Carbohydrate Polymers 96 (2013) 365–370

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Studies on production of fructo-oligosaccharides (FOS) by gamma radiation processing of microbial levan

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ARTICLE INFO

Article history:
Received 15 September 2012
Received in revised form 14 March 2013
Accepted 19 March 2013
Available online 26 March 2013

Keywords:
Levan
Fructo oligosaccharides
Gamma radiation
Acid hydrolysis
GPC

ABSTRACT

Microbial levan, a natural polymer of fructose, was produced and purified by alcohol precipitation from culture supernatants of *Bacillus megaterium* type 1 grown in an optimized liquid sucrose medium. GPC analysis showed that the yield of the major fraction of levan having molecular weight ~5000 D increased with increase in sucrose concentration in the broth. Levan subjected to ^{60}Co-gamma radiation as well as acid hydrolysis was investigated by rheometry, UV–visible spectrophotometry and gel permeation chromatography (GPC) techniques. Unlike most of the polysaccharides, levan powder exhibited good radiation degradation stability up to 150 kGy. Gamma irradiation of 10% levan aqueous solution at 250 kGy yielded 63.0% fructo-oligosaccharide (FOS) with an average molecular weight of 1250 D. Acid hydrolysis of levan using 0.5 N HCl for 60 min treatment time gave rise to the desired FOS with lower yield (23.1%) as compared to that obtained in gamma radiolysis process.

1. Introduction

Levan is a natural polymer and found in different plants and microbial products. Levan is a homopolymer comprising of sugar fructose sub-units that are linked primarily by β-2,6-glycoside bonds and some β-2,1-glycoside bonds in their branches (Fig. 1 inset). It is produced via a trans-glycosylation reaction that occurs in the presence of enzyme levansucrase (EC 2.4.1.10) (Han, 1990). Depending on its source, levan can be either of low molecular weight (plant levans) or of high molecular weight with branching (microbial levans). Microorganisms, such as *Bacillus subtilis* (Elisashvili, 1984), *Zymomonas mobilis*, *Bacillus polymyxa*, *Erwinia herbicola*, *Rahnella aquatilis* (Kim, Kim, Lee, Song, & Rhee, 2000), *Acetobacter xylinum* NCI 1005 (Tajima, Uenishi, Fujiwara, Erata, & Munekate, 1998), etc., are the primary producers of microbial levan.

Levan being a natural polysaccharide finds applications in a wide range of fields such as food, pharmaceutical and cosmetics production (Park et al., 2003). Levan can also be used as an emulsifier, formulation aid, stabilizing thickener, surface-finishing agent, encapsulating agent; a carrier for colours and flavors in the food industry (Jang et al., 2001). Microbial levan has been known for many years; however, it has not been commercially produced and utilized, mainly because of non-availability of large quantity of low-cost source.

Low molecular weight levan, commonly known as fructo-oligosaccharides (FOS), is a non-digestible food fibre. It belongs to a class having 5–9 fructose molecules with molecular weight in the range 720 D–1296 D (Szwengiel, Czarnecka, & Czarnecki, 2004). Although it does undergo a partial depolymerisation under the influence of the stomach juice, it is not digested by the pancreas secretion or small intestine juices (Yamamoto et al., 1999), and, therefore, can be treated as a component of prebiotic nature. In vitro investigations showed that bifidobacteria could utilize FOS as a source of carbon. However, this depends on the degree of polymerization; a molecular weight of approximately 4500 D has been suggested as an upper limit for this purpose (Marx, Winkler, & Hartmeier, 2000). Fructo-oligosaccharide is also characterized by half the sweetness of saccharose. Therefore, it can find application as a sweetener for diabetic patients (Cha, Park, Yang, & Lee, 2001).

Presently, a wide range of polysaccharides are available in the market, which are widely used in the food, agriculture and other industries. Nevertheless, attempts are being made to find polymers, which apart from stabilizing, thickening and other properties, can also fulfill other functional aspects in food production. Microbial FOS, with its favourable prebiotic properties and structure that can qualify them as components of food cellulose, can be used as a superior alternative to the commercially available variants of levan.

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http://dx.doi.org/10.1016/j.carbpol.2013.03.057

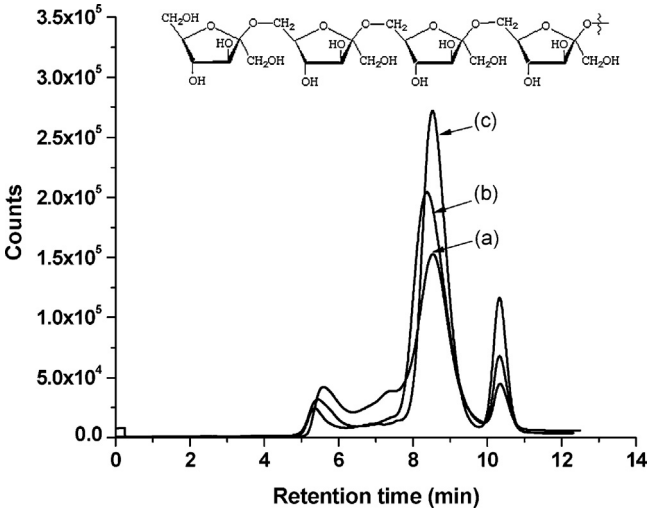

Fig. 1. GPC plots of levan samples produced from different concentrations of sucrose: (a) 10% sucrose, (b) 20% sucrose, (c) 30% sucrose. (Inset: general chemical structure of levan.)

It is well known that ionizing radiation treatment, including gamma irradiation, leads to degradation/depolymerization of polysaccharides, such as starch, cellulose, and pectin by cleavage of the glycosidic bonds and produces lower molecular weight fragments (Ananthaswamy, Vakil, & Sreenvasan, 1970; Charlesby, 1981; Cho, Kim, & Rhim, 2003; Sokhey and Hanna, 1993). The basic advantages of radiation induced degradation of polymers include better process control and elimination of the use of chemical reagents and the need for special equipments/setup for controlling the temperature, environment and additives (Charlesby, 1981). In the present work, to the best of our knowledge, we report herein for the first time, results of studies on gamma irradiation of microbial levan in solid as well as in aqueous solution phase and the feasibility of producing FOS of desired molecular weight. The results have been compared with acid hydrolysis of levan for production of FOS. Radiation processing of microbial levan to produce FOS has potential application in the field of food as sweetener.

2. Materials and methods

Levan was produced by cultivating a previously isolated strain of *Bacillus megaterium* type 1 in a high sucrose (10–30% sucrose w/v) liquid medium and purified as described by Yoshida, Suzuki, and Yagi (1990) and Jang et al. (2006). Briefly, the culture *Bacillus megaterium* type 1 was inoculated into optimized Han's medium containing known concentration of sucrose, 0.2% peptone, 0.2% yeast extract, 0.2% $(NH_4)SO_4$, 0.2% K_2HPO_4, 0.03% $MgSO_4$ and incubated at 30 °C under agitated and aerated conditions. Levan was harvested by centrifugation of the metabolic filtrate at 10,000 × g for 30 min. It was chilled and three volumes of cold ethanol were added. The mixture was homogenized by vortexing for 1 min and then centrifuged at 8000 × g for 10 min at 4 °C. Precipitated material was collected and re-suspended in 10 mL of distilled water and mixed again with three volumes of cold ethanol, for further purification. The microbial levan sample was subjected to multiple re-precipitation treatment in order to ensure maximum purity of the final levan sample. The purity of the microbial levan was tested by TLC method using butanol:ethanol:water (5:5:3) as mobile phase and fructose as a reference standard (Smith, 1958). The TLC result confirms the absence of other compounds in the levan samples. The polysaccharide precipitate was dried at 50 °C to a constant weight.

3. Experimental

3.1. Gamma irradiation

Samples of solid powder levan were taken in stoppered glass vials and then exposed to varying gamma radiation doses at a dose rate of 2.47 kGy h^{-1} using ^{60}Co Gamma Chamber-5000 (BRIT, India). Similarly, 10% aqueous solutions of levan were also irradiated at varying gamma radiation doses at a dose rate of 2.47 kGy h^{-1}.

3.2. Acid hydrolysis

For comparative study, levan sample was also subjected to acid hydrolysis treatment to get lower molecular weight fractions using 0.5 N and 1.0 N HCl at 25 °C. The course of levan hydrolysis with time was monitored using gel permeation chromatography (GPC).

3.3. Viscosity measurement

Viscosities of 10% solutions prepared from gamma irradiated solid levan and un-irradiated levan were determined using Brookfield DVII+ Pro viscometer with an ultra low adapter at 25 ± 1 °C and 85 rpm. For comparison, viscosities of gamma irradiated 10% levan solutions were also measured. Viscosity of distilled water was used as a reference.

3.4. UV–visible spectroscopy

The UV–visible spectra of irradiated and un-irradiated aqueous levan solutions (10%, w/v) were recorded between 200 nm and 500 nm using a double beam UV–visible spectrophotometer (Evolution 300, Thermoelectron, UK) against distilled water as a reference.

3.5. FTIR analysis

The IR spectra of control and gamma irradiated levan samples were recorded on diamond single reflectance ATR assembly in IR Affinity-1 spectrometer (Shimadzu) using resolution of 4 cm^{-1} and with data acquisition run of 25 scans for each sample.

3.6. GPC analysis: estimation of molecular weight of levan

Molecular weight and molecular weight distribution of levan samples were estimated by gel permeation chromatography (GPC) using PL aquagel–OH column (7.5 mm × 300 mm) and refractive index detector (Elite LaChrom, UK, Model L-2490). HPLC grade water was used as mobile phase at a flow rate of 1 mL/min. Solutions of un-irradiated and irradiated levan samples (1%, w/v) were filtered using Sartorius-Minisart filter unit (0.45 μm) and injected to the column through 20 μL loop. Calibration plot was established using standard PEG samples with known molecular weights. Evaluation of the chromatograms was carried out using size exclusion chromatography software (EZ Chrom Elite Software from Scientific Software, Inc.) to determine the molecular weights of the levan samples.

3.7. Particle size analysis

The hydrodynamic diameter of the irradiated and un-irradiated levan samples (2% aqueous solution at 25 °C) was determined using DLS measurements performed on a Malvern 4800 Autosizer employing 7132 digital correlator. The light source was He–Ne laser operated at 632.8 nm with an output power of 15 mW.

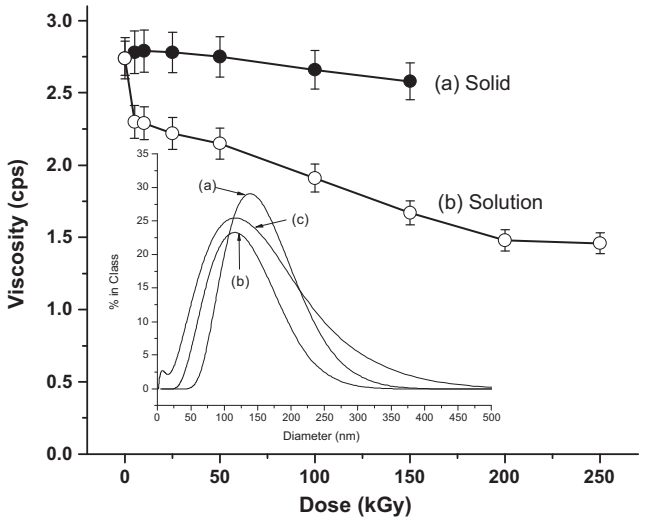

Fig. 2. Viscosity of aqueous solution of levan (10%, w/v) as a function of gamma radiation dose (a) solid state irradiation (b) aqueous solution state irradiation. (Inset: particle size analysis (DLS) of levan samples irradiated in aqueous solution as a function of gamma radiation dose. (a) 0 kGy (b) 50kGy (c) 100 kGy.)

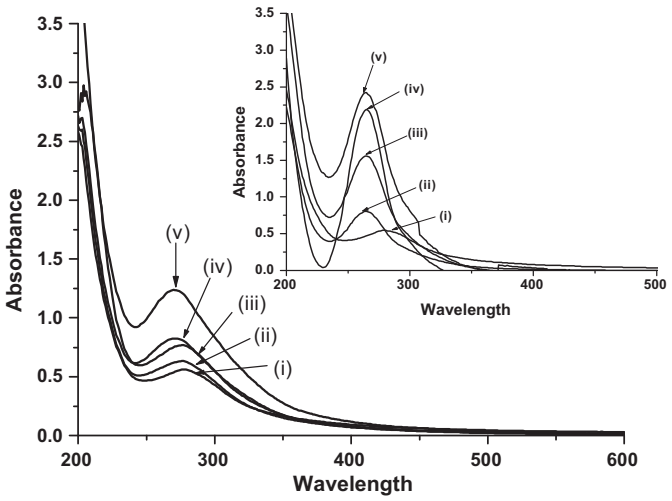

Fig. 3. UV–visible spectra of levan samples irradiated in solid state at varying gamma radiation doses: (i) 0 kGy, (ii) 25 kGy, (iii) 50 kGy, (iv) 100 kGy, (v) 150 kGy. (Inset: UV–visible spectra of levan samples irradiated in aqueous solution state (10%) at varying gamma radiation doses: (i) 0 kGy, (ii) 100 kGy, (iii) 150 kGy, (iv) 200 kGy, (v) 250 kGy.)

3.8. Microbial analysis

Total viable counts (TVC) in irradiated and un-irradiated samples of levan (10%, w/v) were estimated in sterile saline. Serial dilutions were made and plated on sterile Petri plates containing plate count agar (PCA) and incubated at 25 ± 1 °C for 24 h. The final TVC were estimated by colony counting method taking dilution factor in to account and presented as the colony forming unit per gram (CFU/g) of the product.

4. Results and discussion

The analysis of molecular weight of levan produced in Hans medium with various concentrations of sucrose (10–30%, w/v) was carried out using gel permeation chromatography. Fig. 1 presents the GPC plots of levan samples produced at different sucrose concentrations, which shows three basic fractions: a small fraction of high molecular weight levan, a second major fraction of medium molecular weight levan and a third low molecular weight fraction. Since the fraction desired to produce FOS is of medium molecular weight, further analyses were carried out using levan produced by 30% sucrose as it yielded the highest percentage of the medium molecular weight fraction.

4.1. Gamma irradiation of levan

4.1.1. Viscosity analysis of irradiated levan

Fig. 2 shows the viscosity of levan samples irradiated in solid state and dissolved in water to a concentration of 10% (w/v), as a function of radiation dose. The viscosity of the levan solution slowly decreased with radiation dose; 2.74 cps for un-irradiated levan decreased to 2.58 cps for levan irradiated at 150 kGy dose. The drop in viscosity of irradiated solid levan was significantly lower than that observed for many other irradiated natural polysaccharides such as starch, guar gums, etc. This shows good radiation stability of solid levan. However, the viscosity of irradiated aqueous solutions of levan decreased from 2.74 cps to 1.67 cps at 150 kGy and finally to 1.46 cps at 250 kGy. In solution phase, highly reactive radiolysis products of water, namely OH, H and e_{aq}^- react with the levan molecule leading to its degradation. However, in solid phase irradiation, the direct radiation effect is the predominant process, which leads to lower radiation damage to the levan molecule. This result also highlights the non-viability of producing FOS by solid state irradiation of levan owing to the latter's high radiation stability.

4.1.2. Particle size analysis of irradiated levan

Levan is found to exist in nano-spherical form in its aqueous solutions with hydrodynamic diameters of nano-spheres varying between 100 nm and 200 nm. Results of particle size analysis of un-irradiated and irradiated levan samples at different radiation doses are presented in Fig. 2 inset. It was observed that the un-irradiated levan sample exhibits narrow size distribution in the 50–300 nm range with distribution maximum at ~150 nm. Exposure to gamma radiation dose of 50 kGy in solution phase led to decrease in the particle size with distribution maximum at ~100 nm without affecting the narrow size distribution. However, for radiation dose of 100 kGy, in addition to decrease in size (distribution maximum at ~100 nm), a broadening in the size distribution was also observed along with the appearance of a higher particle size tail. These results clearly indicated the gamma radiation induced degradation of levan in solution phase and supports the results of viscosity analysis.

4.1.3. UV–visible spectral analysis of irradiated levan

UV–visible spectra of aqueous solutions made by dissolving irradiated solid levan showed a gradual shift in λ_{max} from 278 nm for un-irradiated levan to 270 nm for sample irradiated to a dose of 150 kGy (Fig. 3). Also, O.D. at λ_{max} increased indicating an increase in concentration of the product formed on irradiation. On the other hand, UV–visible spectra of irradiated aqueous levan solution are shown in Fig. 3 inset, which exhibited a shift in λ_{max} from 278 nm for un-irradiated levan to 265 nm for aqueous levan solution irradiated to a dose of 250 kGy. The increase in O.D. value observed in this case was higher compared to that in the case of gamma irradiated solid levan. The blue shift indicates the formation of radiation induced degradation products of levan. The change in UV/visible spectra of levan after gamma irradiation may be attributed to the radiation induced degradation leading to ring opening and oxidation of levan molecule.

4.1.4. pH analysis of irradiated levan

Gamma irradiation of levan samples leads to decrease in the pH, which may be attributed to the radiation induced degradation

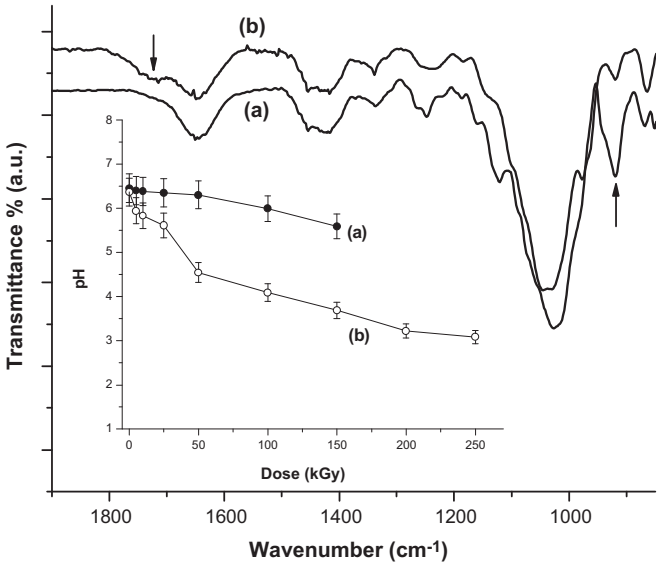

Fig. 4. FTIR spectra of levan samples (a) un-irradiated levan (b) gamma irradiated levan at dose = 150 kGy. (Inset: change in pH of 10% levan solution on gamma irradiation at different doses in (a) solid phase (b) solution phase.)

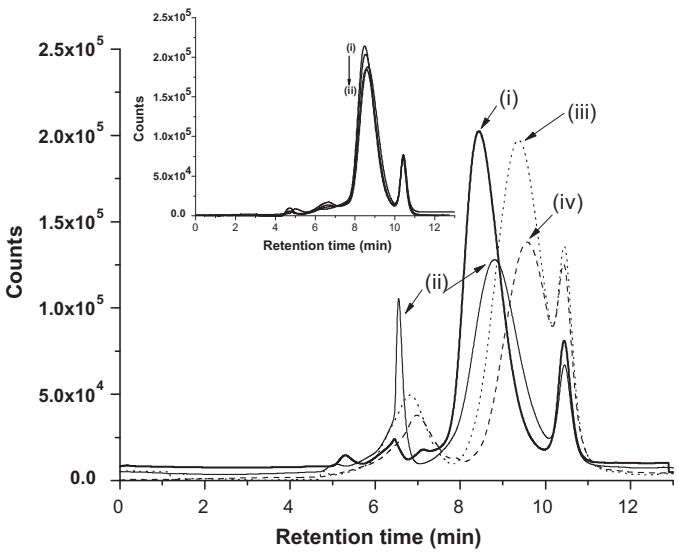

Fig. 5. Gel permeation chromatogram of levan sample irradiated in solution phase (10%, w/v) to different radiation doses: (i) 0 kGy, (ii) 100 kGy, (iii) 200 kGy, (iv) 250 kGy. (Inset: gel permeation chromatogram of levan sample irradiated in solid state to different radiation doses: (i) 0 kGy, (ii) 50 kGy, (iii) 100 kGy, (iv) 150 kGy).

of levan polysaccharide leading to the formation of oxidative products, including organic acids. Fig. 4 inset shows the pH of the levan samples irradiated to different doses in solid and solution phases (10%, w/v), respectively. It can be seen that levan sample irradiated in solution phase exhibited much higher drop in pH as compared to that irradiated in solid phase. In case of solid levan irradiation, the pH decreased from 6.43 to 5.59 after a dose of 150 kGy, whereas, a similar gamma radiation dose of 150 kGy imparted to levan in solution phase (10%, w/v) led to a pH change from 6.43 to 3.69, which further dropped to a value of pH 3.08 at a dose of 250 kGy. The decrease in pH indicated the generation of acids (H^+), which will convert e_{aq}^- to $H^\bullet$ radicals via reaction (1), and consequently increase the *G* value (per 100 eV) of $H^\bullet$ radicals from 0.6 to 3.3.

$$e_{aq}^- + H^+ \rightarrow H^\bullet \quad (1)$$

$H^\bullet$ radical predominantly reacts with organic molecules via H abstraction reaction, and therefore, may lead to H abstraction from the CH_2 groups of the main chain of levan molecule, resulting in the generation of carbon free radical in the main chain. The carbon free radical will subsequently react with oxygen leading to the oxidative degradation of the levan. Therefore, the decrease in the pH would facilitate the radiation induced degradation of levan. These results indicated that levan undergoes higher degradation in aqueous phase irradiation as compared to solid phase irradiation.

4.1.5. FTIR analysis of irradiated levan

Fig. 4 shows the FTIR spectra of un-irradiated levan and 10% (w/v) solutions of levan irradiated to 150 kGy gamma radiation dose. It was observed that, when compared to un-irradiated levan, an additional peak at around 1720 cm^{-1} appeared in the IR spectrum of irradiated levan, indicating formation of carbonyl groups on irradiation of levan. Another important observation made was the decrease in intensity of the peak at around 923 cm^{-1}, corresponding to the intra-ring CO bond stretching of furanose ring, with gamma irradiation, which indicated breakdown of the furanose ring structure of levan molecules. Results showed that gamma irradiation of levan solution leads to oxidation and some extent of ring opening of levan along with the main chain scission reactions, producing molecular species with carboxylic groups. These observations were further supported by decrease in pH of levan solutions after irradiation, as mentioned earlier.

4.1.6. GPC analysis of irradiated levan

Effect of gamma radiation induced radiolysis and acid hydrolysis on the molecular weight of levan was analyzed by gel permeation chromatography (GPC), which revealed varying multifractional distributions in levan samples. Fig. 5 inset presents the GPC plots for levan samples irradiated in solid state and the detailed results are given in Table 1. Un-irradiated levan sample showed three fractions: a small fraction of high molecular weight levan (yield = 1.0%) at a retention time (R_t) 5.01 min (Mol. weight = 900,000 D), a second major fraction of medium molecular weight levan (yield = 86.40%) at R_t 8.40 min (Mol. weight = 5063 D), and a third low molecular weight fraction (yield = 11.90%) at R_t 10.40 min (Mol. weight = 233 D). After gamma irradiation of levan in solid state, the major fraction of medium molecular weight levan did not show a significant shift in R_t. At 150 kGy dose, a marginal shift in R_t to 8.63 min was observed, which corresponds to the decrease in average molecular weight of the fraction from 5063 D to 4400 D (Table 1). Moreover, the yield of the different fractions also did not get affected drastically with the radiation dose.

On the other hand, when 10% (w/v) aqueous solution of levan was irradiated, the retention time of the GPC peak for major fraction of medium molecular weight levan shifted considerably (Fig. 5). On comparing with solid phase irradiation data, it can be seen that for 100 kGy dose delivered to levan in solution phase, a shift in R_t (from 8.40 min to 8.80 min) was achieved, which corresponds to a decrease in molecular weight from 5063 D to 4277 D that could not be achieved in solid state irradiation even at a higher dose of 150 kGy. At still higher doses of 200 kGy and 250 kGy, the average molecular weight of the major fraction reduced to 1730 and 1250 D, respectively. In addition, unlike in the case of solid phase irradiation, the yields of different fractions were found to vary in case of solution phase irradiation of levan. The yield of the middle molecular weight major fraction decreased from 86.40% to 62.78% on applying a 250 kGy radiation dose. These results suggest that in order to achieve the target of producing FOS possessing the desired prebiotic properties and structure that can qualify them as components of food cellulose (i.e., FOS having molecular weight in the

Table 1
GPC results of gamma radiation processed microbial levan.

Dose (kGy)	R_t (min)		% Yield		Av. mol. wt. of the fraction	
	Solid	Solution	Solid	Solution	Solid	Solution
0	5.01	5.01	1.00	1.00	900,000	900,000
	8.40	8.40	86.40	86.40	5063	5063
	10.40	10.40	11.90	11.90	233	233
50	4.70		1.76		1300,000	
	8.50		86.10		5200	
	10.40		12.06		240	
100	6.65	5.08	6.60	0.80	150,000	872,700
	8.56	6.50	81.20	16.80	5100	138,200
	10.40	8.80	11.46	73.30	245	4277
		10.40		8.97		227
150	6.66		6.40		128,900	
	8.63		81.90		4400	
	10.42		11.70		235	
200		6.81		12.16		140,850
		9.36		68.60		1730
		10.44		18.30		208
250		6.92		12.05		133,150
		9.56		62.78		1250
		10.42		24.17		228

range 720–1296) (Szwengiel et al., 2004), irradiation of levan in aqueous solution phase is the most feasible option.

4.2. Acid hydrolysis of levan: GPC analysis

For comparison with the gamma radiolysis process, acid hydrolysis of levan at different acid concentrations was also carried out. Fig. 6 inset presents the GPC chromatogram of levan sample hydrolyzed at different time period using 1 N HCl at 25 °C. Results of acid hydrolysis of levan by 1 N HCl are given in Table 2. Three important observations could be made from the results of 1 N HCl acid hydrolysis of levan: (i) levan is more prone to acid hydrolysis induced degradation as compared to gamma radiation induced degradation, (ii) molecular weight of the major fraction of acid hydrolysis products is very low, (iii) the yield of the desired medium molecular weight fraction is very low. In fact, none of the fractions obtained after 1 N HCl hydrolysis of levan meet the criteria of FOS with prebiotic properties (Szwengiel et al., 2004). FOS obtained in this process was either of a higher (2200 D in 30 min) or a lower (454 D in 60 min) molecular weight than the desired molecular weight of 700–1296 D, with the added disadvantage of low yields, which nullifies the method's viability as a commercial method for FOS production. Moreover, the higher yields fractions have very low molecular weights that fail to fulfill criteria of FOS for practical use; 83 D (yield = 75.50%) and 95 D (yield = 95.90%) obtained after 30 min and 60 min treatment times, respectively (Table 2).

Further, acid hydrolysis experiment was carried out under milder condition using lower acid concentration of 0.5 N HCl at 25 °C. Fig. 6 presents the GPC chromatograms of levan hydrolyzed at different time period using 0.5 N HCl at 25 °C. Results of acid hydrolysis of levan by 0.5 N HCl are given in Table 2. After 0.5 N HCl treatment for 30 min, fraction with molecular weight of 2300 D was obtained with low yield (29.60%), however, the major fraction

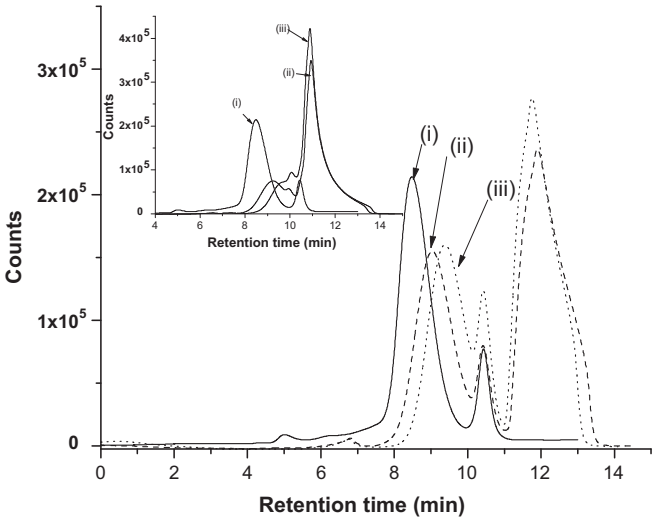

Fig. 6. Gel permeation chromatography of levan hydrolyzed using 0.5 N HCl at 25 °C at different time. (i) Untreated, (ii) 30 min, (iii) 60 min. (Inset: gel permeation chromatography of levan hydrolysed using 1 N HCl at 25 °C at different time. (i) Untreated, (ii) 30 min, (iii) 60 min).

Table 2
GPC results of microbial levan hydrolyzed using HCl at 25 °C.

Time of rxn. (min)	R_t (min)		% Yield		Av. mol. wt. of the fraction	
	1 N	0.5 N	1 N	0.5 N	1 N	0.5 N
Untreated	5.01	5.01	1.00	1.00	900,000	900,000
	8.40	8.40	86.40	86.40	5063	5063
	10.40	10.40	11.90	11.90	233	233
30	9.24	9.03	18.60	29.60	2200	2300
	9.93	10.40	5.20	3.90	450	225
	10.92	11.90	75.50	65.70	83	25
60	10.06	9.37	3.18	23.10	454	1400
	10.87	10.42	95.90	5.20	95	240
	–	11.75		70.30	–	30

with 65.70% yield has very low molecular weight (25 D). Similarly, after 60 min treatment, fraction with molecular weight of 1400 D was obtained with low yield (23.10%), and the major fraction with 70.30% yield has very low molecular weight (30 D). Therefore, 0.5 N HCl hydrolysis of levan for 60 min treatment time gave rise to the desired FOS with low yield as compared to that obtained in gamma radiolysis process.

4.3. Microbial analysis

The initial microbial load in levan was estimated to be 10^9 CFU/g, which was found to increase by 0.3 log units per day of storage at $25 \pm 1\,^{\circ}C$, making the product unfit for storage and consumption. The Decimal reduction dose (D_{10}), which is defined as the dose required to decrease the TVC by one Log cycle, was estimated from the plot of microbial count (CFU/g) versus radiation dose. The D_{10} value for levan samples was estimated to be 2.55 kGy. Using D_{10} value of 2.55 kGy for levan samples, gamma radiation dose required to achieve safety assurance level (SAL) of 10^{-6} was determined to be 38.3 kGy (Gazsó, 2003). The added advantage offered by gamma radiation processing of levan includes the simultaneous hygienisation of the final product-FOS.

5. Conclusion

Radiation processing of microbial levan offers an opportunity to develop FOS of desired molecular weights. As compared to most of the polysaccharides, levan exhibited better radiation stability. Moreover, levan in aqueous solution phase is more susceptible to radiation induced degradation as compared to solid phase. Gamma irradiation of 10% levan aqueous solution at a radiation dose of 250 kGy yielded 63% fructo-oligosaccharide (FOS) with an average molecular weight of 1250 D, which suggested that gamma radiation processing could be used to produce FOS from microbial levan. Although levan was found to be more sensitive to acid hydrolysis induced degradation, the values of molecular weight and yields obtained were found to be inferior to the desired values. Gamma radiolysis of aqueous solution of levan offers a better alternative for production of FOS of desired molecular weights with higher yield as compared to acid hydrolysis process, coupled with the added benefit of providing simultaneous radiation hygienisation of the product. Therefore, the radiation processing of microbial levan to produce FOS has potential application in the field of food as sweetener.

Acknowledgements

The authors are grateful for the support rendered by BRNS Grant No. 2005/35/16/BRNS for carrying out this project. The Authors thank V. Sudarsan and P.A. Hasan, BARC for AFM and DLS analysis, respectively.

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