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Research article

Optimization of oligofructans production from sugarcane juice fermentation using *Bacillus subtilis* TISTR001

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Abstract

Oligofructans are healthy prebiotic compounds linked by β-glycosidic fructose. They have received increasing attention due to their positive health effect. The objective of this research was the optimization of oligofructans fermentation conditions from sugarcane juice using *Bacillus subtilis* TISTR001 that produced fructose polymer enzyme as levansucrase. The cane juice was adjusted to pH 6.8 and supplemented with meat extract (3 g/L), peptone (5 g/L), $(NH_4)_2SO_4$ (2 g/L), and $MgSO_4.7H_2O$ (0.6 g/L). It was found that the oligofructans increased in proportion with the sugarcane juice concentration. The maximum juice concentration was 20 brix, with the highest content of oligofructans in the form of free fructose being 3.79% (w/v) with 0.29 g/g reducing sugar production yield and 0.045 g/L/h productivity. Additionally, 84 hr of fermentation at 30°C were the optimum conditions to produce the highest oligofructans content, which was confirmed by the complete use of the sugars content, sucrose and free fructose.

Introduction

Oligofructans are carbohydrates in the form of fructo-oligosaccharides (FOS) and fructose polymers or fructans, such as inulin or levan, linked by fructose under β-2,1 or β-2,6, respectively (Santos et al., 2013; Bersaneti et al., 2018). The structure of fructo-oligosaccharides is linked with a long chain of fructose with degree of polymerization (DP) from 3 to 10 molecules having a glucose molecule with an α-glycosidic link at the end of the molecular structure (da Silva et al., 2014; Bersaneti et al., 2018). Short chain oligofructans include kestose (DP3) and nystose (DP4) (Flores-Maltos et al., 2016) and long chains of oligofructans (FOS) are used as a sweetener with low calories (1–1.5 kcal/g), having approximately 30% the sweetness of table sugar (Roberfroid, 2000). Furthermore, oligofructans are fiber-like carbohydrate compounds which the human system is unable to enzymatically process in the digestive tract due to the β-linkages of oligofructans. Another characteristic is that the soluble fiber that can be dissolved in water without affecting its overall viscosity, so that although the human body is unable to digest it, it can be dissolved and digested by bacteria in large intestine without requiring oxygen; consequently, it is referred to as prebiotic (Bersaneti et al., 2018; Ninchan and Noidee, 2021). Prebiotic bacteria, especially *Bifidobacterium sp.*, can inhibit the growth of pathogenic bacteria, such as *Clostridium sp.*, *E. coli* and

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Samonella sp.; this is referred to as a bifidogenic or prebiotic effect (Gibson, 1999; Meyer and Stasse-Wolthuis, 2009; Veereman-Wauters et al., 2011). Furthermore, prebiotics are capable of reducing triglyceride and cholesterol, known as the hypocholesterolemic effect (Yamamoto et al., 1999; Santos et al., 2013; Erejuwa et al., 2014; Raveschot et al., 2020) that can have a positive immunostimulatory effect on the body (Niv et al., 2012; Peshev and Van den Ende, 2014), especially for patients with non-communicable diseases (Rolim, 2015). Fructan polymers play a major role in determining the physical characteristics of food products, such as increasing the physical stability of drinking yogurt (Thammarutwasik et al., 2009; Ua-Arak et al., 2017; Witczak et al. 2020) and its low viscosity and good water solubility make it useful as an emulsifier (Kucukasik et al., 2011). Such beneficial effects make it useful for applications in various forms and industries.

Bacillus subtilis can grow in various kinds of substrate, with or without oxygen (Olmos and Paniagua-Michel, 2014). It is regarded as a generally recognized as safe (GRAS) bacterium that has no harmful effects on its host and so it is used to produce important and useful substances, such as antibiotics, antigens, enzymes and several kinds of protein (Olmos and Paniagua-Michel, 2014). Several reports have been published regarding medical applications for humans and livestock, as well as in agricultural industries. Furthermore, *B. subtilis* produces the fructosyltransferase enzyme (levansucrase) which is the primary enzyme for making fructo-oligosaccharides and fructans (Santos et al., 2013; Raga-Carbajal et al., 2016; Ninchan and Noidee, 2021). Levansucrase-producing bacteria include: *B. agaradhaerens* (Kralj et al., 2018), *B. licheniformis, B. megaterium, Acetobacter xylinum, Gluconacetobacter diazotrophicus, Lactobacillus reuteri, Leuconostoc mesenteroides, Rahnella aquatilis, Zymomonas mobilis* (Sangmanee et al., 2016) and *Erwiniaamyl ovara, Halomonas* sp. *Pseudomonas syringace, P. chlororaphis* (Goncalves et al., 2013). The fructosyltransferase enzyme can be produced capably by fungal microbes, such as *Aspergillus* sp., *Aureobasidium* sp., *Artrobacter* sp., *Fusarium* sp. and *Kluyveromyces* sp. (Khanvilkar and Arya, 2015; Sanchez-Martinez et al., 2020). FOS production reached 173.60 g/L under 0.2 vvm aeration and uncontrolled pH using *B. subtilis* natto CCT 7712 (Magri et al., 2019). da Silva et al. (2014) achieved a maximum yield of 54.86 g/L FOS using *B. subtilis* natto fermentation based on response surface methodology from 334 g/L sucrose concentration at pH 6.0 and 45.8°C. Santos et al. (2013) studied levan-production from *B. subtilis* natto and obtained the highest levan concentration of 111.6 g/L using 400 g/L sucrose in 16 hr of culture time.

The main substrate component for oligofructans production is sucrose (Goncalves et al., 2013; Ninchan and Noidee, 2021). Sugarcane juice is composed of sucrose and also contains vital nutrients, such as vitamins and minerals and trace elements that are essential for the metabolism of microorganisms as other sucrose-based substrates such as molasses and date syrup (Khassaf et al., 2019). Thus, the current research focused on the most suitable conditions for oligofructans fermentation using fresh sugarcane juice and *B. subtilis* TISTR001. This strain has not been used to study oligosaccharides production with this substrate. Different levels were investigated of pH, fermentation temperature, the concentration of sugarcane juice and amount of nutrients required for fermentation.

Materials and Methods

Preparation of B. subtilis TISTR001

B. subtilis TISTR001 was supplied by the Thailand Institute of Scientific and Technological Research (Thailand) and was incubated in nutrient broth with yeast extracts at 0.3% (weight per volume, w/v) and peptone at 0.5% (w/v; ATCC medium) at 37°C with shaking at 150 revolutions per minute (rpm; model VS-8480SFN shaking incubator; Vision Scientific Co., Ltd.) for 10 hr until reaching an optical density ($OD_{600\ nm}$) of 0.7–1.0.

Optimization of conditions for oligofructans production by B. subtilis TISTR001

Effect of pH and nutrients broth preparation

Fresh sugarcane juice was locally purchased. The juice had 15.3 brix and was filtered through muslin cloth to remove impurities. Then, 250 mL of juice was diluted to 10 brix for each experiment. The nutrients shown in Table 1 were added (Goncalves et al., 2013; Santos et al., 2013). All chemicals were analytical grade. The diluted juice was adjusted to pH 6.8 and was used in comparison with substrate without pH adjustment. After sterilization at 121°C for 15 min, inoculum of *B. subtilis* at 10% (w/v) was added into sugarcane juice samples that were prepared as shown in Table 1. Samples were fermented at 30°C and mixed using a shaker at 150 rpm (model VS-8480SFN shaking incubator; Vision Scientific Co., Ltd.). Then, samples were collected every 6 hr until 96 hr and were analyzed for the amounts of sugars (sucrose, glucose, fructose) and the activity of levansucrase enzyme and the total amount of oligofructans.

Table 1 Substrate conditions for *Bacillus subtilis* TISTR001 fermentation for oligofructans production

Experiment	Conditions of sugarcane juice as substrate	
	Nutrient	pH
1	No adding nutrients	no adjustment
2	No adding nutrients	pH 6.8
3	2 g/L $(NH_4)_2SO_4$ + 0.6 g/L $MgSO_4.7H_2O$	pH 6.8
4	2 g/L yeast extract	pH 6.8
5	2 g/L yeast extract + 3 g/L meat extract + 5 g/L peptone + 2 g/L $(NH_4)_2SO_4$ + 0.6 g/L $MgSO_4.7H_2O$	pH 6.8

Initial pH of sugarcane juice was 5.23.

Effect of sugarcane juice concentration

Four levels were studied of sugarcane juice concentration (5 brix, 10 brix, 15.3 brix, 20 brix). Concentrations at 5 brix and 10 brix were acquired by dilution. Concentration at 15.3 brix was identified as the actual concentration of the sugarcane juice. Sucrose was added to make the concentration at 20 brix. Initially, the sugarcane was screened through muslin cloth before adjusting the concentration of the sugarcane juice to the desired level. This was adjusted to the optimized conditions for sugarcane juice (based on the pH and nutrients addition) as determined from the previous experiment. The juice was sterilized at 121°C for 15 min before adding inoculum of *B. subtilis* TR001 at 10% (w/v). Fermentation commenced at 30°C with an agitation rate of 150 rpm, and samples were collected every 6 hr for 96 hr and analyzed as in the previous experiment.

Effect of fermentation temperature

Fermentation temperatures (30°C and 37°C) were studied using the optimized substrate and juice concentration as determined in previous experiments. The conditions of fermentation and analysis of the parameters were the same as in the previous experiment.

Analysis of fermentation parameters

Sugars content (sucrose, glucose, fructose) and fructo-oligosaccharide analysis using high performance liquid chromatography

The contents of sugars and short chain fructo-oligosaccharides were analyzed following Ninchan and Noidee (2021), Showa Denko K.K. (2021) and Vertical Chromatography Co., Ltd. (2021). *B. subtilis* TISTR001 was removed from fermented sugarcane juice. Then, the cell-free fermented juice was diluted to the appropriate level of concentration and filtered through a cellulose acetate membrane with a diameter of 0.45 µm. Thereafter, analysis of sugars content was performed using high performance liquid chromatography (HPLC; Shimadzu Corporation; Japan), with a refractive index detector (RID-10A; Shimadzu Corporation; Japan) and a VertiSep™ Sugar CMP column (7.8×300 mm, 8 µm; Vertical Chromatography Co., Ltd.) at 80°C, using deionized water as the mobile phase at a flow rate of 0.4 mL/min, using standard sugars of sucrose, glucose and fructose to study the change of sugar content during fermentation. The quantity analysis of short chain fructo-oligosaccharides was performed with a Shodex Asahipak NH2P-50-4E column (4.6 mm I.D. × 250 mm; Showa Denko America, Inc.) at 40°C with a mobile phase of acetone nitrite per deionized water at a ratio of 70:30 and a flow rate of 1 mL/min, using the two standard fructo-oligosaccharides: kestose (DP3) and nystose (DP4).

Total oligofructans in form of total free fructose

The total oligofructans were determined using a modified procedure from Goncalves et al. (2013) and Ninchan and Noidee (2021). First, samples (1 mL) of fermented sugarcane juice, with separated *B. subtilis,* were mixed with ethanol (2 mL) to precipitate fructo-oligosaccharide and fructans. The precipitate was centrifuge at 10,000 rpm for 10 minutes (Hettich Rotina 35R refrigerated tabletop centrifuge, UK), the supernatant was discarded, the precipitated portion was hydrolyzed with 0.1 N hydrochloric acid at 2 mL, then boiled for 30 min to break the bond between the oligosaccharide and fructan polymers into monosaccharide, before analyzing the quantity of reducing sugar using the Somogyi-Nelson method and calculating the total free fructose according to the total oligofructans.

Levansucrase enzyme activity

The enzyme activity of levansucrase was analyzed following Goncalves et al. (2013) and Ninchan and Noidee (2021). Supernatant or the liquid part from fermentation after *B. subtilis* cell separation was considered as crude enzyme of levansucrase.

The enzyme activity was analyzed by preparing 1 mL of 10 % (w/v) sucrose solution (substrate) in a pH 6.8 phosphate buffer solution and incubating at 30°C for 5 min, later adding crude levansucrase enzyme (1 mL), incubating for 30 min and then stopping the enzyme reaction by boiling in water. The quantity of glucose released from the reaction between the crude enzyme and sucrose was determined using the Somogyi-Nelson method and a standard curve of glucose to calculate the free glucose. The enzyme blank-subtracted assay was determined using distilled water instead of 10 % (w/v) sucrose solution as the reaction substrate. Subtraction of the enzyme blank could obliterate some error of determination due to the remaining glucose in the cell free culture as crude enzyme.

One unit of enzyme was defined as the amount of enzyme that produced 1 µmol/mL of free glucose in 1 min under the conditions used for the enzyme activity analysis.
All experiments were conducted in three replicates.

Statistical analysis

The data were analyzed using analysis of variance followed by Duncan's multiple range test. All tests were considered significant when $p < 0.01$. The Statgraphics XVII-X64 software program was used for analysis.

Results and Discussion

Effect of pH and nutrients addition on production of oligofructans by B. subtilis TISTR001

Changes in each of the sugar content (sucrose, glucose, fructose) were used to indicate the fermentation efficiency of the production of fructo-oligosaccharides and fructans during sugarcane juice fermentation. Sucrose was the primary component of the sugarcane juice that was hydrolyzed as a nutrient substrate to created oligofructans. *B. subtilis* TISTR001 is one of the microorganisms that produce levansucrase enzyme to hydrolyze or digest the bonds of sucrose, a double-molecule sugar, to become a single-molecule sugar (glucose and fructose) or the so-called invert sugar; then, the free fructose was linked in long chains of relevant fructo-oligosaccharides or fructan polymer long chains by this enzyme. Splitting theory for the sucrose molecule suggests that the glucose-to-fructose ratio should be 1:1. However, as shown in Fig. 1, the amount of sucrose continued to decrease while the amount of glucose kept on increasing. The quantity of fructose was minimal and was less than the glucose in every experiment. Due to the free fructose, long chains of oligosaccharides or fructose polymers were formed by the levansucrase enzyme. This might explain the lesser quantity of free fructose as analyzed based on the glucose comparison. The results from Fig. 1 show that the quantity of sucrose relatively reduced in each experiment, especially experiment 5 (sugarcane juice adjusted to pH 6.8 and adding 2 g/L of yeast extract + 3 g/L meat extract + 5 g/L peptone + 2 g/L $(NH_4)_2\ SO_4$ + 0.6 g/L $MgSO_4 \cdot 7H_2O$). In experiment 5, the amount of sucrose rapidly decreased and at 72 hr of fermentation, all the sucrose had been used. In contrast, in experiments 1, 3 and 4, the amount of sucrose rapidly reduced also, but the free sucrose was totally consumed by 96 hr. In experiment 2 (adjusted pH 6.8 without any added nutrients), *B. subtilis* TISTR001 was unable to use all the sucrose and after fermentation had finished, there was still a large amount of sucrose remaining.

Considering only fructose, in experiment 1, there was a minor increase in the quantity of fructose after 84 hr of fermentation, as occurred in experiments 3 and 4 where the quantity of fructose increased after 72 hr of fermentation. In experiment 5, after 72 hr of fermentation, the fructose significantly declined until 84 hr, with the remaining fructose being approximately 0.23% by weight (the free fructose was almost all used). In experiment 2, the hydrolysis of sucrose was minimal compared to the other experiments. Thus, the quantities of glucose and fructose were also less. Consequently, the most suitable conditions for the production of oligofructans were those in experiment 5.

The levansucrase enzyme is known as one of the enzymes responsible for the hydrolyzation of sucrose and linking the molecules of fructose to change to fructo-oligosaccharides and fructan (Santos-Moriano et al., 2015; Bersaneti et al., 2018). As shown in Fig 2A, in experiment 5 (juice adjusted to pH 6.8 and addition of 2 g/L yeast extract + 3 g/L meat extract + 5 g/L peptone + 2 g/L $(NH_4)_2\ SO_4$ + 0.6 g/L $MgSO_4 \cdot 7H_2O$), the amount of levansucrease enzyme was clearly greater than in the other experiments. In addition, the increased levansucrase enzyme was associated with the higher production of fructo-oligosaccharides and fructans. During oligofructans production there was continuous production of kestose (Fig. 2B) and nystose (Fig. 2C) that are short-chain of fructo-oligosaccharides (DP3 and DP4, respectively). During 0–12 hr of fermentation time, there was no apparent activity of levansucrase, with less than 1×10^5 U/mL resulting from the detection of kestose and nystose. However, in experiment 5, more kestose (DP3) was produced than under the other conditions, while there was continuous production of nystose (DP4) then it was inconclusive.

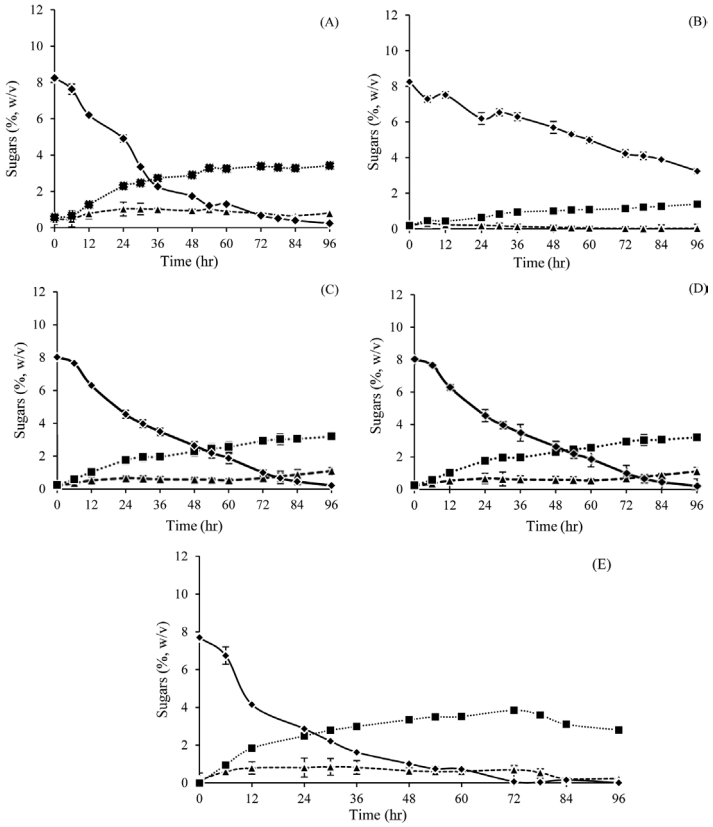

Fig. 1 Changes in sugars contents (sucrose [◆], glucose [■] and fructose [▲])under different nutrients supplemented in culture media of sugarcane juice fermentation at 10 brix for oligofructan production by *Bacillus subtilis* TISTR001 at 30°C for 96 hr: (A) Experiment 1: No adding nutrients-No pH adjustment; (B) Experiment 2: No adding nutrients-pH 6.8; (C) Experiment 3: 2 g/L $(NH_4)_2SO_4$, 0.6 g/L $MgSO_4.7H_2O$, pH 6.8; (D) Experiment 4: 2 g/L yeast extract, pH 6.8; (E) Experiment 5: 2 g/L yeast extract, 3 g/L meat extract, 5 g/L peptone, 2 g/L $(NH_4)_2SO_4$, 0.6 g/L $MgSO_4.7H_2O$, pH 6.8

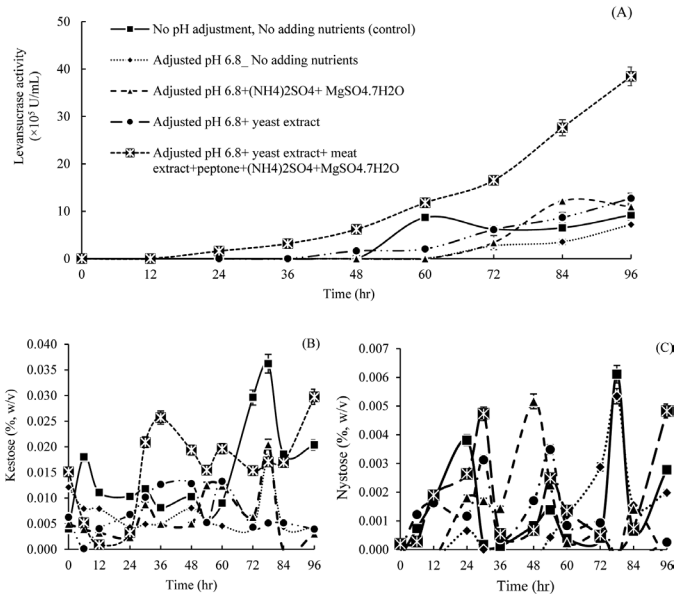

Fig. 2 (A) Levansucrase activity; (B) percentage of kestose produced; (C) percentage of nystose produced during 10 brix sugarcane fermentation using *Bacillus subtilis* TISTR001 at 30°C for 96 hr with different nutrients supplemented in culture media as detailed in figure (A), where w/v = weight per volume

The previous results indicated that experiment 5 used a suitable culturing medium for the production of oligofructans by *B. subtilis* TISTR001. In addition to the good results regarding the consumption of sucrose and fructose, fermentation could be stopped after 84 hr, and thereby reducing the energy cost. The oligofructans content in experiment 5 was the highest content (0.95 % w/v), with quite good results for the consumption of sucrose and fructose in experiments 1, 3 and 4 producing 0.32 %, 0.57% and 0.53% w/v, respectively, of total oligofructans content. Although experiment 5 may have had extra nutrients cost, it had the desirable production goal of the highest content of oligofructans. The pH and nutrients levels were important factors in levansucrase activity and oligofructans production. The higher oligofructans content in experiment 5 was potentially affected by adding some nutrients because Youssef et al. (2014) reported that adding some nutrients (such as $(NH_4)_2SO_4$, peptone, tryptone, and yeast extract) significantly increased levansucrase activity.

Effect of sugarcane juice concentration

Sugarcane concentrations at 5 brix, 10 brix, 15.3 brix and 20 brix were studied under the optimum conditions, resulting from experiment 1 involving pH 6.8 and supplementation with yeast extract (2 g/L), meat extract (3 g/L), peptone (5 g/L), $(NH4)_2SO_4$ (2 g/L) and $MgSO_4 \cdot 7H_2O$ (0.6 g/L)) at a fermentation temperature at 30°C for 96 hr. The optimized concentration of sugarcane juice in fermentation, considering the different kinds of sugar as the composition of sugarcane juice as shown in Fig. 3. The quantity of each monosaccharide to be changed would depend on the primary concentration of sugars. The results showed that *B. subtilis* TISTR001 produced increasingly levansucrase enzyme on longer fermentation time, especially under an amount of nutrient or on more quantity of sugarcane that showed higher sucrose concentration. The quantity of short-chain fructo-oligosaccharide as keystose (DP3) and nystose (DP4), as shown in Figs. 4B−4C, might not be detected. Anyway, these small intermediate oligosaccharides were formed and continuously produced into longer oligofructans.

Furthermore, the result of total oligofructans content under fermentation by hydrolyzing on oligosaccharide and fructan polymers and converting to total free fructose under hydrochloric acid at higher temperature showed in Fig. 5. Sugarcane juice with a high quantity of sucrose or with high brix was positively related to the total fructo-oligosaccharide and fructans, in the form of total free fructose with the high quantity of sugar content. In accordance with quantity in a form of total sugars as invert that was rapidly decreased, while the concentration of sugarcane juice is higher. Additionally, fermentation at 84 hr provided the maximum quantity of total fructo-oligosaccharides and fructans in the form of total free fructose at 20 brix which was the highest level of sugarcane provided in this experiment. The fermentation parameters for this experiment (total oligofructans in the form of total fructose and productivity and production yield) were calculated and are compared for different juice concentrations at 84 hr in Table 2.

Effect of fermentation temperature

A temperature of 37°C optimized *B. subtilis* TISTR001 growth under fermentation with the concentration of sugarcane juice at 20 brix (juice concentration and adjusted pH including adding nutrients as a result of the previous experiment) and was compared with using 30°C. The results on the changes in the sugars (Fig. 6) showed that at 30°C, the sucrose rapidly decreased and was used up at approximately the end of 72 hr fermentation; then, the glucose increased confirming the hydrolysis of sucrose. Furthermore, fructose increased during 12 hr fermentation and then slightly declined before clearly decreasing after 60 hr fermentation. As shown in Fig. 6, the hydrolysis of sucrose at 37°C was clearly less than at 30°C as the higher temperature resulted in fewer fructose molecules to make oligofructose. Consequently, the production of oligofructans at 37°C was less than at 30°C.

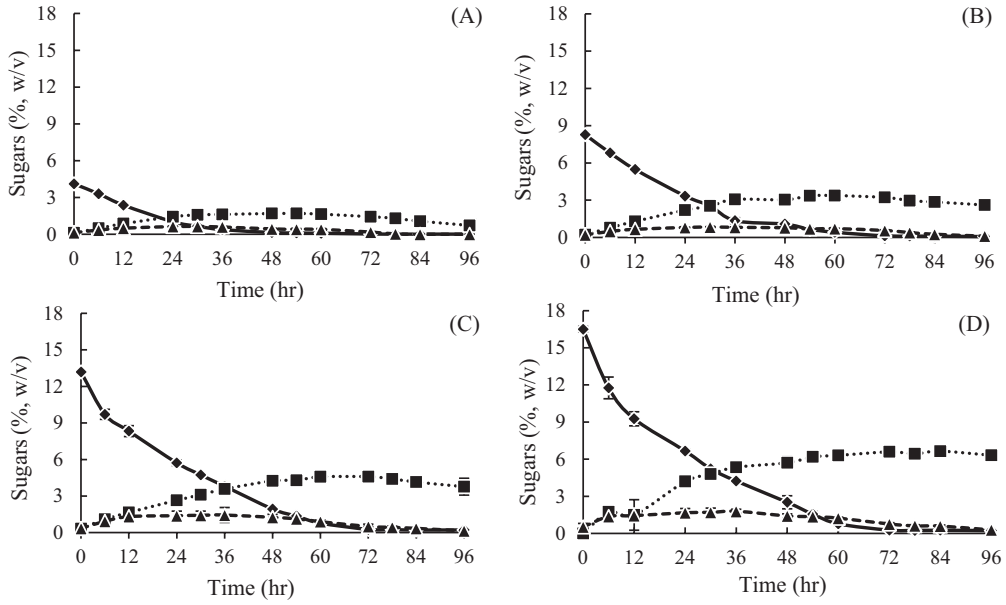

Fig. 3 Changes in sugars contents (sucrose [◆], glucose [■] and fructose [▲]) for oligofructan production by *Bacillus subtilis* TISTR001 at 30°C for 96 hr for different sugarcane juice concentrations at: (A) 5 brix; (B) 10 brix; (C) 15 brix; (D) 20 brix

Table 2 Total oligofructans as total free fructose and yield, and productivity for different sugarcane juice concentrations after 84 hr fermentation time and 30°C

Parameters	Sugarcane juice concentration (brix)			
	5	10	15.3	20
Total oligofructans in form of total free fructose (%, weight per volume)	0.31±0.01[d]	1.03±0.05[c]	2.57±0.05[b]	3.79±0.03[a]
Yield (g /g reducing sugar)	0.09±0.04[c]	0.13±0.01[b]	0.17±0.01[ab]	0.29±0.02[a]
Productivity (g/L/hr)	0.004±0.00[c]	0.012±0.01[b]	0.031±0.01[a]	0.045±0.03[a]

Mean ±SD within each row superscripted with different lowercase letters are significantly ($p < 0.01$) different.

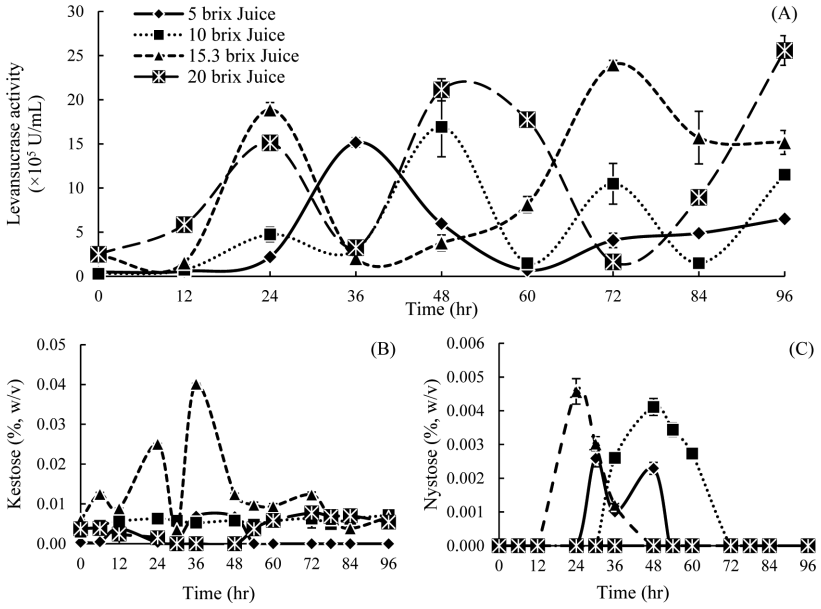

Fig. 4 (A) Levansucrase activity; (B) percentage of kestose produced; (C) percentage of nystose produced during sugarcane juice fermentation using *Bacillus subtilis* TISTR001 at 30°C for 96 hr with different sugarcane juice concentrations as detailed in figure (A)

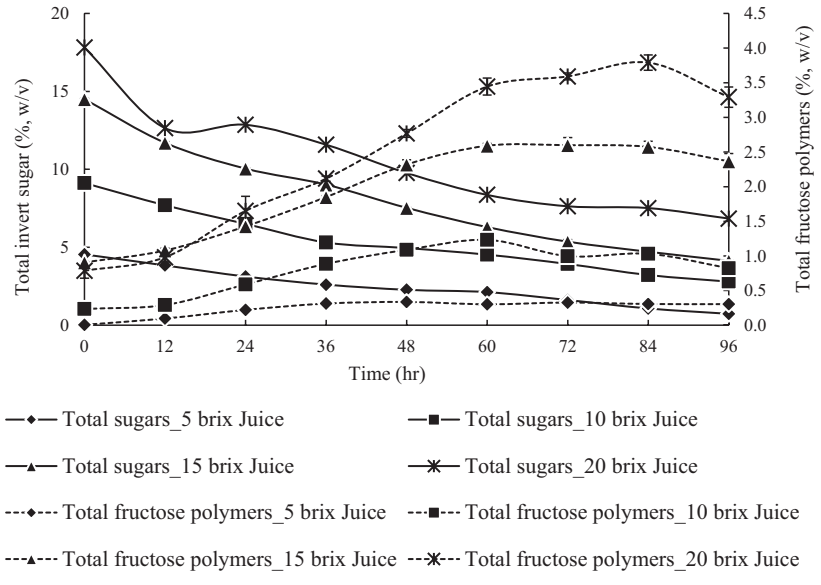

Fig. 5 Total sugars as invert (solid line) and total oligofructans in total fructose polymers form (dotted line) during *Bacillus subtilis* TISTR001 fermentation under different juice concentration at 30°C for 96 hours

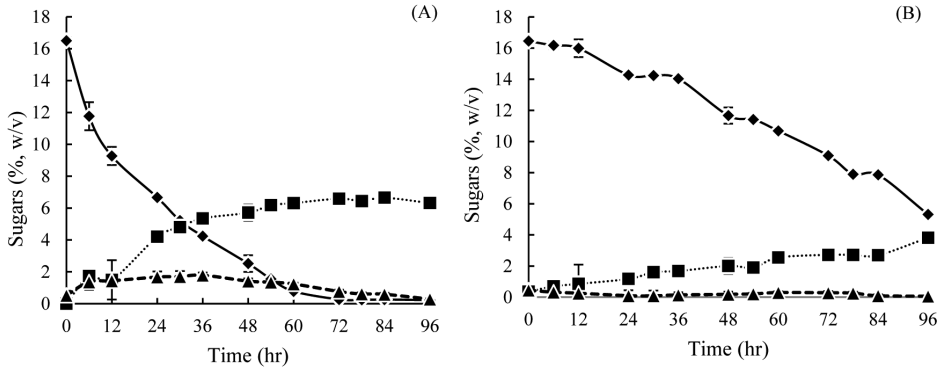

Fig. 6 Change of sugars content (sucrose [◆], glucose [■], and fructose [▲]) under different temperature: (A) 30°C; (B) 37°C at 20 brix sugarcane juice fermentation for oligofructans production by *Bacillus subtilis* TISTR001 for 96 hours

In addition, the amount of levansucrase enzyme at 30°C was higher than at 37°C. This was confirmed by the production of oligofructans (Fig. 7A) as the quantity of enzyme and the efficiency of oligofructans production were positively correlated. The results for the short-chain fructo-oligosaccharides (kestose and nystose) are shown in Figs. 7B and 7C, respectively, were inconclusive. However, the fructose polymers analyzed in the form of total free fructose clearly affected the efficiency of production at 30°C that was preserved to proper conditions for *B. subtilis* TISTR001 was possible to produce oligofructose higher content than 37°C. Moreover, at 30°C seem to be accordingly decreased of in a form of total sugar as invert sugar faster than 37°C as shown Fig. 7D. The results of total oligofructans, yield and productivity are shown in Table 3.

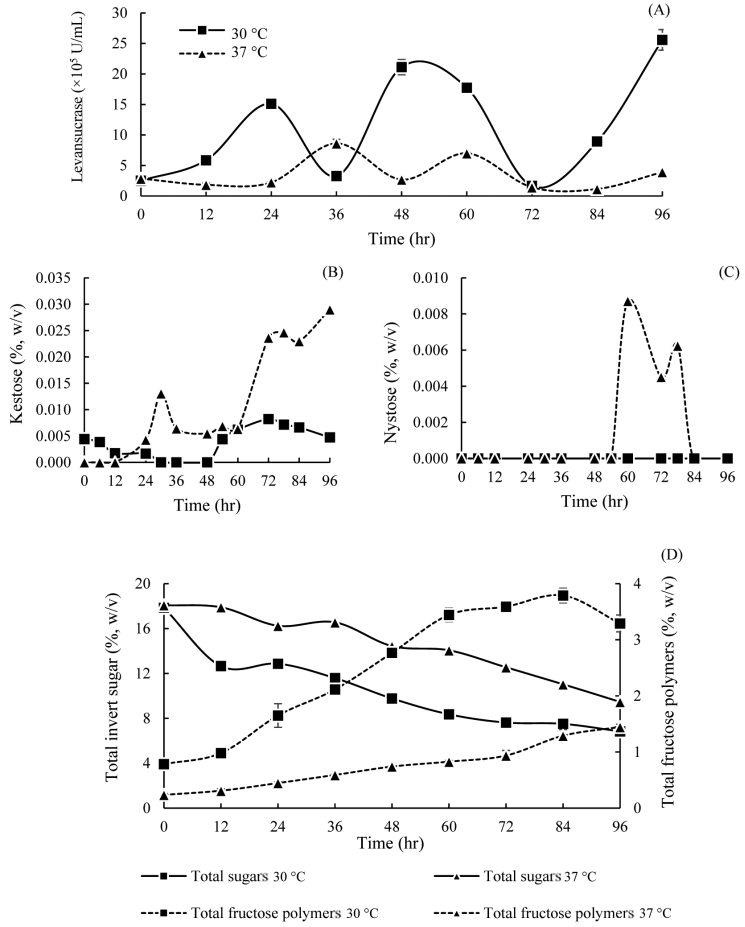

Fig. 7 (A) Levansucrase activity; (B) percentage of kestose produced; (C) percentage of nystose produced; and (D) total sugars as invert (solid line) and total oligofructans in total fructose form (dotted line) during fermentation of 20 brix sugarcane juice using *Bacillus subtilis* TISTR001 for 96 hours under different temperature

Table 3 Total oligofructans as total free fructose and yield and productivity at different temperatures during 20 brix cane juice fermentation for 84 hr

Parameter	Temperature	
	30°C	37°C
Total oligofructans as total free fructose (%, weight per volume)	3.79±0.03[a]	1.29±0.02[c]
Yield (g /g reducing sugar)	0.29±0.02[a]	0.15±0.02[bc]
Productivity (g/L/h)	0.045±0.03[a]	0.013±0.01[c]

Mean ±SD within each row superscripted with different lowercase letters are highly significant ($p < 0.01$) different.

The previous results showed the stronger influence on oligofructans production of levansucrase at 30°C compared to 37°C as the optimum temperature for cell growth. In theory, the roles of levansucrase are in hydrolyzing sucrose and transferring fructose moiety to connect the linkage that is the mechanism of sugar consumption for FOS production. Figs. 6 and 7 confirmed the positive correlation between levansucrase activity and the kinetics of sugar consumption.

Oligofructans were continuously produced during fermentation, resulting in levansucrase also working kinetically to hydrolyze and transform fructose molecules into oligofructans. The fluctuating levansucrase activity was positively related to the cell inoculum and the growth of *B. subtilis* during fermentation. The range of *Bacillus* growth was in the range 10^6–10^8 colony forming units/mL. Based on the ketose (DP3) and nystose (DP4), small intermediate oligosaccharides were formed and continuously produced into longer oligofructans. The high kestose and nystose contents implied the formation of oligofructans that then declined due to the formation of longer oligosaccharides. However, oligofructans-producing enzymes and small oligosaccharides fluctuated during the fermentation time, resulting in some inconclusive trends in the results, as previously discussed.

The maximum oligofructans production yield was 3.79 % (w/v) or 37.9 g/L using 20 brix of sugarcane juice as substrate. This was relatively lower than the result reported by Bersaneti et al. (2018) who achieved 41.3 g/L production of FOS using *B. subtilis* natto with 350 g/L of sucrose. Da Silva et al. (2014) studied the optimum conditions for FOS production by *B. subtilis* natto CCT 7712 and obtained 98.86 g/L of FOS using a sucrose concentration of 300 g/L at pH 7.7 and agitation at 234 rpm. Although there was a lower content of oligofructans produced using fermented fresh cane juice than reported by other researchers, the current research could be further developed to produce a functional drink from fermented fresh cane juice.

Conclusion

The results using *B. subtilis* TISTR001 showed that the optimum conditions for oligofructans fermentation from sugarcane juice, required adjusting to pH 6.8 and supplementing with yeast extract (2 g/L), meat extract (3 g/L), peptone (5 g/L), $(NH_4)_2 SO_4$ (2 g/L) and $MgSO_4 \cdot 7H_2O$ (0.6 g/L) to encourage growth of *B. subtilis* TISTR001 and oligofructans production. The concentration of sugarcane juice was directly correlated to the amounts of fructo-oligosaccharides and fructan or oligofructans. It was found that concentration of sugarcane juice at 20 brix yielded 3.79% (w/v) oligofructans in the form of total free fructose. According to this study, it was possible to achieve a production yield of 0.29 g/g reducing sugar and productivity at 0.045 g/L/h. As the production was directly related to the sugarcane juice concentration, the fermentation of sugarcane juice at 20 brix yielded more product than from fermentation of sugarcane juice at 5 brix, 10 brix and 15 brix. In addition, the fermentation temperature of sugarcane juice at 30°C was suitable for *B. subtilis* TISTR001 as it enabled greater levansucrase enzyme production which resulted in achieving a higher oligofructans content than at 37°C. Fermentation for 84 hr was the most effective period for oligosaccharides and fructose polymer production that was supported by the quantities of sucrose and free fructose being completely used at the end of this period.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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