Continuous Hydrolysis of Soluble Starch by Free β-Amylase and Pullulanase Using an Ultrafiltration Membrane Reactor

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Enzyme hydrolysis of soluble starch by free β-amylase and pullulanase for the production of maltose was done by the simultaneous use of a stirred tank reactor and an ultrafiltration membrane. Higher conversions of starch to maltose were obtained in the permeates than that in a batch reaction. Using the basic mass balance and rate equations, concentrations of maltose, maltotriose, and substrate in the retentates and permeates could be simulated effectively. More than 99.9% of enzyme was found to be rejected by the membrane. The obtained volumetric productivities were several times higher than those reported in other systems. This system was found to have high maltose productivity with a short mean residence time, being easily controlled by transmembrane pressure.

In recent years, many experiments have been done with membrane bioreactors, where bioreactor and membrane technologies are combined.1–3) Membrane bioreactors can be classified into one of three types, mainly from their configuration: a free enzyme membrane reactor (FEMR, a free enzyme is used in a stirred tank reactor with an ultrafiltration (UF) membrane),4) a diffusion membrane reactor (an enzyme is retained in one side of the membrane and transfer of substrate and product is dependent on diffusion),5) and a forced flow membrane reactor (an enzyme is immobilized within a membrane and a substrate is forced through the membrane by pressure).6–8) Among these, the FEMR may be most suitable for enzymatic hydrolysis of high molecular weight substances; continuous production is possible without immobilizing biocatalysts, high productivity can be obtained with high concentrations of biocatalysts and substrate, and low molecular weight products or inhibitors can be continuously removed through a membrane.

Maltose, which is produced by enzymatic hydrolysis of starch, has great commercial value in the food and pharmaceutical industries. The main properties of maltose are low sweetness, high solubility depending on purity, heat stability, and lack of color formation.9) Industrial production of maltose is mainly done in batch reactions. However, a batch system is economically disadvantageous due to the requirement for a large reactor tank, in which separation of enzyme is necessary and the enzyme can be used only once. To overcome these disadvantages, β-amylase and debranching enzyme have been immobilized to some carriers, and an immobilized enzyme column reactor has been constructed and investigated.10–15) Immobilization carriers for β-amylase were ceramic monoliths,10,11) TEAE-cellulose,12) a copolymer of acrylamide–acrylic acid,13–15) cellulose beads,16) alkylamine porous silica,17) cation exchange resin,18) and phenylboronate-agarose.19) However, the efficiency might not be high enough for industrial maltose production, mainly because of low diffusivity in immobilized carriers.

In this study, to get a highly efficient enzyme reactor for maltose production, the FEMR was
used for continuous enzymatic hydrolysis of soluble starch in high concentration using $\beta$-amylase and pullulanase. Mathematical analysis of the system was also done, and productivities of different reactor systems reported were compared.

**Materials and Methods**

**Enzymes and membrane.** Soybean $\beta$-amylase (4000 U (g powder)$^{-1}$, molecular weight 61700, Amano Biozyme M-5), kindly supplied by Amano Seiyaku Co., Ltd., was used. Pullulanase from Bacillus (200 U (cm$^3$ solution)$^{-1}$, Novo Promozyme), kindly supplied by Novo Industry Japan, was used. Low molecular weight substances (e.g., glucose) were removed from the pullulanase solution by a continuous diafiltration technique using an UF membrane (IRIS3038, Rhone Poulenc Chemic Fine, molecular weight cut-off 20,000). The protein concentrations of $\beta$-amylase and pullulanase were 10.1 mg (g powder)$^{-1}$ and 2.2 mg (g crude enzyme solution)$^{-1}$ by the dye-binding method (the method is given below).$^{19)}$

The UF membrane used in this study was a hollow fiber membrane module; MOLSEP FUS-3081 (made by Daicel Chemical Ind. Co., Ltd, effective membrane area 1.4 m$^2$, molecular weight cut-off 30,000, inner and outer diameters of fiber 0.8 and 1.3 mm, module length 408 mm, module volume 2600 cm$^3$, pure water permeate flux of $60 \times 10^{-6}$ m$^3$m$^{-2}$sec$^{-1}$ at 100 kPa transmembrane pressure, and made of polyethersulfone).

The FEMR. A flow sheet of the FEMR (MBR-S, made by Japan Organo Co., Ltd., Japan), is shown in Fig. 1. Soluble starch was supplied to a reactor tank from a feed tank. The retentate was recirculated to the membrane module by a pump (Sanitary Rotary Pump RM4VT, Nakamura Kinzoku Kogyo Co. Ltd, Japan). The enzyme reaction occurred in the reactor tank and the recirculation tube. The product was obtained as a permeate by cross flow filtration. The permeate flux was measured by an electric weight balance. The water level of the tank was controlled by a level sensor and controller (M-1093, Able Co., Ltd., Japan). The same volume of feed solution as that of the permeate was fed from the feed tank by pump (N-Feeder CSD-12, Nihon Feeder Ind. Co., Japan).

The flow rate was measured by an electromagnetic flowmeter (KIC 20A-X, Yamatake Honeywell Co., Ltd., Japan). The transmembrane pressure is determined as $P_t = (P_i + P_o)/2 - P_p$, where $P_t$ is the transmembrane pressure, $P_i$ and $P_o$ are inlet and outlet pressures of the module, and $P_p$ is the permeate pressure (almost equal to atmospheric pressure). Each pressure was measured with a pressure gauge. The temperature in the reactor tank and in the circulation tube were measured, and they were almost identical.

The total working volume of the FEMR was 2500 cm$^3$ (2000, 280, and 220 cm$^3$ of the reactor tank, the membrane module and the other circulation lines such as tubes, pump and flow-meter, respectively).

**Chemical analysis.** The concentrations of maltose and maltotriose were measured by high-performance liquid chromatography (HPLC) with a finely packed SIL-NH$_2$ column (Japan Spectroscopic Co., Ltd.). Both concentrations were converted to unit of carbon concentration (gC m$^{-3}$).

The concentration of the total saccharides was measured by the phenol–sulfuric acid method$^{20]}$: sample solution, 5% w/v phenol, and concentrated sulfuric acid were mixed, and absorbances were measured at 490 nm. The concentration of total saccharides was converted to unit of carbon concentration. The carbon concentration of the total saccharides must be kept constant during hydrolysis while that of each saccharide changes, which was also experimentally confirmed. For simplicity, the substrate concentration during hydrolysis was assumed to be equal

![Fig. 1. Schematic Diagram of the Experimental Apparatus.](https://example.com/image1)

![Fig. 2. Relationship between Retention Time of HPLC and Molecular Weights (MWs) of PEG and Dextran.](https://example.com/image2)
to the total saccharides concentration minus product (maltose plus maltotriose) concentration. The substrate corresponds to the sum of soluble starch and dextrin.

The protein concentration was measured by the dye-binding method: sample solution, and Coomassie brilliant blue G250 (Nacalai Tesque Co., Japan) were mixed, and absorbances were measured at 595 nm, with the calibration curve of bovine serum albumin (Sigma Co., Ltd., U.S.A.).

Distributions of the molecular weight (MW) of substrates in feed, retentate, and permeate were also measured by high performance gel filtration chromatography (GPC, column Asahipak GS-510, Detector RI, calibrated by 1% polyethylene glycol (PEG, Wako Chemicals Co., Ltd., Japan) and dextran having different MWs (Pharmacia Fine Chem., Sweden)). Figure 2 shows the relationship between retention time and mean MWs of dextran and PEG. Good correlations were observed between each dextran or PEG and its retention time. The dextran calibration curve was used over MWs of $10^4$–$5 \times 10^5$.

Batch experiments. Soluble starch (DE 0.90, Wako Chemicals Co., Ltd., Japan) was dissolved in hot water at 70–80°C, the solution was filtered through Filter Paper 5C (Advantec Toyo Co. Ltd., Japan), and the filtrate was used. According to the method of Shiraishi et al., batch experiments were done under conditions of 150 g/l soluble starch concentration, 4 g/l-/β-amylase (activity of 16 U cm$^{-3}$), 64 (cm$^3$-crude enzyme)$^{-1}$ pullulanase (activity of 13 U cm$^{-3}$), 40°C, and pH 4.8 adjusted by acetate buffer. The activity ratio of β-amylase to pullulanase was adjusted to be 0.8, because a high conversion of soluble starch to reducing sugar was reported at activity ratios higher than 0.8.

After the reaction, the enzyme was inactivated by heating in boiling water for 15 min. Concentrations of maltose and maltotriose were measured by HPLC.

FEMR experiments. First the soluble solution in the feed tank was supplied to the reactor tank, then both β-amylase and pullulanase were added, and a batch operation was done for 20 min at 40°C to obtain a conversion higher than 60%.

After a batch operation, a continuous operation was started. A permeate was obtained by crossflow filtration of retentate through an UF membrane at transmembrane pressures of 100, 40, and 20-10 kPa in runs 1, 2, and 3, respectively. The recirculation flow rate was kept at about 40 cm$^3$ sec$^{-1}$ (flow velocity of 5.8 cm sec$^{-1}$) in all runs. The changes in the concentration of substrate and product with time in the retentate and the permeate were measured. The total reaction times were 2, 10, and 30 hr in runs 1, 2, and 3, respectively.

To estimate enzyme leakage through the membrane, the permeate was concentrated about 10 times by diafiltration, and the protein concentration was measured by the dye-binding method.

Results and Discussion

Batch experiments

Figure 3 shows the courses of maltose and maltotriose formation from starch in the batch experiment. Both conversions almost reached their maxima after 20 min, those to maltose and maltotriose being 0.70 and 0.02, respectively, on the basis of carbon concentration. In the case of using β-amylase without pullulanase, the maximum conversions to maltose...
and maltotriose were 0.41 and 0.01, respectively. Thus by the simultaneous use of \(\beta\)-amylase and pullulanase, conversion to maltose from starch increased. From Fig. 3, the time scale for reaction, which was defined to be the approximate time required for sufficient conversion, was 10–20 min. This short time scale for reaction is due to the high enzyme concentration.

**FEMR experiments**

The changes in permeate flux for runs 1, 2, and 3 are shown in Fig. 4. In each run, the permeate flux decreased initially. The stable states in permeate flux were observed after about 1, 4, and 6 hr of operation in runs 1, 2, and 3, respectively. In run 3, the permeate flux decreased to about 40\% with a change from 20 to 10 kPa transmembrane pressure. The stable permeate flux obtained was approximately proportional to the transmembrane pressure. From the stable permeate flux and total working volume, the mean residence times were calculated to be 30, 81, 161, and 472 min in runs 1, 2, 3 (20 kPa), and 3 (10 kPa), respectively. These mean residence times were longer than the time scale for the reaction.

Figure 5 shows the changes in the concentration of the product (maltose plus maltotriose) and the substrate in the retentate and the permeate. The concentration is expressed as carbon concentration, and is normalized by the substrate concentration in the feed solution. The product concentration in the retentate was in a range of 0.70–0.87 in all runs. The product concentration in the permeate was approximately the same as that in the retentate. The substrate concentration in the permeate was about 30\% of that in the retentate. In run 1, the normalized concentrations of total saccharides (open square) and substrate (solid circle) in the retentate increased gradually from 1.0 to 1.6 and from 0.5 to 0.9, respectively. Accumulation of substrate may be due to the short mean residence time. In runs 2 and 3, however, concentrations of both total saccharides and substrate increased initially and reached stable states. The initial increase of each substance was due to rejection by the membrane. The small accumulation of each substance suggests that almost all starch fed into the reactor was hydrolyzed and both product and dextrin passed through the membrane at appropriate rejection. The substrate (soluble starch, dextrin) accumulation in the retentate was found to be negligible by lowering the transmembrane pressure.

The conversion of starch to maltose was determined to be 0.68, 0.74, 0.79, and 0.83 at stable states at transmembrane pressures of 100, 40, 20, and 10 kPa, respectively. Conversion to maltotriose was about 0.03–0.05 in all runs. As the stable permeate flux decreased, conversion increased from 0.68 to 0.83. The highest conversion to maltose [0.83] was observed at 10 kPa transmembrane pressure, which was evidently higher than that of the batch experiment [0.70]. This might be because soluble starch and hydrolyzable dextrin were
retained for a longer time in the reactor than the mean residence time, and some parts of dextrin, of which the hydrolysis rate was very low, were hydrolyzed.

Figure 6 shows the relationship between the reaction rate (production rate of maltose plus maltotriose, gC m\(^{-3}\) sec\(^{-1}\), calculated from the mass balance) and the permeate flux (m\(^3\) m\(^{-2}\) sec\(^{-1}\)) in runs 1, 2 and 3. The reaction rate was approximately proportional to permeate flux. This suggests that the reaction may be limited by the substrate supply, which was affected by the transmembrane pressure.

Figure 7 shows the typical distributions of MW of the substrates in feed, retentates and permeates after 2 and 26 hr operations in run 3. Three main peaks appeared in feed, which were substrates A (corresponding MW is estimated to be larger than 500 kDa from Fig. 2), B (estimated MW 200–500 kDa), and C (estimated MW 20–50 kDa). Substrate D (estimated MW 10–20 kDa) appeared in the retentate after 2h operation. Substrates B, C, and D also appeared in the permeates. The observed rejection (\(R_{obs}\)) by the membrane is given as follows.

\[ R_{obs} = 1 - \frac{A_p}{A_i} \]  
(2)

where \(A_i\) and \(A_p\) are the peak areas of substrates in the retentates and the permeates, respectively. Figure 8 shows the changes in rejection with time for substrates A, B, C, and D, maltose, and maltotriose. Substrate A was completely rejected by the membrane. The observed rejections of substrates B, C, and D were 0.5–0.9, 0.2–0.5, and 0.0–0.2, respectively, which corresponded well with the estimated MWs. The observed rejections changed initially, and became nearly constant after several hours. The observed rejections of maltose and maltotriose were very low [0–0.2].

**Enzyme leakage**

The observed rejection of the enzyme was
found to be more than 99.95%, i.e., almost all enzyme was retained.

A mass balance equation for the enzyme is expressed as follows.

\[ V \left( \frac{dE}{dt} \right) = -JA(1 - RE)E \]  
(3)

where \( E \) is the enzyme concentration in the retentate (g m\(^{-3}\)), \( V \) the total working volume (m\(^3\)), \( t \) the reaction time (sec), \( J \) the permeate flux (m\(^3\)m\(^{-2}\)sec\(^{-1}\)), \( A \) the membrane area (m\(^2\)), and \( RE \) the observed rejection of enzyme (\( RE = 1 - E_p/E_p \)), \( E_p \) the enzyme concentration in the permeate. The solution of Eq. (3) is

\[ \frac{E}{E_0} = \exp\left( -(1 - RE)t/t_m \right) \]  
(4)

where \( E_0 \) is the initial enzyme concentration (g m\(^{-3}\)), and \( t_m \) the mean residence time (sec, \( = V/JA \)). If the value of \( t/t_m \) becomes 10, the ratios of \( E/E_0 \) are 0.368, 0.905, and 0.995 under observed rejections of 0.90, 0.99, and 0.9995, respectively. In the case of \( t/t_m \) is equal to 100, \( E/E_0 \) will be 0.951 at 0.9995 rejection. The very high value of enzyme rejection obtained during the reaction in this study was effective.

Simulation

The mass balance equations for the substrate (soluble starch and dextrin) and the product are given as follows.

\[ V \left( \frac{dS}{dt} \right) = FS_0 - rV - F(1 - R_S)S \]  
(5)

\[ V \left( \frac{dM}{dt} \right) = rV - F(1 - R_m)M \]  
(6)

where \( S \) is the substrate concentration in the retentate (gC m\(^{-3}\)), \( M \) the product (maltose plus maltotriose) concentration in the retentate (gC m\(^{-3}\)), \( V \) total working volume of the reactor (m\(^3\)), \( F \) the feed rate (m\(^3\) sec\(^{-1}\)), \( r \) the reaction rate (gC m\(^{-3}\) sec\(^{-1}\)), and \( R_S \) and \( R_m \) the observed rejections of substrate and product.

Normalizing each parameter in Eqs. (5) and (6) by the initial substrate concentration (\( S_0 \)), the following equations are obtained.

\[ \frac{dC_s}{dt} = C_{rv} - C_r - (1 - R_s)C_{rv}C_s \]  
(7)

\[ \frac{dC_m}{dt} = C_r - (1 - R_m)C_{rv}C_m \]  
(8)

where \( C_{rv} \) is the dilution ratio (sec\(^{-1}\), \( = F/V \)), \( C_s \) the normalized substrate concentration in the retentate (\(- = S/S_0\)), \( C_r \) the normalized reaction rate in the retentate (sec\(^{-1}\), \( = r/S_0\)), and \( C_m \) the normalized product concentration (\(- = M/S_0\)).

The normalized reaction rate (\( C_r \)) was assumed to be as follows from Fig. 6.

\[ C_r = aC_{rv} \]  
(9)

The normalized total saccharides concentration in the retentate (\( C_t \)) is expressed as the sum of \( C_m \) and \( C_s \). The normalized concentrations of the product (\( C_{mp} \)) and substrate in the permeate (\( C_{sp} \)) are given as follows.

\[ C_i = C_m + C_s \]  
(10)

\[ C_{mp} = (1 - R_m)C_m \]  
(11)

\[ C_{sp} = (1 - R_s)C_s \]  
(12)

The initial conditions of \( C_m \) and \( C_s \) were given experimentally. Assuming that \( R_s = 0.68 \) and \( R_m = 0.0 \), the changes in \( C_m \), \( C_s \), \( C_t \), \( C_{mp} \), and \( C_{sp} \) with time were calculated using a microcomputer (Runge-Kutta method).\(^{22}\) The solid lines in Fig. 5 show the simulated results for \( C_t \), \( C_m \), \( C_s \), \( C_{mp} \) (= \( C_m \)) and \( C_{sp} \). The simulated lines agreed well with the experimental results in the three different experiments.

Productivity

Table I summarizes the productivity of different reactor systems. The maltose productivities in runs 1–3 were 30–160 mmol sec\(^{-1}\) per m\(^3\) of working volume of the reactor. From the immobilized enzyme column reactor systems reported by Kusunoki et al.,\(^{11}\) Ohba et al.,\(^{12}\) and Martensson,\(^{15}\) 4–6.4 mmol m\(^{-3}\) sec\(^{-1}\) of productivities were obtained. Martensson also reported 6 mmol m\(^{-3}\) sec\(^{-1}\) with a CSTR (continuous stirred tank reactor).\(^{15}\) The productivities per unit working volume obtained in our study were much higher than those reported in the literature. This is due to a very short mean residence time in the system and high concentrations of the substrate and the enzymes.

Productivities per unit of \( \beta \)-amylase were in
Table I. COMPARISON OF PRODUCTIVITIES OF DIFFERENT REACTOR SYSTEMS FOR MALTOSE PRODUCTION

<table>
<thead>
<tr>
<th>Reactor system</th>
<th>I.E. PFR</th>
<th>I.E. PFR</th>
<th>I.E. Batch</th>
<th>I.E. PFR</th>
<th>I.E. CSTR</th>
<th>FEMR (kPa)</th>
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<tr>
<td>Starch concentration (g l⁻¹)</td>
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<td>50</td>
<td>10</td>
<td>10</td>
<td>150</td>
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<tr>
<td>Productivities</td>
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<td>8</td>
<td>—</td>
<td>4</td>
<td>6</td>
<td>160</td>
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<td>(mmol m⁻³ s⁻¹ (reactor))</td>
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<td>(µmol g⁻¹ s⁻¹ (enzyme))</td>
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<td>Conversion</td>
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<td>maltose + maltotriose</td>
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<td>—</td>
<td>—</td>
<td>—</td>
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<td>0.83</td>
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<td>13–15</td>
<td>13–15</td>
<td>13–15</td>
<td>This study</td>
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I.E., immobilized enzyme; PFR, plug flow reactor; CSTR, continuous stirred tank reactor.

the range of 6.4–32 µmol g⁻¹ sec⁻¹. Previously reported productivities per unit enzyme were 0.09–2.5 µmol g⁻¹ sec⁻¹, which were less than half those in our study.

From these, the maltose production by FEMR method was found to have a higher efficient productivity than other reactor systems. Further investigation on the stability of the FEMR system in long term experiments is needed to improve the system for practical production of maltose.

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