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Exobiopolymer Application of Three Enthomopathogenic Fungal Strains as Prebiotic Used

Wai Prathumpai[1*], *Pranee Rachathewee*[1], *Sutamat Khajeeram*[2], *Pariyada Tanjak*[3] and *Pawadee Methacanon*[3]

[1] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, 113 Thailand Science Park, Klong 1, Klong Luang, Pathumthani 12120, Thailand.*

[2] *Department of Biotechnology, Thammasat University, Klong 1, Klong Luang, Pathumthani 12120, Thailand.*

[3] *National Metal and Materials Technology Center (MTEC), 114 Thailand Science Park, Paholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120, Thailand.*

[*] *Correspondent author: wai.pra@biotec.or.th*

ABSTRACT

Exopolysaccharides of three enthomopathogenic fungal strains namely; *Aschersonia samoensis* BCC 2466, *Ophiocordyceps nipponica* BCC 2092, and *Gibellula pulchra* BCC 2711, were studied for their prebiotic properties. These exopolysaccharides were previously characterized as glucan with molecular weight of 4.34, 3.39 and 4.06 kDa, respectively. The production of these exopolysaccharides was carried out using fermentation in submerged culture and their exobiopolymers were purified. These purified exopolysaccharides were then used as a sole carbon source on their prebiotic study by co-cultivation with probiotic bacteria. *In vitro* prebiotic study shows that all three exopolysaccharides well supported the growth of *Lactobacillus acidophilus BCC 13839 and Bifidobacterium animalis ATCC 25527* after the cultivation of these two bacteria on each exobiopolymer compared with inulin (a commercial prebiotic) and glucose. The highest viability of *L. acidophilus* BCC 13839 was obtained on exobiopolymer of *A. samoensis* BCC 2466 even though its growth was lower when compared that on glucose and inulin. For *B. animalis* ATCC 25527, its cell growth and viability on all exobiopolymers were higher than those on glucose and inulin. These evidences showed well characteristics of the fungal exopolysaccharides on their growth-stimulating prebiotics.

Keywords: prebiotic, exopolysaccharide, enthomopathogenic fungi, *Lactobacillus*, *Bifidobacterium*

1. INTRODUCTION

Non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of useful bacteria in the gastrointestinal tract are defined as prebiotics.(1) The prebiotics have several advantages, but one of the key factor is that they are natural food and feed ingredients. They are generally compounds in the group of oligopolysaccharides, which can resist to stomach acid hydrolysis, fermentation by the intestinal microbiota, and selective stimulation the growth of useful intestinal bacteria (1, 2, 3, 5, 6, 7, 8, 9). The groups of prebiotics including oligosaccharide such as inulin, fructooligosaccharides, galactooligosaccharides, glucooligosaccharides, xylooligosaccharide, and etc. are commonly found in the market (10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21). However, the present commercialized prebiotics are mostly extracted from plants (8, 9) and they have properties limited to be weak in gastric juice. An alternative source of prebiotics from fungi is proposed here in this study, which has their properties as prebiotic requirements. They have been found to be a glucan family with various molecular weight and degree of branching (22, 23). Objectives of this study were to evaluate the *in vitro* prebiotic properties of exopolysaccharides produced from three strains of enthomopathogenic fungi (*Aschersonia samoensis* BCC 2466, *Ophiocordyceps nipponica* BCC 2092, and *Gibellula pulchra* BCC 2711) after optimization. They were tested against two strains of useful probiotics (*Lactobacillus acidophilus* BCC 13839 and *Bifidobacterium animalis* ATCC 25527) and they would promote the growth of these bacteria and could be used as prebiotic in food and feed industries in the near future.

2. MATERIALS AND METHODS

2.1 Fungal strains and cultivation

Entomopathogenic fungi, *Aschersonia samoensis* BCC 2466, *Gibellula pulchra* BCC 2711 and *Ophiocordyceps nipponica* BCC 2092, were used for exobiopolymer production in this study. They are deposited in BIOTEC Culture Collection, which is publicly accessible.

2.2 Bacterial strains and cultivation

Lactobacillus acidoplilus BCC 13839 and *Bifidobacterium animalis* ATCC 25527 were used as probiotic bacteria for the study of their growth on different exobiopolymers as a source of prebiotics compared with a commercial one (inulin).

2.3 Inoculum preparation

Aschersonia samoensis BCC 2466, *Ophiocordyceps nipponica* BCC 2092 and *Gibellula pulchra* BCC 2711 were grown initially on potato dextrose agar (PDA) 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) in a 250 mL Erlenmeyer flask. This liquid seed culture was incubated for 5-7 days at 25 °C on a rotary shaker (200 rpm) (New Brunswick, NJ, USA).

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

Optimization of Exopolysaccharide Production

A full factorial design was used to determine the optimal carbon and nitrogen sources. Twenty $g \times L^{-1}$ carbon (galactose, glucose, lactose, maltose, mannose, sucrose, fructose) and 10 $g \times L^{-1}$ 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,

tryptone) were combined in a basal medium (0.5 g×L^{-1} KH_2PO_4, 0.2 g×L^{-1} K_2HPO_4, 0.2 g×L^{-1} $MgSO_4$×$7H_2O$, 0.14 g×L^{-1} $MnSO_4$×H_2O, and 1 mL×L^{-1} vitamin solution (Blackmores, NSW, Australia). The experiments were conducted in duplicate. Following this selection, the influence of 5-6 quantitative factors (sugar concentration, nitrogen concentration (combination of nitrogen sources), trace element solution, temperature) on exobiopolymer production was evaluated using a fractional factorial design at 2 levels. The experiments were conducted in duplicating with 3 center points. The optimal conditions of each fungal strain selected from the fractional factorial design were evaluated in a 5-L fermenter production in order to obtain the exopolysaccharides for further experiments.

2.5 Enthomopathogenic Fungal Exobiopolymer Production

All fungal strains were cultivated in 5-liter fermenter for exobiopolymer production. The medium used in a 5-liter fermenter (Marubishi Co., Ltd., Pathumthani, Thailand) with a working volume of 4 L had the following composition : for *A. samoensis* BCC 2466; fructose 40 g×L^{-1}, malt extract 5 g×L^{-1}, peptone 7.5 g×L^{-1}, yeast extract 5 g×L^{-1}, $NH_4H_2PO_4$ 5 g×L^{-1}, for *O. nipponica* BCC 2092; mannose 60 g×L^{-1}, corn steep solid 10 g×L^{-1}, $NH_4H_2PO_4$ 10 g×L^{-1}, for *G. pulchra* BCC 2711; Fructose 60 g×L^{-1}, malt extract 10 g×L^{-1}, peptone 15 g×L^{-1}, $NH_4H_2PO_4$ 10 g×L^{-1}, yeast extract 10 g×L^{-1}. The basal medium composition for all recipes contained the following; 0.5 g×L^{-1} KH_2PO_4, 0.2 g×L^{-1} K_2HPO_4, 0.2 g×L^{-1} $MgSO_4$×$7H_2O$, 0.14 g×L^{-1} $MnSO_4$×H_2O, and 1 mL×L^{-1} vitamin solution (Blackmores, NSW, Australia). 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 $ZnSO_4$•H_2O, 2.5 g of $CuSO_4$•$5H_2O$, 0.5 g of $NiCl_2$•$6H_2O$ and 13.8 g of $FeSO_4$•H_2O. About 10% (v/v) seed culture was transferred to each fermenter. Culture was agitated at 300 rpm and aerated at 1 vvm. pH was not automatically adjusted.

2.6 Exobiopolymer purification

Culture filtrate was mixed with four volumes of 95% ethanol, stirred vigorously for 10-15 min and stored at 20 °C for at least 12 h. Precipitated polymer was sedimented at 10,000 *g* for 20 min and lyophilized. Polymer was re-dissolved in distilled water and any insoluble material was removed by centrifugation at 10,000 *g* for 20 min. Supernatant was dialyzed (2 kDa cut off; Spectrum Laboratories, Inc., USA) against 4 L of distilled water for 24 h and lyophilized. The aim of the dialysis is to eliminate salts and sugars, which would interfere with biopolymer characterization.

2.7 Molecular weight determination of the exobiopolymers

The average molecular weight of the polymers was determined by gel-permeation chromatography (GPC) (Waters 600E; Waters, MA, USA); the machine is equipped with a refractive index (RI) detector and an Ultrahydrogel linear column (300´7.8 mm ID; Waters, USA). Universal calibration log (Mp) versus VR, where Mp is the peak molecular weight, was conducted with dextran standards of molecular weights ranging from 4,400 to 401,000. Injection volume was 20 µl and flow rate of the mobile phase (0.05 M sodium bicarbonate buffer) was 0.6 ml/min.

2.8 Cultivation of L. acidophilus BCC 13839 and B. animalis ATCC 25527

The cultivation of the two strain of probiotics, *L. acidophilus* BCC 13839 and *B. animalis* ATCC 25527, were carried out in 1 liters fermentor (Biostat Q, B. Braun, Germany) with a working volume of 700 mL. The medium used for *L. acidophilus* BCC 13839 was MRS medium without carbon sources and the medium used for *B. animalis* ATCC 25527 was Clostridial medium without carbon sources. The carbon sources used in this study were 10 $g \times L^{-1}$ Exobiopolymer (EPS) from 4 strains of enthomopathogenic fungi (*Aschersonia samoensis* BCC 2466, *Gibellula pulchra* BCC 2711 and *Ophiocordyceps nipponica* BCC 2092) compared with glucose and inulin. The cultivation conditions were at 37 ^{o}C, agitation of 100 rpm, no aeration, pH was not controlled and cultivation time was 48 h.

2.9 Biomass determination and Viability counts

The culture broth was collected for the determination of cell turbidity. It was subjected to spectrophotometer at the wavelength of 600 nm. The fresh culture medium was used as blank. Then, the viability counts were performed by serial dilution in fresh medium. The media used for plate counts were MRS and Difco™ Reinforced Clostridial media for *L. acidophilus* BCC 13839 and *B. animalis* ATCC 25527, respectively. They were incubated at 37 ^{o}C for 24-48 h and the colony forming unit was calculated by multiplying with the dilution rate. The petri-dish with 30-300 colonies was determined.

2.10 Metabolite determination

Supernatant was centrifuged at 10,000 *g* for 10 min and filtered through 0.22 µm filter paper. Filtrate was subjected to HPLC analysis using an Aminex column (Bio-Rad, Hercules, Calif.) at 65^{o}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 by refractometry (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA, USA).

2.11 Nondigestibility

Gastric juice hydrolysis

Exopolymers from the studied fungi were investigated for acid resistance by mimicked human gastric juice (HCl solution, pH 1). Each polymer was dissolved and stirred for overnight before adjusting pH using concentrated HCl and the reaction mixture was incubated at 37 °C for 6 h. Sample (0.5 mL) was taken periodically at 0, 1, 2, 4, and 6 h in order to determine reducing sugar liberated by Dinitrosalicylic acid (DNS) assay using glucose as a standard (24).

2.12 Enzymatic hydrolysis

The resistance to a-amylase digestion of the exopolymers derived from fungi was determined using a-amylase (EC. 3.2.1.1) from porcine pancrease (Sigma-Aldrich). Briefly, 2 unit/mL of enzyme was prepared in 20 mM sodium phosphate buffer with 6.7 mM sodium chloride while the polymer solutions (1 %w/v) were prepared in sodium phosphate buffer pH 7. Then, 1 ml of enzyme solution is added into each sample solution (1 mL) with colour reagent (dinitrosalicylic acid, DNS, 1 mL). The reaction mixture was incubated at 37 ^{o}C for 0 and 6 h after that the reaction was stopped by placing in boiling water for exactly 15 min. Then, the mixture was cooled to room temperature before adding with deionized water (9 mL). The reducing sugar was measured by using a Microplate Reader at 540 nm. Finally, the obtained values were calculated from glucose standard curve and converted to % hydrolysis.

3. RESULTS AND DISCUSSIONS

3.1 Exobiopolymer Optimization

Table 1 shows the optimal medium compositions obtained from the fractional factorial design and they were produced in 5-L fermenter. *A samoensis* BCC 2466

(7.3 $g.L^{-1}$) and *G. pulchra* BCC 2711 (5.5 $g.L^{-1}$) produced better exobiopolymer yield on fructose as a carbon source and malt extract, peptone, yeast extract and $NH_4H_2PO_4$ as nitrogen sources. *O. nipponica* BCC 2092 produced 6.2 $g.L^{-1}$ of exobiopolymer on mannose as a carbon source and corn steep solid/ $NH_4H_2PO_4$ as nitrogen sources. Although, these exobiopolymers were not produced on cheap carbon and nitrogen sources, alternative sources of media with lower cost must be justified.

3.2 Molecular weight of exobiopolymers

As shown in Table 1, the molecular weight of exobiopolymers from *A. samoensis* BCC 2466, *G. pulchra* BCC 2711, and *O. nipponica* BCC 2092 was 4.34, 4.06, and 3.39, respectively. These exobiopolymers have been previously characterized and were of different chemical composition, with belonging to the group of glucan. Moreover, it was notable that the molecular weights of exobiopolymers were in the range of oligosaccharides as well as currently available prebiotics such as inulin. Oligosaccharides were found to be more promising although dietary carbohydrates such as fibers as a candidate prebiotics (25).

Table 1 Characteristics of exopolymers from the studied enthomopathogenic fungi

Fungi	Molecular weight at peak (Mp, kDa)	Optimized medium*	Production in 5 L fermentor ($g.L^{-1}$)
A. samoensis BCC 2466	4.34	fructose 40 $g•L^{-1}$, malt extract 5 $g•L^{-1}$, peptone 7.5 $g•L^{-1}$, yeast extract 5 $g•L^{-1}$, $NH_4H_2PO_4$ 5 $g•L^{-1}$	7.3
G. pulchra BCC 2711	4.06	Fructose 60 $g•L^{-1}$, malt extract 10 $g•L^{-1}$, peptone 15 $g•L^{-1}$, $NH_4H_2PO_4$ 10 $g•L^{-1}$, yeast extract 10 $g•L^{-1}$	5.5
O. nipponica BCC 2092	3.39	mannose 60 $g•L^{-1}$, corn steep solid 10 $g•L^{-1}$, $NH_4H_2PO_4$ 10 $g•L^{-1}$	6.2

*Basal medium=0.5 $g•L^{-1}$ KH_2PO_4, 0.2 $g•L^{-1}$ K_2HPO_4, 0.2 $g•L^{-1}$ $MgSO_4•7H_2O$, 0.14 $g•L^{-1}$ $MnSO_4•H_2O$, and 1 $mL•L^{-1}$ vitamin solution

3.3 Nondigestibility

Table 2 shows non-digestibility of all exobiopolymer against gastric juice and α-amylase digestion and the results showed that different polymers have different degrees in non-digestibility. This non-digestibility property is one of the criteria of prebiotic property requirements. In vitro non-digestibility showed that all exobiopolymers possessed high-resistance to gastric juice and enzyme hydrolyses. Exobiopolymer of *O. nipponica* BCC 2092 and *G. pulchra* BCC 2711 had almost complete acid resistance (99.0%), which is higher than that of exobiopolymer of *A. samoensis* BCC 2466 (97% resistance). In contrast, exobiopolymer of *A. samoensis* BCC 2466 is superior resistance to enzyme hydrolysis (99.5%), compared with the other two exobiopolymers. This result showed high potential of these three exobiopolymers to be used as prebiotics.

Table 2 Hydrolysis resistance of fungal exopolymers at 37 °C for 6 h.

Exopolymers from	Acid hydrolysis resistance (%)	Enzyme hydrolysis resistance (%)
A. samoensis BCC 2466	97.0	99.5
G. pulchra BCC 2711	99.0	97.2
O. nipponica BCC 2092	99.0	95.6

3.4 In Vitro Test of Exobiopolymer as Prebiotic

Another criterion of a good prebiotic is capable to promote the growth of probiotics in GI tract, which was studied in vitro for their growth stimulating on two model probiotics (*L. acidophilus* BCC 13839 and *B. animalis* ATCC 25527) compared to glucose and inulin (a commercial prebiotic). The highest growth of *L. acidophilus* BCC 13839 was observed on glucose after 10 h of cultivation with higher significant value of cell turbidity (Figure 1) whereas inulin supported the growth after glucose, which turbidity at 600 nm increased after 10 h of cultivation. Three different exobiopolymers including inulin showed not much difference in cell turbidity. Among the studied exobiopolymers, the highest cell turbidity was obtained on exobiopolymer of *G. pulchra* BCC 2711, followed by that of *A. samoensis* BCC 2466. From Figure 2, the highest cell turbidity of *B. animalis* ATCC 25527 was obtained on the exobiopolymer of *A. samoensis* BCC 2466 after 24 h of cultivation, which is very close to that of *O. nipponica* BCC 2092. The constant cell turbidity of the exobiopolymer of *O. nipponica* BCC 2092 after 18 h of cultivation was observed. Interestingly, the lowest turbidity of *B. animalis* ATCC 25527 was obtained on glucose, which is opposite to the result of *L. acidophilus* BCC 13839. The turbidity of *B. animalis* ATCC 25527 cultivated on inulin was higher than that of glucose and the highest turbidity was obtained after 6 h of cultivation.

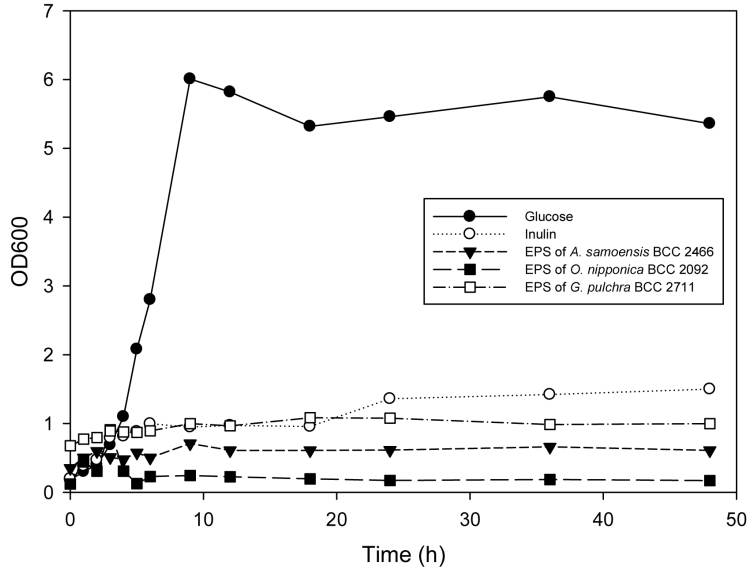

Figure 1 Growth of *L. acidophilus* BCC 13839 after cultivation on different enthomopathogenic fungal exobiopolymers compared with glucose and inulin.

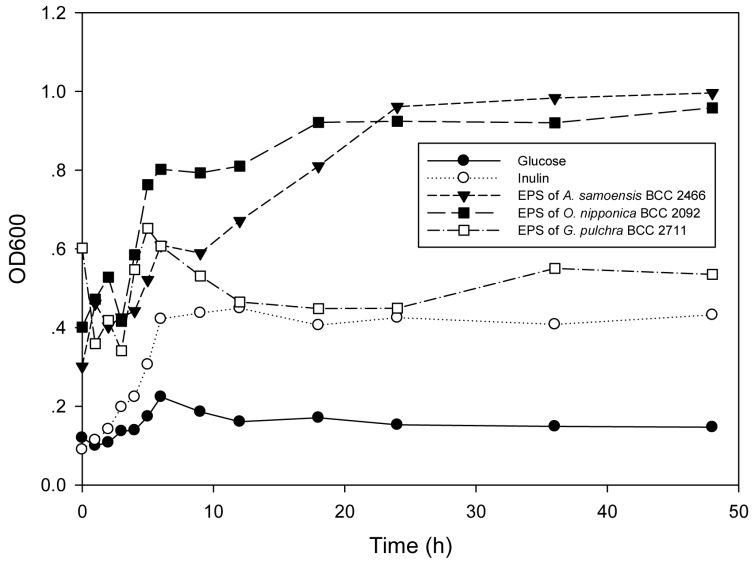

Figure 2 Growth of *B. animalis* ATCC 25527 after cultivation on different enthomopathogenic fungal exobiopolymers compared with glucose and inulin.

The highest viability of *L. acidophilus* BCC 13839 was obtained on the exobiopolymer of *A. samoensis* BCC 2466 (Figure 3) while the viability of *B. animalis* ATCC 25527 on all studied carbon sources were constant after 12 h of cultivation at the similar growth rate (Figure 4). The highest lactic acid production of *L. acidophilus* BCC 13839 was observed on glucose and decreased after 10 h (Figure 5). Lactic acid production on the exobiopolymer of *G. pulchra* BCC 2711 decreased after 24 h of cultivation. There was no lactic acid production on the exobiopolymer of *O. nipponica* BCC 2092. The lowest lactic acid production was detected on the exobiopolymer of *A. samoensis* BCC 2466. It is worth noting that there was no lactic acid production on all exobiopolymers after their co-cultivation with *B. animalis* ATCC 25527. 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 (26).

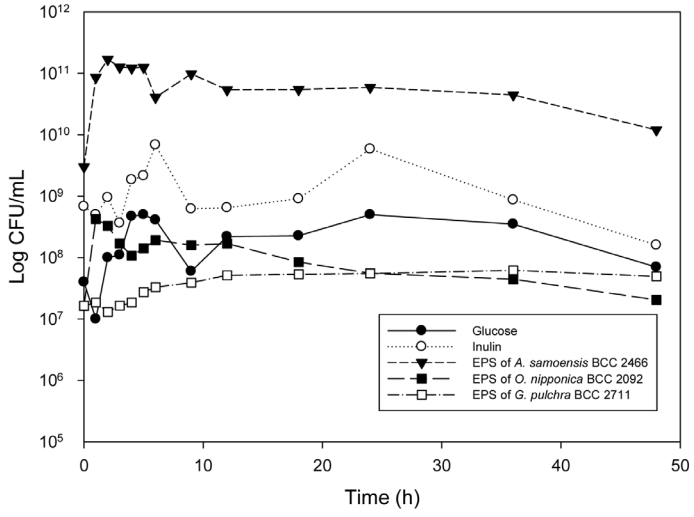

Figure 3 Viability counts of *L. acidophilus* BCC 13839 after cultivation on different enthomopathogenic fungal exobiopolymers compared with glucose and inulin.

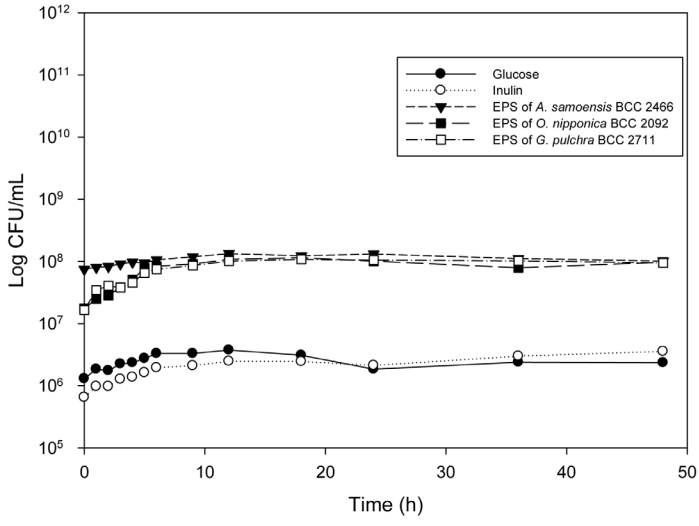

Figure 4 Viability counts of *B. animalis* ATCC 25527 after cultivation on different enthomopathogenic fungal exobiopolymers compared with glucose and inulin.

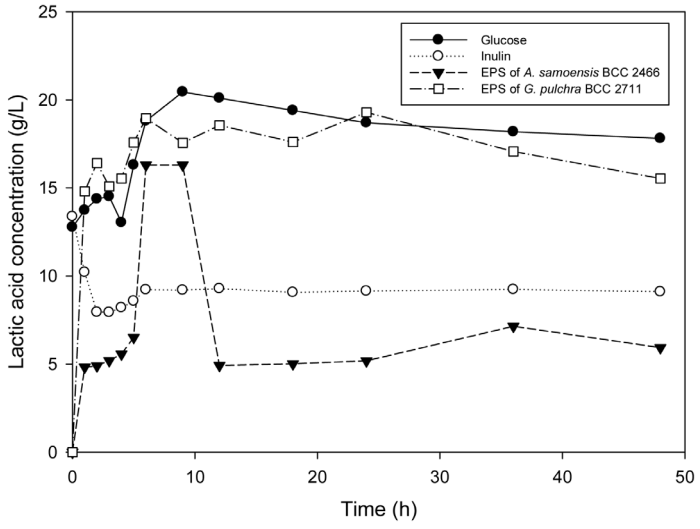

Figure 5 Lactic acid production of *L. acidophilus* BCC 13839 after cultivation on different enthomopathogenic fungal exobiopolymers compared with glucose and inulin.

Many reports show the potential use of prebiotic in food and feed industries (1, 7, 9, 14, 19, 20, 27). This study shows promising prebiotic properties of exopolysaccharides produced by different enthomopathogenic fungi in vitro. Such properties including non-digestibility against gastric juice and α-amylase hydrolyses together with growth stimulating of two probiotics show well characteristic of exobiopolymer as good prebiotics towards their criteria. This characteristic must be confirmed in vivo to prove their prebiotic potential and their feasibility study at industrial scale must be further studied.

4. CONCLUSIONS

In vitro studies of prebiotic of three enthomopathogenic fungi for their resistance to hydrolysis in gastrointestinal system together with their probiotic growth stimulating show high potential use of these exobiopolymers in food and feed industries. With fermentation technology, it will enable high production of these three exobiopolymers with lower cost of production and this will be attractive to food/feed industries after their in-vivo test study.

5. ACKNOWLEDGEMENT

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