Electronic Journal of Biotechnology 49 (2021) 64–71

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage:

Research Article

Enzymatic preparation of manno-oligosaccharides from locust bean gum and palm kernel cake, and investigations into its prebiotic activity

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ARTICLE INFO

Article history:
Received 16 June 2020
Accepted 18 November 2020
Available online 03 December 2020

Keywords:
Bacillus
Glycoside hydrolase Galactomannan
Lactobacillus plantarum
Linear mannan
Locust bean gum
Mannanase
Manno-oligosaccharides
Palm kernel cake
Prebiotic
Sphingomonas
Stability

ABSTRACT

Background: Manno-oligosaccharides (MOS) is known as a kind of prebiotics. Mannanase plays a key role for the degradation of mannan to produce MOS. In this study, the mannanases of glycoside hydrolase (GH) families 5 Man5HJ14 and GH26 ManAJB13 were employed to prepare MOS from locust bean gum (LBG) and palm kernel cake (PKC). The prebiotic activity and utilization of MOS were assessed *in vitro* using the probiotic *Lactobacillus plantarum* strain.

Results: Galactomannan from LBG was converted to MOS ranging in size from mannose up to mannoheptose by Man5HJ14 and ManAJB13. Mannoheptose was got from the hydrolysates produced by Man5HJ14, which mannohexaose was obtained from LBG hydrolyzed by ManAJB13. However, the same components of MOS ranging in size from mannose up to mannotetrose were observed between PKC hydrolyzed by the mannanases mentioned above. MOS stability was not affected by high-temperature and high-pressure condition at their natural pH. Based on *in vitro* growth study, all MOS from LBG and PKC was effective in promoting the growth of *L. plantarum* CICC 24202, with the strain preferring to use mannose to mannotriose, rather than above mannotetrose.

Conclusions: The effect of mannanases and mannan difference on MOS composition was studied. All of MOS hydrolysates showed the stability in adversity condition and prebiotic activity of *L. plantarum*, which would have potential application in the biotechnological applications.

How to cite: Zhang R, Li X-Y, Cen X-L, et al. Enzymatic preparation of manno-oligosaccharides from locust bean gum and palm kernel cake, and investigations into its prebiotic activity. Electron J Biotechnol 2021;49. https://doi.org/10.1016/j.ejbt.2020.11.001

1. Introduction

The reduction in antibiotic-resistant bacteria in food-producing animals is linked to limited antibiotic use in these animals. The use of non-digestible oligosaccharides (NDOs) and probiotics as additives to replace antibiotics can modulate gut ecology and improve animal growth [1,2,3,4]. The NDOs are commonly classified by glycosyl type, such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS) or Manno-oligosaccharides (MOS) [1,5]. MOS are a type of NDO that include the sugar mannopyranose. Several previous studies have shown that MOS promote the proliferation of beneficial bacteria, inhibit pathogens, and improve weight gain in food-producing animals [6,7,8]. Noticeably, MOS had the greatest proliferative effect and adhesive properties more than frequently-used FOS and GOS on *Lactobacillus plantarum in vitro* [9]. In addition, mannose, a monomer MOS, possesses immunological features which have the ability to reduce pathogen colonization of the intestinal tract [10,11,12]. As such, MOS have become some of the most popular NDOs in recent years.

Mannans, the raw material to product MOS, are the constituent of the cell wall hemicellulose fraction [13]. On the basis of sugar backbone composition, they are divided into four types, including linear mannan, glucomannan, galactomannan, and galactogluco-

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso
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https://doi.org/10.1016/j.ejbt.2020.11.001

mannan. Among them, the main chains of linear mannan and galactomannans are both composed of D-mannopyranosyl residues linked with β-1,4 glycosidic bond. Compared with linear mannan, galactomannan was replaced by single 1,6-linked α-D-galactopyranosyl groups along the main chain [14]. Locust bean gum (LBG) has galactomannan, which is commonly commercial substrate to use for the assay of mannanases activity. The renewable biomass palm kernel cake (PKC), rich in linear mannans, is a major agricultural by-product of the palm kernel oil industry [15]. It can contain up to 57% of mannan in dry weight, in the excess of other agro-waste [16]. Therefore, galactomannan from LBG and linear mannan from PKC were used as substrate to study effects on the components of their hydrolyzed products prepared by different mannanases.

β-Mannanases (endo-1,4-β-mannanases, EC 3.2.1.78) are crucial enzymes in the hydrolyzation of mannan, and have greatly influenced the ingredients of hydrolysis products [14]. Mannanases mainly fall into the glycoside hydrolase (GH) families 5 and 26 in the Carbohydrate-Active enZYmes Database [17]. They can randomly hydrolyze mannans with a β-1,4-linked backbone to produce MOS [18,19]. GH5 and GH26 mannanases shared the similar structure and mechanism. However, their substrate specificity had a distinct difference lies between the substrate binding subsites, which could affect the composition of the hydrolyzed products [20]. In the previous study, it was obtained two mannanases with low amino acid sequence identities, GH 5 Man5HJ14 and GH 26 ManAJB13. They were respectively expressed in *Escherichia coli* BL21 (DE3). GH 5 Man5HJ14 exhibited optimal activity at pH 6.5 and 65°C, and GH 26 ManAJB13 had optimal activity at pH 6.5 and 40°C. They share the similar characterizations in salt-tolerant and proteases-resistant, which is benefited to industrial application [18,21].

In the present study, we analyzed differences upon the composition of hydrolysates obtained from LBG and PKC prepared by the purified recombinant GH5 Man5HJ14 and GH26 ManAJB13. Their prebiotic activity and utilization were also determined using the probiotic *L. plantarum* CICC 24202. Given the MOS is a class of compounds, the effect of mannanases and mannan difference on MOS composition is an important question in the biotechnological applications, but it's not clear yet.

2. Materials and methods

2.1. Chemicals and reagents

LBG was purchased from Sigma (St. Louis, MO, USA). PKC was purchased from the Jiahui Feed Company, China. High efficiency silica gel G plates were purchased from the Qingdao Ocean Petrochemical Company, China. Other chemicals and reagents were of analytical grade and commercially available.

2.2. Preparation of refined mannanase enzyme assays

Man5HJ14 was the GH 5 β-mannanase from *Bacillus* sp. HJ14, and ManAJB13 was the GH 26 β-mannanase from *Sphingomonas* sp. JB13. Both of these produced recombinant enzymes were expressed in *Escherichia coli* BL21 (DE3) and purified using a Ni^{2+}-NTA agarose gel column as previously described [18,20]. Mannanase activity was determined using LBG as substrate. The enzyme activity determination method was as detailed in our previous study [18,20]. One unit (U) of mannanase activity was defined as the quantity of enzyme liberating 1 μmol of mannose min^{-1} under assay conditions.

2.3. Production of MOS from LBG and PKC

The galactomannan of LBG was commonly commercial substrate, which did not have to pretreatment. The assay mixture contained 0.5% (w/v) LBG and was incubated in McIlvaine buffer (pH 6.5) with 10 U/ml Man5HJ14 or ManAJB13 at 60°C or 37°C for 8 h, respectively. LBG hydrolysates obtained from Man5HJ14 and ManAJB13 were labelled LBGHL5 and LBGHL26, respectively.

PKC, as the raw material including linear mannan, has to be pretreatment to break its structure before bioconversion. PKC (50 g) was pre-treated with 250 mL of 1% (w/v) NaOH solution at 121°C and 15 lbs pressure for 20 min. The wet biomass was then washed with dilute hydrochloric acid until the pH reached 6.5. The biomass was then harvested and dried to a constant weight. An equal weight of biomass was in McIlvaine buffer (pH 6.5) treated with 10 U/mL Man5HJ14 or ManAJB13 at 60°C or 37°C for 10 h, with a solid: liquid ratio of 1:5. A negative control, using water, was set up under similar conditions. Supernatants were collected by centrifugation at 12,000 × *g* for 5 min at 25°C. PKC hydrolysates obtained from Man5HJ14, ManAJB13, and water were labelled PKCHL5, PKCHL26, and PKCHL0, respectively.

MOS purification was carried out by filtering supernatants through a polyamide ultrafiltration tube (with the cut-off set at 3 kDa, Millipore) to remove any remaining mannan and mannanases. The protein and phenolics compounds in the collected filtrate were removed by chloroform with an equal volume of filtrate and dialysis with Mw of 0.1 kDa (Solarbio), respectively.

2.4. Analysis of MOS

After purification, the hydrolysates were analyzed by thin layer chromatography (TLC) and electrospray ionization mass spectrometry (ESI-MS). TLC was performed according to the procedure of Zhu et al. [22] with a few modifications. At room temperature, the solvent system consisted of *n*-butanol:acetic acid:water (2:1:1), for chromatogram development. The sugars were located using aniline-diphenylamine-phosphoric acid-acetone as the chromogenic reagent. ESI-MS analyses were performed by experts from the Kunming Institute of Botany (Kunming, China). The Agilent 1260 Infinity Series apparatus with Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS was used to detect samples in the range of 0–2000 mass to charge ratio (*m/z*).

Beside LBGHL5 and LBGHL26, there were selected results of hydrolysates released from LBG by diverse mannanases for contrastive analysis. The MOS from LBG were prepared by GH5 mannanases from *Bacillus nealsonii* PN-11 [23], *Gloeophyllum trabeum* ATCC 11539 [24], *Ruminococcus flavefaciens* FD-1 v3 [25] and *Aspergillus kawachii* IFO 4308 [26]. They were produced by GH26 mannanases from *Bacillus circulans* NT 6.7 [27], *Clostridium thermocellum* ATCC 27405 [28] and *Klebsiella oxytoca* KUB-CW2-3 [29].

2.5. Evaluation of MOS stability

The stability of MOS was evaluated by autoclave sterilization. The sample solution was sterilized at 121°C and 15 lbs pressure for 20 min at pH 7.0. Treated samples were cooled down and analyzed by TLC and Image J software. Unsterilized samples were used as controls.

2.6. Prebiotic activity of MOS on L. plantarum CICC 24202

To test for the prebiotic effects of MOS on *L. plantarum* CICC 24202, the protocol by Jagtap et al. was followed, with a few modifications [30]. The basal medium contained Peptone (10 g/L), NH_4Cl (2 g/L), KH_2PO_4 (2 g/L), $MnSO_4$ (100 mg/L), $MgSO_4$ (50 mg/L),

Tween80 (1 ml/L). To this medium, either 0.5% (w/v) MOS, glucose, mannose or normal saline were added as carbon sources. The media were autoclaved at 121°C for 20 min. Activated *L. plantarum* CICC 24202 was inoculated at 1% into 9 mL aliquots of each the 4 media types, separately, in a conical flask. Firstly, each medium containing the strain was then divided into 30 equal parts, which was added respectively into a 96-well deep well plate with the maximum volume of 2 mL. Fermentation was conducted at 37°C under agitation at 180 rpm. Secondly, three out of 30 equal samples of each group were collected aseptically from the 96-well deep well plate to microplate for immediate measurement at one-hour intervals, respectively. The growth of the strain was measured at A_{600} by micro plate spectrophotometer and the measured samples were discard. The Secondly step was repeated 9 times. Data is summarized and the growth curve was plotted. Finally, the ability of *L. plantarum* CICC 24202 to utilize different carbon sources for the 9-hour growth was studied by TLC.

3. Results and discussion

3.1. Characteristics of LBG hydrolysates by Man5HJ14 and ManAJB13

The hydrolysates obtained from LBG prepared by Man5HJ14 and ManAJB13 were named LBGHL5 and LBGHL26, respectively. The composition and content of these MOS were analyzed by TLC and ESI-MS (Fig. 1). In the ESI-MS positive ion mode, the molecular ions of MOS were detected $[M+Na]^+$ [15]. The ESI-MS spectrum of LBGHL5 showed that the molecular ion peaked at *m/z* 203, 365, 527, 689, 851 and 1175 (Fig. 1). The *m/z* value corresponded to the mass of a mannose, mannobiose, mannotriose, mannotetrose, mannopentaose, and mannoheptose residue, with an added 23 mass units corresponding to the sodium ion. The sodiated molecular ion peaks of LBGHL26 detected at *m/z* 203, 365, 527, 689, 851 and 1013 were in conformity with the mass of a mannose, mannobiose, mannotriose, mannotetrose, mannopentaose, and mannohexaose residue, respectively (Fig. 1). By means of molecular weight information of MOS, ESI-MS revealed accurately the MOS composition in LBGHL5 and LBGHL26. Mannoheptose was got from the hydrolysates of LBG by Man5HJ14. However, hydrolysates of LBG by ManAJB13 had mannohexaose. The results showed that Man5HJ14 and ManAJB13 were able to hydrolyze LBG into clearly different degraded products. Beside our study, many previous research results were summarized, which were linked to analysis of hydrolysates released from LBG by diverse mannanases (Table 1). It revealed that the composition and content of MOS obtained from LBG are greatly affected by mannanases. GH5 and GH26 mannanases share the similar TIM barrel fold structures and the same retaining type reaction mechanism. However, amino acid sequence identify between them is very low, usually less than 20%. Just like Man5HJ14 and ManAJB13 exhibited 12.4% amino acid sequence identities with one another. These could lead to striking differences in the catalytic pocket width and substrates binding sites. Measured the distance between the conserved His and Tyr or Trp, cleft widths of GH5 and GH26 mannanases were 11.4 Å and 10.1 Å on average. Except Catalytic residue and the −1 subsite, there are little similarity between the other subsites of them [21]. Therefore, hydrolytic properties of mannanases should be influenced by the difference of the cleft width and distal substrate site [31].

3.2. Characteristics of PKC hydrolysates by Man5HJ14 and ManAJB13

Refined hydrolysates obtained from pre-treated PKC prepared by Man5HJ14, ManAJB13 and water were named PKCHL5, PKCHL26, and PKCHL0, respectively. The composition and content of these MOS were analyzed by TLC (Fig. 2). The results showed that both β-mannases of Man5HJ14 and ManAJB13 were capable of producing MOS from PKC compared with the negative control.

In the ESI negative ion mode, the molecular ions of MOS were detected either as a chloride ion addition or a hydrogen ion removal [15]. The ESI-MS spectrum of PKCHL5 and PKCHL26 in the negative ion mode showed that the chloride molecular ion peaked at *m/z* 215, 377, 539 and 701 (Fig. 2). The *m/z* value corresponded to the mass of a mannose, mannobiose, mannotriose, or mannotetrose residue, with an added 35 mass units corresponding to the chloride ion. The dehydrogenated molecular ion peaks detected at *m/z* 179, 341, and 503 were in conformity with the mass of a mannose, mannobiose, and mannotriose residue (Fig. 2), respectively, confirming that both PKCHL5 and PKCHL26 contained these components.

From the above result, no significant difference was observed in the composition of PKCHL5 or PKCHL26. Similarly to MOS production using mRmMan5A from *Rhizomucor miehei*, all tested samples contained mannose, mannobiose, mannotriose, and mannotetrose [32]. The present results determined that the composition of MOS was similar to that obtained from the enzymatic preparation of PKC, but different to that obtained from LBG. Therefore, various mannanases had little effect on MOS composition from prepared PKC assays. Besides, Man5HJ14 produced MOS from PKC at a notably higher efficiency than did ManAJB13 under the same conditions (data not shown). Accordingly, in fermentation research, Man5HJ14 was selected as the carbon source to produce MOS from PKC *in vitro*.

3.3. Evaluation of stability in MOS produced from LBG and PKC

The manufacturing process are often accompanied by high-temperature and high-pressure environments, such as sterilization, spray drying and puffing granulation. So, it is important that MOS are resistant to adversity conditions during the manufacturing process. In our study, LBGHL5, LBGHL26, and PKCHL5 were routinely autoclaved including high temperature and pressure at their natural pH. Then, the TLC-Image J method was used to quantify oligosaccharides, and it existed a good linear relationship between saccharides concentration and grayscale integration respond [33]. Based on the grayscale integration respond, the *p* values of above MOS between untreated and treated by high temperature and pressure were 0.5~1 by TLC-imageJ analysis method, respectively. It meant that all samples were no significant difference between untreated and treated. The results analysis indicated that LBGHL5, LBGHL26, and PKCHL5 underwent no obvious decomposition when compared with the untreated sample (Fig. 3), thereby confirming the high stability of MOS under adversity conditions. The high molecular (44.5 kDa) galactomannan was obtained from spent coffee ground by means of high temperature [34]. The low molecular XOS (the mixture of xylobiose and xylotriose) from corncob xylan demonstrated stability by being resistant to unfavorable conditions, such as heat and acidity [35]. In addition, LBGHL5, LBGHL26, and PKCHL5 were evaluated stability under acid condition (data not shown). LBGHL26 were stability ranging from pH 2–5. However, the percentage of hydrolysis in LBGHL5 and PKCHL5 was positively correlated with pH value. The result was contrary to non-digestible oligosaccharides of green coffee spent [36]. It needs further in-depth detail research. The present study has proven the applicability of MOS with low molecular as a supplement in thermal and stressful manufacturing processes.

3.4. In vitro fermentation of MOS from LBG and PKC

The effects of MOS on the growth of *Lactobacillus* bacteria differ greatly. For example, *Lactobacillus reuteri* C1 utilized MOS from PKC to produce adequate growth, while *Lactobacillus salivarius* I24

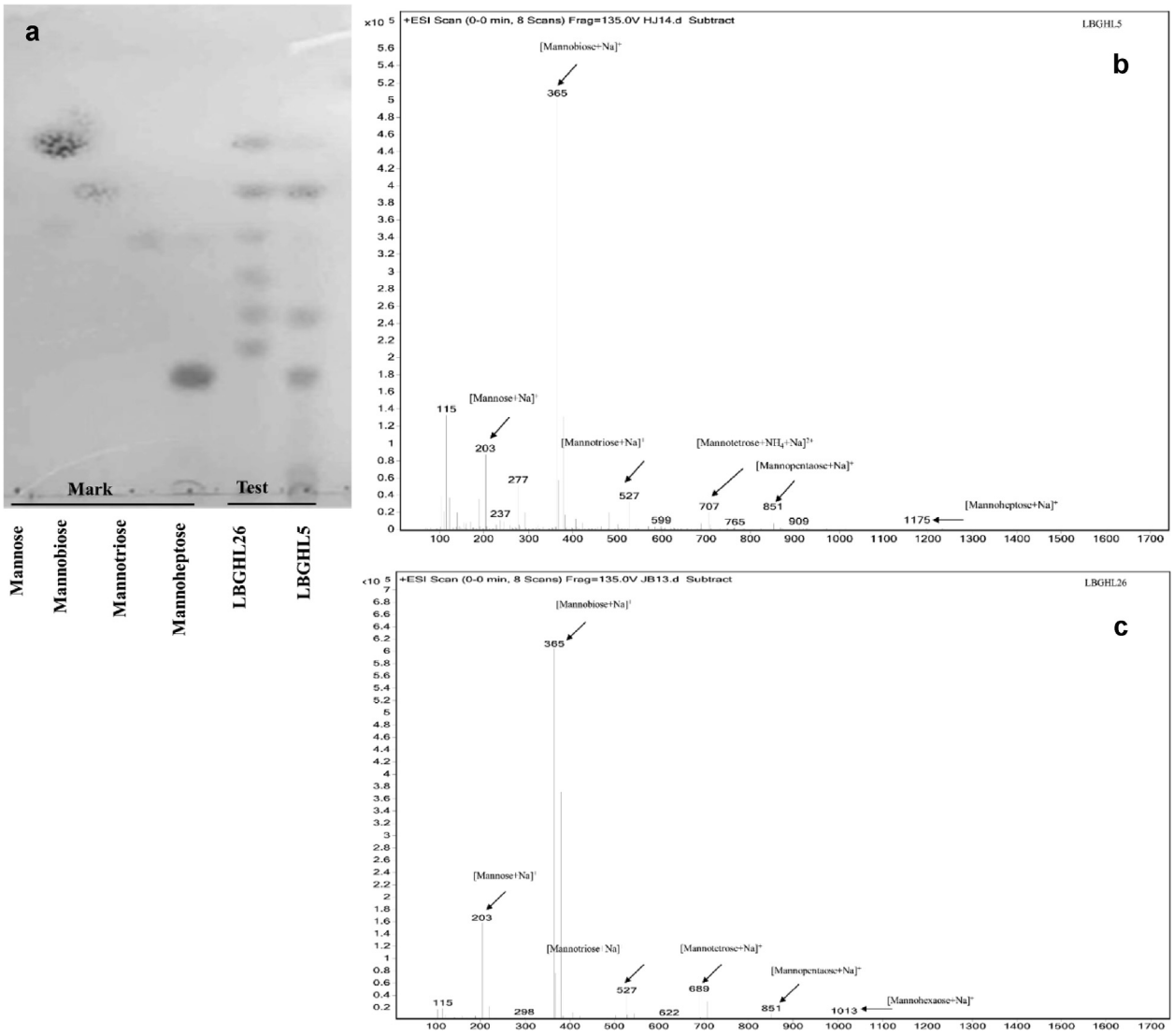

Fig. 1. Analysis of MOS obtained from LBG by TLC and ESI-MS. (a) TLC; (b) ESI-MS of LBGHL5; (c) ESI-MS of LBGHL26.

Table 1
The manno-oligosaccharides from LBG prepared by mannanases from GH5 and GH26.

GH	Origination	Method[a]	Substract	MOS[b,c]						References
				1	2	3	4	5	6	
5	*Bacillus nealsonii* PN-11	HPAEC	LBG	—	11%	23%	20%	18%	—	[23]
5	*Gloeophyllum trabeum* ATCC 11539	HPAEC	LBG	28%	62%	6%	—	2%	—	[24]
5	*Ruminococcus flavefaciens* FD-1 v3	TLC/ESI-MS	LBG	+	+	+	+	+	+	[25]
5	*Aspergillus kawachii* IFO 4308	HPLC	LBG	8%	36%	21%	3%	—	16%	[26]
5	*Bacillus* sp. HJ14	TLC/ESI-MS	LBG	+	+	+	+	+	—	In this study
26	*Bacillus circulans* NT 6.7	HPLC	LBG	—	27%	39%	12%	7%	—	[27]
26	*Clostridium thermocellum* ATCC 27405	HPAEC/TLC	LBG	36%	23%	14%	+	+	—	[28]
26	*Klebsiella oxytoca* KUB-CW2-3	TLC	LBG	—	+	+	+	+	+	[29]
26	*Sphingomonas* sp. JB13	TLC/ESI-MS	LBG	+	+	+	+	+	+	In this study

[a] HPAEC: high-pressure anion-exchange chromatography; HPLC: high performance liquid chromatography.
[b] —: 0% or not detect; number%: the percentage of each MOS component detected by HPAEC or HPLC; +: each MOS component detected by TLC and ESI-MS, because of its only qualitative, all of MOS components were replaced by the same symbol.
[c] 1: Mannose; 2: Mannobiose; 3: Mannotriose; 4: Mannotetrose; 5: Mannopentaose; 6: Mannohexaose.

showed poor growth under similar conditions [15]. MOS from galactomannan in pretreated cassia gum significantly promoted the growth of *Lactobacillus brevis*, *L. delbrueckii,* and *L. reuteri* when compared with *L. acidophilus* and *L. rhamnosus* [37]. NDOs of green coffee spent including MOS showed ability to promoted the growth of *L. plantarum*, *Lactobacillus casei* and *Lactobacillus fermentum* [36]. Only three species of the genus *Lactobacillus*, namely, *L. reuteri*, *L. salivarius,* and *L. casei*, have been used to test for the prebiotic activity of MOS obtained from PKC [15,38]. Although *L. plantarum* is one of the most widespread species of the genus *Lactobacillus*

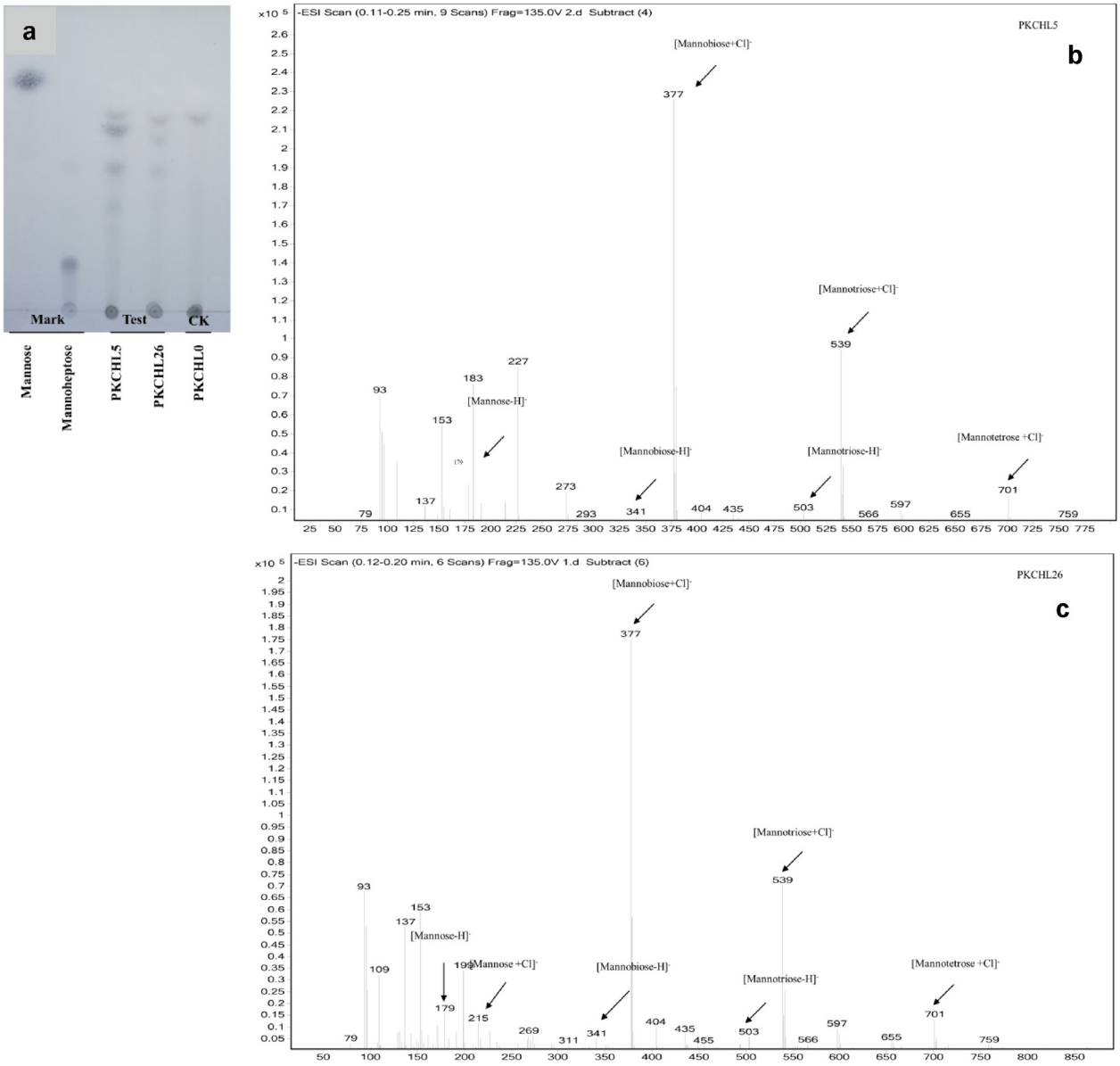

Fig. 2. Analysis of MOS obtained from PKC by TLC and ESI-MS. (a) TLC; (b) ESI-MS of PKCHL5; (c) ESI-MS of PKCHL26.

[39], it has been unknown in the growth situation with MOS as the only carbon source. In our study, MOS from LBG and PKC was used as the carbon source in the fermentation of *L. plantarum in vitro*.

The growth densities (A_{600}) of *L. plantarum* CICC 24202 in basal MRS media containing MOS, positive (glucose and mannose), and negative (normal saline) controls are shown in Fig. 4. At the beginning of the fermentation process, the growth rate of *L. plantarum* CICC 24202 in media containing LBGHL5, LBGHL26, and PKCHL5 were similar to that of the positive control containing glucose, as well as higher than that of the positive control containing mannose. No further increase in *L. plantarum* CICC 24202 density was detected in the medium containing LBGHL5, LBGHL26, and PKCHL5 at 6 h after fermentation, when compared with bacterial density in media with glucose or mannose (positive controls). During the entire fermentation process, *L. plantarum* CICC 24202 in medium containing normal saline did not grow.

The utilization of carbon source in the medium by the bacterial strain were analyzed by TLC, following fermentation (Fig. 5). The results showed that *L. plantarum* CICC 24202 could utilize mannose, mannobiose and mannotriose in LBGHL5 and PKCHL5, but not mannotetrose and above. What's remarkable was that only mannose and manobiose in LBGHL26 were consumed by the strain compared with LBGHL5 and PKCHL5. Galactomannans in prepared LBG are composed of mannopyranosyl residues backbone and substituted by single galactopyranosyl groups at the C-6 position in sugar residues. So, the three types of mannotriose generated by mannanase were substituted mannobiose with galactose at reducing or non-reducing terminal position, and un-substituted mannotriose [40].The interesting phenomenon suggests that composition of oligomeric units from the same raw material could affect the strain to uptake and utilize, which was produced by the different mannanases.

Besides, growth of the bacteria halted at 6 hours after fermentation, indicating that the available carbon source in LBGHL5, LBGHL26, and PKCHL5 was consumed by the strain in the media. Previous research has confirmed that mannobiose and mannotriose are the preferred oligosaccharides among the probiotic *Lactobacillus* sp., rather than functional oligosaccharides with

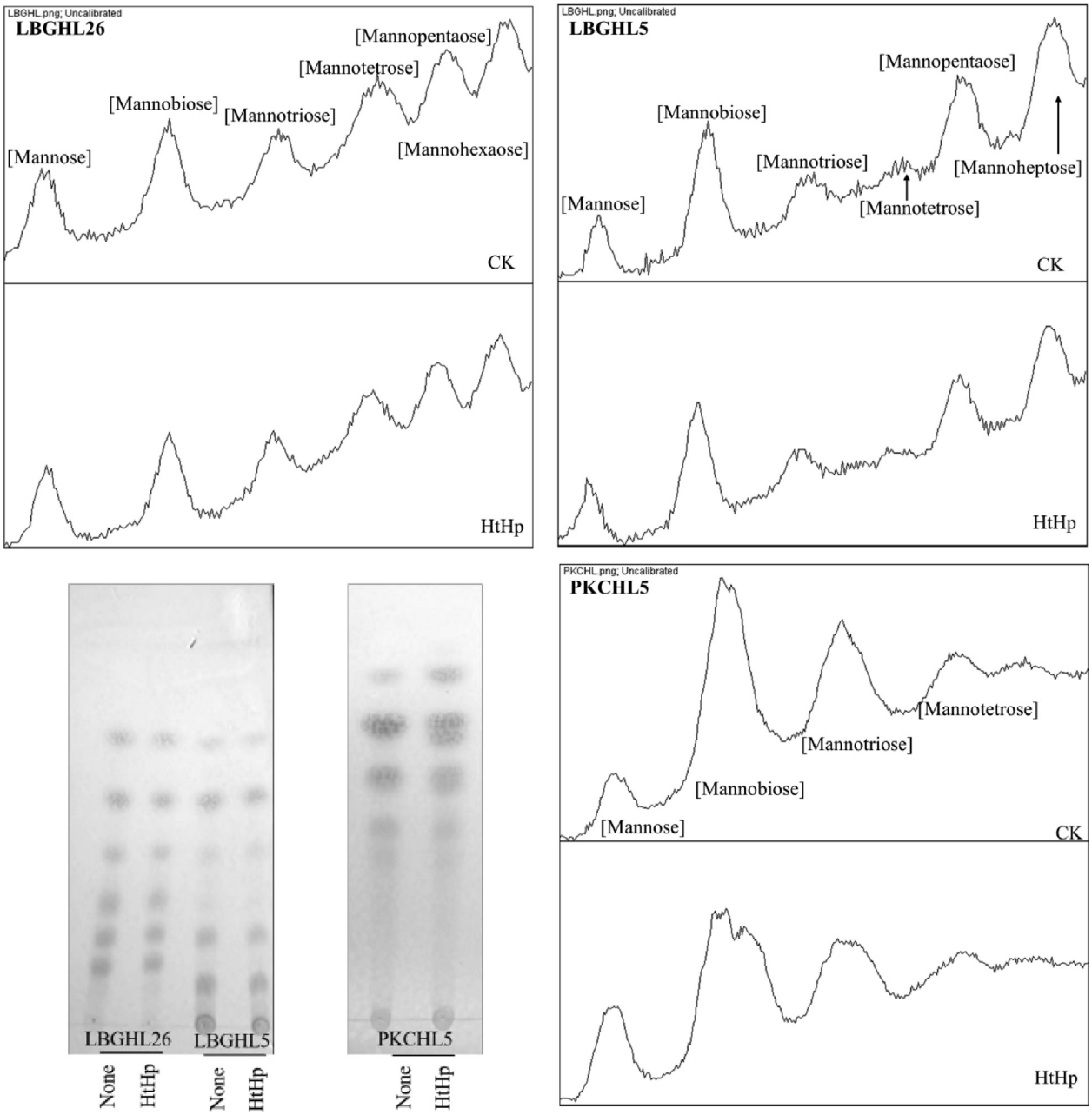

Fig. 3. TLC and ImageJ analysis of MOS treated by high temperature and pressure. None: untreated; HtHp: treated by high temperature and high pressure.

higher degrees of polymerization [37,40]. Therefore, once the available carbon source of MOS was consumed by the strain in the media, the growth of bacteria became limited.

4. Conclusions

In this study, mannans in LBG and PKC were degraded by GH26 ManAJB13 and GH5 Man5HJ14. And then the degradation product was used as the only carbon source for *in vitro* fermentation of *L. plantarum*. The results showed that the hydrolysates of galactomannan from LBG had clearly difference by Man5HJ14 and ManAJB13. However, the hydrolysates of linear mannan from PKC had no significant difference between the MOS components by above mannanases. *In vitro* fermentation, all of tested hydrolysates could efficiently promote the growth of *L. plantarum* CICC 24202. Our results inform the effect of mannanases and mannan difference on MOS composition and the potential for the development of MOS as the synbiotics with *L. plantarum*.

Conflict of interest

The authors declare no potential conflict of interest.

Financial support

This work was supported by the National Natural Science Foundation of China [grant numbers 32060214], the Research and Development Projects in Important Areas of Guangdong Province [grant numbers 2019B020218003], the Yunnan Fundamental Research Projects [grant numbers 202001AS070022] and the National Innovation and Entrepreneurship Training Program for College Students of China [grant numbers 201810681004].

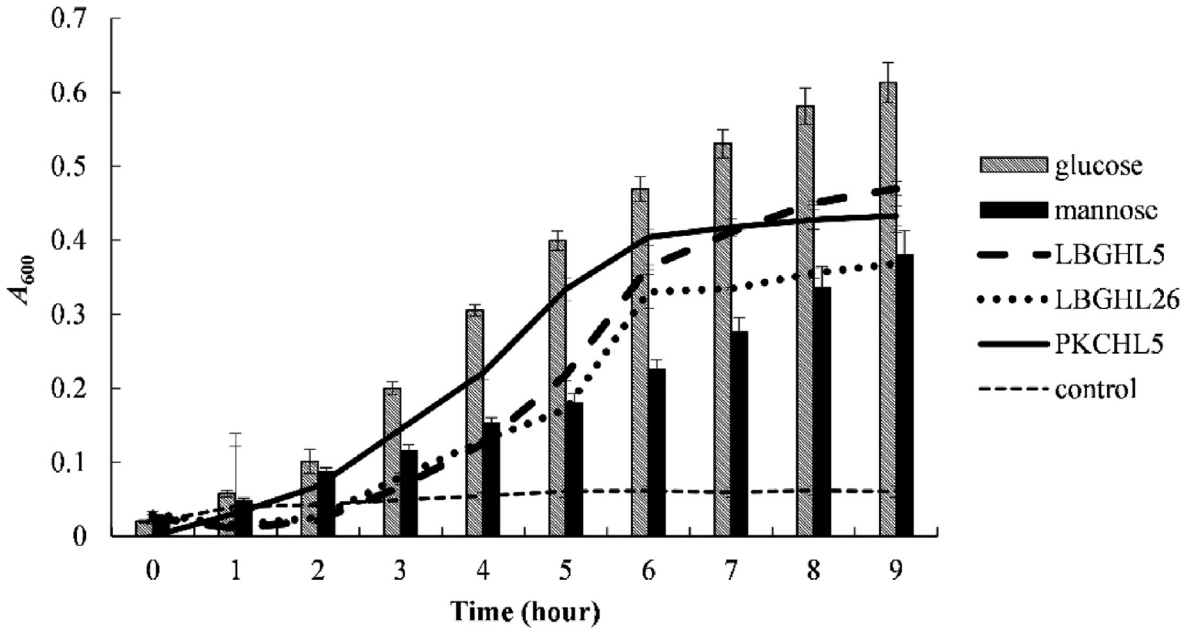

Fig. 4. Growth of *L. plantarum* CICC 24202 in basal medium with MOS as sole carbon source.

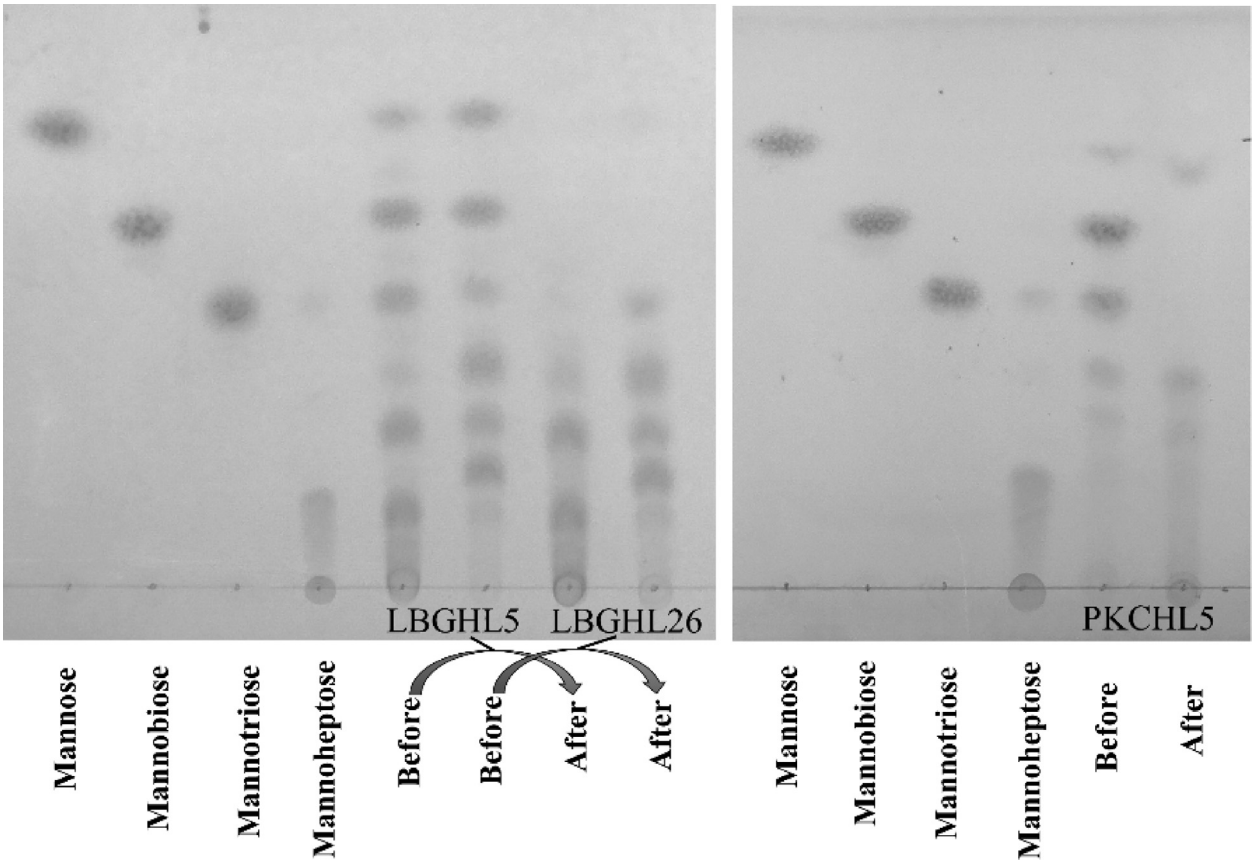

Fig. 5. TLC analysis of carbon source utilization in basal medium before and after fermentation by *L. plantarum* CICC 24202. Before: MOS before fermentation by the strain; After: MOS after fermentation by the strain.

Acknowledgments

The authors acknowledge with thanks Prof. Yang Peilong and Dr. Cai Hongying from the Feed Research Institute of the Chinese Academy of Agricultural Sciences, for *L. plantarum* CICC 24202 strain support.

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