Bioethanol Production from Various Plant Materials via Solid-state Fermentation

Mitsuo HORITA[1]* and Takako KIYOSHI[2]

[1] Institute for Agro-Environmental Sciences, National Agriculture and Food Research Organization, Tsukuba, Japan

[2] Institute of Animal Livestock and Grassland Science, National Agriculture and Food Research Organization, Nasushiobara, Japan

Abstract

Previously, we have reported a novel bioethanol production system based on solid-state fermentation (SSF) and forage rice, which is used in a silage-making process. In this study, we evaluated the productivity of valuable materials via SSF using various plant materials other than forage rice. Italian ryegrass, sorghum, cassava, and other plant materials are also promising for SSF. Laboratory-scale SSF experiments using raw materials of Italian ryegrass yielded a maximum of 99 mg/g of dry matter of ethanol and other valuable materials (organic acids and soluble sugars). The conversion rates were almost the same as that of the forage rice. The nutritional value of the fermented products as livestock feed either improved or was similar to that of silage.

Introduction

The extensive use of fossil reserves has an impact on environmental pollution and global warming. To limit the use of grain resources as a substitute for fossil fuels, the development of technologies that effectively utilize nonedible biomass has been a priority (Sharma et al. 2020, Toor et al. 2020). Previously, we have reported a solid-state fermentation (SSF) method (Kitamoto et al. 2011) that produces a large amount of ethanol in a labor-saving manner by utilizing the process of fermented livestock feed production (silage) from forage crops (McDonald et al. 1991). Plant material is decomposed into soluble sugar and ethanol through fermentation, harnessing moisture (approximately 60%-70%) and nutrients present in the plant material immediately after harvesting, and the action of enzymes (e.g., cellulase and hemicellulase) and fermenting microorganisms (lactic acid bacteria and yeast). After ethanol recovery, the byproduct is used as feed (Horita et al. 2015).

A maximum of 125 L of ethanol per ton of dry weight matter was produced over three months, when feed rice was kept in round bales in the same way as for silage production. Up to 90% of the accumulated ethanol could be recovered using a large-scale distillation apparatus (Horita et al. 2015). The SSF method produces valuable substances other than ethanol (organic acids such as lactic acid and acetic acid, sugars, and other biochemicals) from nonedible or unused biomass other than feed rice (e.g., forage crops, grasses, and plant residue). Research suggests that various biomass resources can be effectively utilized with simple equipment and operations while reducing energy consumption and producing as little liquid waste as possible (Iram et al. 2020, Kitamoto et al. 2011, Sharma et al. 2020).

This study aims to promote the effective use of nonedible biomass in SSF by (1) selecting the types and combinations of biomass-degrading enzymes suitable for decomposition and saccharification of various plant materials; (2) conducting laboratory-scale SSF experiments using plant material, biomass-degrading enzymes, and fermentation microorganisms to investigate and examine the conversion efficiencies using ethanol production as an index; (3) investigating the feasibility of producing valuable substances other than ethanol (e.g., organic acids such as lactic acid) via the SSF process; and (4) evaluating the quality of the fermented feed.

Materials and methods

1. Preparation of materials for the model-scale experiments

Several plant species were selected for the model-scale experiments (Kitamoto et al. 2011). Whole plants of rice (cv. Todorokiwase), wheat (cv. Norin No. 61), sorghum (cv. Hazuki), corn (cv. Nasuhomare), Italian ryegrass (cv. Yayoiwase), and tall fescue (cv. Ushibue) at the heading stage, which were cultivated at Tochigi or Ibaraki Prefecture in Japan, were used. In addition, cassava at the vegetative stage and bagasse (i.e., sugarcane plant residue after juice extraction) produced in Okinawa Prefecture in Japan were included. The collected material was dried in an oven (O-190FDS, Sunaka Rika Kogyo, Tokyo, Japan) at 70°C for 3 days, powdered using a laboratory-scale mill (A11BS1, IKA, Staufen, Germany), passed through a 2-mm mesh, and sterilized using an electron beam (25.0 kGy) using a 5-MeV dynamitron accelerator (Radiation Dynamics Inc., Edgewood, NY, USA).

2. Biomass degradation experiment in the model scale

The biomass-degrading enzymes used in this study were cellulase (ACS, Meiji Seika Pharma, Co., Tokyo, Japan) from *Acremonium cellulolyticus* (0.86-1.72 filter paper unit [FPU]/g dry matter [DM]); glucoamylase (GODO-ANGH, GODO Shusei Co., Tokyo, Japan) from *Aspergillus niger* (0.032-0.32 unit/g DM); and pectinase (Sigma-Aldrich Japan, Tokyo, Japan) from *Aspergillus niger* (2-20 unit/g DM). Enzymes (dissolved in 0.5 mL sterilized distilled water) were mixed with 0.5 g of plant powder and 0.25 mL of 10% (v/v) L-lactic acid (final concentration of 60 mg/g DM) and adjusted to 60% (v/w) moisture in a sterilized 15 mL screw-capped glass tube. The usage rates of these additives were determined during previous studies (Kitamoto et al. 2011, Shinozaki & Kitamoto 2011). Samples for distinct saccharification conditions were prepared in triplicate and incubated at 28°C for 10-11 days.

3. Solid-state ethanol fermentation experiment in the model scale

In addition to the biomass-degrading enzymes and L-lactic acid described above, freeze-dried yeast (*Saccharomyces cerevisiae*, final concentration of 7.5×10^7 colony-forming unit (cfu)/g DM; Saf-instant, S.I. Lesaffre, Marcq, France) was added to 0.5 g of plant powder and adjusted to 60% moisture in a glass tube. The concentration of freeze-dried yeast was determined during previous studies (Horita et al. 2015, Kitamoto et al. 2011). Samples for distinct conditions were prepared in triplicate and incubated at 28°C for 11 or 20 days.

4. Laboratory-scale solid-state fermentation

Two Italian ryegrass cultivars (cvs. Yayoiwase and Kyushu 1) were used in the experiment based on their cultivation characteristics (early growth and high biomass yield) (Arakawa 2021). These were cultivated by the Institute of Animal Livestock and Grassland Science, National Agriculture and Food Research Organization (Tochigi, Japan) from August to November 2019 or from October 2020 to April 2021. Whole Italian ryegrass plants at the heading stage (approximately 83.5% water content) were harvested using a sickle and chopped into 3 cm-5 cm lengths using a hand cutter. Biomass-degrading enzymes (0.86 FPU/g DM cellulase and 2 unit/g DM pectinase), freeze-dried lactic acid bacteria (2×10^5 cfu/g DM, Chikuso No. 1 plus for silage additive supplied by Snowseed Co., Sapporo, Japan), and freeze-dried *S. cerevisiae* (3×10^7 cfu/g DM, Saf-instant) were dissolved in total 45 mL of sterilized distilled water and mixed with the plant material (300 g fresh matter). These were packed in a plastic bag lined with an aluminum film (AL-24, Seisannipponsha Ltd., Tokyo, Japan) together with CO_2-absorbent material (4-15 portions of C-1001P, Mitsubishi Gas Co., Tokyo, Japan). The bag was sealed using a packing machine fitted with a suction pump (V-301, Fuji Impulse, Osaka, Japan). The amounts of these additives were determined either in previous studies (Horita et al. 2015, Kitamoto et al. 2011) or in the present study. As a control, silage was prepared from the same material but with only the addition of lactic acid bacteria. Samples for distinct conditions were prepared in either triplicates or quadruplicates and incubated at 28°C for 10-20 days.

5. Analysis of fermentation products

After incubating the model-scale samples, 4-7 times the initial weight of sterilized distilled water was added to each tube. The samples in the tube were shaken at 25°C for more than 10 min at 120 rpm, transferred to a 1.5 mL microtube, centrifuged (13,000 × *g* for 5 min), and the supernatant was used.

After incubating the laboratory-scale fermentation samples, 100 mL of sterilized distilled water was added to each bag, sealed immediately, and soaked overnight at 4°C. The solution was recovered using filter paper (No. 2, Toyo Roshi Kaisha Ltd., Tokyo, Japan).

The extracted solution was analyzed for sugars (glucose, fructose, and sucrose), ethanol, L-lactic acid, and acetic acid content using an enzymatic method (Catalog Nos.: 139041, 139084, 139106, 148261, and 176290, F-kit, R-Biopharm AG, Darmstadt, Germany)

according to the manufacturer's instructions.

The dry weight % of each sample was determined by drying a 300-g sample of each plant for 72 h at 70°C in a forced-air drying oven (O-190FDS).

6. Analysis of the nutritional value of the fermented sample

Measurements of the nutritional value (acid detergent fiber [ADF], neutral detergent fiber [NDF], lignin, cellulose, hemicellulose, nonfiber carbohydrates [NFC], crude protein, bound protein, neutral detergent insoluble crude protein [NDICP], crude fat, crude ash, potassium [K], calcium [Ca], and total digestible nutrients [TDN]) of the SSF product, silage, and fresh whole Italian ryegrass plant material were performed by the Tokachi Federation of Agricultural Cooperatives (Obihiro, Japan) (Test Result Nos. 2020-06269 to 06274). Before analysis, the samples were dried in a forced-air drying oven (O-190FDS) at 70°C for 72 h and powdered using a laboratory-scale mill (WM-3, Irie Shokai Co., Tokyo, Japan). Components other than K and Ca were investigated according to universal chemical analysis methods (National Agriculture and Food Research Organization 2010). K and Ca contents were assessed using an X-ray fluorescence analyzer (JSX-1000S, JEOL Ltd., Tokyo, Japan) according to the manufacturer's instructions.

The cellulose and hemicellulose contents were calculated as ADF − lignin and NDF − ADF, respectively. NFC content was calculated as 100 − NDF − crude protein − crude fat − crude ash + NDICP. TDN was calculated according to the reports (National Agriculture and Food Research Organization 2010, National Research Council 2001).

Results

1. Saccharification of plant materials in the model scale

Sterilized powders from various plant sources such as rice, wheat, sorghum, corn, Italian ryegrass, tall fescue, cassava, and bagasse were used for the decomposition and saccharification experiments (incubation at 28°C for 11 days).

The release and accumulation of sugars (glucose, fructose, and sucrose) were promoted by adding enzymes to all the samples. For specific plant samples (rice, wheat, sorghum, and cassava), the sugar concentration increased significantly (105-154 mg/g DM) due to the addition of a mixture of cellulase and glucoamylase (Fig. 1).

In grasses, the addition of cellulase significantly increased the sugar concentration (108 and 62 mg/g DM in Italian ryegrass and tall fescue, respectively), whereas glucoamylase did not have any impact. The addition of pectinase slightly improved the sugar concentration in rice, wheat, sorghum, corn, and Italian ryegrass (13-47 mg/g DM) but not in tall fescue and bagasse (Fig. 1).

These results were used to create the optimal condition for biomass degradation for each plant species (Fig. 2). The maximum sugar concentration achieved ranged from 122 to 212 mg/g DM with individual concentrations as follows: corn, 212 mg/g DM; rice, 208 mg/g DM; cassava, 200 mg/g DM; Italian ryegrass, 185 mg/g DM; sorghum, 184 mg/g DM; wheat, 156 mg/g DM; bagasse, 125 mg/g DM; and tall fescue, 122 mg/g DM.

### 2. Solid-state fermentation in the model scale

Yeast was added to the mixture of plant material, enzyme, and lactic acid. A saccharification/ethanol SSF experiment (with incubation at 28°C for either 11 or 20 days, depending on the species) was conducted in a glass tube (Fig. 3). In addition to rice (221 mg/g DM), wheat (111 mg/g DM), and corn (138 mg/g DM) (reported previously by Kitamoto et al. 2011), higher concentrations (> 100 mg/g DM) of ethanol were produced from cassava (393 mg/g DM), Italian ryegrass (165 mg/g DM), and sorghum (122 mg/g DM). Tall fescue (86 mg/g DM) and bagasse (70 mg/g DM) had lower ethanol yields. Ethanol fermentation proceeded over 11 days, except for cassava and tall fescue at 20 days. In the case of corn, Italian ryegrass, tall fescue, and bagasse, a steady amount of ethanol (49-70 mg/g DM) was produced without adding biomass-degrading enzymes.

3. Solid-state fermentation using raw material of Italian ryegrass

Italian ryegrass SSF experiments were conducted by adding a biomass-degrading enzyme and fermenting microorganisms (lactic acid bacteria and yeast) to the harvested and shredded biomass cultivated outdoors.

Repeated tests (at 28°C for 10-20 days) using 300 g (DM weight ratio approximately 16.5%) of fresh plant material resulted in the production of 72-99 mg/g DM of ethanol on the 20th day of fermentation, and accumulation of L-lactic acid (49-73 mg/g DM), acetic acid (30-37 mg/g DM), and sugar (5-43 mg/g DM) were observed (Table 1).

By contrast, silage production experiments using the same biomass resulted in an accumulation of 4-21 mg/g DM of ethanol, 27-79 mg/g DM of L-lactic acid, 12-57 mg/g DM of acetic acid, and 1-12 mg/g DM of sugar.
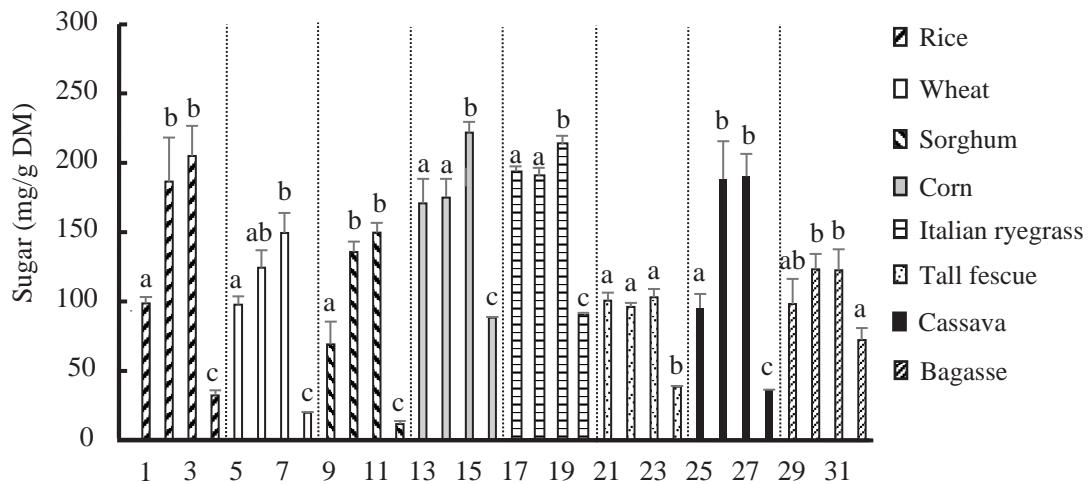
Fig. 1. Biomass degradation experiment in the model scale (1)
Soluble sugar (sum of glucose, fructose, and sucrose) released from plant materials (rice, wheat, sorghum, corn, Italian ryegrass, tall fescue, cassava, and bagasse) with the addition of cellulase (0.86 FPU/g DM) (lanes 1, 5, 9, 13, 17, 21, 25, and 29), cellulase (0.86 FPU/g DM) + glucoamylase (0.32 unit/g DM) (lanes 2, 6, 10, 14, 18, 22, 26, and 30), cellulase (0.86 FPU/g DM) + glucoamylase (0.32 unit/g DM) + pectinase (2 unit/g DM) (lanes 3, 7, 11, 15, 19, 23, 27, and 31), and without any enzyme addition (lanes 4, 8, 12, 16, 20, 24, 28, and 32). Values are expressed as means (bar with standard errors, N = 3). Values with different letters above the bars are significantly different ($P < 0.05$) as determined using an analysis of variance. FPU: filter paper unit; DM: dry matter
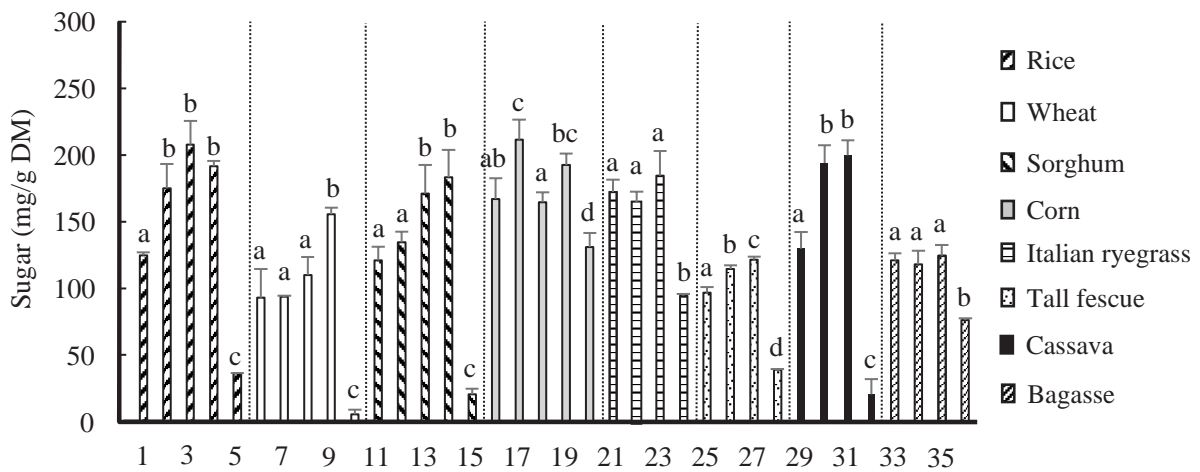
Fig. 2. Biomass degradation experiment in the model scale (2)
Soluble sugar (sum of glucose, fructose, and sucrose) released from plant materials (rice, wheat, sorghum, corn, Italian ryegrass, tall fescue, cassava, and bagasse) with the addition of cellulase (one time amount (×1)) + glucoamylase (×1) + pectinase (×1) (lanes 1, 6, 11, and 16); cellulase (×1) + glucoamylase (×10) + pectinase (×1) (lanes 2, 7, 12, and 17); cellulase (×1) + glucoamylase (×10) + pectinase (×3) (lanes 3, 8, 13, and 18); cellulase (×1) + glucoamylase (×10) + pectinase (×10) (lanes 4, 9, 14, and 19); cellulase (×1) + pectinase (×1, ×3, ×10) (lanes 21, 22, and 23, respectively), cellulase (×1, ×1.5, ×2) (lanes 25, 26, and 27, respectively), cellulase (×1) + glucoamylase (×1) (lanes 29 and 33), cellulase (×1) and glucoamylase (×3) (lanes 30 and 34), cellulase (×1) + glucoamylase (×10) (lanes 31 and 35), and without any enzyme addition (lanes 5, 10, 15, 20, 24, 28, 32, and 36). Cellulase (×1): 0.86 FPU/g DM; glucoamylase (×1): 0.032 unit/g DM; and pectinase (×1): 2 unit/g DM. Values are expressed as means (bar with standard errors, N = 3). Values with different letters above the bars are significantly different ($P < 0.05$) as determined using an analysis of variance. FPU: filter paper unit; DM: dry matter
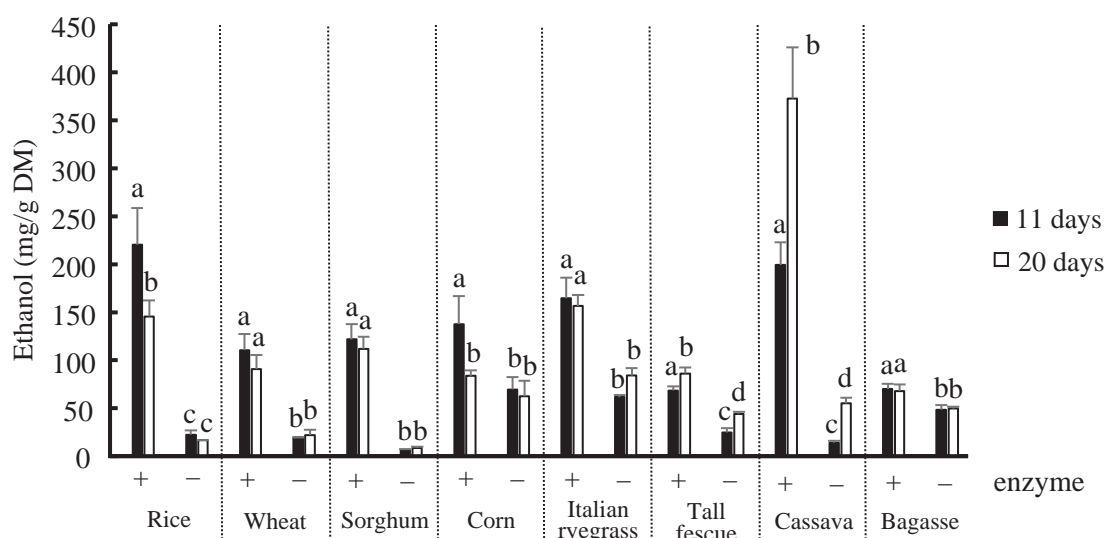
Fig. 3. Ethanol fermentation experiment in the model scale
Ethanol fermentation using plant materials (rice, wheat, sorghum, corn, Italian ryegrass, tall fescue, cassava, and bagasse) with (+) or without (−) the addition of cellulase (one time amount (×1)) + glucoamylase (×10) + pectinase (×3) (rice), cellulase (×1) + glucoamylase (×10) + pectinase (×10) (wheat), cellulase (×1) + glucoamylase (×10) + pectinase (×10) (sorghum), cellulase (×1) + glucoamylase (×10) + pectinase (×10) (corn), cellulase (×1) + pectinase (×1) (Italian ryegrass), cellulase (×2) (tall fescue), cellulase (×1) + glucoamylase (×3) (cassava), cellulase (×1) + glucoamylase (×1) (bagasse). Cellulase (×1): 0.86 FPU/g DM; glucoamylase (×1): 0.032 unit/g DM; and pectinase (×1): 2 unit/g DM. Values are expressed as means (bar with standard errors, N = 3). Values with different letters above the bars are significantly different ($P < 0.05$) as determined by an analysis of variance. FPU: filter paper unit; DM: dry matter

Table 1. Solid-state fermentation (SSF) and silage production tests using raw materials of Italian ryegrass

Test 1 (2019/11/15-2019/12/5)								
Cultivar	Ethanol [a] (mg/g DM)	L-lactic acid [a] (mg/g DM)	Acetic acid [a] (mg/g DM)	Remained sugar [a, b] (mg/g DM)	pH [a]	Conversion rate (%) Ethanol [c]	Total [d]	Survival rate [e] (%)
Yayoiwase (SSF)	76.6 [x]	51.1 [x]	29.9 [x]	43.1 [x]	4.02 [x]	15.0	27.4	44.7
Yayoiwase (silage)	21.2 [y]	42.3 [y]	40.1 [x]	0.7 [z]	4.42 [y]	4.1	12.4	75.7
Kyushu 1 (SSF)	72.0 [x]	49.0 [x]	34.3 [x]	25.9 [y]	4.11 [x]	14.1	25.0	35.4
Kyushu 1 (silage)	16.1 [y]	26.6 [z]	56.6 [y]	0.7 [z]	4.98 [z]	3.2	11.5	68.8
Test 2 (2021/4/16-2021/5/6)								
Cultivar	Ethanol [a] (mg/g DM)	L-lactic acid [a] (mg/g DM)	Acetic acid [a] (mg/g DM)	Remained sugar [a, b] (mg/g DM)	pH [a]	Conversion rate (%) Ethanol [c]	Total [d]	Survival rate [e] (%)
Yayoiwase (SSF)	96.4 [x]	60.4 [x]	37.4 [x]	5.0 [x]	4.09 [x]	18.9	28.7	44.9
Yayoiwase (silage)	3.6 [y]	70.5 [x]	12.2 [y]	7.2 [xy]	3.83 [xy]	1.3	9.7	76.6
Kyushu 1 (SSF)	98.6 [x]	73.4 [x]	31.5 [x]	10.5 [y]	3.93 [xy]	19.3	30.8	49.1
Kyushu 1 (silage)	4.2 [y]	79.0 [x]	11.9 [y]	11.9 [y]	3.72 [y]	0.8	11.1	80.3

Whole Italian ryegrass plants (approximately 3 cm-5 cm cut) after SSF and silage production experiments (20th incubation at 28°C) were used. Either triplicates or quadruplicates were collected, and ethanol, L-lactic acid, acetic acid, and sugar concentration, moisture, and pH were analyzed.
[a]Different alphabetical letters following the numbers indicate significant differences ($P < 0.05$, an analysis of variance) among test samples by dry matter (DM).
[b]Glucose + fructose + sucrose
[c]Ethanol fermentation products (ethanol + CO_2) / materials before fermentation by DM × 100
[d]Total products (ethanol + CO_2 + L-lactic acid + acetic acid + sugar) / materials before fermentation by DM × 100
[e]Residue/materials before fermentation by DM × 100

When comparing the conversion rate (DM weight ratio), 25%-30.8% (14.1%-19.3% ethanol, 5%-7.3% L-lactic acid, 3%-3.7% acetic acid, and 0.5%-4.3% sugar) and 9.7%-12.4% (0.8%-4.1% ethanol, 2.7%-7.9% L-lactic acid, 1.2%-5.7% acetic acid, and 0.5%-4.3% sugar) of biomass were utilized in the SSF and silage production experiments, respectively (Table 1).

4. Nutritional value of the fermented residue

Table 2 lists the composition of the plant material (freshly harvested Italian ryegrass whole plant, cvs. Yayoiwase and Kyushu 1), silage, and SSF products (test 1 in Table 1).

After the fermentation period (20th day), a lower pH (< 4.7) and weight loss (59.9% in SSF and 27.8% in silage) of DM were observed. In addition, a total of 31.5% of the materials were recovered by draining the accumulated solution in the SSF experiment.

Compared with fresh material, major nutritional components (ADF, NDF, cellulose, hemicellulose, NFC, crude protein, NDICP, crude ash, K, Ca, and TDN) of silage and the SSF product decreased with weight loss (as shown in parentheses in Table 2). Among these, NDF, hemicellulose, NDICP, and K decreased at a rate higher

Table 2. Composition of fresh material, silage, and solid-state fermentation (SSF) product of Italian ryegrass

Composition (%)	Fresh material	Silage		SSF product	
Moisture	83.4	85.2		85.6	
pH	6.13	4.65		4.07	
Weight loss [a]	-	27.8		59.9	
Drain [b]	-	0		31.5	
ADF [c, e]	27.1 [x]	32.2 [x]	(23.3 [x]) [d]	26.6 [x]	(10.6 [y]) [d]
NDF [c, f]	53.2 [x]	50.3 [x]	(36.3 [y])	45.7 [x]	(18.1 [z])
Lignin [c]	1.4 [x]	1.8 [x]	(1.3 [x])	3.3 [y]	(1.3 [x])
Cellulose [c, g]	25.7 [x]	30.4 [x]	(22.0 [x])	23.3 [x]	(9.3 [y])
Hemicellulose [c, h]	27.1 [x]	18.1 [x]	(13.4 [y])	19.1 [x]	(7.4 [z])
NFC [c, i]	20.1 [x]	14.9 [x]	(12.5 [y])	17.4 [x]	(9.0 [y])
Crude protein [c]	20.2 [x]	20.3 [x]	(14.6 [y])	19.7 [x]	(8.0 [z])
Bound protein [c]	1.2 [x]	1.7 [y]	(1.2 [x])	1.7 [y]	(0.7 [z])
NDICP [c]	9.5 [x]	3.9 [y]	(2.8 [y])	3.1 [y]	(1.2 [z])
Crude fat [c]	3.3 [x]	4.8 [x]	(3.4 [x])	8.6 [y]	(3.4 [x])
Crude ash [c]	12.9 [x]	13.8 [x]	(10.0 [x])	11.7 [x]	(4.8 [y])
K [c]	5.58 [x]	4.98 [x]	(3.6 [y])	3.39 [y]	(1.37 [z])
Ca [c]	0.57 [x]	0.54 [x]	(0.39 [xy])	0.81 [z]	(0.33 [y])
TDN [c, j]	65.2 [x]	63.9 [x]	(46.2 [y])	69.2 [x]	(27.6 [z])
L-lactic acid [c]	0.1 [x]	3.5 [y]		5.0 [y]	
Acetic acid [c]	0 [x]	4.9 [y]		3.2 [y]	
Ethanol [c]	0 [x]	1.9 [y]		7.5 [z]	

Whole Italian ryegrass plants (cvs. Yayoiwase and Kyushu 1) before/after silage and SSF experiments (20th incubation at 28°C, test 1 in Table 1) were used.

[a] Weight loss by dry matter (DM) during the fermentation process

[b] Solution volume ratio recovered by filtering after fermentation

[c] Percent of DM. The different alphabetical letters following the numbers indicate significant differences ($P < 0.05$, an analysis of variance) among test samples (fresh material, silage, and SSF product).

[d] Numbers in parentheses indicate the composition (%) × (100 − weight loss (%)) / 100.

[e] Acid detergent fiber (ADF)

[f] Neutral detergent fiber (NDF)

[g] ADF − lignin

[h] NDF − ADF

[i] Nonfiber carbohydrates (%) = 100 − NDF − crude protein − crude fat − crude ash + Neutral detergent insoluble crude protein (NDICP)

[j] Total digestible nutrients were calculated according to the reports (National Agriculture and Food Research Organization 2010, National Research Council 2001).

than the weight loss rate. However, the amounts of lignin and crude fat were almost the same.

Discussion

This study evaluated the production of valuable substances using the SSF method and various plant materials. Some of the tested species (Italian ryegrass, sorghum, and cassava) accumulated sugars at a concentration higher than 184 mg/g DM. These sugars were converted to ethanol (122-393 mg/g DM) and other organic materials in the presence of biomass-degrading enzymes and fermentation microorganisms, which would be suitable for the SSF as well as the rice plants (221 mg/g DM) (Figs. 1, 2, 3).

When SSF experiments used whole rice plants, adding a mixture of cellulase and glucoamylase facilitated starch degradation. Cellulase breaks down the cellular plant structure and releases the starch stored inside the cells. The starch can then be degraded by glucoamylase (Horita et al. 2015). Enzymatic saccharification experiments in this study showed that adding glucoamylase positively affects the sugar concentration achieved using starch-storing plants like wheat, corn, and cassava (Figs. 1, 2). Glucoamylase might be effective at facilitating starch degradation, similar to the process in rice plants.

Cellulase from *Acremonium cellulolyticus* used in this study also possesses pectinase activity, which would help degrade the plant structure (Kitamoto et al. 2011, Shinozaki & Kitamoto 2011). In this study, supplementation with commercial pectinase enzyme was effective at increasing sugar accumulation in rice, wheat, sorghum, corn, and Italian ryegrass (Figs. 1, 2) and might facilitate the degradation of the plant structure and subsequently release the soluble carbohydrate stored inside cells.

A laboratory-scale SSF experiment using raw Italian ryegrass plants was conducted. During the model-scale experiments, Italian ryegrass, similar to rice plants, accumulated a high concentration of sugars that was converted to ethanol (Figs. 2, 3). Moreover, Italian ryegrass is a widely cultivated forage grass (from Tohoku to Okinawa area, > 66,000 ha) in Japan (Arakawa 2021) with a high biomass yield (0.6-1.5 t DM/10 a) (Mizuno et al. 2003). The raw material can be obtained two or three times per year (potential harvests in spring, early summer, and late autumn). For our experiments, the plant material was harvested in late autumn (November 2019) and spring (April 2021) and used immediately.

In the SSF experiments, the maximum ethanol concentration (99 mg/g DM) was produced on the 20th day of fermentation, coinciding with L-lactic acid, acetic

acid, and sugar. The conversion rate reached 25%-31% (compared to 10%-12% in silage) (Table 1) and was similar to that of forage rice (total 25%-30%) (Horita et al. 2015).

A steady amount of L-lactic acid (> 49 mg/g DM) was produced and accumulated during the experiments, which is vital to maintain a lower pH (approximately 4.0) and prevent spoilage. Simultaneously, accumulation of acetic acid (> 30 mg/g DM) was observed, suggesting competition between additive fermentation microorganisms (yeast and lactic acid bacteria) and microorganisms associated with the plant material (e.g., acetic acid-producing bacteria) in sugar utilization. Accumulation of sugar (> 26 mg/g DM) was also observed (test 1 in Table 1).

For optimal ethanol production by SSF, a decrease in the conversion rate to other components is necessary. This decrease is brought about by adjusting the additive microorganisms (type and amount) based on the plant species used. Adjustments can also be made to increase the production of lactic acid, acetic acid, or other components. In silage experiments, the maximum amount of acetic acid (57 mg/g DM, test 1) or lactic acid (79 mg/g DM, test 2) was produced when using Italian ryegrass (cv. Kyushu 1) (Table 1).

In this study, we utilized the SSF method that produces ethanol and fermented livestock feed because the conversion rate reached a maximum of approximately 31% (Table 1), and the residues should be effectively used. In the United States, bioethanol is commercially produced from corn, with the residues after ethanol extraction adding value as a concentrate for livestock and fermentation feed (distillers dried grains with solubles) and increasing profitability (Iram et al. 2020). Similarly, if the residues after ethanol extraction have added value, it would become a profitable technology in the future.

Based on nutritional analysis (Table 2), the composition of the SSF product (ADF; NDF; cellulose; hemicellulose, NFC including water-soluble carbohydrates, pectin, and starch; crude protein; crude ash; and TDN) was similar to that of fresh matter and silage. Some components increased (e.g., Ca increased from 0.57% to 0.81%), whereas others decreased (K decreased from 5.58% to 3.39%). Therefore, the SSF product has either improved or maintained its nutritional value as livestock feed, depending on the component considered (> 0.25% in Ca and < 3% in K as the target value) (National Agriculture and Food Research Organization 2017).

Compared with the fresh material, 59.9% dry weight loss occurred after SSF (27.8% loss in silage). When dry weight loss was considered (numbers shown in Table 2's parentheses), the cellulose, hemicellulose, and

NFC contents of the fresh whole plant decreased by 16.4%, 19.7%, and 11.1%, respectively. These results suggest that in SSF with added degradation enzymes, a major part of the sugar (glucose, fructose, and sucrose) was from the degraded cellulose, hemicellulose, and NFC in plants. The sugars were then converted to ethanol, lactic acid, and other materials. In addition, a total weight of 31.5% was discharged as a drain (Table 2), indicating that most of these fermentation byproducts can be recovered in a soluble state, making it easy to extract the materials.

In conclusion, our study demonstrates the feasibility of the SSF method for the production of valuable components such as ethanol, organic acids, and value-added feedstocks from various plant species. Additional experiments using more materials with higher lignocellulose contents are ongoing.

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