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Effects of Alkali Pretreatment on Enzymatic Saccharification of Beet Pulp

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The carbohydrate composition of the beet pulp [the residues of sugar beet (*Beta vulgaris* L.) extracts] was compared among three varieties, Monowhite, Hokkai 57 and Sugarman gold. Sugar composition analysis and sugar-linkage composition analysis showed that the carbohydrates in their alcohol-insoluble materials have features in common. However, total phenolic acid content was found to be different among them. In addition, the conditions for saccharification of beet pulp by commercial enzyme preparations were investigated using 16 kinds of crude enzyme preparations. Upon enzymatic saccharification of beet pulp, the 0.1 M NaOH-pretreatment is very effective to increase the amount of monosaccharides.
Key words: Beet pulp, Saccharification, Phenolic acids, Polysaccharides

1. INTRODUCTION

Ethanol production is one of the major targets for biomass resources [1-5]. We focused on the beet pulp [the residues of sugar beet (*Beta vulgaris* L.) extract] which is mainly used for cattle feeds, as the source of ethanol fermentation. In our previous studies [6, 7], we examined the sugar composition and enzymatic saccharification of beet pulp, and showed that the hydrolysis with a mold pectinase was effective to steadily gain reducing sugars from beet pulp. However, the result suggested that it was necessary to increase the limit of hydrolysis on the enzymatic saccharification of beet pulp [7]. In addition, we reported the chemical structures of two xylose containing polysaccharides, xylan and xyloglucan isolated from the 24% KOH extract of beet pulp [8]. The present work was conducted to compare the carbohydrate composition of three varieties of sugar beet, Monowhite (MW), Hokkai 57 (H57) and Sugarman gold (SG), and to find out a potential condition for saccharification of beet pulp by various enzyme preparations.

2. EXPERIMENTAL PROCEDURE

2.1 General methods

Concentration of the carbohydrate solutions by rotary evaporation was performed under diminished pressure at 35-40°C. Total carbohydrate and total uronic acid contents were determined by the phenol-sulfuric acid method [9] and the carbazole-sulfuric acid method [10], respectively. Reducing power was measured by the Nelson-Somogyi method [11, 12]. Enzymatic hydrolysis of samples was performed in the presence of a few drops of toluene to prevent microbial contamination. Gas-liquid chromatography (GLC) was conducted with a Hitachi G-500 gas chromatograph.

2.2 Analyses of sugar composition, sugar-linkage composition, and phenolic acid content of the alcohol insoluble materials from sugar beets

Sugar beet var. Monowhite (MW), Hokkai 57 (H57) and Sugarman gold (SG) harvested in the autumn of 1991 were provided by the Agricultural Experimental Station of Hokkaido, Sapporo. MW, H57, and SG were individually homogenized. The homogenates were frozen and kept at -20°C. Each frozen sample (200 g) was suspended in 800 ml methanol, and stirred for 24 h at room temperature. The suspension was filtered through Toyo No.2 filter paper. The residue was washed with acetone, and dried to give alcohol-insoluble material (AIM). The alcohol-insoluble materials from MW, H57 and SG were named MW-AIM, H57-AIM, and SG-AIM, respectively. The combined filtrate and washings was concentrated to give alcohol-soluble material (ASM).

Each AIM was suspended in 0.4 ml of 72% sulfuric acid and maintained for 1 h at room temperature. The suspension was then diluted with 3.2 ml of water and hydrolyzed for 4 h at 100°C. After hydrolysis, the hydrolyzate was centrifuged to remove the insoluble material. A portion of the supernatant was subjected to total carbohydrate content and uronic acid content determination. Another portion was subjected to neutral sugar composition analysis by GLC after converting the sugars to alditol trifluoroacetates [13]. GLC was done on a glass column (0.4 × 200 cm) of 1.5% QF-1 on Chromosorb W (AW-DMCS) at 140°C. The retention times of peaks of each sample were compared with those of standard sugars, rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc), and galactose (Gal).

Each AIM was methylated by the method of Hakomori [14], and the neutral sugar-linkage composition was figured out by GLC analysis of the derived alditol acetates [15]. GLC was done on a capillary column (15 m ×0.25 mm) of DB-225. The column oven temperature was raised linearly from 140 to 200°C at a rate of 2°C /min [16].

Each AIM was treated with 4 ml of 1 M NaOH for 17 h at room temp. and centrifuged. Phenolic acid content (ferulic acid equiv.) in the supernatant was figured out by UV absorption at λ max 340 nm as Na-ferulate [17].

2.3 Hydololysis of H57-AIM with Acucelase

Acucelase (a crude cellulase from *Asp. aculeatus* No. F-50) was kindly provided by Dr. R. Sakamoto.

H57-AIMs (100 mg each) were suspended with 10 ml of distilled water, 0.1 M NaOH, 0.5 M NaOH and 1.0 M NaOH, and kept for 7.5 h at room temperature, respectively. Each suspension was adjusted to pH 5.5 with acetic acid, and mixed with 1.0 M Na-acetate buffer (pH 5.5, 10 ml). The mixture was then incubated for 24 h at 40°C with Acucelase (15 mg). After the incubation, the mixture was filtered on Toyo No. 2 filter paper. The residue was washed with distilled water, and dried to give insoluble fraction. The combined filtrate and washings was concentrated to give soluble fraction. A portion of each soluble fraction was subjected to analyses of total sugar content (as Glc equiv.), acidic sugar content (as GalUA equiv.) and reducing sugar content (Glc equiv.).

Another portion of each soluble fraction was applied to a column (1.5×50 cm) of Bio-Gel P-2, followed by filtration through the column with distilled water at room temperature. Fractions of 4 ml each were collected and assayed for carbohydrate by the phenol-sulfuric acid method [9].

2.4 Hydrolysis of H57-AIM with commercial enzyme preparations

A sample (50 mg) was treated for 8 h with 5 ml of distilled water, and then mixed with 1.0 M Na-acetate buffer (pH 4.8, 4.75 ml) and Pectinase G Amano (Amano Enzyme) (10 mg). The mixture was incubated for 24 h at 40°C. Another sample (50 mg) was treated with 8 h with 5 ml of 0.1 M NaOH, adjusted to pH 4.8 with acetic acid, and mixed with 1.0 M Na-acetate buffer (pH 4.8, 4.75 ml) and Pectinase G Amano (10 mg). The mixture was incubated for 24 h at 40°C. After the incubation, each mixture was centrifuged for 0.5 h at 3,000 rpm. The supernatant was assayed for total carbohydrate by the phenol-sulfuric acid method [9]. Degradation rate of H57-AIM by each enzyme was calculated according to the equation: (content of released carbohydrate, mg / initial weight of H57-AIM, 50 mg)×100 (%).

Under the same conditions as described above, H57-AIMs (50 mg each) pretreated with distilled water and 0.1 M NaOH were individually incubated with commercial crude enzyme preparations (10 mg each), Hemicellulase Amano 90 (Amano Enzyme), Cellulase T Amano (Amano Enzyme), Hemicellulase M Tanabe (Tanabe Seiyaku Co. Ltd.), Pectinase Tanabe 2 (Tanabe Seiyaku Co. Ltd.), Pectinase SS (Yakult Pharmaceutical Industry Co. Ltd.), Cellulase Onozuka 3S (Yakult Pharmaceutical Industry Co. Ltd.), Cellulase Y-NC (Yakult Pharmaceutical Industry Co. Ltd.), Sucrase (Sankyo Lifetech), Cellulosin PE60 (HBI Enzymes), Cellulosin HC100 (HBI Enzymes), Cellulosin TP25 (HBI Enzymes), Cellulosin T2 (HBI Enzymes), Ultrazym 40L (Novozymes), Ultraflo L (Novozymes), or Celluclast 1.5 L (Novozymes). In addition, H57-AIMs (50 mg each) pretreated with distilled water and 0.1 M NaOH were individually incubated with and without Acucelase.

2.5 Hydrolysis of H57-AIM, MW-AIM, and SG-AIM with Pectinase G Amano, Cellulase Y-NC and Acucelase and HPLC analysis of the hydrolyzates

H57-AIM (50 mg), MW-AIM (50 mg), and SG-AIM (50 mg) were pretreated for 4 h with 5 ml of distilled water. Another portion (50 mg each) of H57-AIM, MW-AIM, and SG-AIM were pretreated for 4 h with 5 ml of 0.1 M NaOH. Each pretreated sample was adjusted to pH 4.8 with acetic acid (0.1 ml) and mixed with 1.0 M Na-acetate buffer (pH 4.8, 4.75 ml). The mixtures were incubated for 40 h at 40°C with Pectinase G Amano, Cellulase Y-NC and Acucelase, respectively. After the incubation, each mixture was centrifuged for 0.5 h at 3,000 rpm. The supernatant was assayed for total carbohydrate by the phenol-sulfuric acid method [9]. Degradation rate was calculated according to the same equation as described in 2.4.

Analysis of the hydrolyzates of H57-AIM with Pectinase G Amano, Cellulase Y-NC, and Acucelase was carried out by the high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD), Dionex ion chromatography system DX-300 [18]. The retention times of peaks of each sample were compared with those of standard sugars, Fuc, Ara, Rha, Gal, Glc, Xyl and Man.

3. RESULTS AND DISCUSSION

3.1 Comparison of sugar composition, sugar linkage composition, and phenolic acid content among MW-AIM, H57-AIM, and SG-AIM

The homogenized, frozen sugar beet, MW, H57 and SG (200g each) were separately treated with 80% methanol to afford 80% methanol-soluble materials (ASM) and 80% methanol insoluble-materials (AIM). Yields (dry wt.) and total carbohydrate content (as Glc equiv.) of AIM were as follows: MW, 8.727 g and 5.455 g; H57, 7.284 g and 6.012 g; SG, 5.531 g and 4.118 g. Carbohydrate content of ASM was as follows: MW, 28.562 g; H57, 25.496 g; SG, 16.210 g.

MW-AIM, H57-AIM, and SG-AIM were individually subjected to analyses of total carbohydrate content, sugar composition, and total phenolic acid content. Table I shows the results. There is no distinct difference among their sugar composition. But, there are distinct differences among their total carbohydrate content and total phenolic acid content. MW-AIM, H57-AIM and SG-AIM were separately methylated by the method of Hakomori, and their sugar-linkage composition were determined by GLC of the alditol acetates obtained from the acid hydrolyzates of methylated MW-AIM, H57-AIM and SG-AIM. A summary of all the identified derivatives

Table I Total carbohydrate and total phenolic acid contents, and sugar composition of MW-, H57- and SG-AIMs

Sample	Total carbohydrate	Sugar composition (wt%)								Total phenolic acid
	(mg/100mg dry wt.)	U.A.	Rha	Fuc	Ara	Xyl	Man	Glc	Gal	(μg/100mg dry wt.)
MW-AIM	62.5	16.8	2.7	0.5	26.9	3.0	1.7	36.2	12.3	465.5
H57-AIM	82.5	25.8	2.4	0.3	19.7	3.0	1.5	35.3	12.0	251.0
SG-AIM	74.4	21.1	2.3	0.7	22.4	3.1	2.0	37.4	11.0	361.4

presents in MW-AIM, H57-AIM and SG-AIM is shown in Table II. Although a large number of possible structures can be deduced from the data, reference to previous works [19] would suggest that (1→4)-linked xylan (presence of 4-Xyl), arabinan composed a (1→5)-linked arabinan backbone with a single arabinosyl residue as the most prevalent side cahin (the presence of T-Ara, 5-Ara and 3,5-Ara), (1→4)-linked galactan (the presence of 4-Gal), xyloglucan composed of a (1→4)-linked glucose backbone with a single xylose residue (T-Xyl, 4-Glc and 4,6-Glc), rhamnogalacturonan with side chains (the presence of 2-Rha, 2,4-Rha and galacturonic acid) and cellulose (the presence of 4-Glc) were the types of polymers present in MW-AIM, H57-AIM, and SG-AIM. As a matter of fact, arabinan, xylan and xyloglucan had already been isolated and characterized [8, 20]. It is apparent from the data presented here that the carbohydrate in MW-AIM, H57-AIM, and SG-AIM has features in common.

3.2 Hydrolysis of H57-AIM with Acucelase

The cell wall polysaccharides, arabinoxylan, xyloglucan, and pectic(arabino)galactan contain a small amount of phenolic acid (ferulic acid and *p*-coumaric acid), which is present as ester-linked side chains [21]. Such phenols are released from plant cell-walls upon treatment with alkali solutions and the phenolic acid-carbohydrate complex is liberated by a mixture of carbohydrate hydrolases. This means that the ester-linked side chains of phenolic acids inhibit the complete hydrolysis of wall polysaccharides by a mixture of carbohydrate hydrolases.

First, we examined the hydrolysis ratio of H57-AIM with Acucelase which is a crude cellulase from *Asp. aculeatus* No. F-50 before and after alkali-pretreatment. H57-AIMs pretreated with distilled water, 0.1 M NaOH, 0.5 M NaOH and 1.0 M NaOH were incubated with Acucelase, respectively. After the incubation, each sample was fractionated into soluble and insoluble fractions. The dry weight of each insoluble material was determined. A portion of each soluble fraction was subjected to analyses of total sugar content (as Glc equiv.), acidic sugar content (as GalUA equiv.) and reducing sugar content (Glc equiv.). The results are shown in Table III. Another portion of each soluble fraction was subjected to Bio-Gel P-2 chromatography (Fig.1). As can be seen from Table III, the dry weights of

Table II Sugar-linkage composition of MW-AIM, H57-AIM and SG-AIM

Peak No.	Methylated sugar linkage*	Deduced glycosidic	Amount**		
			MW-AIM	H57-AIM	SG-AIM
1	2,3,5-Me3-Ara***	T-Ara	13.0	12.6	10.4
2	2,3,4-Me3-Xyl	T-Xyl	-	0.2	0.3
3	2,3,4-Me3-Fuc	T-Fuc	0.2	0.2	0.2
4	3,5-Me2-Ara	2-Ara	-	-	-
5	3,4-Me2-Rha	2-Rha	0.6	1.0	1.1
6	2,5-Me2-Ara	3-Ara	0.9	1.3	0.7
7	2,3,4,6-Me4-Glc and/or 2,3,4,6-Me4-Man	T-Glc and/or T-Man	0.2	0.2	0.6
8	2,4-Me2-Xyl	3-Xyl	-	-	-
9	2,3-Me2-Ara	5-Ara	15.3	13.1	10.6
10	2,3-Me2-Xyl, 3,4-Me2-Xyl and/or 2,3,4,6-Me4-Gal	4-Xyl, 2-Xyl and/or T-Gal	1.9	2.5	2.3
11	2-Me1-Rha	3,4-Rha	-	-	-
12	3-Me1-Rha	2,4-Rha	1.7	2.2	1.6
13	2-Me1-Ara	3,5-Ara	11.6	10.2	8.3
14	3-Me1-Ara,	2,5-Ara	-	-	-
15	2,4,6-Me3-Gal and/or 2,3,6-Me3-Man	3-Gal 4-Man	3.1	3.4	6.2
16	2-Me1-Xyl and/or 4-Me1-Xyl	3,4-Xyl and/or 2,3-Xyl			
17	2,3,6-Me3-Gal, 3,4,6-Me3-Gal and/or 2,3,4-Me3-Glc	4-Gal, 2-Gal and/or 6-Glc	4.3	4.2	3.5
18	2,3,6-Me3-Glc	4-Glc	36.5	37.9	37.4
19	Ara	2,3,5-Ara	-	-	-
20	2,3,4-Me3-Gal	6-Gal	1.2	1.7	1.4
21	2,6-Me2-Gal	3,4-Gal	0.8	1.0	0.8
22	Unidentified		1.4	0.9	1.6
23	3,6-Me2-Gal	2,4-Gal	1.8	2.2	4.0
24	Unidentified		0.2	0.2	0.5
25	2,3-Me2-Glc	4,6-Glc	3.3	3.4	6.0
26	Unidentified		1.1	0.9	1.0
27	2,4-Me2-Gal	3,6-Gal	0.9	0.9	1.3
			100.0	100.2	99.8

*The numerical prefixes represent the carbon atoms involved in glycosidic linkages in the original polysaccharides. Prefix T indicates sugars linked through C (*O*)-1 only.

** % total area. *** 2,3,5-Me3-Ara = 2,3,5-tri-*O*-methyl-1,4-di-*O*-acetyl-arabinitol, etc.

Table III The carbohydrate content solubilized from H57-AIM (100 mg) by hydrolysis with Acucelase after pre- treatment with H2O, and 0.1, 0.5 and 1.0 M NaOH.

Sample pretreated with	Soluble fraction				Insoluble fraction (mg dry wt.)
	TS[(a)] (mg)		RS[(b)] (mg)	DP[(e)]	
	NS[(c)]	AS[(d)]			
H2O	27.1	19.9	19.1	1.4	55.1
0.1 M NaOH	51.2	24.7	37.6	1.4	20.3
0.5 M NaOH	52.5	21.4	36.3	1.4	19.9
1.0 M NaOH	45.3	21.3	28.9	1.6	28.6

(a) Total sugar. (b) Reducing sugar (as Glc equiv.) determined by the Nelson-Somogyi method. (c) Neutral sugar (as Glc equiv.). (d) Acidic sugar (as GalUA equiv.). (e) Degree of polymerization, TS/RS.

Table IV Degradation rate of H57-AIM by various kinds of enzymes after pretreatment with H2O and 0.1 M NaOH.

Enzymes	Degradation rate (%)		
	Pretreated with		
	H2O (1)	0.1 M NaOH (2)	(2) - (1)
1 . Pectinase G Amano	25.8	44.0	18.2
2 . Hemicellulase Amano 90	21.6	43.3	21.7
3 . Cellulase T Amano	7.9	23.6	15.7
4 . Hemicellulase M Tanabe	8.8	24.0	15.2
5 . Pectinase Tanabe 2	14.8	33.6	18.8
6 . Pectinase SS	16.9	28.4	11.5
7 . Cellulase Onozuka 3S	3.9	33.9	30.0
8 . Cellulase Y-NC	14.1	48.4	34.3
9 . Sucrase	12.5	25.4	12.9
10 . Cellulosin PE60	36.3	50.1	13.8
11 . Cellulosin HC100	12.2	41.5	29.3
12 . Cellulosin TP25	8.2	28.7	20.5
13 . Cellulosin T2	14.9	37.4	22.5
14 . Ultrazym 40L	23.7	36.0	12.3
15 . Ultraflo L	15.3	41.6	26.3
16 . Celluclast 1.5L	12.6	41.8	29.2
17 . Acucelase	18.4	50.6	32.2
18 . No enzyme	4.4	4.2	-0.2

insoluble fractions were decreased and the total sugar contents of soluble fractions were increased about two-fold by treatment with alkali solutions. The increase of total sugar content of soluble fraction are derived from the increase of mono- and oligosaccharides (Fig. 1). These results suggest that upon enzymatic hydrolysis of H57-AIM, the 0.1 M NaOH-pretreatment is very effective to increase the amount of monosaccharides.

3.3 Hydrolysis of H57-AIM with commercial enzyme preparations

Secondly, we examined the effect of 0.1 M NaOH-pretreatment of H57-AIM on the hydrolysis of H57-AIM with 16 kinds of commercial enzyme preparations. In every case, the carbohydrate (Glc equiv.) released from H57-AIM were increased by 0.1 M NaOH-pretreatment (Table IV). The degradation rate of the sample which was pretreated with 0.1 M NaOH and was not treated with enzyme is very small, about 4.2%. This value is almost the same as that (4.4%) of the sample which was pretreated with H2O and was not treated with enzyme. This result strongly suggests that the carbohydrate of H57-AIM was not released by 0.1 M NaOH-treatment. In addition, these results show the effectiveness of alkali-pretreatment applies to the hydrolysis of H57-AIM with not only cellulase but also pectinase or hemicellulase.

3.4 Hydrolysis of H57-AIM, MW-AIM, and SG-AIM with Pectinase G Amano, Cellulase Y-NC, and Acucelase

Finally, we examined the effect of 0.1 M NaOH-pretreatment of H57-AIM, MW-AIM and SG-AIM on hydrolysis of H57-AIM, MW-AIM and SG-AIM with Pectinase G Amano, Cellulase Y-NC and Acucelase. The degradation rate [(the released carbohydrate / initial weight) $\times 100\%$] of H57-AIM, MW-AIM and SG-AIM by Pectinase G Amano, Cellulase Y-NC and Acucelase after treatment with/without 0.1 M NaOH are shown in Table V. In every case, the content of released carbohydrate was increased by alkali-pretreatment. Analysis of neutral monosaccharide composition of the enzymatic hydrolyzates of H57-AIM revealed that the increase of released carbohydrate was mainly as a result of that of arabinose and glucose (Fig. 2). Feruloylated arabinose disaccharide and trisaccharide, feruloylated galactose disaccharide and feruloylated arabinosyl-arabinosyl-xylose were isolated from the Driselase-hydrolyzates of sugar beet pulp [22]. These oligosaccharides were considered to be derived from feruloylated α -(1→5)-arabinan and feruloylated β -(1→4)-galactan which are constituent polysaccharides of cell-walls of sugar beet

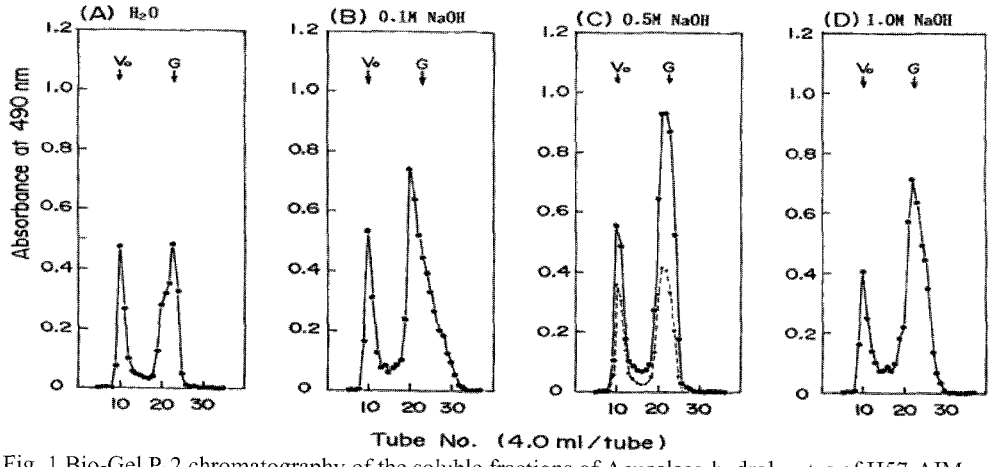

Fig. 1 Bio-Gel P-2 chromatography of the soluble fractions of Acucelase-hydrolyzates of H57-AIM which were pretreated with H_2O, 0.1 M NaOH, 0.5 M NaOH and 1.0 M NaOH, respectively.

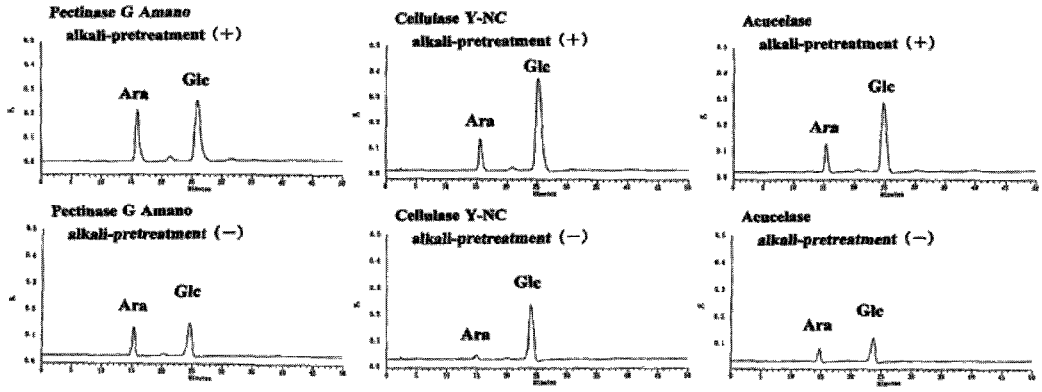

Fig. 2 Analysis of neutral monosaccharides by HPAEC-PAD of the enzymatic hydrolyzates of H57-AIM which were pretreated with (+) and without (-) 0.1 M NaOH.

Table V Degradation rate of H57-AIM, MW-AIM and SG-AIM by Pectinase G Amano, Cellulase Y-NC and Acucelase

Sample	Pretreatment with	Pectinase G Amano	Cellulase Y-NC	Acucelase
H57-AIM	0.1M NaOH	41.8 %	44.6 %	46.6 %
	H2O	23.2 %	9.8 %	25.4 %
MW-AIM	0.1M NaOH	45.0 %	49.0 %	52.0 %
	H2O	30.0 %	11.6 %	32.4 %
SG-AIM	0.1M NaOH	45.2 %	46.6 %	52.6 %
	H2O	24.6 %	13.2 %	27.8 %

It is reasonable that removal of ferulic acid from the polysaccharides by alkali-treatment stimulated the susceptibility of beet pulp to Pectinase G Amano, Cellulase Y-NC and Acucelase, and led to the increase in the recovery of neutral sugar, arabinose and glucose.

From the present works, we can conclude that alkali pretreatment has an effect on enzymatic saccharification of beet pulp.

4. CONCLUSION

Total carbohydrate (as Glc equiv.) and total phenolic acid (as ferulic acid equiv.) contents of the alcohol-insoluble materials from Monowhite (MW-AIM), Hokkai 57 (H57-AIM) and Sugarman gold (SG-AIM) were compared. Total carbohydrate content and total phenolic acid content per 100 mg dry wt. were as follows: MW, 62.5 mg and 465.5 μ g; H57, 82.5 mg and 251.0 μ g; SG, 74.4 mg and 361.4 μ g.

Analyses of sugar composition and sugar-linkage composition suggested that (1→4)-linked xylan, arabinan composed a (1→5)-linked arabinan backbone with a single arabinosyl residue as the most prevalent side chain, (1→4)-linked galactan, xyloglucan composed of a (1→4)-linked glucose backbone with a single xylose residue, rhamnogalacturonan with side chains and cellulose were the types of polymers present in MW-AIM, H57-AIM, and SG-AIM.

Alkali pretreatment had an effect on enzymatic saccharification of MW-AIM, H57-AIM and SG-AIM: the removal of ferulic acid from the polysaccharides in each AIM by 0.1 M NaOH pretreatment stimulated the susceptibility of beet pulp to Pectinase G Amano, Cellulase Y-NC and Acucelase, and led to the increase in the recovery of neutral sugar, arabinose and glucose.

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