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Optimization, characterization and in Vitro evaluation of entomopathogenic fungal exopolysaccharides as prebiotic

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Abstract: Optimization of exopolysaccharides (EPS) produced by three strains of entomopathogenic fungi (Beauveria bassiana BCC 2692, Ophiocordyceps dipterigena BCC 2073, and Paecilomyces tenuipes BCC 2656) was carried out together with analyses of their prebiotic properties. B. bassiana BCC 2692 produced 6.27±0.22 g/L EPS on optimal medium using two-level fractional factorial design and 4.7 g/L EPS in bioreactor. EPS productions of O. dipterigena BCC 2073 were 13.2 g/L and 41.2 g/L in shake flask and bioreactor, respectively. For P. tenuipes BCC 2656, 1.47±0.21 g/L EPS in shake flask and 28.1 g/L EPS in bioreactor were obtained. These EPS were previously characterized as glucan with differences in molecular weights and degree of branching. They were resistant to hydrolysis by both hydrochloric acid and porcine pancreatic α-amylase. Furthermore, when used as the sole carbon source, all three types of EPS supported growth in vitro of two different probiotic bacteria (Lactobacillus acidophilus BCC 13839 and bifid bacterium animals ATCC 25527). A constant viability of L. acidophilus BCC 13839 was maintained throughout the cultivation period (48 hours) on all three entomopatogenic fungal EPS. All EPS also supported better growth and maintained longer growth period of B. animalis ATCC 25527 than glucose or inulin. Thus these entomopathogenic fungi EPS are promising candidates in prebiotic industry, expanding the pool of current commercial prebiotics.

Keywords: Exopolysaccharide, Glucan, Prebiotic, Probiotic, Entomopathogenic Fungi

1. Introduction

It is now well established that gastrointestinal diseases caused by the increase in gastrointestinal pathogens can often be diminished by promotion of the growth of probiotics, such as lactic acid bacteria and bifidobacteria [1-2, 12-14, 18, 27-28, 32, 35-37, 41], thus, there is a great deal of interest in the use of prebiotics. Prebiotics, non-digestible compounds in animals and humans, consist of a group of oligosaccharides that are able to stimulate growth of an essential consortium of bacteria in gastrointestinal tract [6-8, 12, 15-16, 20, 22, 30, 33-34, 37, 39]. Moreover, good prebiotics are required to resist stomach acid hydrolysis, be fermented by the intestinal microbiota, and selectively stimulate growth of useful intestinal bacteria [7-8, 12, 15-16, 22, 30, 33-34, 37]. The population of probiotics in the human's (and animal) gut system can be increased by the consumption of appropriate prebiotics [3, 8, 12, 22, 35-37], which include oligosaccharides, such as inulin, fructo-oligosaccharides, galacto-oligosaccharides, gluco-oligosaccharides, and

xylo-oligosaccharides [1-3, 9-10, 13-16, 20, 29, 32, 39, 41]. There are also several types of prebiotics that have been studied for their functions to promote growth of probiotics [9-10, 18, 27, 29, 38]. However, current prebiotics are limited in their efficacies due to their persistences to the distal colon, while the microbiota known to have saccharolytic metabolism are predominantly in the proximal colon [7-8, 33-34, 37, 39].

Clearly, there are several groups of microorganisms producing exopolysaccharides (EPS) with different properties and applications, such as antitumor agents [5, 31], food packaging materials [25], and wound dressing materials [23, 26]. In particular, EPS produced by fungi serve to protect their cells against desiccation, phagocytosis and phage attack, toxic compounds, predation by protozoans, and osmotic stress, as well as in cellular recognition [42]. There are several reports of prebiotics produced from natural resources, mostly from plants [15, 21], however, no studies on exopolysaccharides from entomopathogenic fungi with their prebiotic properties have been reported. Hence, EPS from fungi are attractive as an alternative source of prebiotics apart from their abundance and diversity in chemical structure.

In this study, prebiotic activities of the EPS secreted from three entomopathogenic fungi, namely, *Beauveria bassiana* BCC 2692, *Ophiocordyceps dipterigen*a BCC 2073, and *Paecilomyces tenuipes* BCC 2656 were investigated. In addition, the optimization of the EPS production was carried out.

2. Materials and Methods

2.1. Fungal Strains

Entomopathogenic fungi, Beauveria bassiana BCC 2692, Ophiocordyceps dipterigena BCC 2073, and Paecilomyces tenuipes BCC 2656, were obtained from BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, Thailand.

2.2. Probiotic Bacterial Strains

Lactobacillus acidoplilus BCC 13839 and Bifidobacterium animalis ATCC 25527 were used as probiotic bacteria for the evaluation of their growths on different EPS in comparison with a commercial prebiotic (inulin; Bio Basic Inc., Toronto, Canada).

2.3. Inoculum Preparation

B. bassiana BCC 2692, *O. dipterigen*a BCC 2073, and *P. tenuipes* BCC 2656 were grown initially on potato dextrose agar (PDA) (Becton, Dickinson and company, MD, USA) at 25 °C for 5-7 days. An agar block (1 cm^3) containing the growing culture was cut into small pieces and transferred to 25 mL of potato dextrose broth (PDB) (Becton, Dickinson and company, MD, USA) and incubated with shaking (200 rpm) for 5-7 days at 25 °C. *L. acidophilus* BCC 13839 and *B. animalis* ATCC 25527 were pre-grown in MRS broth (Merck, Germany) and Difco™ Reinforced Clostridial medium respectively, at 37 °C for 24-48 h.

2.4. Fermentation Conditions

2.4.1. Optimization of EPS Production

A full factorial design was used to determine the optimal carbon and nitrogen sources [19]. Twenty g/L carbon sources (galactose, glucose, lactose, maltose, mannose, sucrose, and fructose) and 10 g/L nitrogen sources ($NH_4H_2PO_4$, $(NH_4)_2SO_4$, malt extract, peptone, yeast extract, meat extract, corn steep solid, $NaNO_3$, NH_4NO_3, and tryptone) were combined in a basal medium (0.5 g/L KH_2PO_4, 0.2 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.14 g/L $MnSO_4 \cdot H_2O$, and 1 mL/L vitamin solution (Blackmore, NSW, Australia)). Experiments were conducted in duplicate. The influence of 6 quantitative factors (carbon (sugar) concentration, nitrogen concentration (combination of four nitrogen sources) and trace element solution) on EPS production was evaluated using a fractional factorial design at 2 levels [19] conducted in duplicate with 3 center points. The optimal condition for each fungal strain selected from the fractional factorial design then was evaluated in a 5-L fermenter in order to produce sufficient exopolysaccharides (EPS) for further experiments.

As the full factorial design at 2 levels with 6 factors requires an evaluation of 64 combinations, a reduced design, known as fractional factorial, with only 32 combinations was used with 3 center points [19]. In these experiments, the production of biopolymer was the main output of the process. In addition, biopolymer and biomass were taken into consideration as primary responses in order to evaluate the relationship between growth and production. Design Expert software (Version 7.0.b1.1, Stat-Ease Inc., and Minneapolis, USA) was used for experimental design selection and data analysis.

2.4.2. Entomopathogenic Fungal EPS Production for Prebiotic Evaluation

The optimized medium used in the 5-L fermented (Marubishi Co., Ltd., Pathumthani, Thailand), with a working volume of 4 L, for *B. bassiana* BCC 2692 consisted of fructose 60 g/L, malt extract 10 g/L, peptone 10 g/L and yeast extract 10 g/L; for *O. dipterigen*a BCC 2073; glucose 60 g/L, malt extract 14 g/L; and for *P. tenuipes* BCC 2656; glucose 60 g/L, corn steep solid 15 g/L. The basal medium composition for all recipes contained 0.5 g/L KH_2PO_4, 0.2 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.14 g/L $MnSO_4 \cdot H_2O$, and 1 mL/L vitamin solution (Blackmore). The vitamin complex consisted of 75 mg vitamin B1 (thiamine hydrochloride), 10 mg vitamin B2 (riboflavin), 50 mg nicotinamide, 25 mg calcium pantothenate, 10 mg vitamin B6 (pyridoxine hydrochloride), 25 mcg vitamin B12 (cyanocobalamin), 15 mcg biotin, 500 mg vitamin C (derived from ascorbic acid 260 mg and calcium ascorbate

290.5 mg), 10 mg choline bitartrate, 10 mg inositol, 10 mg zinc amino acid chelate (zinc 2 mg), 175 mg calcium phosphate, and 75 mg magnesium phosphate. Trace element solution contained (per L) 14.3 g of $ZnSO_4 \cdot H_2O$, 2.5 g of $CuSO_4 \cdot 5H_2O$, 0.5 g of $NiCl_2 \cdot 6H_2O$ and 13.8 g of $FeSO_4 \cdot H_2O$. Fungal seed culture (10% v/v) was transferred to each fermenter and the culture was agitated at 300 rpm and aerated at 1 vvm, with no adjustment of pH.

2.5. EPS preparation

Culture filtrate was mixed with four volumes of 95% ethanol, stirred vigorously for 10-15 min. and kept at 20 °C for at least 12 h. Precipitated EPS was sedimented at 10,000 *g* for 20 min and lyophilized. EPS was redissolved in distilled water (30-50 g wet weight per1 L of distilled water) and insoluble material was removed by sedimentation as described above. The supernatant was then dialyzed (2 kDa molecular weight cut-off; Spectrum Laboratories Inc., USA) against 4 L of distilled water for 24 h and lyophilized once more.

2.6. EPS Characterization

2.6.1. Molecular Weight Determination

The average molecular weight of EPS was determined using gel-permeation chromatography (GPC, Waters 600E; Waters, MA, USA), equipped with a refractive index (RI) detector and Ultrahydrogel column (300×7.8 mm diameter; Waters, USA) calibrated with dextran standard molecular weight markers (4,400 to 401,000 Da). Injection volume was 20 μL and the sample was eluted with 0.05 M sodium bicarbonate buffer at a flow rate of 0.6 mL/min.

2.6.2. ^{13}C-NMR Spectroscopy

^{13}C-NMR spectra of samples in 0.1 M NaOD (50 mg/mL) were recorded at temperature of 298 K using AVANCE 300 MHz Digital NMR spectrometer (Bruker Biospin, AV-500, Germany) at a frequency of 125 MHz. Typical parameters used were: number of scans 20,000, relaxation delay 1 s, spin rate 25 Hz, spectrum size 32 K and time domain points 32 K.

2.6.3. Probiotic Cultivation of L. Acidophilus BCC 13839 and B. Animals ATCC 25527 on EPS as Carbon Source

L. acidophilus BCC 13839 and *B. animalis* ATCC 25527 was MRS and Clostridial medium, respectively. 10 g/L of each EPS from *B. bassiana* BCC 2692, *O. dipterigen*a BCC 2073, *P. tenuipes* BCC 2656, glucose or inulin were used as a carbon source**.**

2.6.4. Biomass and Cell Viability Measurements

Turbidity of cultures was determined by measuring absorbance at 600 nm. Number of viable cells was determined by serial dilutions in fresh medium (MRS for *L. acidophilus* BCC 13839 and Difco™ Reinforced Clostridial for *B. animalis* ATCC 25527) which was incubated at 37 °C for 24-48 h. Colony forming units were determined in Petri-dishes having 30-300 colonies.

2.6.5. Lactic Acid Measurement

Probiotic cell cultures were centrifuged at 10,000 *g* for 10 min and supernatants were filtered through 0.22 μm filters. Filtrates were subjected to HPLC analysis using an Aminex column (Bio-Rad, Hercules, CA) at 65 °C, using 5 mM H_2SO_4 as a mobile phase at a flow rate of 0.6 mL/min and a pressure of 1000-1200 psi. Lactic acid was detected with a refract meter (Waters 410 Differential Refract meter Detector, Millipore Corp., Milford, MA, USA) at retention time of 12.3-12.4 min.

2.7. Digestibility Assay

2.7.1. Gastric Juice Hydrolysis

In order to mimic the acid condition of the stomach, EPS were incubated with 0.1 M HCl at 37 °C and samples (0.5 mL) were interval taken at 0, 1, 2, 4, and 6 h for determination of liberated reducing sugars using dinitrosalicylic acid (DNS) assay using glucose as standard [4].

2.7.2. Enzymatic hydrolysis

Sensitivity of EPS to porcine pancreatic α-amylase (EC. 3.2.1.1) (Sigma-Aldrich) was evaluated by mixing 1 mL of EPS (1% w/v of sodium phosphate buffer pH 7) with an equal volume of enzyme solution (2 units/mL of 20 mM sodium phosphate buffer containing 6.7 mM sodium chloride) and an equal volume of color reagent (dinitrosalicylic acid, DNS), 1 mL), and the mixture was incubated at 37 °C for 0, 1, 2, 4 and 6 h, after which time the reaction was terminated by heating in boiling water for 15 min. The reaction mixture was cooled to room temperature and 9 mL of deionized water were added. The amount of reducing sugar was measured at 540 nm, using glucose as standard.

Table 1. *EPS and biomass production of B. bassiana BCC 2692 using a two-level fractional factorial design with 6 factors (2^{n-1})*

Fructose (g/L)	Malt extract (g/L)	peptone (g/L)	$NaNO_3$ (g/L)	Yeast extract (g/L)	Trace solution (mL/L)	EPS (g/L)	Biomass (g/L)
20	0	0	0	0	1	0	1.78±1.74
60	0	0	0	10	1	0	4.71±1.03
20	10	0	0	10	4	0	14.77±0.56

60	10	0	0	0	4	0	1.89±0.03
20	0	15	0	10	4	0	6.48±1.00
60	0	15	0	0	4	0	4.09±1.12
20	10	15	0	0	1	2.05±0.08	27.27±8.73
60	10	15	0	10	1	6.27±0.22	27.77±10.41
20	0	0	5	0	4	0.93±0.04	7.28±3.27
60	0	0	5	10	4	0	4.18±0.69
20	10	0	5	10	1	2.69±0.33	17.92±0.01
60	10	0	5	0	1	0.47±0.66	4.60±0.35
20	0	15	5	10	1	0.29±0.25	9.49±3.09
60	0	15	5	0	1	0	7.38±0.14
20	10	15	5	0	4	2.27±0.06	22.40±1.06
60	10	15	5	10	4	3.50±1.40	28.09±0.84
40	5	7.5	2.5	5	2.5	5.57±0.97	23.72±3.03

3. Results

3.1. Optimization of EPS Production

Optimal EPS production from *B. bassiana* BCC 2692 (6.27±0.22 g/L) using two-level fractional factorial design was achieved on fructose 60 g/L, malt extract 10 g/L, peptone 15 g/L, yeast extract 10 g/L and trace elements solution 1.0 mL/L (Table 1). For *P. tenuipes* BCC 2656, the highest EPS production (1.47±0.21 g/L) was on glucose 60 g/L, corn steep solid 15 g/L and trace elements solution 1.0 mL/L (Table 2). Furthermore, the highest EPS production of *O. dipterigena* BCC 2073 was 13.2± 0.74 g/L on glucose 60 g/L and malt extract 14 g/L at 25 ^{o}C [19]. The optimal medium composition obtained from the fractional factorial design (Table 3) was evaluated in a 5-L fermenter. After optimization, *B. bassiana* BCC 2692 produced 4.7 g/L EPS, whereas *O. dipterigen*a BCC 2073 and *P. tenuipes* BCC 2656 on glucose-supplemented medium produced higher amounts of EPS (41.2 and 28.1 g/L, respectively).

3.2. EPS Characteristics

Previously we have shown that these fungal EPS belong to the group of β-glucans consisting of β-(1→3) linkage as the main chain with β-(1→6) as branch points [26]. In this study, analysis of the molecular weight showed that the three EPS samples were significantly different, ranging from 4 to 2310 kDa, with that of *P. tenuipes* BCC 2656 being the lowest (in the range of oligosaccharides), and that of *B. bassiana* BCC 2692 having the largest size with a bimodal distribution (49 and 2310 kDa) (Table 3).

The presence of β-D-(1→3, 1→6)-linked glucans in EPS from *O. dipterigena* BCC 2073 and *P. tenuipes* BCC 2656 were confirmed by ^{13}C-NMR (Figure 1). The EPS of *O. dipterigena* BCC 2073 exhibited two anomeric carbon signals at 103 and 100 ppm, clearly showing the presence of β- and α-glucans with a ratio of 2:1. On the other hand, in the spectrum of *P. tenuipes* BCC 2656 EPS, the β-configuration of D-glucosyl residues was evident from the presence of only one anomeric peak at 106 ppm. The other carbon signals in the spectra were tentatively assigned as shown, although some signals were shifted compared to the literature values [11, 17], probably due to different substituents and degree of branching. It is worth noting that the ^{13}C-NMR spectrum of EBP from *B. bassiana* BCC 2692 was similar to that from *O. dipterigena* BCC 2073 but was slightly more complicated due to the existence of two constituents as indicated by MW evaluation (data not shown).

Table 2. *EPS and biomass production of P. tenuipes BCC 2656 using a two-level fractional factorial design with 6 factors (2^{n-1})*

Glucose (g/L)	Malt extract (g/L)	$NaNO_3$ (g/L)	NH_4NO_3 (g/L)	Corn steep solid (g/L)	Trace solution (mL/L)	EPS (g/L)	Biomass (g/L)
20	0	0	0	0	1	0.06±0.08	2.53±0.01
60	0	0	0	15	1	**1.47±0.21**	14.87±8.60
20	10	0	0	15	4	1.09±0.42	13.79±0.16
60	10	0	0	0	4	0.22±0.01	6.55±0.71
20	0	5	0	15	4	0.59±0.01	10.26±0.08
60	0	5	0	0	4	0	5.23±1.21

20	10	5	0	0	1	0.26±0.02	4.70±0.11
60	10	5	0	15	1	0.59±0.83	25.70±4.42
20	0	0	5	0	4	0.19±0.07	3.29±0.07
60	0	0	5	15	4	0	21.34±1.41
20	10	0	5	15	1	0	17.05±0.39
60	10	0	5	0	1	0	5.08±1.32
20	0	5	5	15	1	0	12.47±2.02
60	0	5	5	0	1	0	2.86±0.33
20	10	5	5	0	4	0.34±0.48	4.09±0.83
60	10	5	5	15	4	0.30±0.42	25.69±1.07
40	5	2.5	2.5	7.5	2.5	0.39±0.53	18.82±9.36

Table 3. *Characteristics of EPS from three entomopathogenic fungi*

Fungus	Molecular weight at peak (Mp, kDa)	Optimized medium[a]	Production in 5 L fermentor (g/L)
B. bassiana BCC 2692	2310 and 49	fructose 60 g/L, malt extract 10 g/L, peptone 15 g/L, yeast extract 10 g/L	4.7[a]
O. dipterigena BCC 2073	590	glucose 60 g/L, malt extract 14 g/L	41.2[b]
P. tenuipes BCC 2656	4.3	glucose 60 g/L, corn steep solid 15 g/L	28.1[a]

[a]*Basal medium=0.5 g/L KH_2PO_4, 0.2 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.14 g/L $MnSO_4 \cdot H_2O$, and 1 mL/L vitamin solution* [b]*[19]*

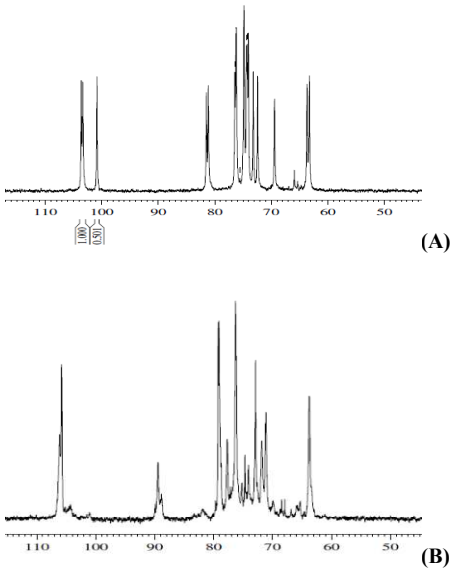

Figure 1. *^{13}C-NMR spectra of EPS from O. dipterigena BCC2073 (A), and P. tenuipes BCC 2656 (B)*

3.3. EPS Prebiotic Characteristics

As one of the criteria for classifying a food ingredient as a prebiotic is resistance to gastric acid digestion and to hydrolysis by mammalian enzymes, in *vitro* treatment with 0.1 M HCl or with porcine pancreatic α-amylase of all three sources of EPS were resistant to such treatments (Table 4). These results indicated that these polysaccharides should reach the intestine almost (> 95%) intact, thus meeting this requirement is part of a prebiotic.

Table 4. *Hydrolysis resistance of fungal EPS*

Fungus	Acid hydrolysis (%)[c]	Enzyme hydrolysis (%)[c]
B. bassiana BCC 2692	0.20	0.84
O. dipterigena BCC 2073	0.00	0.00
P. tenuipes BCC 2656	2.09	0.00

[c]*37 ℃ for 6 hours*

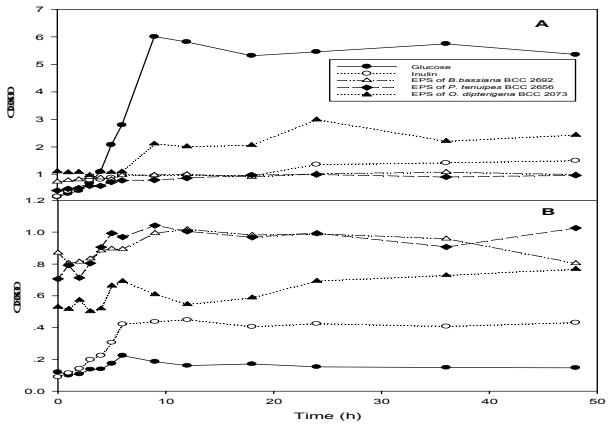

Figure 2*. Growth of L. acidophilus BCC 13839 9A) and B. animalis ATCC 25527 (B) on three entomopathogenic fungal EPS sources, glucose and inulin as sole carbon source. The cultivation was performed in 1 L bioreactor at 37 °C for 48 h under anaerobic condition.*

Another criterion of a prebiotic is the ability to act as nutrient source for intestinal microflora. Thus, two different probiotics were cultivated on the three sources of EPS as carbon source in comparison to glucose and inulin. With L. acidophilus BCC 13839, the highest growth was observed on glucose after 10 h of cultivation (Figure 2A). Among the three EPS samples, that from O. dipterigena BCC 2073 supported the best growth of L. acidophilus BCC 13839, while the other two types of EPS supported lower growth, but comparable to that of inulin. Whereas growth of B. animalis ATCC 25527 on different substrates after 10 h of cultivation was P. tenuipes BCC 2556 EPS ≅ B. bassiana BCC 2692 EPS > O. dipterigena BCC 2073 EPS > inulin > glucose (Figure 2B). However, when viability of the bacteria was examined, that of L. acidophilus BCC 13839 at 10 h of cultivation was on O. dipterigena BCC 2073 EPS > inulin > glucose > B. bassiana BCC 2692 ≅ P. tenuipes BCC 2656 EPS (Figure 3A). In addition, the results showed that viability of the lactic acid bacteria on inulin and glucose noticeably decreased after 24 h growth and 36 h, respectively, whereas viability was steady when using all three sources of EPS. For B. animalis ATCC 25527, its viability when cultivated on glucose, inulin and EPS of O. dipterigena BCC 2073 was comparable, but lower than the other two sources of EPS (Figure 3B). Lactic acid production of L. acidophilus BCC 13839 on various studied substrates at 10 h growth was glucose > P. tenuipes BCC 2656 EPS > B. bassiana BCC 2692 EPS > O. dipterigena BCC 2073 EPS > inulin (Figure 4A). It is notable that on glucose, lactic acid production decreased after 10 h of cultivation, whereas on inulin lactic acid level remained constant after 6 h. Moreover, the production of lactic acid on B. bassiana BCC 2692 EPS continuously decreased after 12 h until no lactic acid was detected at 48 h of cultivation. Regarding B. animalis ATCC 25527, lactic acid production at 10 h is as follows: inulin > B. bassiana BCC 2692 EPS > glucose (Figure 4B). On inulin, lactic acid level remained constant after 12 h of cultivation, whereas on glucose its lactic acid production continuously rose after 5 h. There was no lactic acid detected when EPS of O. dipterigena BCC 2073 and P. tenuipes BCC 2656 used as substrate.

4. Discussion

Prebiotics have to be compounds non-digestible by stomach acid and enzymes and can stimulate growth of essential bacteria in the gastrointestinal tract of animals and humans. Most prebiotics are limited in their persistence to the distal colon and are predominantly fermented in the proximal colon or metabolized by microbial flora saccharolytic enzymes [7-8, 33-34, 37, 39]. This study showed that the exopolysaccharides produced by three different entomopathogenic fungi exhibit prebiotic properties. The chemical structures of these EPS were typical of β-glucans with varying size distributions [19, 23, 26]. All EPS produced by *B. bassiana* BCC 2692, *O. dipterigen*a BCC 2073, and *P. tenuipes* BCC 2656 are non-cytotoxic to human cells [23]. Although it was found that most of the commercialized prebiotics, such as insulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and soybean oligosaccharides have degree of polymerization (DP) in the range of oligosaccharides (DP < 25) [24], some polysaccharides like glucans from fruit bodies of mushrooms have been also reported to possess potential prebiotic activity [40].

The higher production of exobiopolymer of *P. tenuipes* BCC 2656 in bioreactor was obtained in comparison to *B. bassiana* BCC 2692 due to the physical conditions such as aeration, agitation, and dissolved oxygen were not optimized in this study. Furthermore, the production of EPS by *B. bassiana* BCC 2692 in bioreactor was lower than in shake flask (two-level fractional factorial design) due to agitation of the propeller and rotation speed of the shaker might affected the fungal mycelial morphology and influence the production of EPS. Further agitation rate must be optimized for this fungus in order to obtain higher production of EPS.

High production of EPS from *O. dipterigen*a BCC 2073 is very attractive for a fermentation process as its EPS yield under the optimized conditions is 20 times higher than that on potato dextrose broth (PDB, 2.53 g/L and 13.2 g/L on optimal medium) [19]. Although the fermentation in this report was carried out as a batch process, production can be increased using other fermentation processes such as a well mix bioreactor [43], which can reduce problems of high viscosity, low oxygen transfer rate, and low sugar consumption rate. Although growth of *L. acidophilus* BCC 13839 on glucose and inulin were higher than that on entomopathogenic EPS, cell viability was compromised in long term cultivation. For *B. animalis* ATCC 25527, all three entomopathogenic fungal EPS supported and maintained a better bacterial growth in comparison with glucose and inulin. These results indicate that these exopolysaccharides showed prebiotic properties that could maintain the growth of probiotics in gastrointestinal tract of animals and humans. It is worth noting that there was no lactic acid detected when EPS of *O. dipterigena BCC 2073 and P. tenuipes* BCC 2656 used as substrate, which contrast with that when EPS of *B. bassiana* BCC 2692 used as

substrate. This probably attributes to differences in β-glucan structure of these EPS since it has been found that EPS of *B. bassiana* BCC 2692 possesses less branched structure, compared with the other two ones (data not shown). Since EPS with less complex molecular structure might be easily metabolized as carbon source by probiotics.

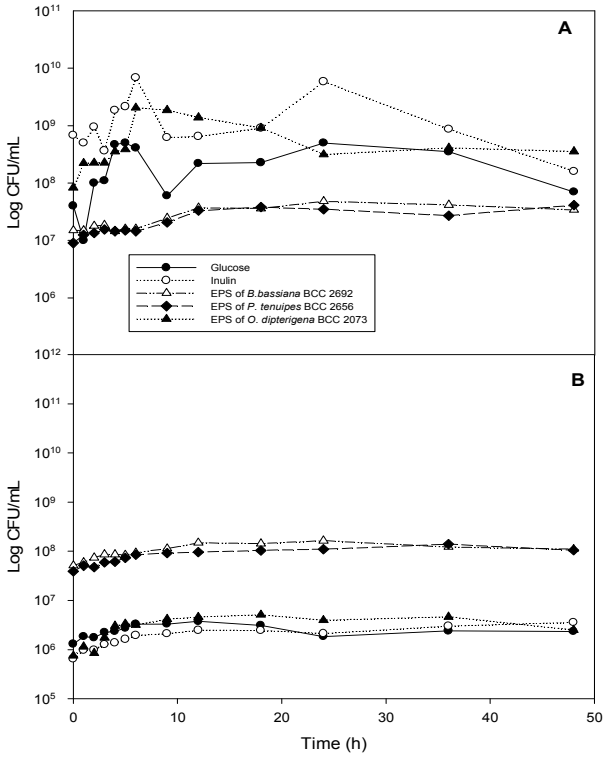

Figure 3. *Viability of L. acidophilus BCC 13839 (A) and B. animalis ATCC 25527 (B) cultivated on three entomopathogenic fungal EPS, glucose and inulin as sole carbon source.*

In addition, the variation in lactic acid production was possibly due to different utilization of carbon sources by the probiotics [38]. *L. acidophilus* BCC 13839 metabolized all carbon sources to produce lactic acid better than *B. animalis* ATCC 25527. The more end-product of lactic acid produced, the more EPS metabolized by the probiotics [44]. Furthermore, the absence of lactic acid produced by *B. animalis* ATCC 25527 on some substrates might be due to the bacteria produced acetic acid rather than lactic acid [44].

5. Conclusion

With the ability to obtain high production using optimized growth medium formulation, these entomopathogenic fungal EPS could be a promising prebiotic in the food and feed industries. β-Glucans from three strains of entomopathogenic fungi demonstrated prebiotic characteristics; non-digestibility by 0.1 M HCl and by porcine pancreatic α-amylase, non-cytotoxic to human cells and ability to promote the *in vitro* growth of two different studied probiotics (*Lactobacillus acidoplilus* BCC 13839 and *Bifidobacterium animalis* ATCC 25527). Thus, these EPS have a potential as alternative sources of prebiotics, which can be produced at an industrial scale through fermentation.

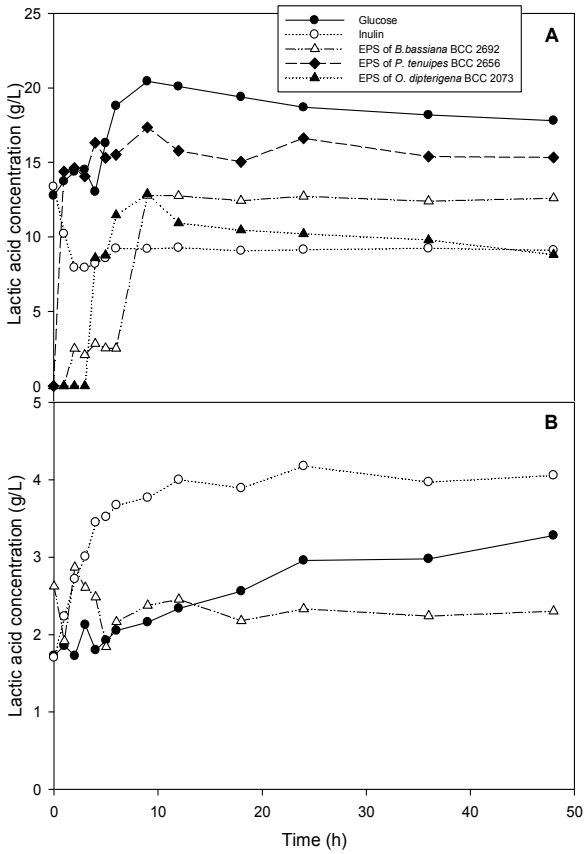

Figure 4. *Lactic acid production of L. acidophilus BCC 13839 (A) and B. animalis ATCC 25527 (B) cultivated on three entomopathogenic fungal EPS, glucose and inulin as sole carbon source.*

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