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Optimization of culture conditions of *Bacillus subtilis* with α-glucosidase inhibitory activity

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Abstract

1-Deoxynojirimycin (DNJ) have been extensively investigated for their α-glucosidase inhibitor on postprandial hyperglycemia, and applied in nutraceuticals and medicine for preventing or delaying progression of type 2 diabetes. However, the amount of DNJ in mulberry leaves is low (about 0.1%), therefore, more effective extraction method is needed. This study was performed to develop microbial DNJ for biological methods of DNJ as an alternative to the chemical methods. In this study, we obtained evidence for *Bacillus subtilis* that produce DNJ in large quantities by high performance liquid chromatography. Inhibition of α-glucosidase activity was determined to DNJ production or non-production. Investigation of the effect of mulberry leaves powder concentration (1~5%), using the DNJ high-production bacteria, provided evidence for microbial mass production of DNJ. When the 4% mulberry leaf powder for 9 days was used, the α-glucosidase inhibitory activity was over the 85%. Also, the results presented in this study confirm DNJ yield's increasement in microbes using the various of nutrients and provide insight of ways to improve DNJ yields in microorganisms.

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Introduction

Diabetes is a common metabolic disease characterized by abnormally high plasma glucose levels, leading to major complications, such as diabetic neuropathy, retinopathy, and cardiovascular diseases. One therapeutic approach to treat diabetes is to retard the absorption of glucose via inhibition of enzymes, such as α-glucosidase (Kim *et al*., 2008; Holman *et al*., 1999). α-glucosidase (EC 3.2.1.20, 3.2.1.10, 3.2.1.48 and 3.2.1.106) are exo-acting carbohydrases distributed widely in microorganisms, plants, and animal tissues, which catalyze release of α-D-glucopyranose from the non-reducing ends of various substrates (Frandsen and Svensson, 1998). As the inhibitor of α-glucosidase activity involved in diabetes, 1-deoxynojirimycin (DNJ) have been obtained in the field of HIV infection (Gruters *et al*., 1987; Mehta *et al*., 1998), Gaucher's disease (Butters *et al*., 2003), and diabetes (Asano *et al*., 1994). This inhibitor combine with intestine α-glucosidase and block the uptake of postprandial blood glucose and have been extensively investigated for their α-glucosidase inhibitory effects on postprandial hyperglycemia, and applied in nutraceuticals and medicine for preventing or delaying progression of type 2 diabetes (Asai *et al*., 2011; Kimura *et al*., 2007; Vichasilp *et al*., 2012).

The potential application of the poly-hydroxylated alkaloid 1-Deoxynojirimycin (DNJ) have stimulated the development

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of methods for producing DNJ and its derivatives for use in functional foods and by the pharmaceutical industry (Asano *et al*., 2001). Generally, the α-glucosidase inhibitors can be isolated naturally from plants or food products. However, the extraction method from plants or food products has the limitations of low amount of DNJ extracted, in consistent yield of DNJ, and requirement for complex purification steps. On the other hand, DNJ can be synthesized chemically or produced by microorganisms. DNJ synthesized by microorganisms is an effective strategy to produce cost-effective and productive α-glucosidase inhibitors. *Streptomyces* (Iwasa *et al*., 1970), *Actinoplanes* (Schmidt *et al*., 1977) and *Flavobacterium saccharophilium* (Kameda *et al*., 1980), were able to synthesize α-glucosidase inhibitors. Also, there is thus an urgent need to develop an alternative DNJ production method such as by microbial fermentation (Hardick *et al*., 1991; Hardick and Hutchinson, 1993). It has been reported that DNJ has long been thought to be produced in several strains of *Bacillus* (Hardick and Hutchinson, 1993, Stein *et al*., 1984) and *Streptomyces* spp. (Ezure *et al*., 1985; Hardick *et al*., 1991, Paek *et al*., 1997).

In this study, we performed to develop microbial DNJ for biological methods of DNJ as an alternative to the chemical methods. It was conducted to determine factors that influence the activity of α-glucosidase inhibitor produced by *Bacillus subtilis*. under various fermentation conditions. Activity of α-glucosidase inhibitor produced by *Bacillus subtilis* using various culture conditions was analyzed to produce cost-effective and productive α-glucosidase inhibitors.

Materials and Methods

Culture condition

The *Bacillus subtilis* isolated from soil was grown on mulberry leaf powder (MLP) media (g/L): K_2HPO_4 14, KH_2PO_4 6g, $MgSO_4 \cdot 7H_2O$ 0.2, $(NH_4)_2SO_4$ 2, $MnSO_4$ 0.0017, $Fe_2(SO_4)_3$ 0.028, $ZnCl_2$ 0.007, $CaCl_2$ 0.15, glucose 50g with each 1% to 5% MLP (Daniel CS *et al*., 1984). Mulberry leaf powder was collected from the Sericulture and Apiculture Division for Department of Agricultural Biology, RDA, Republic of Korea. These samples were kept in plastic bags and stored at 4°C until use and dried at room temperature for few days, ground to powder by a motar and pestle, and passed through 150 μm sieves.

The optimum condition of mulberry leaf powder concentration and time course

Before doing investigation of other nutrient conditions, first of all, the optimum condition of mulberry leaf powder concentration and time course were determined. These were investigated by the following condition for optimum α-glucosidase inhibitory activity. Sterilized growth medium was inoculated with 1% (v/v) of *Bacillus subtilis* culture suspention and incubated at 37°C with shaking 150 rpm for 5 d. The precipitate of culture broth was removed through centrifugation at 6,000 rpm for 10 min. The resulting mixture was dialyzed against distilled water at 4°C overnight and used as sample for α-glucosidase inhibitory activity.

I. Mulberry leaf powder concentration: 0, 1, 2, 3, 4, and 5% (w/v)
II. Time course condition: 3, 5, 7, and 9 d

Effect of various nutrient concentration on α-glucosidase inhibitory activity

To investigate the effect of K_2HPO_4 and KH_2PO_4 concentration, K_2HPO_4 and KH_2PO_4 were added with 0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, and 1.4% (w/v) (K_2HPO_4) and 0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6% (w/v) (KH_2PO_4) in MLP media. Also, $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$ were added with 0, 0.05, 0.1, 0.15, 0.2% (w/v) ($(NH_4)_2SO_4$) and 0, 0.05, 0.1, 0.15, 0.2% (w/v) ($MgSO_4 \cdot 7H_2O$).

Determination of α-glucosidase inhibitory activity

The inhibitory activity of the fermentation broth against was determined by reaction between α-glucosidase and 4-nitrophenyl α-D-glucopyranoside (4-NPG) according to the protocol by Yamaki and Mori (2006). The fermentation broth was serially diluted with an equal volume of distilled water and dispensed into wells of the plates (20 μL per well) followed by the addition of 5 μL of suspension of rat intestine acetone powder (Sigma-Aldrich), 12mM 4-NPG 50 μL as substrate, and 75 μL of 0.1M potassium phosphate buffer (pH 6.8). The mixture was incubated at 37°C for 35 min to allow

α-glucosidase to react with 4-NPG and produce 4-nitrophenol. The reaction was terminated with the addition of Na_2CO_3 (50 μL, 200 mM). Formation of 4-nitrophenol in each well was measured by the intensity of absorbance at 405 nm using a microplate reader (BioTek Instruments Korea Ltd. Model Synergy HT).

< Calculation >

Inhibition (%) = A_{405}(inhibition) – A_{405}(control) / A_{405}(enzyme) - A_{405}(blank) X 100

Sample preparation procedures for crude DNJ

Sterilized growth medium was inoculated with 1%(v/v) of the isolated bacteria culture suspention and incubated at 37°C with shaking 150 rpm 5 days. The precipitate of culture broth was removed through centrifugation at 6,000 rpm for 10 min. Three volumes of cold ethanol were added to the supernatant to precipitate DNJ, which was recovered by centrifugation as above. The precipitate was lyophilized, dissolved in 10 mM Tris/HCl buffer (pH 8.0). The resulting mixture was dialyzed against distilled water at 4 °C overnight and used sample for HPLC analysis.

HPLC analysis for DNJ measurement

The purity of the active compound was determined by a high-performance liquid chromatography (HPLC) method. HPLC analysis was modified based on a method with an amide type column and an evaporative light-scattering detector (ELSD) (Kimura *et al*., 2004). For DNJ content measurement of testing materials, analysis instrument was high performance liquid chromatography, The Luna 3u NH_2 100A column (150 × 2.00 mm, Phenomenex) was used in the SHISEIDO SP3203 HPLC system. The separation was performed using a mixture of acetonitrile and distilled water (81:19, v/v, containing 6.5 mM ammonium acetate; pH 5.5). The flow rate was adjusted to 1 mL/min, and the column temperature was maintained at 70°C. The eluent was split at the postcolumn. The DNJ standard and the purified sample were weighed accurately and dissolved in a mixture of acetonitrile and water (50:50; containing 6.5 mM ammonium acetate; pH 5.5), and 20 μL samples of these solutions were subjected to the HPLC-ELSD system.

Statistical analysis

Each experiment was carried out in triplicate, all data were the average of three independent experiments and analyzed by SPSS (version 18.0), and expressed as mean ± standard deviation (SD). Results were considered significant at $p < 0.05$.

Results and Discussion

The optimum condition of mulberry leaf powder concentration and time course for Bacillus subtilis

This study shows that there is a great variation in α-glucosidase inhibitory activity according to isolated strain *Bacillus subtilis* fermentation. These differences are most probably arisen from the variation in processing techniques and microorganisms used. For using a mulberry leaf powder (MLP) as mulberry leaf powder concentration which inoculated *Bacillus subtilis* DS-21 on becoming a higher the α-glucosidase inhibitory activity, α-glucosidase inhibitory activity of these conditions were monitored (Fig. 1.). When the 4% mulberry leaf powder was used, the α-glucosidase inhibitory activity was over the 80%. Ju *et al* (2015) reported that fermentation of MLP was the best method for producing DNJ. The other studies show that fermented soybeans products possess anti-diabetic properties (Fujita *et al*., 2001; Fujita *et al*., 2003; McCue *et al*., 2005) and the douchi extract demonstrates excellent anti-hyperglycemic effect without causing any side

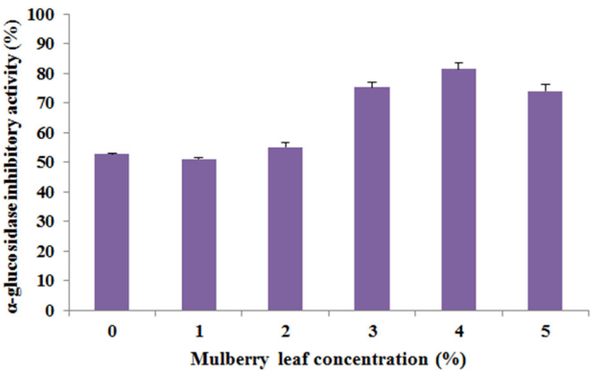

Fig. 1. The α-glucosidase inhibitory activity according to mulberry leaf concentration difference in MLP media. The data represent means±SDs (n=3).

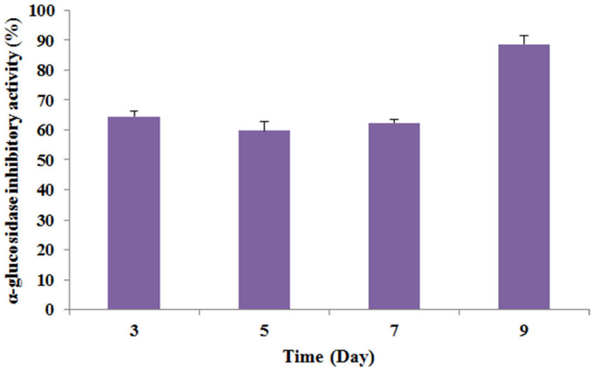

Fig. 2. The α-glucosidase inhibitory activity according to time course in MLP media. The data represent means±SDs (n=3).

effects such as diarrhea, retching and flatulence, which are commonly encountered with the use of currently available α-glucosidase inhibitory therapeutic drugs (Fujita *et al*., 2003). Mulberry leaves (Moraceae) rich in iminosugars such as the glucose analogue 1-deoxynojirimycin (DNJ), N-methyl- DNJ, and 2-O-R-D-galactopyranosyl-DNJ, DNJ being the most abundant and accounting for 50% of the mulberry iminosugars (Asano *et al*., 2001). The infusion of mulberry leaves powder is consumed as antihyperglycemic nutraceutical foods for patients with diabetes mellitus (Kim *et al*., 2003). The incubation time was an important factor for α-glucosidase inhibitory activity. It was a result of α-glucosidase inhibitory activity according to mulberry leaf powder concentration. (Fig. 2.). When the *Bacillus subtilis* was incubated for 9 d, inhibitory activity was high on over 85%. The α-glucosidase inhibitory activity of *Bacillus subtilis* B2 fermentation was increased slightly after 6 d and in the production of commercial douchi by fermentation using *Aspergillus oryzae*, *Actinomucor elegans* and *Rhizopus arrhizus*, very low activity of a-glucosidase inhibitor was found during the first 48 h of fermentation (Zhu *et al*., 2008).

Effect of various nutrient concentration on α-glucosidase inhibitory activity

Results of various nutrient concentration in the MLP broth were reported. The *Bacillus subtilis* was grown on K_2HPO_4 14, KH_2PO_4 6g, $MgSO_4 \cdot 7H_2O$ 0.2, $(NH_4)_2SO_4$ 2, $MnSO_4$ 0.0017, $Fe_2(SO_4)_3$ 0.028, $ZnCl_2$ 0.007, $CaCl_2$ 0.15, glucose 50g with each 1% to 5% MLP. The effect of K_2HPO_4 and KH_2PO_4 on the α-glucosidase inhibitory activity was determined (Fig. 2.). When K_2HPO_4 and KH_2PO_4 were used on 1.2% and 0.6% concentration, respectively, the inhibitory activity was over 70%. As a matter of fact, most of the effective α-glucosidase inhibitors, such as validamycin A, acarbose and validamine, isolated from growth of microorganisms are carbasugars and pseudoaminosugars inhibitors (Iwasa *et al*., 1970; Zheng *et al*., 2006). Seo *et al* (2013) reported that the carbon and nitrogen sources were optimized for DNJ production by *Bacillus amyloliquefaciens* 140N and soluble starch (2%) had the highest effect with 88.9% inhibition.

Fig. 3 was showed the effect of $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$ on the α-glucosidase inhibitory activity. As a results, 0.1% $(NH_4)_2SO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ was the highest α-glucosidase inhibitory activity. The concentration of DNJ of *Bacillus amyloliquefaciens* AS385 may be further improved by optimization of the culture media or by mutagenic treatments, currently under investigation in our laboratory, in order to enable more efficient mass production of DNJ (Ezure *et al*., 1985). It has been suggested that different nitrogen sources may play an important role in the synthesis of α-glucosidase inhibitor because they may affect the synthesis of some enzymes related to the α-glucosidase inhibitor. This result was consistent with a previous report by Zheng *et al* (2006) who reported that different sources of nitrogen could

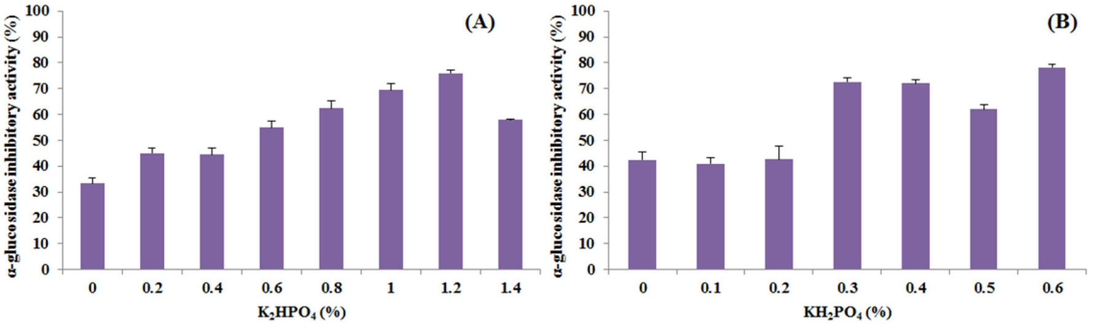

Fig. 3. Effect of K_2HPO_4 (A) and KH_2PO_4 (B) on the α-glucosidase inhibitory activity in MLP media. The data represent means±SDs (n=3).

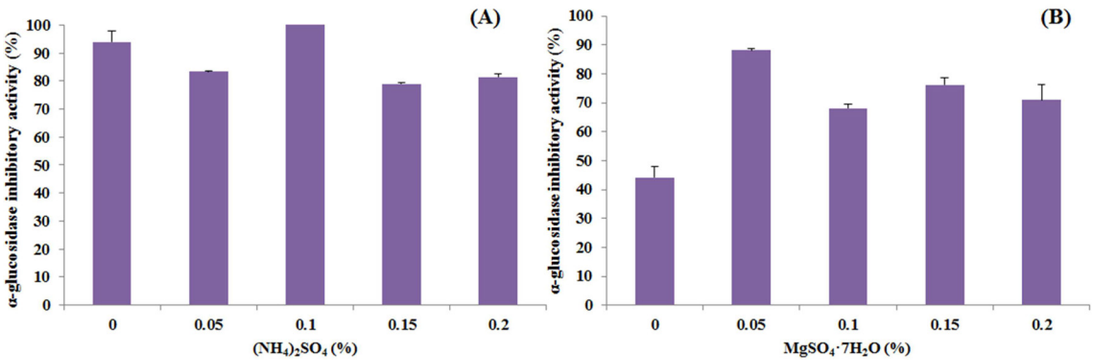

Fig. 4. Effect of $(NH_4)_2SO_4$ (A) and $MgSO_4 \cdot 7H_2O$ (B) on the α-glucosidase inhibitory activity in MLP media. The data represent means±SDs (n=3).

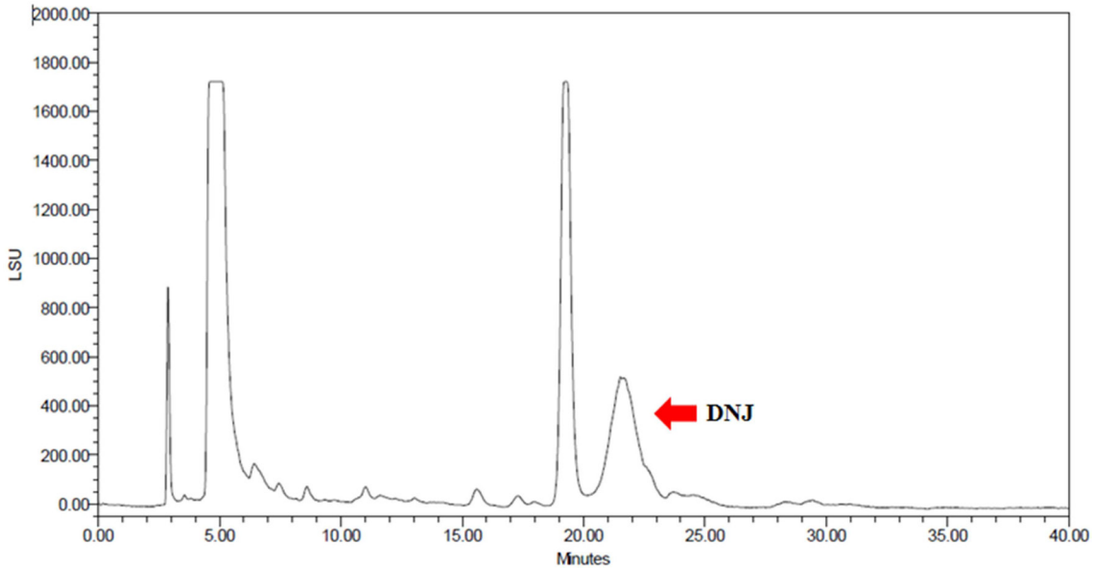

Fig. 5. HPLC chromatograph using ELSD detection. HPLC chromatogram of DNJ produced by optimal culture condition with *Bacillus subtilis*

affect yields of valienamine produced from *Stenotrophomonas maltrophilia*. The large production of DNJ in nongrowing cells might be analogous to the uncontrolled or derepressed synthesis of primary metabolites such as vitamins or amino acids. Therefore, appropriate medium condition is useful for high DNJ content and α-glucosidase inhibitory activity.

HPLC analysis for DNJ contents

Fig. 5 is quantitative analysis of produced DNJ by HPLC chromatogram. When it was measured under optimal medium condition, DNJ was detected on 22 min (retention time). Generally, the DNJ content of mulberry powder, was ranging from 0.32% to 0.47% (Konno *et al*., 2006). DNJ concentration produced on *Bacillus amyloliquefaciens* AS385, which was isolated from soil, it reached a maximum of 460 mg/L. The DNJ produced by *Bacillus subtilis* S10 was detected on 750 mg/L similar to that (Cho *et al*., 2008). In the other reports, the relative DNJ content of parasitic loranthus plants parasitized on a mulberry tree could reach as high as 33.1 to 106.2% of that in their host trees. However, it can be obtained in small quantities by brewing an herbal tea from mulberry leaves. The extraction method from mulberry leaves has the limitations of low amount of DNJ extracted, in consistent yield of DNJ, and requirement for complex purification steps. Therefore, to produce DNJ economically, it is probably necessary to employ a biotechnological fermentation process. In conclusion, the results presented in this study confirm DNJ yield's increasement in

microbes using the various of nutrients and provide insight of ways to improve DNJ yields in microorganisms.

Acknowledgments

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