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Identification of Alkalophilic *Bacillus* sp. S-1013 Producing Non-Cariogenicity Sugar Fuc(1→4)galNAc(2→6)NeuAc and Optimization of Culture Condition for Its Production

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The study was performed to identification of producing microbe Non-Cariogenicity Sugar (NCS; Fuc(1→4) galNAc(2→6)NeuAc) with anti-caries activity, and to optimization of production condition. A typical strain which produced the NCS was identified alkalophilic *Bacillus* sp. S-1013 through the results of morphological, biochemical and chemotaxonomic characteristics and 16S rDNA sequencing. The optimal medium composition for the maximal production of the NCS from alkalophilic *Bacillus* sp. S-1013 was as follow: soluble starch 30 g, dextrin 15 g, yeast extract 5 g, peptone 10 g, K_2HPO_4 2 g in a liter of distilled water. Optimal temperature and pH were 25 and 11.0, respectively. The highest production of NCS was shown 60 hrs cultivation using the optimal medium, and then NCS productivity and dry cell weight of culture broth increased 4.24 and 2.67 time than initial medium, respectively.

Key words: Non-Cariogenicity Sugar, alkalophilic *Bacillus* sp. optimization

INRTODUCTION

The cariogenecity depends on the use of sucrose, one of the most effective ways to prevent dental caries is the substitution of sucrose for other sugars. Many researchers have examined the cariogenicity of several sugars such as lactose and galactose [7, 21] and oligosaccharides such as trehalose, raffinose, stachyose, vervascose, nystose and most of polyols have been determined to have low cariogenicity [9, 10, 11, 18, 19]. Out of monosaccharides and disaccharides, galactose has fermentability by oral microorganisms lower than that of sucrose or glucose [31], but is important in the determination of plaque baseline pH and plaque microorganism. As main component of saliva glycoprotein, galactose may be used solely by the hydrolysis by mucin and oral microorganisms, or consecutively by the fraction of other sugars [8, 14]. Neta *et al.* [24] reported that trehalose is not used as the substrate of glycosyltransferase (GTase), inhibits the synthesis of insoluble glucan by GTase when sucrose exists, and shows acid fermentability lower than that of sucrose by *Streptococcus mutans*. Meanwhile, most oligosaccharides including raffinose, stachyose, vervascose, nystose that is composed of 3~10 units of glycosides, and iso-maltooligosaccharide that is synthesized for commercial purposes are identified not to be metabolized by oral microorganisms [29, 33]. Various polyol such as xylitol are produced by extraction from plants or by synthesis through reduction of sugars. This study started an attempt at finding non-cariogenecity substitutes of sucrose from microorganism. We reported the purification and anti-caries of NCS(Non-Cariogenicity Sugar) from alkalophilic bacteria culture broth [27, 28]. The NCS was identified as Fuc(1→4)galNAc(2→6)NeuAc by FT-IR and NMR analysis. This compound inhibited formation of insoluble glucan which was formed by GTase of obiligate anaerobic oral microbe that colonizes the subgingival plaque. Therefore, the present study was undertaken first to identify of NCS (Fuc(1→4)galNAc(2→6) NeuAc) production microbe. Secondly, to investigate the feasibility of industrial production of NCS, and determined the optimal culture condition for the production by alkalophilic *Bacillus* sp. S-1013.

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MATERIAL AND METHOD

Bacterial Strain

The NCS producing strain S-1013 previously isolated from the tidal flat of the drainage area of the Man Kyung river near Kun-San city was maintained at -20°C in GYP slant (Glucose 10 g/l, Peptone 5 g/l, Yeast extract 5 g/l, KH_2PO_4 1 g/l, $MgSO_4 \cdot 7H_2O$ 0.2 g/l, Na_2CO_3 10 g/l, Agar 1.5 g/l). The strain was subcultured in the media whose composition was same with that of plate medium at 25°C for 3 days. Then, the S-1013 was lyophilized in ampoules to be preserved at -20°C.

Measurement of Cell Growth and Amount of NCS

The cell growth was expressed for dry cell weight. The amount of NCS was determined by HPLC (Shimadzu UV 1240, Kyoto, Japan) with RI detector (RID-10A, Shimadzu). The separation was made with a Pinnecle II C18 column (USP L-1, Shimadzu) eluted isocratically using 80% (v/v) acetonitril (35, 2 ml/min)

Morphological and Physiological Characteristics

The size and form of the isolated strain were observed by light microscope (Nicon, FK-IIA, Japan) after Gram staining based on the method provided by Gerhatdt *et al.* [8]. In order to observe the detailed form, the strain was gold-coated through negative staining by 2.0% phosphotungstic acid to be observed by using electron microscope (Philips, SEM 515, Netherland). The form, size, and color of the strain reared in glucose-nutrient agar medium, skim milk medium, and Luria-Bertani medium, respectively, and as to the movement, the strain that was cultured in semisolid medium (tryptose 1.0%, sodium chloride 0.5%, agar 0.5%) was observed.

Also, casein resolution, starch hydrolysis, gelatin liquefaction, sugar fermentability, indole production, nitrate reduction, and catalase and oxidase production were examined in order to identify the physiologic characteristics of the strains.

Biological Assimilation Test

The biological characteristics of the isolated strains were measured on the based of the methods provided by Bochner *et a. l*[2-4] and Lee *et al.* [15]. The isolated strains were pre-cultured in nutrient limited medium for 24 hours, suspended in sterilized saline to become optimal concentration, and separately injected to BiologTM GP Microplate containing various carbon source, nitrogen source, phosphate source, sulfur source, autotrofic supplement, and inhibitory compounds. The microplates were cultured for 4~24 hours, during which the sources were oxidized by the respiration of cells and the tetrazolium dye in the plates became purple. The patterns were compared with reference strain in similarity by using biology data base.

Chemical Characteristics

The chemical characteristics of the strains were analyzed on the base of the Method in Microbiology provided by Komagata and Suzuki [12, 13] and the Microbial identification system (MIS) provided by Miller [20], which were used to examine the fatty acid type in cell walls, DNA base composition (G+C mole content), and DNA sequence.

Gas chromatography (GC, HP 6890) was used to identify the composition of the whole cell fatty acid of the strains. The calibration standard kit provided by HP (Hewlett-Packard) was used as normal fatty acid. And, a cell fatty acid of strain analyzed by Sherlock program, identification program made by MIDI. The content of G+C mole in the isolated strains was analyzed by HPLC (Hitachi, L-3000, Japan) after the strains were cultivated in tryptic soy broth based on the method provided by Komagata and Suzuki [13] and the crude chromosomal DNA was extracted. The columns used were cosmosil 5C18, and the samples were analyzed at 270 nm by UV/VIS detector (SPD-10A, Shimadzu, Japan) while being eluted 1.0 mL/min at a time by using the solvent system in which 0.2 M $NH_4H_2PO_4$ and acetonitrile were mixed at the rate of 10:1.

16S rRNA Sequencing

As to the 16S rDNA gene analysis, PCR was performed by using Wizard Genomic DNA Prep Kit (Promega). The reaction condition was one minute of extension at 72°C, after one minute of denaturizing at 94°C and one and a half minute of annealing, and such operation was repeated for 30 cycles. The primer used in the operation was 27F (5'AGAGTTTGATCMTGGCTCAG) and 1492r (5'TACGGYTACCTTGTTACGACT T). After amplified fragments (16S rDNA region) was inserted in pGEM T Easy vector (Promega), the samples were transformed by using *E. coli* DH5 as host cell based on the general method of molecular cloning. The mini-preparation of plasmid was conducted by using Wizard plus SV miniprep DNA purification

system, and sequencing was performed by using automated DNA sequencer (ABI3700, Applied Biosystems Inc.). After the sequence was aligned by using Clustal X (ver. 1.8) software with reference to rRNA secondary structure, % similarity was computed and compared with the sequence provided by Genebank in Advanced Blast search. The phylogenetic tree and dendrogram were drawn up by neighbor-joining analysis [30] by using Treeview (ver. 1.66). The scale bar of the tree indicated 0.1 substitutions per site.

Optimization of Culture Condition

The effect of initial temperature, initial pH, carbon source, nitrogen source, phosphate, mineral and growth factor were investigated for optimal production of NCS.

RESULT AND DISCUSSION

Morphological and Physiological Characteristics

In solid medium, S-1013 strain was formed round-shaped, light yellow colonies with swelled and smooth surface in alkaline plates (pH 11.0)(data not shown). Also, the growth in alkaline liquid mediums were fair aerobic without precipitation of cells. Morphologically, the strain was short rod-shaped (as seen in the electron photomicrograph in Fig. 1), the size was relatively small (0.2 × 1.0 μm), and the flagella, though not certain because of surrounded fibers, were observed to be immovable and positive to Gram stain (see Table 1).

Also, Table 1 shows the physiological characteristics of S-1013. The pH range for growth was between 9.0 and 12.0, and the temperature range for growth was between 15

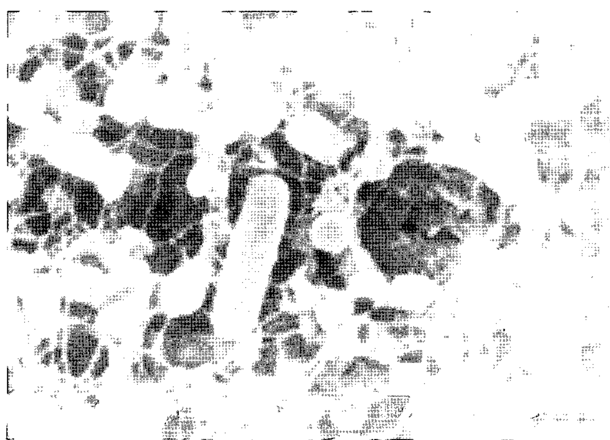

Fig. 1. Electron micrograph of the strain S-1013 by SEM (× 15,000).

Table 1. Morphological and Physiological properties of the strain S-1013.

Shape	Short rod
Cell size	0.2 × 1.0 μm
Gram stain	positive
Spore	Oval, central
Temperature range for growth	15~45°C
pH range for growth	9.0~12.0
NaCl tolerance for growth	≤ 20%
Catalase	+
Oxidase	+
Lecithinase	+
Lipase	−
Phenylalanine deamination	−
Hydrolysis of	
Starch	+
Casein	+
Cellulose	−
Esculin	+
Indole production	+
Levan formation from sucrose	+
NH_3 production from arginin	+
NH_3 production from pepton	+
Gelatin liquefaction	+
Methyl-red test	−
Voges-Proskauer reaction	+
Nitrate reduction	+
Action on milk	
Coagulation	−
Peptonization	−
Oxidation-Fermentation test	Fermentation
Growth on nutrient plate	−

− : negative, + : positive

and 45°C. The strain was grown even in 20% of NaCl, hydrolyzed starch and casein, showed positive to catalase and oxidase, and negative to cellulose hydrolysis. Also, the strain could produce indol, produced NH_3 from arginin and pepton, and was not identified to be grown in neutral nutrient medium.

Biological Assimilation Test

The biological characteristic of the isolated strain was shown in Table 2. The strain showed 13.3% of similarity with *Brevibacillus agri* in Biological Assimilation test using BiologTM GP Microplate, indicating a possibility of new microorganism [5, 22, 24, 31, 32].

Chemical characteristics

The results of the analysis on the cell fatty acid of S-

Table 2. Biological assimilation test of the strain S-1013.

Water	-	α-Methyl-D-galactodise	-	Propionic acid	+
α-cyclodextrin	+	β-Methyl-D-galactodise	-	Pyruvic acid	+
β-cyclodextrin	+	3-Methyglucose	+	Succinamic acid	-
Dextrin	+	α-Methyl-D-glucoside	+	N-acethyl-L-glutamic acid	-
Glycogen	+	β-Methyl-D-glucoside	+	Alaninamic acid	-
Inulin	-	α-Methyl-D-mannoside	-	D-Alanin	-
Mannan	+	Palatinose	+	L-Alanin	-
Tween 40	-	D-Psicose	+	L-Alanyl-glycine	-
Tween 80	-	D-raffinose	+	L-Asparagin	-
N-acethy-D-glucosamin	-	L-Rhamnose	+	L-Glutamic acid	-
N-acethy-D-mannosamin	-	D-Ribose	+	D-xylose	+
Amygdalin	-	Salicin	+	L-Malic acid	+
L-Arabinose	+	Sedoheptulose	-	Glycyl-L-glutamic acid	-
D-Arabitol	+	D-Solbitol	+	L-Pyroglutamic acid	-
Arbutin	+	Stachyose	+	L-Serin	-
D-Fructose	+	D-Tagatose	+	Putrescine	-
D-galactose	+	D-Trehalose	+	2,3-Butanediol	-
D-galacturonic acid	-	Turanose	+	Glycerol	+
Gentiobiose	+	Xylitol	+	Adenosin	-
D-gluconic acid	-	Acetic acid	+	2-Deoxyadenosin	-
α-D-glucose	+	α-hydroxybutylric acid	-	Inosine	-
m-Inositol	-	β-hydroxybutylric acid	+	Thymidine	+
D-fucose	+	γ-hydroxybutylric acid	+	Adenosin-5-monophosphate	-
α-D-Lactose	+	p-hydroxyphenylacetate	-	Thymidine-5-monophosphate	-
Lactutin	-	α-Ketoglutaric acid	+	Fructose-6-phosphate	-
Maltose	+	Lactamide	+	Glucose-1-phosphate	-
Maltotriose	+	D-Lactic acid methylester	-	Glucose-1-phosphate	-
D-Mannitol	+	L-Lactic acid	-	D-L-α-Glycerol phosphate	-
D-Mannose	+	D-malic acid	-	D-melibiose	+
D-Melezitose	+	Methyl pyruvate	-	mono-methyl succinate	+

1013, the principal components were iso-15:0 (63.95%) and anteiso-15:0 (16.17%) of branched type (see Table 3), indicating the representative characteristic of *Bacillus* sp.. The results corresponded to those of the study by Komagata and Suzuki *et al.* [13] reporting that the principal component of the cell fatty acid of *Bacillus* bacteria was 13-methyl tetradecanoic acid (C15:0-iso) and 12-methyl tetradecanoic acid (C15:0-anteiso) of blanched type. However, in the comparison with the reference, the results showed 39.8% to *Bacillus purmillus*, 26.8% to *Bacillus megaterium*, 22.7% to *Bacillus sphaericus*, and 27.0% of similarity to *Brevibacillus agri*, indicating remarkably low reliability. Therefore, the authors performed additional examinations as below. Meanwhile, the comparison of the compositions of cell fatty acid of S-1013 and *Bacillus alcalophillus* AFO 078812 growing in extremely alkaline environment [6] (see Table 3) showed that the compositions of the principal component of the two strains were opposed to each other, though the two grew in the same alkaline condition.

Also, the results of content analysis on G+C mole showed that the content of G was 24.09%, that of C was 30.16%, and that of G+C mole was 54%, similar to the characteristics of *Bacillus* sp. [25].

16S rDNA Sequencing

The results of 16S rDNA gene alignment by using Clustal X (ver. 1.8) software determined the sequence of 1492 bp (data not shown). Meanwhile, the % similarity was compared with the reference (data not shown). The isolated strain was identified to be *Bacillus* genus when such results were considered. The strain may be most similar to the reference strain of *Bacillus alcalophilus* AFO 078812 (95.7% of similarity), but the two strains cannot be considered to be the same species based on the results of the analysis on cell fatty acid and biological

Table 3. Fatty acid composition of total membrane lipid extracted the strain S-1013.

Fatty acid	% of fatty acid in membrane	
	Bacillus alcalopilus AFO 078812 (pH 10.5)	S-1013 (pH 11.0)
C12:0 iso	2	ND
C14:0 iso	1	3.0
C14:0 normal	1	0.75
C15:0 iso	21	63.95
C15:0 anteiso	29	16.17
C15:0 normal	ND[b]	0.31
C16:0 w7c alcohol	ND	1.86
C16:0 iso	6	0.90
C16:0 normal	5	0.66
C16:1 normal	13	ND
C16:1 w11c	ND	1.57
C17:0 iso	12	5.86
C17:0 anteiso	1	2.01
C17:1 w10c iso	2	1.25
C17:1 anteiso	2	ND
C18:0 normal	2	ND
C18:1 normal	1	ND
C18:2 normal	2	ND

ND (not detected)

similarity. Therefore, the strain was considered to be new species of *Bacillus* genus including 75 species, and was named as *Bacillus* sp. S-1013. The phylogenetic tree based on Neighbor-joining method [30] is shown in Fig. 2, the scale bar of the tree meaning 0.1 substitutions per site.

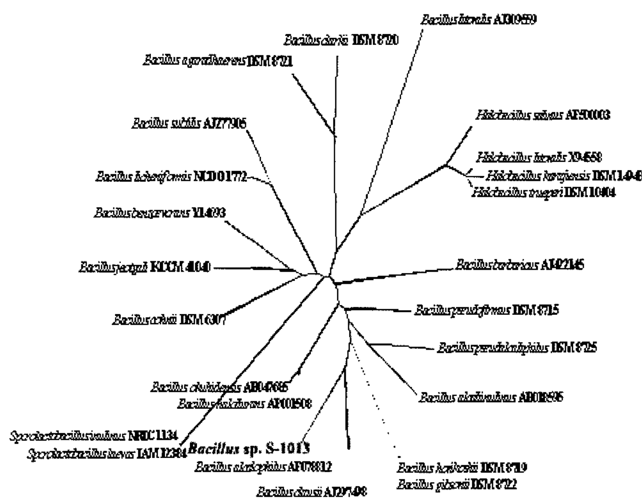

Fig. 2. Phylogenetic tree of the *Bacillus* sp. S-1013.

Optimization of Culture Conditions

Initial Temperature : The effect of the initial temperature on the cell growth and NCS production are shown in Fig. 3 Both the cell growth and NCS production reached to the maximum point at 30°C, and the results are similar to those of Lee *et al.* [16] reporting that the optimal temperature for producing oligosaccharide by *Bacillus cereus* IAM 1072 hit the maximum point at 35°C.

Initial pH : Fig. 4 had shown the effect of initial pH for the cell growth and NCS production, which reached to the maximum points when pH was 11. Such results corresponded to the characteristics of the isolated strain, the alkalophilic bacillus that can grow only in alkaline conditions, not in neutral pH.

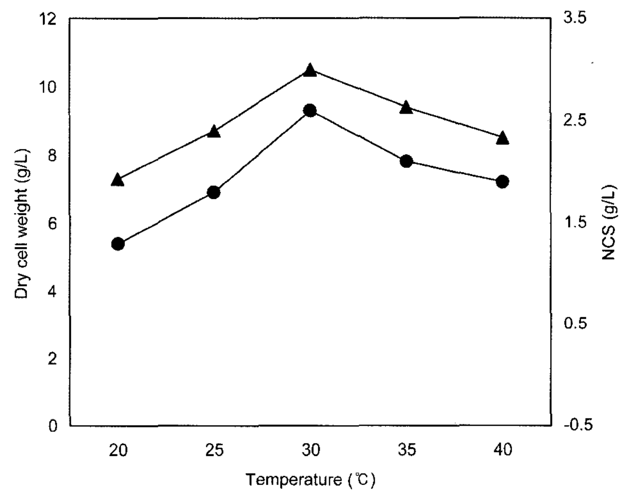

Fig. 3. Effect of temperature on the NCS production and growth of *Bacillus* sp. S-1013. -●- : NCS -▲- : Dry cell weight.

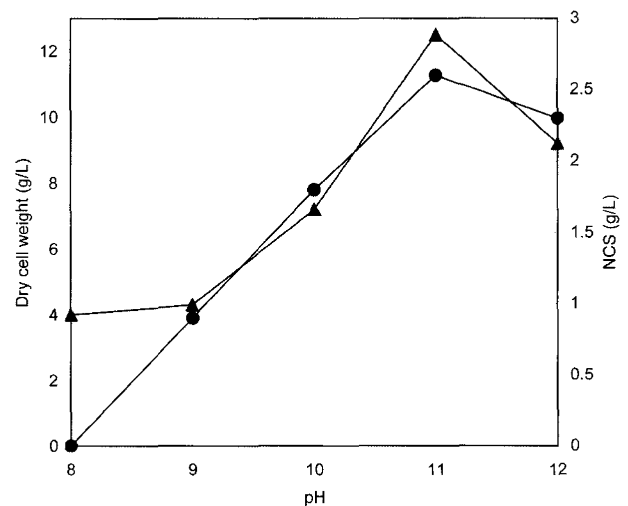

Fig. 4. Effect of pH on the NCS production and growth of *Bacillus* sp. S-1013. -●- : NCS -▲- : Dry cell weight.

Carbon Sources

1.0% of carbon sources were added to the basic medium instead of glucose for 60-hour culture in order to investigate the effect of various carbon sources on the cell growth and NCS production.(data not shown) The cell growth and NCS production provided the highest productivity when soluble starch was added. Such results corresponded to those of Lee *et al.* [16] reporting that the production of oligosaccharide by *Bacillus cereus* showed the highest productivity when soluble starch was added. Adding, monosaccharide such as glucose, galactose and fructose or disaccharide such as lactose also decreased the production considerably. On the other hand, adding polysaccharides except CMC showed relatively superior NCS productivity, indicating that the strain produced NCS by using polysaccharides. Also, had shown the results of the experiments in which soluble starch and dextrin having the highest NCS were reached to the maximum point when 3% of soluble starch and 2.5% of dextrin was added, respectively (see Fig. 5). Meanwhile, when 3% of soluble starch and 2.5% of dextrin were mixed, rather than when each carbon sources were solely added. The cell growth and NCS production are increased by 10%. Ahn *et al.* [1] reported that 12% of dextrin provided the maximum results in the production of saccharide by *Bacillus* sp. A29, Lee *et al.* [17] reported that 1.5% of chitin provided the maximum results in the production of N-acetyl-chitooligosaccharide by *S. marcescen*. The strain in this study showed favorable growth and effectively produced NCS in the medium containing relatively low-concentration carbon sources.

Nitrogen Sources

The organic nitrogen sources such as yeast extract induced favorable results in the production, while inorganic nitrogen sources including KNO_3 provided relatively low productivity for NCS. Out of organic nitrogen sources, yeast extract, peptone and tryptone remarkably increased the NCS productivity (data not shown). As seen in Fig. 6, the cell growth were reached to the maximum point when 1% yeast extract, 1% tryptone and 1% peptone were added, respectively (see Fig. 6). The mixed addition of yeast extract 0.5%, tryptone 1%, and peptone 1% produced somewhat better results than the sole addition of each nitrogen source. Lee *et al.* [17] reported that the production of oligosaccharide was considerably increased when yeast extract, peptone and tryptone were added.

Phosphate and Mineral Source

Fig. 7 had shown the effect of phosphate on the cell growth and NCS production. 0.2% of K_2HPO_4 induced the highest productivity. On other hand, the productivity was rapidly reduced when metal salts such as $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 4H_2O$, $MnCl_2 \cdot 4H_2O$, $FeCl_3 \cdot 6H_2O$, and $CoCl_2 \cdot 6H_2O$ except $BaCl_2 \cdot 2H_2O$ were used.(data not shown) Such results were similar to those of Petronella *et al.* [26] reporting that using K_2HPO_4 increased the productivity of

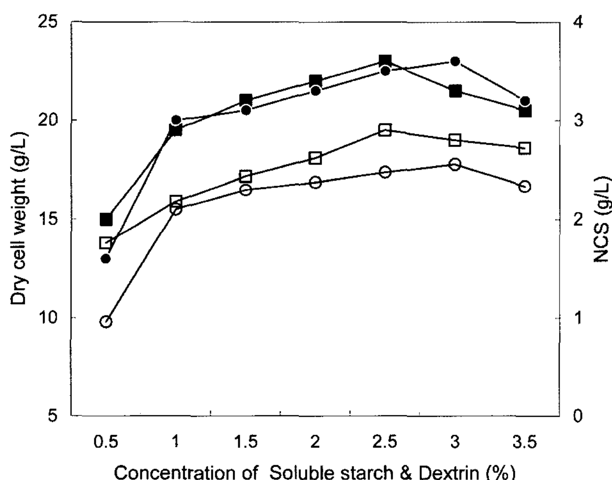

Fig. 5. Effect of soluble starch and dextrin concentration on the NCS production and growth of *Bacillus* sp. S-1013. -●- : NCS at soluble starch, -○- : NCS at dextrin, -◆- : Dry cell weight at soluble starch, -◇- : Dry cell weight at dextrin.

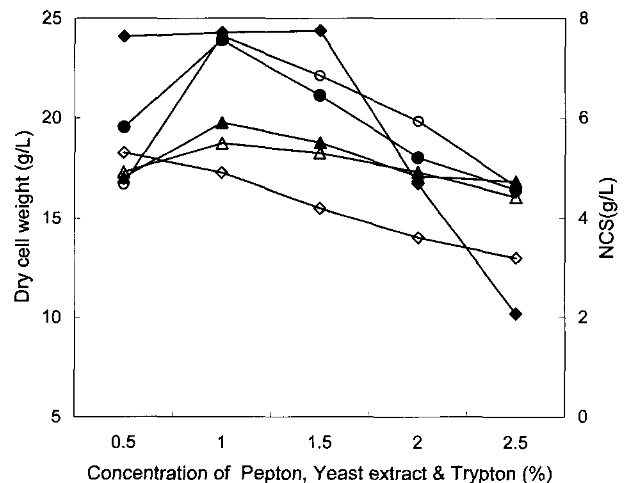

Fig. 6. Effect of pepton, yeast extract and trypton concentration on the NCS production and growth of *Bacillus* sp. S-1013. -●- : NCS at pepton, -○- : Dry cell weight at pepton, -◆- : NCS at yeast extract, -◇- : Dry cell at weigh tyeast extract, -▲- : NCS at trypton, -△- : Dry cell weight at trypton.

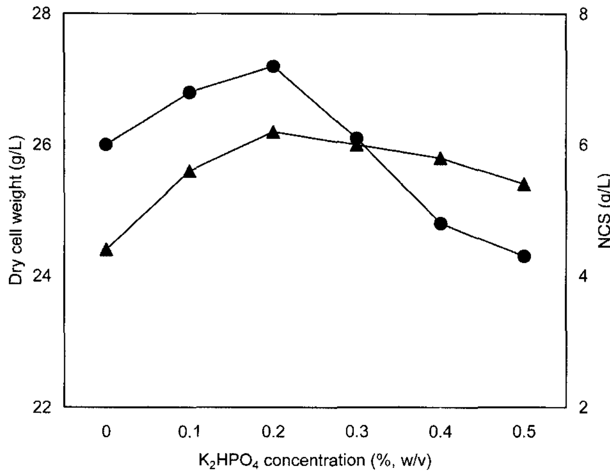

Fig. 7. Effect of K_2HPO_4 concentration on the NCS production and growth of *Bacillus* sp. S-1013. -●- : NCS, -▲- : Dry cell weight.

Lactococcus lactis exo-polysaccharide and using metal salts such as Cu^{2+} decreased the productivity. Also, Growth Factor and Amino Acids hardly affected the NCS production of the strain used in this study.

Table 4 had shown the optimal composition for the medium for producing NCS by *Bacillus* sp. S-1013.

Production of NCS

The cell growth and NCS production reached to the stationary phase after 60 hours on the optimal medium. Meanwhile, the NCS production and cell growth in basic medium and optimal medium were 1.7 g, 9.8 g and 7.2 g, 26.2 g, respectively. (see Fig. 8) Therefore, both NCS production and cell growth were increased more in the optimal (4.24 and 2.67 times) than in the basic medium.

Table 4. Optimal culture condition and medium Composition for NCS production and cell growth.

Ingradient	%
Soluble starch	3
Dextrin	2.5
Yeast extract	0.5
Pepton	1
K_2HPO_4	0.2
Temperature	30°C
pH	11
Inoculum size	5
Working volume	20
Agitation speed	50 rpm
Culture Time	60 hrs

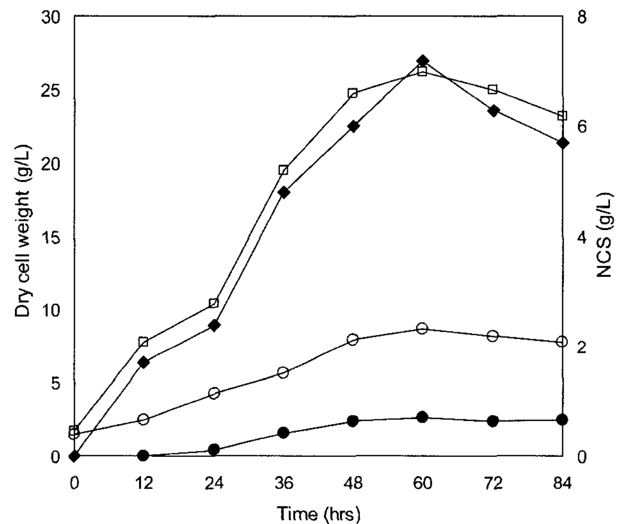

Fig. 8.Time course of the NCS production and growth of *Bacillus* sp. S-1013 on the basal and optimal condition. Basal condition : -●- : NCS, -○- : Dry cell weight, Optimal condition : -■- : NCS, -□- : Dry cell weight.

ACKNOWLEDGMENT

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초 록

비우식성 당 Fuc(1→4)galNAc(2→6)NeuAc를 생산하는 호알칼리성 *Bacillus* sp. S-1013의 동정 및 생산조건의 최적화

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비우식성 당 NCS(Fuc(1→4)galNAc(2→6)NeuAc)를 생산하는 미생물을 동정하고 이 미생물이 생산하는 비우식성

당의 생산조건을 최적화하기 위하여 본 연구를 수행하였다. NCS를 생산하는 균주는 형태학적, 생화학적, 화학분 류학적 특성 및 16S-rRNA 염기서열 결정을 통하여 *Bacillus* sp. S-1013로 동정되었다. 또한, 이 균주에 의한 NCS의 최적 배지조성은 soluble starch 3.0%, dextrin 1.5%, yeast extract 0.5%, pepton 1.0%, K_2HPO_4 0.2%였으며, 최적온도 및 pH는 각각 25와 11.0이였다. 최적배지에서 NCS 및 균체의 생산은 기본배지에 비해 각각 4.24배 및 2.67배 증가하였다.

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