Paper

Porous Alumina Ceramics for Immobilization of Soy Sauce Yeast Cells

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醤油用酵母の固定化に適した多孔性アルミナセラミックス

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Alumina ceramics with controlled pores were prepared to immobilize soy sauce yeast cells. The pore size appropriate for the immobilization was selected on the basis of ethanol productivity in batchwise fermentation. The ceramics with an average pore diameter of about 4-5 times of yeast cells provided the highest ethanol formation. Through the ceramic granules with yeast cells, the repeated ethanol fermentation of solid-free solutions from soy sauce mash was performed by means of a batchwise column reactor. The porous alumina ceramics was confirmed to be available as a cell support for the fermentation of soy sauce.

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1. Introduction

In food industries, various kinds of food additives are added in the manufacturing process. Over the last decade, natural additives have been preferred to artificial ones by the food customers, and ethanol is provided for soy sauce instead of sodium benzoate¹⁾ which has been usually added. The addition of ethanol into soy sauce, however, resulted in the rise of the manufacturing cost. Provided more ethanol is produced in soy sauce fermentation process, it would become possible to diminish the quantity of ethanol added. From such a point of view, the studies^{2),3)} have been undertaken for promoting and controlling ethanol formation by means of adding yeast cell slurry into soy sauce mash during the fermentation process. The large-scaled cultivations of yeast cells, however, were required each time soy sauce mash was fermented. We hence believe that immobilized yeast cells which can be used for the repeated fermentation is more effective than the yeast cell slurry.

An investigation to reduce the fermentation time of soy sauce⁴⁾ has been undertaken with immobilized yeast cells and lactic acid bacteria in biocatalytic reaction. Various kinds of mater-

ials, i.e. gelatinous organic compounds⁵⁾ and porous ceramics,⁶⁾ have been reported to be available for the immobilization. To choose the material appropriated for the immobilization of soy sauce yeast cells, the food hygiene safety is essentially desired in order not to contaminate the fermented products. The structure of netlike open pores exerts a favorable influence on the contact of cells with reactant and the resulting promotion of cell growth. In addition, the long life and the proper price of the material become important factors in the practical application. Porous ceramics satisfies such requirements. Glasses⁷⁾ with controlled pores are known to have an affinity with protein, however, the fabrication process is complicated and the resulting market price is high, while porous alumina ceramics⁸⁾ is used as a bioceramics and have a proper price. *Saccharomyces cerevisiae* and *S. amurcae*, the yeast, are known to have the adaptable pore diameter⁹⁾ of ceramics for the population growth. The suitability of soy sauce yeast cells with ceramics, however, is scarcely reported.¹⁰⁾

In this study, the vitrified-bonded alumina ceramics consisted of complicated and controlled open pores was prepared by controlling the grain

size and the composition of raw materials in the fabrication process. The pore suitability for cell growth was evaluated by batchwise ethanol fermentation. The highest ethanol productivity was realized in the ceramics with average pore diameter of about 4–5 times of yeast cells. The repeated ethanol fermentation of solid-free solutions from soy sauce mash is subjected by a batchwise column reactor. Soy sauce yeast cells immobilized inside ceramics are realized to be available for repeated 5 times ethanol fermentation. The vitrified-bonded alumina ceramics was proved to be one of the most effective material as a support of yeast cells in soy sauce fermentation.

2. Experimental procedures

2.1 Preparation of porous ceramics

To prepare the pore-controlled ceramics easily at a low cost, fused alumina abrasive grains which were manufactured by crushing electro-fused alumina and separating to various grain size (Nippon Kenmazai Kogyo Co., Ltd., Osaka) were used as the raw material. The distribution of abrasive grain size was measured by the method defined by JIS R 6002 and shown in Fig. 1. To investigate the effect of grain size, four kinds of vitrified-bonded ceramics were fabricated by the following procedures:

The mixture of feldspar (55%), pottery stone (30%), and Kibushi clay (15%) was used as ceramic binder. These natural substances have

Fig. 1. The grain size distribution of four kinds of alumina abrasives (The number is as same as in Table 2).

Table 1. Chemical composition of ceramic binder.

SiO ₂	Al ₂ O ₃	Fe ₂ O ₃	CaO	MgO	K ₂ O	Na ₂ O	Ig. Loss
66.81	20.27	0.25	0.33	0.09	6.79	2.28	3.44

(Weight %)

been used as the raw materials for traditional ceramic products and these food hygiene safety has been confirmed when vitrified. The chemical composition of the mixture was presented in Table 1. As temporary binder, the dextrin from potatoes (Wako Junyaku Kogyo Co., Ltd., Osaka) was adopted.

Abrasive grains (85%), ceramic binder (13%) and dextrin (2%) were mixed completely with a small quantity of water (1.5%), and pressed into green pellets. The pellets were heated in an electric furnace at the rate of 2.5°C/min to 1300°C, maintained at this temperature for 1 h, then allowed to cool down to room temperature. These vitrified pellets were crushed and sifted out the ceramic granules of 3–4 mm ϕ .

The ceramics prepared in such a process were composed of grains, bonds and open pores. The volumetric ratios of these elements were measured by the water absorption method defined by JIS R 6122. The ceramic surface was observed by a JEOL JSM-840 scanning electron microscope (SEM).

2.2 Yeast cells and cultivation

The yeast cell SR-2²¹ which was isolated from soy sauce mash and identified as *Zygosaccharomyces rouxii* was adopted. The cells from agar slant cultures containing yeast extract, peptone, glucose and sodium chloride were transferred by sterile loops to 10 ml of liquid culture medium in test tubes. The medium contains 56 ml of soy sauce, 432 ml of saturated NaCl solution, 465 ml of water, 48 g of glucose and 1 g of K₂HPO₄, per liter. After growing on a reciprocal shaker at 28°C in water bath for 2 days, the cells were transferred to 1 l Sakaguchi flasks with the same medium as in the test tube and cultured in the same way for 2 days. These cells were harvested by centrifugation, washed with distilled water and suspended in chilled water to yield the yeast cell slurry of concentration 8×10^9 cells/ml.

2.3 Immobilization of yeast cells

The ceramic granules sterilized by heating were soaked in the slurry of yeast cells. The cells were immobilized physically inside the pores of ceramics under reduced pressure. The air included in ceramic granules was removed, and the pores were filled with the slurry. The initial amount of immobilized cells was calculated at the volumetric ratio of the pores inside ceramics and the slurry absorbed.

2.4 Preparation for fermented solution

Complex culture medium was prepared to contain 50 g of glucose, 1.0 g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 0.1 g of CaCl₂·H₂O, 4.0 g of casamino acid, 1.0 g of yeast extract and 150 g of

NaCl, per liter.

Feed solution in the reactor required to be a solid-free state and obtained by pressing soy sauce prefermenting mash (*Moromi*): the enzymatical hydrolyzate of *koji* in salt water. The *koji* was prepared by the solid-state fermentation of steamed soybean and parched wheat with the seed spores of *Aspergillus sojae* and/or *A. oryzae*. The solid free solution was sterilized by filtration with 0.45 μm membrane (Advantec Toyo, Membrane filter), fermented with lactic acid bacteria to bring close in conventional mash before ethanol fermentation, and filtrated out lactic acid bacteria through 0.45 μm membrane.

The pH of these solution was adjusted to 5.0 by 1 molar NaOH solution according to the method of Ando and Yamashita;²¹ they presented that the initial pH of 5.0-5.2 was the most desirable for the growth of the yeast cell SR-2.

2.5 Fermentation

To examine the pore dependence of ceramics on ethanol productivity, the fermentation test was performed under static condition with 200 ml complex culture medium in 500 ml Erlenmeyer flask containing 10 g cell immobilized granules at $30 \pm 1^\circ\text{C}$ for 12 days. The batchwise column reactor (volume: 500 ml), as illustrated in Fig. 2, was used to evaluate the ethanol fermenta-

Fig. 2. The experimental apparatus of batchwise column reactor (Volume: 500 ml) 1: Ceramic granules with immobilized yeast cells, 2: Reservoir, 3: Magnetic stirrer, 4: Water bath, 5: Temperature controller, 6: Peristaltic pump, 7: Sample cock.

Fig. 3. The linear velocity related to glucose consumption through packed ceramics in batchwise column reactor (Maximum velocity as 1.0).

tion with yeast cells immobilized in the ceramics selected in this study. Feed solution in the reactor (reactant) was recirculated and fermented through the column with ceramic granules at $30 \pm 0.5^\circ\text{C}$. The glucose consumption in reactant was dependent on the linear velocity in column as shown in Fig. 3; the linear velocity reached plateau over 0.7 cm/min. Reactant was therefore recirculated at the velocity of 0.7 cm/min. The fermentation test was repeated 5 times to examine the activity of immobilized cells. Fermented solution was taken out of the reactor after the end of log phase in the fermentation. Feed solution was added into the reactor and ethanol fermentation was started again.

2.6 Analysis

The amount of cells in yeast slurry was measured by Thoma haemocytometer. Fermented products were withdrawn at appropriate time intervals and sterilized by filtration with 0.45 μm membrane. Ethanol was measured by a gas chromatography (Type GC-6 A, Shimadzu Co., Ltd., Kyoto) with a flame ionization detector (FID) and a digital integrator (Type ITG 4 A). Glucose was determined by enzymatic method with F-Kit (Boehringer Mannheim-Yamanouchi Co., Ltd., Tokyo).

3. Results and discussion

3.1 The structure of vitrified-bonded ceramics

The structure of vitrified-bonded alumina ceramics is composed of three elements of grains, bonds and open pores. The region of three elements in this type of ceramics resembled with that in conventional vitrified grinding wheel as shown in Fig. 4.

Grains were coated with vitreous ceramic binder, and the ceramics was made of the three-dimensional framework of the grains and the consequent intricate open pores. Provided ceramic grains had the shape of a sphere, the

Fig. 4. The volumetric pore (P)-bond (B)-grain (G) composition of prepared ceramics (region 1) and conventional vitrified grinding wheel (region 2).

thickness of the vitreous substance was calculated to ca. $2.0\ \mu\text{m}$ in the case of the grains with average diameter of $66\ \mu\text{m}$ (No. 2 in Table 2) from the difference of densities between ceramic grains (3.9) and vitrified-bonded ceramics (3.8). The surface of the grains in the vitrified-bonded alumina ceramics was hence confirmed to be coated with rather thin vitreous substance.

As observed in SEM photograph in Fig. 5, the pores inside vitrified-bonded alumina ceramics had complex geometries. The properties of the pores was listed in Table 2. With increasing the grain size of the ceramics, average pore diameter increased proportionally. The pores inside such a vitrified-bonded alumina ceramics fabricated in this study can be easily controlled by the grain size.

3.2 The ceramics appropriated for the immobilization of yeast cells

The change of ethanol concentration with fermentation time was shown in Fig. 6. Ethanol formation was found to be related with the type of vitrified-bonded alumina ceramics, and the highest productivity was obtained in the fermentation using the ceramics with average pore diameter of $21.4\ \mu\text{m}$.

The yeast cells¹¹⁾ is known to ferment glucose in soy sauce to ethanol associated with cell population growth. Provided the cell population growth

inside the pores of prepared alumina ceramics was identical, ethanol formation was to be dependent on the number of the cells immobilized inside the ceramic pores.

The initial number of the cells immobilized in prepared ceramics was in a range of $0.9\text{--}1.2 \times 10^8$ cells/g-ceramics, the ethanol productivity hence was to be about the same. The fact, however, was not the case. The cell population growth inside the pores of the vitrified-bonded alumina ceramics was found to be influenced by the type of the ceramics.

Messing and Oppermann⁹⁾ presented that cell population growth in porous ceramics was dependent on both the dimension of cells and the pore diameter of ceramics, and that the optimum pore diameter for the growth of yeast cells lays in the range of one to four times of the cell dimension. They used polyisocyanate in acetone solution as a coupling reagent in the procedure of cell immobilization with ceramics of various composition.

In this study, soy sauce yeast cells were immobilized physically inside the pores of ceramics with the same type of composition without a coupling procedure. The yeast cells have the size of $4\text{--}5\ \mu\text{m}$ under a microscope, then the average pore diameter with the fastest cell growth can lead to the size of 4–5 times of the yeast cells. This result is in fair agreement with that of Messing and Oppermann.

3.3 Fermentation

The repeated batch fermentation of feed solution was undertaken in the column reactor, and the variation in the concentration of ethanol and glucose with time was shown in Fig. 7. The first

Table 2. Pore properties of ceramic granules.

Grain size	Average pore diameter μm	Pore volume $\text{cm}^3\ \text{g}^{-1}$	Specific pore surface area $\text{cm}^2\ \text{g}^{-1}$
No 1	16.8	0.26	820
2	21.4	0.27	500
3	67.9	0.29	170
4	156.0	0.22	56

Fig. 5. The typical scanning electron micrograph of ceramic surface prepared.

Fig. 6. The change of ethanol concentration with time in the fermentation of complex culture medium (The number is as same as in Table 2).

Fig. 7. The variation in concentration of ethanol and glucose with time in the repeated fermentation of feed solution.

ethanol formation reached at stationary state over a period of 180 h. After the second fermentation, the lag phase of ethanol formation became shorter and the consequent fermentation time of feed solution was reduced. The yield factor which equals to the total mass of ethanol divided by the theoretical one from mass of glucose consumed was calculated to about 0.70 from the chemical stoichiometry indicated in Eq. (1).

The yield factor fairly agreed with the value 0.72 obtained in conventional batch fermentation for 2 months using free yeast cells.³¹

During the fermentation process, the free cells leaked from the supports was observed and measured to be ca. 1×10^6 cells/ml in the stationary state. Initial cells immobilized inside ceramic pores were calculated to be 4×10^8 cells/ml. This reactor system hence regarded as a prolific cell immobilized one.

The repeated ethanol fermentation with yeast cells immobilized in vitrified-bonded alumina ceramics was consequently found to be effective about the fermentation time and the yield factor as compared with the conventional fermentation of soy sauce. In addition, having a high thermal and chemical stability and a strong mechanical strength,

the vitrified-bonded alumina ceramics prepared in this study are more desirable for the immobilization of cells than organic ones⁴¹ which have been used as a material to entrap cells and enzymes.

4. Summary

To prepare the ceramics suitable for the immobilization of soy sauce yeast cells, vitrified-bonded alumina ceramics were fabricated, and the activity of cells inside ceramics was examined by the repeated batchwise fermentation.

The vitrified-bonded alumina ceramics which had average pore diameter of about 4–5 times of a cell dimension showed the best suitability for the immobilization. Cells in ceramic pores was realized to ferment feed solution more than 5 times. The fermentation with the ceramics as a support of soy sauce yeast cells was proved to be effective judging from the cell activity, the fermentation time and the ethanol yield factor.

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