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Enzymatic hydrolysis of apple pulp followed by lactic acid fermentation

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Apple pomace was fractionated into the water-soluble and water-insoluble materials (apple pulp). The effective saccharification method of apple pulp using PECTINASE HL from *Aspergillus* sp. (PECase) and CELLULASE "ONOZUKA" 3S (CELase) from *Trichoderma viride*, was investigated in addition to a screening of favorable microorganism for lactic acid fermentation from *Lactobacillus amylophilus* (NBRC15881), *Rhizopus oryzae* (NBRC5384) and *Kluyveromyces thermoteleran* (AOK A0357-23). On the basis of the results, lactic acid production by *R. oryzae* from the mixture of water-soluble materials of apple pomace and the materials solubilized by hydrolysis of apple pulp with a mixture of PECase and CELase was carried out. Lactic acid was produced at a concentration of 572 g from 46.8 kg (wet wt.) of apple pomace at 7 day. The ratio of D- and L-lactic acids was 56:44.
Key words: Apple pomace, Apple pulp, Lactic acid fermentation, Saccharification

1. INTRODUCTION

Bio-plastic, poly-lactic acid production is one of the major targets for biomass resources. We focused on the residues (apple pulp) of apple fruit extract as the source of lactic acid fermentation. In our previous studies[1)] on the sugar composition and enzymatic saccharification of apple pulp, we showed that the approximate ratio of carbohydrate in the mono- and oligo-saccharide, water-soluble polysaccharide, pectic substances, hemicellulose, and cellulose fractions of apple pomace was 56:1:17: 15: 11, and that about 31% of apple pulp which corresponds to pectic substances, hemicelulose and cellulose was hydrolyzed to be soluble by PECTINASE HL from *Aspergillus* sp., and about 9% was hydrolyzed to be soluble by CELLULASE "ONOZUKA" 3S from *Trichoderma viride*. The present work was conducted to investigate the effective saccharification method with these two enzymes, a screening of microorganism for lactic acid fermentation, and a trial of lactic acid fermentation of the saccharified apple pomace.

2. MATERIAL AND METHODS

2-1. Materials

The apple pomace mainly derived from a cultivar "Fuji" was kindly provided by Gold Nouen, Hirosaki, Aomori. The sample was consisted of water (78%), the water soluble mono- and oligo-saccharide (10%), the cell-wall polysaccharide (8%) and the others (4%).

2-2. Enzymes

PECTINASE HL from *Aspergillus* sp. (PECase) and CELLULASE "ONOZUKA" 3S (CELase) from *Trichoderma viride* were purchased from Yakult Pharmaceutical Industry Co. Ltd. and used for saccharification of apple pulp.

2-3. Microorganisms

Lactobacillus amylophilus (NBRC15881), *Rhizopus oryzae* (NBRC5384) and *Kluyveromyces thermoteleran* (AOK A0357-23) were used for the production of lactic acid.

2-4. General methods

Determination of total carbohydrate and uronic acid, and analyses of the constituent mono- and oligo-saccharides in the samples were carried out as reported previously[2,3)]. D/L-Lactic acid was determined by a kit of D/L-lactic acid-UV- method (Roche).

2-5. Stepwise hydrolysis of apple pulp by PECase and CELase

Apple pomace (wet wt. 400.1 g) was mashed and mixed with two volumes of water, and centrifuged to fractionate into the water-soluble materials (mono- and oligo-saccharides and water-soluble polysaccharides) and the water-insoluble materials (apple pulp, wet wt. 222.3 g). The apple pulp was mixed with two volumes of water and autoclaved for 20 min at 121°C. The autoclaved apple pulp was incubated with PECase (1.1 g) at 45°C. After 48 hr, the materials solubilized by PECase (PEC-M) were removed by centrifugation. The insoluble materials (precipitate) was mixed with two volumes of water, and incubated with CELase (0.5 g) for 24 hr at 45°C. The resulting sample was centrifuged to fractionate into the materials solubilized by CELase (CEL-M) and the insoluble materials (final residue, wet wt. 56.1 g). Total carbohydrate contents and the sugar compositions of PEC-M and CEL-M were determined respectively.

2-6. Hydrolysis of apple pulp by a mixture of PECase and CELase

Apple pomace (wet wt. 400.5 g) was mashed and mixed with two volumes of water, and centrifuged to fractionate into the water-soluble materials (mono- and oligo-saccharides and water-soluble polysaccharides) and the water-insoluble materials (apple pulp, wet wt. 226.7 g). The apple pulp was mixed with two volumes of water and autoclaved for 30 min at 121°C. The autoclaved apple pulp was incubated with a mixture of PECase (1.1 g) and CELase (1.1 g) for 48 hr at 45°C. The resulting sample was centrifuged to fractionate into the materials (PEC-CEL-M) solubilized by a mixture of PECase and CELase, and the insoluble materials (final residue, wet wt. 50.0 g). Total carbohydrate contents and the sugar compositions of PEC-CEL-M were determine-ed.

2-7. Comparison of L-lactic acid production by various microorganism.

The pH of water-soluble materials (WS-M) obtained from apple pomace and the materials solubilized by hydrolysis of apple pulp with a mixture of PECase and CELase (PEC-CEL-M) were adjusted to 6.5 and 5.5 with NaOH for lactic acid fermentation by *L. amylophilus* and by *K. thermoteleran* and *R. oryzae*, respectively. Then each sample was autoclaved for 20 min at 121°C. Each microorganism (about 10^8) was inoculated into 5 ml of the WS-M (48.6 mg as Glc equiv.) and into 5 ml of the PEC-CEL-M (163.7 mg as Glc equiv.). Fermentation was carried out for 7 days at 25°C. The content of D/L- lactic acid, and the total sugar content and sugar composition of each sample were determined.

2-8. Lactic acid production by *R. oryzae* from the mixture of WS-M and PEC-CEL-M.

Apple pomace (wet wt. 46.8 kg) was mashed and mixed with equal volume of water, and the water-soluble materials (WS-M) (yield: 3,546 g as Glc equiv.) were squeezed out by ASAHI PRESS. The water-insoluble materials (apple pulp, wet wt. 39.3 kg) was mixed with 2 volumes of water and heated for 30 min at 95°C. After cooling, the apple pulp was incubated with a mixture of PECase (197 g) and CELase (197 g) for 48 hr at 45°C. The resulting sample was squeezed by ASAHI PRESS to separate the soluble- (PEC-CEL-M, 5,835 g as Glc equiv.) and insoluble- materials (wet wt. 1.4 kg). The WS-M and PEC-CEL-M were combined. The pH of the combined materials was adjusted to 5.5 with NaOH. *R. oryzae* was inoculated into the combined materials. Fermentation was carried out for 7 days at 25°C. These procedures are summarized in Fig.1. The content of D/L-lactic acid during fermentation, and the total sugar content and sugar composition before and after fermentation were determined.

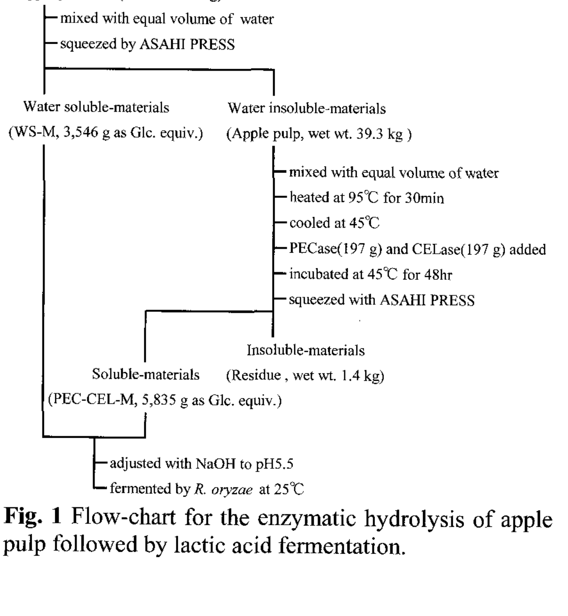

Fig. 1 Flow-chart for the enzymatic hydrolysis of apple pulp followed by lactic acid fermentation.

3. RESULTS AND DISCUSSION

3-1. Saccharification of apple pulp.

The influence of different hydrolysis method on the saccharification of apple pulp was examined. Apple pomace was fractionated into the water-soluble (mono- and oligo-saccahrides and water-soluble polysaccha-rides) and the water-insoluble materials (apple pulp). First, the apple pulp (222.3 g) was treated stepwise with PECase and CELase. Next, the apple pulp (226.7 g) was treated with a mixture of PECase and CELase. Table I shows the yields and sugar composition of the materials solubilized by the enzymatic treatments. There is no distinct deference on the carbohydrate content and sugar composition between them. Considering the cost including water and heating expenses, the treatment of apple pulp by a mixture of PECase and CELase is more efficient than that of apple pulp by stepwise hydrolysis with PECase and CELase.

Table I Yields and sugar composition of the materials solubilized from apple pulp by the enzymatic treatments.

Solubilized materials	Yield (g)	Sugar compositions (%) Fuc.	Ara	Rha.	Gal.	Glc.	Xyl.	Man	U.A.
PEC-M	28.4	2.5	23.1	trace.	16.6	42.3	10.2	0.2	5.3
CEL-M	17.3	1.4	9.7	trace.	8.0	67.4	10.4	0.2	2.8
PEC-M+CEL-M	45.7	2.1	18.0	trace.	13.3	51.8	10.3	0.2	4.3
PEC-CEL-M	50.5	1.7	12.7	trace.	9.3	58.3	10.0	0.3	7.6

Fuc., Ara., Gal., Glc., Xyl., Man. and U.A. indicate fucose, arabinose, galactose, glucose, mannose and uronic acid, respectively.

3-2. Comparison of L-lactic acid production by various microorganism.

To examine which microorganism most effectively produce L-lactic acid, WS-M and PEC-CEL-M were fermented for 7 days using *L. amylophilus*, *R. oryzae*, and *K. thermoteleran*, respectively. The content of D/L-lactic acid, and the residual sugar content and sugar composition were determined. Table II shows the content of residual sugar and the sugar composition in 7-days-fermented PEC-CEL-M. Table III shows the yields of D/L-lactic acid of 7-days-fermented WS-M and PEC-CEL-M. The results indicated that *R. oryzae* was effective for the production of L-lactic acid without any other nutrients.

Table II Content of residual sugar and the sugar composition of 7-days-fermented PEC-CEL-M

Fermentation with	Residual sugar (mg)	Sugar compositions (%) Fuc.	Ara.	Rha.	Gal.	Glc.	Xyl.	Man.	U.A.
None	163.7	1.7	12.7	trace.	9.3	58.3	10.0	0.3	7.6
A	129.3	1.9	11.6	trace.	7.8	50.3	13.1	1.0	14.2
B	37.7	1.9	14.4	0.3	9.5	14.7	18.4	4.9	35.9
C	62.2	2.4	14.0	4.5	10.6	21.4	18.0	4.7	24.4

A : *L. amylophilus* , B : *R. oryzae* , C : *K. thermoteleran*

Table III Yields of D/L-lactic acid of 7-days-fermented WS-M and PEC-CEL-M

Fermentation with	Materials	Yield (mg) L-Lactic acid	D-Lactic acid
None	WS-M	0.08	0.08
	PEC-CEL-M	0.03	0.03
L. amylophilus	WS-M	0.94	0.10
	PEC-CEL-M	3.40	0.40
R. oryzae	WS-M	6.53	0.04
	PEC-CEL-M	9.40	0.20
K. thermoteleran	WS-M	5.47	0.06
	PEC-CEL-M	1.60	0.20

3-3. Lactic acid production by *R. oryzae* from the mixture of WS-M and PEC-CEL-M.

Apple pomace was fractionated into the water-soluble materials (WS-M) and the water-insoluble materials (apple pulp). The apple pulp was hydrolyzed for 48 hr by a mixture of PECase and CELase. Table IV shows the changes in the content of solubilized carbohydrates and their sugar composition during enzymatic hydrolysis of apple pulp. The solubilized carbohydrate increased with the increase in hydrolysis time. Finally 4,658 g of carbohydrates were solubilized from 39.3 kg (wet wt.) of apple pulp. The final solubilized carbohydrates (PEC-CEL-M) consisted of Fuc, Ara, Rha, Gal, Glc, Xyl, Man and UA in the molar ratio of trace:13.4:trace:8.8:62.4: 7.1:trace: 8.3.

Table IV Changes in the content of solubilized carbohydrates and their sugar composition during enzymatic hydrolysis of apple pulp

Hydrysis time (hr)	Solubilized carbohydrate (g)	Sugar composition (%) Fuc.	Ara.	Rha.	Gal.	Glc.	Xyl.	Man.	U.A.
0	480	trace.	0.1	trace.	0.3	41.3	11.7	trace.	46.5
1	2,409	trace.	2.1	trace.	2.7	55.2	8.2	trace.	31.8
2	2,765	trace.	3.5	trace.	5.0	57.6	2.8	2.6	28.5
3	2,944	trace.	4.1	trace.	5.6	57.0	2.5	3.3	27.5
6	3,538	trace.	9.8	0.4	7.3	64.0	4.4	trace.	14.1
9	3,444	trace.	8.6	0.9	10.0	59.0	3.7	trace.	17.8
24	4,010	trace.	14.3	trace.	9.9	60.2	5.2	trace.	10.4
48	4,658	trace.	13.4	trace.	8.8	62.4	7.1	trace.	8.3

WS-M which corresponds to solbitol, glucose, fructose and sucrose, and the 48 hr-hydrolyzate (PEC- CEL-M) were combined, and the combined materials were subjected to lactic acid fermentation by *R. oryzae*. Figure 2 shows the time course of production of lactic acid. In addition, Table V shows the changes in the content of residual carbohydrates and their sugar composition of a mixture of WS-M and PEC-CEL-M during fermentation by *R.oryzae*. Lactic acid increased with the increase in fermentation time, and the carbohydrate decreased with the increase in fermentation time. Sugar composition analysis revealed that glucose, fructose and sucrose were mainly converted to lactic acid (Table V). Lactic acid was produced at a concentration of 572 g from 46.8 kg (wet wt.) of apple pomace at 7 days (Fig. 2). The ratio of D- and L-lactic acids was 56:44. Further studies will be necessary to produce pure L-lactic acid from apple pomace.

Table V Changes in the content of residual carbohydrates and their sugar composition of a mixture of WS-M and PEC-CEL-M during fermentation by *R.oryzae*.

Fermentation days	Residual carbohydrate (g)	(%)	Sugar composition (%) Sol.	Fuc.	Ara.	Rha.	Gal.	Glc.	Xyl.	Man.	Frc.	Suc.	U.A.
0	11,016	100.0	1.7	trace.	7.8	1.1	6.0	54.5	8.0	1.1	10.9	5.3	5.3
1	4,916	44.6	4.3	trace.	9.8	1.8	8.9	32.4	7.2	2.4	12.3	8.9	12.0
3	1,532	13.9	28.5	trace.	5.7	1.2	4.5	5.6	6.2	1.5	8.3	3.2	35.3
5	965	8.8	39.8	trace.	4.6	0.7	2.6	2.1	0.5	0.6	0.2	0.6	48.3
7	198	1.8	44.7	trace.	1.1	0.5	0.7	0.4	0.2	0.1	trace.	trace.	52.4

Sol., Frc. and Suc. indicated sorbitol, fructose and sucrose, respectively.

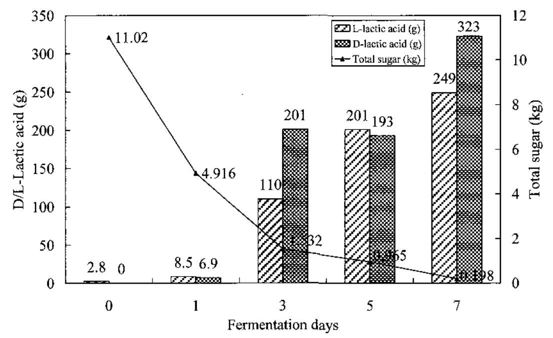

Fig. 2 Production of lactic acid from the mixture of WS-M and PEC-CEL-M by *R. oryzae*.

4. ACKKNOWLEGEMENTS

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