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The structure and physical properties of glucuronic acid oligomers produced by a *Gluconacetobacter hansenii* strain using the waste from beer fermentation broth

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Abstract

The aim of this work was to study the chemical structure and physical properties of water-soluble oligosaccharides (WSOS) produced by *Gluconacetobacter hansenii* PJK using the waste from beer fermentation broth as a basal medium. The analysis of the hydrolyzed products and the spectroscopic studies of the native WSOS showed that it is a mixture of oligomers all having a single sugar α-linked glucuronic acid as building blocks with an *O*-acetyl and *O*-methyl group, in the terminal unit of the non-reducing end. The thermal studies displayed a progressive degradation of WSOS without a major weight loss throughout a range of temperatures. The melting point and pyrolysis temperature were found to be 130.16 and 275.25 °C, respectively. The optimum concentration of WSOS for a maximum emulsifying ability was found to be 0.10% (w/v). The resulting emulsions, however, did not demonstrate a noteworthy stability.

Keywords: Oligosaccharides; Glucuronic acid; Waste from beer fermentation broth; *Gluconacetobacter hansenii*; Physical properties

1. Introduction

Oligosaccharides can be found in important medicinal, food and agricultural applications. They are indicated in infectious and inflammatory diseases, transplantation, neutralization of toxins, cancer immunotherapy, metabolic and cardiovascular diseases and in drug delivery (Koizumi, 2003; Simon, 1996). Oligosaccharides are less sweet, have less calories than sucrose and thus, are useful as bulking agents in diet food. They also act as a pre-biotic (Anonymous, 1997). Similarly, oligosaccharides activate the plant cell machinery leading to defence reactions or to plant developmental processes. They are also used as fertilizers (Delattre, Michaud, Lion, Courtois, & Courtois, 2005). In view of these examples, it appears necessary to produce new oligosaccharides quickly.

Oligosaccharides can be obtained by way of chemical syntheses, extraction from plants, or by using microbial fermentation. Oligo- and polysaccharides have complex structures and their stereospecificity and regiospecificity are difficult to control which hamper their efficient synthesis (Khan, Park, & Kwon, 2007; Koizumi, 2003). The production of these products from plants and seaweed suffer from limitations due to geographical and climatic conditions and variation in seasons (Khan, Park, et al., 2007; Selbmann, Onofri, Fenice, Federici, & Petruccioli, 2002). By contrast, microbial production is independent of such limitations so product recovery and purification is easy. Microorganisms exhibit a high growth rate and thus, exhibit a high product yield. Moreover, appropriate strains can be genetically modified in order to acquire a product with the desired properties (Khan, Park, et al., 2007). Several microbial oligo- and polysaccharides have been reported, but only a few have been developed on a commercial scale. The underlying reasons may include the pathogenic nature of the producer organism, high production costs and diffi-

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culty in maintaining the product quality or the product may not pass regulatory standards (Sutherland, 2001).

Our previous studies have shown that *Gluconacetobacter hansenii* PJK, a non-pathogenic strain, is capable of producing glucuronic acid-based oligosaccharides from the defined medium (GAO) (Jung, Park, & Chang, 2005; Park, Khan, & Jung, 2006). These oligosaccharides were found to be free from bacterial cells' contamination, possess good emulsifying properties and have sufficient thermal stability in order to be developed commercially (Khan, Khan, & Park, 2007). However, their production from a chemically-defined medium is expensive. Therefore, it is important to look for alternative, cheap sources that can be used as medium for bacterial growth in order to facilitate the production of these potentially valuable products.

In our recent investigations, it was revealed that *G. hansenii* PJK could produce enormous quantities (112.65 g/l) of water-soluble oligosaccharides (WSOS) from the waste from beer fermentation broth (WBFB) under optimized conditions (Khan, Hyun, & Park, 2007). WBFB contains 68.11 g/l carbon, 17.19 g/l nitrogen and 116.14 g/l hydrogen (Khan, Hyun, et al., 2007). It also contains 29.44 g/l total carbohydrate, 0.67 g/l total protein and 4.58% ethanol (Khan, Hyun, et al., 2007). The composition of the culture medium and fermentation conditions (pH, temperature, oxygen concentration, agitation) greatly influence the chemical structure, composition and viscosity of the microbial polysaccharides (Duta, França, & Lopes, 2006). For the production of WSOS from the WBFB, similar fermentation conditions were applied that were used in the production of GAO (Jung et al., 2005; Khan, Hyun, et al., 2007), but the change in the medium composition was obvious. In this scenario, it seemed important to learn about the modifications in the chemico-physical properties of the WSOS that were produced by this new medium (WBFB).

For this purpose, the WSOS from the WBFB were subjected to detailed structural analyses using various analytical and spectroscopic techniques while the various physical properties investigated included morphology, thermal characteristics and emulsifying activities compared with the physico-chemical properties of GAO. This study will be helpful in determining if the obtained WSOS from the WBFB are appropriate for commercial applications.

2. Experimental

2.1. Production and isolation of WSOS

The WSOS from the WBFB synthesized and isolated in previous investigations (Khan, Hyun, et al., 2007) were used in the present study. The colonies of *G. hansenii* were inoculated in a 50 ml medium in a 250 ml flask, shaken at 200 rpm and cultured at 30 °C for 24 h. This culture broth (150 ml) was inoculated into 3 L of the WBFB in a 5 L jar fermenter (Kobiotech Co., Korea), which was equipped with a six flat-blade turbine impeller. The fermentations were carried out at 30 °C with an agitation rate of 600 rpm and an aeration rate of 1 vvm. For the isolation of WSOS from the WBFB, the supernatant obtained by centrifuging the culture broth for 20 min at 3580*g*, was treated twice with 5 volumes of ethanol for 1 h at 4 °C. Both times, the precipitates were separated by centrifugation and finally dried at 60 °C in an oven until a constant weight was achieved.

2.2. The hydrolysis of the WSOS and an analysis of the monosaccharides

The dried WSOS from the WBFB were hydrolyzed with HCl as reported previously (Park et al., 2006). The monosaccharide composition of the hydrolysates was determined with an HPLC (model 600E, Waters Co.) that was equipped with a Sugar-Pak I column (6.5 × 300 mm) and a RI-detector (Model 410, Water Co). The Ca-EDTA buffer (0.5%) was used as the mobile phase and the analysis was carried out at a flow rate of 0.5 ml min^{-1} at 30 °C. The optical rotation of the hydrolyzed product was measured with a polarimeter (AP-100, ATAGO, USA) at 27 °C.

2.3. Spectroscopic studies

The FT-IR spectrum of the WSOS from the WBFB was acquired using a Bruker (Germany) FT-IR-IFS120HR/FRA106. The WSOS from the WBFB sample was pressed into KBr pellets and the spectra were recorded in a transmittance mode over a wavelength range between 4000 and 400 cm^{-1}.

The ^{1}H and ^{13}C NMR spectra of the WSOS from the WBFB were obtained in D_2O (99.8% atom D; Merck, Germany) using a FT-NMR spectrometer (Varian Inova, USA), at 500 and 125 MHz, respectively. The chemical shifts were referenced to acetone.

The matrix-assisted, laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrum of the WSOS from the WBFB was obtained with a PE Voyager DE-STR biospectrometry work station with a N_2 laser (337 nm, 0.5-ns pulse width, 20-Hz repetition rate) (Applied Biosystems). α-Cyano-4-hydroxycinnamic acid was used as the matrix.

2.4. Morphology and thermolytical studies

To determine the morphology and surface topography, the micrographs of the platinum-coated WSOS were taken with a field-emission scanning electron microscope (S-4300; Hitachi Co., Japan).

A thermal gravimetric analysis (TGA) of the WSOS from the WBFB was carried out using a thermo gravimetric/differential thermal analyzer (Seiko Instruments Inc.), under a nitrogen atmosphere between 30 and 800 °C with temperature increments of 20 °C min^{-1}.

The pyrolysis pattern and energy level of the WSOS from the WBFB was measured with a differential scanning calorimeter (TA4000/Auto DSC 2910 system; TA instruments), between 30 and 300 °C with a temperature increase of 20 °C min^{-1}.

2.5. Emulsifying properties

The emulsions of the WSOS from the WBFB solutions (0–0.3% w/v in a 0.067 M sodium phosphate buffer, pH 7.4) in olive oil were prepared and diluted as reported previously (Khan, Khan, et al., 2007). The emulsifying ability of the WSOS from the WBFB was determined from the absorbencies of the resulting emulsions obtained at 500 nm with a UV–VIS spectrophotometer (UV-1201; Shimadzu, Japan). The stability of the emulsion (prepared with 0.1% WSOS) was evaluated by observing the separation of the aqueous and oil phases of the emulsion incubated at ambient temperatures (Khan, Khan, et al., 2007).

3. Results and discussion

3.1. The chemical structure of the WSOS

The chemical structure of the isolated WSOS from the WBFB was investigated by the analysis of the hydrolyzed products and spectroscopic studies (FT-IR, ^{1}H and ^{13}C NMR and MALDI-TOF MS) of the native WSOS from the WBFB.

3.1.1. Analysis of the hydrolyzed products of WSOS

Hydrolyzed products yielded a single peak in the HPLC chromatogram with a well-matched retention time (5.37 min) and a shape to the peak for standard D-glucuronic acid (retention time 5.36 min) (Fig. 1). Moreover, the hydrolyzed products showed a positive optical rotation, which confirmed the dextro (D) configuration for the glucuronic acid. In our previous studies (Park et al., 2006), the oligosaccharides that were produced from a defined medium (GAO) by *G. hansenii* PJK also yielded D-glucuronic acid upon hydrolysis. The native product was found to be a mixture of homo-oligomers of α-D-glucuronic acid. The major oligomer contained an unsaturation at the non-reducing terminus (Fig. 4b), which was reduced to D-glucuronic acid upon hydrolysis (Park et al., 2006). Similarly, several other microbial polysaccharides have been reported to be the homo-polymers of glucuronic acid (Courtois, Courtois, Heyraud, Colin-Morel, & Rinaudo, 1992; Courtois et al., 1993; Valla & Kjosbakken, 1981) or to contain glucuronic acid moiety in their hetero-polymer structures (Michaud et al., 1994).

3.1.2. Spectroscopic studies of the native WSOS

The FT-IR spectrum of the WSOS from the WBFB was nearly the same as that reported for GAO (Park et al., 2006). The assignments to the important absorption bands that appeared in the IR spectrum of WSOS are presented in Table 1, which revealed the presence of hydroxyl (hydrogen bonded) and carbonyl functions and glycoside linkages in the molecules (Table 1).

There were two major differences between the ^{1}H NMR of the WSOS from the WBFB (Fig. 2) and GAO (Park et al., 2006); (i) the presence of signals around 2 ppm and (ii) the upfield shift of the peak for an anomeric proton from 5.639 to 5.381 ppm. Signals around 2 ppm are characteristic for *O*-acetyl group(s) and thus, the WSOS from the WBFB might be partly acetylated in contrast to GAO (Park et al., 2006). Furthermore, the proton resonance at 5.381 ppm ($J = 3.62$ Hz) can be assigned to H-1 of *O*-methyl-α-glucuronic acid, suggesting that some glucuronosyl units of the WSOS are *O*-methylated. This

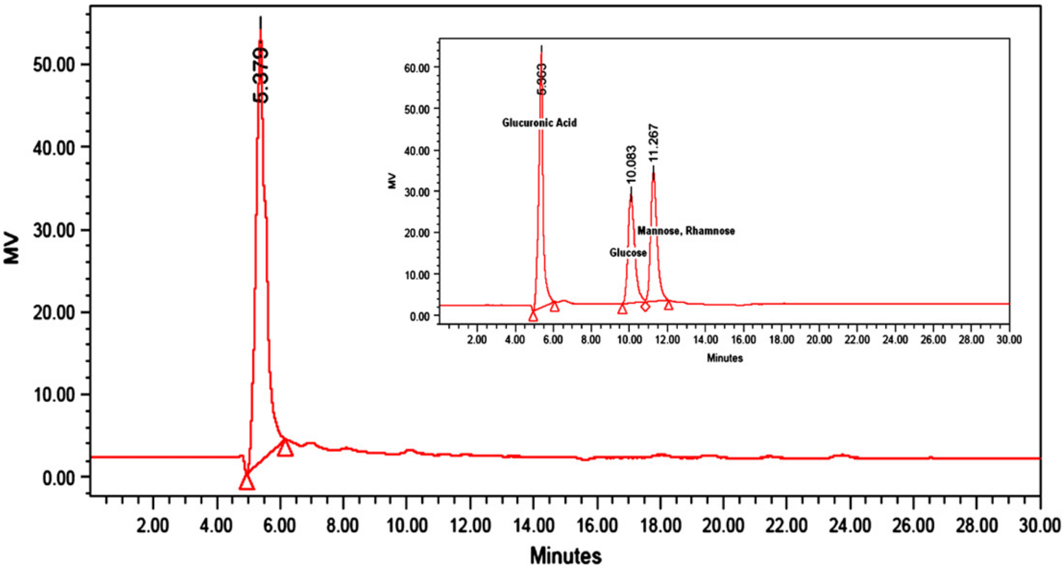

Fig. 1. A HPLC profile of the hydrolysates of WSOS that were produced from the waste from beer fermentation broth by *G. hansenii* PJK. The peaks in the upper right show standard monosaccharides.

Table 1
Assignments to the important absorption bands in the FT-IR spectrum of WSOS, which were produced using the waste from beer fermentation broth by *G. hansenii* PJK

Wavenumber (cm^{-1})	Assignments
3434	O—H stretching (hydrogen-bonded)
2921	C—H stretching
1635	C=O asymmetric stretching
1418	C=O symmetric stretching
1027	C—O—C stretching

O-methylation could be at the *O*-4 position (Kardošová, Matulová, & Malovíkovà, 1998; Park et al., 2006; Shatalov, Evtuguin, & Neto, 1999) of the terminal glucuronosyl unit(s).

The ^{13}C NMR spectrum of the WSOS from the WBFB (Fig. 3) displayed a single anomeric peak at 102.53 ppm, indicating that only one type of sugar is present in the structure of WSOS from the WBFB. Moreover, this peak was in good agreement to that of the ^{1}H NMR data and revealed the α configuration of the glucuronic acid. Similarly, two other peaks appeared at 177.3 and 175.09 ppm due to the C-6 and C=O of the acetyl group(s). The ring protons appeared in the region of 60–80 ppm and *O*-CH$_3$ carbons were found at 50–60 ppm. The peaks at 26.516 and 24.079 ppm could be due to the methyl of the acetyl groups.

From the MALDI-TOF mass spectrum, the WSOS appeared to be a mixture of oligosaccharides. The peak at *m/z* 1044 could be the molecular ion peak [M+H]$^{+}$ for the major oligosaccharide in which all carboxylic functions, except the terminal glucuronosyl unit, are present in a sodium salt form. The molecular weight of 1043 was found to be consistent with the structure shown in Fig. 4a, which is derived from the combination of data of the HPLC analysis of the hydrolyzed products, and the FT-IR, NMR and MALDI-TOF mass spectrum data of the native WSOS. The base peak in the MALDI-TOF mass spectrum was observed at *m/z* 793 due to the loss of the terminal glucuronosyl unit (C1) from the molecule. The fragmentation pattern (Fig. 4a) and important fragments of this compound are displayed in Table 2 and these are consistent with those reported for acidic oligosaccharides (Park et al., 2006; Schiller, Arnhold, Benard, Reichl, & Arnold,

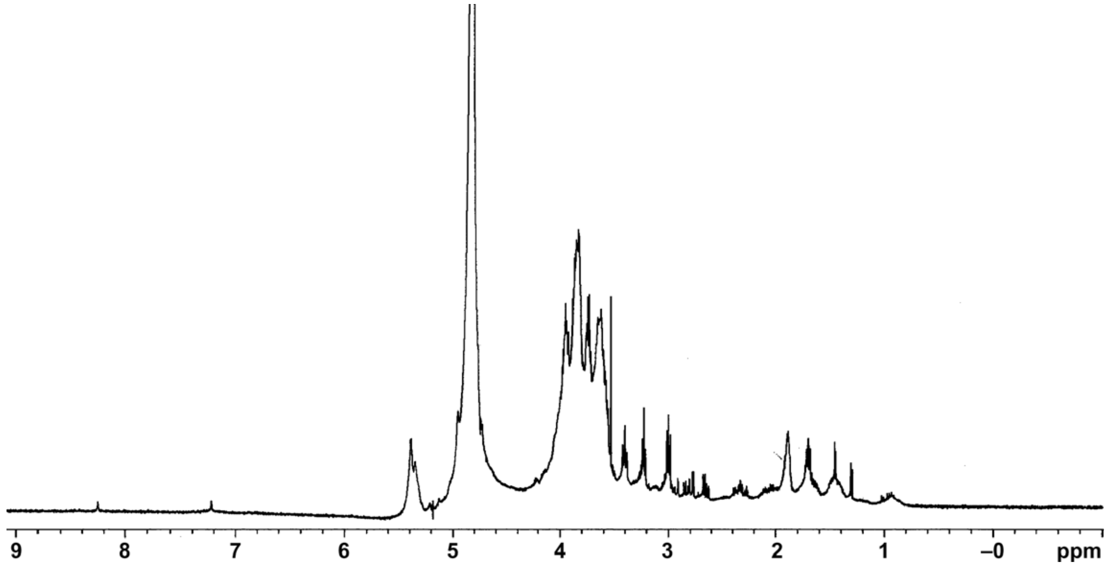

Fig. 2. The ^{1}H NMR spectrum of WSOS that were produced from the waste from beer fermentation broth by *G. hansenii* PJK.

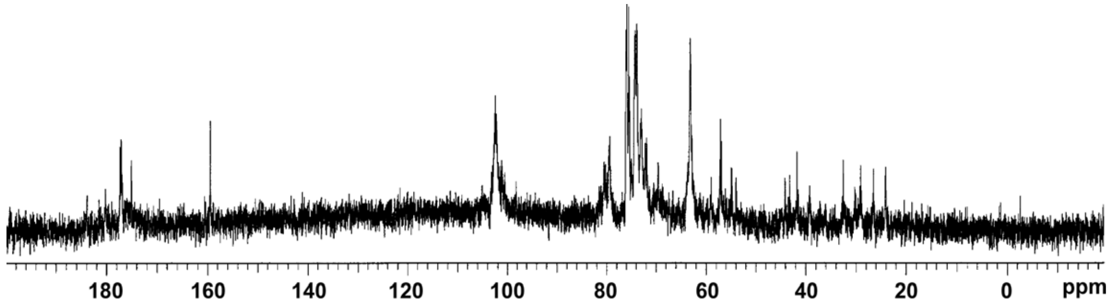

Fig. 3. The ^{13}C NMR spectrum of WSOS that were produced from the waste from beer fermentation broth by *G. hansenii* PJK.

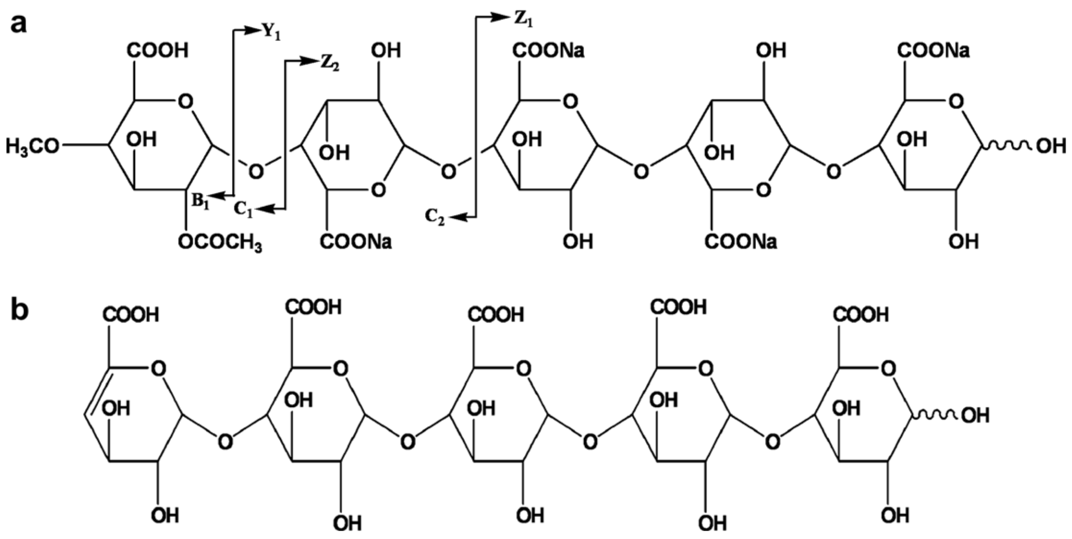

Fig. 4. The chemical structures of the major glucuronan oligosaccharides produced by *G. hansenii* PJK from (a) the waste from beer fermentation broth and (b) a chemically-defined medium. The (a) also shows the fragmentation scheme for the major oligoglucuronate in the mixture.

Table 2
A fragmentation analysis of the major oligoglucuronate found in the mixture of WSOS, which were produced from the waste from beer fermentation broth by *G. hansenii* PJK

S.No.	*m*/*z*	Species	Type of fragment
1	1044	$C_{33}H_{43}O_{32}Na_4$	$[M+H]^+$
2	1028	$C_{32}H_{40}O_{32}Na_4$	$[M-CH_3]^+$
3	969	$C_{30}H_{37}O_{30}Na_4$	$[M-CH_3-OAc]^+$
4	953	$C_{30}H_{37}O_{29}Na_4$	$[3+2H]-H_2O$
5	905	$C_{29}H_{34}O_{27}Na_4$	$[M+H]-OAc-COOH-CH_3-H_2O$
6	810	$C_{24}H_{29}O_{25}Na_4$	$[M-B1]$
7	793	$C_{24}H_{28}O_{24}Na_4$	$[M-C1]$
8	701	$C_{24}H_{28}O_{24}$	$[M-Cl-4Na]$
9	685	$[8+2H]-H_2O$	$[8+2H]-H_2O$
10	527	$C_{18}H_{22}O_{18}$	$[M-C2]$
Other possible oligomers			
11	452	Acetylated dimer	
12	236	Acetylated monomer	
13	175	Glucuronosyl unit	

1999). The presence of other oligomers has also been determined from the same spectrum (Table 2).

From these spectroscopic studies, in combination with the HPLC analysis of the hydrolyzed products, it is apparent that WSOS from the WBFB are a mixture of oligomers all having α-glucuronic acid as a building block. The major oligosaccharide is in the form of sodium oligoglucuronate with *O*-acetyl and *O*-methyl groups in the terminal unit of the non-reducing end (Fig. 4a). The HPLC analysis of the hydrolysates revealed the presence of only D-glucuronic acid, which may be due to the substitution of H^+ for Na^+ and the cleavage of the attached *O*-acetyl and *O*-methyl groups during the acid hydrolysis, thus all monomers are yielded as glucuronic acid. In a nutshell, WSOS from the WBFB have only a few structural differences from GAO (Park et al., 2006). The major dissimilarities include the presence of *O*-acetyl and *O*-methyl groups and a lack of unsaturation in the terminal unit of the non-reducing end of WSOS as shown in Fig. 4a and b.

The ^1H, ^{13}C NMR and MALDI-TOF MS spectra revealed some unidentified peaks, which may be due to the presence of impurities or to the complex nature of the mixture.

3.2. The physical properties of the WSOS

3.2.1. Morphology

The FE-SEM micrographs of the WSOS from the WBFB (Fig. 5a and b) revealed morphological characteristics similar to those reported for GAO (Khan, Khan, et al., 2007; Park et al., 2006). They were flake-type of different sizes and shapes with no attached bacterial cells (Fig. 5a). The WSOS from the WBFB had porous surfaces and appeared to be composed of small agglomerates (Fig. 5b). The similarities in the physical structure of the WSOS from the WBFB to GAO (Khan, Khan, et al., 2007) may be due to their chemical structures and molecular weight range (Park et al., 2006).

3.2.2. Thermal characteristics

The TGA thermogram of the WSOS from the WBFB (Fig. 6) displayed consistent degradation without a major weight loss throughout the temperature range. Compared to GAO (Khan, Khan, et al., 2007), the WSOS from the WBFB showed three major differences; (i) minor weight loss in the first step, (ii) faster initiation of the degradation of the main polymer structure and (iii) overall better thermal stability up to 650 °C. The weight loss in the first step is due to the elimination of the initial moisture present in a polysaccharide due to carboxyl functions (Kumar, Joo, Choi, Koo, & Chang, 2004). The carboxyl functions in

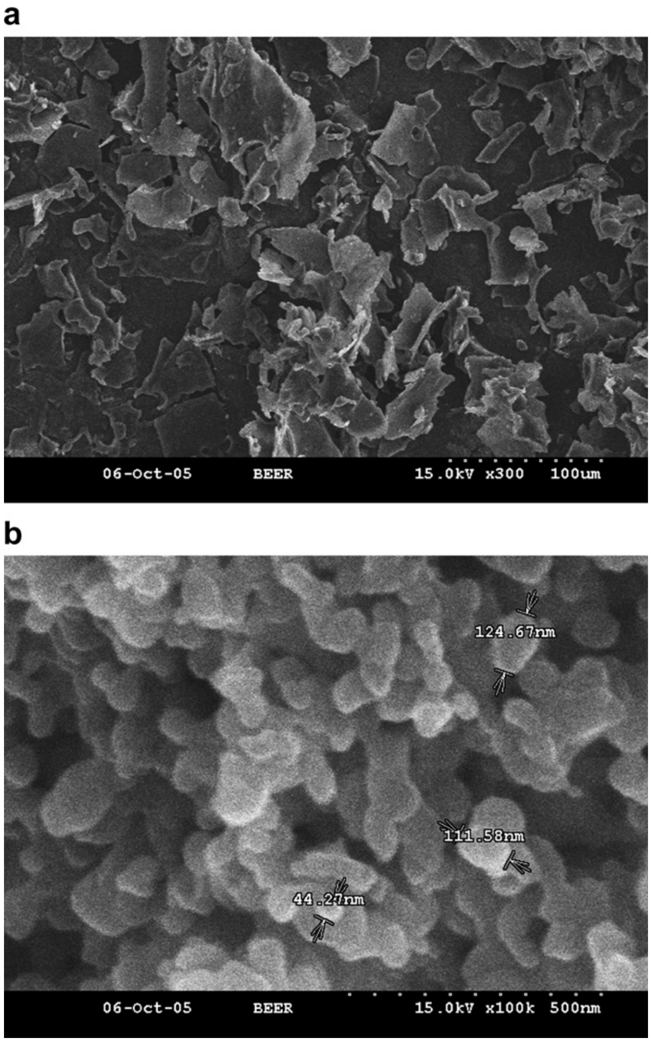

Fig. 5. SEM photographs of the WSOS produced from the waste from beer fermentation broth by *G. hansenii* PJK.

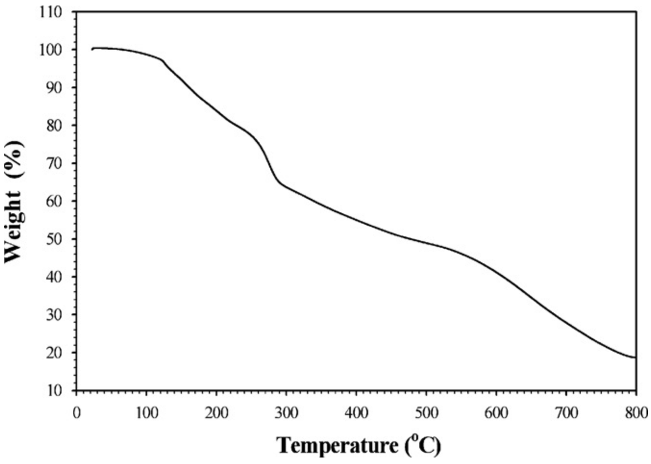

Fig. 6. The thermal gravimetric analysis (TGA) curves of WSOS that were produced from the waste from beer fermentation broth by *G. hansenii* PJK.

the WSOS from the WBFB are in a sodium salt form and thus, are expected to have a low water holding capacity. The true initiation of the thermal degradation of WSOS from the WBFB seemed to occur at 120–130 °C, which is much lower than the temperature (~230 °C) required for the initiation of the degradation of GAO (Khan, Khan, et al., 2007). Gröndahl, Teleman, and Gatenholm (2003) reported that water in samples promotes hydrolysis through 4-*O*-methyl glucuronic acid, which speeds up the decomposition process. As the WSOS from the WBFB possess a 4-*O*-methyl glucuronic acid unit in their structure, a faster onset of the thermal degradation may occur compared to GAO which have no such moiety in their structure (Khan, Khan, et al., 2007; Park et al., 2006). The WSOS from the WBFB have an overall better thermal stability than that of GAO (Khan, Khan, et al., 2007). According to Gröndahl et al. (2003) the thermal stability of glucuronoxylan is improved by acetylation. The WSOS from the WBFB contains acetyl group(s) which may enhance their thermal resistance.

In the DSC thermogram (Fig. 7), the melting point of the WSOS from the WBFB appeared at 130.16 °C with endothermic calories of 104.6 J/g, while the pyrolysis temperature was found to be 275.25 °C with an enthalpy of 43.15 J/g. The pyrolysis temperature values of the WSOS from the WBFB are closer to those reported previously for GAO (278 °C) (Khan, Khan, et al., 2007). The endothermic peak at around 92 °C, denoting the loss of water from the molecule, which appeared for GAO (Khan, Khan, et al., 2007) was absent in the WSOS from the WBFB thermogram. As evident from the TGA, the WSOS from the WBFB do not contain a significant amount of water and thus, there was no respective peak in the DSC thermogram (Fig. 7).

3.2.3. Emulsifying properties

The emulsifying ability of the WSOS from the WBFB (0–0.3% w/v) (Fig. 8) displayed nearly the same pattern

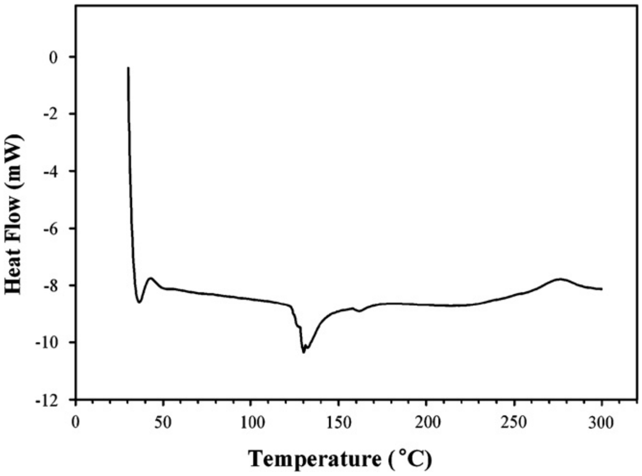

Fig. 7. Differential scanning calorimetry (DSC) curves of WSOS that were produced from the waste from beer fermentation broth by *G. hansenii* PJK.

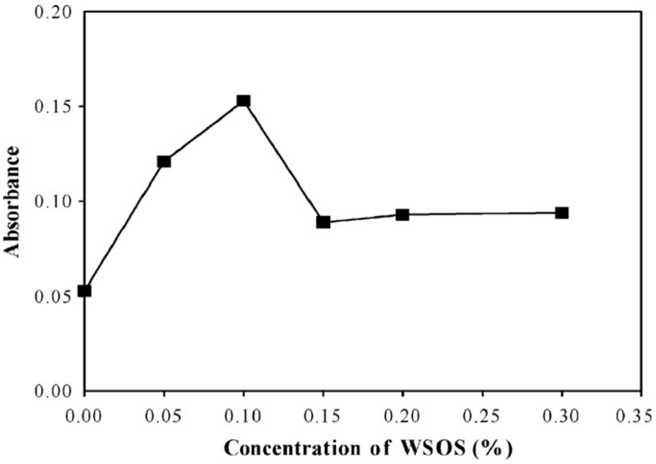

Fig. 8. The emulsifying ability of WSOS that were produced from the waste from beer fermentation broth by *G. hansenii* PJK.

and trend to those reported for GAO (Khan, Khan, et al., 2007). However, the WSOS from the WBFB required a lower concentration for maximum emulsification and they suffered a more abrupt decrease in their emulsion ability beyond the optimum concentration. The optimum concentration was found to be 0.1% in contrast to 0.15% for GAO (Khan, Khan, et al., 2007). The emulsions prepared with an optimum concentration (0.1%) of WSOS from the WBFB were unstable in the same way as those prepared with GAO (Khan, Khan, et al., 2007). The initial emulsion was homogeneous, but soon started to break up into oil and aqueous layers. A complete partition of two layers was observed after 24 h. The weak emulsion stability could be attributed to the oligomeric nature of WSOS from the WBFB and being free of any protein contamination (Dickinson, 2003).

4. Conclusions

From these results, we can conclude that waste from beer fermentation broth is a valuable alternate and a cheap source that can be used to obtain glucuronic acid oligomers. These oligomers have only few structural differences from GAO. The major dissimilarities include the presence of *O*-acetyl and *O*-methyl groups and a lack of unsaturation in the terminal unit of the non-reducing end of WSOS. The WSOS from the WBFB have similar or even better thermal and emulsifying characteristics than GAO and thus, they have the potential to be developed for commercial applications.

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