https://doi.org/10.1016/j.ijbiomac.2018.01.052

Submitted to: *International Journal of Biological Macromolecules*

(Original Research MS) *Revised* IJBIOMAC_2017_3097

Bifidogenic effects of *Cordyceps sinensis* fungal exopolysaccharide and konjac glucomannan after ultrasound and acid degradation

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Short running title: **Prebiotic effects of partially degraded polysaccharides**

Abstract

The bifidogenic effects of exopolysaccharide (EPS) of a medicinal fungus (*Cordyceps sinensis*) and a well-known food polysaccharide konjac glucomannan (KGM) with different molecular weight (MW) ranges were evaluated through *in vitro* experiments in liquid cultures of *Bifidobacteria*. Native EPS and KGM were partially degraded with power ultrasound (US) to improve the water solubility, and further hydrolysed with trifluoroacetic acid to much lower MW. The acid-hydrolysed fractions (EPS-AH and KGM-AH) supported the growth of all five tested bifidobacterial species, while the US-degraded high MW fractions, EPS-US and KGM-US, could only slightly supported the growth of some species. All EPS fractions increased the acetic acid production of most bifidobacterial species. Most remarkably, the high MW EPS-US, EPS-AH and KGM-US fractions significantly enhanced the cell viability with much higher colony forming unit (CFU) counts, suggesting a protective effect of these high MW polysaccharides for the bacterial survival. The results have shown that MW was a significant factor on the bifidogenic properties of partially degraded EPS and KGM.

*Keywords: Bifidobacteria***;** Polysaccharide; Prebiotic; Molecular weight; Partial degradation

1. Introduction

The gut microbiota or microflora plays an important role in human health and disease. Prebiotics are non-digestible food ingredients such as carbohydrate fibres that can selectively stimulate the growth and metabolic activity of certain bacteria in the colon in a way beneficial to the host health [1]. The undigested carbohydrate fibres enter the large intestine or colon and

may be utilized as the carbon sources by the gut bacteria via anaerobic metabolism [2]. The chief end metabolite products of bacterial fermentation in the gut are short chain fatty acids (SCFAs) including acetate, propionate and butyrate, which have important physiological functions and health benefits to the host. The ingestion of prebiotic foods and supplements has been increasingly taken as a viable measure for restoring and maintaining the healthy balance of gut microbiota so as to improve human health [3, 4]. However, only a small number of carbohydrate polymers or oligomers are generally accepted and applied as probiotic ingredients including fructooligosaccharides (FOS), galactooligosaccharides (GOS), and inulin extracted from chicory root [1, 4].

Natural polysaccharides from edible plants and fungi are regarded as potential sources of prebiotics [5]. Because of the important role of gut microbiota in human health and disease and the increasing demand for prebiotic products, it is of significance to evaluate the prebiotic function of new and alternative probiotic materials. A common and feasible approach for evaluating the prebiotic function of samples is assessment of their effects on the *in vitro* growth and SCFA production of probiotic bacterial species, mainly *Bifidobacteria* and *Lactobacilli* in culture. Many previous studies have evaluated the effects of natural polysaccharides from food sources on the cell growth and production of SCFAs during *in vitro* fermentation of probiotic bacteria. Among the various molecular properties molecular weight (MW) is an important factor affecting the fermentability and prebiotic activity of natural polysaccharides and their hydrolysed low-MW derivatives [6]. In general, carbohydrate fibres with lower MW were more fermentable in bacterial cultures as most of the prebiotic fibers belong to low to medium MW

carbohydrate polymers. Therefore, high MW natural polysaccharides have been hydrolyzed by enzymes or acids to improve the prebiotic activities in many previous studies [3].

Konjac glucomannan (KGM) is a water-soluble polysaccharide from the root of *Amorphophallus konjac* plant which has been traditionally used as a food ingredient in China and some other Asian countries. KGM or konjac flour has been widely used as a thickening and gelling additive to food products because of its desirable physical properties. KGM is a linear copolymer of (1→4) linked β-D-mannose and β-D-glucose at molar ratio of 1.6:1 [7]. More recently, KGM has been recognized as a dietary fibre and functional food supplement for relieving constipation and lowering the cholesterol level and the risk of type 2 diabetes and obesity [8, 9], and other health benefits [10]. As a major source of dietary fibre, the health benefits of KGM are most probably associated with its potential prebiotic function [11]. Enzymatic hydrolysates of KGM have been shown to promote the growth of *Lactobacilli* and *Bifidobacteria* in culture [12].

Edible and medicinal fungi or mushrooms provide a rich source of health promoting polysaccharides with notable antitumor, immunomodulatory and other bioactivities. Most of the bioactive polysaccharides from mushrooms are non-starch with complex structures and various glycosidic linkages and are usually non-digestible in the human gastrointestinal tract and can be potential prebiotic fibres for the gut microbiota [13]. Therefore, mushroom has been recognized as a potential source of prebiotics [5]. However, the potential of mushroom as a valuable source of prebiotics remains to be widely explored. To date only a small number of polysaccharides from mushrooms have been evaluated for prebiotic function compared to the many of bioactive polysaccharide documented in the literature. *Cordyceps sinensis* generally

called Chinese caterpillar fungus or Dong-Chong-Xia-Cao is a special and highly-valued medicinal fungus which has been used in traditional Chinese medicine mainly as a tonic with numerous health benefits [14]. Polysaccharides are the major bioactive constituents of *C. sinensis* fungus with antitumor, immunomodulatory and some other medicinal properties [15]. As natural *C. sinensis* caterpillar fungus is very rare and expensive, mycelial fermentation is mainly applied for commercial production of *C. sinensis* biomass and polysaccharides. We have established the mycelial culture of a *C. sinensis* fungus Cs-HK1 and applied it to liquid fermentation for the production of exopolysaccharide (EPS) [15, 16]. However, little or no information is available on the prebiotic activity of polysaccharides originated from the *C. sinensis* species.

This study was to evaluate the bifidogenic effects in relationship to the MW range of KGM and the EPS of Cs-HK1 based on their utilization as an alternative carbon sources to glucose in liquid cultures of several common bifidobacterial species. The native KGM and EPS were first degraded with high-intensity power US for higher water solubility, and then further hydrolysed with trifluoroacetic acid to much lower MW range. Their effects on the growth, viability and SCFA production of the bifidobacteria were measured in comparison with some known prebiotic OS.

2. Materials and methods

2.1. Production and recovery of EPS from Cs-HK1 mycelial fermentation

Cs-HK1 is a fungus species which was isolated from a wild *Cordyceps sinensis* fruiting body by Wu's group and has been maintained in mycelial culture as reported previously [16].

As described previously, the liquid medium for Cs-HK1 mycelial fermentation consisted of 40 g/L glucose, 5 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 10 g/L yeast extract. The liquid fermentation was carried out in 1 L Erlenmeyer flasks each filled with 200 mL of the liquid medium at 20 °C in a shaking incubator at 200 rpm for 7 days. The mycelial fermentation liquid was then centrifuged at 12,000 rpm (21,612×g) for 15 min and the supernatant medium was collected for recovery of EPS. Ethanol (95%) was added into the solid-free liquid medium at 5:1 volume ratio and maintained at 4 °C overnight for precipitation. The precipitate was separated by centrifugation at 12,000 rpm (21,612×g) for 15 min and then freeze-dried, yielding EPS. The whole experimental procedure for isolation and purification of EPS and the preparation of partially degraded EPS fractions is shown with a flowchart in Supplemental data (Fig. 1).

2.2. Ultrasonic degradation of EPS and KGM

The native EPS produced by the Cs-HK1 fermentation and the KGM attained from the commercial supplier had very high MW and low water solubility, they were exposed to power ultrasound (US) for partial degradation to lower MW and higher solubility. Ultrasonic degradation of EPS and KGM and the recovery, and purification of the degraded fractions were carried out as reported previously with minor changes [17, 18]. Power US was generated with a VCX 750 processor with a fixed frequency of 20 kHz and a maximum output power of 750 W (Sonics and Materials Inc., Newton, USA). For preparation of US-degraded of EPS, the supernatant from centrifuged Cs-HK1 mycelial fermentation liquid (1 L) was transferred into a glass beaker and the US probe (with a tip diameter 13 mm) was inserted into the sample liquid

for irradiation. During the US treatment, the sample beaker was surrounded with ice to avoid overheating. Based on our preliminary test, the US power was fixed at 80% amplitude and the treatment period was 30 min. After the US treatment, ethanol precipitation was performed and the precipitate was recovered and dried as described above, yielding the US-degraded EPS, EPS-US. The crude EPS-US was further purified through a series of steps as shown in Supplemental data (Fig. 1). The crude EPS-US (5 g/L) was first deproteinized by Sevage treatment (1-butanol/chloroform at 1:4 v/v) at 25 ^{o}C with constant stirring for 30 min and then centrifuged to remove denatured protein. This step was repeated several times until no protein layer was visible. The deproteinized EPS-US solution was dialysed using a 3.5 kDa membrane against water for 48 h. The purified EPS-US solution was concentrated by vacuum evaporation and then lyophilised.

The raw KGM material with a purity over 90% was attained from Hubei Konson Konjac Gum Co., Ltd. (Hubei, China) and used without purification. The KGM solution (10 g/L in deionized water) was treated by US for 30 min in the same conditions as for the Cs-HK1 EPS. The US-degraded KGM was recovered and purified to attain the KGM-US fraction through the same procedure as for the EPS-US.

2.3. Acid hydrolysis of ultrasound-degraded EPS and KGM

For more extensive degradation of the polysaccharides into much lower MW fractions, the purified EPS-US and KGM-US were hydrolysed with trifluoroacetic acid (TFA). Each 0.15 g of the PS sample mas mixed with 60 mL of 2 M TFA in a 100 mL round-bottomed flask at 70 ^{o}C for 4 h with vigorous stirring. The liquid was evaporated to dryness at 40 ^{o}C under vacuum

in a rotary evaporator, washed with methanol, and then re-dissolved in 10 mL water. The solution was freeze dried to give the acid hydrolysed EPS (EPS-AH) and KGM (KGM-AH).

2.4. Chemical composition and molecular weight analysis

The total carbohydrate content of all polysaccharide samples (native and degraded EPS and KGM) was determined by Anthrone test, involving the acid hydrolysis of polysaccharide samples in the presence of anthrone agent to yield a yellow green colour at 100 ^{o}C [19]. The absorbance of sample solution was measured at 620 nm with a spectrophotometer and glucose was used as a standard. The protein content was determined by Lowry method [20] through the reaction with copper ions at 100 ^{o}C under alkaline condition to generate a blue colour. The absorbance of sample solution was measured at 750 nm using bovine serum albumin (BSA) as a standard.

Monosaccharide composition was analysed by the 1-phenyl-3-methyl-5-pyrazolone high-performance liquid chromatograph (PMP-HPLC) method as described previously (Siu et al., 2014) with minor modifications. The analysis was performed with an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm) on an Agilent 1100 instrument at 25 ^{o}C. Potassium phosphate buffered saline (0.05 M, pH 6.9) containing 15 % (solution A) and 40% acetonitrile (solution B) was used as the mobile phase. The sample (~5 mg) was hydrolysed with 2 mL of 2 M TFA at 110 ^{o}C for 4 h, followed by vacuum evaporation under. The residual solid was re-dissolved in 2 mL DI water and mixed 0.5 M PMP solution in methanol and 0.3 M NaOH solution at an equal volume (450 μL) and maintained at 70 ^{o}C for 30 min. The reaction was

stopped by adding 450 μL of 0.3 M HCl, followed by washing thrice with chloroform, and the aqueous layer was collected for the HPLC analysis.

Molecular weight (MW) of the polysaccharide samples was determined by high-pressure gel permeation chromatography (HPGPC) as reported previously [21]. The HPGPC instrument consisted of a Waters 1515 isocratic pump, a 2414 refractive index detector and three columns in series, Ultrahydrogel 120, 250 and 2000 (7.8 mm × 300 mm) (Waters Co., Milford, MA, USA). The column temperature was fixed at 50 ºC. Milli-Q water was used as the mobile phase at a flow rate of 0.6 mL/min. All samples were dissolved in Milli-Q water (0.2 mg/ml for EPS-US and KGM-US and 3 mg/ml for EPS-AH and KGM-AH), centrifuged at 6000 rpm (4020×g) for 15 min, and filtered through 0.45 μM membrane before injection into the HPGPC system. Calibration curve was derived with nine dextran MW standards ranging from 1 to 670 kDa and computed with the Breeze V3.3 software.

2.5. Bacterial species, culture conditions and inoculum preparation

Five species of *Bifidobacteria* from China Centre of Industrial Culture Collection (CICC) were used in this study including *B. adolescentis* (CICC 6070), *B. bifidum* (CICC 10395), *B. breve* (CICC 6079), *B. infantis* (CICC 6069) and *B. longum* (CICC 6186). The bacterial species were routinely stored in 30% glycerol at -80ºC. For experiments the bacteria were cultured in liquid Reinforce Clostridial Medium (RCM) broth containing 10.0 g/L beef extract, 10.0 g/L peptone, 3.0 g/L yeast extract, 1.0 g/L soluble starch, 5.0 g/L glucose, 0.5 g/L cysteine HCl, 5.0 g/L sodium chloride, 3.0 g/L sodium acetate, and 0.5 g/L agar [22]. To initiate the liquid culture, the bacterial strain from the storage was inoculated and incubated on solidified RCM

broth with 1.45% (w/v) agar (A 4675, Sigma-Aldrich, USA) for about 2 days. A single colony spot taken from the solid culture was inoculated into 5 ml of RCM broth liquid medium in a 10 ml centrifuge tube and incubated with shaking at 200 rpm for about 22 h. The culture period for the starter culture was chosen based on our preliminary experiments when the culture was in the stationary phase at a stable concentration. The final bacterial suspension was inoculated at 1% (v/v) into RCM liquid broth under the same conditions for the culture experiments. All bacterial cultures were maintained at 37 ^{o}C under anaerobic condition in air-tight jar with anaerobic gas-generating sachets (AnaeroGen TM, Thermo Scientific Oxoid, USA) [22].

2.6. Medium preparation for experiments on EPS and KGM

For the experiments on various carbohydrates as the alternative carbon sources to glucose for the bacterial growth, all the carbohydrate samples were added at 5 g/L (~0.5% w/v) to a glucose-free RCM broth (5 mL) in 10 mL centrifuge tubes and stirred at room temperature for overnight. Undissolved portion of EPS and KGM, if any, was removed by centrifugation at 4000 rpm (1780×g). Galactooligosaccharide (GOS, 5 g/L) was used as a prebiotic reference and glucose-free RCM broth was used for the control group. In the experiments on combination of EPS or KGM fraction with glucose or GOS, the two carbohydrates were each added at 5 g/L to make a total of 10 g/L in the culture medium. The glucose-free RCM broth was sterilized by autoclaving at 121^{o}C for 20 min while all the carbon sources including glucose, EPS, KGM and GOS were sterilized (in a thin layer in petri dish) with ultraviolet (UV) light inside a biosafety cabinet for 60 min (30 min on each side). Complete sterilization was assured by free

of contamination after incubating RCM agar containing the UV-sterilized carbohydrate sources for several days.

2.7. Measurement of bacterial growth and viability

The growth of bacterial cultures on various carbon sources was evaluated based on the final concentration and the colony-forming unit (CFU) (viability) of bacteria in the culture medium over a selected culture period. Bacterial concentration in the liquid culture medium was determined by measuring the optical density (OD) at 600 nm with a spectrophotometer. For CFU measurement, the bacterial suspension was diluted with 0.85% (w/v) saline sequentially from 10^{-1} to 10^{-10}, and then inoculated onto a solid RCM agar plate. After incubation in anaerobic atmosphere at 37 ^{o}C for 48 h, the number of viable colonies on the plate was counted, and valid counts in the range of 20-300 colonies were recorded.

2.8. Measurement of medium pH and SCFAs

The culture medium pH was measured before and after bacterial fermentation with a pH meter. Short chain fatty acids (SCFAs) in the bacterial culture medium were analysed by gas chromatograph (GC) according a documented method with minor modifications [23]. In brief, the bacterial culture liquid was diluted with four volumes of milli-Q water and the pH was adjusted to 2-3 with 1 M HCl, followed by centrifugation at 5000 rpm (2790×g) for 20 min. The supernatant was added with 2-ethylbutyric acid as an internal standard at 1 mM final concentration before injection into the GC system. The GC analysis was performed on an Agilent 7980B GC system equipped with a flame ionization detector (FID) (Agilent

Technologies Inc., USA) and a fused-silica capillary column (dimension 30 × 0.32 mm) coated with a free fatty acid phase of 0.25 film thickness (DB-FFAP 123-3232, Agilent Technologies Inc.). Nitrogen gas was used as the carrier gas flowing at 0.6 mL/min. The initial oven temperature was 80 °C, maintained for 2 min, then raised gradually at 6 °C/min to 180 °C, and held for 4 min. The detector was controlled at 220 °C and the sample liquid (1 μL) was injected at 200 °C. Several SCFA standards (Aladdin®, Shanghai, China) were used for identification and quantification including acetic acid (A116165), propionic acid (P110443), *n*-butyric acid (B110439), *i*-butyric acid (I103521), *n*-valeric acid (V108269), and *i*-valeric acid (I108280).

3. Results and discussion

3.1. Chemical composition and physical properties of EPS and KGM

Table 1 shows the major chemical contents of EPS, KGM and their partially hydrolysed products. The EPS samples had a similar carbohydrate content (~70%) but different protein contents, which were lowest in the native EPS (6.97%) and highest in EPS-US (18.4%). A possible explanation for the relatively low protein content of the native EPS was that EPS with a very high MW had a high viscosity and formed large aggregates which trapped the protein molecules. The US treatment of EPS disrupted the aggregates and lowered the viscosity, freeing the protein molecules [17]. In contrast, all native and degraded KGM samples had the similar carbohydrate content of 65-70% and protein content of ~2.5%. The HPLC analysis (Supplemental data, Fig. 2) shows that EPS contained three monosaccharide constituents including mannose, glucose and galactose at a molar ratio of 1.73:1.81:1, and KGM contained

two, mannose and glucose at molar ratio of 1.93:1, which were consistent with those reported previously [15, 24].

Fig. 2 shows the GPC profiles of native and partially degraded EPS and KGM samples. The native EPS exhibited several peaks, a large broad peak group, peak1 around 27.167 min with very high MW (~10^8) and several small peaks, e.g. peak 2 and peak 3 at 42.333 min and 50.867 min, respectively. The native KGM exhibited two major peaks at 26.283 min (MW~10^8) and 30.883 min, respectively. The GPC results indicate that both native EPS and KGM were composed of different MW fractions. Compared to the native EPS, the US-degraded EPS-US also had three major peaks but fewer small peaks, implying a more uniform MW distribution. In the GPC of EPS-AH, the high MW group peak 1 had a delayed elution time and reduced peak area; the two lower MW peaks peak 2 and 3 had similar elution time, and peak 3 became sharper and higher. The changes in the GPC profile and MW distribution of KGM after US and acid treatment were similar to those of EPS. All these changes could be attributed mainly to polymer degradation and reduction of MW, moderately by US and dramatically by acid hydrolysis.

Table 2 presents the MW distribution data of all EPS and KGM factions derived from the GPC analysis. Both native EPS and EPS-US consisted mainly of high MW fractions (over 10^8) and a small fraction of reducing sugar (MW 210-220) based on the relative peak areas. The acid hydrolysis resulted in significant reduction of the maximum MW (to ~7×10^5) and increase in the percentage of low MW fractions. Compared with EPS, the MW distribution and composition of KGM were affected more significantly by US degradation and acid hydrolysis with notable MW reduction and increase in the percentage of low MW fractions. In particular,

nearly 100% of KGM-AH was composed of low MW oligomer about 1400 [25]. Overall, the MW results showed that US is only effective for moderate reduction of MW and acid hydrolysis is a much more effective method for extensive degradation of high MW polysaccharides to small molecules such as oligomers.

3.2. Bacterial growth on glucose

Fig. 2 shows the growth curves of the five bifidobacterial species in the normal RCM liquid medium containing glucose as the major carbon source, which were all in a sigmoid pattern. Most of the bacterial species started with a lag growth phase for about 6 h with little or no increase in the cell concentration and then underwent a rapid growth period for 10-12 h with a notable increase in the cell concentration. Four of the bacterial species reached the stationary phase in about 18 h post inoculation, while *B. bifidum* reached the stationary after 24 h. The maximum cell concentration (OD) as well as the average growth rate differed among the bacterial species, being the highest (~1.0) with *B. adolescentis* and the lowest with *B. bifidum* (<0.5), and ~0.7 with the other three species. The maximum OD of all species remained at the similar level after 48 h of culture (data not shown).

3.3. Bacterial growth on degraded EPS and KGM as alternative carbon sources

Fig. 3 shows the results from the growth experiments of bifidobacterial species on different carbon sources. The acid hydrolysed polysaccharide fractions EPS-AH and KGM-AH supported a better growth of all five bacteria species (reaching a higher OD or bacterial concentration) compared with the control without a carbon source ($p < 0.05$ or $p < 0.01$). The

US-degraded fractions, EPS-US and KGM-US, only supported the growth of some species, i.e. EPS-US by *B. adolescentis* and *B. bifidum* ($p < 0.05$), and KGM-US by *B. bifidum* ($p < 0.05$). The results suggest that lower MW fractions from acid hydrolysis were more easily utilized or fermentable by the bifidobacteria. However, these partially hydrolysed polysaccharides were much less favourable for bacterial growth than glucose and the prebiotic GOS. In addition, GOS was more favourable than glucose for most of the bifidobacterial species especially *B. bifidum* and *B. breve* to grow to higher concentrations. When the acid hydrolysed EPS and KGM fractions were applied to the medium containing glucose or GOS (i.e. Glc or GOS+EPS-AH or KGM-AH), the growth of some bacterial species were enhanced, suggesting the ability of the bacterial species to metabolize different carbohydrates. However, addition of the US degraded EPS-US and KGM-US fractions significantly suppressed the growth of most bacterial species.

The better growth of bifidobacteria with lower MW KGM fractions derived from acid hydrolysis is in agreement with that reported previously on acid hydrolysed glucomannans as the carbon sources for growth of probiotic bacteria [11, 26, 27]. As reported by Chen et al. [28], acidic hydrolysates of glucomannans increased the cecal bifidobacterial counts in mice more significantly than the native glucomannans. Although KGM-AH could increase the growth of bifidobacteria in the present study, the effect was not so significant as in many previous reports. This may be attributed to the different bacterial strains used. Moreover, most previous studies were carried out in faecal cultures or animal models instead of pure cultures of single bacterial strains as in the present study. The cooperation and interaction among different bacterial

species may contribute to a better use of polysaccharides in mixed faecal cultures than in pure bacterial cultures with limited number of polysaccharide degrading enzymes.

The fermentability of a polysaccharide by the gut bacteria is species specific and may require the collaborative action of different species to provide all the enzymes for cleaving the different glycosidic linkages. Therefore, the complex carbohydrate polymers are less likely to be metabolized by a single bacterial species in pure culture. As no enzymes for hydrolyzing the carbohydrate polymers have been found in the extracellular media, polysaccharides and oligosaccharides are mainly metabolized inside the bacterial cells, probably after being hydrolyzed by cell-associated glycoside hydrolases into monosaccharides. The uptake of carbohydrates by the bifidobacterial cell is mediated by certain carbohydrate transport mechanisms such as ATP-binding cassette (ABC) transporters, permeases and proton symporters [29, 30]. The high MW and complex structure can make the carbohydrate more difficult to be transported into and metabolized by the cell . The suppressed bacterial growth by EPS-US or KGM-US combined with glucose or GOS was due probably to the interference of these high MW polymers with the transport of nutrients to and through the bacterial cell.

3.4. Effects of EPS and KGM fractions on bacterial viability

Table 3 shows the results of CFU measurement from bacterial cultures with different carbon sources. OD is proportional to the total bacterial cell concentration including both viable and dead cells. Colony forming unit (CFU) is a reliable measurement of the viable cells in the culture or the cell viability. Although the OD of all the species cultured with glucose or GOS as the carbon source was much higher than that in control, the CFU values of most bacterial

species were similar or only slightly higher. This means that the bacteria cultured in glucose and GOS medium contained a higher proportion of non-viable cells at the time of CFU measurement. This may be attributed to the depletion of limiting nutrients and accumulation of inhibitory metabolites in these cultures. Another possible cause for the higher cell death rate in these cultures is the more significant drop in the medium pH because the survival of probiotic bacteria is very sensitive to pH change during fermentation [31]. As shown in Table 4, the initial pH of RCM medium was about 6.6-6.8 and was notably lower in most bacterial cultures after fermentation for 48 hours, and the lowest in the cultures with glucose and GOS for most bacteria species. The pH drop was mainly attributed to acid production from anaerobic fermentation, which is more rapid at a higher growth rate.

More interestingly, most bacterial species cultured in medium with the partially degraded EPS and KGM fractions as the carbon sources attained a much higher CFU (Table 3). Among these EPS and KGM fractions, EPS-US was the most effective for maintaining bacterial viability, giving rise to the highest CFU for all five species. For most bacterial species, KGM-US was more favourable than KGM-AH to support a higher viability. The high MW polysaccharides (EPS-US and KGM-US) appeared very beneficial for the survival of bacterial cells, though they were not well utilized as a carbon source for the bacterial growth based on the above OD measurement. The results suggest that the EPS and KGM fractions have a protective effect on the survival and viability of probiotic bacteria and the protective effect is stronger at a higher MW.

Similar to the protective effect of EPS and KGM on the bifidobacteria in the present study, a previous study has shown that β-glucans produced by *Pediococcus parvulus* significantly

increased the survival of *Lactobacillius paracasei* in the acidic environment during gastrointestinal passage [32]. A few previous studies have also investigated the protective effect of natural polysaccharides from different sources on probiotic bacteria in the gastrointestinal condition [33-35]. The protective effect of high MW EPS and KGM fractions on the bifidobacteria may share the similar mechanisms as for the EPS produced by *Lactobacilli* or lactic acid bacteria (LAB) [36]. The bacterial EPS may be beneficial to the probiotic bacteria in adapting to extreme environments and protect the bacterial cells against biotic and abiotic stresses [37]. A possibility is that the EPS bind to the bacterial cell surface to form a physical barrier, protecting the cell against the environment stresses [38].

3.5. Short chain fatty acids (SCFAs)

The chief SCFA produced during the bacterial fermentation was found to be acetic acid (Table 5). For most bacterial species, the acetic acid content was highest in the cultures using glucose as the carbon source and lowest in the control culture without a carbon source. The higher acid concentrations in the cultures supplied with the partially degraded EPS and KGM than the control suggest their utilization and metabolism by the bacteria as a carbon source. Overall, there was a weak correlation between the acetic acid contents and the OD values (Fig. 3) for most of the bacterial species. Specifically, the US-degraded EPS and KGM increased the acetic acid production of most species except *B. adolescentis* though they did not increase the cell concentration (OD) compared with the control. Together with the above CFU results, it can be suggested that the high MW EPS-US and KGM-US were effective to sustain the bacterial survival and metabolic activity.

4. Conclusions

In the present study, partially degraded EPS and KGM fractions in different MW ranges were tested as alternative carbon sources to glucose for *in vitro* culture of five bifidobacteria species. The growth, viability and short chain fatty acid production of bacterial cultures were dependent both on the bacterial species and the MW of EPS and KGM fractions. In general, the lower MW fractions derived from acid-hydrolysis were more favourable for the bacterial growth (with a notable increase in cell concentration). All EPS/KGM fractions, irrespective of their MW, were able to increase the acetic acid production of some bifidobacterial species. The most remarkable effect of the EPS and KGM fractions was a dramatic enhancement of the cell viability (with a much higher CFU), especially by the high MW polysaccharide fractions. These results indicated that the water-soluble EPS and KGM fractions have prebiotic or bifidogenic activity. It is of significance to assess the prebiotic function of EPS and KGM in mixed cultures of faecal flora and to analyse quantitatively their fermentability, utilization and metabolism, and to investigate the protective effect of EPS and KGM on the probiotic bacteria.

Acknowledgments

This work was supported by the Shenzhen Basic Research Program Project [grant number JCYJ20160531184200806; JCYJ20151030164008764]; and The Hong Kong Polytechnic University.

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Table 1 Major chemical contents of EPS, KGM and their partially degraded products

Sample	Carbohydrate (wt %)	Protein (wt %)
EPS	71.34 ± 3.82	6.97 ± 0.15
EPS-US	70.72 ± 3.10	18.43 ± 1.44
EPS-AH	70.07 ± 6.41	13.04 ± 2.82
KGM	64.51 ± 3.36	2.38 ± 0.78
KGM-US	67.44 ± 1.04	2.43 ± 1.56
KGM-AH	70.32 ± 1.13	2.56 ± 2.05

Table 2 The major molecular weight (MW) peaks on the GPC profiles of EPS and KGM samples.

Sample	Retention time (min)	MW (Da)	% Area
EPS	27.167	2.768×10^{8}	85.75
	42.333	1.858×10^{5}	4.61
	50.867	213	9.65
EPS-US	28.633	2.252×10^{8}	82.75
	42.833	1.226×10^{4}	5.10
	50.069	217	12.15
EPS-AH	35.719	7.098×10^{5}	25.85
	40.353	5.641×10^{4}	27.94
	45.700	3,981	19.02
	50.868	214	20.82
KGM	26.283	8.773×10^{8}	69.83
	30.883	2.781×10^{7}	27.88
KGM-US	26.600	8.885×10^{7}	4.29
	29.100	2.009×10^{7}	1.39
	34.100	1.774×10^{6}	78.04
KGM-AH	47.367	1,369	98.89

Table 3 Colony forming units (CFU) 10^8/mL of *Bifidobacteria* cultured with different carbon sources (culture period 48 h; C source concentration 5 g/L)

C source	*B. adolescentis*	*B. bifidum*	*B. breve*	*B. infantis*	*B. longum*
Control	0.77 ± 0.315	0.22 ± 0.09	(2.50 ± 0.46) × 10^3	0.70 ± 0.34	0.17 ± 0.14
Glc	0.50 ± 0.39	0.24 ± 0.04	(2.69 ± 0.43) × 10^3	1.8 ± 0.45	0.55 ± 0.07
GOS	0.78 ± 0.13	0.25 ± 0.10	(2.12 ± 0.54) × 10^3	1.53 ± 0.35	0.71 ± 0.33
EPS-US	**> 3 × 10^5**	**10.9 ± 5.72**	**> 3 × 10^5**	**> 3 × 10^5**	**(1.72 ± 0.38) × 10^4**
EPS-AH	**1.07 ± 0.44**	**1.24 ± 0.83**	**> 3 × 10^5**	**1.20 ± 0.35**	**19.43 ± 9.34**
KGM-US	**19.5 ± 7.09**	0.20 ± 0.04	**> 3 × 10^5**	**(1.48 ± 0.06) × 10^3**	**(5.30 ± 3.90) × 10^3**
KGM-AH	**0.81 ± 0.20**	**2.65 ± 0.62**	(1.56 ± 0.19) × 10^3	**1.3 ± 0.69**	**0.19 ± 0.10**

Bod-faced numbers highlight the significantly higher CFUs over other cultures.

Table 4 pH of the culture medium for *Bifidobacteria* with different carbon sources after 48 h fermentation (initial medium pH 6.6-6.8; C source concentration 5 g/L)

C source	*B. adolescentis*	*B. bifidum*	*B. breve*	*B. infantis*	*B. longum*
Control	5.71 ± 0.21	5.88 ± 0.08	4.93 ± 0.05	5.52 ± 0.06	5.65 ± 0.02
Glc	4.51 ± 0.09	4.92 ± 0.07	4.35 ± 0.03	4.39 ± 0.03	4.35 ± 0.08
GOS	4.40 ± 0.03	4.73 ± 0.26	4.14 ± 0.09	4.24 ± 0.07	4.41 ± 0.10
EPS-US	5.07 ± 0.06	5.72 ± 0.06	4.96 ± 0.12	5.02 ± 0.04	5.13 ± 0.01
EPS-AH	5.09 ± 0.07	5.66 ± 0.08	4.88 ± 0.12	4.96 ± 0.08	5.07 ± 0.12
KGM-US	5.91 ±0.07	6.21 ± 0.03	5.23 ± 0.17	5.86 ± 0.24	6.09 ± 0.03
KGM-AH	5.15 ± 0.07	5.41 ± 0.06	4.87 ± 0.01	5.03 ± 0.04	5.11 ± 0.05

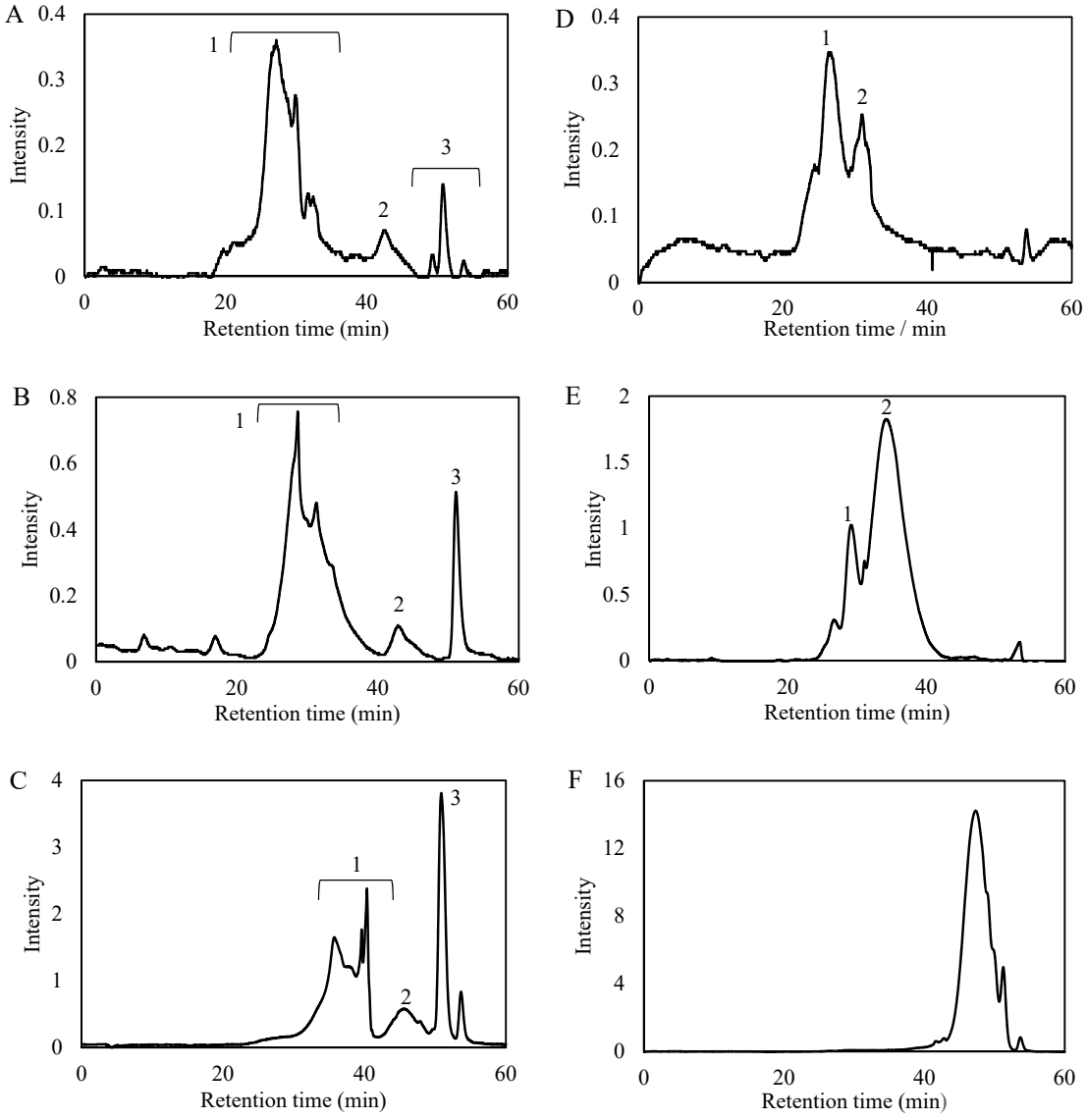
Table 5 Acetic acid accumulation (mM) in liquid cultures of *Bifidobacteria* with different carbon sources (C source at 5 g/L; no C source in control group; 48 h culture in RCM medium).

C source	*B. adolescentis*	*B. bifidum*	*B. breve*	*B. infantis*	*B. longum*
Control	29.75 ± 2.22	26.85 ± 1.82	32.00 ± 2.94	25.70 ± 1.78	26.41 ± 1.67
Glc	72.19 ± 7.06	70.68 ± 1.24	68.28 ± 4.09	77.90 ± 0.89	73.04 ± 3.88
GOS	55.75 ± 3.42	68.86 ± 3.32	55.18 ± 4.36	59.20 ± 5.37	58.91 ± 1.33
EPS-US	42.68 ± 10.82	39.97 ± 2.68	39.81 ± 3.37	42.24 ± 2.69	34.39 ± 3.23
EPS-AH	34.23 ± 3.17	34.85 ± 1.20	41.93 ± 2.64	35.56 ± 4.10	31.10 ± 5.11
KGM-US	34.02 ± 3.01	35.31 ± 2.17	38.67 ± 2.43	36.33 ± 3.67	34.09 ± 3.77
KGM-AH	28.97 ± 1.49	40.76 ± 3.47	44.18 ± 2.77	30.17 ± 2.23	29.87 ± 2.27

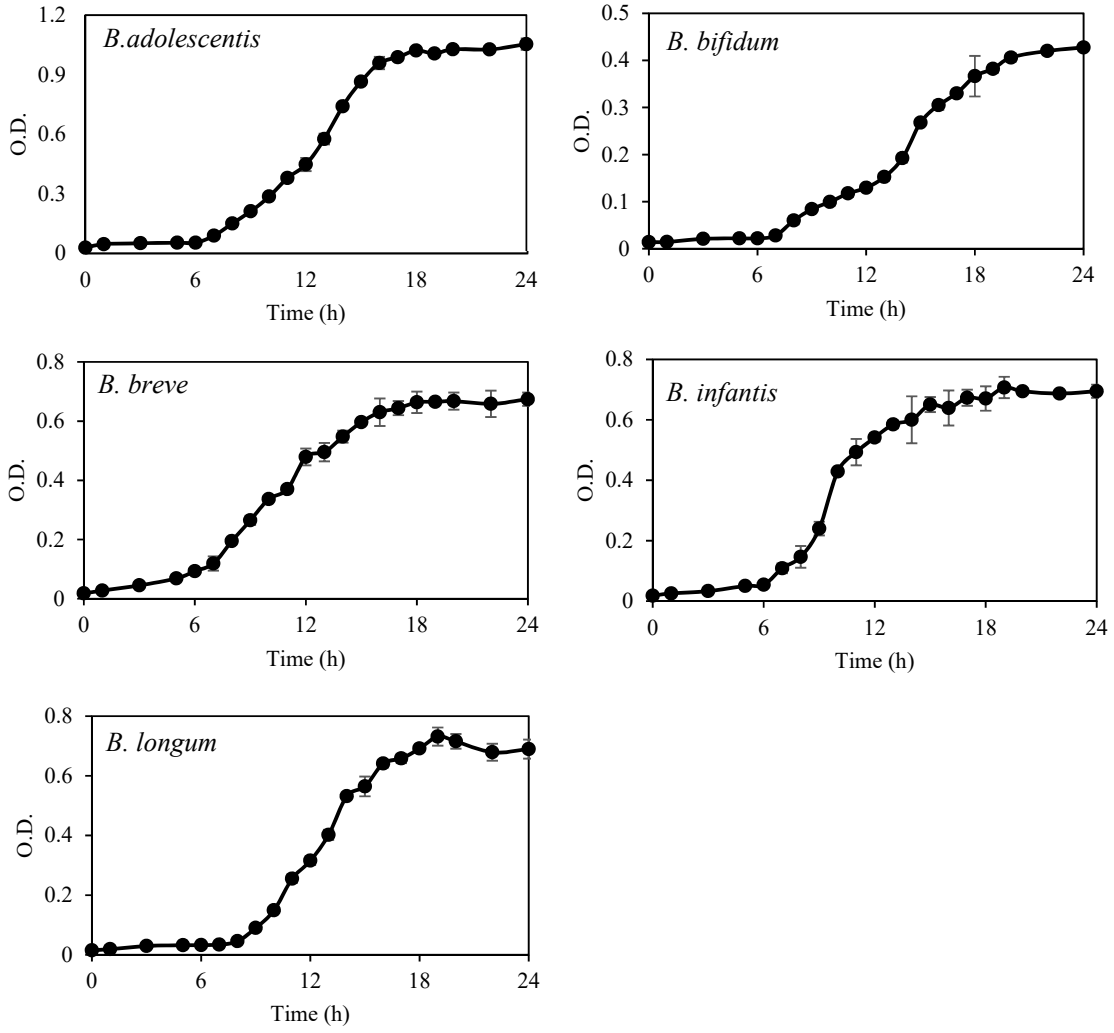
Fig. 1. GPC profiles of (A) EPS, (B) US degraded EPS (EPS-US), (C) acid hydrolyzed EPS (EPS-AH), (D) KGM, (E) US degraded KGM (KGM-US), and (F) acid hydrolyzed KGM (KGM-AH).

Fig. 2. Growth curves of five *Bifidobacteria species* cultured in RCM medium.

Fig. 3. Optical density (600 nm) of five *Bifidobacteria* species cultured in RCM medium with different carbon sources (no C source in control group; concentration of single C source 5 g/L; total concentration of mixed C source 10 g/L and ratio of two C source 1:1; 48 h cultured period; *: $p < 0.05$; **: $p < 0.01$ by student *t*-test).



<Fig. 1. Int. J. Biol. Macromol, Song et al>



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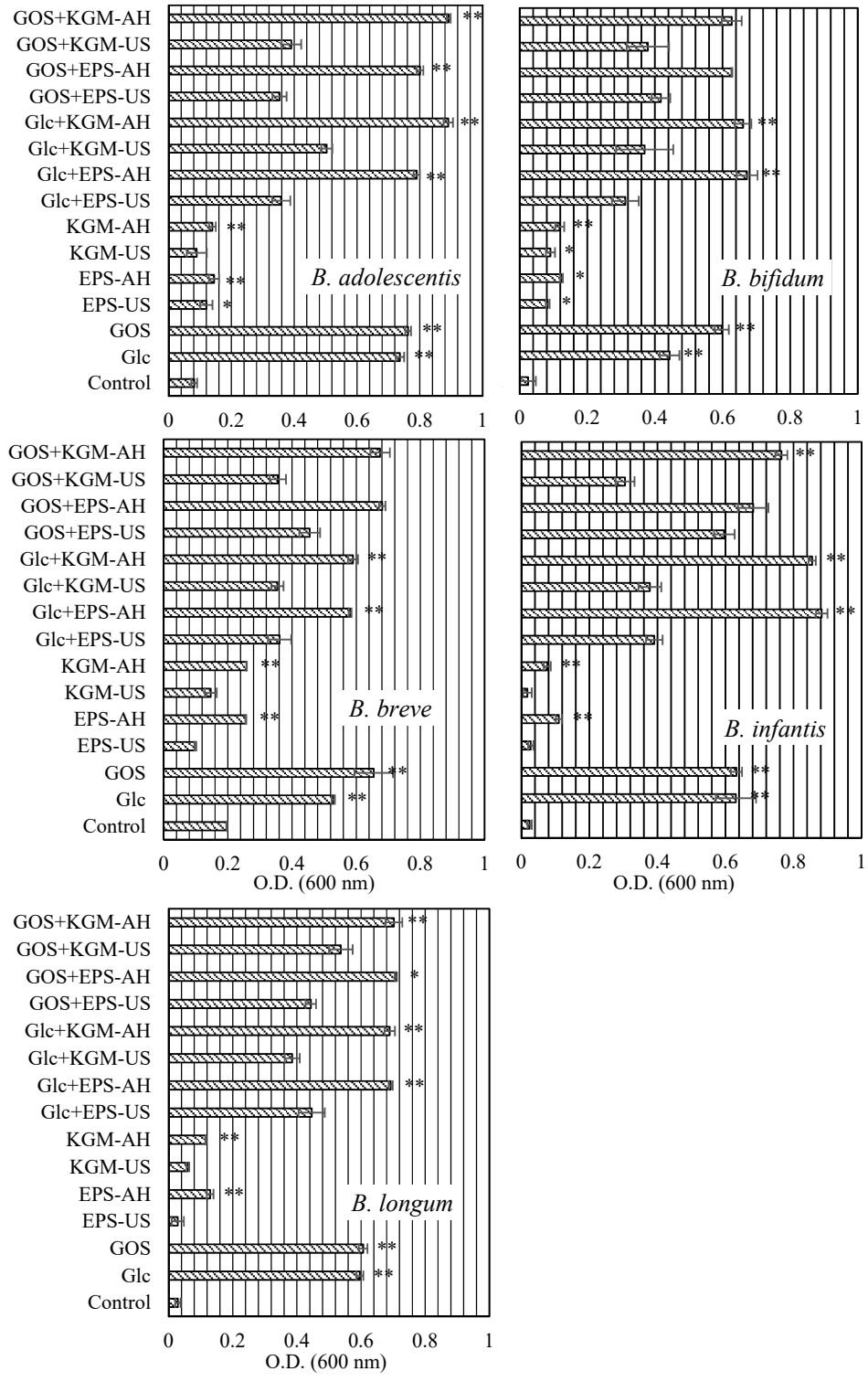

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Supplemental data

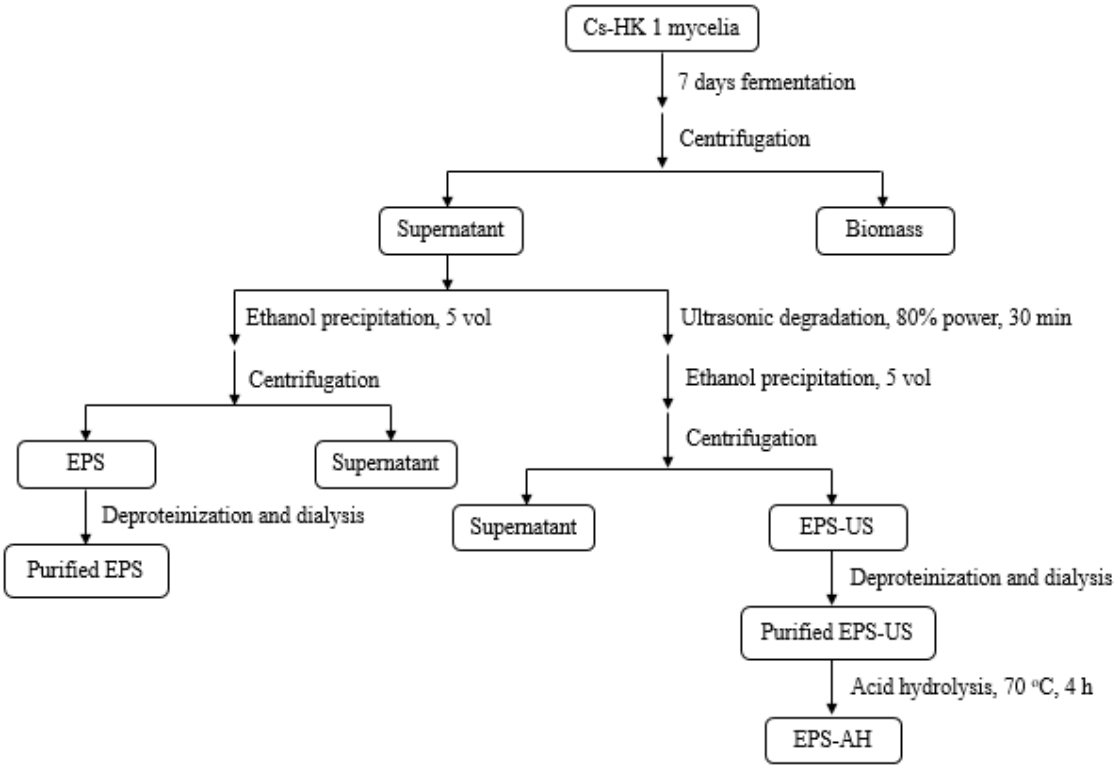

Fig. 1 Experimental procedure for isolation of EPS from Cs-HK1 mycelial fermentation medium and for preparation and purification of US-degraded and acid-hydrolysed EPS fractions.

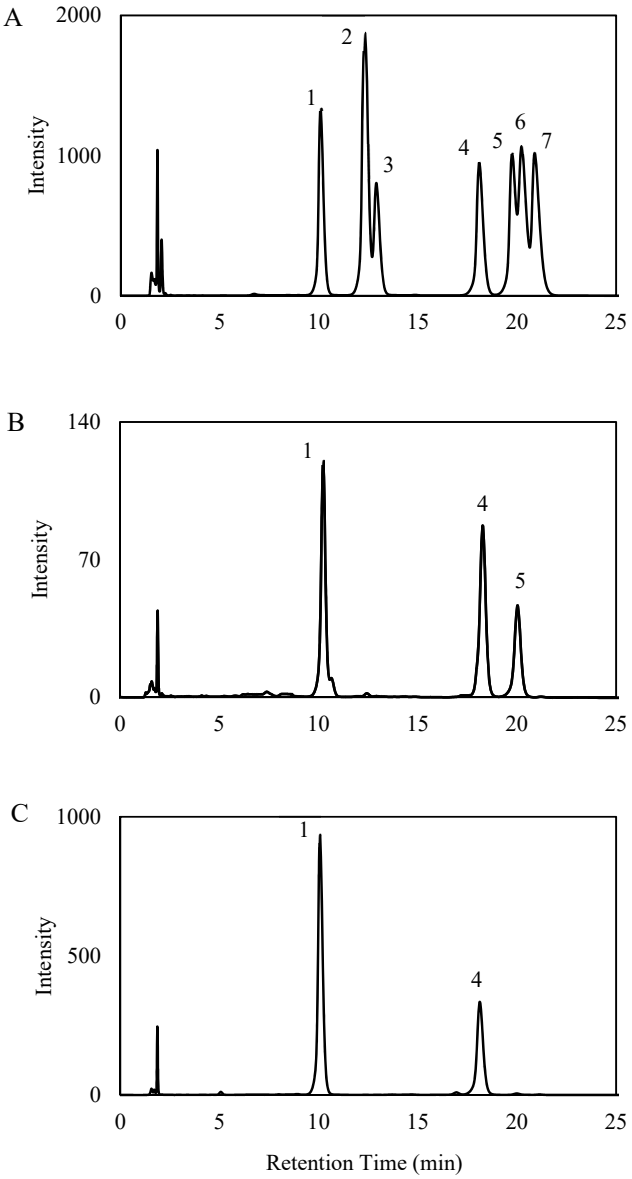

Fig. 2 HPLC profiles of (a) monosaccharides standards, (b) EPS and (c) KGM (Peaks: 1, mannose; 2, ribose; 3, rhamnose; 4, glucose; 5, galactose; 6, xylose; 7, arabinose).