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Anti-Cariogenicity of NCS (Non-Cariogenicity Sugar) Produced by Alkalophilic *Bacillus* sp. S-1013

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Abstract The NCS inhibited the activity of glucosyltransferase which was produced by *Streptococcus mutans* JC-2, and the rate of inhibition at 100 μM and 200 μM were 74.0% and 99.8%, respectively. It was stable in alkali condition, but unstable in acid condition. It was also stable up to 80°C. The kinetic study of the inhibition by NCS was carried out by Lineweaver-Burk plot and Dixon plot. It was non-competitive inhibition, determined by the two plots and K_i and K_i' values were 15 μM and 19.3 μM, respectively. The NCS did not show cytotoxicity against human gingival cells at K_i (15 μM, 150 μM, and 1,500 μM) concentrations. It had less cytotoxicity than chlohexidin, which has usually been used as the agent of anticaries. To evaluate the industrial applicability of the NCS, human pluck tooth was used. The inhibitory rates of tooth calcification and calcium ion elution by the NCS were 41% and 2.5 times, respectively. These results suggested that NCS from *Bacillus* sp. S-1013 is an efficient anticaries agent.

Key words: *Bacillus* sp. S-1013, glucosyltransferase, cytotoxicity, acid production

Many studies have been conducted on the prevention of dental caries due to sugar, since sugar seems to be inevitably associated with dental caries, and sorbitol, mannitol, hydrogenated glucose syrup, maltitol, xylitol, and lactitol have been used as dental caries inhibitors since 1982. [21] It was found that these polyols have low dental caries activities, and xylitol is a non-caries inducer. However, many studies showed that acids production was rapidly reduced, when xylitol was added to cell cultures obtained from plaque-inducing bacteria [3, 11, 12, 15]. According to numerous investigations, however, polyols such as sorbitol, maltitol, and mannitol, which are used in industries as low calorie, low cariogenic sweeteners, are fermented by oral microorganisms, including Streptococci [4, 5, 7, 10, 13, 32], Lactobacilli [7, 10], Actinomyces [7, 31], and Propionibacterium [31]. Birkhed [3] reported that the acid production was swiftly increased when oral cavity was washed frequently with sorbitol. Also, polyols except erythritol are difficult to be absorbed in intestines [2], and have problems in that the enteric fermentation of unabsorbed polyols induces other gastrointestinal disturbances, as seen in the study by Ranbaud and Flourie [29]. To prevent plaque formation, Okami *et al.* [26] in 1981 isolated a substance from heat-treated culture solution to inhibit GTase of *S. mutans* and reported ribocitrin as the GTase inhibitor (adhesive, inhibitor of insoluble synthetic glucan synthesis) of *Streptomyces nsyawaensis* MF 980-CFI. Newburn *et al.* [25] searched α-glucosidase inhibitors from microorganisms for a GTase inhibitor of *S. mutans* and confirmed that compounds such as 1-desoxynojirimycin, N-methyl-desoxynojirimycin, and acarbose have GTase inhibitory activity. Endo *et al.* [9] found that mutastein, which is a glycoprotein, has a strong inhibitory activity on glucan synthesis by culturing *Aspergillus terreus,* and they even commercialized this substance, however, there were problems in efficacies.

Therefore, based on this design, the present study was undertaken first to investigate the adaptation of dental plaque to NCS. Secondly, the effect of NCS on acid production was examined. Finally, the cytotoxixity against human gingival cell and inhibitory rate of calcification against human pluck teeth were investigated.

Materials and Methods

Materials

The NCS(Non-cariogenicity sugar) used in the present study was purified from *Bacillus* sp. S-1013, as previosly reported [30], and then stored at 4°C until use.

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The Growth Inhibitory Effect of NCS on *Streptococcus mutans* JC-2

The effect of the NCS on the fermentation of *S. mutans* JC-2 was determined after adding the NCS of various concentrations (0.1-200 μM) to BHI broth (Difco, U.S.A.) in a screw-cap tube. After the test strain was inoculated to the concentration of 4% (v/v) and cultured for 48 h in a CO_2 incubator at 37°C, antimicrobial growth was spectrophotometrically measured by determining optical density (Varian DMS 200, U.S.A.) at 620 nm.

The Inhibitory Effect of NCS on Acid Production in *Streptococcus mutans* JC-2

In order to measure pH changes due to various organic acids produced during the culture of *S. mutans* JC-2 in the presence of the NCS, the pH of the culture medium was measured by placing a pH meter (ORION SA 720, U.S.A.) directly into the culture medium. Simultaneously, Stephan curve was used to measure pH after the purified sweetener in increasing concentrations was added. In other words, after measuring change of pH by adding 0.1 μM NCS, 1 M urea was added to readjust the pH to 7.0. Then, 10 μM isolated sweetener was added to measure the change of pH for 20 min. This process was repeated until the final concentration reached 200 μM.

Measurement of Membrane Permeability of *Streptococcus mutans* JC-2

In order to measure the effect of NCS on the membrane permeability of *S. mutans* JC-2, Various amounts of the NCS were added to BHI broth. *S. mutans* JC-2 was then inoculated and cultured at 37°C in a CO_2 incubator for 24 h. Subsequently, according to the method of Chun *et al.* [6], the cultured medium was centrifuged (10,000 ×*g*, 20 min, 4°C) to collect the strain, which was resuspended in 0.1 M potassium phosphate buffer solution (pH 7.0), and the suspension was left at 37°C for 10 min and membrane permeability was colorimetrically quantified at 260 nm.

GTase Inhibitory Activity

GTase activity was measured according to the method of Torii and Hamada [34], in which glucan produced from sucrose as the substrate was measured with DNS. In place of water used in the control, 0.1 ml of NCS (10, 50, 100, 200 μM) was used and the final volume was brought to 1 ml, and absorbance was read. The % inhibitory rate was calculated from the following equation.

$$\% \text{ Inhibition rate} = \frac{A - B}{A} \times 100$$

Here, A is OD_{550} value of adhesive insoluble glucan formed in the control, B is OD_{550} value of adhesive insoluble glucan in the presence of the purified non-caries-inducing sweetener. The buffer for the substrate was prepared by adding 12.5 g of sucrose and 0.25 g of sodium azide to 1 l of 0.0625 M potassium phosphate buffer (pH 6.5).

Kinetic Study of Glucosyltransferase Suppression

In order to calculate the Michaelis-Menten constant (K_m) of the GTase for sucrose as the substrate, GTase activity was measured by carrying out the reaction for 15 min at 37°C with 4.0 units of the enzyme. The apparent K_m value was obtained by the Lineweaver-Burk plot. In order to determine the type of inhibition of NCS on GTase, various concentrations of NCS were added. After incubation of the reaction mixture at 37°C for 30 min without adding sucrose, GTase activity was measured after incubation for 30 min while varying the substrate concentration, and Dixon plot was used to determine different inhibitory type and inhibition coefficient (K_i and K_i') from a coordinate of the X-axis and the intersection of various substrate concentrations.

Decalcification Ratio of Teeth Enamel

To determine the effect of NCS on tooth tissues, teeth extracted from healthy people in their 20's to 30's were fixed in epoxy resin. Then, the sample was prepared so that the tooth exposure surface would be 2 mm in width and height. The initial hardness was measured 6 times, using a Knoop Hardness tester. Thus, each sample was placed in a solution containing 200 μM NCS and washed with distilled water. After repeating this process for one week, the decrease in initial hardness was measured using the decalcification ratio according to the following equation.

$$\% \text{ Decalcification ratio} = \frac{(\text{Initial hardness} - \text{Hardness after t time})}{\text{Initial hardness}} \times 100$$

Quantification of Calcium Ion Eluted from Teeth

To measure the amount of calcium ion eluted from teeth, the teeth extracted from healthy people in their 20's and 30's were prepared so that the tooth exposure surface would be 2 mm in width and height. The sample was placed in a solution containing 200 μM NCS for 30 min and washed 5 times with 1 ml of distilled water, and the washings were centrifuged at 5,000 ×*g* for 20 min to discard the precipitate and collect the supernatant. Calcium concentration in this supernatant was determined by an atomic spectrophotometer.

Cytotoxicity Against Human Gingival Cells

The method by Chun *et al.* [6] was used to determine the effect of NCS on human gingival fibroblasts. Human gingival fibroblasts activated by subculture were suspended in Trypsin EDTA (0.05% trypsin, 0.53 mM EDTA, Gibco/BRL, U.S.A.) solution and the suspension was centrifuged to collect single cells. The collected cells were incubated

in microtest plate wells (1×10^4 cell/well) with DMEM (Dulbecco's Modified Eagle Medium, Gibco/BRL, U.S.A.) containing 1% FBS (fetal bovin serum, Gibco/BRL, U.S.A.) in a CO_2 incubator at 37°C in 5% CO_2 and 100% humidity for 72 h. In order to discard cells not suspended, the well was washed with DMEM containing no FBS and cultured in DMEM (containing 1% FBS) with certain concentrations of NCS added. After culturing, 300 μl of MTT (3-4,5-dimethylthiazol-2-yl, 5-diphenyl tetrazolium bromide, Sigma Co., U.S.A.) dissolved in physiologic saline solution was added to each well and the wells were incubated for 4 h in a CO_2 incubator. After MTT solution was removed from the culture, crystallines formed were dissolved by adding DMSO (dimethyl sulfoxide, Sigma Co., U.S.A.) by an increment of 200 μl, and placed in a 96-well plate. Then, absorbance was measured, using as ELISA analyzer (spectra MAX 250, Molecular DEvices Co., U.S.A.), by reading at 540 nm to evaluate the toxicity. The % cellular activity was calculated according to the following equation.

$$\text{Cellular activity (\%)} = \frac{\text{Absorbance of test well}}{\text{Absorbance of control well}} \times 100$$

Then, statistical analysis was done using SAS system.

RESULTS AND DISCUSSION

The Growth Inhibitory Effect of NCS on *Streptococcus mutans* JC-2

The fermentation characteristics of *S. mutans* JC-2 in purified NCS were determined by measuring growth of *S. mutans* after placing BHI broth (Difco, U.S.A.) into a screw-cap tube and adding NCS at concentrations ranging from 0.1 to 200 μM. As shown in Fig. 1, the lag phase was

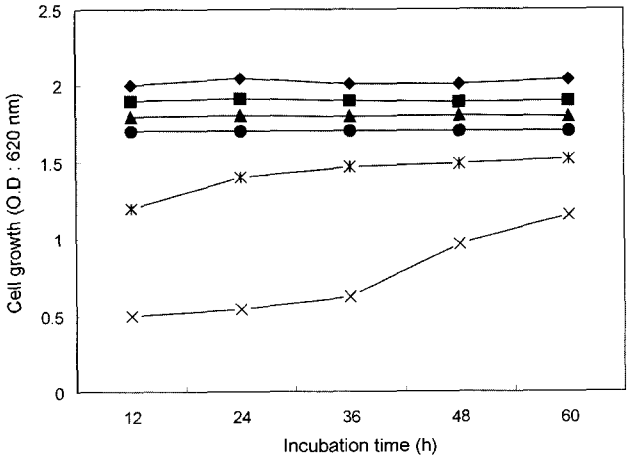

Fig. 1. Effect of NCS on growth of *S. mutans* JC-2.
-◆-: Control; -■-: 0.1 μM; -▲-: 10 μM; -●-: 50 μM; -*-: 100 M; -×-: 200 M.

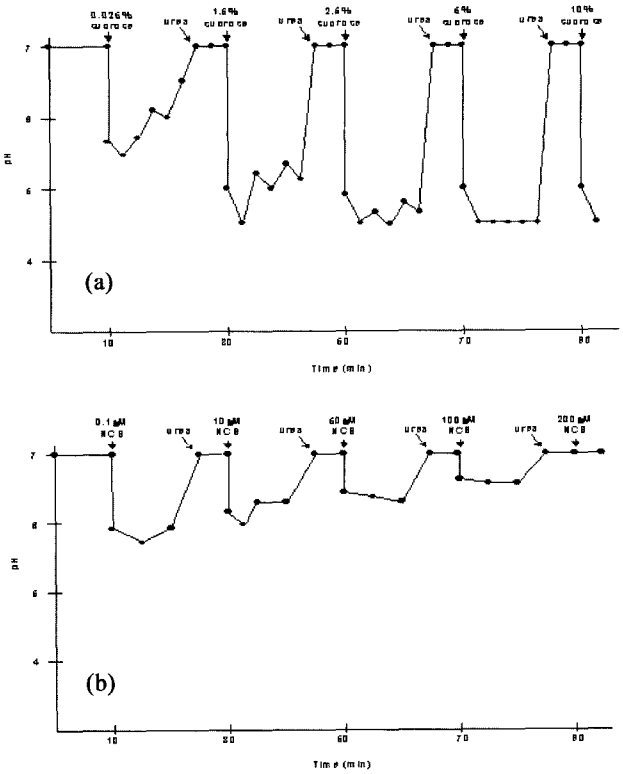

Fig. 2. Stephan curves of pH change when sucrose (a) and (b) NCS were added.

increased even at 100 μM NCS and growth in the logarithmic phase was decreased. In 200 μM NCS broth system, growth was clearly delayed, in which the lag phase was increased and growth was significantly decreased during the logarithmic phase until 36 h of culturing, thus definitely showing delayed growth, probably due to the fact that *S. mutans* JC-2 could not use NCS.

Inhibition of Acid Production by NCS on *Streptococcus mutans* JC-2

Measurement of acid production ability of this strain along with measuring growth are of great importance, since organic acids produced by *S. mutans* JC-2 are direct causes of dental caries, destroying the enamel and cement. Thus, after adding NCS at the concentrations ranging from 0.1 to 200 μM, which would inhibit the growth of *S. mutans* JC-2, the changes of pH in *S. mutans* JC-2 culture medium were measured. As shown in Fig. 2, the degree of pH decrease was lower as the concentration of NCS was increased according to the Stephan curve used. In the case of sucrose used as the control, on the other hand, the degree of pH decrease was higher as the concentration of NCS was increased, showing that dental caries would be prevented because of pH decrease within plaque. This was in good agreement with the result of Susanne and Gunther [33] who showed that the degree of pH decrease was

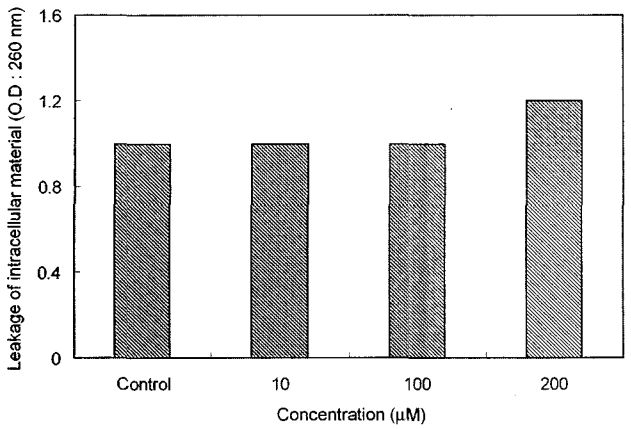

Fig. 3. Effect of NCS on the cellular permeability of *S. mutans* JC-2.

significantly lower with the addition of 50 mM nystose, which is a neosugar produced from sucrose by fungal fructosyltransferase and composed of 2 fructose molecules connected to sucrose and fructose through β,1→2 glycosidic linkage. Furthermore, nystose was more efficient in preventing dental caries, compared with disaccharide, trehalose, which is composed of 2 glucose molecules connected with α-1,1 bonding and is a sugar known to cause little dental caries [24].

Measurement of the Membrane Permeability of *Streptococcus mutans* JC-2

In order to measure the effect of NCS on the membrane permeability of *S. mutans* JC-2, various concentrations of the extract were added, and the cytoplasmic component eluted was measured colorimetrically. The results are shown in Fig. 3. At 0.1 μM, 1 μM, and 10 μM concentrations, the values were similar to that of the control, but increased at the concentration of 200 μM. This result indicates that NCS produced by *Bacillus* sp. S-1013 inhibited the growth of *S. mutans* JC-2, but did not elute the cytoplasmic component. Thus, NCS would not function as an antibiotic that elutes cytoplasmic component by inhibiting the synthesis of cell membrane. Streptomycin, kanamycin, capreomycin, and neomycin inhibit growth by lowering liposome protein synthesis through lowering m-RNA function. Among various actions of aminoglycoside antibiotics, some are involved in cell membrane (Jang *et al.* [16]), in cell surface receptors (Lanford *et al.* [20]), or in ion channel transport (March *et al.* [23]). Thus, unlike the general belief that aminoglycoside antibiotics participate in inhibiting protein synthesis and cell membrane permeability, their actions are probably due to innutrition of *S. mutans* JC-2, rather than the actions of antibiotics. The elution of some cytoplasm at high concentrations was probably due to autolysis resulting from apoptosis rather than the action of NCS.

Table 1. Inhibitory effect of NCS on GTase activity from *S. mutans* JC-2.

Concentration (μM)	Synthesized insoluble glucan (O.D. 550 nm)	Inhibition (%)
0	0.642±0.005	-
10	0.524±0.010	19.39
50	0.486±0.010	25.30
100	0.198±0.005	70.16
200	0.001±0.001	99.85

Mean±standard deviation.

Inhibitory Effect on GTase

As an extracellular enzyme secreted by *S. mutans* JC-2, GTase forms plaque by synthesizing glucan, which is an insoluble adhesive polysaccharide, from foods left in the mouth. Thus, the development of a non-caries-inducing sweetener could prevent it in the initial stage by inactivating GTase or inhibiting its activity. The effect of NCS, isolated from the new microorganism, on the activity of *S. mutans* GTase is shown in Table 1. GTase activity was inhibited by NCS, and the amount of insoluble glucan synthesized was decreased. The rate of inhibition was also increased as the concentration increased. In other words, when the amount of insoluble glucan produced was measured by measuring absorbance at 550 nm, the absorbance was 0.586±0.010 with the addition of 50 μM NCS, 0.168±0.005 with 100 μM, and 0.001±0.001 with 200 μM. Compared with the control, the rate of insoluble glucan synthesis was inhibited by 25.30, 70.16, and 99.8% with the addition of 50, 100, and 200 μM of NCS, respectively. Consequently, no glucan synthesis and enzyme activity were present with the addition of 200 μM NCS.

This GTase activity is an important up-stream target in the pathological cascade in which GTase participates in 2 series of reactions. In other words, it binds sucrose, fructose, and enzyme to break the glucosyl site, and transfers the fructose residue to the glucose residue of glucan; i.e., to the C-3/C-6 site. The GTase has a relatively independent domain and is biosynthesized by *S. mutans* [14]. It is known that *S. mutans* produces 3 types of GTase, including GTase-S1 synthesizing soluble glucan with a low molecular weight, GTase-1 synthesizing soluble glucan connected by alc α-1,3, and GTase-Se synthesizing insoluble glucan connected by α-1,6 [35]. GTase-1 has a signal 34 amino acid residues peptide at the N-terminus and is composed of 1,475 amino acids. Its sequence participating in sucrose binding is DSIRVDAVD (446- 454 residues), and 451 Asp is one of the centers of catalytic action. The binding site with glucan at the C-terminus is composed of 6 repeating units, each having 65 amino acid residues. More than 3 units are needed in glucan synthesis [1]. Among these, it is known that aminoglycoside antibiotics would work on the 451 Asp site to inhibit the enzyme activity. Park *et al.* [28]

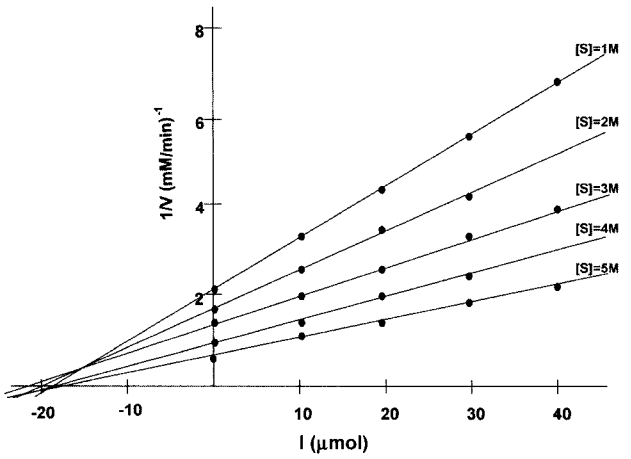

Fig. 4. Dixon plot of GTase inhibition by NCS.

reported that (+)-catechin at 1,000 μg/ml concentration inhibited the synthesis of insoluble glucan by 99.8%, Kim [17] reported that propolis purified from bee hives inhibited the enzymatic activity at 1,600 μg/ml concentration by 93.9%, and Park and Shin [27] reported that Phellodendron amurense Rupr Water-Extract at 1,000 μg/ml concentration inhibited the enzymatic activity by 63.3%. Furthermore, David and William [8] reported that sorbitol, xylitol, 1-deoxynojirimycin, and sucarose(1,4',6'trideoxy-trichloro-galacto sucrose) at 200 mM concentration inhibited GTase activity by +2%, 11%, 87%, and 57%, respectively. The sugar alcohols such as sorbitol and xylitol slightly inhibited GTase activity in *S. mutans*. In the case of 200 mM sorbitol, the enzymatic activity instead increased. Xylitol at 200 mM inhibited the enzymatic activity by 11%, showing better effect than sucrose. Thus, NCS effectively inhibited GTase activity even at low concentrations, unlike other substances reported in the literature.

Kinetic Study of GTase Suppression by NCS

The affinity index (K_m) of GTase for sucrose was 6.66 mM (data not shown). Furthermore, in order to actually compare the affinity index of GTase to NCS produced by *Bacillus* sp. S-1013, to the double reciprocal plot of Dixon was performed, and the result indicated a noncompetitive inhibition (Fig. 4). According to the figure, K_i and $K_{i'}$ values were 15 μM and 19.3 μM, respectively, suggesting that the affinity is better for the composition of EI complex and similar to the action of the acarbose reported by Kim *et al.* [18]. Furthermore, they reported that K_i and $K_{i'}$ of GTase in acarbose were 2.03 and 3.09 μM, respectively. The inhibition coefficient of NCS was lower compared with these values, probably due to a difference in chemical structure and properties.

Measurement of Decalcification Ratio in Dental Enamel

Table 2 shows the decalcification ratio of NCS at the concentrations ranging from 10 to 200 μM. As shown in the table, when 200 μM NCS was added, the decalcification ratio was 41%, being slightly lower than the 43% reported by Chun *et al.* [6] with the addition of a neomycin derivative at 2.5×10^{-7} μM, but higher than the 38% reported by Lucky Co. [21].

Table 2. Effect of NCS from *Bacillus* sp. S-1013 on the tooth decalcification.

Concentration (μM)	Decalcified ratio of tooth (%)
Control	1.47
200	0.60
100	0.97
50	1.31

Quantification of Calcium Ion Eluted from Teeth

Table 3 shows the results of the amount of calcium ion eluted from the extracted teeth in the presence of NCS at the concentrations ranging from 0 to 200 μM. As shown in the table, the amount of calcium ion eluted was 2.5 times higher in the presence of 200 μM NCS, compared with the control. Thus, we concluded that NCS isolated and purified from *Bacillus* sp. S-1013 has a relatively superior anticaries activity and possibly a commercial value.

Toxicity for Human Gingival Cells

Table 4 shows the results of cellular activity measured to evaluate the toxicity of NCS on human gingival fibroblasts. As the concentration of NCS increased, cellular activity decreased somewhat, however, the degree of decrease was low. The addition of 205 mM, which resulted in a 1,000-fold decrease of inhibition coefficient K_i, decreased the relative cellular activity to 71.77% by day 1 of culturing and to 79.27% by day 2 of culturing. At the concentrations lower than 15 mM, no significant difference was observed, compared with the control. High cellular toxicity has been the common problem in using antibiotics to treat dental caries and periodontal diseases. For example, chlorhexidine, having the antibacterial effect at 20 μg/ml and inducing cellular toxicity on human gingival cells, is the most widely used drug to treat dental caries and periodontal diseases [19]. However, Jang *et al.* [16] reported that chlorhexidine at 1.0 μg/ml and 10.0 μg/ml concentrations decreased the activity of human gingival cells by 75.2% and 99.7%, respectively. Thus, the NCS isolated and

Table 3. Effect of NCS from *Bacillus* sp. S-1013 on elution of calcium ion from tooth.

Concentration (μM)	Eluted calcium ion (%)
Control	0.38
200	0.14
100	0.33
50	0.38

Table 4. Cell activity of NCS on human gingival fibroblast cell.

	Time (h)	1	2	3	Average	%	variation	error
0	24	1.458	1.523	1.557	1.513	100	0.050	0.036
	48	4.163	4.163	4.154	4.160	100	0.005	0.004
20 mM	24	1.001	1.006	1.250	1.086	71.77	0.142	0.101
	48	3.308	3.246	3.339	3.298	79.27	0.047	0.033
2 mM	24	1.620	1.397	1.354	1.457	96.32	0.143	0.101
	48	4.149	4.163	4.015	4.109	98.77	0.081	0.058
200 μM	24	1.447	1.498	1.579	1.508	99.69	0.067	0.047
	48	4.022	4.163	4.152	4.112	98.85	0.078	0.055
20 μM	24	1.511	1.591	1.421	1.508	99.67	0.085	0.060
	48	4.055	4.143	4.163	4.120	99.05	0.057	0.041
0.2 μM	24	1.566	1.477	1.499	1.514	100.09	0.046	0.033
	48	4.035	4.163	4.163	4.120	99.05	0.074	0.052

purified from *Bacillus* sp. S-1013 in this study could be used in place of chlorhexidine, which poses many side effects including tooth discoloration, peeling of oral epithelial layer, and taste dysfunction. Furthermore, the present NCS could possibly replace non-caries-inducing sweeteners, including xylitol used in the place of sucrose. Also, the NCS produced by *Bacillus* sp. S-1013 could be applied in various fields as a sugar compound.

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