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Biogenic Amine Degradation by *Bacillus* Species Isolated from Traditional Fermented Soybean Food and Detection of Decarboxylase-Related Genes

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Biogenic amines in some food products present considerable toxicological risks as potential human carcinogens when consumed in excess concentrations. In this study, we investigated the degradation of the biogenic amines histamine and tyramine and the presence of genes encoding histidine and tyrosine decarboxylases and amine oxidase in *Bacillus* species isolated from fermented soybean food. No expression of histidine and tyrosine decarboxylase genes (*hdc* and *tydc*) were detected in the *Bacillus* species isolated (*B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10), although substantial levels of amine oxidase gene (*yobN*) expression were observed. We also found that the three selected strains, as non-biogenic amine-producing bacteria, were significantly able to degrade the biogenic amines histamine and tyramine. These results indicated that the selected *Bacillus* species could be used as a starter culture for the control of biogenic amine accumulation and degradation in food. Our study findings also provided the basis for the development of potential biological control agents against these biogenic amines for use in the food preservation and food safety sectors.

Keywords: *Bacillus* spp., biogenic amines, degradation, fermented soybean pastes, histamine, tyramine

Introduction

Biogenic amines (BAs), important compounds for metabolic and physiological functions in all living organisms, are basic nitrogenous organic bases of low molecular weight that occur naturally in many foods, and are mainly produced by the microbial decarboxylation of precursor amino acids *via* substrate-specific enzymes [3]. The presence of BAs in food is undesirable because it can result in adverse effects in consumers, such as headache, respiratory distress, heart palpitations, hyper- or hypotension, and several allergic disorders, when ingested in high concentrations [8, 29].

BAs are structurally classified into aliphatic amines (putrescine, cadaverine, spermine, and spermidine), aromatic amines (tyramine, phenylethylamine, and serotonin), and heterocyclic amines (histamine and tryptamine) [27]. Among the BAs, histamine and tyramine are most extensively studied owing to their potential physiological actions and toxicological effects [25]. Histamine is a key mediator of immediate hypersensitivity reactions, and may cause urticaria, hypotension, tachycardia, flushing, and abdominal cramps, whereas tyramine is a potent vasoconstrictor with effects on healthy individuals usually limited to headache or migraine [24, 29].

Histamine synthesis is regulated by histidine decarboxylase (HDC), the enzyme that catalyzes the decarboxylation of L-histidine; therefore, this enzyme is considered a specific marker for the biosynthesis of histamine [18]. Tyrosine decarboxylase (TYDC) is an important enzyme for the production of tyramine and serves as an essential mediator for its biosynthesis. At the transcription level, high levels of expression of the genes encoding HDC and TYDC precede a sharp increase in the intracellular concentration of histamine and tyramine [9, 10].

BAs are frequently found in high concentrations in

fermented food, such as dairy and soybean products, and can cause intoxication symptoms in humans when ingested in excessive amounts [26]. Thus, a risk assessment study of BAs such as histamine and tyramine in fermented foods is important for the detection of these toxic compounds and as spoilage indicators. To control the production of BAs, various approaches have been investigated, including heat, low temperature storage, modified atmosphere packaging, irradiation, high hydrostatic pressure (HHP), microbial modeling, and addition of preservatives [20]. However, these methods can potentially have some adverse effects such as a decrement in the nutritional value of food, food functionality, and organoleptic properties. Mbarki *et al*. [19] observed that polyunsaturated fatty acids were reduced significantly and maximum lipid oxidation rates were induced with high radiation doses. Some recent studies have suggested that non-amine forming (amine-negative) or amine-oxidizing bacterial starter cultures for manufacturing of various fermented products may be applied to the research and development of industrial-scale fermented foods [20, 21]. In addition, emerging approaches for the control of BA production involve combinations of existing methods, such as the BA-negative bacterial starters and HHP [15]. A few strains of food fermenting microorganisms, such as isolated *Bacillus subtilis* and *Lactobacillus* spp., were found to exhibit the highest capacity for amine degradation [29]. *B. subtilis*, a broad-spectrum biological control agent, contains an amine oxidase gene (*yobN*) that catalyzes the oxidation deamination of a wide range of BAs, including various amine neurotransmitters, histamine, tyramine, and xenobiotic amines. Owing to these beneficial properties, *B. subtilis* has been widely used to prevent BA formation or to reduce BA levels, particularly during the production of fermented foods [20].

In this study, we determined the effects of *Bacillus* spp., isolated from Korean fermented soybean food, on the expression of biosynthetic genes encoding histidine and tyrosine decarboxylases (*hdc* and *tydc*, respectively) and the formation of BAs (histamine and tyramine).

Materials and Methods

Bacterial Strains and Culture Conditions

Bacterial strains were grown in Luria-Bertani (LB) broth (Difco, Becton-Dickinson, Sparks, MD, USA) or on LB agar medium at 30°C. The strains were subcultured in LB broth for 24 h at 30°C and streaked onto nutrient agar plates, and were then incubated at 30°C for 24 h before use. To test for the presence of strains producing biogenic amines (histamine and tyramine), the bacterial strains were grown in LB supplemented with 0.5% histidine, 0.5% tyrosine, and 0.005% pyridoxal-5-phosphate (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 30°C for 24 h. The biogenic amine-degrading ability of the strains was assessed at 30°C for 24 h in LB broth containing 0.25% histamine and 0.25% tyramine (Sigma-Aldrich).

Decarboxylase Activity

The decarboxylase activity of the isolated bacterial strains was measured by incubating strains in modified decarboxylase agar base containing tyrosine and histidine, as previously described [1]. After a period of incubation, the color changes of the medium were monitored at 30°C for different time intervals (12, 24, 36, 48, 60, and 72 h). The appearance of a purple color in the medium around the colonies indicated a positive decarboxylase reaction, whereas a yellow color indicated a negative reaction.

Reverse Transcription PCR (RT-PCR) and Quantitative Real-Time PCR (qPCR) Analysis

Total RNA from cultured bacterial cells was isolated using the RNeasy plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of the isolated RNA was measured using a Synergy Mx microplate reader (BioTek Instruments, Winooski, VT, USA). First-strand cDNA was synthesized from 1 μg of isolated RNA template using an amfiRivert Platinum cDNA synthesis Master Mix (GenDEPOT; Barker, TX, USA). A subset of genes was amplified with amfiEco Taq DNA polymerase (GenDEPOT) and the gene-specific primers listed in Table 1. The amplified products were separated on a 1% agarose gel with Safe-Pinky DNA gel staining solution (GenDEPOT). qPCR analyses were performed using a C1000 Thermal Cycler equipped with a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) in a total volume of 10 μl containing 5 μl of iQ SYBR Green Supermix (Bio-Rad), 200 nM of each of the primers listed in Table 1, and 2 μl of cDNA. The cycling conditions were as follows: initial denaturing at 95°C for 5 min, followed by 35 cycles each consisting of 95°C for 45 sec denaturing, 48°C for 45 sec annealing, and 72°C for 2 min of extension. Final extension was performed at 72°C for 5 min. Data were normalized to 16S rRNA expression.

Total Protein Extraction and Western Blot Analysis

For the expression of histidine decarboxylase and histamine H3 receptor in bacterial strains, bacteria were grown under the conditions described above for 3 h at 30°C. The cultures were collected by centrifugation at 10,000 ×*g* for 5 min at 4°C. The cell pellet was resuspended in 40 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and was then boiled at 95°C for 10 min. Equal amounts of sample proteins were resolved on 12% SDS-PAGE gels and were then subjected to western blot analysis. After electrophoresis, the separated proteins in the gel were transferred onto a nitrocellulose membrane by electroblotting (120 V, 1 h) using a Mini Trans-Blot

Table 1. Primer sequences.

Primers	Sequence	Description
tydc-L[a]	5′-ACATAGTCAACCATRTTGAA-3′	RT-PCR, qPCR
tydc-R[a]	5′-CAAATGGAAGAAGAAGTAGG-3′	RT-PCR, qPCR
hdc-L[b]	5′-GATGGTATTGTTTCKTATGA-3′	RT-PCR, qPCR
hdc-R[b]	5′-CAAACACCAGCATCTTC-3′	RT-PCR, qPCR
yobN-L	5′-CCGTGTCTGCACKTTGAGATC-3′	RT-PCR, qPCR
yobN-R	5′-ACATCCGCCAATTTCTTKCWGG-3′	RT-PCR, qPCR
16s-rRNA-L	5′-AGAGTTTGATCCTGGCTCAG-3′	RT-PCR, qPCR
16s-rRNA-R	5′-GGCTACCTTGTTACGACTT-3′	RT-PCR, qPCR

[a]Primer sequences have been described previously [6].

[b]Primer sequences have been described previously [5].

K = G or T; R = A or G; W = A or T; Y = C or T; S = C or G; M = A or C; D = A, G, or T; N = A, G, C, or T.

Electrophoretic Transfer Cell (Bio-Rad). These membranes were then blocked with 3% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 h, and were then incubated for 1 h with the following primary antibodies: polyclonal anti-histidine decarboxylase (1:1,000; Abcam Inc., Cambridge, MA, USA) and polyclonal anti-histamine H3 receptor (1:1,000; Signalway Antibody LLC, College Park, ML, USA). After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated goat IgG secondary antibodies (1:3,000; Bio-Rad) for 1 h. Blots were developed using a BM chemiluminescence blotting substrate (POD; Roche, Mannheim, Germany).

Derivatization with Benzoyl Chloride

Derivatization and quantification of biogenic amines, such as histamine and tyramine, was performed using a modification of methods that have been described previously [12, 22]. Standard solutions of biogenic amines (histamine and tyramine; Sigma-Aldrich) were dissolved separately to 10 mg/ml in distilled water. These standard solutions were stored in glass containers at 4°C and contained 1 mg of the free-base form of the biogenic amine in 1 ml of solution. An internal standard solution contained 2 mg of 1,7-diaminoheptane in 1 ml. To prepare a mixture of standard biogenic amines, suitable volumes of standard solutions were mixed and diluted to 10 ml with distilled water immediately prior to use. After incubation, the cells were centrifuged at 6,000 ×*g* for 15 min at 4°C; the supernatant was used for ultra-performance liquid chromatography (UPLC) analysis and stored at 4°C until required for use.

To a 4 ml aliquot of a mixture of standard biogenic amines and sample in a glass tube, 2 ml of 2 M sodium hydroxide solution and 100 µl of benzoyl chloride were added. The mixture was vortex-mixed for 3 min and then incubated at 30°C for 45 min in a water bath. After incubation, the reaction was stopped by adding 3 ml of a saturated sodium chloride solution. The resulting supernatants were extracted with 4 ml of diethyl ether by vortexing for 3 min and were then centrifuged at 2,500 ×*g* for 15 min at 4°C. Following centrifugation, 3 ml of the upper organic phases was transferred to another glass test tube and was evaporated to dryness under a steam of nitrogen gas. The solid residue was dissolved in 1 ml of acetonitrile, filtered through a 0.2-µm-pore-size syringe filter (Millipore, Billerica, MA, USA) and injected directly into the UPLC system.

UPLC Analysis of Biogenic Amines

Chromatographic separation was performed on a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) equipped with an autosampler, a binary gradient pump, and a photodiode array detector. The chromatographic column used was Acquity UPLC HSS C18 (1.8 µm particle size, 2.1 mm × 75 mm; Waters Corp., Milford, MA, USA). All data were acquired and processed with Waters Empower-2 chromatography data software. Gradient elution was performed with a mobile phase consisting of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B) (Merck, Darmstadt, Germany). The elution profile was as follows: 0 min: 50% A, 50% B; 0.2 min: 60% A, 40% B; 1 min: 50% A, 50% B; 2 min: 50% A, 50% B; 3 min: 45% A, 55% B; 4 min: 40% A, 60% B; 5 min: 35% A, 65% B; and 6 min: 40% A, 60% B). The flow rate was 0.4 ml/min, and the injection sample volume was 2 µl. The chromatographic column temperature was maintained at 30°C and detection was performed at 254 nm. The levels of histamine and tyramine were identified in the sample by comparison of retention times and peaks with those of the standard. Quantification was carried out using a calibration curve.

Quantification of Histamine and Tyramine by ELISA

The production of the biogenic amines tyramine and histamine in cultivated bacterial strains was assessed by quantitative histamine and tyramine enzyme-linked immunosorbent assays (ELISAs), as previously described [11, 28]. Briefly, bacterial strains were grown as described above in LB broth supplemented with 0.25% histamine and 0.25% tyramine. Cultures were harvested by centrifugation (1,500 ×*g* for 10 min) after 24 h of incubation at 30°C, and were filter-sterilized using 0.22-µm-pore-size polyvinylidene

fluoride filters (Millipore). Samples were diluted in phosphate-buffered saline, and histamine concentrations were determined using a histamine ELISA kit (Neogen, Lexington, KY, USA) and tyramine concentrations were measured by ELISA using an anti-tyramine antibody (Abcam Inc.), according to the manufacturer's instructions. After development of the colorimetric reaction, the absorbance was measured with a Synergy Mx microplate reader (BioTek Instruments) at 450 nm. A calibration curve (or standard curve) was generated by plotting the mean absorbance for each standard concentration (X-axis) against the target protein concentration (Y-axis). The concentration of tyramine and histamine in the samples was determined by comparing the absorbance spectrum of the samples with the standard curve.

Statistical Analysis

All statistical analyses were based on data from duplicate samples from experiments that were repeated three times. One-way analysis of variance (ANOVA) using SPSS ver. 12.0 was used to determine whether differences between the strains were significant. The means were compared with Duncan's multiple-comparison test, and $p < 0.05$ was considered to indicate statistical significance.

Results and Discussion

Histidine- and Tyrosine-Decarboxylase Activities of Isolated Bacteria

We have isolated strains of bacteria from buckwheat *sokseongjang*, a Korean traditional fermented soybean food, which do not produce BA and which are capable of degradation of BAs, as shown by thin-layer chromatography (TLC). Based on the TLC screening results, three BA-reducing strains (*B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10) and one BA-producing strain (*B. subtilis* H'J53-3) were selected for further investigation of their ability to remove biogenic amines (data not shown).

We confirmed the histidine- and tyrosine-decarboxylase activities of the isolated strains by evaluating color changes in the decarboxylase medium. Bacterial strains that possess decarboxylase activity generate alkaline amines; the subsequent pH change in the medium is indicated by a purple color [1]. Whereas a positive decarboxylase reaction is indicated by a deep purple color, a negative result is indicated by the absence of color or a bright yellow color for glucose-fermenting microorganisms. As shown in Fig. 1A, for the selected strains, *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10, there was no change in the color of the agar surrounding the colonies, but the *B. subtilis* H'J53-3 strain formed large grayish colonies with purple discoloration of the surrounding medium, indicative of histidine- and tyrosine-decarboxylase activities. The

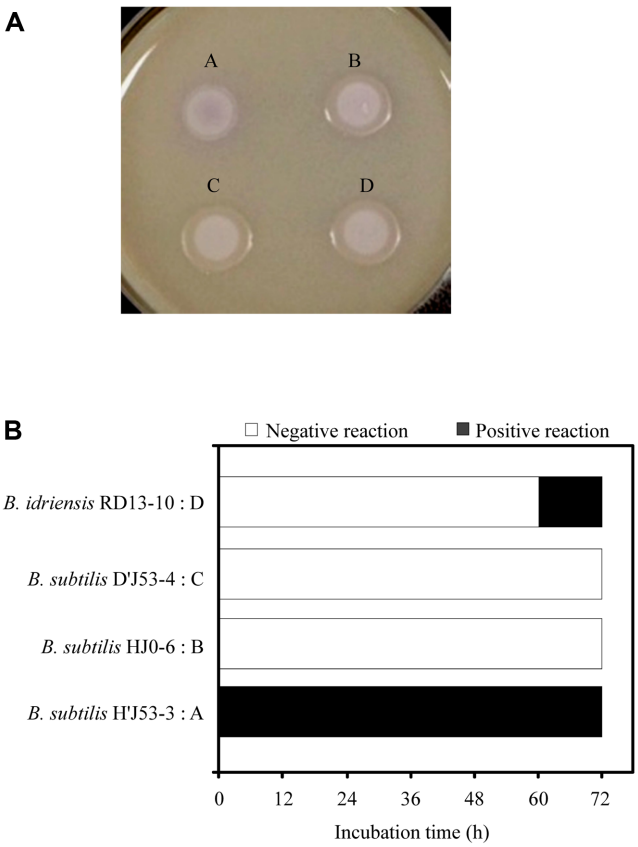

Fig. 1. Selection of biogenic amine-reducing strains in the decarboxylase broth.

Color development on the synthetic medium containing histidine, tyrosine, and cresol red as pH indicator during incubation of biogenic amine-producing bacterial strains. Negative reaction: yellow, Positive reaction: purple. Strains: A, *B. subtilis* H'J53-3. Biogenic amine-non-producing *Bacillus* strains isolated in this study: B, *B. subtilis* HJ0-6; C, *B. subtilis* D'J53-4; and D, *B. idriensis* RD13-10.

positive decarboxylase reaction of *B. subtilis* H'J53-3 and the negative activity of *B. subtilis* HJ0-6 and *B. subtilis* D'J53-4 were continuously maintained over time, whereas the *B. idriensis* RD13-10 strain showed a positive decarboxylase reaction after 60 h (Fig. 1B). In this study, we used *B. subtilis* H'J53-3 as the positive control because this strain is capable of producing BA and is positive for decarboxylase activity. The results indicated that *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10 exhibited no or weak decarboxylase activity against the BAs histidine and tyrosine.

Rodriguez-Jerez *et al*. [23] observed that *Bacillus* strains isolated from commercial samples of Spanish semi-preserved anchovies were able to prevent accumulation of BAs that lead to histamine food poisoning; this paper

investigated the effect of selecting strains of *Bacillus* for development as a means of reducing BA accumulation. In addition, *B. subtilis* MC138 isolated from a Korean traditional fermented food, *cheonggukjang*, showed a lower concentration of BAs such as histamine and tyramine; thus, this *B. subtilis* strain was used as a starter culture to inhibit BA production [4]. Thus, similarly, the bacterial strains isolated here, *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10, can be used as potential candidates for control of BA formation in food applications.

Expression of Genes Encoding Histidine- and Tyrosine-Decarboxylases and Amine Oxidase

To examine the expression of genes *hdc*, *tydc*, and *yobN* in the selected bacterial strains containing no or low decarboxylase activity, we performed transcription and translation analyses.

We determined whether expression of the BA-associated genes *hdc* and *tydc* in the selected strains could be detected, but found no expression in *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, or *B. idriensis* RD13-10, whereas *B. subtilis* H'J53-3, the BA-producing positive control, showed marked expression of these genes (Fig. 2A). Consistent with the RT-PCR data, the qPCR data showed that decarboxylase-related genes *hdc* and *tydc* were not expressed in *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, or *B. idriensis* RD13-10, but was expressed in *B. subtilis* H'J53-3 (Fig. 2B). Based on these results, we accepted that *B. subtilis* H'J53-3 can be used as a BA-producing bacterial strain, and that the remaining three strains produced little or no BAs.

We subsequently examined protein expression levels of histidine decarboxylase by using an antibody specific to histidine decarboxylase and synthesis of histamine. The histidine decarboxylase is a universal homodimeric enzyme

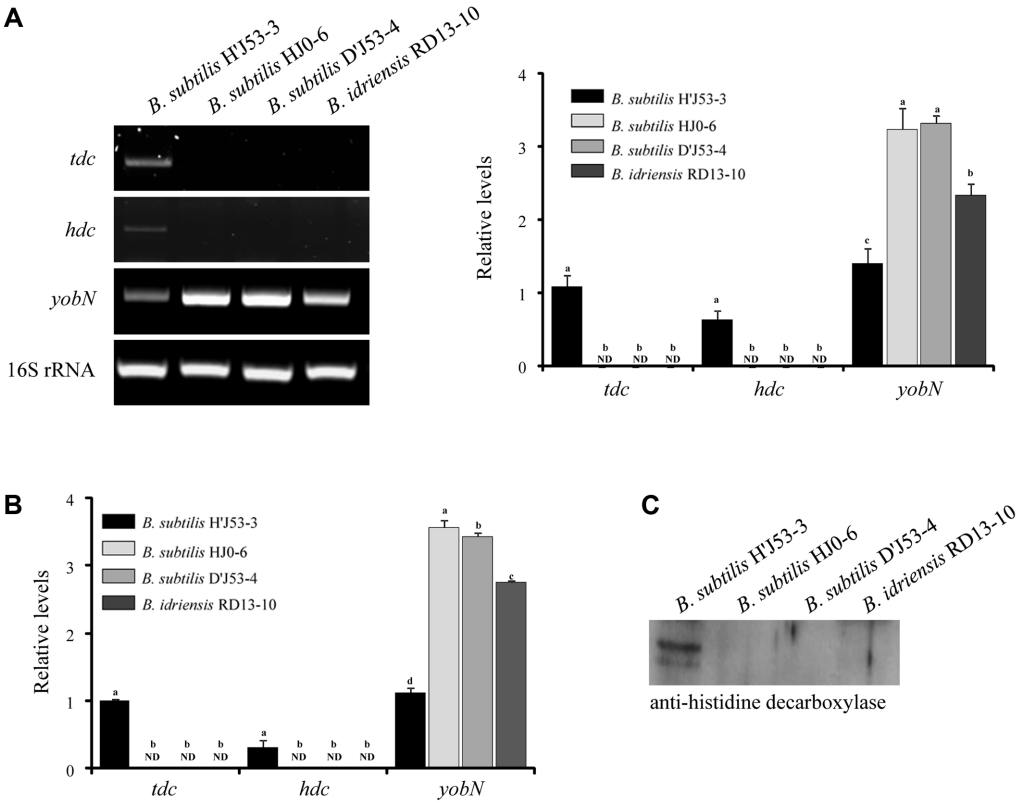

Fig. 2. Expression of *hdc*, *tydc*, and *yobN*, and histidine decarboxylase levels in selected bacterial strains.

Expression of *tydc*, *hdc*, and *yobN* mRNA was determined by RT-PCR (**A**) and qPCR (**B**). Band intensities were quantified by densitometry and normalized to 16S rRNA. qPCR expression data were normalized to 16S rRNA levels and reported relative to the expression level in the H'J53-3 positive control. Graphs represent relative mRNA levels for *tydc*, *hdc*, and *yobN*. Each value is the mean ± SD of three replicate analyses, and within each row means with different superscripts letters are statistically significantly different, $P < 0.05$ (one-way ANOVA, followed by Duncan's multiple comparison test). (**C**) The histidine decarboxylase levels in whole-cell extracts were detected by immunoblotting with an anti-histidine decarboxylase antibody. The experiment was performed at least three times, and representative data are shown.

involved in the biosynthesis of histamine from histidine; it is a pyridoxal phosphate-dependent decarboxylase and is highly specific for its histidine substrate. The antibody is used to detect the expression of the histidine decarboxylase enzyme in human, animal, and food samples. Fig. 2C shows that expression of the histidine decarboxylase protein was not detectable in *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, or *B. idriensis* RD13-10, but the BA-producing *B. subtilis* H'J53-3 exhibited marked levels of histidine decarboxylase.

Amine oxidases are ubiquitous enzymes that catalyze the oxidation of amines, producing aldehyde, ammonia, and hydrogen peroxide, and have a broad range of functions, including cell survival, differentiation, wound healing, detoxification, and cell signaling processes [14]. Normally, the amine oxidase enzyme system breaks down BAs, such as histamine and tyramine, and prevents excessive resorption, suggesting that bacterial amine oxidases are useful tools for the rapid quantification of BAs in foods [16]. Some lactic acid bacteria, such as *Lactobacillus casei*, isolated from different fermented foods, have been shown to degrade BAs through the production of amine oxidase enzymes [7]. Therefore, we assessed whether the selected strains can induce the production of *yobN* at the transcriptional level. As shown in Fig. 2A, *yobN* transcripts were detected in *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10, and their levels were higher than those observed in *B. subtilis* H'J53-3. The qPCR data confirmed the RT-PCR results, showing marked increases in the expression of *yobN* in the three strains (*B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10; Fig. 1B). These results demonstrated that the non-production of histidine and tyrosine decarboxylases might be regulated by the expression of the amine oxidase gene *yobN*, which leads to the breakdown of BAs in *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10. Thus, *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10 can be used as potential starter strains that do not produce BAs, in order to reduce the toxic levels of BAs in food.

Production of Biogenic Amines Histamine and Tyramine

Strains possessing histidine- and tyrosine-decarboxylase genes (*hdc* and *tydc*) generate histamine and tyramine in processed food products; these BAs causes sensitivity reactions and affect the safety of food, and are of particular concern in fermented foods owing to the intensive microbial activity with potential for BA formation [17]. Based on the mean content in foods and consumer exposure data, the food categories showing the highest mean values of histamine and tyramine are fermented sausages (23.6 and 136 mg/kg), fish sauce (197 and 107 mg/kg), cheese (62 and 104 mg/kg), fermented fish (31.2 and 47.9 mg/kg), and fermented vegetables (42.6 and 47.4 mg/kg) [2]. In patients with BA (histamine and tyramine) intolerance, even small amounts of BA in ingested food may cause harm or adverse health effects, so that only levels below detection limits can be considered as safe. However, to date, it has not been possible to conduct quantitative risk assessment of BAs, individually or in combination.

UPLC analysis was used to quantify the production of BAs (histamine and tyramine) in the culture supernatants of the above-mentioned four bacterial strains. As shown in Table 2, the concentrations of tyramine in the selected strains, *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10, were decreased by approximately 0.89 mg/l, 2.12 mg/l, and 2.53 mg/l, respectively, when compared with the positive control *B. subtilis* H'J53-3 (8.51 mg/l). A tyramine level of 1.09 mg/ml was detected in the culture medium, consisting of basal medium without bacteria, used as a negative control. In addition, no histamine was detected in supernatants of the *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10 strains, in comparison

Table 2. Contents of biogenic amines produced by selected strains using ultra-performance liquid chromatography analysis.

Sample	Biogenic amines (mg/l ± SD)[1)]		
	Histamine	Tyramine	Total
Medium	ND[2)]	1.09 ± 1.23[d]	1.09[d]
Bacillus subtilis H'J53-3	4.51 ± 0.34[a]	8.51 ± 1.23[a]	13.02[a]
Bacillus subtilis HJ0-6	ND	0.89 ± 0.08[e]	0.89[e]
Bacillus subtilis D'J53-4	ND	2.12 ± 0.16[c]	2.12[c]
Bacillus idriensis RD13-10	ND	2.53 ± 1.01[b]	2.53[b]

Determination of biogenic amines was performed after cultivation at 30°C for 24 h in LB broth containing 0.25% histidine and tyrosine.

[1)]Each value is the mean ± SD of three replicate analyses, and values within each row with different superscript letters are statistically significant at $p < 0.05$ (one-way ANOVA, followed by Duncan's multiple comparison test).

[2)]Not detected.

with the positive control *B. subtilis* H'J53-3 strain (4.51 mg/l; Table 2). Among the three selected strains, *B. subtilis* HJ0-6 strain had the lowest BA (histamine and tyramine) accumulation; consequently, this strain could be considered a good starter culture for controlling BA production in the food industry.

The best risk management strategies for BA are hygienic measures aimed at reducing the occurrence of BA-producing bacteria in raw material, use of additional microbial controls, and use of BA oxidase or BA-non-producing starter cultures. Kim *et al*. [13] reported that *B. subtilis* and *Bacillus amyloliquefaciens* isolated from traditional fermented soybean products did not produce BAs, and were found to reduce BAs, including histamine, tyramine, putrescine, and cadaverine. These findings suggest that the use of selected strains could be a potential control measure in manufacturing traditionally fermented soybean products in which it is difficult to control BA levels. Thus, these results demonstrated that the BA (histamine and tyramine)-non-producing strains (*B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10) that harbor the amine oxidase gene can be employed in strategies for the reduction of BA-associated risks.

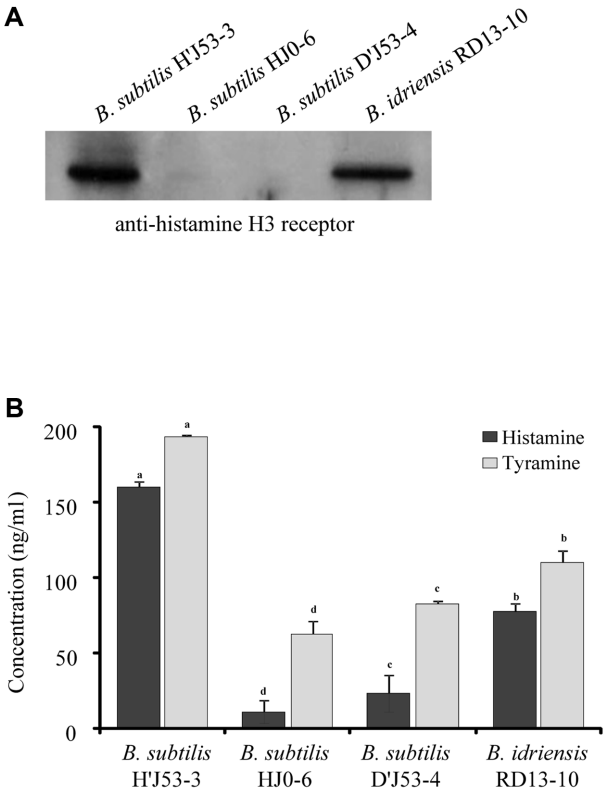

Fig. 3. Levels of histamine and tyramine produced by the selected bacterial strains.

(**A**) The expression levels of histamine in the whole cell from all strains were determined by western blotting using primary antibody specific for the histamine H3 receptor. Data are representative of three independent experiments. (**B**) Quantification of histamine and tyramine levels in cell supernatants using ELISA. Histamine and tyramine concentrations were increased in the *B. subtilis* H'J53-3 strain compared with that in other strains. All experiments were performed at least three times, and representative data are shown. Bars correspond to means ± SD. The sample means were compared by one-way ANOVA followed by Duncan's multiple comparison tests. Mean of different samples labeled with different letters are statistically significantly different ($p < 0.05$).

Degradation of Histamine and Tyramine by Selected Strains

We tested whether the selected strains that contained no or weak decarboxylase activity against histidine and tyrosine were able to degrade these BAs.

To examine the capacity for histamine degradation in the selected strains, we performed western blot analysis using an anti-histamine H3 receptor antibody for the detection and quantification of histamine. Fig. 3A shows that histamine H3 receptor expression decreased in *B. subtilis* HJ0-6 and that *B. subtilis* D'J53-4 exhibited a lower level of expression of the histamine H3 receptor than the *B. subtilis* H'J53-3 control, suggesting that histamine degradation was apparently mediated by suppression of histamine H3 receptor expression. However, expression of the histamine H3 receptor was markedly increased in the *B. idriensis* RD13-10 strain; thus, this strain had a lower histamine degradation rate than the *B. subtilis* HJ0-6 and *B. subtilis* D'J53-4 strains (Fig. 3A).

Presently, UPLC analytical methods enable the simultaneous and highly sensitive quantification of all BAs in food and food products; hence, these are best suited for monitoring and control purposes. Therefore, we performed UPLC-based quantification to determine the effect of the selected bacterial strains on the degradation of histamine and tyramine. Histamine and tyramine degradation rates in culture supernatants from *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10 were increased by approximately 19–47% compared with the *B. subtilis* H'J53-3 control strain (degradation rates of 2% and 1%). Consistent with previously reported results, the highest histamine and tyramine degradation activity was observed in *B. subtilis* HJ0-6, suggesting that using this strain as a positive starter culture can largely prevent BA accumulation in food products, especially in fermented food (Table 3).

To gain more comprehensive insights into the BA-

Table 3. Degradation rates of biogenic amines by selected strains using ultra-performance liquid chromatography.

Sample	Biogenic amine degradation rate (% ± SD)[1)]	
	Histamine	Tyramine
Bacillus subtilis H'J53-3	2.54 ± 0.66[a]	1.01 ± 0.99[a]
Bacillus subtilis HJ0-6	47.60 ± 1.57[d]	33.53 ± 2.23[c]
Bacillus subtilis D'J53-4	28.02 ± 0.44[c]	34.45 ± 1.16[c]
Bacillus idriensis RD13-10	19.48 ± 0.47[b]	26.23 ± 1.31[b]

Determination of biogenic amines was performed after cultivation at 30°C for 24 h in LB broth containing 0.25% histamine and tyramine.

[1)]The degradation rate is expressed as a percentage of the control lacking this strain. Each value is the mean ± SD of three replicate analyses, and within each row, means with different superscripts letters are statistically significantly different at $p < 0.05$ (one-way ANOVA, followed by Duncan's multiple comparison test).

degrading activities of these strains, we next analyzed the degradation of histamine and tyramine by these strains using ELISA. As shown in Fig. 3B, the histamine concentrations in culture supernatants were significantly reduced in *B. subtilis* HJ0-6 and *B. subtilis* D'J53-4, whereas it was increased by approximately 14- and 7-fold in the *B. subtilis* H'J53-3 control strain. Consistent with the histamine ELISA results, concentrations of tyramine were approximately 3- and 2-fold lower in *B. subtilis* HJ0-6 and *B. subtilis* D'J53-4 strains, respectively, than in the *B. subtilis* H'J53-3 control, suggesting that the significantly lower concentrations of histamine and tyramine in the bacterial-treated samples were related to the high degradation rates of these BAs (Fig. 3B). *B. idriensis* RD13-10 demonstrated relatively high histamine and tyramine concentrations, compared with the other two strains (*B. subtilis* HJ0-6 and *B. subtilis* D'J53-4). These results indicated that the selected three strains (*B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10) were able to degrade histamine and tyramine; in particular, *B. subtilis* HJ0-6 had the highest BA-degrading capability. Together, these results suggested that the three *Bacillus* strains *B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10 might affect the synthesis and degradation of BAs, such as histamine and tyramine, by decreasing the expression of histidine and tyrosine decarboxylase-related genes (*hdc* and *tydc*).

In conclusion, *Bacillus* species (*B. subtilis* HJ0-6, *B. subtilis* D'J53-4, and *B. idriensis* RD13-10) isolated from buckwheat *sokseongjang*, a Korean traditional fermented soybean food, showed significant inhibition of BA accumulation owing to their reduced expression of *hdc* and *tydc*, and abundant expression of *yobN*. The isolated bacterial strains may be used as effective biological control agents in various food products to degrade the undesired accumulation of toxic biogenic amines during food manufacturing, without causing qualitative changes and inhibiting the fermentation of *Bacillus* spp., and may have other uses in the food, agricultural, biotechnology, and pharmaceutical industries. Further studies are required to optimize food safety risks based on the control of BA formation in foods; such studies should include the establishment of criteria for hygienic processing and food safety, and validation of the analysis of novel emerging methods.

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