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The Effect of Corn (*Zea mays*) Cob Extract on the Growth of Bifidobacteria

Ribka Ananda Sejati[1], Catarina Aprilia Ariestanti[1*], Charis Amarantini[1]

[1]Faculty of Biotechnology, Duta Wacana Christian University
Jl. Dr. Wahidin Sudirohusodo no. 5-26 Kotabaru, Gondokusuman, Yogyakarta 55224, Indonesia
Email: catarina.ariestanti@staff.ukdw.ac.id **Corresponding author*

Abstract

Corncob is a source of dietary fiber, contains xylan polysaccharides (12.4%–31.94%), and is classified as the highest xylan-producing source compared to other agricultural wastes. Human digestive enzymes cannot degrade xylan but can be enzymatically degraded by lactic acid bacteria (LAB) through fermentation. This research aimed to evaluate the effect of corncob extract on the growth of *Bifidobacterium*. Corncob extract is obtained through alkaline extraction. The growth of *Bifidobacterium bifidum* FNCC 0462 and *Bifidobacterium longum* FNCC 0463 was observed through *in vitro* fermentation for 48 h using the total plate count (TPC) method. *Bifidobacterium bifidum* FNCC 0462 showed the ability to ferment corn cob extract for 48 h of fermentation with the highest growth at 8 h of fermentation (4,08 log10 CFU/mL) while *B. longum* FNCC 0463 was able to grow up to 16 h of fermentation only. The results indicated that corncob extract could support *B. bifidum* FNCC 0462 growth.

Keywords: alkaline, *Bifidobacterium*, corn cob, total plate count, xylan

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Introduction

Aside from rice and wheat, corn (*Zea mays*) is one of the most consumed food crops worldwide as a source of carbohydrates. Various uses of corn result in corncob waste. Nowadays, corncob is used as a component in animal feed (Wachirapakorn et al., 2016), furfural products (Hidayat et al., 2019; Wang et al., 2019; Li et al., 2018), metal ions and carbon adsorbents (Liu et al., 2021; Christica & Julia, 2018), and bioenergy (Blandino et al., 2016). On a larger scale, corncobs are usually disposed off and burned, causing environmental pollution (Anukam et al., 2017).

Corncob mainly contain lignin, cellulose, and hemicellulose, which consist of xylan polymers (Van Eylen et al., 2011). Xylan is a biopolymer of a non-starch polysaccharide group that cannot be broken down by enzymes naturally found in human digestion and consists

The characteristic of being resistant to low pH allows xylan to reach the large intestine, which is the living place of microbiota that can break down fiber through a fermentation process. This microbiota includes bacteria from the genus Bacteroides, Prevotella, of xylan, arabinose, acetyl, and glucuronic acid as side chain sugars (Sporck et al., 2017). Chemical hydrolysis, acid hydrolysis, autohydrolysis, and enzymatic hydrolysis by several microorganisms capable of producing key enzymes such as xylanase A, xylanase β-xylosidase, and endo-β-1-4-xylanase can support the degradation of xylan to xylose fractions.

Microorganisms that specifically produce hydrolytic enzymes include *Aspergillus foetidus* (Chapla et al., 2012), *Trichoderma reesei* (Ike and Tokuyasu, 2018), and bacteria that are naturally found in the human large intestine, namely *Ruminococcus albus* and *Ruminococcus flavefaciens*. In the colon, *Bifidobacterium adolescentis* ATCC15703 is also known to be able to produce carbohydrate-active enzymes (CAZymes) in the human digestive system as fibrinolytic bacteria (Flint et al., 2012).

Ruminococcus, Treponema, Succinivibrio, Bifidobacterium, and Lactobacillus, where the composition and varieties of the microbiota are affected by environmental lifestyle and the dietary intake (Arumugam et al., 2011; De Filippo et al., 2010; Schnorr et al., 2014).

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Xylan fermentation products have benefits for human health, especially in controlling obesity, and gastrointestinal diseases, and maintaining intestinal microbiota in both humans and livestock (Baker et al., 2021) that makes xylan potentially a prebiotic (Mendis and Simsek, 2013; Broekaert et al., 2011). The previous study result of Ariestanti et al. (2022) showed the growth-promoting properties of corncob waste extract for *Bifidobacterium longum* FNCC 0210. This research introduces a new approach by integrating locally cultivated corncobs to assess whether the extract similarly affects the growth of *Bifidobacterium longum* FNCC 0462 and *Bifidobacterium bifidum* FNCC 0463. Bifidobacterium was specifically chosen for this study due to its prevalent presence in a healthy gut and its potential health benefits through the production of short-chain fatty acids (SCFAs) (Vlasova et al., 2016). The research objective was to observe the effect of corncob extract on the growth of Bifidobacterium.

Research Methods

Materials

Corncob from harvested 2.8–3.2 months old corn was obtained collectively from Tani Makmur Farmers Group, Sendangsari Village, Kulon Progo, Special Region of Yogyakarta, Indonesia. *Bifidobacterium bifidum* FNCC 0462 and *Bifidobacterium longum* FNCC 0463 were purchased from Center for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta, Indonesia. All chemicals and reagents used in this study were of analytical grade.

Sample preparation

Sample preparation was carried out based on the method used by Ariestanti et al. (2022) with modification. Corncobs are washed under clean running water, brushed, and cut into pieces about 0.5–1 cm thick. The corncobs were dried at 60°C for 48 h, ground, sieved using 200 mesh into powder, then stored in an airtight container.

Alkaline extraction of corn cobs

Corncob powder (50 g) were soaked in 575 mL of 0.5% NaOCl solution for 5 hours at 28°C for delignification process and removal of organisms and other impurities.

The sample was rinsed with distilled water twice and the solid was allowed to subside. The distilled water was discarded and the remaining solid was centrifuged at 4000 rpm for 30 minutes. The solid was dried at 35°C for 24 hours and soaked in 400 mL of 10% NaOH solution for 24 hours at 35°C to precipitate cellulose under alkaline conditions. The pH of the filtrate was measured, neutralized by adding 6N HCl, and centrifuged at 4000 rpm for 30 minutes to separate the supernatant from the remaining pellet. The dissolved carbohydrates in the supernatant were separated by adding 95% ethanol with a supernatant: ethanol in the ratio of 1:3 (v/v). The precipitate formed was separated from the ethanol, then dried in an oven at 50°C for 24 hours until there was no ethanol remaining, ground into a fine powder, and stored in a closed container (Richana et al., 2007 with modification).

Bacterial isolate maintenance

Bifidobacterium bifidum FNCC 0462 and *B. longum* FNCC 0463 were subcultured in de Man, Rogosa, and Sharpe (MRS) broth and incubated at 37°C for 48 hours. For a longer storage period, it can be done by inoculating the isolate in MRS agar with the stab culture method (Süle et al., 2014 with modification).

Starter culture preparation

Starter cultures were prepared according to the method of Jaskari et al. (1998) with modifications. Stock bacterial isolates in MRS broth were vortexed and centrifuged at 6000 rpm for 15 minutes. The supernatant was removed and the pellet was washed using 0.9% physiological saline and repeated twice until the pellet was clean from the remaining medium. The pellet was added with 0.9% NaCl, vortexed, and the cell density was standardized using a 0.5 McFarland standard or equal to 1.5×10^8 CFU/mL. A 0.5 McFarland standard has an absorbance value of 0.08–0.1 at 600 nm which will also be used as a requirement for the OD value of the initial growth starter culture in the *in vitro* fermentation process. Density validity was tested by the OD value using an Ultraviolet-Visible (UV-Vis) spectrophotometer.

In vitro fermentation of corncob extract

In vitro fermentation of the sample extract was done using the method by Chapla *et al*. (2012) with modification. This step was performed using a batch fermentation process with the following treatments: (i) positive control, the starter culture was inoculated in the medium supplemented by glucose, and (ii) the treatment group, the starter culture was inoculated into the medium supplemented by the corncob extract. Fermentation medium for bacterial culture consists of (g/50 mL): 0.5 g glucose, 0.1 g soya peptone, 0.25 g urea, 0.02 g sodium bicarbonate ($NaHCO_3$), 0.4 mL calcium chloride ($CaCl_2$), 1 mL magnesium sulfate ($MgSO_4$), 4 mL sodium chloride (NaCl), 2 mL monopotassium phosphate (KH_2PO_4), and 2 mL dipotassium phosphate (K_2HPO_4). The treatment group was designed by replacing glucose with 0.5 g corncob extract. The ingredients were put into a 100 mL Erlenmeyer flask, added 50 mL of distilled water, was heated using a laboratory hot plate, and stirred until dissolved. The medium was sterilized in an autoclave at 121°C for 15 minutes, and allowed to stand at room temperature until cool before being inoculated with 5% (w/v) of each Bifidobacterium strain aseptically and incubated at 37°C for 48 hours under static condition. Sampling was done at 0, 8, 16, 24, 32, 40, and 48 h fermentation.

Bacterial enumeration using total plate count (TPC) method

The samples from the fermentation for 48 hours were diluted using 0.9% physiological saline with dilution series 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. Dilution was done by taking 1 mL of fermentation broth and adding it to 9 mL of sterile 0.9% physiological saline to maintain the ionic balance of the microorganism cells. 1 mL of sample bacteria from 10^{-1} dilution series was added in 9 mL of sterile 0.9% physiological saline for the 10^{-2} dilution series. The same technique was performed for further dilutions up to the 10^{-6} dilution series. The spread plate technique was done in duplicate on MRS agar. One percentage of $CaCO_3$ was added for the characterization of bacterial colonies. Colonies that grew on each plate were counted for bacterial growth curve data (Sharah et al., 2015 with modification).

Result and Discussion

Corncob extraction

In the research conducted, 585.3 g of corncobs can be converted into an extract with a yield of 17.50 g or 2.99% of the raw materials used. This total yield is 8.97% less than the results of the extraction according to the study from Richana et al. (2007). This was caused by the difference in the surface area of the corncob powder used, in which this study used a sieve with a mesh particle size of No. 10 (200 mesh size) that produces ≤2.0 mm powder particle, while previous research used a 40 mesh size sieve which means that the powder has a larger particle sizes. The fine particle size allows the insoluble extract to be easily removed unintentionally during the extraction process, especially in the supernatant and pellet separation step. The dried and ground extract that is used as a carbohydrate source in *in vitro* fermentation by *B. bifidum* FNCC 0462 and *B. longum* FNCC 0463 can be seen in Figure 1.

As reported by Richana et al. (2007), corncob extract by using alkaline extraction contains xylan with an average yield of 10.95% (g xylan/g corncob). Using the same method, it can be concluded that the expected carbohydrates contained in the sample extracts in this study are xylan. However, a further step to identify and collect data about the types of carbohydrates contained in corncob extract was not performed in this study. As a result, the amount of xylan present in the extract remained undetermined.

Corncob extract is also insoluble in the media. Xylan structure is characterized into two, glucuronoarabinoxylan with fewer side chains which makes it insoluble in water, and heteroxylan which has more side chains. The large number of heteroxylan side chains gives water more access to bind to existing molecules, thereby determining the polarity of the compound (Oliveira, 2010). In corncob, water-insoluble xylan is more available and localized evenly throughout the lignified part, while xylan that dissolves in water is only available in a small part of the corncob (Takada, 2018). The presence of lignin residue also causes the corncob extract to have low polarity and tend to form precipitates when dissolved in the fermentation medium. Notwithstanding, its low polarity allows the extract to get through the physiological environment of the stomach and small intestine, so it can increase its potential as

a prebiotic candidate in the colon (Levantovsky et al., 2018).

The Growth of Bifidobacterium in 1% Glucose-Supplemented Medium

The growth curve in this study was determined based on the CFU/mL log number value obtained from enumeration of bacterial colonies using the TPC procedure. The colony observed of *B. bifidum* FNCC 0462 and *B. longum* FNCC 0463 that grew in fermentation medium using glucose as a carbon source are shown in Figure 2. Bifidobacterium colonies are characterized by round-shaped colony with varying diameters, convex, shiny white or pale pigmentation, and the edges are generally entire. This characteristic is in accordance with the study by Ballongue (2004).

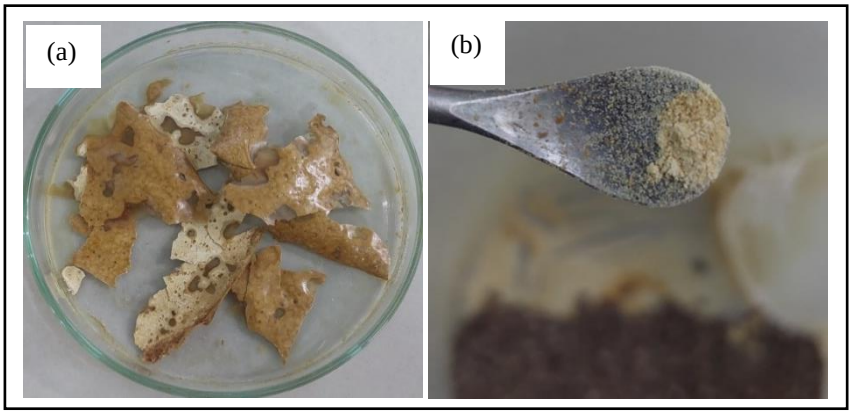

Figure 1. Dried corncob extract from alkaline extraction (a) and corncob extract powder (b)

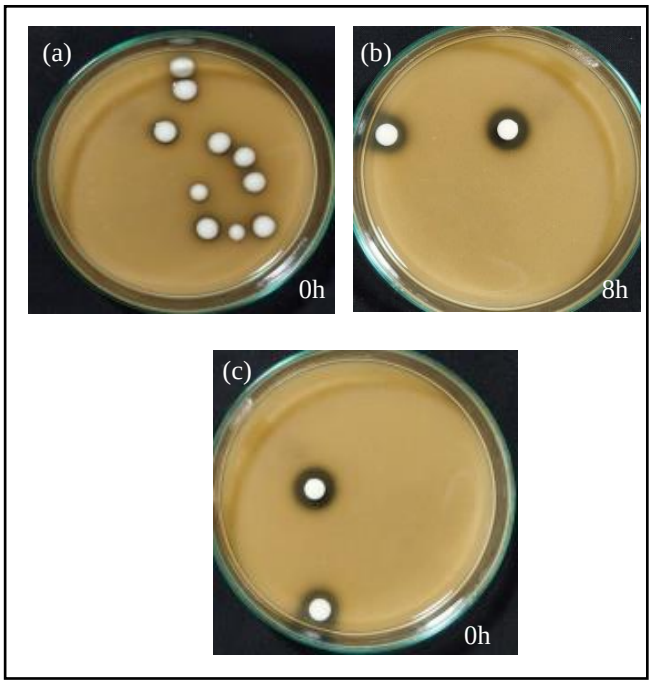

Figure 2. The colony observations of *Bifidobacterium bifidum* FNCC 0462 (a) and *Bifidobacterium longum* FNCC 0463 (b) in MRS agar during 48 h incubation in glucose-supplemented medium

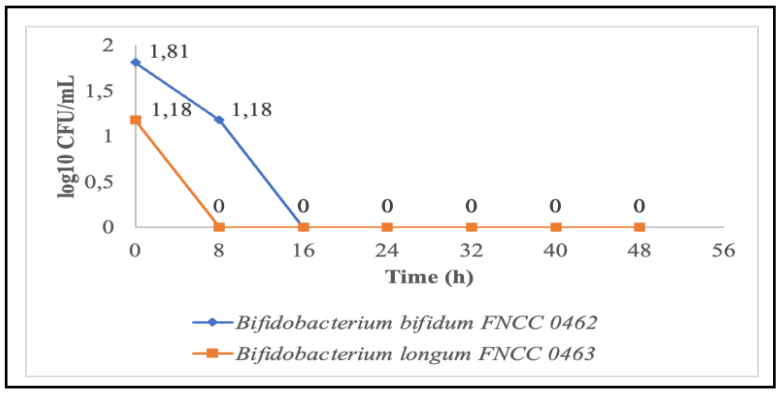

Figure 3. Growth curves of *Bifidobacterium bifidum* FNCC 0462 and *Bifidobacterium longum* FNCC 0463 in 1% glucose-supplemented medium based on TPC enumeration data

The presence of healthy bacterial cells from the starter culture at the start of fermentation (0 hours) can be observed through the colonies on the agar medium during enumeration process using the TPC method (Figure 2). In this study, growth curves of *B. bifidum* FNCC 0462 and *B. longum* FNCC 0463 in 48 hours fermentation with 8 hours of sampling intervals obtained through data enumeration with TPC procedure presented in Figure 3. with no growth detected since 8 hour fermentation for *B. longum* FNCC 0463 and 16 hour fermentation for *B. bifidum* FNCC 0462.

Based on Figure 3, it shows that the growth of both of *B. bifidum* FNCC 0462 and *B. longum* FNCC 0463 which was supported using glucose as a carbohydrate source (positive control) experienced a death phase at 0 to 16 hours fermentation. Normally, glucose is the simplest sugar often used as a source of energy for the growth of lactic acid bacteria in the pure lactic acid fermentation process (Mıdık et al., 2020; Wang et al., 2021). The discrepancy between the statement mentioned and the results of this study can be caused by several factors, namely the low number of bacterial cells that live in the starter culture and the presence of oxygen during the fermentation process and the incubator used to grow the culture that prevents complete anaerobic condition.

The presence of oxygen in growth medium of Bifidobacterium that live anaerobically will be responded with the production of antioxidant superoxide dismutase (SOD) which functions as a counter to free radicals (Rahmawati et al., 2014). In its oxygen metabolism, SOD will bind to oxygen and become hydrogen peroxide (H_2O_2). An excessive accumulation of H_2O_2 will damage the cells. According to Ruiz et al. (2011), an optimal culture environment for Bifidobacterium involves a medium with specific and low range of oxygen levels, temperature, and acidity. Anaerobic condition can be acquired by setting the suitable fermentation system, sampling technique, and incubation for the culture.

Growth of Bifidobacterium in 1% Corncob Extract-Supplemented Medium

The colony observed of *B. bifidum* FNCC 0462 and *B. longum* FNCC 0463 that grew in fermentation medium using corncob extract as a carbon source are shown in Figure 4. Additionally, the growth curve resulted from colony enumeration can be seen in Figure 5. Bifidobacterium colonies are characterized by round-shaped colony with varying diameters, convex, shiny white or pale pigmentation, and the edges are generally entire. This characteristic is by the study by Hendrati et al. (2017).

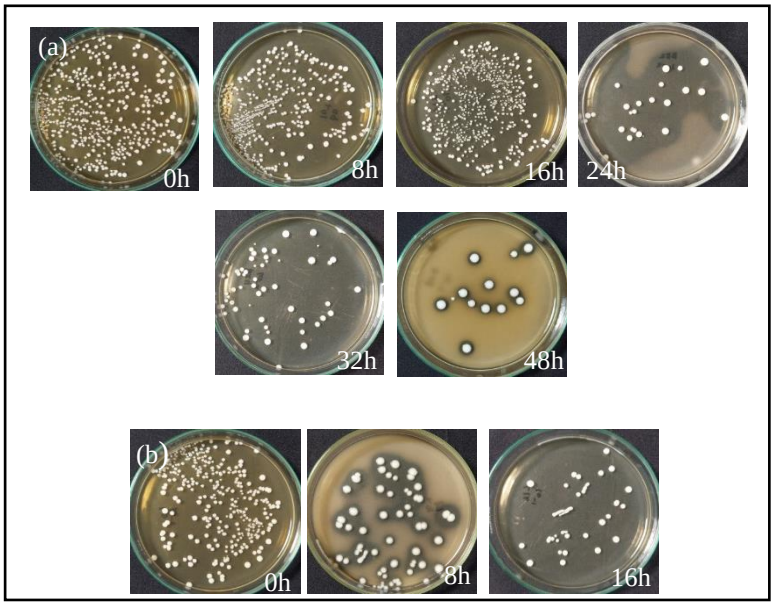

Figure 4. The colony observations of *Bifidobacterium bifidum* FNCC 0462 (a) and *Bifidobacterium longum* FNCC 0463 (b) in MRS agar during 48 h incubation in 1% corncob extract-supplemented medium

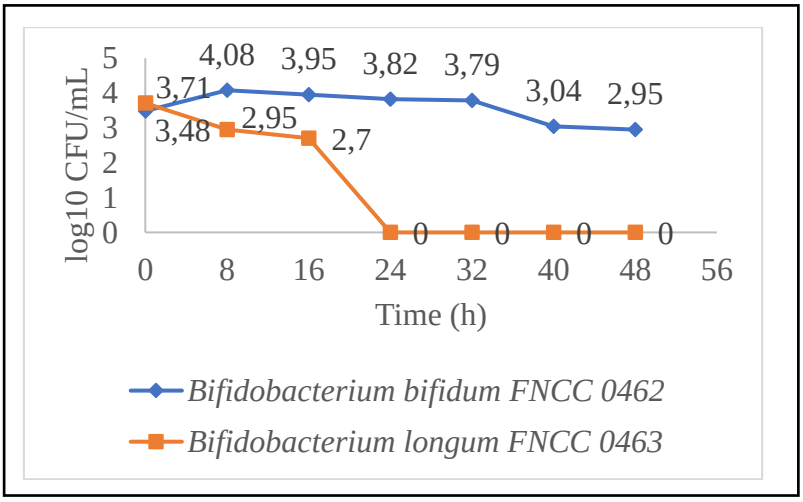

Figure 5. Growth curves of *Bifidobacterium bifidum* FNCC 0462 and *Bifidobacterium longum* FNCC 0463 in 1% corncob extract-supplemented medium based on TPC enumeration data

Based on Figure 5, the growth curve shows that *B. bifidum* FNCC 0462 has the ability to adapt in the fermentation medium supplemented by corncob extract which is expected to contain xylan and simpler fractions. The data shows that the lag phase is possible to occur at 0 to 8 h of fermentation. The *B. bifidum* FNCC 0462 strain lacks the ability to further utilize the available substrate, possibly due to factors such as nutrient limitations, unfavorable conditions (pH, temperature, oxygen), and the accumulation of toxic substances from its by-products (Atolia et al., 2020). Consequently, the cells enter the stationary phase and tend to die. Sasmitaloka et al. (2019) also reported a significant amount of sodium chloride (NaCl) in the extract. NaCl can be lethal to microorganisms because of their hygroscopic nature, allowing them to absorb water (cytoplasm), which can lead to bacterial cell death. *B. longum* FNCC 0463, on the other hand, does not show growth during the fermentation process. *Bifidobacterium longum* FNCC 0463 showed a tendency to decrease in growth that began at 0th to 8th hour and lasted until the 16th hour before there was no colony

growth found in the following hours. The biological condition of the inoculum is thought to be not ready for adaptation and metabolism for its growth. This decreasing growth can also be caused by unfavorable inoculum conditions for the fermentation process. Mostly, this condition is related to the availability of nutrient in the starter culture medium and the temperature (Gonzalez and Aranda, 2023).

In keeping with the study from Grootaert (2007) and Richana et al. (2007), the utilization of xylan by bacteria as a carbon source is likely facilitated when xylan is obtained from corncobs through alkaline extraction. This utilization process involves the production of two key enzymes: (i) xylanase, an enzyme that plays a crucial role in breaking down the complex xylan chains into simpler sugars, primarily xylose; and (ii) β-xylosidase, that complements this process by cleaving the xylose chains into individual xylose molecules.

The xylose will have a role as a carbon source for the growth of microorganisms. In terms of its metabolic process, Egan and Van Sinderen (2018) stated that xylose enters the fructose-6-phosphate phosphoketolase or "bifid shunt" pathway as xylulose-5-phosphate. However, it is possible for several factors prohibit microorganisms from effectively utilizing xylan as a carbon source. These factors include: (i) microorganisms lacking necessary enzymes like xylanase and β-xylosidase needed to break down xylan; (ii) pH and temperature influencing the activity of enzymes (Robinson, 2015); (iii) oxygen availability impacting aerobic and anaerobic microorganisms differently, affecting their growth and metabolism (Couvert, 2015); (iv) microorganisms tend to express the enzymes only for the preferred carbon source if other carbon sources readily available (Wang et al., 2019); (v) variability in xylan structure based on the source it's extracted from, allowing certain microorganisms to use it for their growth (Bajpai, 2014).

Conclusion

Bifidobacterium bifidum FNCC 0462 showed the ability to adapt and grow on medium supplemented with corncob extract. In contrast, *Bifidobacterium longum* FNCC 0463 was unable to grow in the medium supplemented by corncob extract as good as *B. bifidum* FNCC 0462. Further study to determine the optimum conditions for xylan fermentation by *B. bifidum* FNCC 0462 and *B. longum* FNCC 0463 should be performed.

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