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NOTE

Bioethanol from sea lettuce with the use of crude enzymes derived from waste

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Abstract The mid-gut gland of the scallop contains saccharification enzymes such as cellulase and amylase, and these enzymes have been disposed of together with the mid-gut gland after the removal of the adductor muscle, an edible part of the scallop. We used a drip from the mid-gut gland of the scallop, obtained by squeezing the gland, as an inexpensive enzyme mixture and tried to produce bioethanol from the glucans present in sea lettuce by the method of simultaneous saccharification and fermentation (SSF) with the use of baker's yeast. The ethanol concentration attained was as high as 7.2 g/L, which corresponded to ~37% of the conversion of glucans in sea lettuce in the solid-state SSF. Furthermore, we ascertained that the drip and sea lettuce contain nutrients that are indispensable for maintaining the yeast activity, and, thus, the SSF did not require any additional nutrients, such as yeast extract or peptone, the use of which increases the cost of fermentation to a high level.

Keywords Bioethanol · Sea lettuce · Simultaneous saccharification and fermentation · Crude enzymes derived from waste

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Introduction

The biomass-based production of bulk chemicals, which are used as raw materials for chemical processing, has attracted attention to biorefinery. The production of lactic acid, which is used as a raw material for biodegradable plastic, and ethanol are two examples of biorefinery, although ethanol is used not only as a raw material for chemicals, but also as an alternative to petroleum-derived fuel. Ethanol production from inedible biomass or biomass that people do not find palatable has long been considered a crucial requirement for the effective utilization of such material. Thus, many studies on the production of ethanol from cellulosic materials [1–5], and other biomass wastes containing starch, such as food waste [6] and winery waste [7], have been published.

The bioconversion of glucan—polysaccharide consisting of glucose, such as cellulose and starch—to ethanol involves two steps: the hydrolysis of glucan to glucose, followed by the fermentation of glucose to ethanol. The hydrolysis process currently used is either acid hydrolysis or enzymatic hydrolysis. Enzymatic hydrolysis is often applied; however, the use of commercial enzyme tends to involve a high cost for ethanol production because commercial enzyme, particularly cellulase, itself is highly expensive. To save on the cost of enzyme, we attempted to use scallop mid-gut gland drip as an inexpensive source of saccharification enzymes in the present study. It is known that the cellulase [8] and amylase [9] are present in the mid-gut gland and digestive gland of the scallop, respectively.

For ethanol production in this study, we used sea lettuce (*Ulva pertusa* Kjellman), a type of green algae seaweed, as a raw material. Only a small proportion of sea lettuce is consumed as seasoning in Japan. We attempted to produce ethanol from the cellulose and starch contained in sea

lettuce by using the mid-gut gland drip of the scallop as a crude enzyme solution. Sea lettuce poses a nuisance to fishery by accumulating on the shores of seas and lakes, and putrefying to produce materials inhibitive to fish and aquatic organisms. Moreover, the putrefaction of sea lettuce affects the esthetic appeal of the shore, and, thus, has an impact on sightseeing and similar activities. Therefore, the effective use of waste sea lettuce has been of interest for a long time.

The bioconversion of seaweed to biomass energy as a means of methane gas recovery has been well studied, and the giant kelp [10] and *Ulva* spp. [11] have been used as raw materials for methane fermentation. In addition, although the purpose of their research was not biorefinery, Uchida and Murata [12] observed the simultaneous production of lactic acid and ethanol from *Ulva* spp. when they investigated the food and dietary applications of sea lettuce while utilizing expensive commercial cellulase. Furthermore, a paper on ethanol production from the waste of another type of seaweed, *Laminaria japonica*, following alginate extraction has been published; however, commercial cellulase was used in this study [13]. We know of no previous studies that describe the low-cost production of ethanol as an alternative energy source through inexpensive crude enzymes derived from waste.

Materials and methods

Mid-gut gland drip of scallop, sea lettuce, and yeast

Mid-gut gland drips from scallops were collected in a local fisheries factory in Yubetsu-cho, Hokkaido prefecture, Japan, and used as an enzyme mixture (crude enzymes derived from waste) for digesting seaweed. The mid-gut glands were first dissected from scallop viscera by hand during the manufacture of scallop adductor muscle products. The mid-gut glands were then put on a 2 × 2-mm mesh, and drips were collected by squeezing with ~5 g/cm pressure. The drips including the saccharification enzymes were stored at −30°C until use. The composition of the mid-gut gland drip of the scallop is shown in Table 1. The mid-gut gland drip is composed of ~94% water and contains a concentration of protein as high as 2.7%.

Dried powder of sea lettuce produced as a seasoning in Fukushima prefecture, Japan, was purchased from a local market in Hakodate, Hokkaido prefecture, since we could use the same sea lettuce for all experiments and, thus, obtain reproducible data, though it has been ascertained that the concentration of the ethanol obtained from fresh sea lettuce is greater than that obtained from dried sea lettuce powder. The composition of the sea lettuce is shown in Table 1. The carbohydrate content in the sea lettuce measured as nitrogen-free extract (NFE) by the method of Japan Food Analysis was 50.5%. The total glucan content in the sea lettuce was 14.0%; this was determined by first hydrolyzing the glucan using the method described in the National Renewable Energy Laboratory (NREL) Chemical Analysis and Testing Procedure [14], then measuring the glucose with high-performance liquid chromatography (HPLC) equipped with an L-3300 RI Monitor (Hitachi Ltd., Japan) and a SUGAR SH1011 column (Shodex, Japan). The content of starch, which is one of the constituents of glucans, was found to be 8.0% by using the F-kit starch (R-Biopharm AG, Germany), a commercial analytical kit system based on the measurement of the reaction product of amyloglucosidase. One of the advantages of utilizing the sea lettuce as a raw material for ethanol production is that it contains not only cellulose but also starch in a high concentration.

Table 1 Compositions of mid-gut gland drip from scallop and sea lettuce

	Mid-gut gland drip	Sea lettuce
Glucan (%)	–	14.0
Starch (%)	–	8.0
Crude fiber (%)	0	4.8
NFE (%)	0.6	50.5
Moisture content (%)	93.9	–
Crude protein (%)	2.7	17.4
Crude fat (%)	0	0.6
Crude ash (%)	2.8	26.7

Baker's yeast (*Saccharomyces cerevisiae*), which was maintained on the YM agar medium (glucose 10 g; Polypepton 5 g; yeast extract 3 g; malt extract 3 g; agar 20 g; distilled water 1 L; pH 6.2) at 30°C, was used in the fermentation experiment of sea lettuce hydrolysate.

Effects of pH and temperature on the activities of cellulase and amylase

In order to investigate the pH dependency of the cellulase activity, McIlvain buffer solutions of seven different pH values, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, were used. Enzyme solutions with different pH values were prepared by mixing the mid-gut gland drip of the scallop with an equal quantity of a twofold concentration of McIlvain buffer solutions, and 20 g/L of cellulose powder (Merck, Germany) was added to 50 mL of each enzyme solution in a 100-mL Erlenmeyer flask. The enzymatic hydrolysis was conducted at 35°C for 4 h with shaking at 100 rpm using a rotary shaker. The glucose concentration was measured by using HPLC, and the cellulase activity was determined by calculating the production rate of glucose from cellulose

powder for 4 h. The temperature dependence of the cellulase activity was checked under a set pH value of 5.5 with the temperature varied at four different set points, 25, 30, 35, and 40°C.

The pH and temperature dependencies of amylase activity were determined by calculating the production rate of glucose from 20 g/L of soluble starch (Wako Pure Chemical Industry Ltd., Japan) for 4 h. In order to determine the temperature dependence of amylase, similar experimental conditions to those for the cellulase were used, whereas the pH dependence of the amylase activity was investigated by adjusting the pH values at four different points, 5.0, 5.5, 6.0, and 6.5, at the constant temperature of 35°C.

Saccharification of sea lettuce

To ascertain the ability of the mid-gut gland drip of the scallop to decompose sea lettuce, sea lettuce was hydrolyzed at pH 5.5 at 35°C for 72 h. The concentration of sea lettuce in the mid-gut gland drip was adjusted to 20 g/L, and the same experimental method described in the previous section was used for saccharification. The concentration of the glucose produced was measured by HPLC after the sample was filtered through a 0.45-μm membrane filter.

Liquid-state SSF

The enzyme solution was prepared by mixing the mid-gut gland drip of the scallop with an equal quantity of 0.2 M citrate buffer solution with the pH adjusted to 5.5. In order to maintain the high activity of the yeast, 3 g/L of yeast extract and 5 g/L of peptone were added to the enzyme solution. This solution was filtered through a 0.45-μm membrane (Toyo Roshi Kaisha Ltd., Japan) after centrifugation at 20,000 rpm for 20 min. The sea lettuce powder was sterilized beforehand by autoclaving at 121°C for 20 min and then suspended in the enzyme solution at a concentration of 60 g/L. The baker's yeast precultured on the YM agar plate at 30°C for 36 h was collected using a sterilized spatula and inoculated into the enzyme solution at around 10^7 CFU/mL. A liquid-state simultaneous saccharification and fermentation (SSF) experiment was carried out in a 100-mL Erlenmeyer flask containing 30 mL of enzyme solution at 35°C for 72 h with a shaking condition of 100 rpm using a rotary shaker. The cellulase and amylase activities of the enzyme solution used in the liquid-state SSF corresponded to 11 and 85 units/L, respectively. During the SSF experiment, the cell density of the yeast was determined by the dilution plating method, and the concentrations of glucose and ethanol were measured by HPLC.

Solid-state SSF

The sea lettuce powder was sterilized once by autoclaving and then added to the mid-gut gland drip of the scallop supplemented with 3 g/L of yeast extract and 5 g/L of peptone, at a concentration of 240 g/L. The moisture content of this raw material was found to be ∼77%. The precultured baker's yeast was inoculated into the raw material at around 10^8 CFU/mL. Solid-state SSF experiment was carried out in a 100-mL Erlenmeyer flask containing the raw material of 26.8 g at 35°C for 72 h under static conditions. The cellulase and amylase activities of the enzyme solution used in the solid-state SSF corresponded to 22 and 170 units/L, respectively. During the SSF experiment, the sea lettuce powder was thoroughly mixed with a sterilized spatula every 24 h in order to maintain the homogeneity of the solid material, and the concentrations of glucose and ethanol were measured by HPLC after obtaining the water extract of the sample, which was filtered through a 0.45-μm membrane filter.

Solid-state SSF without the addition of organic nutrients, the yeast extract and the peptone, was also carried out in order to ascertain whether the nutrients such as the protein indigenous to the mid-gut gland drip of the scallop and sea lettuce were sufficient for maintaining the yeast activity. The production cost of ethanol can be reduced if the SSF does not require any additional nutrients.

Results and discussion

Effects of pH and temperature on the activities of cellulase and amylase

The pH values of the enzyme solutions were maintained around the expected values after the mid-gut gland drip of the scallop was mixed with McIlvain buffer solutions of each pH value from 5.0 to 8.0. The effects of pH and temperature on the activities of cellulase and amylase are shown in Fig. 1. The optimum pH levels for the activities of cellulase and amylase were around 5.3 and 4.7, respectively, although there is a possibility that the optimum pH for the amylase activity may be lower than 4.7, judging from the pH dependence curve of amylase activity. However, we did not carry out the experiment using pH levels lower than 4.7, since the activity of the yeast significantly decreases in such low pH conditions, and, thus, controlling the low pH value is not practical for the present case of SSF. The optimum temperature for both cellulase and amylase activities was found to be 35°C. Therefore, the optimum pH and temperature for both enzymes were rather similar, i.e., a pH around 5 and a temperature of 35°C. The activities of cellulase and amylase, which were calculated

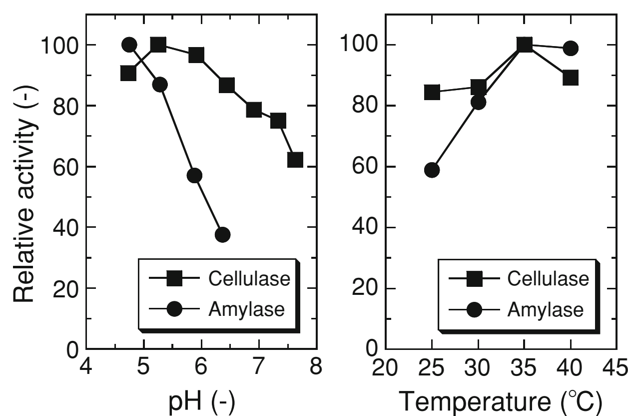

Fig. 1 Effects of pH and temperature on the activities of cellulase and amylase

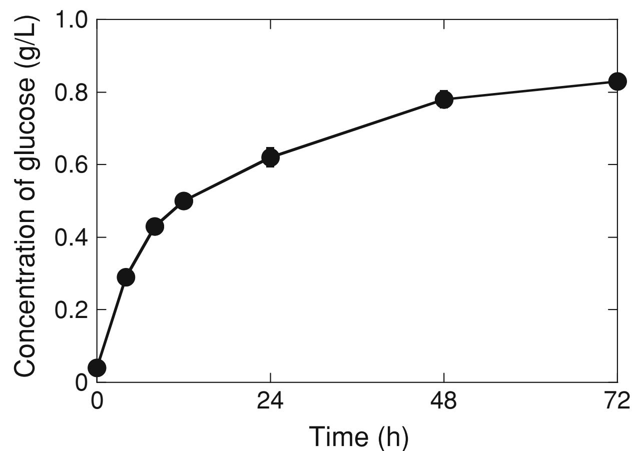

Fig. 2 The course of the concentration of glucose during the saccharification of the sea lettuce using the mid-gut gland drip of the scallop. The error bar on the concentration of glucose indicates the 95% confidence interval for the averaged values ($n = 3$), though this is not visible since the intervals were too narrow

as the production rate of glucose from cellulose powder and soluble starch 4 h from the start of saccharification at pH 5.3 and 35°C, were determined as 0.12 and 0.92 g glucose/L/h, respectively. One unit of cellulase or amylase activity was defined as the amount of the respective enzyme required for the release of 1 μmol of glucose per minute [15]; the cellulase and amylase activities of the mid-gut gland drip were calculated to be 22 and 170 units/L mid-gut gland drip, respectively. The protein concentration of the mid-gut gland drip was measured with the Lowry method, and the specific activities of cellulase and amylase in the mid-gut gland drip were 1.3 and 9.8 units/g protein, respectively. The activities were compared with those of commercially available cellulase (Meicelase; Meiji Seika Kaisha Ltd., Japan) and α-amylase (α-amylase from *Bacillus subtilis*; Wako Pure Chemical Industry Ltd., Japan). The specific activity of Meicelase was 81 units/g protein (51 units/g enzyme). In our previous study [16], 1 g/L of Meicelase was required for successful cellulose hydrolysis; 1 g/L of Meicelase corresponds to 51 units/L Meicelase. Thus, the specific activity of cellulase contained in the mid-gut gland drip of the scallop is about 1/60 times lower than that of Meicelase, but the cellulase activity of the mid-gut gland drip is rather high and is almost half that of the Meicelase solution.

The specific activity of the α-amylase purchased from Wako was 3,200 units/g protein (840 units/g enzyme). In earlier studies, concentrations of α-amylase (different from that used in our study) ranging from 0.19 [17] to 1 g/L [18] were used for the production of kefiran and L-lactic acid from starches. The activity of α-amylase solution may reach 160 units/L when α-amylase concentrations as low as 0.19 g/L are used. Therefore, the amylase activity of the mid-gut gland drip of the scallop, i.e., 170 units/L, can be considered as rather high, although the specific activity of amylase contained in the mid-gut gland drip is 1/330 times lower than that of α-amylase.

Saccharification of sea lettuce

The time course of changes in the concentration of glucose released as a result of the saccharification of the sea lettuce by the mid-gut gland drip of the scallop is shown in Fig. 2. The final glucose concentration was 0.83 g/L, which corresponded to a 27% conversion of the glucans in the sea lettuce when glucan conversion was defined as the ratio of the amount of glucose produced by saccharification to the potential amount of glucose in the sea lettuce:

$$\text{Conversion of glucans}\ (\%) = \frac{[\text{Glucose}]_f}{1.11 f_G [\text{Biomass}]} \times 100 \qquad (1)$$

where $[\text{Glucose}]_f$ = final glucose concentration (g/L); [Biomass] = dry biomass concentration at the beginning of saccharification (g/L); f_G = glucan fraction in raw dry biomass; 1.11 = theoretical conversion factor from glucan to glucose. This result indicates that the mid-gut gland drip of the scallop can hydrolyze the glucans in the sea lettuce.

Liquid-state SSF

The courses of liquid-state SSF are shown in Fig. 3. The ethanol concentration continued to increase even in the later stages of the SSF when the glucose concentration was 0. This indicated that glucose was converted to ethanol immediately after glucose production. Therefore, it can be concluded that glucan hydrolysis is the rate-limiting step of SSF.

The final ethanol concentration was 1.6 g/L, and this corresponded to ~29% conversion of the glucans contained in the sea lettuce. The conversion of glucans was calculated as follows:

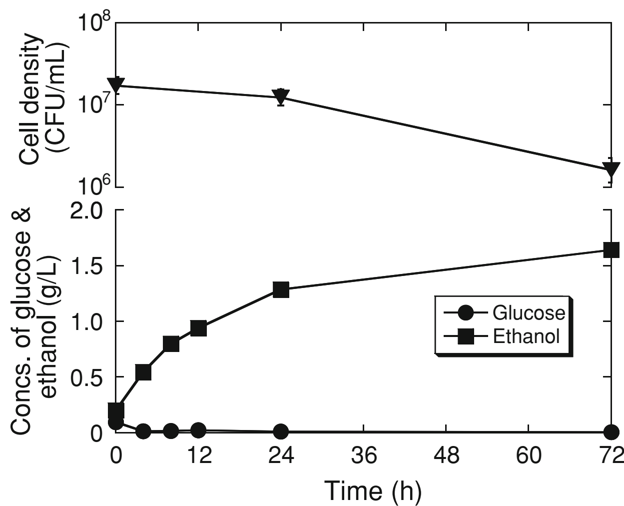

Fig. 3 The courses of cell density and concentrations of glucose and ethanol during liquid-state simultaneous saccharification and fermentation (SSF). The error bars on the cell density and concentrations of glucose and ethanol indicate 95% confidence intervals for the averaged values ($n = 3$)

$$\text{Conversion of glucans}\ (\%) = \frac{[\text{EtOH}]_f - [\text{EtOH}]_i}{0.568 f_G [\text{Biomass}]} \times 100 \tag{2}$$

where $[\text{EtOH}]_i$ = initial ethanol concentration (g/L); $[\text{EtOH}]_f$ = final ethanol concentration (g/L); [Biomass] = dry biomass concentration at the beginning of SSF (g/L); f_G = glucan fraction in raw dry biomass; 0.568 = theoretical conversion factor from glucan to ethanol.

The cell density of baker's yeast was maintained near the initial value, $\sim 10^{7.2}$ CFU/mL, until 24 h, and it then decreased by one order of magnitude. Most of the glucose formed as a result of the hydrolysis of glucan was converted to ethanol because the baker's yeast did not grow during the SSF. The reason why the cell density of baker's yeast decreased at the later stages of the SSF has not clarified yet; however, the deficiency of glucose might bring about the decrease in the cell density.

Solid-state SSF

Figure 4 compares the solid-state SSF with and without the addition of the yeast extract and the peptone in terms of the concentration of ethanol produced. Glucose did not accumulate after 72 h in both experiments, and the ethanol concentrations were 7.2 g/L in the case of SSF with the addition of organic nutrients and 6.6 g/L in the case of SSF without organic nutrients. The conversions of glucans in the sea lettuce for SSF with and without the addition of organic nutrients were 37 and 34%, respectively. The ethanol concentrations and the conversions of glucans for the SSF with and without the addition of organic nutrients did not differ significantly. We, therefore, confirmed that the application of solid-state SSF was effective for producing a high concentration of ethanol using the mid-gut gland drip of the scallop, and, further, the mid-gut gland drip of the scallop and sea lettuce could provide valuable nutrients for the yeast activities. It should be possible to increase the concentration of the ethanol obtained by partially purifying the mid-gut gland drip of the scallop and/or supplementation with a small amount of commercial enzyme for increasing the conversion of sea lettuce.

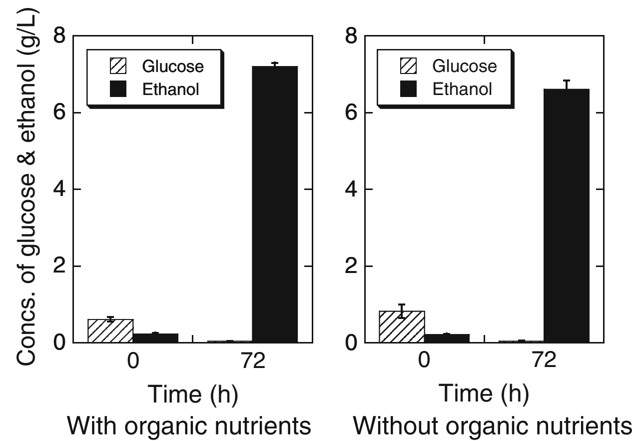

Fig. 4 Comparison of concentrations of glucose and ethanol in the solid-state SSF with and without the addition of organic nutrients. The error bars on the concentrations of glucose and ethanol indicate 95% confidence intervals for the averaged values ($n = 3$)

Conclusions

In this study, we could produce ethanol by combining two waste materials—one, a waste biomass comprising sea lettuce, which contains glucans like cellulose and starch, and the other, crude enzymes derived from waste, mid-gut gland drip of the scallop. We confirmed that scallop mid-gut gland drip contains saccharification enzymes such as cellulase and amylase, and that these enzymes can hydrolyze sea lettuce glucans. Ethanol could be produced from sea lettuce by the method of soild-state simultaneous saccharification and fermentation (SSF) using scallop mid-gut gland drip together with baker's yeast; the ethanol concentration attained was as high as 7.2 g/L. Furthermore, we ascertained that scallop mid-gut gland drip and sea lettuce contain nutrients that are indispensable for maintaining yeast activity during fermentation, and, thus, the SSF process did not require any additional nutrients. These results indicate that bioethanol can be produced at a low cost by using a combination of these two waste materials.

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