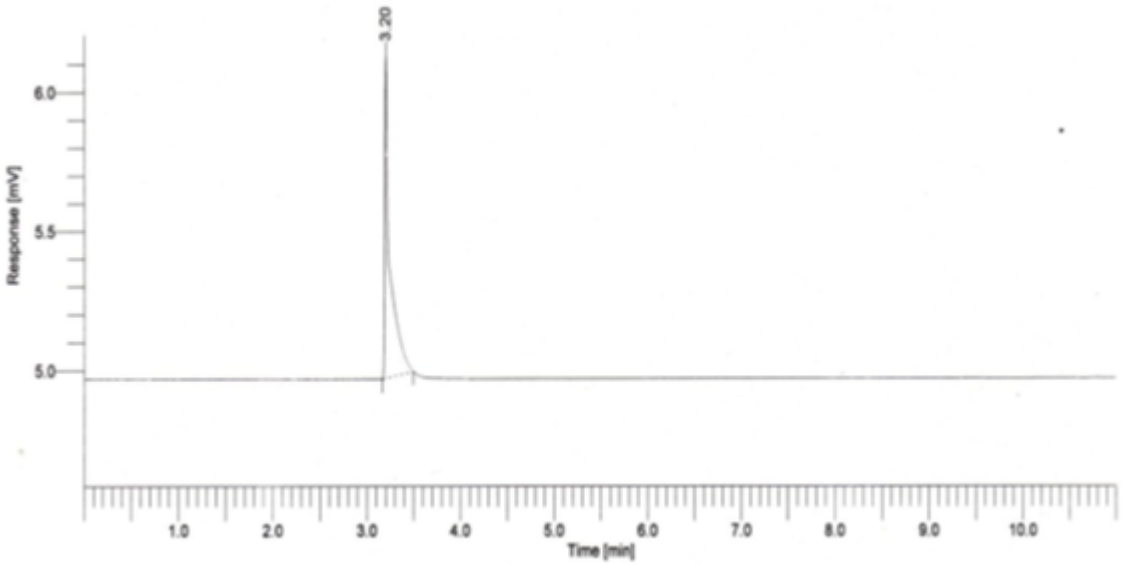

Fig 6. Chromatogram of GC-FID of fermentation products after distillation with condition of Na-alginate : yeast of 2:4 (w/w)

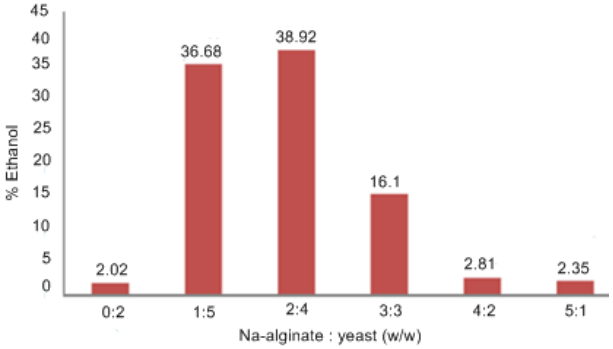

Fig 7. Ratio of Na-alginate : yeast (w/w) vs ethanol content

maximum glucose content about 2.09% was obtained for 360 min (6 h) of glycoside cracking of acidic hydrolysis of polysaccharide occurred maximally for 360 min.

Immobilization using Ca-alginate and Optimization of Fermentation

In this study, media chose was Ca-alginate made from Na-alginate that is hydrophilic substance and able to form gel with calcium ions. The benefit of using natural of polymer alginate is non toxic, non allergic and biodegradable. The Ca-alginate is formed by a process of chelate bonding between Na-alginate and $CaCl_2$ through interchain mechanism. Solution of Na-alginate with the present of $CaCl_2$ is able to form water-insoluble gel that acts as chelating agent. Ca^{2+} ion in the gel can interact with oxygen atom of carboxyl group. Cells of *Saccharomyces cerevisiae* was entrapped in the gel media of Ca-alginate (see Fig. 5). This affects *Saccharomyces cerevisiae* survives longer during fermentation process.

Preliminary treatment of detoxification of hydrolysate with $Ca(OH)_2$ was aimed to remove the inhibitor substances disturbing fermentation process such as furan derivatives, aliphatic acids, formic acid, lyvulinic acid and phenolic acids. Fermentation process in the study was adjusted at pH 4-5 by addition citrate buffers. Ethanol as main products and CO_2 as main metabolite product was theoretically yielded from the fermentation process. *Saccharomyces cerevisiae* is one of strains that are able to produce zimace and invertace enzyme. In anaerob circumstance, zimace enzyme acts as sucrose cracker that yields monosacarida of glucose and fructose. Invertace enzyme acts to convert glucose to ethanol and releases energy and CO_2.

Optimization of Na-alginate : Yeast (w/w) and Fermentation Time

A fine chromatogram of ethanol analysis using GC-FID was shown on Fig. 6. From the Fig. 7, the highest ethanol content of the fermentation was obtained of Na-alginate : yeast with ratio 2:4 (equivalent with 1 g Na-alginate and 2 g yeast). By this composition, the yeast quantity trapped in the gel medium was conditioned sufficiently for the fermentation. Beside, the yeast was not easily to dissolve in the water solution. In the gel of Na-alginate : yeast with ratio 1:5 (w/w), the hardness of granule form was insufficient to hold the yeast to dissolve. In this condition, the yeast was not easy to maintain their life in

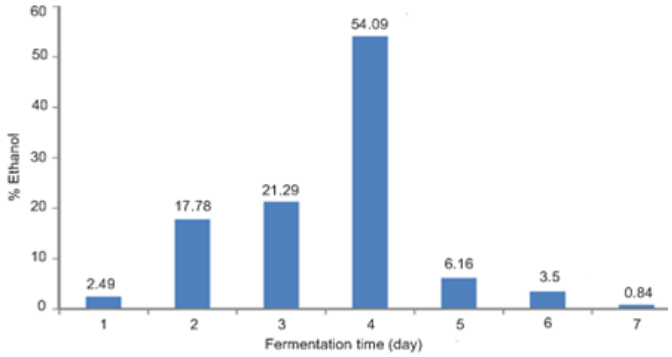

Fig 8. Fermentation time (day) vs ethanol content

Table 3. Ethanol yielded from two methods, with and without immobilization gel

Method	Ethanol content (%) GC-FID response
Without immobilization gel of Ca-alginate	2.01
With immobilization gel of Ca-alginate	38.92

the solution, and converted less ethanol from glucose. In the case with the ratio of 3:3 or higher, the gel media formed became too hard. It affected the pore size and flexibility of the gel decrease. In this condition, the activity of *Saccharomyces* in converting glucose to ethanol was disturbed. This study also showed that the fermentation with Na-alginate : yeast of ratio 2:4 yielded higher content of ethanol compared to 2 g yeast content in a fermentation without immobilization media. Table 3 was shown that the ethanol content yielded from both fermentation methods was significant different. Immobilization technique affords better result with 38.92% ethanol content than 2.01% without immobilization one.

In this study, fermentation was varied within 1, 2, 3, 4, 5, 6, 7 days of Na-alginate : yeast of ratio 2:4 (w/w). Ethanol yielded versus fermentation time (day) was shown on Fig. 8. The highest ethanol content about 54.09% (GC base) was obtained with fermentation time for 4 days. The diagram showed that the ethanol content from first day increased up to fourth day and then significantly decreases until seventh day. The first until third day of fermentation, *Saccharomyces cereviseae* was in the adaptation with the circumstance and grew. In this stage, the ethanol content was not high until fourth day. In the fifth until seventh day of fermentation, *Saccharomyces cereviseae* reached a stationary growth and the strain growth was less than strain death. In this stage, ethanol content began to decrease. Generally, fermentation time influences the ethanol products yielded. More time settled for the process should be more products yielded. However, the increasing alcohol products will reach maximum until alcohol content hinders the strain growth, except for the strains that has good resistance and capability of growing with high alcohol content.

CONCLUSION

The best ratio of Na-alginate : yeast for the fermentation of green algae (*Spirogyra majuscula* Kuetz) was 2:4 (w/w). The best fermentation of the algae was conditioned within four days with ethanol content approximately 54.09%. Fermentation using immobilization technique of Ca-alginate of the best condition affords higher ethanol content approximately 38.92% compared to 2.01% without immobilization.

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