Development of the High-Performance Bioethanol Fermenting Reactor

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Bioethanol, as an alternative for fossil fuels, is produced by fermenting sugar components of plant materials. It is important to bring the sugar-containing source material into efficient contact with condensed microorganisms to attain faster ethanol fermentation. In the study, high microbial concentration was achieved in a fermentation reactor using a flocculent strain of yeast. The yeast particles were continuously fluidized by upward current generated by the fermentation gas. During more than 30-day operation, high ethanol productivity was performed steadily. It was clearly demonstrated that the system is applicable to high-performance bioethanol production.

1. Introduction

In order to reduce dependence on fossil fuels, trials have been conducted to ferment the sugar components of plant materials to mass produce ethanol as a fuel for transportation. It is necessary to take certain measures to keep the fermenting microorganisms at high concentrations in a reaction tank to produce as much ethanol as possible in a short time. In general, microorganisms disperse in a liquid and easily flow out of a fermentation reactor. Baptista et al. produced ethanol from sugar made from sugarcane by attaching yeast (*Saccharomyces cerevisiae*) used in the production of sparkling wine to the surface of microorganism carriers with a particle diameter of approximately 1 mm.[1] Liu et al. succeeded in increasing the production rate by fixing yeast using sponge.[2] Although these methods enable the microorganisms to be kept at high concentrations in a reaction tank and contribute to higher ethanol productivity, problems often arise such as the microorganisms detaching from the carriers, making it difficult to maintain stable continuous operation.[3] Furthermore, use of increased amounts of carriers results in an increase in the ethanol production cost.

In order to increase the concentration of yeast in a fermentation tank without depending on the above-mentioned carriers, there is one technique receiving attention in which a strain of yeast that flocculates and forms particles is accumulated as flocculence inside a fermentation tank.[4, 5] Cells attach to each other and grow to become flocculent with good settleability. By improving the settleability of the flocculence, it becomes possible to maintain the concentration of microorganisms in a fermentation reactor and maintain excellent ethanol productivity.

Such flocculence with good settleability tends to remain in the fermentation tank. Once it settles at the bottom of the fermentation tank, it does not effectively contact the source material and the reaction speed slows down. Therefore, it is very important to develop a technique to accelerate the contact between the flocculence and the source material. Loosdrecht et al. formed flocculent microorganisms with fine sand as the core and generated upward current by aeration to maintain the fluidized state.[6] Vellinga et al. effectively obtained methane gas generated by an anaerobic reaction and used the gas lift effect to fluidize granular sludge in a fermentation reactor.[7]

When using flocculence, there is concern that it may become more difficult for the substrate material to penetrate to the center of flocculence as the flocculent particle diameters become larger. Li et al. carried out experiments and numerical analyses to examine the amount of substrate supplied from the surface to the center of microorganism flocculence, and their findings suggest that the substrate is more unlikely to reach the center of flocculence as the concentration of substrate in the bulk liquid declines and the particle diameter of flocculence increases.[8] When part of the flocculence becomes unable to react with the substrate, the number of microorganisms contributing to

fermentation decreases and the ethanol production rate declines.

In this study, we prepared several types of flocculence with different average particle diameters and measured the ethanol production rates for each type of flocculence, in order to understand the relationship between the rate of ethanol production and the particle diameter of flocculence. We then carried out continuous fermentation for more than 30 days and demonstrated stable ethanol production using flocculence and a reactor having both mixing and fluidizing functions, using the gas lift effect produced by the buoyancy of carbon dioxide generated by fermentation.

2. Materials and methods

2.1 Microorganisms

S. cerevisiae NBRC 1953, an ethanol-producing yeast, was used. This strain is used in the production of beer and easily settles at the bottom of a fermentation tank during brewing, so it is considered to have excellent flocculating properties. Using a culture fluid with the composition listed in **Table 1**, we cultivated the freeze dried yeast in a reciprocal shaker, then stored the yeast in a frozen state at −80°C using a 2% glycerol solution.

2.2 Formation of flocculence of different particle diameters

Approximately 1 m*l* of the frozen yeast was thawed at room temperature and immediately added to the round-bottom flask shown in **Fig. 1**. During pouring of the sterilized source material solution with the composition listed in **Table 2**, flocculence formed under the following conditions : 300 m*l* of liquid in the reaction zone, a rotation rate in the range of 20 to 200 rpm, a temperature of 30°C, and a hydraulic retention time of 2 hours. The fermentation reactor was rotated in batch sequencing mode ; namely, the following operations were repeated: the source material was continuously poured into the flask for three minutes while the flask was rotated around the center axis of its cylindrical part, the flask was kept stationary for 20 seconds to settle the flocculence, and supernatant fluid was removed for 40 seconds.

We accelerated flocculation by rotating the round-bottom flask in such a way as to roll the flocculence around the bottom of the flask, and altered the rotation rate to change the particle diameters of the flocculence. We operated the reactor at a constant rotation rate for more than 48 hours and visually confirmed that the flocculence had uniform particle diameter distribution. To ensure that the flocculence fermented the source material, we measured the concentration of ethanol in the effluent using gas chromatography (FID-GC).

Table 1 Medium composition for yeast growth

Constituent	Unit	Content
Glucose	g	10
Yeast extract	g	5
Polypeptone	g	3
Malt extract	g	3
Distilled water	*l*	1

Table 2 Feed medium composition for sequencing batch reactor

Constituent	Unit	Content
Sucrose (cane sugar)	g	100
Yeast extract	g	5
Ammonium chloride	g	2
Potassium dihydrogen phosphate	g	2
Magnesium sulfate	g	0.1
Calcium chloride dihydrate	g	0.1
Tap water	*l*	1

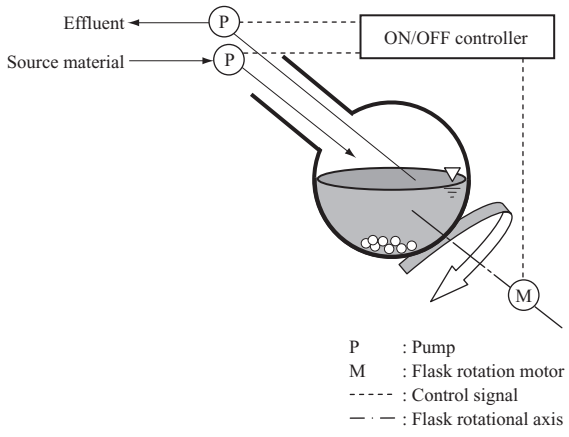


Fig. 1 Schematic of a sequencing batch reactor with a round shape flask

2.3 Ethanol production rate measurement

We extracted a suitable quantity of flocculence of consistent particle diameter from the round-bottom flask shown in **Fig. 1**. Using a closed vessel with a capacity of approximately 1 *l* as shown in **Fig. 2**, we added enough flocculence and sterilized medium to bring the amount of liquid to 40 m*l* with the composition listed in **Table 3**. We

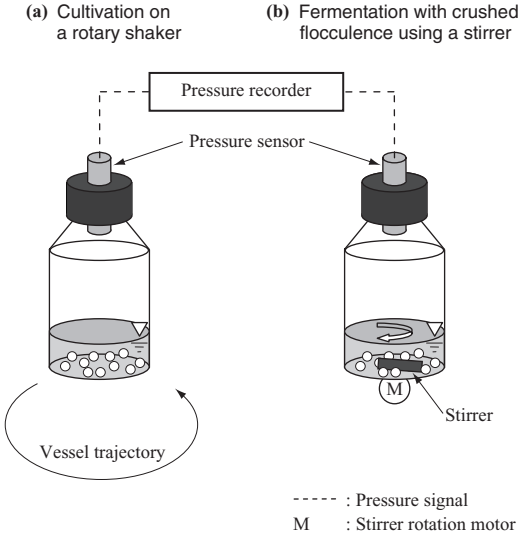


Fig. 2 Schematic of batch fermentation with pressure sensor

Table 3 Medium composition for batch reactions

Constituent	Unit	Content
Sucrose	g	Approx. 10 - 13
Yeast extract	g	5
Ammonium chloride	g	2
Potassium dihydrogen phosphate	g	2
Magnesium sulfate	g	0.1
Calcium chloride dihydrate	g	0.1
Tap water	*l*	1
Flocculence	g	5 - 8

measured the concentration of flocculence by collecting the flocculence using a glass filter of pore size 1 μm, drying the flocculence at 105°C for 2 hours, then measuring the dry mass.

Immediately after preparing the reaction solution, we substituted the gas phase in the vessel with nitrogen gas and carried out cultivation on a rotary shaker using a batch reactor at a temperature of 30°C (**Fig. 2-(a)**). We visually confirmed that the flocculence had almost the same particle diameter before and after the batch reaction. We also performed an experiment using a system in which a stirrer (500 rpm) was used for high-speed mixing to positively crush the flocculence into fine pieces, instead of cultivation on the rotary shaker (**Fig. 2-(b)**).

After the batch reaction, we took samples of the reaction solution and recorded images of the flocculence directly using a CCD camera. We chose 30 pieces of flocculence at random from each sample and measured the longest and shortest diameters. We calculated the average of the two diameters and evaluated the particle diameters of flocculence.

We determined the changes in concentrations of ethanol and sucrose in the closed vessel by converting the time course of the pressure in the closed vessel. Equation (1) represents the ethanol fermentation reaction by flocculence.

$$C_{12}H_{22}O_{11}+H_2O \rightarrow 4CH_3CH_2OH+4CO_2 \quad \cdots\cdots\cdots (1)$$

Since ethanol and carbon dioxide are generated in the course of fermentation, we assumed that the changes in pressure in the closed vessel were caused by the generation of carbon dioxide only, and determined the amount of ethanol generated by stoichiometry. We also measured the concentrations of ethanol and sucrose in the reaction solution before and after batch fermentation, and confirmed that the same moles of ethanol as carbon dioxide were generated. We measured the ethanol using the method described above and measured the sucrose using the phenol-sulfuric acid method.[9]

2.4 Continuous fermentation using a fluidized bed reactor

We performed continuous fermentation using a transparent plastic fluidized bed reactor with a reaction zone capacity of 8.6 *l* as shown in **Fig. 3**. In order to supply flocculence formed in the round-bottom flask to the fluidized bed

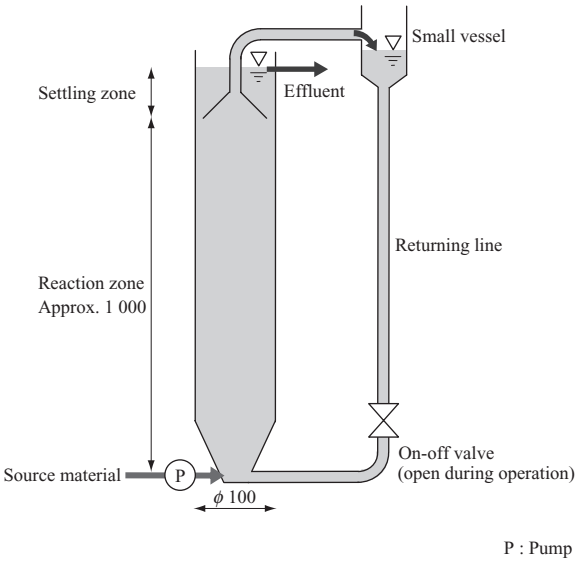


Fig. 3 Schematic of fluidized bed reactor (unit : mm)

reactor, the effluent outlet shown in **Fig. 1** and the source material inlet shown in **Fig. 3** were connected in series ; approximately 1 m*l* of the frozen yeast was added directly into the round-bottom flask before continuous operation began. We cultivated the yeast by supplying the source material into the round-bottom flask at a flow rate of approximately 5.4 *l*/h, at a temperature of 30°C, and at a constant rotation rate of the round-bottom flask of 100 rpm. **Table 4** shows the composition of the source material. When the concentration of flocculence in the fluidized bed reactor exceeded the concentration of flocculence in the round-bottom flask, we disconnected the round-bottom flask, switched to single operation of the fluidized bed reactor at the same flow rate as the source material, and commenced continuous operation for one month. Assuming that actual operation would use glucose obtained by saccharification of wood-derived cellulose or similar material, we used a non-sterilized source material composed mainly of glucose as shown in **Table 4**, instead of using sucrose. We did not adjust the pH of the source material and the content of the reaction zone. Each time the flocculence grew too much and stagnated in the settling zone above the reaction zone, we removed the flocculence from the settling zone.

In the reaction zone, the flocculence causes the reaction given in Equation (2) to produce ethanol and carbon

Table 4 Feed medium composition for fluidized bed reactor

Constituent	Unit	Content
Glucose	g	100
Yeast extract	g	5
Ammonium chloride	g	2
Potassium dihydrogen phosphate	g	2
Magnesium sulfate	g	0.1
Calcium chloride dihydrate	g	0.1
Tap water	*l*	1

dioxide from glucose.

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad \cdots\cdots\cdots\cdots\cdots\cdots \quad (2)$$

Bubbles of carbon dioxide generated in this reaction rise up and are caught by an umbrella positioned in the lower part of the settling zone. The carbon dioxide bubble that accompanies the surrounding solution and flocculence moves to the small vessel positioned above the settling zone. Gas is separated from the liquid entering the small vessel, and liquid containing flocculence is returned to the bottom of the reaction zone. A series of these operations maintain generation of upward current in the reaction zone, prevent flocculence from settling at the bottom of the reaction zone, and at the same time, accelerate contact between the source material and the flocculence.

In order to evaluate the operation results, we measured the ethanol concentration, glucose concentration, and pH of the effluent overflowing from the top of the settling zone. To gain an understanding of the reaction speed per biomass of flocculence, we measured the flocculence concentration, and used the methods described in **Sections 2.2 and 2.3** for measuring the concentrations of flocculence and ethanol. We used the phenol-sulfuric acid method as in the case of sucrose measurement to measure the glucose concentration.

3. Results and discussion

3.1 Influences of flocculence particle diameters on the ethanol production rate

In the operation shown in **Fig. 1** in which the round-bottom flask was rotated at a rate of 100 rpm or higher, the flocculence rolled and grew at the bottom of the flask and formed particles with a uniform particle diameter distribution. **Figure 4** shows the flocculence formed at a rotation rate of 100 rpm and 200 rpm, and flocculence crushed by a stirrer. **Figure 5** shows the results of measurement of the particle diameters of these types of flocculence. These figures clearly show that the diameters of the flocculence particles crushed by the stirrer, particles formed at 200 rpm, and particles formed at 100 rpm became larger : approximately 0.5 mm, 0.8 mm and 1.5 mm, respectively.

Figure 6 shows the results of measurement of time course in the amount of ethanol produced using these types of flocculence and the batch reactor shown in **Fig. 2**. These

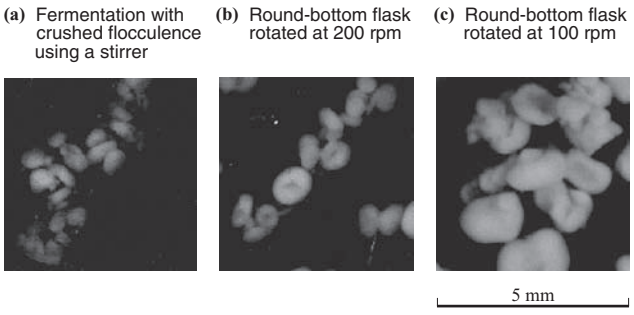
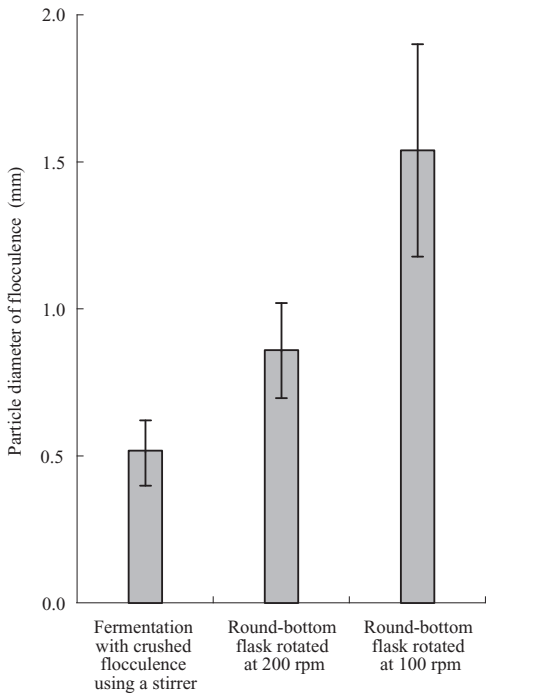
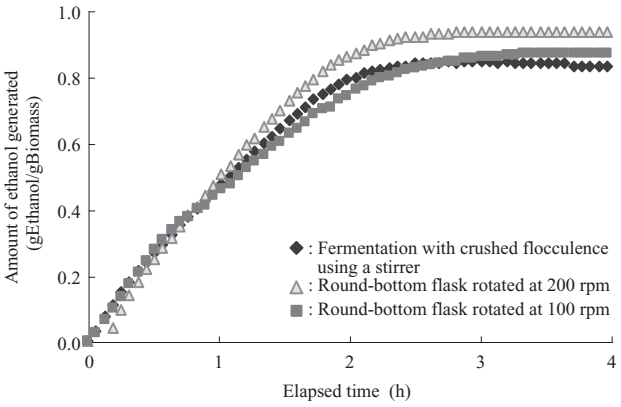


Fig. 4 Flocculent particles of *S. cerevisiae*



(Note) Error bars indicate standard deviations.

Fig. 5 Flocculent particle size



(Note) The concentration of flocculence in the batch reactions ranged from 5 to 8 g/*l*.

Fig. 6 Effects of particle size on ethanol productivity by yeast flocculent

results indicate that the amount of ethanol produced per biomass of flocculence remains almost the same even when the average particle diameter of flocculence changes in the range of approximately 0.5 to 1.5 mm.

Initially there was concern that the sucrose would not penetrate the flocculence entirely and the ethanol production rate would decrease as the particle diameters of enlarged flocculence became larger and the concentration of sucrose in the liquid declined. However, it was apparent that the flocculence particle diameters have little effect on the ethanol production rate even immediately prior to the end of the reaction when the concentration of sucrose in the liquid decreases and the amount of sucrose reaching the center of flocculence tends to decline due to progress of the

batch reaction.

During the continuous fermentation in the reaction zone described in **Section 3.2** below, the sugar concentration of the liquid in contact with the flocculence decreased to the same level as that of the effluent, in the same way as immediately prior to the end of the batch reaction. This indicates that stable ethanol fermentation can be achieved in a continuous reaction when flocculence has a particle diameter of 1.5 mm or smaller.

When we operated the batch reactor by changing the rotation rate of the round-bottom flask in the range of 20 to 80 rpm, most flocculence settled on the bottom of the flask and formed irregular-shaped masses. These masses grew while sliding around the spherical surface of the glass, and as a result, the boundaries between the particles became unclear, making it difficult to determine the particle diameters. While the sequencing batch reactor was operated under stable conditions for more than one week at a rotation rate of 100 rpm or higher, producing ethanol at the concentration of more than 40 g/*l* in the effluent, operation was unstable at a rotation rate of 80 rpm or lower, producing just 20 to 35 g/*l* of ethanol (data not provided in this report). It is likely that the reactor performance decreased at the low rotation rate of the round-bottom flask because the flocculence could not be stirred and mixed in the reactor at such a low rotation rate and the source material did not penetrate the flocculence entirely.

The above results indicate that the fluidized bed reactor (**Fig. 3**), which fluidizes flocculence for fermentation of ethanol, can accelerate contact between the source material and flocculence, and thus it can be considered suitable for producing high rates of ethanol.

3.2 Continuous ethanol fermentation

We began continuous operation while supplying flocculence from the round-bottom flask into the fluidized bed reactor. On the seventh day of operation, the concentration of flocculence in the reaction zone of the fluidized bed reactor reached approximately 10 g/*l* and exceeded the concentration of flocculence in the round-bottom flask. Next we disconnected the round-bottom flask and commenced single operation of the fluidized bed reactor. **Figure 7** shows the results of the continuous fermentation from single operation of the fluidized bed reactor. The pH value of the effluent was stable in the range of 3.3 to 3.6. During continuous operation, carbon dioxide bubbles generated in the reaction zone caused continuous upward current, thereby maintaining the fluidized state. The ethanol production rate per unit volume of the reaction zone was almost constant at slightly higher than 25 gEthanol/(*l*·h). This value is equal to the target value of the EU-funded

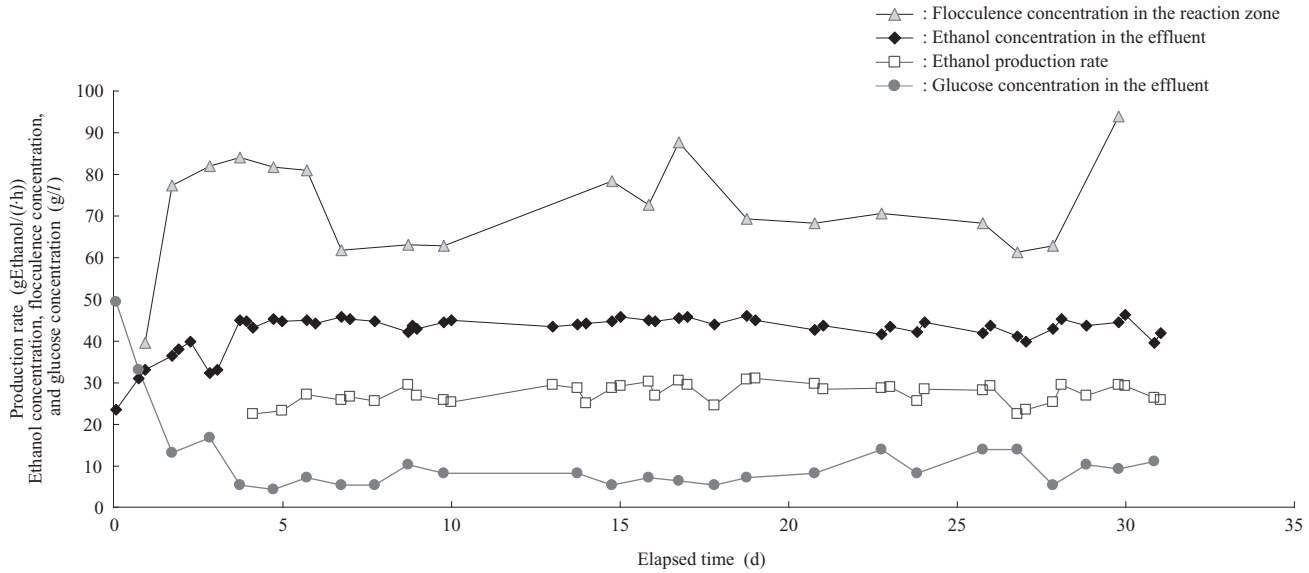


Fig. 7 Continuous fermentation in fluidized bed reactor

Table 5 Fermentation parameters for ethanol production by flocculent yeast

Production method	Reaction zone volume (*l*)	*S. cerevisiae* strain number	Type of sugar in material	Reaction temperature (°C)	Ethanol production rate (gEthanol/(*l*·h))	Ethanol yield (gEthanol/gSugar)	Literature number
Two vertical reactor towers connected in series with yeast returned from the settler and upstream tower aerated at the bottom	0.45	IR-2	Brown sugar	32.5	35	0.46	(11)
Vertical reactor with yeast returned from the settler and aerated at the bottom	0.23	HA-2	Molasses	30	25	0.42	(12)
Vertical reactor with yeast returned from the settler and aerated at the bottom	1.8	NCYC 1119	Glucose Sucrose	30	16	0.47	(4)
Stirred by an impeller	6.0	KF-2	Molasses	30	6.6	0.44	(13)
This study (fluidized bed method)	8.6	NBRC 1953	Glucose	30	25	0.44	—

FERMATEC Project.[10]

Table 5 shows examples of ethanol production at high rates achieved using flocculating yeast as in this study. Kuriyama et al. and Kida et al. achieved an ethanol production rate of 35 and 25 gEthanol/(*l*·h), respectively, in the reaction zone by combining a tower reactor of capacity of 1 *l* or less and a gas aerating system at the bottom, with a settling zone to return overflowed yeast to the reaction zone.[11), (12)

This method, however, requires pressurization of the aeration gas to or above the water pressure at the bottom when a large, tall reactor is used, thus increasing equipment and electric power costs. The use of an electric mixing impeller in the entire reactor zone is also possible.[13] However, a larger reactor requires a greater stirring force, and this may cause the flocculence to be crushed into particles by a stronger shearing force, as shown in **Figs. 4 and 5**. When flocculence is crushed into particles that are too small, it becomes difficult to fully recover the particles in the settling zone. This causes the amount of flocculence held in the reaction zone to decrease and the ethanol production rate per unit volume of the reaction zone to decline. The fluidized bed reactor used in this study requires no external power for maintaining the fluidized state and does not use a stirrer that could excessively crush the flocculence. For these reasons, this technique is considered effective for the mass-production of bioethanol.

In order to confirm the importance of maintaining the fluidized state, we closed the valve installed in the returning line during continuous stable operation and observed the changes that occurred. After approximately one minute, the upward current in the reaction zone ceased and the flocculence stopped flowing and settled at the bottom of the reaction zone. After two more minutes, a gas phase formed, probably due to fermentation gas, among the settling flocculence, and most of the flocculence rose and thickened in the umbrella below the settling zone by buoyancy. When the valve of the returning line was reopened, the flocculence concentrated in the umbrella gradually dispersed and stable operation resumed. This observation indicates that maintaining the fluidized state allows the flocculence to disperse in the entire reaction zone and accelerates contact between the flocculence and the material, thereby avoiding clogging in the narrow tube.

The approximate particle diameters of the flocculence in the reaction zone measured using a CCD camera from outside of the reaction zone were always around 1.5 mm and smaller. As shown in **Fig. 6**, the speed of reaction to sucrose does not decrease when the particles have diameters in this range. Glucose, which is the main constituent of the source material for continuous ethanol fermentation, has a smaller molecular weight and diffuses better than sucrose used in the batch reaction shown in **Fig. 2**. These results indicate that the reaction speed does not decrease due to enlargement of flocculence in the reaction zone of the fluidized bed reactor.

The ethanol production rate per biomass of flocculence in the reaction zone is calculated as approx. 0.4 gEthanol/(gBiomass·h) when the concentration of ethanol in the effluent is approximately 43 g/*l*, the hydraulic retention time of the reaction zone is approximately 1.6 hours, and the concentration of flocculence is 60 g/*l*, which was the minimum value during continuous operation. This value is equal to the amount of ethanol produced per biomass of flocculence after a lapse of one hour in the batch reaction shown in **Fig. 6** (0.4 gEthanol/gBiomass). The concentration of flocculence in the fluidized bed reactor was 60 g/*l* or higher, which is more than eight times as high as the concentration of flocculence in the batch reaction shown in **Fig. 6** (5 to 8 g/*l*), however, the reaction speed per biomass of flocculence at the same level was achieved with the fluidized bed reactor. It appears that the fluidized bed reactor allows efficient contact between the flocculence and source material as in the case of cultivation on a rotary shaker and mixing cultivation using a stirrer, without causing any deterioration in performance due to poor mixing in the reaction zone.

When the concentration of flocculence in the reaction zone changed in the range of 60 to 90 g/*l*, the ethanol production rate per volume remained constant at 25 gEthanol/(*l*·h). A flocculence concentration of 60 g/*l* was sufficient to achieve this production rate. Thus, the ethanol production rate achieved using this reactor can be further increased by consistently maintaining a flocculence concentration of 90 g/*l*.

4. Conclusion

In order to produce bioethanol from the sugar components of plant materials at high rates, we studied the reaction characteristics of flocculent yeast (*S. cerevisiae*) and conducted one-month continuous fermentation using a reactor in which the fermentation gas of flocculence was utilized for mixing and fluidizing. Our experimental results revealed the following :

(1) When the average particle diameter of flocculence was in a range of 0.5 to 1.5 mm, the ethanol production rate per biomass of flocculence was constant independent of the flocculent size.

(2) In the continuous fermentation using the fluidized bed reactor, stable operation was achieved with ethanol produced in the reaction zone at a rate of approximately 25 g/(*l*·h). It was important to maintain the fluidized state of flocculence by upward current in the reaction zone to achieve stable operation. The flocculent particle size was approximately 1.5 mm or smaller, thus indicating that the reaction speed does not decrease even when the flocculent particles are overgrown.

(3) The flocculent concentration required to achieve an ethanol production rate of 25 gEthanol/(*l*·h) in the reaction zone was approximately 60 g/*l*. At a higher concentration of flocculence, it is possible that the ethanol production rate can be further increased in the reaction zone.

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