

Improvement of Bioethanol Production by Using *Saccharomyces cerevisiae* [Meyen ex E.C. Hansen] Immobilized on Pretreated Sugarcane Bagasse

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Abstract

Pretreated of sugarcane bagasse was used as a carrier for immobilization of Saccharomyces cerevisiae. Pretreatments were carried out by steaming, pressurized steam, and combination both of procedure. The objectives of this research was to investigate the effect of pretreatment on sugarcane bagasse to cells adsorption and bioethanol production. Immobilization process was conducted in a ratio of 2.5 g carrier/50 mL cell suspension. Whole cell biocatalyst as much as 1% (w/v) was used as inoculum for bioethanol fermentation. The best pretreated sugarcane bagasse for carrier of immobilized cells was obtained using steam treatment for 30 minutes. Those treatment improved the physical properties of carrier and increased the cell retention up to 10.05 mg/g. The use of whole cell biocatalyst after steaming pretreatment also enhanced ethanol yield 1.5 times higher than control.

Keywords: bioethanol; immobilization; pretreatment; steam treatment; sugarcane bagasse

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INTRODUCTION

Ethanol production using immobilized cells has been investigated during the last decades. Productivity of bioethanol by using immobilized cells becomes two or three times higher along with the increasing of cell density (Singh *et al.*, 2013). Cells immobilization is defined as a form of physical entrapment or localization of whole cell into a specific space (Kourkoutas *et al.*, 2004). This technology is known as an alternative to enhance productivity of bioethanol

with minimum of production cost (Santos *et al.*, 2008; Razmovski and Vucurovic, 2012; Kridponpattara and Phisalaphong, 2013).

Technology of cells immobilization is divided into four types, namely flocculation, encapsulation, entrapment into porous matrix, and adsorption on solid substrate (Kourkoutas *et al.*, 2004; Verbelen *et al.*, 2006). Immobilization process by adsorption cell on lignocellulosic biomass is considered to be

reasonable than cell entrapment into porous matrix (Yu *et al.*, 2007).

Singh *et al.*, (2013) reported that bioethanol production using immobilized cells on sugarcane bagasse was higher than using alginate and agar as a carrier. Bioethanol production using immobilized cell on sugarcane bagasse, alginate, and agar as a carrier were 15.4, 11.8, and 9.4 g/L, respectively. Anita *et al.* (2016) also reported that the bioethanol yield for 24 h fermentation by using immobilized cells on sugarcane bagasse was three times higher than the free cells system.

Sugarcane bagasse is potential to be used as a carrier for immobilized cells due to the porous of surface structure and the availability in nature. Approximately, 5.4×10^8 dry tons of sugarcane bagasse was produced throughout the world (Singh *et al.*, 2013). In Indonesia production of sugarcane bagasse was about 2.9×10^6 ton per year (Hermiati *et al.*, 2010). Rajawali II was one of the largest sugar factory in West-Java, Indonesia. Sugarcane milling capacity of this factory was about 1.35×10^6 tons resulting 1.35×10^5 tons of sugarcane bagasse per year (Hermiati *et al.*, 2010).

The surface characteristics of carriers such as pore size, moisture content, hydrophilic properties (water retention rate), and water absorption index are the main factors that influence the efficiency of attachment, characteristics of immobilized yeast cell, and also their productivity (Yu *et al.*, 2010; Razmovski and Vucurovic, 2012). Pretreatment of lignocellulosic biomass as a carrier for immobilized cells aims to increase the affinity of cell microorganism to substrate (Santos *et al.*, 2008), prevent the adsorption of non-productive cells, and allow immobilization of viable cells (Kilonzo *et al.*, 2011). Furthermore, pretreatment can decrease lignin content and cellulose crystallinity. Thus, enhancing porosity of substrate (Cardona *et al.*, 2010). Enzymatic pretreatment on sorghum bagasse could increase cell retention from 0.87 g/g to 1.35 g/g of dry cell weight (Yu *et al.*, 2010). Combination pretreatment, steam and biological, on sugarcane bagasse also could decrease lignin content to 27.44% (Fitria *et al.*, 2011). Pressurized steam pretreatment was reported can enhance porosity and retention of substrate (Yi Zheng *et al.*, 2009) which was indicated by increasing of water retention and cell adsorption (Vucurovic and Razmovski, 2012). To determine the effect of sugarcane bagasse physical properties to cell adhesion as well as bioethanol production, modification of carrier through pretreatment process need to be observed (Anita *et al.*, 2016).

This research focuses on utilization of pretreated sugarcane bagasse as a carrier of immobilized cells for bioethanol production. In this experiment, the effect of steam, pressurized steam, and combination pretreatment of sugarcane bagasse on cells adsorption and bioethanol production are investigated.

MATERIAL AND METHODS

The research was conducted at the Microbiology and Biomass Conversion Laboratory, Research Center for Biomaterials, LIPI. Sugarcane bagasse was obtained from the Rajawali Sugar Factory, Subang, West Java. *Saccharomyces cerevisiae* LIPI MC 0070 was obtained from Research Center for Biology, LIPI. Peptone was obtained from Hi-Media Chemical, while others were purchased from Merck (Germany).

Carrier Preparation and Characterization

Sugarcane bagasse from Rajawali II sugar factory, Pasir Bungur, Subang, West Java-Indonesia was used as a carrier for immobilization process. Sugarcane bagasse fiber with 1-2 cm in length was washed for several times until wash water colorless. Biomass was then air-dried and kept at room temperature (Singh *et al.*, 2013).

For treatment, distilled water was added onto 20 g of sugarcane bagasse in comparison 1:3 (w/v) and then, steaming at 100°C for 30 min, pressurized-steam at 121°C for 15 min, as well as the combination of both procedure. Physical characteristic of carrier i.e. lignin content, water retention (H), and water absorption index (WAI) before and after pretreatment were analyzed. Lignin content was determined according to NREL/TP-510-42618 (Sluiter *et al.*, 2008). Water retention was conducted according to Vucurovic and Razmovski (2012) and calculated as the weight of water retained per dry weight of sugarcane bagasse. WAI was determined according to Mussatto *et al.* (2009).

Microorganism and Mediums

Saccharomyces cerevisiae LIPI MC 0070 was maintained on yeast extract peptone dextrose (YEPD) agar slant (glucose 20 g/L, yeast extract 10 g/L, peptone 20 g/L, and agar 25 g/L) and stored at 4°C, and sub cultured every 2 weeks (Yadav *et al.*, 2011).

For starter culture preparation, one to two loop of 48-h yeast was cultivated into 30 mL starter medium (Nikolic *et al.*, 2009) that consist of (g/L): glucose, 10; yeast extract, 3; peptone, 3.5; KH₂PO₄, 1; MgSO₄·7H₂O, 1; (NH₄)₂SO₄, 1; pH 5, in 100 mL Erlenmeyer flask. It was cultured at room temperature (30 ± 2°C) in a rotary incubator (120 rpm) for 24 h. The content was transferred into 270 mL of medium production in 500 mL Erlenmeyer flask, and cultured under the same condition. Yeast cells were aseptically separated by centrifugation at 3000 rpm for 10 minutes. As much as 100 mg yeast cells were suspended in 50 mL 0.9% NaCl and used as an inoculum for immobilization process (Razmovski and Vucurovic, 2012).

Immobilization and Fermentation Condition

Pretreated sugarcane bagasse as much as 2.5 g was hydrated by adding 93.5 mL of distilled water and

incubated for 24 h at room temperature (Vucurovic and Razmovski, 2012). After that, the liquid was decanted and the solids were autoclaved at 121°C for 15 min.

For immobilization process, flask that consist carrier was inoculated by transferring 50 mL of yeast suspension and incubated for 24 h at room temperature ($30 \pm 2^\circ\text{C}$) in a rotary incubator at 120 rpm (Razmovski and Vucurovic, 2012). After immobilization, cell retention of whole cell biocatalyst/WCB (carrier containing cells) was measured according to Singh *et al.* (2013) and used for ethanol fermentation.

Fermentation medium as much as 300 mL, consist of (g/L) glucose, 100; yeast extract, 3; peptone, 3.5; KH_2PO_4 , 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2; $(\text{NH}_4)_2\text{SO}_4$, 1; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; pH 5 (Nikolic *et al.*, 2009) was inoculated with 1% (w/v) of whole cell biocatalyst (WCB) in 500 mL Erlenmeyer flask and covered by bubble traps. The flask was incubated for 24 h at room temperature ($30 \pm 2^\circ\text{C}$) in static condition. Samples were taken every 3 h for analysis.

Evaluation of Immobilization Parameters

Cell biomass was determined according to LAP/TP-510-42630 (Dowe and McMillan, 2001) on the absorbance at 600 nm with a UV-visible spectrophotometer and converted to dry cell weight on standard curve. The concentration of immobilized cells, concentration of free cells, and efficiency of immobilization were determined and calculated according to Vucurovic and Razmovski (2012).

Evaluation of Fermentation Parameters

Glucose concentration was determined using Somogyi-Nelson method (Wrolstad *et al.*, 2005). Samples of absorbance were measured using a UV-Vis spectrophotometer at 500 nm.

The concentration of ethanol was determined by using Shimadzu GC 14B gas chromatography with carbowax 20M column and a flame ionization detector. The ethanol productivity, ethanol yield, sugar conversion, and efficiency of sugar conversion to ethanol were calculated according to Singh *et al.*, (2013).

Scanning Electron Microscopic (SEM)

Micrograph of the whole cell biocatalysts (after washing two times with 10 mL distilled water and drying at room temperature for 24 hour) were obtained by using scanning electron microscopy (SEM) FE-SEM FEI INSPECT F50.

The dried samples were fixed on specimen holder with tape and then sputtered with gold in sputter-coater under high vacuum condition. Each sample was examined at 4000-fold magnification.

Statistical Analysis

All experiments were conducted in triplicates and analyzed for statistical significance by analysis of variance (ANOVA). The differences among the

samples were tested using analysis of significant difference (LSD) with a confidence level of 95% ($P = 0.05$).

RESULT AND DISCUSSION

Effect of Pretreatment to Physical Properties of Carrier

Physical properties of carrier such as water retention, water absorption index (WAI), and lignin content were the main factor that influence adsorption of cells onto carrier and efficiency of immobilization process, as well as ethanol productivity (Razmovski and Vucurovic, 2012; Genisheva *et al.*, 2011). Table 1 shows the physical properties of sugarcane bagasse before and after pretreatment process.

Table 1. Physical properties of sugarcane bagasse as a carrier before and after pretreatments

Carrier	Water content (%)	Water retention (g/g)	Water content after hydration process (%)	WAI (g/g)	Lignin content (%)
Control	7.77 ± 0.35 ^a	4.80 ± 0.44 ^a	84.27 ± 1.38 ^a	8.58 ± 0.22 ^a	24.40 ± 1.52 ^a
Steam	70.68 ± 3.13 ^{ab}	7.66 ± 1.05 ^{ab}	88.32 ± 1.52 ^{ab}	8.73 ± 1.20 ^a	21.71 ± 1.19 ^a
Pressurized-steam	71.63 ± 3.24 ^{ab}	7.39 ± 1.19 ^{ab}	87.90 ± 1.86 ^{ab}	9.28 ± 1.10 ^a	22.52 ± 2.61 ^a
Combination	70.90 ± 6.44 ^{ab}	6.13 ± 0.45 ^{ab}	85.93 ± 0.92 ^a	8.77 ± 0.12 ^a	22.38 ± 0.75 ^a

Description: ab = statistically significant difference ($P < 0.05$).

Addition of water in the pretreatment process caused a significant increases ($P < 0.05$) of sugarcane bagasse water content which was about ten times higher than the control. Sugarcane bagasse showed a high ability to absorb water. Water affected the growth of microorganisms. Low water content reduced a nutrient solubility thus extending the lag phase of microorganisms. Conversely, a high water content reduced the porosity of substrate and the oxygen transfer into the substrate (Prior *et al.*, 1992)

Steam pretreatment could increase the water retention of carrier from 4.80 ± 0.44 g/g to 7.66 ± 1.05 g/g. Water retention indicated the hydrophilic properties of carrier to absorb water (Razmovski and Vucurovic, 2012). In this study, pretreatments that used did not give a significant effect on water absorption index ($P > 0.05$) (WAI). Water absorption index (WAI) indicated the quantity of water that can be absorbed by the substrate. Sugarcane bagasse has a higher value of WAI than others such as fresh sugar beet pulp (6.59 g/g) (Vucurovic and Razmovski, 2012), coffee husk (7.76 g/g), and corn cobs (3.77 g/g) (Mussatto *et al.*, 2009).

Lignin content of lignocellulosic biomass was also important in selecting an appropriate substrate to

be used as a carrier. Lignin, on lignocellulosic biomass, covered the hemicellulose and cellulose component causing cell wall appears smoother (Yu *et al.*, 2010). Pretreatment aimed to reduce the lignin content that making it easier for the cells to immobilize on substrate. However, lignin was the component that gives stability and rigidity to the lignocellulosic biomass. Lignin content, that was too low, affected the strength of substrate as a carrier for cell immobilization (Plessas *et al.*, 2007; Escobar *et al.*, 2012). However, steam and pressurized steam as well as combination of both pretreatment decreased the lignin content of sugarcane bagasse. From the result, the pretreatment is not only can decrease the lignin content but also can maintain the strength of carrier.

The changes of the carrier structure and surface are presented in Figure 1. The surface of untreated carrier was smoother while pores were fewer and smaller than pretreated ones. Steam pretreatment caused the surface of carrier become rougher and very porous. While the surface structure of carrier by using pressurized and combination pretreatment become more damage.

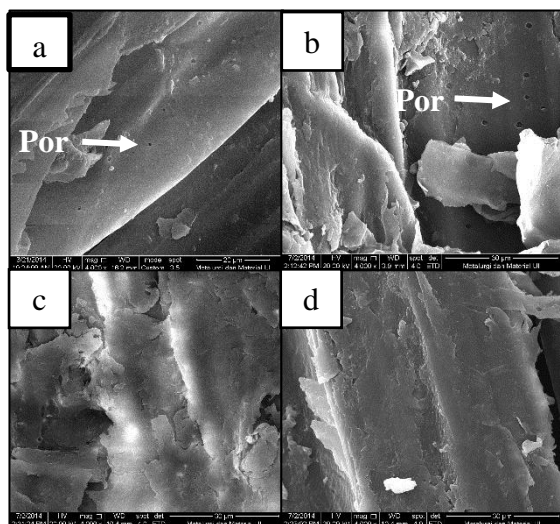


Figure 1. Scanning electron micrograph (4000x) of sugarcane bagasse as a carrier: (a). untreated, (b). steam treatment, (c). pressurized steam treatment, (d). combination treatment.

Steam pretreatment was reported can remove and redistribute lignin on fiber surfaces thereby increasing the pore volume of the pretreated material (Jørgensen *et al.*, 2007). Water in pretreatment process was absorbed into the carrier and then filled the pores of biomass. When steaming process occurs, the water evaporated rapidly and caused the changes of pores volume and fiber fragmentation (Chandel *et al.*, 2009). The increasing of pore volume could enhance the material porosity that was indicated by the high value of water retention (Yi Zheng *et al.*, 2009). In this study, steam pretreatment showed better result on the improving of sugarcane bagasse physical properties than other pretreatment.

Immobilization of Yeast Cells to Pretreated Carrier

After pretreatment, sugarcane bagasse was used as a carrier for immobilization process. Pretreatment gave a positive impact on cell retention. The retention of *S. cerevisiae* cells onto pretreated carrier is significantly different ($P < 0.05$) than control (Table 2). The highest cell retention of 10.05 ± 0.17 mg of dry cell weight/g of dry carrier weight was obtained by using steam treatment or almost two times higher than the control.

Table 2. Cell retention on sugarcane bagasse.

Carrier	Immobilized cell weight (mg)	Cell retention (mg/g)
Control	13.69 ± 2.55	5.41 ± 1.06^a
Steam	25.21 ± 0.44	10.05 ± 0.17^{ab}
Pressurized-steam	21.46 ± 0.38	8.56 ± 0.15^{ab}
Combination	17.67 ± 0.97	7.05 ± 0.39^{ab}

Description: ab = statistically significant difference ($P < 0.05$).

The steam pretreated carrier was very hydrophilic so that cells can easily absorb into the carrier. Yeast cells penetrated into pores of carrier and be embedded. Interaction between cells and carrier surfaces allowed the immobilization process to occur. The process of cell adsorption into the carrier occurred not only by natural entrapment mechanisms but also by physical or chemical adhesion between cells and carrier surface. Natural entrapment occurred due to the surfaces of carrier that were very rough and porous (Verstrepen and Klis, 2006). Physical adhesion was due to the hydrophobic interactions or Van der Waals forces, while chemical adhesion occurred as a result of covalent, hydrogen, and ionic bonds between cells and carrier (Verbelen *et al.*, 2006; Verstrepen and Klis, 2006; Plessas *et al.*, 2007).

The mechanism of cell adsorption into the carrier could be explained through the three stages of the process. First, the process of cell absorption into the porous carrier that was determined by the retention properties of material. Then the cells formed aggregates which adhered to each other. After the formation of aggregates, cell colonies attached to the carrier (Escobar *et al.*, 2012). Vucurovic and Razmovski (2012) also explained that the adhesion of cells was depend on electrostatic interaction between positively charge of carrier binding site and negatively charge of cells surface. Attachment process was mediated by specific proteins on the cell surface i.e. adhesin or flocculin. This protein could increase the hydrophobicity of the cell surface that triggers hydrophobic interactions between cells and abiotic surfaces (Lavoie *et al.*, 2011).

Table 3 shows that the concentration of free and immobilized cell on untreated whole cell biocatalyst (WCB) are better and significantly different ($P < 0.05$) than pretreated ones after 18-21 h

fermentation. Efficiency of immobilization control also showed a higher value compared to pretreated WCB. The highest value of $98.58 \pm 0.37\%$ was obtained by using untreated WCB after 21 h fermentation. However, this value is not significant statistically than pretreated WCB ($P > 0.05$).

Table 3. Immobilization parameter on various whole cell biocatalyst

Whole cell biocatalyst (WCB)	Concentration of free cell (mg/mL)	Concentration of immobilized cell (mg/mL)	Total cell concentration (mg/mL)	Efficiency of immobilization (%)
Control	0.04 ± 0.01^a	2.19 ± 0.58^a	2.22 ± 0.59^a	98.58 ± 0.37^a
Steam	0.09 ± 0.05^a	0.99 ± 0.54^{ab}	1.08 ± 0.60^{ab}	95.98 ± 3.79^a
Pressurized steam	0.04 ± 0.01^a	0.73 ± 0.10^{ab}	0.77 ± 0.12^{ab}	96.01 ± 3.30^a
Combination	0.10 ± 0.08^a	0.88 ± 0.30^{ab}	0.98 ± 0.38^{ab}	91.28 ± 5.23^a

Description: ab = statistically significant difference ($P < 0.05$).

The highest efficiency of immobilization showed that the immobilized cell concentration in the carrier was higher than the free cell concentration. Decreasing the efficiency of immobilization indicated that the cell released from the carrier. The absence of barrier between cells and solution allowed the release or relocation of cells from the carrier (Verbelen *et al.*, 2006).

Fermentation using Whole Cell Biocatalyst (WCB)

Carrier that contains cell, whole cell biocatalyst (WCB), was then used as an inoculum for fermentation. Fermentation parameters such as sugar conversion, ethanol concentration, ethanol productivity ethanol yield were analyzed in this study. The highest sugar conversion was $90.02 \pm 0.66\%$ and obtained by steam pretreated WCB during 24 h fermentation. However, that value was not significantly different than untreated WCB. Percentage of sugar conversion during the fermentation process can be seen in Figure 2.

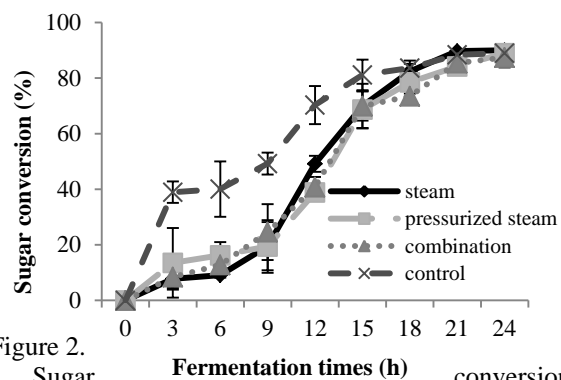


Figure 2. Sugar conversion on various whole cell biocatalyst during fermentation.

Ethanol concentration and ethanol productivity are presented in Figure 3. The highest values were

obtained by using pretreated WCB and significantly different compared to control. The highest ethanol concentration was 37.04 ± 0.73 g/L with ethanol productivity of 1.54 ± 0.03 g/L/h.

The highest ethanol yield and efficiency of sugar conversion were obtained by steam pretreated WCB (Table 4). Efficiency of sugar conversion was calculated as the percentage of ethanol yield produced during fermentation to the theoretical ethanol yield (0.51 g/g) (Singh *et al.*, 2013). The results showed that the ethanol yield with steam pretreated WCB was about 1.5 higher than the control.

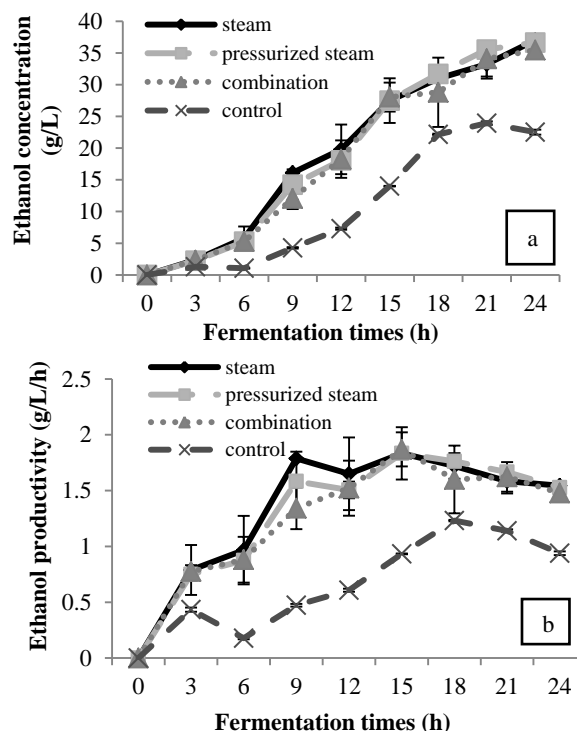


Figure 3. Ethanol concentration (a) and ethanol productivity (b) during fermentation

Table 4. Ethanol yield and efficiency of sugar conversion during fermentation

Whole cell biocatalyst (WCB)	Ethanol yield (g/g)	Efficiency of sugar conversion (%)
Control	0.26 ± 0.01^a	50.71 ± 0.59^a
Steam	0.40 ± 0.01^{ab}	77.52 ± 1.52^{ab}
Pressurized steam	0.39 ± 0.01^a	76.98 ± 0.12^a
Combination	0.38 ± 0.01^a	75.28 ± 0.12^a

Description: ab = statistically significant difference ($P < 0.05$)

Cell concentration in pretreated WCB was lower than untreated ones (Table 3). However, the ethanol production occurred vice versa. These result indicated that the mass transfer was one of the main factor that affected productivity during ethanol fermentation using immobilized cell systems. The surface structure of sugarcane bagasse was rougher and more porous after pretreatment process. So that the mass transfer occurred easier. Yu *et al.* (2010) also

reported that the concentration of cells immobilized on a carrier after hydrolysis using cellulase for one day was higher than the carrier after hydrolysis for 3 days. However the ethanol production was lower. It explained that cellulase for pretreatment process of carrier changed the structure of the carrier thus accelerating the mass transfer during the fermentation process.

CONCLUSIONS

Cell immobilized system by adsorption method of *S. cerevisiae* on sugarcane bagasse can be used as an alternative method for ethanol fermentation. Steam pretreatment improved the physical properties of carrier, i.e. water retention and water absorption index to 7.66 g/g and 8.73 g/g, respectively. Moreover, steam pretreatment increased the cell retention up to 10.05 mg/g and the ethanol yield 1.5 higher than the control.

Ethanol fermentation by using whole cell biocatalyst immobilized on lignocellulosic biomass was suitable to use on fermentation systems with a ready of fermentable sugars such as molasses and sugarcane juice, simultaneous saccharification and fermentation (SSF) system with starchy substrate, and separate enzymatic hydrolysis and fermentation (SHF) system with lignocellulosic biomass substrates.

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