Production of α -Glucosidase Inhibitor and 1-Deoxynojirimycin by *Bacillus subtilis* MORI

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Galactose and soybean meal were selected as the best carbon and nitrogen sources, respectively, for the efficient production of α -glucosidase inhibitor (AGI) and 1-deoxynojirimycin (DNJ) by a newly isolated *Bacillus subtilis* MORI. The optimal concentrations of the galactose and soybean meal for the production of AGI and DNJ were investigated by response surface methodology. For the production of AGI, the optimal galactose and soybean meal concentrations were 4.3% (w/v) and 3.2% (w/v), respectively, and for DNJ, 4.5% (w/v) and 3.0% (w/v), respectively. The nearly identical optimal concentrations of galactose and soybean meal for the production of both AGI and DNJ indicated a close correlation between the production of AGI and DNJ. The maximum production of AGI (50,880 GIU/ml) and DNJ (824 μ g/ml) obtained from the statistically optimized medium in a jar fermenter was 2.33 and 2.38-fold, respectively, higher than those (21,798 GIU/ml and 346 μ g/ml, respectively) of the pre-optimized medium. The production of both AGI and DNJ was greatly increased by jar fermentation (AGI of 38,524 GIU/ml and DNJ of 491 μ g/ml) compared with flask fermentation.

Keywords: α -Glucosidase inhibitor, 1-deoxynojirimycin, production, *Bacillus subtilis* MORI, response surface methodology

Introduction

Type 2 diabetes is a genetically heterogeneous metabolic disorder characterized by both insulin deficiency and peripheral insulin resistance [1]. α -Glucosidases (AGIs) (EC 3.2.1.10, 3.2.1.20, 3.2.1.48, and 3.2.1.106) are exo-acting carbohydrases that catalyze the release of α -glucopyranose from non-reducing ends of various carbohydrate substrates [2] and that are used to prevent or treat type 2 diabetes [3–6]. The presence of AGI in the diet can inhibit the activity of human intestinal α -glucosidases and can reduce the absorption of dietary carbohydrates. Agents such as voglibose and acarbose, that have α -glucosidase inhibitory activity, delay carbohydrate digestion in the small intestinal tract and thereby reduce meal-induced increases in postprandial blood

glucose and plasma insulin levels [7, 8].

Although powerful synthetic AGIs are available, they can cause hepatic disorders and other gastrointestinal symptoms [9]. Therefore, natural AGIs from food sources have become an attractive therapeutic approach for treating postprandial hyperglycemia. Soybean and soybean products are considered health foods because of their valuable nutritional and medicinal attributes. For example, the intake of soybean foods has been associated with the prevention and treatment of chronic diseases, such as cardiovascular disease and cancer [10]. Historically, the incidence of type 2 diabetes has been lower in Asian populations compared with those in Western countries. One possible reason for the lower incidence among Asian populations is the consumption of fermented soybean products, which are unique to the traditional Asian diet [11].

AGIs can be isolated from plants [12] and food products [10], synthesized chemically [14] or produced by

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microorganisms, including species of *Streptomyces* [15], *Actinoplanes* [16], and *Flavobacterium saccharophilum* [17]. Recently, *Bacillus subtilis* B2, which produced an efficient AGI, was isolated from Meitauza (a Chinese traditional fermented okara) [18, 19]. The use of different carbon and nitrogen sources has been suggested to play an important role in the synthesis of AGIs from microbes [19].

1-Deoxynojirimycin (DNJ), a typical naturally occurring azasugar, is a D-glucose analog with an NH-group substituting for the oxygen atom of the pyranose ring [16]. DNJ and some of its derivatives have been previously shown to be effective AGIs with promising biological activities, such as potential antidiabetic, antitumor, and anti-HIV activities [20]. DNJ has been isolated from the root [21] and leaves [22] of mulberry trees and from silkworms. DNJ is also produced by several *Bacillus* [23, 24] and *Streptomyces* [25, 26] species.

Many studies have focused on the isolation and screening of microorganisms for AGI and DNJ production [26, 27]; however, few reports have investigated culture medium optimization. Medium optimization can be approached by either empirical or statistical methods. Previously, the composition of the medium was optimized empirically by using a 'one-factor at a time' variation process [18, 28]. Unlike conventional optimization, statistical optimization methods take into account the interactions of more than one variable in generating the process response [29]; therefore, response surface methodology (RSM) is a more efficient technique for optimization [30]. This method had been successfully applied to the optimization of medium composition [31] and to extraction [32] and fermentation process conditions [33]. Using this method, the concentration of each medium component is compared with a base value and is altered in a specific pattern. This pattern is designed using statistical methods to yield the most information by a minimum number of experiments.

Previously, we have isolated a new strain of *B. subtilis* MORI, which produced both AGI and DNJ in high quantities, from a traditionally fermented Korean soybean [34]. Unlike other AGI and DNJ producing microbial strains such as *Streptomyces* sp. [25, 26], *B. subtilis* is edible and can be used to prepare functional foods.

In the present study, the most efficient carbon and nitrogen sources for the production of AGI and DNJ in

the culture medium of *B. subtilis* MORI were first selected for production of AGI and DNJ, and then the optimal concentration of each selected source was statistically investigated using RSM. The production of both AGI and DNJ was also examined by jar fermentation and compared with flask fermentation. The production of DNJ was again studied using a 50 L pilot scale fermenter.

Materials and Methods

Microbial strain and seed culture

The new strain *B. subtilis* MORI (The Korean Culture Collection of Microorganisms No. 10450, Korea), isolated from a Korean traditional fermented soybean Chungkookjang [34], was used for this study. Single loop culture grown on a Difco™ YM agar plate [Becton, Dickinson and Company (BD, USA), MD 21152, USA] for 1 day at 37°C was inoculated into Bacto™ Tryptic Soy Broth (50 ml), in a 250 ml Erlenmeyer flask and the flask was incubated at 37°C for 17 h with shaking at 180 rpm.

Selection of carbon source

Fermentation broths (50 ml in a 250 ml Erlenmeyer flask) containing basal medium and a carbon source were inoculated with 1 ml of the seed culture. Basal medium consisted of 0.5% (w/v) yeast extract, 0.5% (w/v) soybean meal, 0.05% (w/v) KH₂PO₄ and 5% (w/v) (NH₄)₂SO₄. The following 13 carbon sources were separately added to the basal medium at 1% (w/v): corn starch, fructose, galactose, glucose, pectin, lactose, maltose, molasses, potato starch, raffinose, sorbitol, sucrose, and xylose. The inoculated fermentation broths, each containing a single carbon source, were incubated at 37°C with shaking at 180 rpm. After 4 days, each fermented broth was centrifuged at 14,000 g for 10 min at 4°C, and the supernatant was filtered through a 0.45 µm membrane syringe filter (Sartorius AG, Germany). The filtrates were used for the analysis of AGI activity using rat intestinal α-glucosidase (Sigma Chemical Co., USA) to select the best carbon source.

Selection of nitrogen source

One milliliter of the seed culture broth was inoculated

into a 250 ml Erlenmeyer flask containing 50 ml of fermentation broth containing one nitrogen source and basal medium. Basal medium consisted of 1.0% galactose, 0.05% KH_2PO_4 and 0.05% $(\text{NH}_4)_2\text{SO}_4$. The following 13 nitrogen sources (1.0%, w/v) were separately added to fermentation broth: beef extract, corn steep liquor (C.S.L.), corn steep powder, fish meal, malt extract, polypeptone, soybean meal, soy pulp, soypeptone, soytone, sodium nitrate, whey powder, and yeast extract. Each test broth was incubated at 37°C for 4 days with shaking at 180 rpm, and then was centrifuged at 4°C and filtered. The filtered supernatants were used for the analysis of AGI activity using rat intestinal α -glucosidase to select the best nitrogen source.

Experimental design for shake-flask cultures

RSM was used to determine the optimal concentrations of galactose and soybean meal in shake flask cultures using the following equation (1) [35]:

$$x_i = (X_i - X_0)/\Delta X_i \quad i = 1, 2, 3, \dots, j \quad (1)$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change value.

The behavior of the system was explained by the following second-order polynomial equation (2):

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

where y is the predicted result, β_0 (offset term), β_i (linear effect), β_{ii} (squared effect), and β_{ij} (interaction effect) are constant coefficients; x represents the coded level of the independent variable. The SAS 9.1 package (SAS Institute Inc., USA) was used for regression analysis of the experimental data and to estimate the regression equation coefficients.

Determination of AGI activity

The AGI activity of the fermented broth filtrates was determined by reaction between α -glucosidase and 4-nitrophenyl- α -D-glucopyranoside (4-NPG, Sigma Chemical Co., USA) and was carried out in microtiter plates (SPL, Korea) as described by Matsui *et al.* [36]. The filtrates were serially diluted from 1×10^{-2} to 5×10^{-7} fold with an equal volume of 0.1 M phosphate buffer, and then the filtrates were dispensed into wells of the microtiter plates

(10 μ l per well) followed by the addition of 10 μ l of rat α -glucosidase (16 mg/ml) (Sigma Chemical Co.), 100 μ l of 4-NPG (1.2 μ g/ml) and 80 μ l of 0.1 M phosphate buffer (pH 6.8). The mixture was incubated at 37°C for 30 min to allow the reaction of α -glucosidase with 4-NPG and the production of 4-nitrophenol. The reaction was terminated with an addition of 100 μ l of 0.2 M sodium carbonate. The formation of 4-nitrophenol in each well was measured by the intensity of absorbance at 405 nm using a microplate reader (Molecular Device, USA). The AGI activity of the filtrate was then analyzed using BioDataFit 1.02 software (Molecular Devices). The production of AGI was expressed as α -glucosidase inhibitor unit (GIU/ml), which was defined as the equivalent amount of inhibitor that inhibits 50% of the original activity by this assay method [25].

Determination of DNJ production

The amount (μ g/ml) of DNJ in the fermented broth samples was determined by the method of Kim *et al.* [38] using 9-fluorenylmethyl chloroformate (FMOC-C, Fluka Chemie AG, Switzerland) as a derivatizing agent. First, 10 μ l of DNJ standard solution or the filtrate of each fermented broth sample was mixed with 10 μ l of 0.4 M potassium borate buffer (pH 8.5) in a 1.5 ml microtube. FMOC-Cl in CH_3CN (5 mM, 20 μ l) was added with immediate mixing, and the reaction was allowed to proceed at 20°C for 20 min in a water bath (Lab Companion, Korea). Glycine (0.1 M, 10 μ l) (Sigma, USA) was added to terminate the reaction by quenching the remaining FMOC-Cl. The mixture was diluted with 950 μ l of 0.1% (v/v) aqueous acetic acid (Sigma) to stabilize the DNJ-FMOC, and then the mixture was filtered through a 0.2 μ m syringe filter (Sartorius Minisart RC 4, Germany). Aliquots (10 μ l) of the resulting filtrates were used for the HPLC analysis, which was carried out using a Nanospace SI-2 HPLC system (Shiseido, Japan), consisting of a 3010 degasser, a 3101 pump, a 3023 autosampler, a Capcell Pak C_{18} MG Column (250 $\times$ 4.60 mm ID, 5 μ m), an FL3013 fluorescence detector (excitation 254 nm, emission 322 nm). SMC21 (version 5.0) data processing software was used for the data analysis. The analytes were eluted with a mobile phase of acetonitrile 0.1% aqueous acetic acid (1:1, v/v) at 1.0 ml min⁻¹ for 40 min, and the DNJ content was determined.

Comparison of the statistically optimized medium and pre-optimized medium in a jar fermenter

The production of AGI and DNJ using a statistically optimized medium was compared with the production using the pre-optimized medium. Fermentation was carried out in a 2.5 L jar fermenter (Kobiotech, Korea) containing 1 L of the optimized medium [galactose 43 g/l, soybean meal 32 g/l, KH_2PO_4 0.5 g/l, $(\text{NH}_4)_2\text{SO}_4$ 0.5 g/l] or pre-optimized medium [galactose 10 g/l, soybean meal 10 g/l, KH_2PO_4 0.5 g/l, $(\text{NH}_4)_2\text{SO}_4$ 0.5 g/l] at 37°C, 1.0 vvm and 300 rpm. The samples were taken aseptically every 24 h and were further analyzed for AGI and DNJ production.

50 L pilot fermenter culture

For massive production of AGI and DNJ in a 50 L fermenter, *B. subtilis* MORI was inoculated in a 1 L Tryptic Soy broth and then cultured with shaking at 37°C for 24 h. After inoculation into 30 L of the statistically optimized medium in the 50 L fermenter (Boksung, Korea), it was cultured at 37°C with agitation speed 100 rpm and 0.5 vvm aeration for 6 days, and the AGI (GIU/ml) and DNJ content ($\mu\text{g/ml}$) in the culture supernatant was measured by taking 30 ml of the culture solution every 24 h. The pH and residual sugar (% w/v) of the culture medium were measured. To determine the number of viable cells, 1 ml of the culture solution was serially diluted with saline (0.85% NaCl), spread onto a TSB agar plate, and the number of colonies was measured after incubation at 37°C for 2 days.

Results and Discussion

Carbon source selection

AGI production by the *B. subtilis* MORI was examined using various carbon sources, and the results are shown in Fig. 1. The fermented medium containing galactose as the carbon source produced the highest level of AGI (8,491 GIU/ml). The carbon source that had the second-highest level of AGI production (6,632 GIU/ml) was pectin. Raffinose, corn starch, and potato starch also produced relatively high levels of AGI production. The remaining carbon sources caused low AGI production, less than 3,000 GIU/ml. On the basis of these results, galactose was selected as the best carbon source for AGI production.

Fig. 1. The effect of various carbon sources on the production of AGI.

Nitrogen source selection

AGI production was assayed using various nitrogen sources, and the results are shown in Fig. 2. The fermented medium containing soybean meal exhibited the highest level of AGI production (9,252 GIU/ml). The nitrogen source that showed the second and the third-highest levels of AGI production were corn steep powder (4,696 GIU/ml) and soybean residue (3,985 GIU/ml), respectively. The other sources investigated had low levels of AGI production, less than 3,000 GIU/ml. Thus, soybean meal was selected as the best nitrogen source for AGI production. In previous studies of AGI production, culture broths containing soybean meal have been

Fig. 2. The effect of various nitrogen sources on the production of AGI.

shown to produce higher AGI levels compared with broths containing other nitrogen sources [10, 18, 27, 29]. In further studies, most amino acid levels either increased or remained almost constant with fermentation time, but glutamate was decreased by fermentation, suggesting that glutamate may be the preferred nitrogen source for microorganisms [38]. This finding suggests that glutamate, the richest amino acid in soybean, may play an important role in the synthesis of AGI.

Optimization of carbon and nitrogen sources by RSM

The optimal concentrations of culture medium for AGI and DNJ production were determined using the culture medium containing 3% (w/v) galactose and 2% (w/v) soybean meal. Galactose and soybean meal concentrations were analyzed against AGI and DNJ production levels using the statistical package SAS 9.1.

The experiment was performed using two independent variables, galactose (X_1) and soybean meal (X_2), in a 2^2

Table 1. Experimental variables used and the results of optimization of AGI and DNJ production using galactose and soybean meal.

Factors	Symbol	Coded values				
		-1.414	-1	0	+1	+1.414
Galactose	X_1 (% w/v)	0.17	1	3	5	5.83
Soybean meal	X_2 (% w/v)	0.59	1	2	3	3.42

Runs	Variable		AGI activity (GIU/ml)		DNJ (μ g/ml)	
	X_1	X_2	Measured	Predicted	Measured	Predicted
1	-	-	9,070	6,807	152	120
2	+	-	5,134	5,613	116	131
3	-	+	19,523	19,185	311	282
4	+	+	31,591	33,994	499	517
5	-1.414	0	8,140	10,009	144	184
6	1.414	0	21,644	19,634	382	356
7	0	-1.414	2,285	3,575	53	62
8	0	1.414	33,822	32,391	444	449
9	0	0	26,888	25,899	427	398
10	0	0	25,895	25,899	368	398
11	0	0	25,565	25,899	407	398
12	0	0	25,250	25,899	389	398

Table 2. Statistical analyses for the model of AGI production at different concentrations of galactose and soybean meal.

Source	Sum of squares	Degrees of freedom	Mean square	F-value	$P > F$
Model	1237761890	5	247552378	61.91	<0.0001
Error	23990957	6	3998493		
Corrected total	1261752847	11			

Coefficient of variation (CV) = 10.22; coefficient of determination (R^2) = 0.98.

Model term	Parameter estimate	Degree of freedom	Computed t	P ($P > t $)
Intercept	25900		25.9	<.0001
X_1	3404	1	4.81	0.0030
X_2	10190	1	14.41	<.0001
$X_1 * X_2$	4001	1	4.00	0.0071
X_{11}	-5540	1	-7.01	0.0004
X_{22}	-3959	1	-5.01	0.0024

Table 3. Statistical analysis for the DNJ production model at different concentrations of galactose and soybean meal.

Source	Sum of squares	Degrees of freedom	Mean square	F-value	<i>P</i> > <i>F</i>
Model	241145.7527	5	48229.1505	44.12	0.0001
Error	6558.9139	6	1093.1523		
Corrected total	247704.6667	11			

Coefficient of variation (CV) = 10.74632; coefficient of determination (R^2) = 0.973521

Model term	Parameter estimate	Degree of freedom	Computed t	<i>P</i> (<i>P</i> > <i>t</i>)
Intercept	398		24.06	<0.0001
X_1	61	1	5.22	0.0020
X_2	137	1	11.71	<0.0001
$X_1 * X_2$	56	1	3.39	0.0147
X_{11}	-64	1	-4.89	0.0027
X_{22}	-71	1	-5.45	0.0016

full factorial design with four star points ($\alpha = \pm 1.414$) and four replicates at the center point. The experimental design and the results after 4 days of fermentation are presented in Table 1. A regression analysis was performed to fit the response function to the experimental

data (Table 2, 3). The coefficient of determination ($R^2 = 0.98$ and 0.97) was high, indicating that the response equation provided a suitable model for the response surface of AGI and DNJ production, and the coefficient of variation (CV = 10.22 and 10.75) was low,

Fig. 3. Three-dimensional plot and contour plot of the calculated response surface on the production of AGI (A, C) and DNJ (B, D).

indicating the high degree of accuracy and reliability of the experiment [32, 39]. The following response equations were obtained:

$$Y_1 = 25900 + 3404X_1 - 5540X_1^2 + 10190X_2 - 3959X_2^2 + 4001X_1X_2$$

$$Y_2 = 398 + 61X_1 - 64X_1^2 + 137X_2 - 71X_2^2 + 56X_1X_2$$

where x_1 = coded value of galactose, x_2 = coded value of soybean meal, and Y_1 = glucose inhibitor unit (GIU/ml), Y_2 = DNJ (μ g/ml).

Fig. 3 shows a contour plot and 3D plot of the calculated response surface. The observed values were in agreement with the predicted values (Fig. 4). The optimal values of galactose (X_1) and soybean meal (X_2) for maximum AGI production were determined to be 0.67 and 1.25, respectively. The optimal galactose and soybean meal concentrations were 4.3% (w/v) and 3.2% (w/v), respectively. The maximal value for AGI production predicted by the model was 36,492 GIU/ml after 4 days of fermentation. The optimal values for maximal DNJ production were also determined and were 0.76 for galactose (X_1) and 0.98 for soybean meal (X_2). The optimal galactose and soybean meal concentrations for DNJ production were 4.5% (w/v) and 3.0% (w/v), respectively. The maximal value for DNJ production predicted by the model was 526 μ g/ml after 4 days of fermentation. To confirm the optimization results, culture experiments were performed using a medium containing the predicted optimal concentrations for AGI and DNJ production. The results show that values for AGI production of

38,524 GIU/ml and that values for DNJ production of 491 μ g/ml were obtained after 4 days of fermentation in a 250-ml Erlenmeyer flask. The levels of AGI and DNJ production were close to the predicted values of 36,492 μ g/ml and 526 μ g/ml, respectively.

Our results showed that the optimal concentrations of galactose and soybean meal for the production of AGI and DNJ were similar, indicating a strong co-relationship between the production of AGI and of DNJ. Previously, Yatsunami *et al.* [40] also reported that a strong correlation existed between AGI activity and DNJ content ($R^2 = 0.9419$) in mulberry leaves. In another report, DNJ was shown as the main substrate for AGI activity produced by *B. subtilis* B2 [41].

Comparison of the statistically optimized medium and pre-optimized medium using a jar fermenter

For verification of the adequacy of the response surface model for predicting AGI and DNJ production, the statistically optimized medium and pre-optimized medium were compared in a jar fermentor-scale experiments. As shown in Fig. 5, AGI production peaked at 120 h of fermentation. The maximum AGI production (50,880 GIU/ml) obtained from the statistically optimized medium was 2.33-fold higher than that (21,798 GIU/ml) of the pre-optimized medium. The maximum DNJ production obtained from the optimized medium was also 2.38-fold higher (824 μ g/ml) than that (346 μ g/ml) of the pre-optimized medium. The production of both AGI and DNJ was greatly increased by jar fermentation compared with flask fermentation (AGI of 38,524 GIU/

Fig. 4. The linearity of the calculated response surface on the production of AGI (A) and DNJ (B).

Fig. 5. Comparison of the time course of AGI and DNJ production between statistically optimized medium (●) and pre-optimized medium (○). Fermentation was carried out in a 2.5 L jar fermenter with 1 L working volume at 37 °C 1.0 vvm, and 300 rpm. The optimized culture medium consisted of galactose (43 g/l), soybean meal (32 g/l), KH_2PO_4 (0.5 g/l), and $(\text{NH}_4)_2\text{SO}_4$ (0.5 g/l). The pre-optimized culture medium consisted of galactose (10 g/l), soybean meal (10 g/l), KH_2PO_4 (0.5 g/l) and $(\text{NH}_4)_2\text{SO}_4$ (0.5 g/l).

ml and DNJ of 491 $\mu\text{g/ml}$ more). This increase may have been caused by an enhanced aeration rate and agitation speed in the jar fermenter. The correlation between the predicted and experimental results verified the validity of the model and the existence of an optimal point.

AGI and DNJ production using a 50 L pilot fermenter

Mass production of AGI and DNJ was conducted using a 50 L fermenter. A working volume of 30 L culture was used with the statistically optimal medium (Fig. 6). The initial reducing sugar concentration [4.3% (w/v)] gradually decreased to 0.14% at 144 h. The initial pH 7.5 was

lowered to pH 6.3 after 24 h, then increased continuously to become 9.2 at 144 h. The number of viable cells in the culture was about 10^9 CFU/ml after 24 h of culture and maintained thereafter. The AGI production reached 44,540 GIU/ml of maximal production at 144 h. Likewise, the DNJ production increased rapidly and reached 600 $\mu\text{g/ml}$ of maximal production at 120 h. Compared with the results of jar fermenter, AGI and DNJ production decreased by 1.1 and 1.4 times. In the 50 L fermenter, in order to avoid excessive foaming, the aeration flow (0.5 vvm) and agitation speed (100 rpm) were lowered for the fermentation. It was assumed that AGI and DNJ

Fig. 6. Mass production of AGI and DNJ by *B. subtilis* MORI in a 50 L pilot fermenter.

production were lowered due to the decrease in agitation speed and aeration flow as the culture volume increased. In batch culture, culture conditions such as agitation speed and aeration amount were known as essential factors [42].

In the future, maximal AGI and DNJ productivity for commercial mass production can be expected by establishing additional optimal culture conditions in a scaled-up fermentation tank using the RSM. Among the various carbon sources that affect productivity, corn starch and pectin are considered to be economically advantageous. In particular, in the case of pectin, economical production is possible by using fruit peel by-products such as mandarin orange peel, which is rich in pectin. In order to produce economical AGI and DNJ, we are considering a medium optimization using by-products such as mandarin orange peel.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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