




Conversion of sacha inchi (*Plukenetia volubilis* L.) residues into potential prebiotic oligosaccharides

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Abstract

Optimal conditions for the production of oligosaccharides from sacha inchi shell (SL) and defatted kernel cake (KC) were studied by combining chemical and enzymatic treatments. Growth enhancement of *Lactobacillus* spp. and resistance to digestion under simulated gastrointestinal conditions were also assessed. SL and KC powders were subjected to alkali, delignification, and combined alkali and delignification pretreatments. The pretreated materials were then hydrolyzed by the commercial cellulases cellulase SS (CSS) and cellulase XL-531 (CXL). Pretreating SL with delignification and pretreating KC with alkali before hydrolysis by CSS gave the highest content of reducing sugars and various types of oligosaccharides. Response surface methodology gave optimized conditions for SL oligosaccharide (SLO) as 60 °C, 4 h, and pH 5.14 with an enzyme concentration of 1.52% (v/v), while optimized KC oligosaccharide (KCO) was recorded at 45.68 °C, 4.5 h, and pH 4 with an enzyme concentration of 1.05% (v/v). The crude oligosaccharides were then purified by *Saccharomyces cerevisiae*, which removed 80.80% and 99.97% of glucose from SLO and KCO, respectively. Both SLO and KCO profiles were analyzed by thin layer chromatography (TLC), containing cellobiose, xylobiose, and xylotriose. The oligosaccharides were evaluated for their prebiotic properties—enhancement of the growth of *Lactobacillus brevis*, *L. delbrueckii* subsp. *bulgaricus*, and *L. plantarum*. SLO stimulated probiotic growth more than KCO. The oligosaccharides also promoted growth of *L. brevis*, with a highest specific growth rate similar to that of commercial xylo-oligosaccharides. SLO withstood in vitro digestion (77.89% being retained), demonstrating the prebiotic potential of SL for oligosaccharide production.

Keywords Sacha inchi residues · Oligosaccharide · Prebiotic · Probiotic

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1 Introduction

Sacha inchi (*Plukenetia volubilis* L.) is a perennial plant of the Euphorbiaceae family. The fruits are green and ripen to blackish brown with capsules of four to seven lobes. Sacha inchi seeds have protein content of 27% and oil content of 35% to 60%. The oil is rich in essential fatty acids including omega-3, omega-6, vitamin A, and vitamin E with widespread industrial oil extraction [1]. Following the sachu inchi oil squeezing process, the remaining by-products such as sachu inchi shells (SL) and defatted kernel cake (KC) account for up to 50% of the raw material, with no current value-added benefits.

Recently, research has focused on extraction of the protein in sachu inchi by-products that are also high in fiber. SL contains 85.45% fiber, while KC contains 25.26%, but research to support the utilization of dietary fiber from these by-products is lacking [2]. Nondigestible carbohydrates, including dietary fiber, oligosaccharides, and resistant starch, serve a variety of physiologic activities, and many nondigestible carbohydrates have an effect on well-being, improved health and disease prevention [3]. Soluble and insoluble dietary fibre from plants consists of lignocellulosic material such as wheat straw, corn stover, orange peel, cassava, and almond shell and can be converted into oligosaccharides as prebiotic ingredients. A prebiotic can be defined as a functional food, carbohydrate or non-carbohydrate, that withstands digestion and absorption in the upper gastrointestinal tract, encourages beneficial microorganisms as probiotics in the colon, and provides health benefits in the gastrointestinal tract and throughout the body [4]. The gut microbial diversity could be changed according to the nutritional behavior [5]. The essential final components of carbohydrate metabolism are short-chain fatty acids, in particular acetic acid, propionic acid, and butyric acid, which are fermented by probiotics and used as an energy source by the host organism.

Oligosaccharides can be produced by three different methods, the isolation of plant resources, enzymatic synthesis, and enzymatic hydrolysis of polysaccharides [6]. Using enzymes and chemically assisted treatment are good ways to produce large amounts of high-purity oligosaccharides due to specific site activity [7]. Lignocellulosic materials mainly consist of the three polymers cellulose, hemicellulose, and lignin. These polymers are inter-associated in a complex hetero-matrix formed by intrapolymer and interpolymer linkages, connected by strong bonds that are highly resistant to attack by enzymes. The pretreatment process is one of the steps in the conversion of lignocellulose to oligosaccharides by increasing the surface contact area of the material, making it more accessible to enzymatic reactions that increase sugar yields with high-purity

oligosaccharide production. This research investigated the optimal conditions for producing oligosaccharides from SL and KC by combining chemical and enzymatic treatments. Growth enhancement of *Lactobacillus* spp. and resistance to digestion under simulated gastrointestinal conditions were also assessed.

2 Materials and methods

2.1 Materials

SL and KC were obtained from The Ultimate Bangkok Ltd., Thailand. The materials were ground and passed through a 60-mesh sieve. The samples were then dried at 50 °C until the moisture content was lower than 10% (w/w). Commercial cellulase SS and cellulase XL-531 were purchased from Nagase ChemteX Corporation, Tokyo, Japan. All chemicals and solvents used in this study were of analytical grade.

2.2 Chemical and structural carbohydrate composition analysis

The methods of the Association of Official Analytical Chemists were used to determine the moisture, crude fiber, protein, fat, ash, and carbohydrate content [8]. Holocellulose and insoluble lignin were assessed by the TAPPI T222 om-98 method. Determination of structural carbohydrates in SL followed the National Renewable Energy Laboratory method. In brief, 400 mg of SL was mixed with 4.5 mL of 72% sulfuric acid and ground for 30 min. The solution was added to 85 mL of distilled water and heated in an autoclave at 121 °C for 1 h. After completion, the hydrolysate was cooled and filtered. Structural carbohydrate analysis was performed using barium carbonate to neutralize each sample to pH 5.0–6.0 and then passed through a 0.45 µm cellulose acetate membrane. Next, HPAEC-PAD analysis was performed. Briefly, a Dionex CarboPac PA-1 column (250 mm × 4 mm) with a guard column (50 mm × 4 mm) was used at a flow rate of 1.0 mL/min. The post-column pump had a controlled flow rate of 0.5 mL/min. A stepwise linear gradient was applied over 20 min. Peaks of monosaccharides were assigned using xylose (Merck), arabinose (Sigma), mannose (Merck), galactose (Sigma), and glucose (Sigma) standards at 1–5 ppm.

2.3 Pretreatment methods

For the alkali pretreatment method, SL and KC powders were soaked in 0.5 M sodium hydroxide solution at a ratio of 1:10 with continuous stirring for 6 days and then washed with distilled water three times and filtered through a mesh sieve filter. The pH was adjusted to neutral by 1 M hydrochloric acid followed by drying at 50 °C [9].

For the delignification method, 50 g of SL and alkali-pretreated SL powder were applied to 1600 mL of distilled water, and the mixture was stirred to ensure uniformity for 15 min. Then, 10 mL of acetic acid and 30 g of sodium chlorite were added to the suspension every hour, eight times with continuous stirring. The suspension was placed in a water bath at 70 °C in a chemical hood to contain chlorine dioxide gas from the reaction of lignin in the SL. The delignified suspension was soaked in a cold-water bath for 20 min, then filtered to separate the liquid, and washed with hot water 3–4 times until the sample became white. Finally, the sample was precipitated twice with 95% ethanol and once with acetone before drying at 50 °C.

For deproteinization, KC and alkali-pretreated KC powder were added to distilled water at a ratio of 1:10. The mixture was adjusted to pH 9.0 with 2 M sodium hydroxide and soaked for 2 h. The pH was then adjusted to neutral with 1 M hydrochloric acid followed by drying at 50 °C.

All pretreated samples were also hydrolyzed with commercial cellulase to determine whether the pretreatment process improved oligosaccharide production. The commercial cellulases used in this study were cellulase SS (CSS) and cellulase XL-531 (CXL) (Nagase ChemteX Corporation, Japan). CSS contains cellulase, β -glucanase, β -1,3 glucanase, and xylanase (1936 U/g, 394,575 U/L, 535 U/L, and 755 U/g, respectively), while CXL contains cellulase, β -1,3 glucanase, and xylanase (1559 U/g, 995 U/L, and 1451 U/g, respectively). In brief, pretreated samples were added to 1 M sodium acetate buffer pH 5.0 at a ratio of 1:20. The commercial cellulases were used at an enzyme concentration of 0.5% (v/v) and incubated at 50 °C with continuous shaking at 170 rpm for 0, 8, and 24 h. Next, the reducing sugar content was analyzed using the colorimetric dinitrosalicylic acid (DNS) method with glucose as a standard [10]. The sugar profile was analyzed using thin layer chromatography (TLC) performed on Merck TLC silica gel 60 (20 × 20 cm aluminum sheets). The plates were developed once with 1-butanol:acetic acid:water in the ratio 2:1:1. Spots were verified by spraying with 10% sulfuric acid in ethanol with 0.2% orcinol and then heated at 110 °C and compared with standard mixtures of the xylo-oligosaccharides (XOS) xylobiose

(X₂), xylotriose (X₃), xylo-tetraose (X₄) and xylopentaose (X₅) (Wako, Japan), cellobiose (C₂) (Sigma), and glucose (G) (Megazyme, Ireland).

2.4 Optimization of oligosaccharide production

For oligosaccharide production, temperature, time, pH, and enzyme concentration were considered important factors influencing sugar production. A Box–Behnken design (BBD) was used to investigate the interaction between the studied parameters affecting the amount of sugar generated. The BBD experiments were established based on references, with maximum and minimum values for temperature (F₁), time (F₂), pH (F₃), and enzyme concentration (F₄) [11]. BBD coded values and experimental data for oligosaccharide production are presented in Table 1. Optimal conditions producing the highest sugar yields were determined using response surface methodology (RSM). The calculations were carried out using Design Expert 7.0. The four distinct variables were studied at the same concentration of substrate (50 mg/mL) in 1 M sodium acetate buffer. After completion, the crude oligosaccharide was freeze-dried and analyzed for reducing sugar content using the DNS method.

2.5 Purification of oligosaccharides

Saccharomyces cerevisiae (TISTR 5019) and *Candida pulcherrima* (JCM 1631) were used for the purification of crude oligosaccharides. Briefly, 1% of 6 log CFU/mL initial starter culture was added to yeast and mould medium consisting of 0.4% peptone, 0.1% yeast extract, 0.1% potassium dihydrogen phosphate, and 0.05% magnesium sulfate in 100 mL of distilled water. The culture was incubated at 30 °C for 24–72 h. After completion, the culture was centrifuged at 6000 rpm to separate the cells, filtered through a 0.2 μ m filter syringe, and freeze-dried. The purified oligosaccharide was analyzed for sugar profile by TLC and for monosaccharide content by HPAEC.

Table 1 Levels of variables for construction of the Box–Behnken design

Factor	Variation	Levels (SL)			Levels (KC)		
		–1	0	+1	–1	0	+1
Temperature (°C)	F ₁	40	50	60	40	50	60
Time (h)	F ₂	4.0	8.0	12.0	0.5	2.5	4.5
pH	F ₃	4.0	5.0	6.0	4.0	5.0	6.0
Enzyme concentration (v/v)	F ₄	0.75	1.25	1.75	0.25	0.75	1.25

SL, sacha inchi shell; KC, defatted sacha inchi kernel cake

2.6 Evaluation of prebiotic properties

2.6.1 Growth enhancement of lactic acid bacteria

The probiotics used in this research were strains of *Lactobacillus* spp.: *Lactobacillus plantarum* (JCM1149^T), *Lactobacillus delbrueckii* subsp. *bulgaricus* (JCM1002), and *Lactobacillus brevis* (TISTR860). Activated *Lactobacillus* spp. were added to modified 1% (v/v) De Man, Rogosa, and Sharpe medium (MRS) consisting of 10 g of peptone, 10 g of beef extract, 5 g of yeast extract, 2.6 g of dipotassium hydrogen phosphate, 5 g of sodium acetate, 1.7 g of ammonium monohydrogen citrate, 0.2 g of magnesium sulfate, 0.05 g of manganese sulfate, and 1 mL of Tween 80 in 1000 mL of distilled water. The obtained oligosaccharide was used as a carbohydrate source instead of dextrose. Glucose was used as a positive control and modified MRS without a carbohydrate source was used as a negative control. Each carbohydrate source was added at 2% (w/v) to modified MRS and compared to commercial XOS. The culture medium was incubated at 37 °C for 48 h, and optical density was measured at 600 nm every 10 min. The culture medium was spread in MRS agar to determine the microbial growth curve at 0–40 h incubation time.

2.6.2 Resistance to digestion under simulated human gastrointestinal tract conditions

Simulated gastric fluid buffer (SGF) and simulated intestinal fluid buffer (SIF) were prepared as described by Hongpattarakere et al. [12]. Ten milliliters of the obtained oligosaccharide and commercial XOS (2%, w/v) were mixed with 7.5 mL of SGF buffer and 1.6 mL of 25,000 U/mL pepsin. The pH of these mixtures was adjusted to 2.0 with 1 M HCl, and they were incubated at 37 °C for 2 h. Then, 20 mL of the mixtures was mixed with 11 mL of SGF buffer, 0.5 mL of 800 U/mL porcine pancreatin, and 2.5 mL of 160 mM bile salt. The pH of these mixtures was adjusted to 6.9 with 1 M NaOH, and they were incubated at 37 °C for 2 h. All samples were collected at 2 and 4 h and analyzed for reducing sugar content using the DNS method and for total sugar [13] content using the phenol–sulfuric acid method. The percentage of hydrolysis is calculated as

$$\% \text{hydrolysis} = \frac{(\text{final reducing sugar} - \text{reducing sugar released}) \times 100}{\text{total sugar content} - \text{initial reducing sugar content}} \quad (1)$$

2.7 Experimental design and statistical analysis

A completely randomized design (CRD) with three replications and analysis of variance (ANOVA) were used to compare mean values by Duncan's multiple range test (DMRT) at 95% confidence level.

3 Results and discussion

3.1 Chemical and carbohydrate composition of sacha inchi residues

The chemical composition of sacha inchi residues was determined (Table 2). SL contained 47.20% carbohydrate and 37.20% fiber, consisting of 0.14% soluble fiber and 37.06% insoluble fiber. KC contained protein and fat as the major components. Sathe et al. [14] reported the extraction of protein from sacha inchi seeds to produce a protein powder to increase its value. KC contained 7.17% fiber consisting of 1.39% soluble fiber and 5.78% insoluble fiber. The composition of SL is substantially less well known compared to KC. de Souza et al. [15] reported that the seed shell of sacha inchi contains 1.24% total lipids and is low in saturated fatty acids and high in α -tocopherol, consisting of n-3 fatty acids at 438.7 mg/g of total lipid. Sacha inchi shell biomass extract has been used to synthesize silver nanoparticles with silver nitrate solution [16]. Sacha inchi shells are high in phenolic compounds and antioxidants; one study reported a total phenolic compound content of 74.56 mg/g, with condensed tannins (93.1%) accounting for the majority of the phenolic compounds in the shell [17]. The lipid content of sacha inchi oil press-cake is reported as 5–25%. Previous studies reported that sacha inchi oil press-cake contains protein 32–62% and 11.1–25.3% total dietary fiber [2, 18, 19]. Many researchers have studied the extraction of protein from sacha inchi KC. Chirinos et al. [20] reported that alkali and enzyme-assisted protein extraction from defatted sacha inchi cake meal provided a maximum protein yield of 46% at an enzyme (Alcalase 2.4L) concentration of 5.6%, extraction

Table 2 Chemical and carbohydrate composition (mean and standard deviation) of SL and KC

Composition	Content (wt%)	
	SL	KC
Moisture	8.51 ± 0.25	5.44 ± 0.10
Ash	1.44 ± 0.05	4.28 ± 0.03
Fat	1.55 ± 0.34	26.85 ± 0.16
Protein	4.10 ± 0.23	35.99 ± 2.81
Carbohydrate	47.20	20.27
Fiber	37.20 ± 0.75	7.17 ± 0.15
Soluble fiber	0.14	1.39
Insoluble fiber	37.06	5.78
Lignocellulose		
Cellulose	13.37 ± 0.06	17.71 ± 0.18
Hemicellulose	12.17 ± 0.07	3.18 ± 0.17
Lignin	16.56 ± 0.48	-

SL, sacha inchi shell; KC, defatted sacha inchi kernel cake

time of 40.4 min, meal:solvent ratio of 1:50 (w/v), pH 9.0, and 50 °C. The essential amino acid composition of sacha inchi press-cake includes lysine, histidine, and leucine as well as high amounts of isoleucine, valine, tryptophan, and phenylalanine [19]. Sacha inchi hydrolysates obtained with Alcalase-Neutrase for 4 h at 50 °C demonstrated the lowest ACE inhibition and IC₅₀ value, with high antioxidant activity determined by the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) method of 1.19 µmol Trolox equivalent/mg [1]. However, the literature contains no evidence of the use of dietary fiber from sacha inchi residue from sacha inchi oil manufacturing.

Lignocellulose is the most abundant biopolymer found in waste biomass and is commonly used for biofuel production. The transformation of lignocellulose into functional sugar depends on its nutritional properties. Hence, this study focused on the oligosaccharides obtained from lignocellulose degradation of sacha inchi residues. SL consisted of 13.37% cellulose and 12.17% hemicellulose, and KC consisted of 17.71% cellulose and 3.18% hemicellulose, while lignin was 16.56% of SL but not found in KC. The results showed that cellulose was the main component of SL and KC. Commercial cellulase enzymes are used in the production of oligosaccharides. Table 2 shows the chemical composition of sacha inchi residues. SL contained high amounts of lignin (16.56%), and this should be removed before the production of oligosaccharides. Conversely, KC did not contain lignin, but the protein in KC should be eliminated. Therefore, selection of the pretreatment method is critical to maximize the conversion of lignocellulosic materials into sugars by enzymatic

hydrolysis [21]. Previous reports indicate that an alkaline reagent improves the degradation of lignocellulose [22].

The monosaccharide composition of sacha inchi residues is shown in the HPAEC chromatogram (Fig. 1). SL consisted of two main monosaccharides, glucose (14.49%), and xylose (5.90%), while KC consisted of 3.68% glucose and 1.27% arabinose. Glucose was the main component in SL and KC. As a result, the predicted oligosaccharides produced were divided into three categories: cello-oligosaccharide, XOS, and arabinoxylo-oligosaccharide. Polysaccharides previously extracted from sacha inchi seeds consisted of 69.76% glucose, 14.86% mannose, 10.53% arabinose, 2.42% galactose, 1.23% ribose, 0.27% rhamnose, and 0.93% xylose [23]. Non-isothermal autohydrolysis of peanut shells at 210 °C presented an XOS yield of 48.24% [24]. Almond shells have a high xylan content, with an XOS yield of 63% for autohydrolysis at 190 °C for 19 min [25]. The highest XOS production reported in the literature was achieved from hazelnut shell by autohydrolysis at 190 °C for 15 min, yielding 62% of the initial xylan [26].

3.2 Effect of pretreatment on reducing sugar content and sugar profile

Because of differences in their chemical composition, the methods of pretreating SL and KC were specific, as shown in Table 2. SL pretreated with alkali appeared as a dark-brown powder, while SL pretreated by delignification and alkali-delignification was a light powder (Fig. 2a–c). KC pretreated with alkali gave a light-brown powder and deproteinization provided a light-yellow powder while alkali-deproteinization

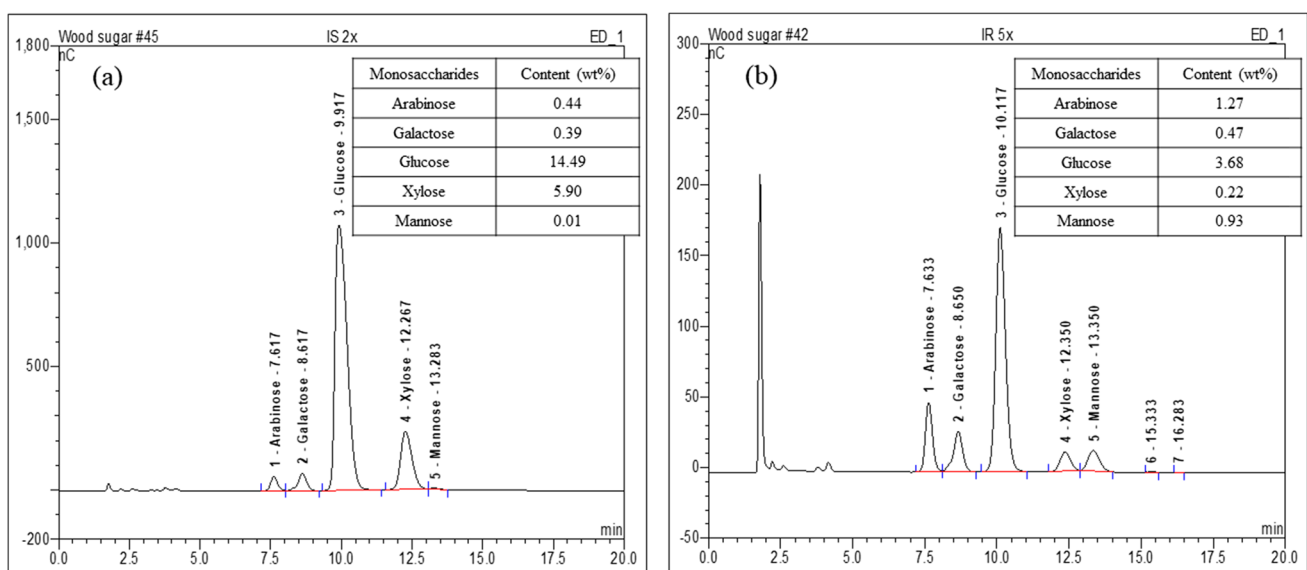


Fig. 1 HPAEC chromatograms showing monosaccharide compositions of SL (a) and KC (b)

Fig. 2 SL images pretreated with alkali (a), delignification (b), and alkali-delignification (c), and KC pretreated with alkali (d), deproteinization (e), and alkali-deproteinization (f)



provided a dull-brown powder (Fig. 2d–f). Pretreatment is an important stage in the cellulose and hemicellulose conversion process since it prepares the cellulosic components for enzymatic reaction [21]. During lime pretreatment at mild temperatures of 60–90 °C, lignin and hemicellulose were preferentially removed but cellulose was unaffected. Alkali pretreatment generally requires lower temperatures, pressures, and residence periods depending on the nature of the biomass feedstock, particularly its lignin content [27]. Saponification of intermolecular ester linkages, cross-connected xylan hemicelluloses and other components during alkaline hydrolysis, and the porosity of the lignocellulosic biomass increase as the cross links are removed [28]. Alkali pretreatment increases the susceptibility of poplar wood to enzymatic hydrolysis due to partial degradation of the lignin which prevents cellulase penetrating the biomass [29]. Furthermore, alkali treatment decomposes lignin preferentially without affecting the polysaccharide component of lignocellulose [30]. According to Zheng et al. [31], 6% w/w NaOH is the strongest alkali chemical, achieving an 85% increase in glucose yield by enzymatic hydrolysis from rice straw in 24 h at 25 °C. Delignification with sodium chlorite has been studied to reduce the lignin content of lignocellulosic material. Enzymatic hydrolysis improves with an increase of accessible pore capacity to a specific majority after removal of lignin by sodium chlorite delignification. Sodium chlorite delignification results in substantial enzymatic conversion when the lignin content is reduced to 15%, corresponding to 0.30–0.35 g/g accessible pore volume in forest biomass [32].

Pretreated SL and KC were used as precursors for the production of oligosaccharides by CSS and CXL. Increasing the incubation time increased the reducing sugar content in all samples. The reducing sugar content of SL pretreated

with alkali, delignification, and alkali-delignification then hydrolyzed with CSS for 24 h was 2655.0, 84,900.0, and 9025.0 ppm, respectively, while hydrolysis with CXL gave 3552.5, 6077.5, and 5630.0 ppm, respectively (Table 3). SL pretreated by delignification had the highest reducing sugar content, followed by that pretreated with alkali-delignification, while SL pretreated with alkali provided the lowest reducing sugar content. Delignification pretreatment removed 44.2% of the lignin content and gave a yield of 84.0% (data not shown). The reducing sugar content of KC pretreated with alkali, deproteinization, and alkali-deproteinization then hydrolyzed with CSS for 24 h was 20,680.0, 6805.0, and 7105.0 ppm, respectively, while hydrolysis with CXL gave 11,510.0, 12,010.0, and 7130.0 ppm, respectively. KC pretreated with alkali had the highest reducing sugar content, followed by that subjected to deproteinization and alkali-deproteinization, respectively. Alkali pretreatment removed 94.2% of the protein content (data not shown).

Oligosaccharide profiles were observed by TLC (Fig. 3). Fewer monosaccharides should be present in the optimal oligosaccharide profile because monosaccharides can be absorbed in the small intestine and used by pathogens in the colon, resulting in fewer prebiotic properties. SL pretreated with delignification and alkali-delignification produced oligosaccharides with a degree of polymerization (DP) greater than 2, while the main product of SL pretreated with alkali was DP 2 oligosaccharide. By contrast, KC pretreated with alkali and hydrolyzed with CSS gave the highest reducing sugar content and an oligosaccharide DP greater than 2. Furthermore, the two commercial enzymes used in this study were compared. CSS provided greater oligosaccharide variety than CXL, while monosaccharide amounts were lower with both materials. The different composition of the

Table 3 Reducing sugar content (mean and standard deviation) of pretreated SL and KC after hydrolysis by commercial cellulases

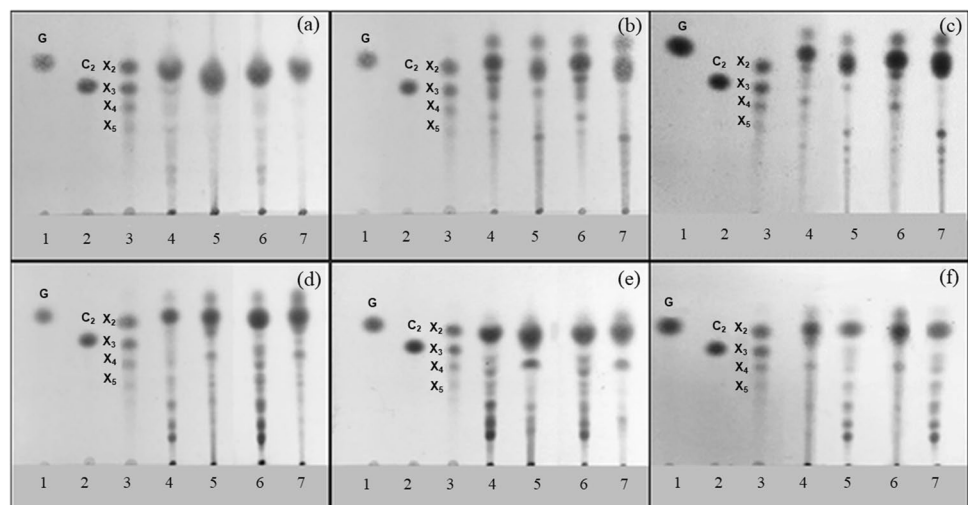
Raw material	Pretreatment method	Com- mercial enzyme	Reducing sugar content (ppm)		
			0 h ^C	8 h ^B	24 h ^A
SL	Alkali	CSS	15.3 ± 0.0 ^b	152.9 ± 0.1 ^e	2655.0 ± 0.0 ^d
		CXL	15.3 ± 0.0 ^b	152.8 ± 0.1 ^e	3552.5 ± 0.0 ^d
	Delignification	CSS	19.0 ± 0.0 ^a	8490.0 ± 123.7 ^a	84,900.0 ± 176.8 ^a
		CXL	17.8 ± 1.8 ^a	6165.0 ± 53.0 ^b	6077.5 ± 35.4 ^c
	Alkali-delignification	CSS	15.3 ± 0.0 ^b	5677.5 ± 70.7 ^c	9025.0 ± 353.6 ^b
		CXL	15.3 ± 0.0 ^b	1877.5 ± 35.36 ^d	5630.0 ± 176.8 ^c
KC	Alkali	CSS	81.5 ± 8.8 ^a	7152.0 ± 141.1 ^b	20,680.0 ± 35.4 ^a
		CXL	77.8 ± 7.1 ^b	9340.0 ± 123.7 ^a	11,510.0 ± 282.8 ^c
	Deproteinization	CSS	69.9 ± 1.8 ^c	1065.0 ± 17.7 ^f	6805.0 ± 212.1 ^e
		CXL	76.5 ± 8.8 ^{b,c}	1615.5 ± 53.0 ^e	12,010.0 ± 707.1 ^b
	Alkali-deproteinization	CSS	30.3 ± 3.5 ^d	5677.0 ± 106.1 ^d	7105.0 ± 141.4 ^d
		CXL	19.0 ± 1.8 ^e	6640.0 ± 123.7 ^c	7130.0 ± 176.8 ^d

^{A–C}Values with different superscript uppercase letters are significantly different for time of incubation

^{a–f}Values with different superscript lowercase letters are significantly different for pretreatment method and commercial enzyme used ($p < 0.05$)

SL, sachu inchi shell; KC, defatted sachu inchi kernel cake; CSS, cellulase SS; CXL, cellulase XL-531

Fig. 3 TLC chromatograms of sugar profiles obtained from SL pretreated with alkali (a), delignification (b), alkali-delignification (c), and oligosaccharide obtained from KC pretreated with alkali (d), deproteinization (e), and alkali-deproteinization (f) after hydrolysis by commercial cellulases at pH 5.0, 50 °C for 8, and 24 h. Lane 1, standard glucose; lane 2, standard cellobiose; lane 3, mixture of standard XOS; lane 4, CSS at 8 h; lane 5, CXL at 8 h; lane 6, CSS at 24 h; lane 7, CXL at 24 h



commercial enzymes caused the variety of oligosaccharides produced, with CSS containing cellulase, glucanase and xylanase, and CXL containing mostly cellulase and xylanase. Thus, SL pretreated with delignification and KC pretreated with alkali hydrolyzed by CSS, having the highest reducing sugar content and lower monosaccharide content, were further optimized for effective oligosaccharide production by RSM. However, the purity of the oligosaccharides was reduced due to the presence of monosaccharides that have reduced prebiotic potential because of their ability to rapidly absorb through the upper part of the small intestine [33]. Díaz et al. [34] reported that enzymatic hydrolysis of banana pseudostem pulp provides the highest cello-oligosaccharide content using a low dosage of cellulase, slowing the

rate of glucose production. As a result, reducing the monosaccharide content of the mixture is critical for improving the quality of the final oligosaccharide product from enzymatic hydrolysis [35, 36].

3.3 Optimization of oligosaccharide production by enzyme hydrolysis

The concentrations of reducing sugars obtained using the generated BBD experimental conditions for both SK and KC are reported in Supplementary Table 1. Concentration ranges of sugars from SL and KC, respectively, were 8641.27–37,176.70 and 4913.23–20,514.30 ppm. Based on the experimental data and the resulting amount of sugars,

polynomial equations for reducing sugar production using SL and KC are presented as follows:

$$\begin{aligned}
 Y_{SL} = & -2.84 \times 10^5 + 3520.86F_1 + 12,646.06F_2 + 63,184.26F_3 \\
 & + 10,815.38F_4 - 263.33F_1F_2 \\
 & + 170.90F_1F_3 + 137.51F_1F_4 + 66.14F_2F_3 \\
 & + 1960.71F_2F_4 - 2190.48F_3F_4 - 24.17F_1^2 \\
 & - 193.45F_2^2 - 6843.99F_3^2 - 193.45F_4^2
 \end{aligned} \quad (2)$$

$$\begin{aligned}
 Y_{SL} = & -2.02 \times 10^5 + 5290.16F_1 + 21,202.28F_2 + 22,448.20F_3 \\
 & + 30,084.41F_4 - 151.91F_1F_2 + 64.86F_1F_3 \\
 & + 351.75F_1F_4 - 1465.08F_2F_3 - 3747.09F_2F_4 \\
 & - 1888.30F_3F_4 - 57.31F_1^2 \\
 & - 495.26F_2^2 - 2159.04F_3^2 - 18,228.48F_4^2
 \end{aligned} \quad (3)$$

The RSM contour plots in Fig. 4 reveal the interaction between the studied variables affecting the amount of reducing sugar. The contours were parabola-shaped with the crown identifying the maximum values of the produced sugar. The optimal values were in the middle of the generated contours, implying that the established BBD models are applicable for the investigation of optimal conditions. For oligosaccharide production using SL, the optimal conditions were 60.00 °C, 4 h, pH 5.14, and an enzyme concentration of 1.52%, resulting in a reducing sugar content of 35,187.30 ppm (R^2 0.9094; R^2 adjusted 0.8036). Conversely, the optimal conditions for sugar production using KC were 45.68 °C, 4.5 h, pH 4.00, and an enzyme concentration of 1.05%, resulting in a reducing sugar content of 18,641.27 ppm (R^2 0.9177; R^2 adjusted 0.8217). To experimentally validate the optimization results, the sugar production processes were carried out using the obtained

optimal conditions, resulting in a reducing sugar content of 35,187.30 and 18,641.27 ppm, respectively, for SL and KC with error values of 0.84 and 3.22, respectively.

3.4 Purification of oligosaccharides

Impurities of SL oligosaccharide (SLO) and KC oligosaccharide (KCO) included glucose and other monosaccharides. A biological approach was used in this study for purification due to the low economic cost and ease of processing. Another method of purifying oligosaccharides is to use yeast to ferment unintended monosaccharide impurities into ethanol, which increases XOS purity [37]. *S. cerevisiae* and *C. pulcherrima* are two yeast strains that effectively use monosaccharides as a carbon source. Instead of glucose, 2% crude oligosaccharides were added to the yeast culture medium and incubated at 30 °C for 24–72 h. The glucose band on TLC disappeared in SLO purified with *S. cerevisiae* and was substantially reduced in SLO purified with *C. pulcherrima* (Fig. 5), while both yeasts removed the glucose band in KCO. Furthermore, the glucose band was still apparent after 1 day of incubation, while it was removed, and the other sugar profiles remained the same after 48 and 72 h of incubation (data not shown). *S. cerevisiae* is the most commonly used microorganism for industrial ethanol production from hexoses. It is also widely used as a cell factory to make advanced biofuels, chemicals, and pharmaceuticals. According to Lian et al. [38], *S. cerevisiae* utilizes 50 mM glucose and galactose completely within 24 h. *S. cerevisiae* has also been used to purify gluco-oligosaccharides, despite the fact that only the monosaccharide can be removed [39]. In a previous study, *C. pulcherrima* eliminated 6.5% of xylose within 4 days but did not assimilate xylobiose and the DP of the sugar remained constant [40]. As a result, *S. cerevisiae*

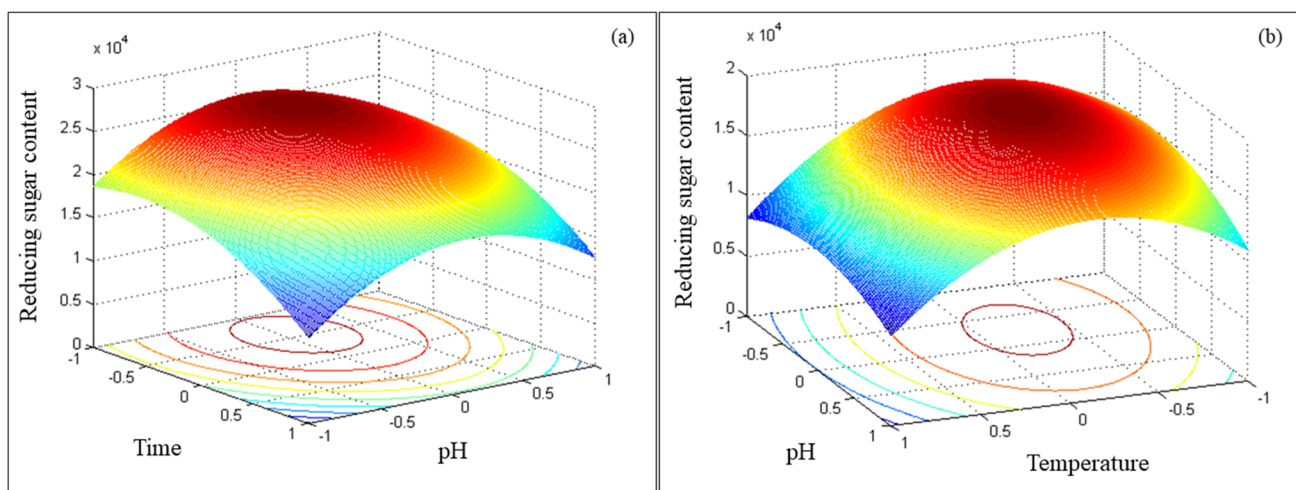


Fig. 4 RSM contour plots for SL showing interaction between time (F_2) and pH (F_3) (a) and for KC showing interaction between temperature (F_1) and pH (F_3) (b) affecting the amount of sugar produced

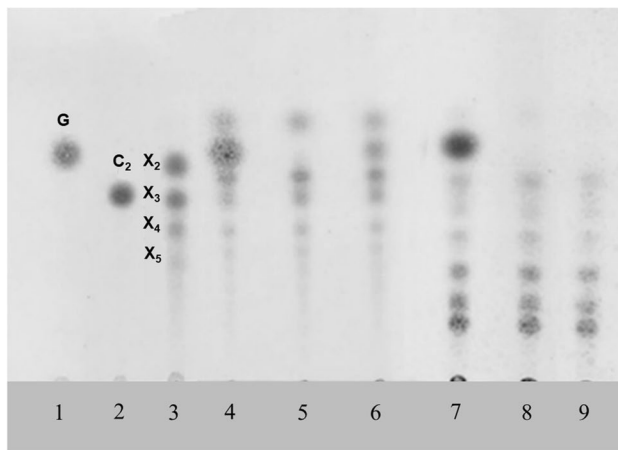


Fig. 5 TLC chromatograms of sugar profiles obtained from crude SLO and KCO after purification by fermentation with *S. cerevisiae* and *C. pulcherrima* at 30 °C for 48 h. Lane 1, standard glucose; lane 2, standard cellobiose; lane 3, mixture of standard XOS; lane 4, crude SLO; lane 5, SLO purified by *S. cerevisiae*; lane 6, SLO purified by *C. pulcherrima*; lane 7, crude KCO; lane 8, KCO purified by *S. cerevisiae*; lane 9, KCO purified by *C. pulcherrima*

was employed for purification for 2 days due to its ability to remove the glucose in both SLO and KCO.

The purified SLO and KCO were analyzed for removal of monosaccharides by HPAEC. SLO contained glucose as a major monosaccharide (71.59% w/w), as shown in Table 4. Purification of SLO with *S. cerevisiae* removed 80.80% of the glucose and all of the mannose and galactose. SLO contained small amounts of xylose and arabinose that *S. cerevisiae* was unable to remove. KCO had total monosaccharides less than SLO but also contained glucose as a major monosaccharide (43.24% w/w). Purification of KCO with *S. cerevisiae* removed all the glucose, mannose, and galactose, while xylose and arabinose were slightly reduced. According to Subtil et al. [41], D-glucose reduces the simultaneous utilization of pentoses primarily by inhibiting pentose uptake, while intracellular D-glucose does not inhibit pentose utilization. D-glucose catabolism also impedes pentose

Table 4 Monosaccharide content before and after purification by *S. cerevisiae* for 48 h

Sugar	Monosaccharide content (%wt)			
	SLO	Purified SLO	KCO	Purified KCO
Glucose	71.59	13.75	43.24	0.02
Mannose	1.81	0.00	2.13	0.00
Galactose	6.75	0.00	0.17	0.00
Xylose	6.67	5.93	1.12	1.03
Arabinose	0.05	0.04	0.17	0.14

SLO, saccha inchi shell oligosaccharide; KCO, defatted kernel cake oligosaccharide

utilization. L-arabinose utilization in *S. cerevisiae* has been accomplished through the use of multi-step oxidoreductive fungal or bacterial pathways. The fungal L-arabinose utilization pathway, like the fungal D-xylose utilization pathway, employs NADPH- and NADH-dependent redox reactions, resulting in severe co-factor imbalances [42].

3.5 Prebiotic properties of obtained oligosaccharides

Purified SLO and KCO were used to promote the growth of three species of lactic acid bacteria, *L. brevis*, *L. delbrueckii* subsp. *bulgaricus*, and *L. plantarum*. Lactic acid bacteria were cultured in modified MRS medium at 37 °C for 48 h, and absorbance was measured at 600 nm every 10 min. Glucose, originally contained in normal MRS medium, was replaced by SLO, KCO, and commercial XOS. The positive control was normal MRS, and the negative control was modified MRS without a carbon source. The positive control provided the highest specific growth rate for all lactic acid bacteria, followed by commercial XOS and SLO, while KCO had a low specific growth rate similar to that of the negative control (Table 5). *L. brevis* is found in milk, cheese, sauerkraut, sour bread, silage, cow manure, feces, and the oral and digestive tracts of humans and rats [43]. It is one of the few lactobacilli known to be capable of growing on and using XOS of low molecular weight [44, 45]. *L. brevis* displays high growth with XOS consumption, preferring an average DP of 2 for XOS [46]. *L. brevis* strains also grow well on isomalto-oligosaccharide but growth is lower than on XOS [47]. *L. plantarum* is found in various foods and in the human gastrointestinal tract [48]. *L. plantarum* cultured with malto-oligosaccharides grows better than that cultured in fructo-oligosaccharides or galacto-oligosaccharides [49]. *L. plantarum* prefers to hydrolyze DP7 isomalto-oligosaccharides to produce DP5, rather than using oligosaccharides

Table 5 Specific growth rate (mean and standard deviation) of lactic acid bacteria cultured with SLO and KCO at 37 °C for 48 h

Carbon source	Specific growth rate		
	<i>L. brevis</i>	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	<i>L. plantarum</i>
Glucose ¹	0.27 ± 0.01 ^a	0.20 ± 0.01 ^a	0.22 ± 0.01 ^a
Negative control ²	0.03 ± 0.01 ^d	0.02 ± 0.00 ^e	0.01 ± 0.00 ^e
Commercial XOS	0.20 ± 0.01 ^b	0.15 ± 0.01 ^b	0.18 ± 0.01 ^b
SLO	0.09 ± 0.01 ^c	0.08 ± 0.01 ^c	0.07 ± 0.01 ^c
KCO	0.03 ± 0.02 ^d	0.03 ± 0.00 ^d	0.03 ± 0.00 ^d

¹Normal MRS medium; ²MRS medium without carbon source

^{a-e}Values with different superscript letters indicate a significantly different specific growth rate of *Lactobacillus* spp. on each carbon source ($p < 0.05$)

with a low DP [50]. The strain *L. delbrueckii* subsp. *bulgaricus* is used industrially for yogurt production [51]. Growth of *L. delbrueckii* subsp. *bulgaricus* strains is significantly different on oligosaccharides with different DPs and including gluco-oligosaccharides, fructo-oligosaccharides, and galacto-oligosaccharides [52].

As a result, SLO was chosen to investigate the growth curve of the three lactic acid bacteria. The three species of cultured lactic acid bacteria were sampled to count the number of cells using the spread plate technique. The highest number of *L. brevis* cells was cultured in glucose, followed by SLO, commercial XOS, and the negative control (9.0, 8.2, 8.1, and 5.7 log CFU/mL, respectively), while the growth of *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* on SLO was similar to that on the negative control (Fig. 6a–c). In comparison to other species, *L. brevis* is substantially promoted by XOS produced from rice by-products utilizing hydrothermal treatment-assisted enzymatic hydrolysis [53]. In addition, *L. brevis* cultured in glucose has a log phase between 10–14 h, whereas SLO and commercial XOS have faster log phases at 8–12 h. When bacteria reach the stationary phase, the substrate becomes depleted, which may result in a lower number of *L. brevis* cells being promoted by SLO and commercial XOS than glucose [54]. XOS produced from microwave-assisted enzymatic hydrolysis of rice straw supports *L. brevis* growth at a slower rate when compared to glucose, but with the same population in the stationary phase [55]. Lactic acid bacteria can produce lactic acid associated with their growth rate; thus, probiotics with a fast log phase can inhibit pathogen activity rapidly [56]. *L. brevis* produces significant amounts of lactate during cell growth, with linear arabino-oligosaccharides and debranched sugar beet arabinan after 48 h [57]. Spent coffee ground polyphenol extracts can induce the growth of *L. brevis* as a unique carbon source at a similar level to dextrose, which has a high total phenolic content with antioxidant and anti-pathogenic activity [58]. *L. brevis* strains exhibit a higher survival rate in gastric conditions and antimicrobial activity against various foodborne pathogens including *Listeria monocytogenes*, *Escherichia coli* O157:H4, *Staphylococcus aureus*, and *Salmonella* Enteritidis [59]. Brevicin is a natural biopreservative produced by *L. brevis* NS01, which presents good antimicrobial activity [60]. A previous study reported final populations of *L. brevis* and *L. plantarum* fermented with soybean milk after 24 h of 8.98 and 9.03 log CFU/mL, respectively [61]. *L. plantarum* has good probiotic properties and can tolerate the simulated digestive tract environment. It can metabolize and synthesize bacteriocins, which have a strong inhibitory effect on the growth of both Gram-positive and Gram-negative bacteria [62]. The growth of *L. plantarum* is rapid between 24 and 48 h of fermentation and produces 3-phenyllactic acid, an antifungal compound with high activity [63]. Although the

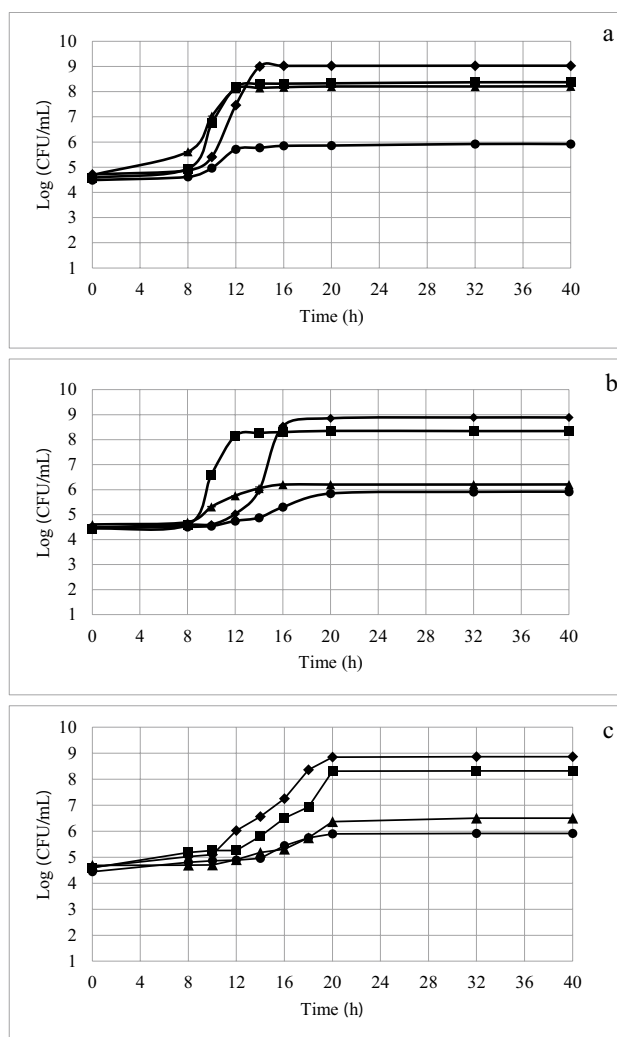


Fig. 6 Growth of *L. brevis* (a), *L. delbrueckii* subsp. *bulgaricus* (b), and *L. plantarum* (c) on oligosaccharides obtained from SL (triangle) compared with glucose (diamond), non-carbon source (circle), and commercial XOS (box) at 37 °C for 40 h

heat sensitivity of *L. plantarum* was increased under a severe food process, pressurized carbon dioxide treatment [64], oligosaccharides could enhance growth rate and stress tolerance of the beneficial bacteria [65]. *L. delbrueckii* subsp. *bulgaricus* induces growth of neoagaro-oligosaccharides and fructo-oligosaccharides at a concentration of 7.2 log cfu/mL [66]. Oligosaccharides extracted from soy sauce lees strongly promote the proliferation of *L. delbrueckii* subsp. *bulgaricus*, with the concentration reaching 9.67 log cfu/mL [67]. The antimicrobial substances of *L. delbrueckii* subsp. *bulgaricus* are specific against Gram-negative bacteria [68].

A 2% (w/v) concentration of SLO solution was continuously tested for resistance to digestion in simulated gastrointestinal conditions; 90.4% of commercial XOS withstood simulated digestion, while 83.66% of SLO remained undigested in the stomach simulation system, and 77.89%

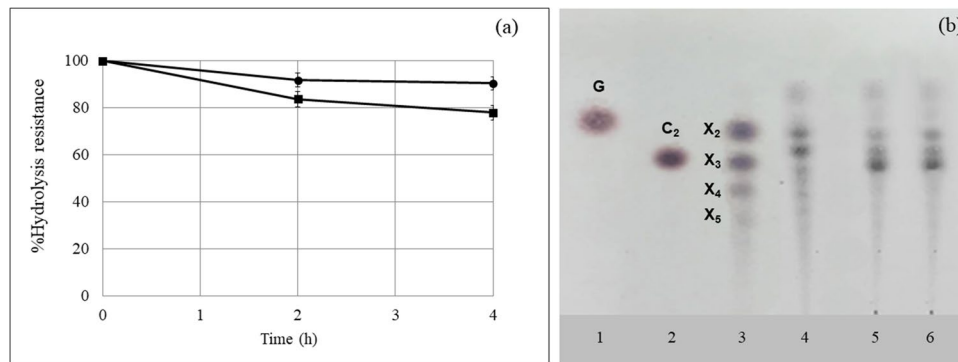


Fig. 7 Percentage carbohydrate digestion of SLO (box) and commercial XOS (circle) (a) and sugar profile of SLO (b) after hydrolysis in vitro simulated gastric phase and intestinal phase digestion. Lane 1, standard glucose; lane 2, standard cellobiose; lane 3, mixture of

standard XOS; lane 4, SLO; lane 5, SLO after incubation with simulated gastric fluid; lane 6, SLO after incubation with simulated intestinal fluid. G, glucose; C₂, cellobiose; X₂, xylobiose; X₃, xylotriose; X₄, xylotetraose; X₅, xylopentaose

remained undigested in the small intestine simulation system (Fig. 7a). The sugar type was determined using the TLC technique after SLO was digested under acidic and enzymatic simulated human gastrointestinal conditions for 0, 2, and 4 h. Digested oligosaccharides had a 2–3 times higher DP than undigested oligosaccharides (Fig. 7b). The stomach plays a greater role in hydrolysis via hydrochloric acid, which hydrolyzes the carbohydrate structure randomly [69]. Pancreatic amylase is the major carbohydrate-hydrolyzing enzyme in the intestine [70]. Pancreatic amylases degrade the α -1,4-glycosidic linkages of linear glucans to maltose [71]. Brush border enzymes in the mammalian small intestine also hydrolyze α -1,4- and α -1,6-glycosidic linkages [72]. Cello-oligosaccharides have β -1,4-glycoside linkages formed by linear D-gluco-oligomers, which involve glycoside linkages largely resistant to hydrolytic degradation by human digestive enzymes [73]. Various microorganisms express these enzymes either singly or associated in a macromolecular complex known as the cellulosome [74]. Nevertheless, cello-oligosaccharides enhance cell density by up to 4.1 times in *Clostridium butyricum*, *Lactococcus lactis* subsp. *lactis*, *L. paracasei* subsp. *paracasei*, and *L. rhamnosus*, with reduced efficiency for *Bifidobacterium* sp. [75]. Cello-oligosaccharide is effectively expressed in lactic acid bacteria and shows direct production of D-lactic acid with lengths ranging from cellobiose to cellohexaose [76].

4 Conclusions

Sacha inchi by-products from the oil industry are lignocellulosic materials that can be used for polysaccharide conversion. Oligosaccharides are by-products of polysaccharide conversion that have functional properties and can increase the value of these by-products. Oligosaccharides produced from various materials have potential for consumers, but

information on production of oligosaccharides from saccha inchi by-products is limited. The key to oligosaccharide production via enzymatic hydrolysis is lignocellulose pretreatment, which breaks down the complex structure of the substrate and increases the surface area for enzymatic catalysis. Because of their chemical composition, SL and KC require different pretreatment methods. SL and KC were able to produce oligosaccharides using commercial cellulases. A BBD experimental design was used to determine the optimal conditions for oligosaccharide production. *S. cerevisiae* removed more than 80% of glucose from SLO and KCO. However, KCO was unable to promote lactic acid bacteria as a probiotic, whereas SLO was able to promote *L. brevis* at the same level as commercial XOS and withstand simulated digestion (90.4% was retained). The study results suggested that oligosaccharides with prebiotic properties can be produced from saccha inchi by-products.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Declarations

Conflict of interest The authors declare that the research was conducted with no known competing financial interests or personal relationships that could be construed as a potential conflict of interest.

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