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RESEARCH PAPER

Culture Optimization Strategy for 1-Deoxynojirimycin-producing *Bacillus methylotrophicus* K26 Isolated from Korean Fermented Soybean Paste, *Doenjang*

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Abstract 1-Deoxynojirimycin (1-DNJ) is an α-glucosidase inhibitor that is used for the treatment of type 2 diabetes. In this study, we isolated *Bacillus methylotrophicus* K26 with α-glucosidase inhibition (AGI) activity from Korean fermented soybean paste (*Doenjang*) and confirmed that the genome harbored the DNJ biosynthesis genes including *gabT1*, *yktc1*, and *gutB1* by PCR screening, while 1-DNJ production was confirmed by ultra-performance liquid chromatography–quadrupole time-of-flight–mass spectrometry. To increase 1-DNJ production by *B. methylotrophicus* K26, culture conditions were optimized with one-factor-at-a-time (OFAT) and response surface methodology (RSM) approaches. Screen of 11 carbon and 9 nitrogen sources by the OFAT method identified sucrose and yeast extract as optimal culture components. Sucrose concentration (X_1), yeast extract concentration (X_2), and culture temperature (X_3) were selected as independent variables for central composite design. The coefficient of determination (R^2) for the model was 0.927, and the probability value of the regression model was highly significant. RSM predicted the optimal conditions for 1-DNJ production by *B. methylotrophicus* K26 as sucrose and yeast extract concentrations of 4.61% and 7.03%, respectively, at a temperature of 34°C. Under these conditions, AGI activity was experimentally measured as 89.3%, which was close to the predicted value of 91.9%.

Keywords: 1-deoxynojirimycin, *Bacillus methylotrophicus*, α-glucosidase inhibitor, optimization, response surface methodology

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1. Introduction

Type 2 diabetes, which accounts for 90% of cases of diabetes mellitus, is caused by insulin deficiency due to insulin resistance or insufficient secretion of insulin from pancreatic β-cells, resulting in high blood glucose levels [1]. This can be controlled by oral hypoglycemic agents including sulfonylurea, non-sulfonylurea, biguanide, thiaxolinedione, and α-glucosidase inhibitor [2]. The latter hydrolyzes carbohydrate to glucose in the small intestine, thereby reducing the rate of carbohydrate degradation leading to postprandial hypoglycemia [3]. Accordingly, α-glucosidase inhibitors such as acarbose [4], miglitol [5], and voglibose [6] have been developed as an oral anti-diabetic drug [7]. However, these have side effects such as abdominal bloating and vomiting, and therefore many efforts have been made in recent years to develop a novel α-glucosidase inhibitor [8-10].

1-Deoxynojirimycin (1-DNJ) as one of the known α-glucosidase inhibitors is a poly-hydroxylated alkaloid in which the glucose oxygen is substituted with nitrogen [11]. 1-DNJ has anti-diabetic effects and has also been used to treat human immunodeficiency virus infection and gonorrhea [12]. 1-DNJ can be chemically synthesized or extracted from mulberry [13,14]; however, these methods are not cost effective due to its low and inconsistent production yield. Microorganisms such as *Bacillus* and *Streptomyces* have been proposed as factories for the biosynthesis of 1-DNJ [15-17] from glucose through amination, dephosphorylation, and oxidation [18], which encode the GabT1, Yktc1, and GutB1 genes, respectively [19]. Additionally, 1-DNJ is produced by the *B. subtilis* strains DSM704 [17], B2 derived from meitauza (fermented okara) [20], and S10 derived from soil [21], as well as *B. amyloliquefaciens* strains HZ-12 from soil [22] and 140N from Korean fermented foods [23].

The one-factor-at-a-time (OFAT) culture optimization method has been used to increase 1-DNJ production by microorganisms [17,22,24] along with response surface methodology (RSM) [25,26], which predicts that 1-DNJ production by *Streptomyces lawendulae* can be increased by as much as 19% [25]. In fact, the α-glucosidase inhibition (AGI) activity of *B. subtilis* B2 producing 1-DNJ was increased by the RSM method [26].

In this study, *B. methylotrophicus* K26, which inhibits α-glucosidase, was isolated from Korean fermented soybean paste (*doenjang*). It was performed for the presence of 1-DNJ biosynthetic gene cluster using PCR and the 1-DNJ production was also confirmed by ultra-performance liquid chromatography–electrospray ionization–quadrupole time-of-flight–mass spectrometry (UPLC-ESI-Q-TOF-MS) analysis. The OFAT and RSM methods were then used to optimize 1-DNJ production by *B. methylotrophicus* K26.

2. Materials and Methods

2.1. Isolation and identification of microorganisms

Bacteria were isolated from five traditional Korean fermented foods including kimchi, soybean paste (Doenjang), red pepper paste, salted shrimp, and pickled cucumbers. The food samples were diluted in 0.85% NaCl solution on tryptic soy broth (TSB) nutrient broth (NB) agar (BD Biosciences, Franklin Lakes, NJ, USA) plates at 37°C for 24 h. The 16S rRNA gene of isolated bacteria was amplified by colony PCR using universal primers (27F, 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R, 5'-GGT TAC CTT GTT ACG ACT TC-3'). The fragments were sequenced by Cosmo Genetech Co. (Seoul, Korea) and sequence alignment and identification were performed using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ezbiocloud (http://www.ezbiocloud.net/). A phylogenetic tree was constructed using MEGA6 software (http://www.megasoftware.net/).

2.2. Determination of AGI activity

To evaluate AGI activity, isolated bacteria were cultured at 37°C with shaking at 180 rpm in YG broth composed of 1% glucose, 1% yeast extract, 0.05% KH_2PO_4, and 0.05% $(NH_4)_2SO_4$. After 5 days, the culture was heated for 10 min at 100°C and then centrifuged for 10 min at 10,000 rpm. AGI activity in the supernatant was determined based on the reaction between α-glucosidase (Sigma-Aldrich, St. Louis, MO, USA) and *p*-nitrophenyl α-glucopyranoside (*p*NPG; Sigma-Aldrich). A supernatant (147 μL) was dispensed into a 96-well plate, followed by addition of 30 μL of 0.1 M phosphate buffer (pH 7.2), 30 μL of *p*NPG (10 mM), and 3 μL of α-glucosidase (100 U/mL). The absorbance of the mixture was measured at 405 nm with a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Analysis of 1-DNJ biosynthesis gene cluster

To identify the 1-DNJ biosynthetic gene cluster, genomic DNA extracted using the HiYield Genomic DNA Mini kit (RBC Bioscience Corp., New Taipei City, Taiwan) was used for PCR amplification of the full open reading frame of GabT1 (MJS-23, 5'-ATG GGA ACG AAG GAA ATC ACG AAT CCA-3' and MJS-24, 5'-TCA CTT GAT TTC CTC CAA TAG CTT GCG-3'); Yktc1 (MJS-19, 5'-GTG AGA GAC TAT ATC ATY GRG CTT GGA-3' and MJS-20, 5'-TTA GGA GTC CAG ACC AAC GCC TTC ATA-3'); and GutB1 (MJS-21, 5'-ATG AAG GCG TTG GTC TGG ACT CCT AAT-3' and MJS-22, 5'-TTA TAA AAG TTY CGG ATC AGA CAC RAG-3'). The cycling protocol was as follows: 94°C for 5 min; 35 cycles at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min; and 72°C for 10 min [23].

2.4. UPLC-ESI-Q-TOF-MS analysis

1-DNJ production was measured by UPLC-ESI-Q-TOF-MS using an Agilent 1290 Infinity UPLC system and Agilent 6520 Q-TOF mass spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Cultures were centrifuged at 10,000 rpm for 10 min, and the supernatant was extracted with methanol (1:1, v/v) and passed through a 0.22-μm polyvinylidene difluoride filter. Chromatographic separation was carried out using a hydrophilic interaction LC (HILIC) column (ZORBAX HILIC Plus, 2.1 × 100 mm, 3.5 μm; Agilent Technologies). The flow rate was 0.3 mL/min and the injection volume was 1 μL, with the mobile phase consisting of solvent A (5 mM ammonium acetate in

water) and solvent B (0.1% formic acid in acetonitrile). The following elution gradient was used: 90% B at 0 min, 90% B at 0.1 min, 60% B at 9.9 min, 100% B at 1 min, 100% B at 2 min, 90% B at 1 min, and 90% B at 6 min. The eluates were detected with N_2 gas at a temperature of 325°C and gas flow rate of 8 l/min. The fragment voltage was 70 V, and the capillary voltage was 4000 V [23]. Commercial 1-DNJ (Sigma-Aldrich) was used as a standard.

2.5. OFAT-based optimization of carbon and nitrogen sources

Carbon and nitrogen sources were selected with an OFAT approach. The 1% carbon sources (glucose, galactose, sucrose, maltose, fructose, corn starch, potato starch, soluble starch, sorbitol, lactose, and xylose) and 1% nitrogen sources (yeast extract, malt extract, casamino acid, soybean flour, soytone, tryptone, polypeptone, beef extract, and corn steep liquor) were added instead of glucose and yeast extract to a 200-mL Erlenmeyer flask containing 80 mL YG medium for enhanced 1-DNJ production with carbon and nitrogen sources, which was confirmed by measuring AGI activity.

2.6. RSM

To evaluate the effects of different variables on 1-DNJ production by *B. methylotrophicus* K26, we performed RSM optimization using a central composite design model with three variables—i.e., sucrose (1%, 3%, and 5%), yeast extract (3%, 6%, and 9%), and culture temperature (30°C, 35°C, and 40°C). Parameters for culture optimization were selected based on OFAT optimization; these were the critical variables and were designated as X_1, X_2, and X_3 (Table 1). Data were evaluated by analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC, USA). Each experiment was carried out in triplicate for statistical analysis.

Table 1. Experiment design for the α-glucosidase inhibition activity of the culture condition by Central Composite Design (CCD)

No.	Coded variable levels and range			*Y*, AGI activity[a] (%)
	X_1 (sucrose conc., %)	X_2 (yeast extract conc., %)	X_3 (culture temperature, °C)	
1	0 (5)	1 (9)	1 (40)	76.9%
2	0 (3)	0 (6)	0 (35)	88.0%
3	0 (3)	0 (6)	0 (35)	87.2%
4	-1 (1)	-1 (3)	-1 (30)	76.8%
5	-1 (1)	1 (9)	1 (40)	41.8%
6	0 (3)	0 (6)	0 (35)	81.8%
7	0 (3)	0 (6)	1 (40)	80.9%
8	0 (3)	-1 (3)	0 (35)	75.0%
9	-1 (1)	-1 (3)	1 (40)	58.2%
10	1 (5)	1 (9)	-1 (30)	88.6%
11	-1 (1)	0 (6)	0 (35)	77.9%
12	0 (3)	0 (6)	0 (35)	90.1%
13	1 (5)	-1 (3)	-1 (30)	70.2%
14	0 (3)	0 (6)	0 (35)	89.8%
15	1 (5)	-1 (3)	1 (40)	82.0%
16	0 (3)	0 (6)	0 (35)	88.8%
17	-1 (1)	1 (9)	-1 (30)	70.3%
18	0 (3)	0 (6)	-1 (30)	86.7%
19	1 (5)	0 (6)	0 (35)	89.0%
20	0 (3)	1 (9)	0 (35)	88.4%

[a]AGI activities were determined from 0.5-fold diluted supernatant.

3. Results and Discussion

3.1. Isolation and identification of 1-DNJ-producing *Bacillus*

Traditional Korean fermented foods were plated on TSB NB agar plates, and 372 colonies of different shapes were selected and screened for AGI activity by inoculating into YG media for 5 days. Of the 372 bacterial strains, 35 showed 73% or greater AGI activity (data not shown); these were identified as *Bacillus* (n = 29), *Staphylococcus* (n = 3), *Enterobacter* (n = 1), *Kocuria* (n = 1), and *Klebsiella* (n = 1) based on the 16S rRNA gene sequence (Fig. 1). Among them, we focused on the members of genus *Bacillus* including *B. subtilis* and *B. methylotrophicus* which could be directly applicable to fermented food products in this study. In addition, *Bacillus* species including *B. subtilis* [17,27] and *B. amyloliquefaciens* [22,23] have been known to produce 1-DNJ as one of α-glucosidase inhibitors. Thereafter, 29 *Bacillus* strains were PCR-amplified with *gabT1*, *yktc1*, and *gutB1* genes which are essential for 1-DNJ biosynthesis [19] to screen 1-DNJ biosynthetic gene cluster-harboring strains, resulting seven strains to be selected. Based on the putative amino acid sequences, *gabT1*, *yktC1*, and *gutB1* were identified as acetyl ornithine aminotransferase, inositol-1-monophosphatase, and sorbitol dehydrogenase, respectively. Six strains (K26, K50, K68, JGYJ1, OEZHJ4, and JK332) showed 96% ~ 100% homology to *B. amyloliquefaciens* FZB42, whereas one strain (DJHJ8) showed 98% homology to *B. atrophaeus* 1942 (Table 2). Among them, *B. methylotrophicus* K26 was finally selected due to its plausible capability to produce 1-DNJ by harboring three requisite genes for 1-DNJ biosynthesis and its highest AGI activity (86.7%) which was slightly high compared to that (78.9%) of the previously reported 1-DNJ producer, *B. amyloliquefaciens* 140N under same culture conditions [23].

1-DNJ production by *B. methylotrophicus* K26 was analyzed by UPLC-ESI-Q-TOF-MS in the positive-ion mode. The mass spectrum showed a peak with an *m/z* for

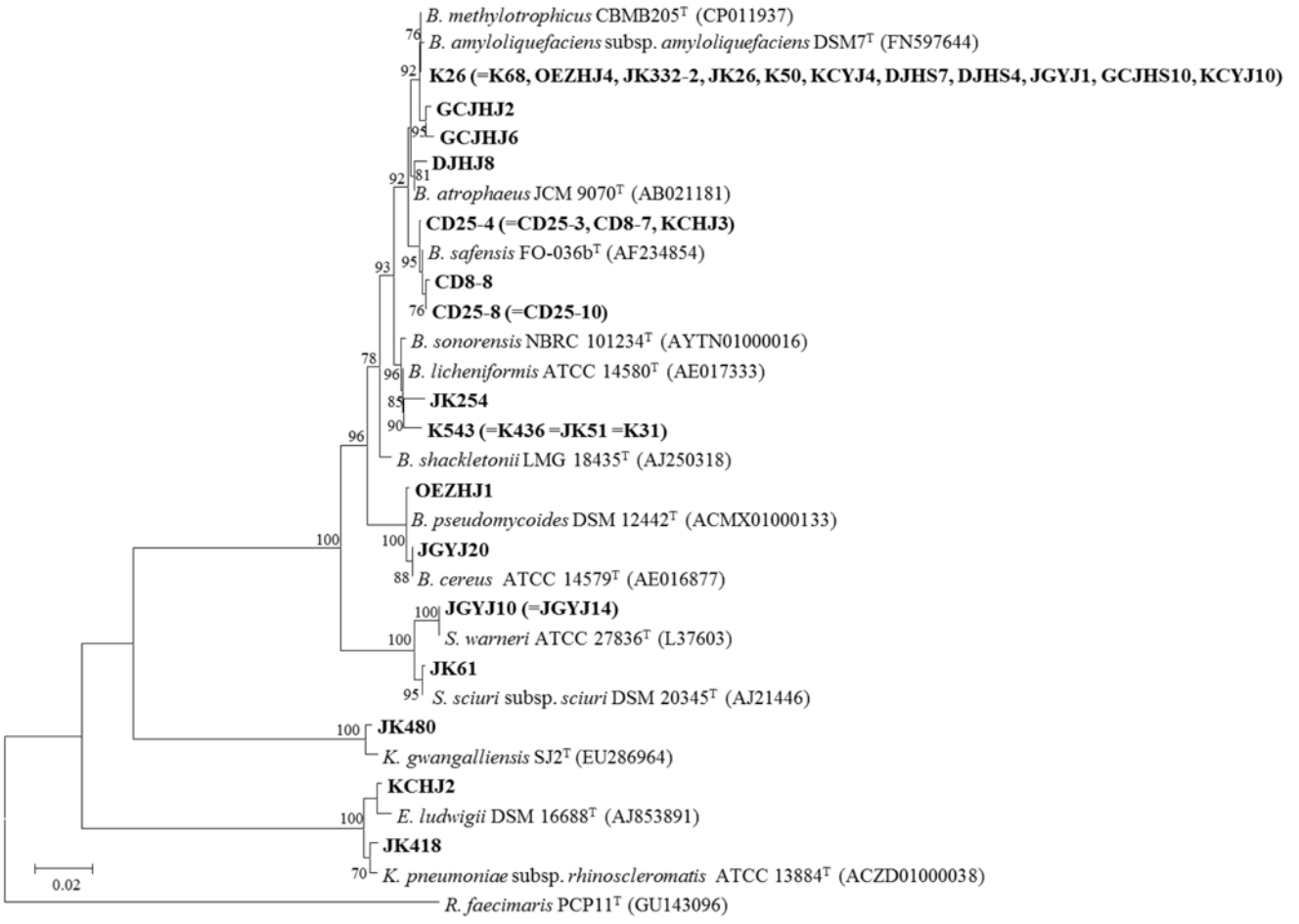

Fig. 1. Neighbor-joining phylogenetic tree of the 16S rRNA gene sequence of bacterial strains isolated from Korean fermented foods as well as of other related taxa. Numbers at branch points indicate bootstrap values (1000 replications; values > 70% are shown based on the neighbor-joining algorithm). Bar = 0.02 accumulated changes per amino acid.

$[M+H]^+$ of 164.09 at a retention time of 5.9 min, which corresponded to the standard used for 1-DNJ ($C_6H_{13}NO_4$, M.W. 163.08). This confirmed that 1-DNJ was produced by strain K26 (Fig. 2).

3.2. Culture optimization for 1-DNJ-producing *B. methylotrophicus* K26 by the OFAT approach

The culture conditions were optimized to increase 1-DNJ production by *B. methylotrophicus* K26 with the OFAT method; 11 carbon and 9 nitrogen sources were evaluated for their ability to stimulate AGI activity, which was determined by measuring 1-DNJ level. Although 1-DNJ production can also be confirmed by HPLC [21], HILIC-MS/MS [28], and other methods, these are more complex and time-consuming. We used AGI activity which can be confirmed quickly and easily.

The carbon source inducing the highest AGI activity was sucrose (78.25%), followed by glucose (71.4%) and sorbitol (65.07%). Therefore, sucrose was determined to be the optimal carbon source, since it was associated with higher AGI activity than glucose, a constituent of the basic medium (Fig. 3A). Yeast extract—the nitrogen source of the basic medium—induced the highest AGI activity followed by polypeptone (58.5%), with the other seven nitrogen sources resulting in less than 50% AGI activity (Fig. 3B). The optimal carbon and nitrogen sources for other *Bacillus* strains include sorbitol for *B. subtilis* DSM 704 [17]; galactose and polypeptone for *B. subtilis* S10 [21]; and soluble starch and tryptone for *B. amyloliquefaciens* 140N [23]. The optimal carbon and nitrogen sources identified by the OFAT method (sucrose and yeast extract, respectively) in the present study were set as the independent variables in RSM.

3.3. Culture optimization by RSM

RSM was performed to maximize 1-DNJ production by *B. methylotrophicus* K26. The factors affecting 1-DNJ production were the independent variables selected by the

Table 2. Comparison of the predicted proteins in the putative DNJ biosynthetic genes with *B. amyloliquefaciens* FZB42 and *B. atrophaeus* 1942

Strains	(Proteins)	*B. amyloliquefaciens* FZB42[a] (% identity/similarity)	*B. atrophaeus* 1942[b] (% identity/similarity)	AGI activity[c] (%)
DJHJ8	GabT1	92.1/96.6	98.8/99.5	73.8
	Yktc1	88.6/94.3	98.7/99.3	
	GutB1	85.9/94.2	98.8/99.4	
K26	GabT1	99.2/99.7	92.4/96.9	86.7
	Yktc1	98.7/99.0	87.9/93.9	
	GutB1	99.1/99.7	85.6/94.2	
K68	GabT1	99.5/99.7	92.8/96.9	75.5
	Yktc1	99.3/99.6	88.6/94.6	
	GutB1	99.1/99.7	85.6/94.2	
JGYJ1	GabT1	99.0/99.7	92.1/96.9	79.0
	Yktc1	99.0/99.3	88.2/94.3	
	GutB1	100/100	86.2/94.5	
OEZHJ4	GabT1	99.2/100	92.4/97.1	73.7
	Yktc1	96.8/98.4	87.3/93.3	
	GutB1	99.4/100	85.6/94.5	
JK332	GabT1	99.2/100	92.4/97.1	79.0
	Yktc1	98.7/99.0	87.9/93.9	
	GutB1	99.7/100	85.9/94.5	
K50	GabT1	99.0/99.5	92.1/96.6	75.3
	Yktc1	99.0/99.6	88.9/94.6	
	GutB1	99.4/99.7	85.9/94.2	

[a]GabT1 accession number (ABS72608), Yktc1 (ABS72609), and GutB1 (ABS72610).
[b]GabT1 accession number (ADP34799), Yktc1(ADP34800), and GutB1 (ADP34801).
[c]AGI activities were determined from 0.5-fold diluted supernatant.

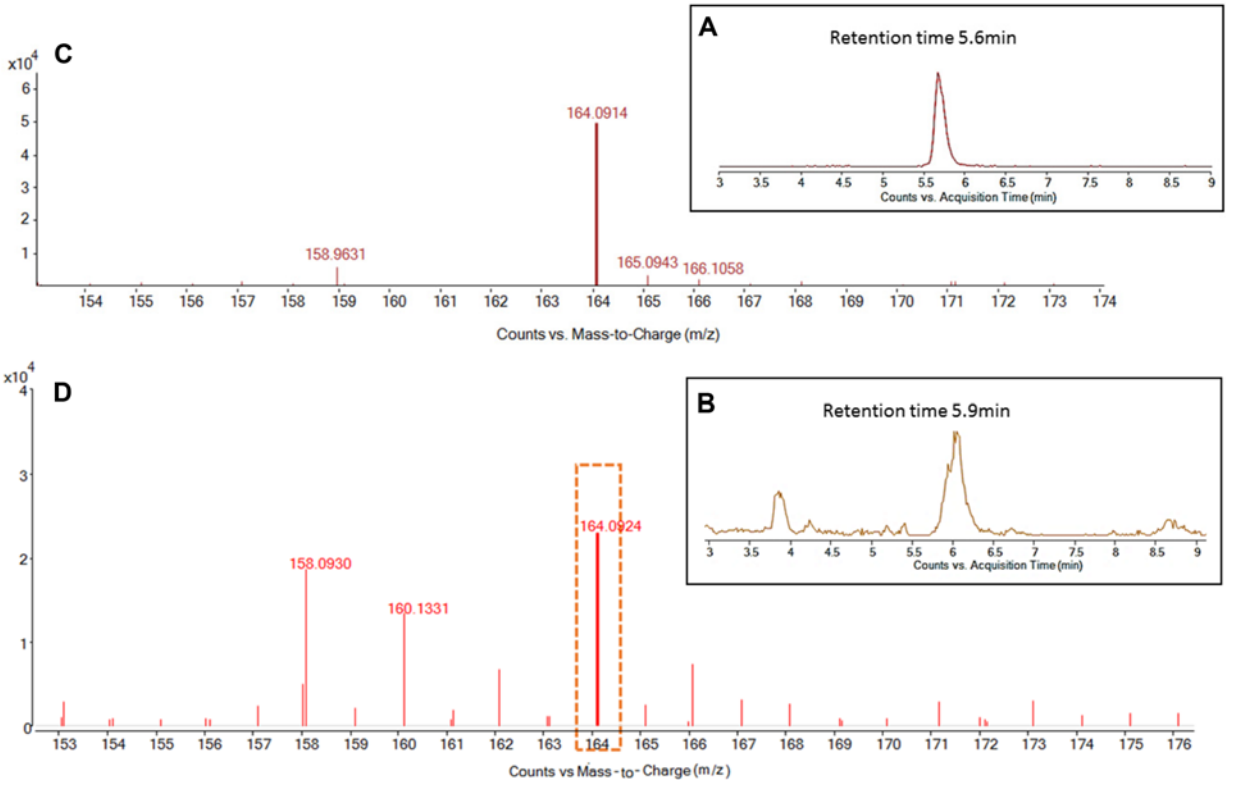

Fig. 2. UPLC-ESI-Q-TOF-MS analysis of 1-DNJ standard (A, C) and culture supernatant of *B. methylotrophicus* K26 (B, D), and MS data (C, D) with *m/z* for $[M + H]^+$ of 164.09.

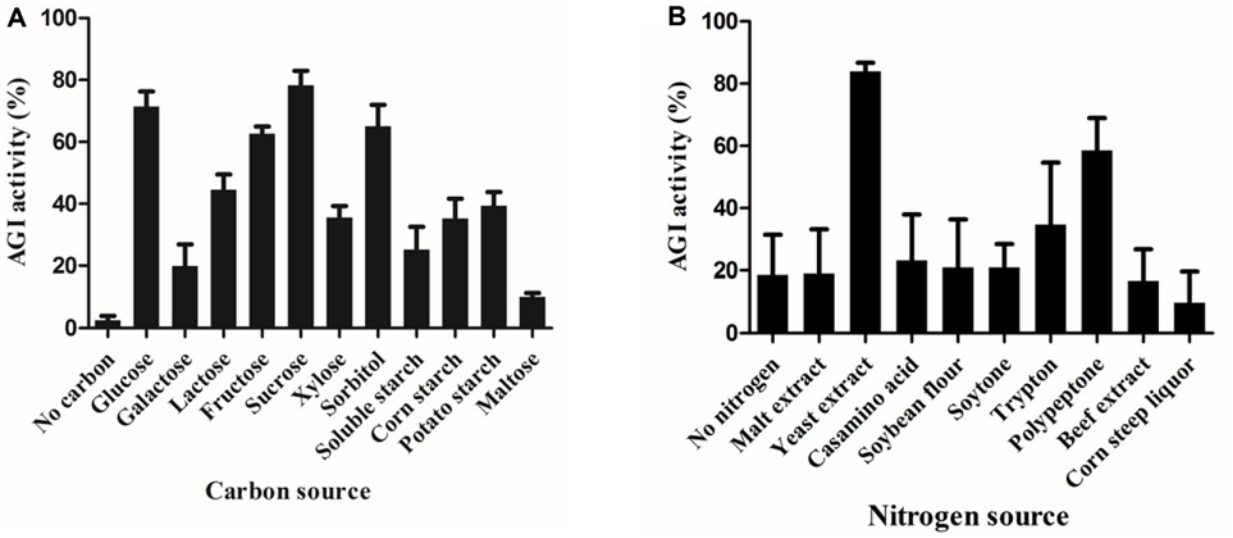

Fig. 3. Effects of carbon (A) and nitrogen (B) sources on the AGI activity of *B. methylotrophicus* K26 using the OFAT approach.

OFAT method—namely sucrose (X_1), yeast extract (X_2), and culture temperature (X_3); their relationship to 1-DNJ production is represented by Eq. 1.

$$Y = -153.58 - 13.14\ X_1 + 17.00\ X_2 + 12.7\ X_3 - 1.32\ X_1^2 - 0.78\ X_2^2 + 0.19\ X_3^2 + 0.75\ X_1X_2 + 0.59\ X_1X_3 - 0.27\ X_2X_3 \quad \text{(Eq. 1)}$$

The statistical significance of equation 1 was evaluated by ANOVA (Table 3). The coefficient of determination (R^2) was 0.927, indicating a high correlation between experimental and predicted values. The coefficient of variation of 5.7% suggested a high degree of interaction between and an independence of the variables. At a fixed culture temperature of 34°C, AGI activity increased with sucrose (carbon) and yeast extract (nitrogen) concentrations (Fig. 4A). The interactions between sucrose concentration and culture temperature and between yeast extract concentration and culture temperature influenced AGI activity, which increased as a function of all three variables (Fig. 4B, C). The maximum AGI activity was predicted by RSM to be 91.95% at a temperature of 34°C and sucrose and yeast extract concentrations of 4.61% and 7.03%, respectively.

We evaluated the AGI activity of *B. methylotrophicus* K26 under the optimal culture conditions determined by RSM by inoculating *B. methylotrophicus* K26 into the optimized medium. The experimentally measured AGI activity was 89.3%, which was close to the value of 91.9% predicted by RSM, confirming the reliability of the experimental design method. It was previously reported that soluble starch and tryptone were the optimal carbon and nitrogen sources, respectively, for maximal 1-DNJ production by *B. amyloliquefaciens* 140N as determined by the OFAT approach, yielding 88.9% AGI activity [23]. Previous optimization studies have mainly used the OFAT method, and few have attempted to enhance 1-DNJ production using an RSM approach, which can bring together many factors. RSM involving five factors (extraction solvent, ratio of extraction solvent to mulberry sample, ultrasonic power, extraction temperature, and extraction time) [29] and four factors (ethanol concentration, extraction temperature, extraction time, and ratio of solvent to sample) [30] has been applied to increasing 1-DNJ production in mulberry leaves. Another study used RSM

Table 3. Analysis of variance (ANOVA) for the DNJ production

Factors	Sum of squares	DF	Mean square	*F* value	Prob > *F*
Model	2,631.907	9	292.434	14.26	0.0001
X_1	1,187.021	4	296.755	14.47	0.0004
X_2	441.375	4	110.343	5.38	0.0142
X_3	764.090	4	191.022	9.32	0.0021
Lack of fit	158.536	5	31.707	3.41	0.102
Pure error	46.488	5	9.297		
Correlation total	205.025	10	20.502		

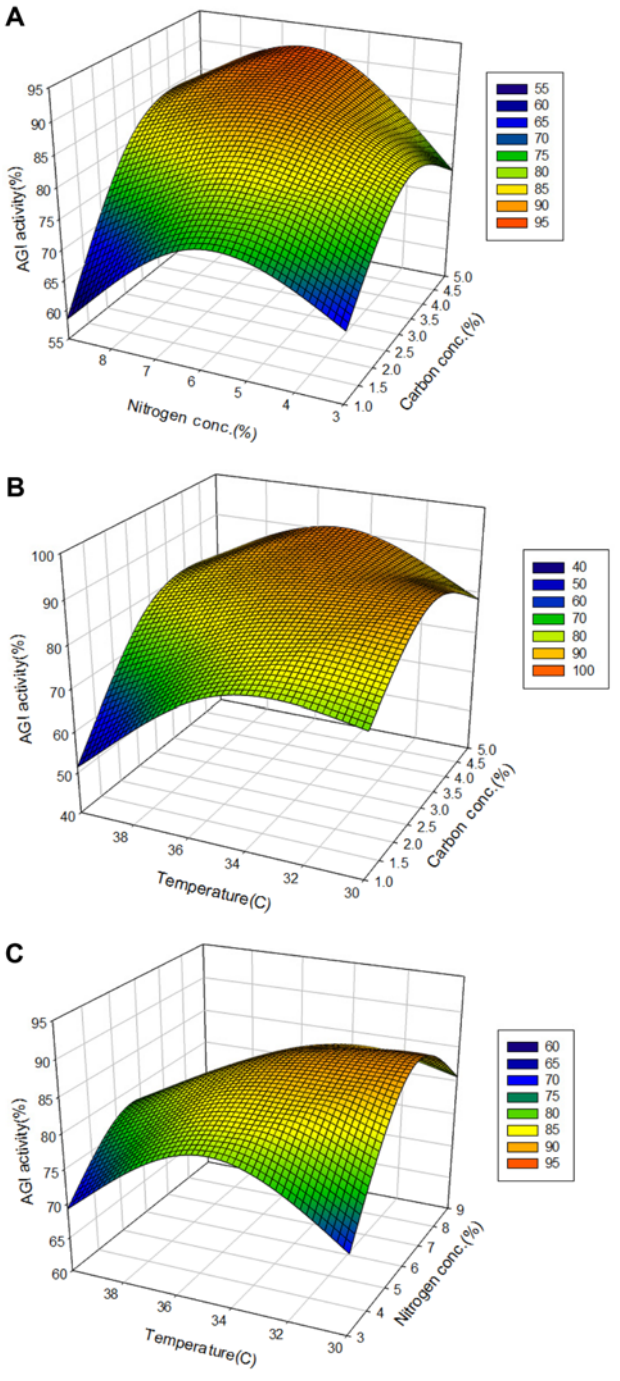

Fig. 4. Three-dimensional response surface plot of predicted AGI activity (Y, %) against sucrose concentration (X_1, %), yeast extract concentration (X_2, %), and culture temperature (X_3, °C). (A) Response surface plot representing the effect of sucrose (X_1) and yeast extract (X_2) on AGI activity (Y). (B) Response surface plot representing the effect of sucrose (X_1) and culture temperature (X_3) on AGI activity (Y). (C) Response surface plot representing the effect of yeast extract (X_2) and culture temperature (X_3) on AGI activity (Y).

with three factors (pH, potassium nitrate content, and inoculum volume) to increase 1-DNJ production by *Ganoderma lucidum*-mediated fermentation of mulberry leaves [31]. AGI activity in *B. subtilis* B2 was increased by 21% by fermentation time and temperature of 65.6 h and 39°C, respectively, at a pH of 7.11 and okara concentration of 4.5% [26]. Additionally, 1-DNJ production by *S. lavendulae* TB-412 was 42.875 mg/L for fermentation time and temperature of 11 days and 27°C, respectively, at an initial pH of 7.5 and soluble starch concentration of 8% [25]. Thus, RSM is expected to be useful for the development of functional foods through fermentation by *B. methylotrophicus* K26, such as mulberry tea with high 1-DNJ content [32].

4. Conclusion

B. methylotrophicus K26 was isolated from traditional Korean fermented soybean paste (*Doenjang*) and the gene cluster associated with 1-DNJ biosynthesis was shown to share 98% ~ 99% homology with those of other strains. 1-DNJ production through the AGI activity of *B. methylotrophicus* K26 was confirmed by UPLC-ESI-Q-TOF-MS analysis. We optimized the culture conditions to maximize 1-DNJ production with the OFAT and RSM approaches. Sucrose and yeast extract were identified as the optimal carbon and nitrogen sources, respectively. Under conditions of 4.61% sucrose, 7.03% yeast extract, and a culture temperature of 34°C, the experimental value for AGI activity was 89.3%, which is close to the predicted value of 91.9%. These results demonstrate that RSM can be used to determine the optimal *B. methylotrophicus* K26 culture conditions that will increase 1-DNJ yield, which is important for large-scale production of this compound for medicinal purposes.

Acknowledgments

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