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Production, purification and fecal fermentation of fructooligosaccharide by FTase from Jerusalem artichoke

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Abstract

Fructooligosaccharides (FOS) has been used as prebiotic that serves as a substrate for microflora in the large intestine. FOS are produced by fructosyltransferase (FTase) derived from some plants such as Jerusalem artichoke, chicory, asparagus, banana, dragon fruit and onion. It was found that Jerusalem artichoke cultured in tropical region for 3-5 months showed good source of FTase. It had the highest crude enzyme activity of 0.253±0.003 U/ml. Optimal conditions for purification of FTase by chromatography techniques with anion exchangers showed the highest specific activity which increased from 1.411 to 2.240 U/ml. Optimum conditions for production of FOS were 20% sucrose, reaction time of 96 h and 1 U/ml FTase. It was found that highest FOS (35%) consisted of 27.5% 1-kestose (DP 2) and 7.5% nystose (DP 3). Fructooligosaccharide was further purified by yeast fermentation using 2.5% *Saccharomyces cerevisiae* TISTR5019 for 36 h. It could decrease sucrose from 46.1% to 28.7%. The chemical composition of partially purified freeze-dried FOS was analyzed by HPLC. It consisted of 35 % FOS, 17.4% sucrose, 7.6% glucose and 40% fructose. The prebiotic property of the partially purified FOS was evaluated by cultivation of human fecal microflora in batch culture. It was found that the FOS had prebiotic property with PI value of 2.33. There was also stimulated growth of *Lactobacillus* and *Bifidobacterium*. It also produced lactic acid, acetic acid, propionic acid and butyric acid at concentration of 1287.92, 20.97, 10.34 and 18.67 µg/ml, respectively and vitamins B1 and folic acid were produced at concentration of 19.19 and 20.00 µg/ml, respectively.

Introduction

Fructooligosaccharides (FOS) are among the most extensively studied prebiotics and include a diverse family of fructose polymers which vary in length and can either be derivatives of simple fructose polymers or fructose moieties attached to a sucrose molecule (Wichienchot and Chinachoti, 2011). Fructooligosaccharides consist mainly of 1-kestose, nystose, and 1F-fructofuranosyl nystose which have 1~3 units of fructose. FOS are one in non-digestible and fermentable group that are the most popular compounds which gained specific interest because it had been reported to possess significant prebiotic properties such as galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), gentio-oligosaccharides, gluco-oligosaccharides, isomalto-oligosaccharides (IMO), mannan-oligosaccharides, chito-oligosaccharides (COS), melibiose, pectic oligosaccharides (POS), and xylo-oligosaccharides (XOS) (Gibson *et al*., 2004; Roberfroid, 2007; Fastinger *et al*., 2008; Calame *et al.*, 2008). FOS can be found in large quantity in natural food, such as asparagus, banana, garlic, onion, wheat and dragon fruit. In FOS production process, enzymes responsible for catalyzing this reaction is extracted from microbial source, but in this present time an enzyme for FOS production is extracted from plants such as Jerusalem artichoke tubers. Jerusalem artichoke is a famous plants that can produce high FOS and they grow well in tropic regions. Jerusalem artichoke is classified in the genus *Helianthus* in the family Asteraceae (Aster or Daisy family), in the order Asterales. Species in the genus *Helianthus* are native to North America, but have very different distributions, varying from restricted (e.g., *H. argophyllus* and *H. ludens*) to widespread (e.g., *H. annuus* and *H. tuberosus*). Jerusalem artichoke tubers contain little or no starch, virtually no fat, and have a relatively low calorific value. The small amount of fat present, trace amounts of monounsaturated and polyunsaturated fatty acids have been reported, but

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no saturated fatty acids (Whitney and Rolfes, 1999). The polyunsaturated fatty acids linoleic (18:2 cis, cis n-6) and α-linoleic acid (18:3 n-3) have been recorded as present at 24 mg and 36 mg·100 g^{-1} of raw tuber, respectively (Fineli, 2004). The tubers are a good source of dietary fiber, beca use of the presence of fructooligosaccharides and inulin. The inulin content of tubers ranges from 8 and 21% of fresh weight (van Loo *et al.*, 1995) and fructooligosaccharides content between 5-6 % of fresh weight. The total carbohydrate about 16.7 g, dietary fiber 0.6 g per 100g fresh weight that was calculated by difference (minus protein, fat, water, and ash). FOS and scFOS are resistant to digestion in the stomach and small intestine (Ellegard *et al*., 1997) and thus are able to reach the colon. In the human colon where they are selectively fermented by beneficial bacteria, the microbe that ferment carbohydrate in the human colon (Bouhnik *et al*., 1999; Le Blay *et al.*, 1999) has diverse microbial ecology which is an important food source for the microbes. As the diet is passed from mouth to the small intestine and large intestine, respectively, the waste food remain about 200 grams with around 60% of its dry weight as microbes. The cecum has low pH which is very good for feeding and enhances microbial growth, the colon is the longest part of large intestine and its pH is neutral (Macfarlane *et al*., 1998; Egert *et al*., 2006). Subsequently there is a growth spurt among the populations of bifidus bacteria (such as bifidobacteria and lactic acid bacteria) that eventually outgrow the pathogenic and/ or the putrefactive bacteria (such as colon bacilli) (Gibson and Roberfroid, 1995). Therefore there is a decrease in toxic fermentation products. FOS also aid in lowering the cholesterol and blood sugar levels, and enhance absorption of mineral elements.

Materials and Methods

Chemicals and microorganism

Reagents for purification of enzyme, production of fructooligosaccharides, analysis of oligosaccharides and sugar fermentation were purchased from Sigma-Aldrich Co. Ltd. Reagents for studying in artificial colon system for batch culture and evaluation of prebiotic index were purchased from Sigma-Aldrich Co. Ltd and Panreac AppliChem Co Ltd. The microbes from fresh human feces was obtained from a healthy donor who has not received antibiotics, food supplement and other health food such as dairy mix probiotic or prebiotic in the last 3 months.

Preparation of plant materials

Jerusalem artichokes were obtained from Phetchaboon research station, hosted by Kasetsart University, Thailand. Jerusalem artichoke was grown in nursery tree bag and watered every 2 days. After a month, the plant was transferred to a tree pot. Then the tubers were harvested at 3.5, 4, 5 and 6 months and stored at -20°C until use. The relationship between tuberization stage of Jerusalem artichoke and fructosyltransferase activity (FTase) were investigated during 3.5-6 months.

Extraction of crude enzyme

Ten gram of the tubers was homogenized in 10 ml of 0.007 M potassium phosphate buffer (pH 7.0) containing 0.5% Triton X-100 and 0.5% cysteine and stored for 16 h at 4°C. The homogenate was filtered through a cheese cloth and centrifuged (12,000 x g, 20 min, 4°C) to remove insoluble matter.

Determination of protein content

Protein content of the sample was determined using Bradford method (Bradford, 1976). Solution was prepared at dilution of 1:4 (dye: DI water) and solution was filtered with filter paper then stored in a amble bottle before use. The calibration curve was prepared by pipette at concentrations of 0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml BSA. Distilled water was used for blank. Pipette 200 µl of Bradford dye to each microtubes and mixed well by inversion or gentle vortex mixing. Avoid foaming, which will lead to poor reproducibility. Absorbance of the sample was measured at 595 nm and a graph of absorbance versus protein content was plotted.

Determination of fructosyltransferase activity

Pipette 100 µl of clear supernatant solution to make reaction with 0.1 M potassium phosphate buffer (pH 5.4) in presence of 0.575 M sucrose and incubated for 24 h at 34°C. The reaction was terminated by heating to 95°C, 2 min. Sample was stored at -20°C before it was analyzed with Somogyi–Nelson and GOD-POD method. Mixed Nelson reagent and diluted crude enzyme sample or distilled water for blank in microtubes. Microtube was inserted in boiling water for 10 min. Addition of 100 µl of arsenomolybdate reagent and allowed to stand for at least 5 min at room temperature. Samples and standards were transferred from microtubes to microplate and measure absorbance at 500 nm. Protein determination by glucose oxidase-peroxidase (GOD-POD) method was used. Pipette 20 µl standards glucose (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mg/ml), diluted crude enzyme samples or distilled water (for blank) were transferred to microplate and added 200 µl GOD-POD solution then kept in the dark for 40

min. Absorbance of samples were measured at 596 nm.

Purification of FTase enzyme

Crude enzyme sample was loaded though Q sepharose fast flow column which was equilibrated by buffer A (50 mM potassium phosphate buffer, pH 6.5) with flow rate of 2 ml/min. Protein was eluted protein by buffer B (50 mM potassium phosphate buffer + 0.6 M NaCl, pH 6.5) and buffer C (50 mM potassium phosphate buffer + 0.6 M NaCl, pH 6.5). Fractions that had high activity were poolled, salt was removed and the protein was concentrated by ultrafiltration (MWCO 10 kDa). After that, the protein content and enzyme activity were determined.

Production of fructooligosaccharides

Mixed 2 ml of purified enzyme with 0.46 M sucrose solution in 0.1 M potassium phosphate buffer pH 5.4 (8 ml). Incubation was carried out for 24 h at 35°C and reaction was terminated at 95°C for 2 min. Oligosaccharides and sugars were analyzed by HPLC.

Analysis of oligosaccharides and sugars

Sample was diluted to obtain optimal concentration before filtered through 0.22 µm syringe filter. Filtered sample was injected (5 µl) into the Rezek RNM carbohydrate column (diameter 7.8 x 300 mm) and flow rate of mobile phase (HPLC water) was 0.4 ml/min, 45°C using RI detector.

Preparation of fecal slurry

The inoculum represents the intestinal microorganisms using feces from healthy volunteers without gastrointestinal tract disease and that have not taken antibiotics in the last 3 months. Preparing feces at 10% for use as an inoculum in fecal fermentation (batch culture). The fresh stool was weighed and diluted with phosphate buffered saline (0.1 M, pH 7) and mixed using stomacherfor 2 min.

Batch fecal fermentation

Sterilize basal media (100 ml) was poured into a glass vessel, pH and temperature was controlled by pH controller and circulating water at 6.8 and 37°C, respectively. Anaerobic condition was maintained by flushing with nitrogen gas. Two gram of sample (FOS in this study, commercial FOS or mixed sugars) was added, and fermentation was allowed for 24 hours. The samples were collected at 0, 3, 9, 12 and 24 hours.

Microbial colony counting with fluorescent in situ *hybridization (FISH) technique*

The sample (375 µl) was added by 1,125 µl of 4% (w/v) paraformaldehyde solution (pH 7.2), mixed and kept at 4°C overnight to fix bacterial cells. Then the cell was washed with PBS 2 times and thawed back with 150 µl of PBS and added with 150 µl of cool ethanol 96% (v/v), the mixture was tored at 20°C for at least 1 hour or until use, but should not be stored over 3 months. The fixed cell (20 µl) at optimal dilution was spreaded on the whole slide with coating TEFLON/Poly-L-lysine. The slide was placed on slide warmer at 45°C for 10-12 minutes until the sample dried. The slide was dipped in ethanol concentration at 50, 80 and 96% (v/v).

Pre-warmed hybridization buffer at the specific temperature for hybridization of each probe. Bacteroides (Bac303) had sequence CCAATGTGGGGGACCTT and optimum hybridization temperature at 48°C; Bifidobacteria (Bif164) had sequence CATCCGGCATTACCACCC; Lactobacillus (Lab158) had sequence GGTATTAGCA(T/C)CTGTTTCCA; Clostridia (Chis150) had sequence TATGCGGTATTAATCT(C/T) CCTTT; Eubacteria (Eub 338) had sequence GCTGCCTCCCGTAGGAGT (Rycroft *et al*., 2001) Sample (45 µl) was mixed with a specific DNA probe (5 µl). The Bif 164 probe was used for analyses of *Bifidobacterium*, *Bacteroides* was Bac 303, *Lactobacillus/Enterococcus* was Lab 158, *Clostridium* was Chis 150, Eubacteria or total bacteria was Eub 338 (Rycroft *et al*., 2001). The samples were dipped onto the slide and incubated in hybridization oven with optimum temperature of each probe for 4 hours. The slide was washed with 50 ml washing buffer in the optimum temperature of each probe (Bac303 at 48°C; Bif164 at 50°C; Lab158 at 50°C; Chis150 at 50°C and Eub338 at 48°C) for 15 minutes. Then the slide was washed with 50 ml of distilled water, the slide was dried and 5 µl of antifade agent was added. The slide was covered with slide lid and the bacterial number was counted by fluorescence microscope, 15 fields per hole. Prebiotic index (PI) was calculated by the following equation (Palframan *et al*., 2003).

$$\text{Prebiotic index (PI)} = \alpha + \beta - \gamma - \delta$$

$\alpha = (Bif_{24}/Bif_0)/Total$, $\beta = (Lac_{24}/Lac_0)/Total$, $\gamma = (Bac_{24}/Bac_0)/Total$, $\delta = (Clos_{24}/Clos_0)/Total$, $Total = Eub_{24}/Eub_0$

Eub_0, Eub_{24} is the cell number of Eubacteria or total bacterial count at time 0 hour and after 24 hours

Bif_0, Bif_{24} is the cell number of *Bifidobacterium*

Table 1. Purification steps of fructosyltransferase (FTase) from Jerusalem artichoke tuber cultivated for 3.5 months

Purification steps	Activity (U/ml)	Total activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/ml)	Purification (fold)	Yield (%)
Crude enzyme extract	1.905	38.105	1.857	37.138	1.025	1.000	100.000
Q sepharose + ultrafiltration	2.455	9.821	0.455	1.821	5.393	5.257	25.773

at start time and after 24 hours

Lac_0, Lac_{24} is the quantity of *Lactobacillus* at time hour and after 24 hours

Bac_0, Bac_{24} is the cell number of Bacteroides at time 0 hour and after 24 hours

$Clos_0$, $Clos_{24}$ is cell number of *Clostridium* at time 0 hour and after 24 hours

Analysis of short chain fatty acid (SCFA)

The sample from batch culture was centrifuged at 13,680×g for 10 minutes to obtain supernatant. Then the sample was filtered through 0.22 µm nylon filter. Short chain fatty acids (acetic, propionic, butyric acid) and lactic acid were analyzed by High Performance Liquid Chromatography (HPLC). HPLC colum was BIO-RAD Aminex HPX-87 H ion exclusion with diameter 7.8 mm, length 300 mm. Mobile phase was 0.005 M H_2SO_4, flow rate of 0.6 ml/min, temperature at 50°C and UV detector at 215nm was used (Judlyn *et al*., 1999).

Analysis of vitamin B1, B2 and folic acid

The sample from batch culture was centrifuge at 13,680 ×g for 10 minutes to obtain supernatant. Then, the sample was filtered through 0.22 µm nylon. Vitamin was analyzed by HPLC equipped with Inertsil Diol (5 µm resin) column with diameter 4.6 mm, length 250 mm. Mobile phase was using acetonenitrile (CH_3CN), water and trifuroacetic acid (TFA) as mobile phase with ratio as CH_3CN: H_2O: TFA at 90: 10: 0.1. Flow rate of mobile phase was 1.0 ml /min, 40°C with the UV detector at 254 nm (Xueyan, and Denis, 2006).

Results and Discussion

Cultivation time and enzyme activity

The relationship between tuberization stage of Jerusalem artichoke and fructosyltransferase activity were investigated during 3.5-6 months cultivation period. Jerusalem artichoke had no tuber from the 1-2 months of cultivation, and after 2-3 months the stem was initiated till 3.5-4 months, from 3.5-4months tuber and flowers developed and were harvested. After 5-6 months they withered. In this experiment we studied the relationship between tuberization stage of Jerusalem artichoke and FTase activity. Result of the enzyme activity in 3.5, 4, 5, and 6 months of cultivation were 0.103, 0.098, 0.069 and 0.033 U/ml, respectively. 3.5 month of cultivation had the highest enzyme activity. However, there was no activity before 3.5 months due to absence of tuber.

Isolation of crude enzyme by anion exchange chromatography

In 3.5 months, tubers extract had highest enzyme activity obtained by anion exchange chromatography that had specific activity at 1.025 U/ml. After purification of fructosyltransferase by anion exchange chromatography that passed through Q sepharose column and concentrated with ultrafiltration. This method obtained higher enzyme activity 5.393 U/ml (Table 1). But the total protein in the enzyme that passed through Q sepharose column decreased when compared to the crude enzyme. It revealed that purified enzyme have higher specific activity which is similar to the report of Koops and Jonker (1996) (0.27 U/ml specific activity).

Production of fructooligosaccharides

During the production of fructooligosaccharide, FTase reacted with sucrose in potassium phosphate buffer (pH 5.4) and final concentration of sucrose at 0.46 M (157.32 mg/ml), enzyme concentration at 0.1, 0.2, 0.4 and 0.6 U/ml and the mixture was incubated at 35°C (Figure1). FTase reacted with sucrose in potassium phosphate buffer (pH 5.4) and final concentration of sucrose at 0.46 M (157.32 mg/ml), enzyme concentration at 0.6 U/ml the mixture was incubated at 35°C for 24h. Chemical compositions of

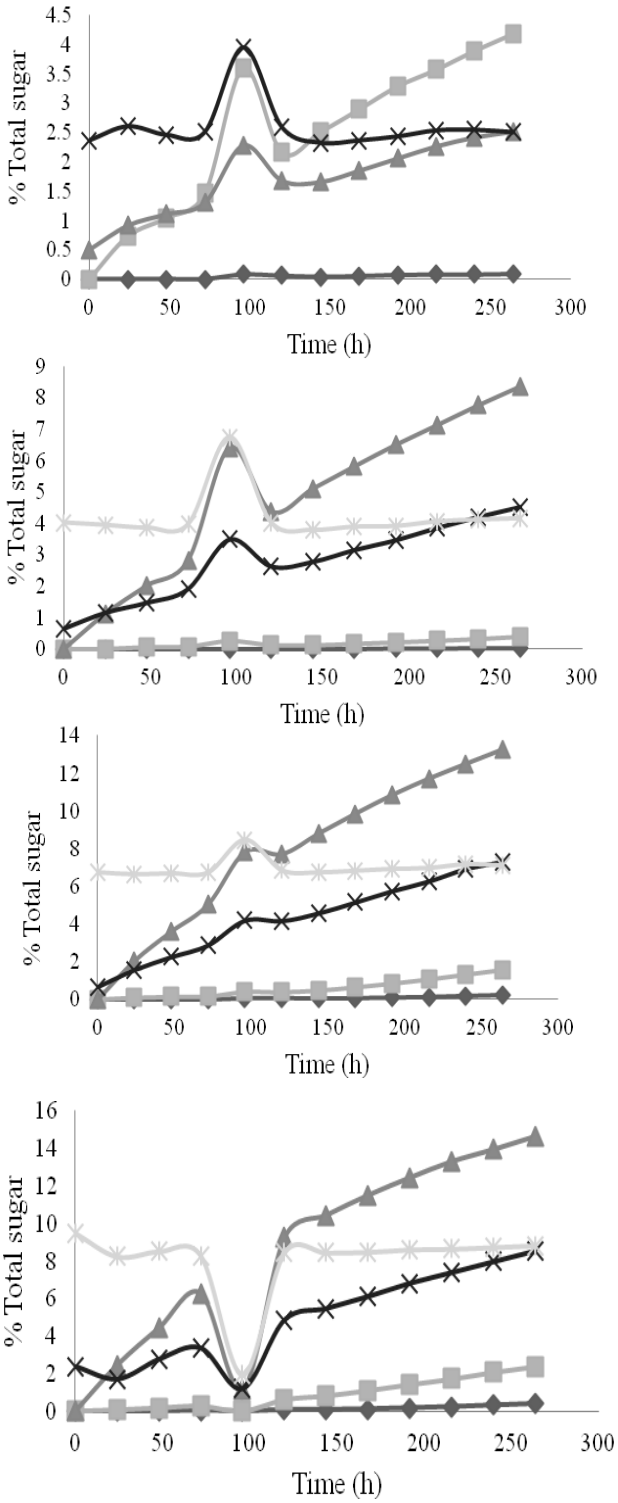

Figure 1. Fructooligosaccharide production with 0.46 M sucrose in potassium phosphate buffer (pH 5.4) at various FTase concentrations; 0.1 U/ml (a), 0.2 U/ml (b), 0.4 U/ml (c), 0.6 U/ml (d); ◆fructofuranosylnystose, ■ nystose, □ 1-kestose,✕ glucose, *fructose

FOS was analyzed by HPLC. It consisted of 13.95% 1-kestose (DP2), 2.10% nystose (DP3) and 0.32% fructofuranosylnystose (DP4). The maximum yield of FOS synthesized by FTase was 16.37 %. The action of FTase on FOS synthesis from sucrose was

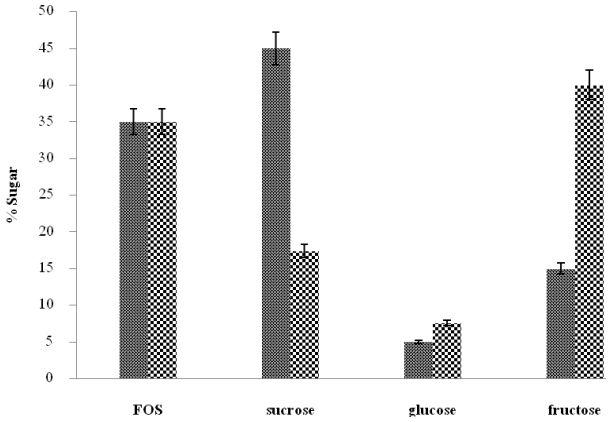

Figure 2. Purification of the fructooligosacchaide by fermentation with *Saccharomyces cerevisiae* TISTR5019; (▩) 0 h and (▩) 36 h

proposed. Sucrose was used as substrate because it was found in high concentration at 0 h and FOS synthesis at the beginning. The enzyme acted on sucrose and hydrolyzed it to glucose and fructose moieties, the fructose moiety was attached to another fructose molecules. Glucose, fructose and FOS were found in the reaction. the reaction was carried out for 24 h. FOS produced after 24h consisted of 13.95% 1-kestose, 2.10% nystose and 0.32% fructofuranosylnystose. To obtained higher purity of FOS, removal of glucose and fructose residues should be further carried out. Commercial production of FOS is preferred using β-fructofuranosidase (FFase) from bacteria *(Aureobasidum pullulans)* and fungi *(Aspergillus niger)* with about 60% yield (Yoshikawa *et al*., 2008). However, the main component of FOS from different sources are difference in term of degree of polymerization (DP). Interestingly, the scFOS in this study has mixed DP with very high in DP5. So that the scFOS produced in this study confirmed to have different in prebiotic property.

Purification of FOS by yeast fermentation

The fructooligosaccharide produced was further purified by yeast *Saccharomyces cerevisiae* TISTR5019. The chemical compositions of the mixture were 32.9% FOS, 46.1% sucrose, 4.7% glucose and 15.3% fructose. The mixture was incubated at room temperature for 36 hours. The chemical composition of the product after yeast fermentation was 35% FOS, 17.4% sucrose, 7.6% glucose and 40% fructose (Figure 2). It could be explained that the sucrose was decreased by 28.7%, whereas glucose and fructose were slightly increased. *S. cerevisiae* could convert sucrose into glucose and fructose. Finally, the purified FOS was freeze dried and analyzed by HPLC. Freeze drying could increase purity of FOS from 16.37% to 35%.

Table 2. Prebiotic index of the FOS produced, commercial FOS and mixed sugars

Sample	Composition (%)				Prebiotic index (PI)
	FOS	Sucrose	Glucose	Fructose	
FOS (in this study)	35.01	17.36	7.61	40.02	2.33
Commercial FOS	93.68	3.65	2.42	0.24	1.64
Sucrose + Glucose + Fructose	0	17.36	7.61	40.02	-7.3

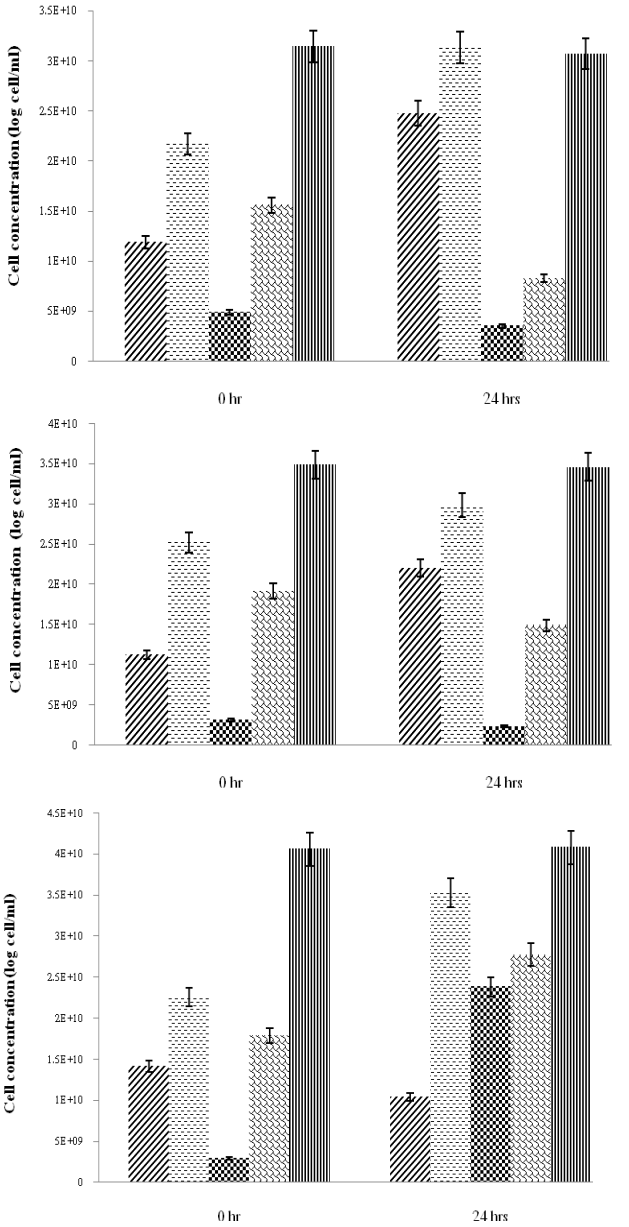

Figure 3. Cell concentration in fecal batch culture using the FOS produced (a), commercial FOS (b), mixed sugar (c) at 0 and 24 hours; (▨) *Lactobacillus*, (▤) Bifidobacteria, (▩) Bacteroides, (▧) Clostridia and (▥) Eubacteria.
Different letters were statistically significant ($p<0.05$)

Evaluation of prebiotic properties in batch system

Fermentation of FOS in batch system for evaluation of prebiotic index in fecal batch culture was carried out by using produced FOS that had content 35% of total sugar, commercial FOS and mixed sugar (17.36% sucrose, 7.6% glucose and 40.02% fructose) as carbon source in batch culture. The result showed that, produced FOS and commercial FOS increased Lactobacilli, Bifidobacteria and decreased Clostridia groups significantly ($P<0.05$). Mixed sugars increased Bifidobacteria and Clostridia but decreased Lactobacilli significantly ($p<0.05$) (Figure 3). Prebiotic index could be calculated from formula by using number of bacterial count with fluorescent in situ hybridization (FISH) technique. The microbial groups counted were Lactobacilli, Bifidobacteria, Clostridia, Bacteroides and Eubacteria. The result showed positive value for both produced FOS and commercial FOS. The produced FOS had prebiotic index of 2.33 and commercial FOS had prebiotic index of 1.64 but mixed sugar showed negative value of -7.3, this showed that mixed sugar had no prebiotic property (Table 2).

Analysis of short chain fatty acids

Short chain fatty acids were produced from fermentation process in fecal batch culture. It was found that produced FOS promoted the production of lactic acid and acetic acid. Propionic acid was produced during first period of fermentation and decreased in later fermentation time. Butyric acid decreased in 24 h. Concentrations of lactic acid, acetic acid, propionic acid and butyric acid at 24 h were 1287.92, 20.97, 10.34 and 18.67 µg/ml, respectively. Commercial FOS showed promotion in production of lactic acid and acetic acid of 1,156.47 and 22.48 µg/ml, respectively at 24 h. Mixed sugar could produce acetic acid but lactic acid, propionic acid and butyric acid were decreased at 12 h. Lactic acid and acetic acid concentration were 12.00 and 13.16 µg/ml, respectively at 24 h fermentation.

Analysis of vitamins

It was found that fecal fermentation of FOS produced in this study could produce vitamin B1 and folic acid of 19.19 and 20.00 µg/ml, respectively. Meanwhile commercial FOS and mixed sugar had no effect on vitamin production in fecal batch culture.

Conclusions

The highest activity of FTase in Jerusalem artichoke tubers was found at 3.5 months of cultivation. Optimal conditions to obtain high enzyme activity were temperature and pH of 35°C and 5.4, respectively. Metal ions and reagents such as pyridoxal-HCl, $MnCl_2$ and KNO_3 increased enzyme activity but $MgCl_2$, $CuCl_2$, KI, LiCl, NaCl, $CaCl_2$ and KCl inhibited enzyme activity. The K_m values of FTase using sucrose as substrate was 0.31 M and V_{max} was 1.14 µM/sec. Optimal conditions for production of FOS were 0.60 U/ml FTase and 25% sucrose at 35°C for 24 h. Chemical compositions of FOS were analyzed by HPLC. It consists of 13.95% 1-kestose, 2.10% nystose and 0.32% fructofuranosylnystose. The maximum yield of FOS synthesized by FTase was 16.37% before purification and it was increased to 35% after purification by yeast fermentation. The prebiotic property of the obtained FOS was evaluated by cultivation of human fecal microflora in batch culture. It was found that produced FOS had prebiotic property with PI value of 2.33. There was also SCFA produced particularly lactic acid and acetic acid. The vitamins produced are vitamin B1, B2 and folic acid.

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