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High-Yield Levan Produced by *Bacillus licheniformis* FRI MY-55 in High-Sucrose Medium and Its Prebiotic Effect

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A high levan-producing strain of *Bacillus licheniformis* FRI MY-55 was isolated from a brackish water fish pond. The effects of carbon and nitrogen sources, initial pH of the medium, sucrose concentration, and temperature on levan production by this strain; effects of sucrose concentration and temperature on the molecular weight of levan; and prebiotic effect of levan were investigated. The maximum levan yield was obtained in the presence of sucrose and soytone at an initial pH of 7.0. *B. licheniformis* FRI MY-55 produced a remarkable level of 145.94 g/L of levan in 50% sucrose-containing medium. The maximum levan production rate was 3.92 g/L/h, which is the highest rate reported for any strain till date. The molecular weight of levan decreased with increasing temperature or sucrose concentration and ranged from 13.45 to 2.96 kDa. The produced levan had prebiotic effect.

Key words: *Bacillus licheniformis;* Levan; Sucrose; Temperature; Prebiotic effect.

Levan, a β(2→6)-linked fructose polymer with occasional β(2→1) branching, is found in many plants and microbial products[1]. Microbial levan production is of a considerable industrial importance; it has major applications in cosmetic, pharmaceutical, and food industries[1]. Levan has some potential pharmaceutical applications because of its anticarcinogenic and hypocholesteroloemic properties[2–6]. Levan can affect the immune system through tumor suppression, enhancement of leukocyte antitumor activity, and stimulation of lymphocyte proliferation in the spleen[7–9]. Levan has been shown to inhibit hyperglycemia and oxidative stress induced by diabetes[10, 11]. Black bean *Rhynchosia molubilis* and levan treatment is known to be effective against bone loss in rats. Furthermore, intake of black bean and levan has been shown to increase trabecular bone mineral density and serum calcium concentration[12]. Levan can be used in food as a gum, a sweetener, an emulsifier, a thickener, an encapsulating agent, a flavor vehicle, and a fat substitute[1].

Gupta *et al*. have reported that levan at 1.25% can be used as a dietary immunostimulants for *Labeo rohita* Hamilton juveniles in aquaculture[13]. The relative survival percentage of juveniles after challenge with *Aeromonas hydrophila* was found to be the highest in the fish fed a diet supplemented with levan[13]. Increased level of heat shock protein (HSP) 70 concentrations have been observed in fish fed a diet supplemented with 1.25% levan. A recent study has shown that

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levan ameliorates the effects of thermal stress in *L. rohita* juveniles[14]. Gupta *et al*. have suggested that microbial levan is an ideal immunostimulant in aquaculture[15].

Microbial levans are produced from sucrose-based substrates by transfructosylation of levansucrase (beta-2, 6 fructan: D-glucose-fructosyl transferase, EC 2.4.1.10) by various microorganisms, such as *Aerobacter aerogenes, Bacillus subtilis*, *Erwinia herbicola*, and *Zymomonas mobilis*[1, 16].

Prebiotics are defined as "nondigestible food ingredient(s) that beneficially affect(s) host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon"[17]. Various lactobacilli and bifidobacteria are part of the resident intestinal bacteria stimulated by prebiotics. Inulin and related oligosaccharides are generally acknowledged to be efficient prebiotics[18]. Levan has been less investigated, and very few studies have demonstrated its prebiotic properties[19, 20].

To find a new levan with appropriate properties, Huang *et al*. studied a bacterial strain T1 with high levan-producing abilities, thriving in high salt concentrations (7.0% NaCl), and isolated from the biofilter tank of a brackish water tilapia culture system[21]. They have shown that hydrolysates derived by acid and heating treatments from levan produced by the strain T1 mainly contain fructose. ^{1}H NMR and ^{13}C NMR spectra of the polysaccharide have very similar peak positions for levan produced. To investigate the suitability of strain T1 for efficient levan production and its application, this strain was identified and the effects of various carbon and nitrogen sources, initial pH of the medium, sucrose concentration, and temperature on levan production were examined in this study. In addition, the effects of sucrose concentration and temperature on the molecular weight of the produced levan and the prebiotic effect of levan were also examined.

MATERIALS AND METHODS

Reagents, bacteria strain and culture medium

Reagents for cultivation such as tryptic soy broth (TSB), de Man, Rogosa, and Sharpe (MRS), reinforced clostridial broth (RCB), nutrition broth (NB), skim milk, yeast extract, peptone, casein, soytone, tryptose, beef extract were purchased from Difco (Difco Laboratories, Detroit, MI, USA). NaCl, KH_2PO_4, $MgSO_4$ÿ$7H_2O$, sucrose, raffinose, lactose, maltose, glucose, galactose, fructose, sorbitol, urea, and ammonium sulfate were obtained from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). All other reagents used were of the highest grade available unless indicated otherwise.

The strain T1 was isolated from a biofilter tank of a brackish water tilapia culture system[21]. The purified isolate was cultivated in TSB supplemented with 2.5% (w/w) NaCl at 28°C for 24 h. To preserve the strain, 15% glycerol was added to part of the culture and the frozen at "85°C. The culture was centrifuged at 10,000 ×*g* for 20 min; 10% skim milk was added to the precipitate and the mixture was freeze dried. Unless otherwise specified, the sucrose medium for levan production contained yeast extract (5 g), peptone (5 g), KH_2PO_4 (1 g), $MgSO_4$,$7H_2O$ (0.2 g), NaCl (20 g), sucrose (200 g, sterilized separately), and distilled water (770 mL). The pH was adjusted to 7.0 before autoclaving. Before testing, the culture was transferred to TSB and incubated at 28°C for 24 h.

Identification of the isolate

The isolate was examined for its biochemical and physiological characteristics[22], and characterized further by performing 16S rRNA gene sequence analysis according to the method of Weisburg *et al.*[23]. The nearly full-length 16S rRNA gene sequence was amplified by polymerase chain reaction using the universal primers 16S_F: [52 -AGAGTTTGATCATGGCTCAG-32] and 16S_R: [52 -GGTTACCTTGTTACGACTT-32]. Sequence analysis was performed using the BLAST program[24] and the phylogenetic tree was constructed by the neighbor-joining method using Accelrys Gene v2.5 program (Accelrys Inc., California, USA)[25].

Analytical methods

The biomass of the culture broth was determined by measuring the optical density (OD) using a photoelectric colorimeter and at 600 nm (WPA CO75, Linton Cambridge, UK). Levan was harvested from the culture broth by precipitation with ethanol[26, 27]. The culture broth was centrifuged at 10,000 ×*g* for 20 min at 4°C. Three volumes of cold (4°C) ethanol (99%) were added to 1 volume of culture supernatant. The mixture was stored

overnight at 4°C. The precipitate was collected by centrifugation at 10,000 ×*g* for 15 min at 4°C and resuspended in 1 volume of deionized water. After repeating the procedure 3 times, the pellet was freeze dried. The levan yield was determined by measuring the dry weight of the precipitate. The number average molecular weight of levan was measured by gel permeation chromatography using a Waters 1515 system controller (Waters corporation, Milford, MA, USA) equipped with Ultrahydrogel™ series columns (250 and 125, 7.8 × 300 mm) and a refractive index detector (Waters, 2414). Dextran standards (Polymer Standards Service-USA Inc., Warwick, RI, USA) of 5.2, 11.6, 23.8, 48.6, 148, 273, 410, and 668 kDa were used to construct a calibration curve. The eluant flow rate of deionized water was 1 mL/min, and the column was maintained at 80°C.

Effect of the carbon source, nitrogen source, and the initial pH of the medium on cell growth and levan production

In carbon source and nitrogen source experiments, sucrose media containing 20% of different carbon sources and 0.5% of different nitrogen sources was used, respectively. The sucrose medium was used in the experiment of pH. The carbon sources examined were sucrose, raffinose, lactose, maltose, glucose, galactose, fructose, and sorbitol, and the nitrogen sources were yeast extract, peptone, casein, soytone, tryptose, beef extract, urea, and ammonium sulfate. The medium was examined at initial pHs of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0. The strain T1 was transferred from -85°C into TBS and cultivated at 28°C for 24 h with shaking at 150 rpm. The inoculum consisted of a small sample (1%) of actively growing cells (OD_{600} = 0.3) incubated at 23°C for 36 h with shaking at 150 rpm. The biomass of culture broths was determined by measuring OD at 600 nm. The culture supernatants after centrifugation were used for the determining the levan yield using the abovementioned method.

Effect of the temperature and sucrose concentration on cell growth, levan production, and molecular weight of produced levan

To determine the effect of temperature on growth, the levan yield and the molecular weight of produced levan, the medium supplemented with 20% (w/w) sucrose was used. The test temperatures were 18°C, 23°C, 30°C, 35°C, 40°C, 45°C, 50°C, and 55°C, and bacteria were cultivated at pH 7.0 with shaking at 150 rpm for 168 h. To study the effect of sucrose concentration on growth, the levan yield, and the molecular weight of produced levan, sucrose concentrations were varied at 1%, 5%, 10%, 20%, 30%, 40%, and 50% (w/w), and the cells were cultivated at 40°C, pH 7.0, with shaking at 150 rpm for 144 h. The biomass of the culture broth was determined by measuring OD at 600 nm. After centrifugation, the culture supernatant was used to determine the levan yield as well as the molecular weight of produced levan using the abovementioned methods.

Evaluation of levan for use as prebiotic

Levan produced by the strain T1

Mass levan production was performed by slight modification of a previously described method[28]. After cell separation, the culture liquid was treated with ethanol to precipitate levan together with the extracellular levansucrase. The levan–levansucrase sediment was used as a biocatalyst for levan production in a high-sucrose solution.

The strain T1 was transferred from -85°C into TSB and cultivated at 28°C for 24 h with shaking at 150 rpm. The inoculum consisted of a small sample (1%) of actively growing cells (OD_{600} = 0.3) on medium containing 20% sucrose incubated at 23°C for 36 h with shaking at 150 rpm. The biomass was separated by centrifugation. Ethanol (99%) was added to the cell-free culture liquid (3.0:1.0) by mixing for 12 h at 4°C. The sediment was then separated by centrifugation. The sediment obtained from 200 mL culture liquid was incubated with 100 mL of 60% sucrose at 45°C for 48 h. Levan was purified from the incubation solution by repeated ethanol precipitation, dissolved in distilled water, and freeze dried. Freeze-dried levan was quantified by weighing the dried precipitate, and the molecular weight was determined using the abovementioned method.

Evaluation of levan for use as prebiotic

Prebiotic activity scores and bacterial utilization of levan were determined by slight modification of a previously described method[29]. The assay was performed by adding 1% (v/v) of an overnight culture of each probiotic, enteric, or pathogenic strain to separate tubes containing the appropriate reconstituted broth without glucose and with 1% (w/v) fructose or levan. The tested

strains and incubation conditions are listed in Table 1. The enteric mixture was composed of *Escherichia coli* BCRC 11549, *E. coli* BCRC 13055, and *E. coli* BCRC 11634 in a 1:1:1 ratio. The prebiotic activity score and percent utilization of levan by the probiotic, enteric, and pathogenic strains were determined using the following equation:

Percent utilization

(final bacterial OD_{600} on levan - initial bacterial OD_{600} on levan)/(final bacterial OD_{600} on fructose - initial bacterial OD_{600} on fructose) x 100%

Prebiotic activity score

{(final probiotic OD_{600} on levan - initial probiotic OD_{600} on levan)/(final probiotic OD_{600} on fructose - initial probiotic OD_{600} on fructose)} - {(final enteric mixture OD_{600} on levan " initial enteric mixture OD_{600} on levan)/(final enteric mixture OD_{600} on fructose - initial enteric mixture OD_{600} on fructose)}

RESULTS AND DISCUSSION

Identification of the isolate

The strain T1 with high levan-producing abilities was isolated from the biofilter tank of a brackish water system for tilapia culture. The isolate was identified by examining its biochemical and

Table 1. Tested bacterial strains in this study

Strain	Incubation condition	Source
Anaerobic	Anaerobic	
Bifidobacterium adolescentis BCRC 14606	MRS/37°C + 0.05% cysteine	BCRC[1]
Bifido. bifidum BCRC 11844	MRS/37°C + 0.05% cysteine	BCRC
Bifido. breve BCRC 11846	MRS/37°C + 0.05% cysteine	BCRC
Bifido. longum subsp. *infantis* BCRC 14602	MRS/37°C + 0.05% cysteine	BCRC
Bifido. longum subsp. *infantis* BCRC 15416	RCB/37°C	BCRC
Bifido. longum subsp. *longum* BCRC 11847	RCB/37°C	BCRC
Clostridium sporogenes BCRC 11259	RCB/37°C	BCRC
Aerobic	Aerobic	
Aeromonas hydrophila	TSB/28°C	Dr. Chin-I Chang[2]
Edwardsiella tarda BCRC 10670	TSB/28°C	BCRC
Enterobacter aerogenes BCRC 10370	NB/30°C	BCRC
Escherichia coli BCRC 11634	NB/37°C	BCRC
E. coli BCRC 13055	TSB/37°C	BCRC
E. coli BCRC 11549	TSB/37°C	BCRC
E. coli serotype O:157 H:7	TSB/37°C	FDA[3]
Lactobacillus acidophilus BCRC 10695	MRS/37°C	BCRC
Lb. acidophilus BCRC 16099	MRS/37°C	BCRC
Lb. casei subsp. *casei* BCRC 12272	MRS/37°C	BCRC
Lb. delbrueckii subsp. *bulgaricus* BCRC 16053	MRS/37°C	BCRC
Lb. plantarum BCRC 10069	MRS/37°C	BCRC
Lb. plantarum BCRC 12250	MRS/37°C	BCRC
Lb. rhamnosus BCRC 16000	MRS/37°C	BCRC
Lactococcus garvieae BCRC 17074	TSB/28°C	BCRC
Lactococcus lactis subsp. *lactis* BCRC 10791	MRS/37°C	BCRC
*Pediococcus pentosaceu*s BCRC 14024	MRS/30°C	BCRC
Staphylococcus aureus BCRC 10451	TSB/37°C	BCRC
Streptococcus thermophilus BCRC 12268	MRS/37°C	BCRC
Vibrio anguillarum BCRC 12908	TSB/28°C	BCRC
V. damsela	TSB/28°C	Dr. Chin-I Chang
V. harveyi	TSB/28°C	Dr. Chin-I Chang

[1]BCRC: Bioresources Collection and Research Center (BCRC), Food Industry Research and Development Institute, Hsinchu, Taiwan.

[2]Dr. Chin-I Chang: Fisheries research institute, COA, Taiwan.

[3]FDA: Food and Drug Administration, Department of Health, Executive Yuan, Taiwan.

Table 2. The effect of different carbon sources on cell growth and levan production of *B. licheniformis* FRI-MY55 in 20% carbon-containing media (23°C, pH 7.0, with shaking at 150 rpm for 36 h)

Carbon source	OD_{600}	Levan yield (g/L)
Control	1.45 ± 0.06	ND[a]
Fructose	0.09 ± 0.04	ND
Galactose	0.24 ± 0.05	0.07 ± 0.05
Glucose	0.92 ± 0.04	ND
Lactose	0.63 ± 0.12	0.09 ± 0.08
Maltose	0.81 ± 0.03	ND
Raffinose	0.45 ± 0.07	ND
Sorbitol	0.80 ± 0.06	0.07 ± 0.06
Sucrose	1.18 ± 0.03	71.78 ± 5.58

Levan was purified from the culture supernatants by repeated ethanol precipitation, dissolved in distilled water, freeze dried, and quantified by weighing the dried precipitate (n = 3; 500 mL).
[a] Not detectable.

Table 3. The effect of various nitrogen sources on cell growth and levan production of *B. licheniformis* FRI-MY55 grown in the medium containing 20% sucrose at 23°C for 36 h with pH 7.0 and shaking at 150 rpm

Nitrogen source	OD_{600}	Levan yield (g/L)
Tryptose	0.47 ± 0.04	61.89 ± 5.60
Soytone	0.83 ± 0.04	71.91 ± 2.79
Yeast extract	0.61 ± 0.10	51.02 ± 3.14
Casein	0.01 ± 0.02	17.85 ± 0.32
Beef extract	0.48 ± 0.02	43.17 ± 1.31
Peptone	0.60 ± 0.08	22.86 ± 3.92
Ammonium sulfate	0.14 ± 0.04	2.10 ± 1.20
Urea	0.05 ± 0.03	0.44 ± 0.34

Levan was purified from the culture supernatants by repeated ethanol precipitation, dissolved in distilled water, freeze dried, and quantified by weighing the dried precipitate (n = 3; 500 mL).

Table 4. The effect of the initial pH on cell growth and levan production of *B. licheniformis* FRI-MY55 grown in the medium containing 20% sucrose at 23°C for 36 h with shaking at 150 rpm

Initial pH of medium	OD_{600}	Levan yield (g/L)
4.0	0.01 ± 0.01	0.09 ± 0.09
5.0	0.03 ± 0.03	0.17 ± 0.19
6.0	0.34 ± 0.07	0.11 ± 0.07
7.0	0.87 ± 0.09	68.12 ± 2.97
8.0	1.12 ± 0.14	53.03 ± 8.27
9.0	0.55 ± 0.02	0.07 ± 0.07
10.0	0.03 ± 0.01	ND[a]
11.0	0.03 ± 0.02	ND

Levan was purified from the culture supernatants by repeated ethanol precipitation, dissolved in distilled water, freeze dried, and quantified by weighing the dried precipitate (n = 3; 500 ml).
[a] Not detectable.

physiological characteristics and 16S rRNA gene sequence. The colonies of strain T1 had mucoid appearance on solid sucrose medium. Microscopic examination revealed Gram-positive, rod-shaped, and spore-forming cells. The strain produced arginine hydrolase, was positive for nitrate reduction and acid production from glucose, and gave positive results in Voges–Proskauer test. Hence, strain T1 was tentatively identified to belong to *Bacillus* sp. The partially sequenced 16S rRNA gene sequence of this strain exhibited high similarity (99%) to that of *B. licheniformis.* Phylogenetic analysis (Fig. 1) revealed that strain T1 was most closely related to *B. licheniformis*. Therefore this strain was identified as *B. licheniformis* and named *B. licheniformis* FRI MY-55. *B. licheniformis* has been previously described as a levan producer[3, 8, 30].

Production of levan by *B. licheniformis* FRI MY-55

Bacillus licheniformis FRI MY-55 produced a large quantity of extracellular polysaccharide when grown on media containing 20% sucrose. Sucrose initially present in the medium was rapidly transformed to levan, glucose, fructose, kestose, and nystose. The total amount of 18.2 g/L of levan was detected after 24 h of cultivation at 23°C. Levan production increased until the end of the logarithmic growth phase, reaching a maximum of 66.84 g/L after 36 h at 23°C, and then gradually decreased. The initial pH of 7.0 decreased to 5.8 after 24 h and remained in the range of 5.4–5.7 (data not shown).

The growth dynamics and characteristics of levan production differ in various bacterial strains. Studies of levan production in cultures of *B. subtilis* (natto) Takahashi and *B. licheniformis* have shown that levan production occurs in all phases of growth, but proceeds most actively at the beginning of the stationary phase[27, 30]. In *B.*

Table 5. Comparison of molecular weight of levans produced by various microorganisms

Microorganism	Molecular weight of levan (kDa)	Sucrose (%)	Temperature (°C)	Cultivation time (h)	Reference
Bacillus licheniformis FRI MY-55	3	50	40	60	This study
B. licheniformis FRI MY-55	4	20	50	24	This study
B. subtilis NRC 33a (crude enzyme)	<5	40	30	24	34
B. subtilis C4 (purified enzyme)	10	32	37	8	33
B. subtilis (natto) Takahashi	11	20	37	21	27
B. licheniformis FRI MY-55	12	10	40	48	This study
B. licheniformis FRI MY-55	13	20	18	72	This study
B. licheniformis 8-37-0-1	28	10	30	60	8
B. subtilis NRC 33a (crude enzyme)	43	5	40	24	34
B. subtilis NRC 33a (crude enzyme)	59	5	15	24	34
B. subtilis NRC 33a (crude enzyme)	60	5	30	24	34
B. subtilis C4 (purified enzyme)	~1,000	32	37	8	33
Halomonas sp.	>1,000	5	39	95	31
B. subtilis (natto) Takahashi	1,794	20	37	7	27
B. polymyxa	2,000	15	30	240	26

Table 6.

Comparison of levan yield of various microorganisms

Microorganism	Levan yield (g/L)	Sucrose (%)	Yield on available sucrose (%)	Temperature (!)	Cultivation time (h)	Levan production rate (g/L/h)	Reference
Erwinia herbicola	15	5	30	25	72	0.21	16
Bacillus licheniformis ATCC 9945	18	5	36	30	24	0.75	30
B. polymyxa	36	15	24	30	240	0.15	26
B. licheniformis 8-37-0-1	38	10	38	30	60	0.63	8
Zymomonas mobilis	41	20	21	7	696	0.06	35
B. subtilis (natto)Takahashi	40-50	20	20-25	37	21	1.90-2.38	27
B. subtilis (natto)Takahashi	50-60	25	20-24	37	24	2.08-2.50	39
B. licheniformis FRI MY-55	50	20	25	40	24	2.07	This study
B. licheniformis FRI MY-55	67	20	34	23	36	1.86	This study
B. licheniformis FRI MY-55	94	30	31	40	24	3.92	This study
B. licheniformis FRI MY-55	132	40	33	40	36	3.66	This study

polymyxa and *Halomonas* sp., the maximum levan production has been reported after the onset of the stationary phase[26, 31]. In *Pseudomonas caryophylli*, levan production correlates with bacterial growth and a peak is obtained toward the stationary growth phase. Nevertheless, these bacteria also possess levanase activity, and thus, levan is hydrolyzed in the later stages[32]. *B. licheniformis* FRI MY-55 has the similar pattern. In the medium containing 20% sucrose, levan production increased until the end of the logarithmic growth phase, and gradually decreased. Hydrolysis of levans by levansucrase has been reported by Euzenat *et al*.[33]; similarly, the hydrolysis of levan in the later stages of growth of *B. licheniformis* FRI MY-55 may be caused by levansucrase or levanase activity.

Effect of different carbon sources on cell growth and levan production

The ability of *B. licheniformis* FRI MY-55 to use different carbon sources for growth and levan production was analyzed by growing the cells in media containing different carbon sources. *B. licheniformis* FRI MY-55 produced the maximum levan yield of 71.78 g/L (Table 2) in the presence of sucrose. Although the maximum biomass concentration was observed in the medium without a supplementary carbon source, high biomass concentrations were also achieved with glucose, maltose, and sorbitol as carbon sources. However, in all these cases the levan yield was very low compared with that produced in the presence of sucrose.

Shih *et al*.[27] have reported that *B. subtilis* (natto) Takahashi produces a high concentration of levan when cultivated in the presence of sucrose, but produced none when cultivated in presence of lactose, maltose, glucose, and fructose. Fourteen different carbon sources (at 10 g/L) were examined for their effect on growth and levan production of halophilic *Halomonas* sp.[31]. The results have shown that maximum levan production (1.073 g/L) is observed when *Halomonas* sp. cells are cultivated in the presence of sucrose, with lower yields achieved in the presence of xylose (0.267 g/L) and raffinose (0.206 g/L). The use of glucose, maltose, fructose, and galactose as carbon sources has resulted in high biomass concentrations but low levan yield were compared with those observed in medium containing sucrose. Han has

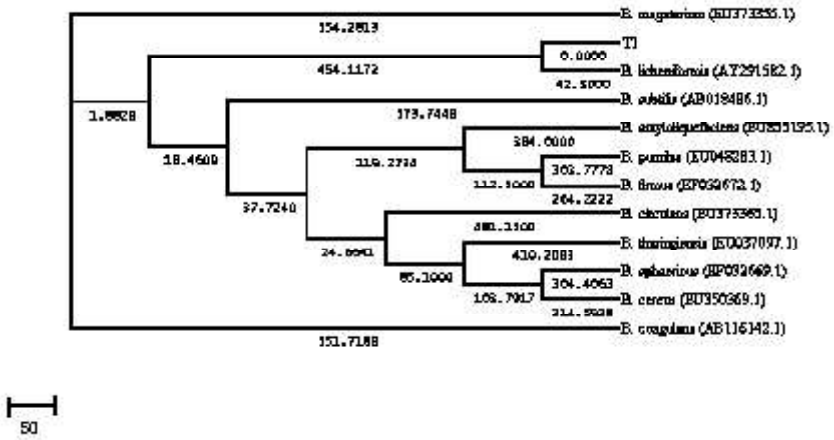
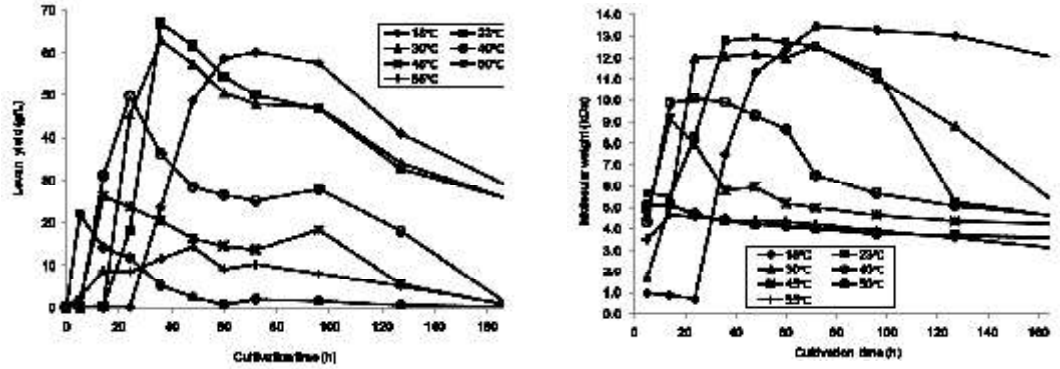


Fig. 1. Phylogenetic tree showing the 16S rRNA relationships of the isolate with other relevant species. The rectangular cladogram presents the results of the neighbor-joining method. The sequences were obtained from the GenBank databases and their nucleotide sequence accessions numbers are in brackets. The isolate and the closest species are boxed and indicated with an arrow. The nearly full-length 16S rRNA gene sequence was amplified by polymerase chain reaction using the universal 16S primers. Sequence analysis was performed using the BLAST program. Phylogenetic analysis was performed by the neighbor-joining method using Accelrys Gene v2.5



(a) (b)

Fig. 2. The effect of different cultivation temperatures on the levan yield (a) and the molecular weight of the produced levan (b). *Bacillus licheniformis* FRI-MY55 was grown in 20% sucrose-containing medium at pH 7.0 with shaking at 150 rpm (n = 3; 500 mL)

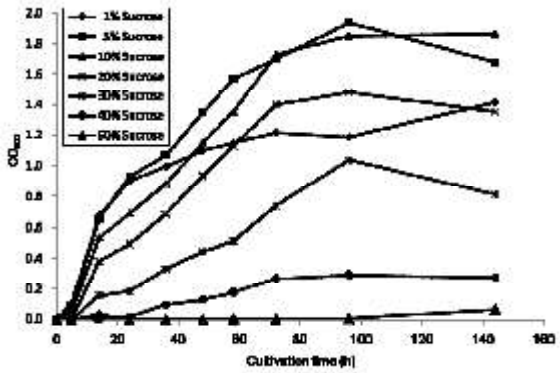


Fig. 3. Time course of cell growth for *B. licheniformis* FRI-MY55 grown in media containing various sucrose concentrations at 40°C, pH 7.0 with shaking at 150 rpm (n = 3; 500 mL)

reported that levan yield in medium containing raffinose was approximately one-third of that obtained in the presence of sucrose[1]. However, in this study, no levan was produced when the *B. licheniformis* FRI MY-55 was cultivated in the presence of raffinose.

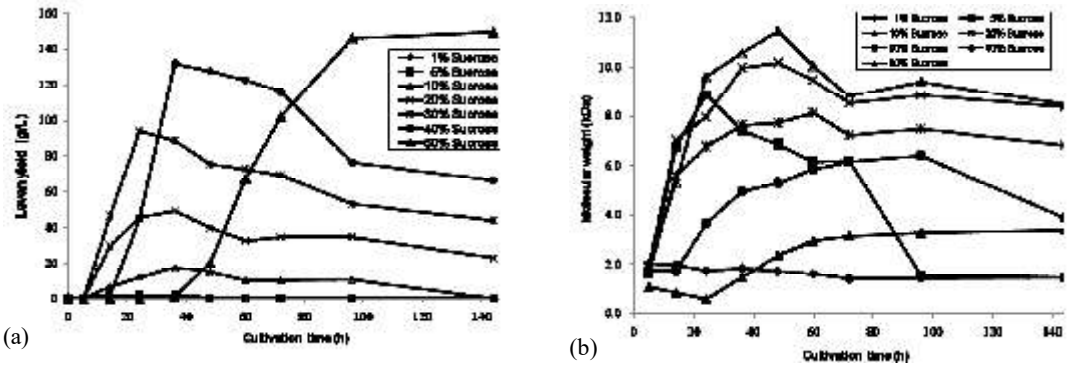


Fig. 4. The effect of various sucrose concentrations on the levan yield (a) and the molecular weight of the levan (b) produced by *Bacillus licheniformis* FRI-MY55 grown at 40°C and pH 7.0 with shaking at 150 rpm (n = 3; 500 mL)

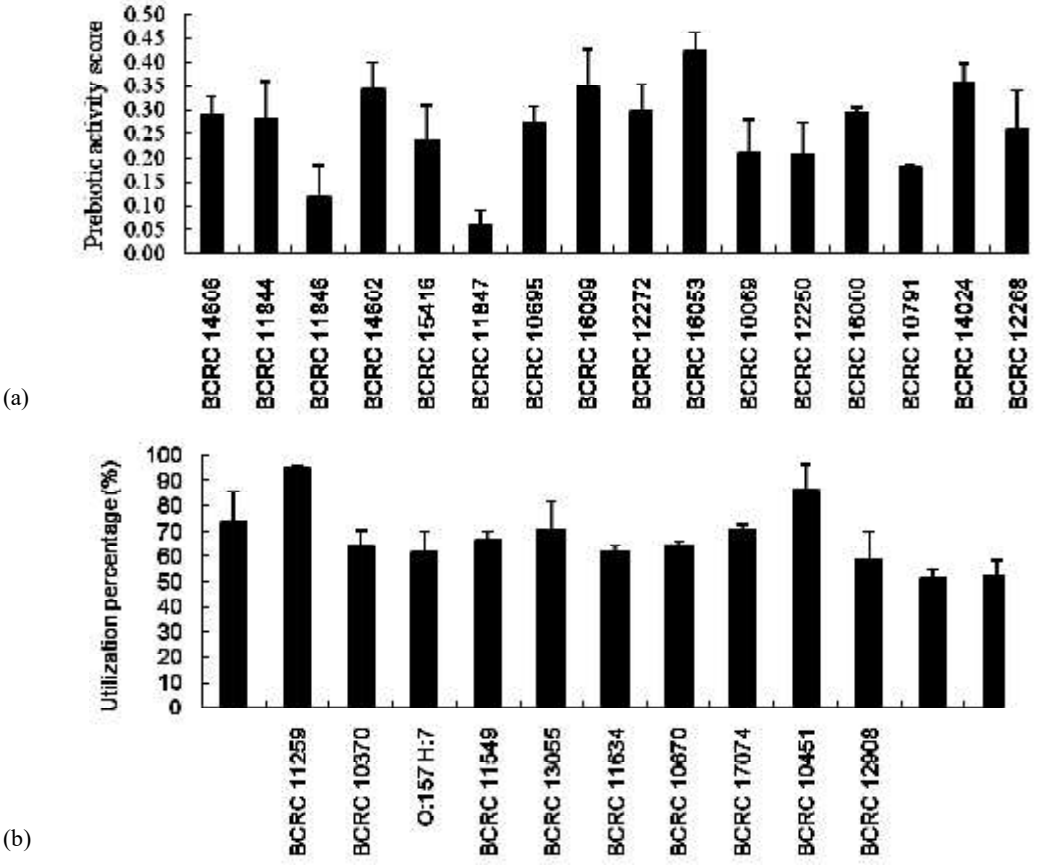


Fig. 5. Prebiotic activity scores with different probiotics (a) and percent utilization by enteric and pathogenic strains (b) of the levan produced by *Bacillus licheniformis* FRI-MY55 (n = 3; 100 mL)

Effect of different nitrogen sources on cell growth and levan production

The cell growth of the isolate in the sucrose medium and its levan production are presented in Table 3 as a function of the nitrogen source type. In these experiments, sucrose concentration in the medium was 20%, while that of nitrogen was 0.5%. *B. licheniformis* FRI MY-55 utilizes a broad range of organic compounds. The maximum biomass concentration (OD_{600} = 0.83) was obtained in the presence of soytone, followed by that in the presence of yeast extract and peptone. The medium containing soytone was the most favorable for levan production (71.91 g/L), followed by that containing tryptose (61.89 g/L), yeast extract (51.02 g/L), and beef extract (43.17 g/L). The use of inorganic compounds, ammonium sulfate or urea, in the culture medium led to low levan production.

Baker's yeast (0.02% N) and yeast extract (0.25%) increase levansucrase activity in *B. subtilis* NRC 33a, followed by that in the presence of soybean[34]. Muro *et al*. have reported favorable effect of ammonium sulfate (0.5 g/L) on levan production by *Z. mobilis*.[35]

Effect of the initial pH on cell growth and levan production

Cell growth and levan production by *B. licheniformis* FRI MY-55 were highly dependent on pH of the medium. Cells grew most actively when the initial pH was 8.0 (OD_{600} = 1.12), followed by growth at pH 7.0 (OD_{600} = 0.87) and pH 9.0 (OD_{600} = 0.55). When the pH of the medium was below 5.0 or above 10.0, cell growth was poor. The initial pH of 7.0 was most suitable for levan production (68.12 g/L), followed by pH 8.0 (53.03 g/L) (Table 4). Although high biomass concentration was observed when the initial pH was 6.0 or 9.0, the levan yield under this condition was very low.

The previously reported optimum initial pH for cell growth and levan production of *B. subtilis* (natto) Takahashi is 6.0[27]. *B. licheniformis* FRI MY-55 has a higher initial optimal pH (8.0 and 7.0) than *B. subtilis* (natto) Takahashi for growth and levan production; this is most likely caused by the marine origin (pH 6.6–8.3) of *B. licheniformis* FRI MY-55. Levan production by alginate-immobilized *B. subtilis* (natto) Takahashi is maximum at pH 5.0–6.0[36]. The results in this study show that a pH close to 5.8 is optimal for levansucrase activity in *B. licheniformis* FRI MY-55; it is close to the optimal pH for alginate-immobilized *B. subtilis* (natto) Takahashi.

Effect of temperature on cell growth, levan production, and the molecular weight of produced levan

The maximum biomass concentration was observed when *B. licheniformis* FRI MY-55 was cultivated at 30°C or 40°C for 48 h (OD_{600} = 1.34), followed by that at 23°C (OD_{600} = 1.18). Although high biomass concentration was observed when the strain was cultivated for 5 h at 45–55°C (OD_{600} = 0.41), after 14 h of cultivation at those temperatures growth decreased (OD_{600} = 0.85–1.27). At 18°C, the biomass concentration was the lowest (OD_{600} = 0.86) compared to that at other temperatures (data not shown).

The effect of various temperatures (18–55°C) on levan production by *B. licheniformis* FRI MY-55 was investigated. Levan production in the medium containing 20% sucrose progressively increased and reached 66.84 and 62.81 g/L at 23°C and 30°C, respectively, after 36 h of cultivation but decreased thereafter (Fig. 2a). At 50°C and 55°C, the levan yield was much lower (by 83%) than in cells grown at 23°C or 30°C. The level of levan reached 49.73 g/L at 40°C after 24 h of cultivation and then gradually decreased. The cultivation time needed for mass production (60.17 g/L) at 18°C was 60 h. For mass production of levan by *B. licheniformis* FRI MY-55 grown in the medium containing 20% sucrose (62.81–66.84 g/L), production must be terminated after 36 h of cultivation at 23°C or 30°C to maintain a high degree of sucrose transformation to levan; the maximum production rate of levan was 2.07 g/L/h at 40°C after 24 h of cultivation.

Euzenat *et al*.[33] have reported the low levan conversion over time as a result of levan hydrolysis by levansucrase, and a decrease in levan production at 50°C and 55°C. This decrease is presumably due to the increased hydrolysis of sucrose, frequent dissociation of the enzyme from its product under these conditions, and partial hydrolysis of levan molecules at high temperature. Han has reported the optimum temperature for cell growth and levan production by *B. polymyxa* as 30°C[1], while Shih *et al*.[27] have shown that for *B. subtilis* (natto) Takahashi, 25–40°C is the optimum

temperature, in the medium containing 20% sucrose. The optimal temperature for levansucrase production by *B. circulans* is 35°C[37]. For *B. subtilis* NRC 33a, 30°C is the optimum temperature for levansucrase activity and levan production in the medium containing 20% sucrose[33]. Ernandes and Garcia-Cruz have reported the optimum temperature for cell growth and levan production by *Z. mobilis* CCT 4494 as 35°C and 30°C, respectively[38]. In this study, for *B. licheniformis* FRI MY-55 cultivated in the medium containing 20% sucrose, the optimum temperature for cell growth and levan production was 30–40°C and 23°C, respectively.

The enzymatic synthesis of levan by *B. subtilis* NRC 33a has been studied using 60% acetone fraction of its crude extract[34]. The reported maximal conversion of the fructose moiety of sucrose occurs at 30°C (84%) and decreases to 60% and 20.8% at 35°C and 40°C, respectively (testing range of 15–40°C). Euzenat *et al*. have shown that levan production by levansucrase from *B. subtilis* C4 varies with the temperature of reaction[33]. They demonstrated a levan productivity of 29.4% (total sugars) at 37°C, decreasing to 15.8% at 60°C, in the reaction solution containing 32% sucrose.

The study of the effect of cultivation temperature on the molecular weight of produced levan demonstrated that the molecular weight decreased with increase in temperature from 18 to 55°C (Fig. 2b). The highest molecular weight of 13.45 kDa was observed at 18°C after 72 h of cultivation, followed by that at 23°C (12.79 kDa, 36 h), 30°C (11.99 kDa, 24 h), and 40°C (9.91 kDa, 14 h). The molecular weight of produced levan at 50°C and 55°C was in the range of 5.21–3.10 kDa. The product was also affected by the fermentation time, except at 50°C and 55°C. After 5–14 h of cultivation, the low molecular mass product (smaller than 5.67 kDa) predominated. As the fermentation proceeded, the concentration of the high molecular weight product generated at 18–45°C increased, but it gradually decreased thereafter. At 45°C, 40°C, 30°C, and 23°C, the molecular weight of produced levan gradually decreased after 14, 36, 72, and 72 h of cultivation.

Currently, little information is available about the effect of cultivation temperature on the molecular weight of levan produced by bacteria. The enzymatic production of levan by *B. subtilis* NRC 33a has been studied using 60% acetone fraction of its crude extract[34]. They found that the highest molecular weight of levan (58.52 kDa) was obtained at 15°C and gradually decreases at higher temperature (43.0 kDa, 40°C). The enzymatic production of levan by *B. circulans* has been studied using 50% ethanol fraction of its crude extract. The highest molecular weight of levan was obtained at 30°C (38 kDa), and it was lower at higher temperature (5 kDa, 50°C) (testing range of 25–50°C)[37]. Our study demonstrated that the molecular weight of the produced levan decreased with increase in temperature. These results are consistent with those reported by Euzenat *et al*.[33]; the temperature should be considered an effective factor controlling the molecular weight of the produced levan.

It has been previously reported that when cultivated in the presence of 20% sucrose at 37°C, *B. subtilis* (natto) Takahashi produces two fractions of levan with different molecular weights (1794 and 11 kDa), and the product distribution is affected by the fermentation time[27]. In this study, we demonstrated the effect of temperature and fermentation time on the molecular weight of produced levan.

Effect of sucrose concentration on cell growth, levan production, and molecular weight of produced levan

The maximum biomass concentration was observed when *B. licheniformis* FRI MY-55 was cultivated in a medium containing 5% sucrose (OD_{600} = 1.31, 48 h), followed by that in media containing 10% (OD_{600} = 1.16, 48 h), 1% (OD_{600} = 0.95, 48 h), and 20% sucrose (OD_{600} = 0.83, 48 h). The biomass concentration decreased with increasing concentration of supplemented sucrose (up to 50% sucrose). OD_{600} of media containing 30%, 40%, and 50% sucrose was 0.42, 0.13 and <0.06, respectively, after 48 h of cultivation (Fig. 3).

Levan production by *B. licheniformis* FRI MY-55 depends on the sucrose concentration and no levan was found in the absence of sucrose. In the medium with sucrose, the levan yield increases with increasing sucrose concentration (10%–50% sucrose, Fig. 4a). If the initial sucrose concentration is 10%, this strain produces 17.09 g/L of levan. For a 2.0-, 3.0-, or 4.0-fold increase in sucrose

concentration, the levan yield increases approximately 2.88-, 5.50-, and 7.71-fold to give 49.28, 94.07, and 131.76 g/L of levan, respectively. Although *B. licheniformis* FRI MY-55 grew poorly in the medium containing 50% sucrose, the number of cells was 1.2×10^7 CFU/mL, this medium yielded the maximum levan production. High levan productivity (131.76 and 145.94 g/L) was obtained in media containing 40% and 50% sucrose at 40°C after 36 and 96 h of cultivation, respectively. The cultivation time required for levan production increased with increasing initial sucrose concentration. At lower sucrose concentrations, the levan yield decreased. When cells were cultivated in a medium with 1% or 5% sucrose, the levan yield was less than 1–2.19 g/L. Increasing the cultivation time decreased the levan yield. In the medium containing 20%–40% sucrose, the levan yield gradually decreased from 48 h until the end of the experiment. The levan producing rate the maximum (3.92 g/L/h) in the medium containing 30% sucrose after 24 h of cultivation.

The levan production by *B. subtilis* (natto) Takahashi varies with the sucrose concentration. The maximum levan productivity (49.40 g/L, 37°C) has been obtained in the medium containing 20% sucrose, whereas it decreased at the higher (30%) and lower sucrose concentrations (2%–10%)[27]. The highest sucrose concentration used for maximum levan production is 20% for most bacteria. However, in this study, the maximum levan yield produced by *B. licheniformis* FRI MY55 was found in the medium containing 50% sucrose. It's possible that since *B. licheniformis* FRI MY55 was isolated from brackish water, its osmosis resistance is high, and the efficiency of levansucrase at high sucrose concentrations is excellent. To the best of our knowledge, this is the first report of high levan productivity of *B. licheniformis* FRI MY55 in a medium containing 50% sucrose.

The molecular weight of produced levan decreased from 11.46 to 2.96 kDa when sucrose concentration increased from 10% to 50% (Fig. 4b). Increasing the cultivation time from 48 and 96 h to 144 h led to a decrease in the molecular weight of produced levan (by 1.5–2.0 kDa for media containing 10%–30% and 40% sucrose), after which the molecule weight remained constant until the end of the experiment. The cultivation time had no effect on the molecular weight of levan produced in the medium containing 50% sucrose; the average molecular weight was approximately 2.96 kDa. The levan yield was very low when the cells were cultivated in the medium containing 1% or 5% sucrose; the molecular weight of levan produced varied from 1.43 to 8.86 kDa.

The enzymatic production of levan (30°C, 24 h) by *B. subtilis* NRC 33a has been studied using its 60% acetone fraction[34]. The study has shown that the molecular weight of levan decreases with increased sucrose concentrations, from 60 kDa (for 2.5% sucrose) to 2 kDa (40% sucrose). A similar study has been conducted for *B. circulans* using 50% ethanol fraction of its crude extract[37], showing that levan with the highest molecular weight is produced in presence of 5% sucrose (38 kDa), and the molecule size gradually decreases at higher sucrose concentrations (3 kDa for 32% sucrose). The results of this study conclude that the sucrose concentration is an effective factor controlling the molecular weight of produced levan.

Comparison of molecular weight of produced levan and levan yield of various microorganisms

Previous studies have shown the low molecular weight of the levan produced by various microorganisms ranges from 5 to 70 kDa. Molecular weights of several million kDa have also been reported (Table 5). The sizes of levan products produced by *B. licheniformis* FRI MY-55 were in the range of 3–13 kDa, thus belonging to the low molecular weight group. It should be noted that the degree of polymerization of levan largely depends on the conditions for the levansucrase reaction.

For *B. licheniformis* FRI MY-55, under the optimal cultivation conditions, 24 and 36 h of cultivation (30% and 40% sucrose, 40°C) were usually required to obtain the maximum levan yield (94 and 132 g/L). Shih *et al*. have reported that *B. subtilis* (natto) Takahashi produces a large quantity of levan, with the maximum productivity (40–50 and 50–60 g/L) obtained in the medium containing 20% and 25% sucrose after 21 and 24 h of cultivation (37°C), respectively [27, 39] (Table 6). The levan production rate of *B. licheniformis* FRI MY-55 in the medium containing 30% and 40% sucrose (at 40°C) was 3.92 g/L/h and 3.66 g/L/h, respectively. It is higher than the rate achieved by *B. subtilis* (natto) Takahashi (2.50 g/L/h) in 20% sucrose-containing medium, the most efficient levan-producing strain reported to date[39].

Evaluation of levan for use as prebiotic

The levan yield of the levan–levansucrase sediment from *B. licheniformis* FRI-MY55 used as a biocatalyst was very high, reaching 160 g/L. The levan produced by this procedure had molecular weights of 2.33 kDa (61.92%), 14.88 kDa (36.66%), and 540.80 kDa (1.42%) (data not shown).

The percent utilization was used to monitor the consumption of levan and fructose by probiotic, enteric, and pathogenic bacteria of humans or fish. The percent utilization by 16 probiotic strains, including six strains of bifidobacteria, seven strains of lactobacilli, *Lactococus lactis* subsp. *lactis* BCRC 10791, *Pediococcus pentosaceus* BCRC 14024, and *Streptococcus thermophilus* BCRC 12268, of the levan produced by *B. licheniformis* FRI MY-55 was in the range of 92.73%–114.07%, except for *Bifidobacterium breve* BCRC 11846 (83.68%) (data not shown). The prebiotic activity assay was based on the change in cell biomass after 24 h of growth of a probiotic strain on 1% prebiotic or fructose relative to the change in cell biomass of a mixture of enteric strains grown under the same conditions. Prebiotic activity scores for the 16 probiotic strains are shown in Fig. 5a. The highest prebiotic activity scores were obtained for *Lactobacillus delbrueckii* subsp. *bulgaricus* BCRC 16053 (0.42), followed by *P. pentosaceus* BCRC 14024, *Lb. acidophilus* BCRC 16099, and *Bifido. longum* subsp. *infantis* BCRC 14602 (0.36, 0.35, and 0.34, respectively). In contrast, the lowest score was obtained for *Bifido. longum* subsp. *longum* BCRC 11847 (0.06). All the tested probiotic strains had prebiotic activity scores above zero when grown on the levan produced by *B. licheniformis* FRI MY-55. This reflects the extent to which the levan produced by *B. licheniformis* FRI MY-55 promotes selective growth of probiotic strains in the presence of competitors unable to utilize the levan.

The percent utilization by 13 human and fish enteric and pathogenic strains of the levan produced by *B. licheniformis* FRI MY-55 was in the range of 51.20%–73.51%, except for *Clostridium sporogenes* BCRC 11259 and *Staphylococcus aureus* BCRC 10451 (95.11% and 86.30%, respectively)(Fig. 5b). Thus, the utilization of levan by the enteric and pathogenic strains was less than that by the abovementioned probiotic strains.

In this study, the levan produced by *B. licheniformis* FRI MY-55 displayed interesting prebiotic characteristics in terms of its ability to support the growth of 16 probiotic bacterial strains. However, Yamamoto *et al*. reported that when the pH of the culture medium was changed to judge the utilization of levan by eight bifidobacteria and *Lb. acidophilus* shown, low-molecular-weight levan (6 kDa) was not fermented by *Bifido. infantis*, *Bifido. liberorum*, *Bifido. lactentis*, *Bifido. animalis*, *Bifido. breve*, *Bifido. longum*, *Bifido. bifidum*, *Bifido. adolescentis,* or *Lb. Acidophilus*[40].

Bello *et al*. studied the prebiotic properties of levan produced by *Lb. sanfranciscensis* and *Erwinia herbicola* cultures inoculated with human feces and reported that the numbers of bifidobacteria increased (approximately 100-fold) exclusively with the levan produced by *Lb. sanfranciscensis*, whereas the numbers of lactobacilli decreased concomitantly. Neither bifidobacteria nor lactobacilli were enriched on the levan produced by *E. herbicola*[19]. Semjonovs and Zikmanis reported that high-molecular-weight levan (2,000 kDa) was fermented by *Bifido. lactis*, *Lb. acidophilus*, and *S. thermophilus* in MRS medium and milk[20].

In this study, the levan produced by *B. licheniformis* FRI MY-55 displayed interesting prebiotic characteristics in terms of its ability to support the growth of 16 probiotic bacterial strains. Levan utilization by the enteric and pathogenic strains was less compared with that by the probiotic strains. *In vivo*, the levan produced by *B. licheniformis* FRI MY-55 demonstrated potential prebiotic properties in the spotted grouper *Epinephelus coioides*. It is well known that prebiotics reach the large intestine and are fermented by the colonic microflora to produce short-chain fatty acids. The ingestion of levan was shown to increase the amount of short-chain fatty acids in the feces. Furthermore, the relative percent survival of the spotted groupers after challenge with *Vibrio harveyi* was the highest in the fish fed a diet supplemented with levan (personal communication).

CONCLUSION

In this study, a high levan-producing strain of *B. licheniformis* FRI MY-55 was isolated from a fish pond. *B. licheniformis* FRI MY-55 could

produce remarkable levels of levan in 50% sucrose-containing medium. The maximum levan production rate was 3.92 g/L/h. This is higher than the rate achieved by *B. subtilis* (natto) Takahashi (2.50 g/L/h), the most efficient levan-producing strain reported till date. The levan produced by *B. licheniformis* FRI MY-55 had prebiotic effect. *B. licheniformis* FRI MY-55 could represent an alternative efficient source of levan polymer when grown on high-sucrose medium, with potential industrial applications.

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