DOI:10.1021/jf101994u

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Metabolomic Assessment of Fermentative Capability of Soybean Starter Treated with High Pressure

Bong-Kuk Ko,[†] Ki Myong Kim,[‡] Young-Shick Hong,*[,†] and Cherl-Ho Lee*[,†]

[†]School of Life Science and Biotechnology, Korea University, Seoul 136-701, Republic of Korea, and [‡]Jeonnam Biofood Technology Center, 15-1 Dongsu dong san, Naju City, Jeollanamdo, Republic of Korea

Meju, a brick of dried fermented soybean naturally inoculated with microorganisms, is a starter used for producing traditional Korean fermented soybean products such as soybean paste (*doenjang*) and soy sauce (*ganjang*). In order to reduce aging time during production of soybean paste and soy sauce, high pressure (HP) treatment was applied to the *meju* starter at 500 MPa of pressure for 10 min at 15 °C. Fermentative behaviors of normal and HP-treated *meju* were assessed and compared through physicochemical and ^{1}H NMR-based metabolomic analysis. All *mejues* were incubated for 3 weeks at 30 °C. At 1 week of incubation, total bacterial population decreased mainly due to a reduction of water content by spontaneous evaporation during the incubation period. As the incubation time increased, glutamate, proline, betain, choline, and phosphocholine levels increased in both normal and HP-treated *mejues*, indicating that microorganisms in the *mejues* synthesize these metabolites to endure intracellular hyperosmotic stress induced by the reduction in water content. Through 3 weeks of incubation, the amino-type nitrogen contents and neutral protease activities in HP-treated *meju* were significantly higher ($p < 0.05$) than in normal *meju*, even though total bacterial content in HP-treated *meju* was 2 or 3 times lower. Moreover, marked increases in glycerol, acetate, tyrosine, and choline levels were observed in HP-treated *meju* compared to normal *meju*. In particular, higher levels of tyrosine in HP-treated *meju* were consistent with the increased neutral protease activities compared to normal *meju*, indicating an improvement in enzyme stability with HP treatment. These findings highlight a new or better understanding of the influence of the HP or physical treatments on fermentative products in food processing, such as those associated with soybean paste and soy sauce, regarding metabolic behaviors in fermentative starter induced by HP treatment.

KEYWORDS: Metabolomics; NMR; *meju*; starter; fermentation; soy sauce

INTRODUCTION

Fermented soybean products are important in Korean, Chinese, and Japanese diets as a nutrient source as well as a substance that provides palatability to the diet (*1*). In particular, soybean paste (*doenjang*) and soy sauce (*ganjang*) are representative products of soybean fermentation manufactured with *meju* molded with cooked and crushed soybeans and used as a fermentation starter (*2*). In general, traditional Korean *meju* is produced with only soybeans and fermented with various beneficial microflora, typically *Bacillus subtilis* and *Aspergillus oryzae*, from naturally inoculated rice straw (*3*). However, at least 1 year is required for producing high quality soybean products with *meju* starter (*3*). In attempts to reduce fermentation time and improve the quality and functionality of soybean products, various methods have been evaluated, for example, gamma irradiation and hydrostatic pressure (*4*−*10*).

High pressure (HP) treatment has been considered as one of the diverse ways to improve the qualities of food products during processing. The benefits of HP included the fact that it is a nonthermal technology capable of eliminating pathogenic microorganisms and extending shelf life while maintaining high sensory quality and improving safety (*7*, *8*). Yokohama et al. (*9*) filed a patent for acceleration of cheese ripening using HP, in which they reported that Cheddar cheese could be ripened in 3 days by treatment at 50 MPa. This finding showed that HP treatment is very effective in reducing ripening time especially that of the conventional Cheddar cheese, where a ripening period of 6 to 9 months is needed. Saldo et al. (*10*) also reported that HP-treated goat milk cheeses at 400 MPa for 5 min were found to have increased levels of total free amino acids, indicating acceleration of the cheese ripening process.

Metabolomics or metabonomics in the "-omics" era is a promising new approach aimed at improving our understanding of metabolic perturbations in drug toxicity (*11*, *12*), disease status or risk (*13*, *14*), dietary intervention (*15*, *16*), and plant metabolism (*17*, *18*). Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are used to generate global metabolite profiles in metabolomic studies (*19*). An advantage of NMR is that the samples do not require any physical or chemical treatment prior to analysis, whereas MS usually requires the metabolites to be separated from the sample before detection by liquid chromatography (*20*). Pattern recognition methods, such as principal components analysis (PCA), partial least-squares discriminant

*To whom correspondence should be addressed. Y.-S.H.: e-mail, chtiger@korea.ac.kr; tel, +82-2-929-2751; fax, +82-2-927-5201. C.-H.L.: e-mail, chlee@korea.ac.kr; tel, +82-2-3290-3414; fax, +82-2-929-5201.

 Published on Web 07/21/2010

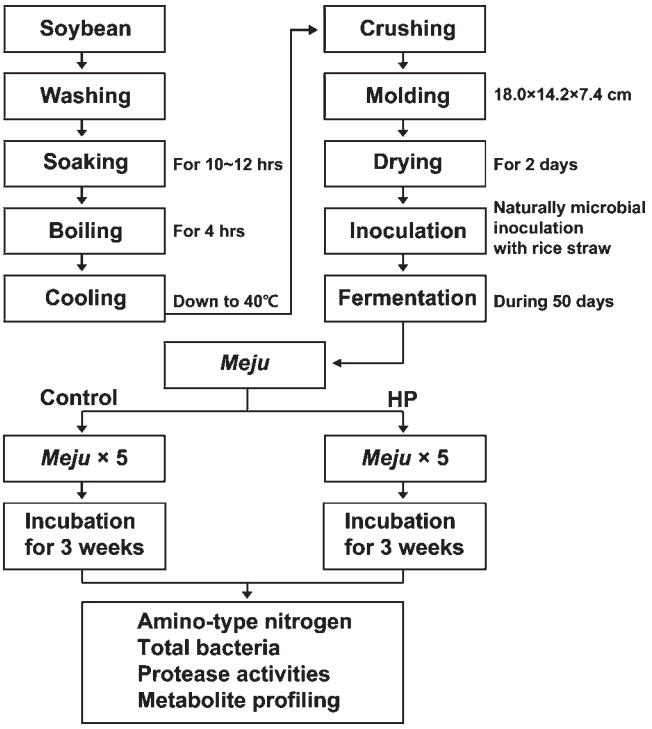

Figure 1. Manufacturing process of traditional Korean *meju* and experimental scheme.

analysis (PLS-DA) and orthogonal-projection to latent structures discriminant analysis (OPLS-DA), can be used to discern significant patterns in complex data sets, resulting in a reduction of the dimensionality of multivariate data and in visualization of inherent patterns in the data. Recently, ^{1}H NMR-based metabolomic studies have been applied in the food sciences (*21*−*24*). In particular, we reported on metabolic dependence on aging periods in soy sauce using ^{1}H NMR-based metabolomic studies (*3*). Since *meju* starter also consists of metabolites originating from soybean and produced by fermentative microorganisms inoculated naturally, comprehensive profiling of metabolites in *meju* could provide a better understanding of the effects of HP treatment on fermentative behavior in *meju* starter.

In the present study, we evaluated the effect of HP treatment on *meju* starter and the fermentative behavior of the HP-treated *meju*, through physicochemical and ^{1}H NMR-based metabolomic approaches, prior to further study on investigation of the HP-treatment effect on the acceleration of aging in fermented soybean products, soybean paste and soy sauce, manufactured with HP-treated *meju* starter.

MATERIALS AND METHODS

Soybean. Soybeans (*Glycine max*) harvested in 2009 were purchased from the National Agricultural Cooperative Federation (Changpyeong-myeon branch in Damyang-gun, Jeollanam-do, Korea) to make traditional Korean *meju* starter.

Preparation of *Meju*. The method used for producing traditional Korean *meju* is described in **Figure 1**. In brief, dried soybeans were washed and soaked in water at 20 °C for 10 to 12 h, and boiled for 4 h. The boiled soybeans were cooled down to 40 °C and then pounded to making a paste. This paste was then molded into hexagonal bricks with a constant size of 18.0 × 14.2 × 7.4 cm. After drying under the sunlight for about 2 days, the blocks were tied up with rice straw, moved to a warm place, and hung to speed up fermentation, which takes 50 days. During this stage, various beneficial bacteria and molds inoculated naturally on the rice straw transform into the blocks; referred to as *meju* (*3*).

High Pressure Treatment. Five *mejues* were packed separately in a polyethylene bag under vacuum. The packaged samples were loaded into a cylindrical container and treated at 500 MPa for 10 min at 15 °C. HP treatments were performed using QUINTUS Type QFP 35 L-600 (Avure Technologies, Sweden). The maximum operating pressure of the Quintus pressure vessel was 600 MPa. This pressure vessel has a 35 L capacity and the internal dimensions of 190 mm (*D*) × 1,220 mm (*H*). After high pressure treatment, the *meju*es were cut and ground to pass through a 2 mm sieve, which included the normal *mejues*. The ground *mejues* were put into Petri dishes and incubated at 30 °C and 45% RH for 3 weeks. For the incubation, a total of 40 Petri dishes were prepared from each 5 experimental replicate of normal and HP-treated *mejues*, which were ground, at 0, 1, 2, and 3 weeks of incubation and 10 Petri dishes corresponding to normal and HP-treated *meju* starter were taken each week for analysis.

Moisture Contents and pH Value. Ground *meju* (5 g) was mixed with distilled water (20 mL), and the pH was measured using a pH meter (Orion 3 Star, Beverly, MA). Moisture contents of *meju* were determined at 105 °C by a drying method (*25*).

Amino-Type Nitrogen. The analysis of amino-type nitrogen was performed using the formol titration method (*26*). *Meju* (5 g) was mixed with distilled water (95 mL) and homogenized by stirring for 1 h and then centrifuged at 10,000 rpm (15230*g*) for 5 min. Supernatants (25 mL) were added to distilled water (20 mL), and neutral formalin (20 mL) was subsequently added to the solution. The pH of the formalin was adjusted to 8.4 by adding 0.1 N NaOH. The amount of amino-type nitrogen was calculated using the following equation:

$$\text{amino-type nitrogen (\%)} = [\text{sample titration (mL)} - \text{blank test (mL)}] \times 0.0014 \times F \times D \times 100/S$$

where the constant 0.0014 is the amount (g) of amino-type nitrogen equal to 1 mL of 0.1 N NaOH, *F* is the factor of 0.1 N NaOH, *D* is the dilution factor, and *S* is the sample amount (g). All data were calculated using the dry weight of each sample.

Neutral and Alkaline Protease Activities. Neutral and alkaline protease activities were measured using the method described by Kim et al. (*6*). *Meju* (5 g) was mixed with distilled water (45 mL). The mixtures were extracted by shaking for 5 h at 30 °C (shaking incubator, VS-8498SF, Vision Scientific Co. Ltd., Seoul, Korea) and centrifuging at 12,000 rpm (14650*g*) for 10 min. The supernatants were used to measure protease activity. The substrate was casein (1% Hammersten casein, USB Corporation, Cleveland, OH) dissolved in 0.1 M phosphate buffer (neutral) in borate buffer (0.2 M, pH = 7 for neutral protease, pH = 10.0 for alkaline protease). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine mL^{-1} min^{-1}. All data were calculated using the dry weight of each sample.

Total Number of Bacteria. Total viable cell counts were determined on plates onto which had been poured 1 mL of diluted *meju* samples using plate count agar (Difco). The plates were incubated for 3 days at 30 °C. Results were reported as log CFU (colony forming units) per gram of *meju*.

^{1}H NMR Spectroscopic Analysis of *Meju*. *Meju* (2 g) was mixed with distilled water (8 mL) and incubated with shaking for 1 h at 30 °C (shaking incubator, VS-8408SF, Vision Scientific Co. Ltd., Seoul, Korea) and then centrifuged at 13,000 rpm (15870*g*) for 10 min. The supernatant (120 μL) was mixed with phosphate buffer (480 μL, 0.1 M sodium phosphate in H_2O/D_2O = 10/90, pH 7.0). The mixture (550 μL) was transferred into 5 mm NMR tubes. D_2O provided a field frequency lock. ^{1}H NMR spectra were acquired on a Varian Inova-500 MHz NMR spectrometer (Varian Inc., Palo Alto, CA) operating at 499.899 MHz ^{1}H frequency and a temperature of 298 K, using a 5 mm HCN triple resonance indirect probe. A NOESY PRESAT pulse sequence was applied to suppress the residual water signal. For each sample, 64 transients were collected into 32 K data points using a spectral width of 8012.6 Hz with a relaxation delay of 2 s, an acquisition time of 2.00 s, and a mixing time of 400 ms. Chemical shift was referenced to that of lactate (^{1}H, δ 1.32). A 1.0 Hz line-broadening function was applied to all spectra prior to Fourier transformation (FT). Signal assignment for representative samples was facilitated by acquisition of two-dimensional (2D) total correlation spectroscopy (TOCSY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), and spiking experiments, as described in our previous study (*3*).

NMR Data Preprocessing and Multivariate Statistical Analysis. All NMR spectra were manually phased and baseline corrected by VnmrJ software 2.1B (Varian Inc., Palo Alto, CA) and then converted to ASCII

Table 1. Changes in pH, Amino-Type Nitrogen (%), Water Content (%), Enzyme Activity (unit/g) and Total Bacteria (cfu/g $\times 10^{10}$) in Normal (N) and High Pressure (HP)-Treated *Meju* during 3 Weeks of Incubation

	0 week	1 week	2 weeks	3 weeks
pH				
N	7.44 ± 0.06 a,A[a]	6.83 ± 0.29 b,A	6.78 ± 0.14 b,A	6.75 ± 0.07 b,A
HP	7.50 ± 0.16 a,A	6.96 ± 0.12 b,A	6.65 ± 0.10 c,A	6.66 ± 0.07 c,A
amino-type nitrogen				
N	0.92 ± 0.11 a,A	1.06 ± 0.16 a,B	1.00 ± 0.14 a,B	0.94 ± 0.10 a,B
HP	1.04 ± 0.04 c,A	1.32 ± 0.05 a,A	1.22 ± 0.06 ab,A	1.15 ± 0.04 bc,A
water contents				
N	35.34 ± 2.43 a,A	11.38 ± 2.56 b,A	8.48 ± 0.35 c,A	8.25 ± 0.44 c,A
HP	36.42 ± 0.49 a,A	11.55 ± 1.84 b,A	8.73 ± 0.28 c,A	8.92 ± 0.52 c,A
neutral protease				
N	44.53 ± 2.39 ab,A	47.80 ± 3.71 a,B	43.31 ± 2.31 b,B	42.87 ± 1.80 b,B
HP	46.83 ± 1.23 b,A	53.05 ± 2.67 a,A	47.25 ± 1.30 b,A	45.48 ± 0.98 b,A
alkaline protease				
N	43.04 ± 2.89 b,A	51.40 ± 3.67 a,A	53.61 ± 2.62 a,A	53.19 ± 3.69 a,A
HP	44.92 ± 1.56 b,A	52.77 ± 2.48 a,A	55.97 ± 4.14 a,A	54.19 ± 1.50 a,A
total bacteria				
N	0.46 ± 0.04 d,A	9.68 ± 1.31 a,A	3.30 ± 0.74 c,A	4.83 ± 0.92 b,A
HP	0.10 ± 0.01 c,B	5.38 ± 0.92 a,B	1.74 ± 0.41 b,B	1.81 ± 0.30 b,B

[a] The same lowercase letter in a row indicates no significant difference at $P < 0.05$. The same uppercase letter in a column indicates no significant difference at $P < 0.05$.

formatted files which were imported into MATLAB (R2006a, Mathworks, Inc., 2006). NMR spectral data were reduced into 0.001 ppm spectral buckets. The region corresponding to water (4.7−5.0 ppm) was removed prior to normalization to the total spectral area. After the normalization, all spectra were aligned using the recursive segmentwise peak alignment (RSPA) method to reduce variability in the peak positions (*27*). The resulting data sets were then imported into SIMCA-P version 12.0 (Umetrics, Umeå, Sweden) for multivariate statistical analysis.

The mean center was applied for all multivariate analysis by SIMCA-P version 12.0 (Umetrics, Sweden). Principal components analysis (PCA), an unsupervised pattern recognition method, was performed to examine intrinsic variation in the data set. To maximize separation between samples, orthogonal partial least-squares discriminant analysis (OPLS-DA) was applied. OPLS-DA can be described as the regression extension of PCA that gives the maximum covariance between measured data (*X* variable, metabolites in NMR spectra) and the response variable (*Y* variable, classifier on group). Hotelling's *T*2 region, shown as an ellipse in the scores plots, defines the 95% confidence interval of the modeled variation (*28*). The quality of the model was described by R^2x and Q^2 values. R^2 is defined as the proportion of variance in the data explained by the model and indicates goodness of fit, and Q^2 is defined as the proportion of variance in the data predictable by the model and indicates predictability (*22*).

Statistical Analysis. All data were analyzed by independent-sample *t*-test and analysis of variance (ANOVA). Significant differences in mean values among data were determined at $P < 0.05$ by Duncan's multiple range test and *t*-test using SPSS 12.0 (SPSS Inc., Chicago, IL).

RESULTS

Change in pH, Water Contents, and Amino-Type Nitrogen, Protease Activity, and Total Bacteria. A marked reduction in total bacteria in HP-treated *meju* starter was observed compared to that found in normal *meju* starter, indicating an inactivation of microorganism by HP treatment (**Table 1**). The largest amount of amino-type nitrogen and the highest activity of proteases were observed at 1 week of incubation in both normal and HP-treated *meju* starters, which is consistent with the findings that total bacteria was the greatest at 1 week. After 1 week, total bacteria were markedly reduced due to the decreased water content of less than 10% (w/w) by spontaneous evaporation during the incubation. The reduction in total bacteria may result in no changes in pH, amino-type nitrogen contents and protease activities in both normal and HP-treated *meju* starters as incubation time increases. However, amino-type nitrogen contents and neutral protease activity were significantly higher in HP-treated *meju* starter, although total bacteria in HP-treated *meju* starter was 2 or 3 times less than normal *meju* starter.

^{1}H NMR *Meju* Spectra. Representative one-dimensional (1D) ^{1}H NMR spectra of normal and HP-treated *mejues* incubated at 0 and 3 weeks are shown in **Figure 2**. Forty metabolites were identified through analysis of 2D NMR and spiking experiments as well as information from our previous study (*3*). The ^{1}H NMR spectra of *mejues* were dominated by a number of metabolites: leucine, isoleucine, valine, lactate, alanine, acetate, arginine, betaine, tyramine, phenylalanine, formate, α-glucose, *β*-glucose, lysine, *γ*-aminobutyrate (GABA), fucose, ethanol, succinate, tyrosine, proline, choline, uracil, aspartate, methionine, pyroglutamate, glutamate, glycine, trimethylamine, malonate, phosphocholine, butyrate, oligosaccharide (O1), oligosaccharide (O2), oligosaccharide (O3), glycerol, hypoxanthine, histamine, histidine, tryptophan, and fumarate.

Visual inspection revealed marked decreased levels of glucose and oligosaccharides and increased levels of betaine in *mejues* after 3 weeks of incubation. To examine the significant variations in metabolites during the incubation, and between HP-treated and normal *mejues*, pattern recognition methods such as PCA and OPLS-DA were applied to each entire data set. The PCA and OPLS-DA score plots showed clear metabolic dependences in both normal (**Figure 3A**,**B**) and HP-treated (**Figure 3C**,**D**) *mejues* according to incubation times. Movements of the score plots from the right to the left indicate a continuous metabolic change in HP-treated and normal *mejues* during the incubation.

Changes in Metabolites of *Meju* Starters during Incubation. To identify the metabolites changed during incubation, an OPLS-DA model was generated in normal *meju* incubated for 0 and 3 weeks (**Figure 4**). OPLS-DA score plot showed clear differentiation of normal *meju* at 0 and 3 weeks, with high statistics as indicated by the statistical values of $R^2X = 0.83$, $R^2Y = 0.98$ and $Q^2 = 0.90$ (**Figure 4A**). To validate the OPLS-DA model, we performed permutation tests with 200 random permutations in PLS models with the same components as the OPLS models and cross-validated (CV)-ANOVA test (*29*, *30*). Most R^2Y and Q^2Y values of the permuted models were lower than the corresponding values of the original model of normal *meju* at 0 and 3 weeks, thus validating the model (**Figure 4C**). The OPLS-DA loading plot reveals the metabolites that contributed to the differentiation in the complementary OPLS-DA score plot (**Figure 4B**). The upper section of the loading plot indicates relatively higher levels of metabolites in

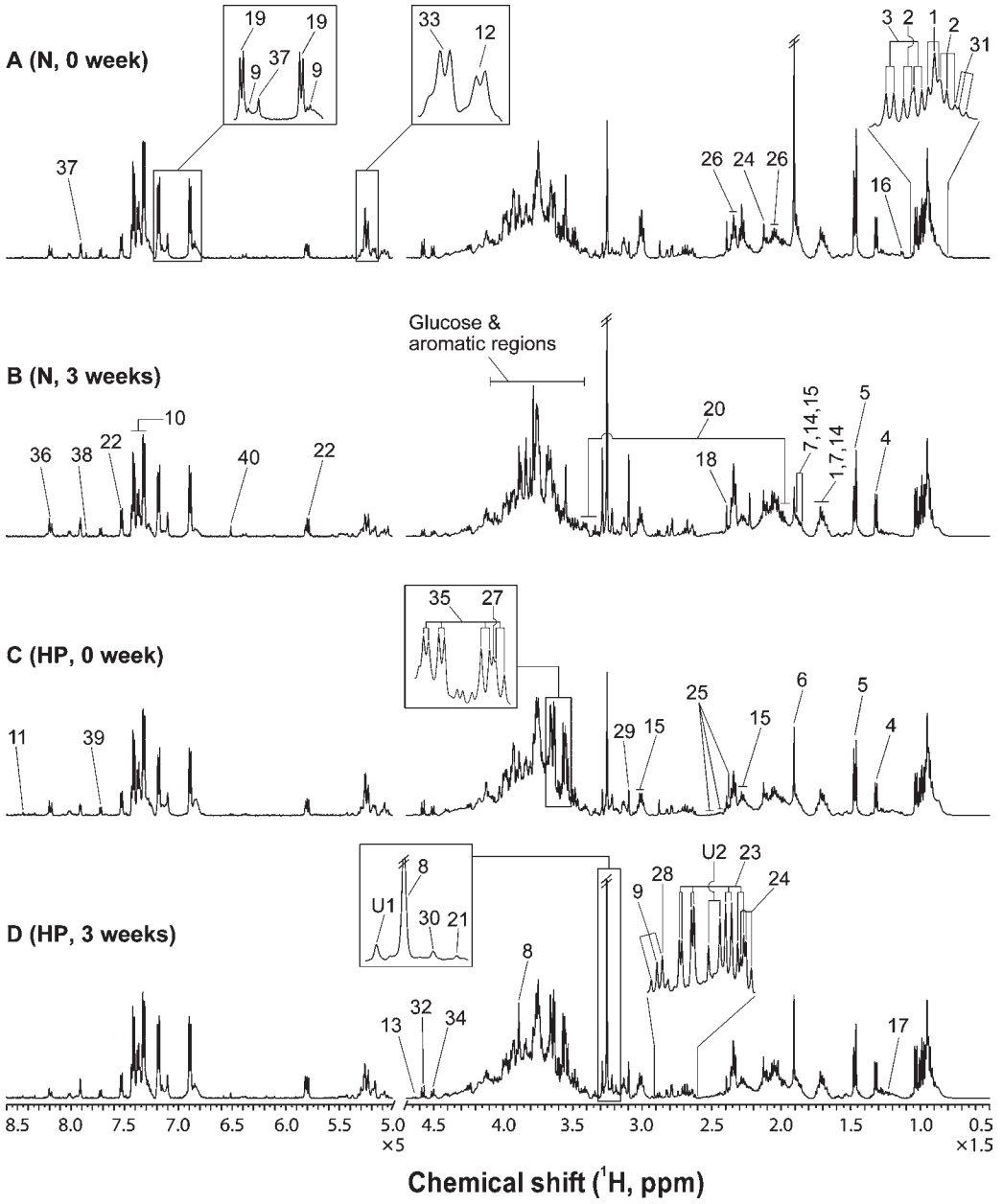

Figure 2. A typical 500 MHz ^{1}H NMR spectra of normal *meju* incubated for 0 week (**A**) and 3 weeks (**B**) and of high pressure (HP)-treated *meju* incubated for 0 week (**C**) and 3 weeks (**D**). Key: 1, leucine; 2, isoleucine; 3, valine; 4, lactate; 5, alanine; 6, acetate; 7, arginine; 8, betaine; 9, tyramine; 10, phenylalanine; 11, formate; 12, α-glucose; 13, β-glucose; 14, lysine; 15, γ-aminobutyrate (GABA); 16, fucose; 17, ethanol; 18, succinate; 19, tyrosine; 20, proline; 21, choline; 22, uracil; 23, aspartate; 24, methionine; 25, pyroglutamate; 26, glutamate; 27, glycine; 28, trimethylamine; 29, malonate; 30, phosphocholine; 31, butyrate; 32, oligosaccharide (O1); 33, oligosaccharide (O2); 34, oligosaccharide (O3); 35, glycerol; 36, hypoxanthine; 37, histamine; 38, histidine; 39, tryptophan; 40, fumarate; U1 and U2, unknown compounds.

normal *meju* at 3 weeks compared to 0 week, whereas the lower section indicates lower levels. The differentiation between normal *meju* at 0 and at 3 weeks was caused by higher levels of betaine, proline, uracil, methionine, glutamate, malonate, phosphocholine, fumarate, and unknown compound (U1) and by lower levels of leucine, isoleucine, valine, alanine, acetate, arginine, tyramine, phenylalanine, α-glucose, lysine, γ-aminobutyrate (GABA), fucose, succinate, tyrosine, oligosaccharide (O1), oligosaccharide (O2), oligosaccharide (O3), and tryptophan in normal *meju* at 3 weeks (**Figure 4B**). The significance of metabolite changes was verified by column loading plots (*22*) and is shown typically in Figure S-1 (in the Supporting Information). Metabolites having significant changes were indicated by corresponding numbers in the loading plots throughout the present study.

Figure 5A also shows clear differentiation between HP-treated *meju* incubated for 0 and 3 weeks, with high statistics of $R^2X = 0.81$, $R^2Y = 0.95$ and $Q^2 = 0.85$. We performed permutation tests as mentioned previously and thus validated the model (**Figure 5C**). HP-treated *meju* at 3 weeks incubation was characterized by higher levels of betaine, tyrosine, choline, methionine, glutamate, malonate, phosphocholine, fumarate, and U1, as shown in the OPLS-DA loading plot (**Figure 5B**). In addition, levels of leucine, alanine, acetate, α-glucose, fucose, trimethylamine, oligosaccharide (O1), oligosaccharide (O2), oligosaccharide (O3), glycerol, and hypoxanthine were decreased in HP-treated *meju* at 3 weeks of incubation compared to those in HP-treated *meju* at 0 week.

Differences in Metabolites between Normal and HP-Treated *Meju* Starters. To investigate the effects of HP treatment on

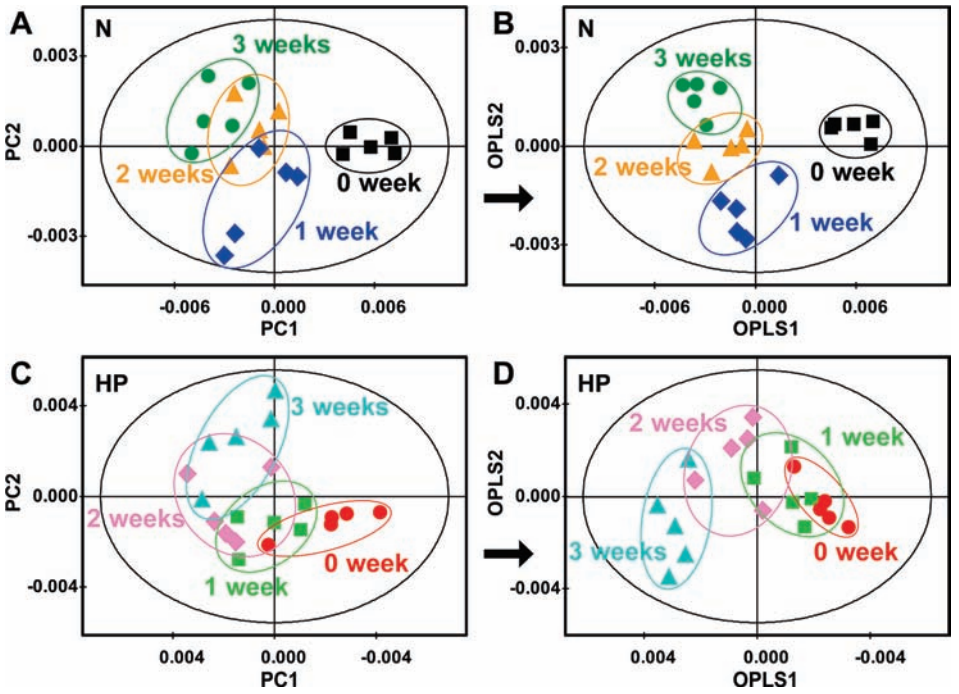

Figure 3. PCA (**A** and **C**) and OPLS-DA (**B** and **D**) score plots derived from the ^{1}H NMR spectra of normal (N) and high pressure (HP)-treated *meju*, indicating metabolic evolution during 3 week incubation.

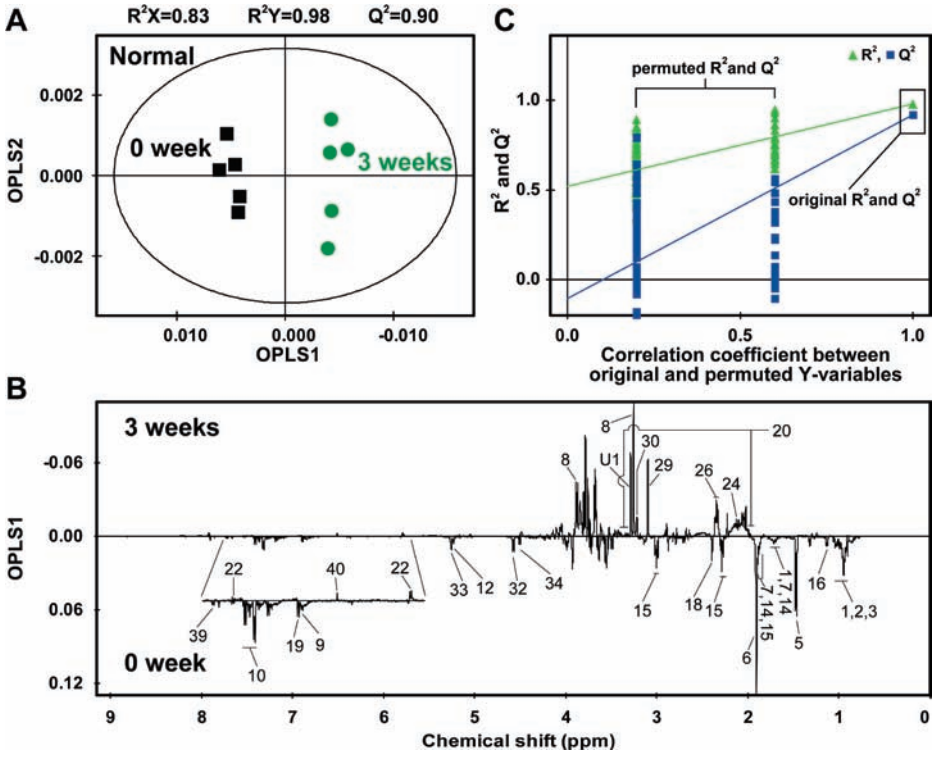

Figure 4. OPLS-DA score (**A**), loading plots (**B**), and permutation test (**C**) derived from the ^{1}H NMR spectra of normal *meju* incubated for 0 week and 3 weeks. Key: 1, leucine; 2, isoleucine; 3, valine; 5, alanine; 6, acetate; 7, arginine; 8, betaine; 9, tyramine; 10, phenylalanine; 12, α-glucose; 14, lysine; 15, γ-aminobutyrate (GABA); 16, fucose; 18, succinate; 19, tyrosine; 20, proline; 22, uracil; 24, methionine; 26, glutamate; 29, malonate; 30, phosphocholine; 32, oligosaccharide (O1); 33, oligosaccharide (O2); 34, oligosaccharide (O3); 39, tryptophan; 40, fumarate; U1, unknown compound.

meju metabolites, OPLS-DA models were applied to ^{1}H NMR data sets obtained from normal and HP-treated *mejues* incubated at each week. OPLS-DA score plots showed clear differentiation between normal and HP-treated *mejues* at 0, 1, 2, and 3 weeks, with the statistical values of $R^2X = 0.80$, $R^2Y = 0.96$ and $Q^2 = 0.70$, $R^2X = 0.63$, $R^2Y = 0.91$ and $Q^2 = 0.74$, $R^2X = 0.59$, $R^2Y = 0.96$, and $Q^2 = 0.76$, and $R^2X = 0.62$, $R^2Y = 0.93$ and $Q^2 = 0.64$, respectively (**Figure 6**). To validate the OPLS-DA models, we also performed permutation tests as mentioned previously (Figure S-2 in the Supporting Information). In fact, the model between normal and HP-treated *mejues* at 1 week did not validate because one sample from normal *meju* starter at 1 week overlapped, mainly due to

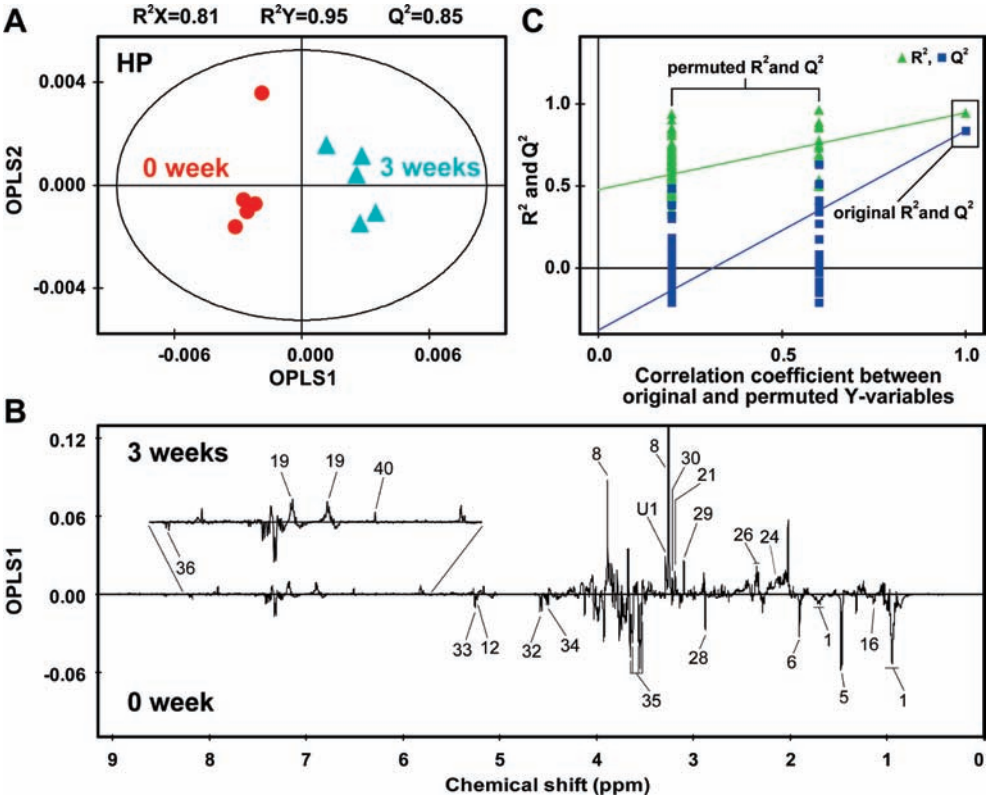

Figure 5. OPLS-DA score (**A**), loading plots (**B**), and permutation test (**C**) derived from the ^{1}H NMR spectra of high pressure (HP)-treated *meju* incubated for 0 week and 3 weeks. Key: 1, leucine; 5, alanine; 6, acetate; 8, betaine; 12, α-glucose; 16, fucose; 19, tyrosine; 21, choline; 24, methionine; 26, glutamate; 28, trimethylamine; 29, malonate; 30, phosphocholine; 32, oligosaccharide (O1); 33, oligosaccharide (O2); 34, oligosaccharide (O3); 35, glycerol; 36, hypoxanthine; 40, fumarate; U1, unknown compound.

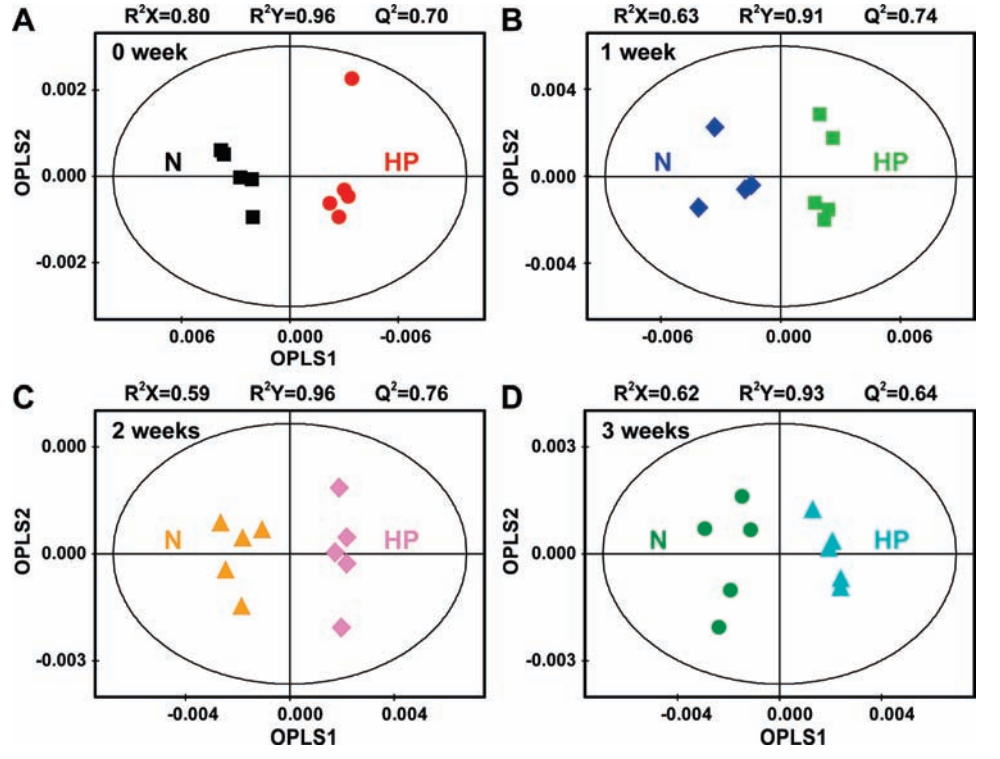

Figure 6. OPLS-DA score plots derived from the ^{1}H NMR spectra of normal (N) and high pressure (HP)-treated *meju* at each incubation time of 0 (**A**), 1 (**B**), 2 (**C**), and 3 (**D**) weeks, showing the comparisons of metabolites between normal and HP-treated *meju* starters at each incubation time.

misalignments in several regions of the ^{1}H NMR spectrum (data not shown). Therefore, we excluded the overlapped sample and regenerated the OPLS-DA model between normal and HP-treated *meju* starters at 1 week, resulting in improvement of the predictability of the model as indicated by increases in Q^2 value from 0.41 to 0.74 and validation (Figure S-2B in the Supporting Information).

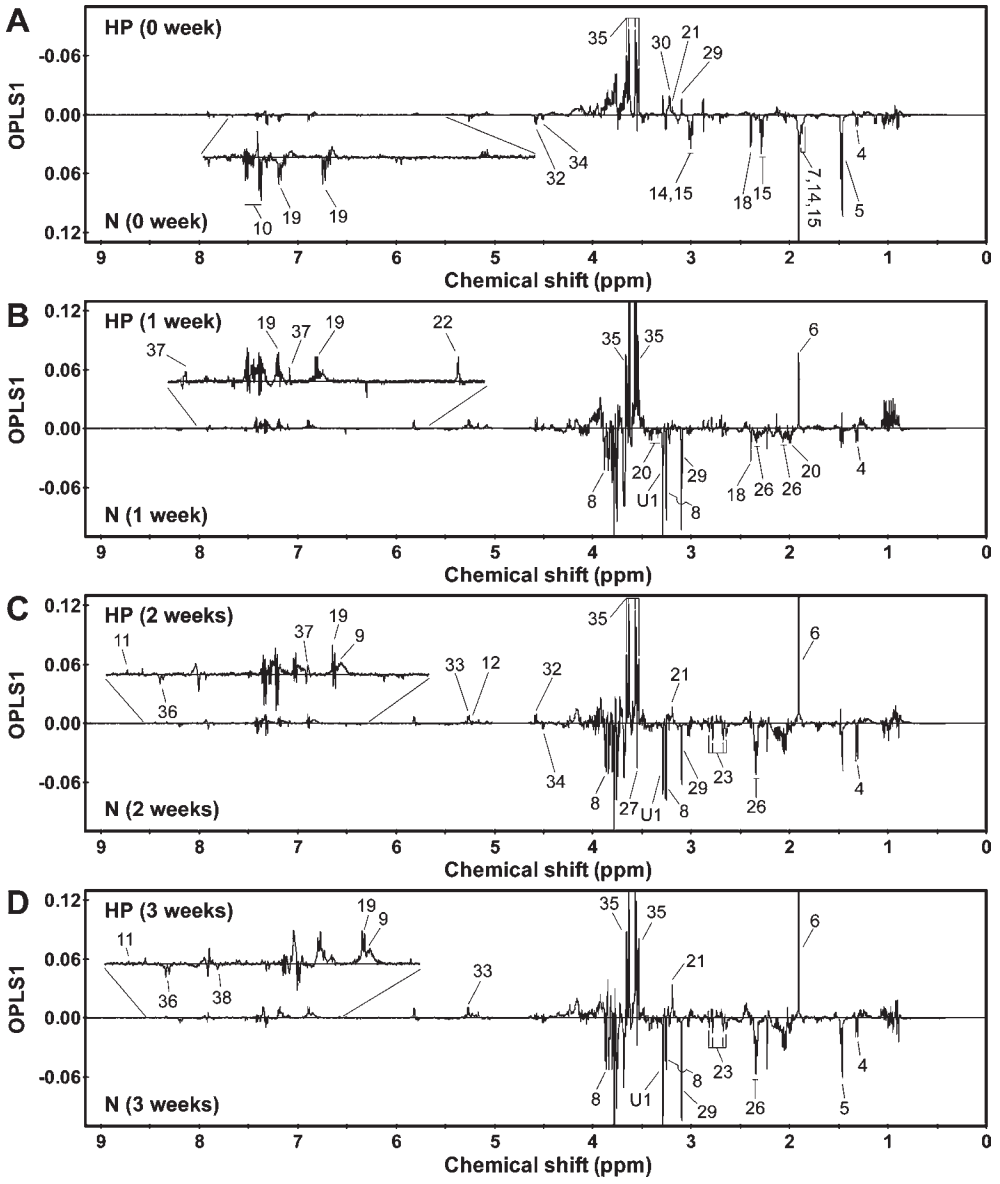

Figure 7. Identifications of metabolites responsible for the differentiations of normal (N) and high pressure (HP)-treated *meju* at incubation times of 0 (**A**), 1 (**B**), 2 (**C**) and 3 (**D**) weeks, through OPLS-DA loading plots in **Figure 6**, which correspond to OPLS-DA score plots. Key: 4, lactate; 5, alanine; 6, acetate; 7, arginine; 8, betaine; 9, tyramine; 10, phenylalanine; 11, formate; 12, α-glucose; 14, lysine; 15, γ-aminobutyrate (GABA); 18, succinate; 19, tyrosine; 20, proline; 21, choline; 22, uracil; 23, aspartate; 26, glutamate; 27, glycine; 29, malonate; 30, phosphocholine; 32, oligosaccharide (O1); 33, oligosaccharide (O2); 34, oligosaccharide (O3); 35, glycerol; 36, hypoxanthine; 37, histamine; 38, histidine; U1, unknown compound.

To identify the metabolites influenced by HP treatment in *meju*, OPLS-DA loading plots were generated (**Figure 7**). The differentiation between normal and HP-treated *meju* at 0 week was caused by higher levels of choline, malonate, phosphocholine, and glycerol and by lower levels of lactate, alanine, arginine, phenylalanine, lysine, γ-aminobutyrate (GABA), succinate, tyrosine, oligosaccharide (O1), and oligosaccharide (O3) in HP-treated *meju* at 0 weeks (**Figure 7A**). Higher levels of acetate, tyrosine, uracil, histamine, and glycerol in HP-treated *mejues* and lower levels of lactate, betain, succinate, proline, glutamate, malonate, and U1 were responsible for the differentiation between normal and HP-treated *mejues* at 1 week incubation (**Figure 7B**). Upon comparison between normal and HP-treated *mejues* at 2 weeks, we found higher levels of acetate, tyramine, formate, α-glucose, tyrosine, choline, oligosaccharide (O1), oligosaccharide (O2), glycerol, and histamine, together with lower levels of lactate, betaine, aspartate, glutamate, glycine, malonate, oligosaccharide (O3), hypoxanthine, and U1 in HP-treated *meju* (**Figure 7C**). Higher levels of acetate, tyramine, formate, tyrosine, choline, oligosaccharide (O2), and glycerol in HP-treated *meju* were responsible for the differentiation between normal and HP-treated *mejues* at 3 weeks incubation (**Figure 7D**). On the other hand, normal *meju* at 3 weeks incubation was dominated by higher levels of alanine, betaine, aspartate, glutamate, malonate, hypoxanthine, histidine, and U1, compared to HP-treated *meju* at 3 weeks of incubation.

DISCUSSION

We assessed the effect of HP treatment on fermentative behaviors in *meju*, a fermentative starter for soybean paste and soy sauce, by using physicochemical changes and global metabolite profiling with ^{1}H NMR spectroscopy. Furthermore, the metabolic changes and fermentative behaviors in both normal and HP-treated *meju* starters during incubation were also investigated.

Physicochemical Changes of Normal and HP-Treated *Mejues*. One of the main applications of HP in the food industry is to extend shelf life and eliminate microbial pathogens (*31*). According to

McClements et al. (*32*), pressure causes changes in cell morphology, inhibition of genetic mechanisms, and disruption of ribosomes, although the primary site for pressure-induced microbial inactivation is the cell membrane by altering its permeability and ion exchange. Moreover, Linton et al. (*33*) reported that high hydrostatic pressure causes denaturation of proteins, including key enzymes, leading to modifications in biochemical reactions. Typically, significant inactivation of vegetative bacteria, yeasts, and mold viruses can be observed within minutes at room temperature and pressures higher than 300 MPa (*34*). Arroyo et al. (*35*) reported a reduction of initial microbial population with 1 log unit on lettuce, tomato, asparagus, onions, cauliflower and spinach processed between 200 and 400 MPa for 30 min at 5 °C. Our finding on the reduction of total viable bacteria by HP treatment in the present study was consistent with those reports (**Table 1**). In addition to the inactivation or elimination of microbial pathogens, HP treatment could be used for acceleration of cheese ripening (*9*, *10*), mainly due to increased proteolysis. However, in our preliminary study, no effect of HP treatment on soy sauce manufactured with normal *meju* starter was observed during 6 months of aging, which was evaluated using the same approaches in the present study of physicochemical and ^{1}H NMR-based metabolomic analysis. Interestingly, HP treatment in *meju* starter affected the fermentative behaviors of the starter in the present study, demonstrating that fermented soybean products such as soybean paste and soy sauce could be influenced by HP treatment when the products are manufactured with HP-treated *meju* starter under study by these authors.

During the incubation of normal and HP-treated *mejues* for 3 weeks, the neutral protease activity was significantly higher in HP-treated *meju* than in normal *meju* ($p < 0.05$), but the alkaline protease activity was not different (**Table 1**). Higher activity of the neutral protease in HP-treated *meju* compared to normal *meju* was consistent with the result that amino-type nitrogen contents were higher in HP-treated *meju* (**Table 1**). HP can be utilized to stabilize or increase activity of many enzymes from various sources and in various solvent systems (*36*). The proteolytic enzyme α-chymotrypsin (CT) has been reported to facilitate the hydrolysis of peptide bonds and is used to aid protein digestion (*36*). HP also increased stability of CT. CT is readily inactivated (< 5 min half-life) at 0.1 MPa, while at 180 MPa CT remains active for 30 min (*37*). Mozhaev et al. (*37*) found that an increase in pressure at 20 °C resulted in a 6.5-fold increase in activity at 470 MPa versus 0.1 MPa. Stability and activity of CT was also improved by pressure in organic media (*38*). Moreover, the activity and stability of the partially purified enzyme from a hyperthermophilic barophilic *Methanococcus jannashii*, which is an extremely thermophilic deep-sea methanogen, increased with pressure. Raising the pressure increased the enzyme reaction rate 3.4-fold and the thermostability 2.7-fold at 50 MPa at 125 °C, compared to 0.1 MPa (*39*). Pennisto (*40*) suggested that the activities of monomeric enzymes were stimulated, while the activities of multimeric enzymes were inhibited by application of high hydrostatic pressure. However, at least 15 dimeric and tetrameric enzymes have been reported to be activated by pressure (*41*). Explanations for pressure-induced changes in the rate of enzyme-catalyzed reactions can be classified into (1) direct changes in the structure of an enzyme; (2) changes in the reaction mechanisms, for example, a change in the rate-limiting step; and (3) changes in the substrate or solvent physical properties (e.g., pH, density, viscosity, phase) that affect enzyme structure or the rate-limiting step (*36*). Therefore, it is likely that HP treatment of *meju* starter led to increases in the activity or stability of enzymes such as proteases in the *meju* starter in the present study.

Variation in Metabolites in Normal and HP-Treated *Meju* Starters. It was likely that decreased levels of α- and β-glucose and oligosaccharides (O1, O2, and O3) during the incubation were due to consumption by microorganisms (**Figures 4** and **5**). Reduction in water content by spontaneous evaporation during incubation of the *meju* starters could not be avoided in the present study, without completely sealing the Petri dishes used in the incubation. However, we did not seal them because microorganisms in the *meju* starters grew rapidly and became uncountable in our preliminary study. Microorganisms must cope with reductions in water availability in their milieu since the concentration of solutes within the cell is higher than that in the environment with normal water availability. However, microorganisms do not possess active transport mechanisms for water; hence, turgor is adjusted by controlling the pool of osmotically active solutes in the cytoplasm (*42*), through the accumulation of intracellular concentrations of ions (*43*, *44*) and osmotically active compounds of compatible solutes including sugars (e.g., trehalose), polyols (e.g., glycerol and glucosylglycerol), free amino acids (e.g., proline and glutamate), quaternary amines and their sulfonium analogues (e.g., betaine, carnitine and dimethylsulfoniopropionate), sulfate esters (e.g., choline-*O*-sulfate), and *N*-acetylated diamino acids and small peptides (e.g., *N*-acetylornithine and *N*-acetylglutaminylglutamine amide), which are known as osmoprotectants (*43*−*48*).

The key physiological role of the osmoprotectant accumulation as an adaptive response of *B. subtilis* to high osmolality environments has been firmly established (*49*, *50*). *B. subtilis* responds to a sudden increase in external osmolality through an initial rapid uptake of K^+ followed by the accumulation of large amounts of the osmoprotectant proline through de novo synthesis (*50*, *51*). Influx of K^+ is essential for the recovery of turgor, increased proline biosynthesis, and the resumption of growth subsequent to an osmotic challenge. The nature of the counterion for K^+ in *B. subtilis* is unclear since, in contrast to *Escherichia coli*, glutamate levels increase only slightly after osmotic upshock. Proline plays a particularly important role in the osmostress response because it is accumulated by osmoregulating *B. subtilis* cells by both de novo synthesis and uptake from the environment. Unlike *E. coli*, which uses the disaccharide trehalose as the endogenously synthesized compatible solute (*52*), *B. subtilis* employs the amino acid proline for this purpose (*50*). Proline biosynthesis in many bacteria is frequently regulated through feedback inhibition of the first biosynthetic enzyme (γ-glutamyl kinase). Hence, salt-stressed *B. subtilis* cells require an adjustment of the proline biosynthetic pathway to sustain high-level proline production under hypertonicity. This could be accomplished either by producing enzymes that are refractory to feedback inhibition or by using two distinct sets of proline biosynthetic genes to meet the different demands for this amino acid under low- and high-osmolality growth conditions. The molecular and enzymatic mechanisms leading to the remarkable rise in proline content in *B. subtilis* at high osmolality are unclear, but it is known that both activation of pre-existing proline biosynthetic enzymes and their increased synthesis are involved (*50*).

Betaine (trimethylglycine or glycine betaine) can be synthesized as an osmoprotectant by *E. coli*. A limited number of bacteria can produce this substance de novo via stepwise methylation of glycine (*43*). *E. coli*, however, does not belong to this group of bacteria; rather, it synthesizes betaine by a two-step oxidation of the precursor choline with glycine betaine aldehyde as the intermediate (*53*, *54*). In our previous study, large accumulations or synthesis of betaine in soy sauce aged over 10 years have been found leading to the conclusion that microorganisms involved in the aging of soy sauce can survive through regulating their cellular hyperosmolality by accumulation of the osmoregulator, betaine, induced by the high salt content of soy sauce (*3*). Therefore, it is very likely that increased levels of glutamate, proline, and betaine were caused by the hyperosmotic environment induced from the markedly reduced

water contents in both normal and HP-treated *meju* starters (**Figures 4** and **5**).

Choline and phosphocholine levels were also increased in *meju* during incubation. These compounds might act as precursors of osmoprotectant molecules of betaine (*49*, *55*). Choline and phosphocholine could be enzymatically converted into betaine, as microorganisms respond to high-osmolality stress. In addition, methionine levels were increased along with betaine in *meju* during the incubation in the present study, demonstrating that methionine is also associated with cellular hyperosmolality, because betaine donates a methyl group in the conversion of homocysteine to methionine in mammals (*55*, *56*).

Effect of HP Treatment on Metabolic Behavior of *Meju* Starter. Perturbations in amino acids levels between normal and HP-treated *meju* starters were observed (**Figure 7**). Although the levels of alanine, arigine, lysine, GABA, tyrosine, phenylanine, and tyrosine were higher in normal *meju* starters at 0 week of incubation, isoleucine, leucine and valine levels were not different between normal and HP-treated *meju* starters. After 1 week of incubation, these amino acids levels were still higher in normal *meju* starter, except for tyrosine. These findings may indicate that higher total bacteria contributed to the increased amino acids levels in normal *meju* starter through synthesis as they grow since HP treatment led to a reduction in total bacteria, as shown in **Table 1**. However, glycerol and choline levels were clearly higher in HP-treated *meju* than normal *meju*. Unfortunately to date, elevation and variation of glycerol levels by HP treatment has not been reported. Considering that glycerol production by osmotolerant yeast such as *Candida glycerinogenes* (*57*) and *Pichiu furinose* (*58*) has received much attention recently, HP-treatment could be a useful way to increase glycerol levels. Furthermore, since glycerol serves as the osmoprotectant, increased glycerol levels in HP-treated *meju* may suggest that a large amount of osmotolerant yeast could be involved in fermentation of HP-treated *meju* compared to normal *meju*. However, glycerol levels in HP-treated *meju* starters did not change as incubation periods increase to 3 weeks, but were higher in HP-treated *meju* starter than in normal *meju* starter at each incubation time. This may indicate that glycerol levels were increased by physical degradation of soybean material with high pressure rather than by synthesis or accumulation by osmotolerant microorganisms. Large amounts of glycerol in industrial Japanese soy sauce have been found compared to traditional Korean soy sauce mainly due to involvement of yeast such as *Zygosaccharomyces rouxii* in soy sauce fermentation as described in our previous study (*3*). The industry selected the yeast with high enzyme activity to enhance the fermentation. Moreover, glycerol is a byproduct in grape wine through alcoholic fermentation with yeast and contributes to the richness of taste and aroma perception (*59*, *60*). The glycerol content in grape wines is used for quality scaling in several European countries. Therefore, increased glycerol in *meju* starter by high pressure, even physical degradation, could improve sensory characteristics of soybean products with a *meju* starter such as soy sauce or soybean paste.

In the fermentative point of view, since total bacterial numbers were 2 or 3 times higher in normal *meju* starter than in HP-treated *meju* starter, variations in amino acid levels between two starters were expected in this study mainly due to synthesis of amino acids or enzymatic degradation from protein or peptide during bacterial growth. However, there were no differences in isoleucine, leucine and valine levels between normal and HP-treated *meju* starters, indicating increased degradation of protein to amino acids in HP-treated *meju* starters by increased stability or activity of protease induced by the HP treatment. These phenomena were consistent with the increased contents of amino-type nitrogen and the increased activities of neutral proteases, resulting from/in degradation of protein to peptides and amino acids. Furthermore, higher levels of tyrosine in HP-treated *meju* starters at all incubation times and increased tyrosine levels in HP-treated *meju* starters with increasing incubation time provided further direct evidence of the increased stability or activity of protease.

In conclusion, a metabolomic approach together with physicochemical analysis of *meju* starter revealed metabolic variations of glutamate, proline, betaine, and choline, which are related to hyperosmotic stress induced by low water availability and showed the increased tyrosine, glycerol, acetate and choline levels by HP treatment, which demonstrate the improvement in proteolytic enzyme stability or activity. This study highlights that metabolomics with global metabolite profiling could useful for evaluating the influence of food processing and the food quality.

Supporting Information Available: OPLS-DA line and column loading plots derived from ^{1}H NMR spectra of normal *meju* and permutation tests of OPLS-DA models of normal and high pressure treated *meju*. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Lee, C. H. Fermentation Technology in Korea. Korea University Press 2003.

(2) Kim, J. K.; Chang, H. G.; Seo, J. S.; Lee, A. J. Character impact compounds in flavors of Korean soy sauce manufactured with the traditional and the improved *meju*. *J. Microbiol. Biotechnol.* **1993**, *3*, 270–276.

(3) Ko, B. K.; Ahn, H. J.; van den Berg, F.; Lee, C. H.; Hong, Y. S. Metabolomic insight into soy sauce through H^{1} NMR spectroscopy. *J. Agric. Food Chem.* **2009**, *57*, 6862–6870.

(4) lm, M. H.; Choi, J. D.; Chung, H. C.; Lee, S. H.; Lee, C. W.; Choi, C.; Choi, K. S. Improvement of *meju* preparation method for the production of Korean traditional *kanjang* (soy sauce). *Korean J. Food Sci. Technol.* **1998**, *30*, 608–614.

(5) Kim, Y. S.; Park, C. W.; Kim, S. J.; Park, S. J.; Ryu, C. H.; Cho, H. J.; Kim, J. O.; Lim, D. K.; Ha, Y. L. Preparation of mushroom myceliacultured traditional meju with enhanced anticaricinogenicity and sensory quality. *J. Korean Soc. Food Sci. Nutr.* **2002**, *31*, 986–993.

(6) Kim, D. H.; Lee, K. H.; Yook, H. S.; Kim, J. H.; Shin, M. G.; Byun, M. W. Quality characteristics of gamma irradiated grain shape improved *meju*. *Korean J. Food Sci. Technol.* **2000**, *32*, 640–645.

(7) Goodridge, L. D.; Willford, J.; Kalchayanand, N. Destruction of *salmonella enteriditis* inoculated onto raw almonds by high hydrostatic pressure. *Food Res. Int.* **2006**, *39*, 408–412.

(8) Promatec Food Ventures. 2009. Available from http://www.p0romatecfoodventures.com/kk_1100018.html. Accessed May 13, 2010.

(9) Yokoyama, H.; Sawamura, N.; Motobayashi, N. Method for accelerating cheese ripening. *European Patent application*, **1992**, EP 0469857.

(10) Saldo, J.; McSweeney, P. L. H.; Sendra, E.; Kelly, A. L.; Guamis, B. Proteolysis in caprine milk cheese treated by high pressure to accelerate cheese ripening. *Int. Dairy J.* **2002**, *12*, 35–44.

(11) Coen, M.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. NMR-based metabolic profiling and metabonomic approaches to problems in molecular toxicology. *Chem. Res. Toxicol.* **2008**, *21*, 9–27.

(12) Coen, M.; Hong, Y. S.; Clayton, T. A.; Rohde, C. M.; Pearce, J. T.; Reily, M. D.; Robertson, D. G.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. The mechanism of galactosamine toxicity revisited; A metabonomic study. *J. Proteome Res.* **2007**, *6*, 2711–2719.

(13) Holmes, E.; Loo, R. L.; Stamler, J.; Bictash, M.; Yap, I. K. S.; Chan, Q.; Ebbels, T.; De Iorio, M.; Brown, I. J.; Veselkov, K. A.; Daviglus, M. L.; Kesteloot, H.; Ueshima, H.; Zhao, L. C.; Nicholson, J. K.; Elliott, P. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature* **2008**, *453*, 396–U50.

(14) Holmes, E.; Wilson, I. D.; Nicholson, J. K. Metabolic phenotyping in health and disease. *Cell* **2008**, *134*, 714–717.

(15) Rezzi, S.; Ramadan, Z.; Martin, F. P. J.; Fay, L. B.; van Bladeren, P.; Lindon, J. C.; Nicholson, J. K.; Kochhar, S. Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals. *J. Proteome Res.* **2007**, *6*, 4469–4477.

(16) Wang, Y. L.; Tang, H. R.; Nicholson, J. K.; Hylands, P. J.; Sampson, J.; Holmes, E. A metabonomic strategy for the detection of the metabolic effects of chamomile (Matricaria recutita L.) ingestion. *J. Agric. Food Chem.* **2005**, *53*, 191–196.
(17) Verhoeven, H. A.; Ric De Vos, C. H.; Bino, R. J.; Saito, H. K. Plant Metabolomics Strategies Based upon Quadrupole Time of Flight Mass Spectrometry (QTOF-MS). In *Biotechnology in Agriculture and Foresty, Plant Metabolomics*; Saito, K.; Dixon, R. A.; Willmitzer, L., Eds.; Springer-Verlag: Berlin, Germany, 2006; Vol. 7, pp 33−47.
(18) Hall, R. D. Plant metabolomics: from holistic hope, to hype, to hot topics. *New Phytol.* **2006**, *169*, 453–468.
(19) Lenz, E. M.; Wilson, I. D. Analytical strategies in metabonomics. *J. Proteome Res.* **2007**, *6*, 443–458.
(20) Nicholson, J. K.; Lindon, J. C. Systems biology—Metabonomics. *Nature* **2008**, *455*, 1054–1056.
(21) Son, H. S.; Hwang, G. S.; Kim, K. M.; Ahn, H. J.; Park, W. M.; Van Den Berg, F.; Hong, Y. S.; Lee, C. H. Metabolomic Studies on geographical grapes and their wines using H^1 NMR analysis coupled with multivariate statistics. *J. Agric. Food Chem.* **2009**, *57*, 1481–1490.
(22) Son, H. S.; Hwang, G. S.; Kim, K. M.; Kim, E. Y.; van den Berg, F.; Park, W. M.; Lee, C. H.; Hong, Y. S. H^1 NMR-based metabolomic approach for understanding the fermentation behaviors of wine yeast strains. *Anal. Chem.* **2009**, *81*, 1137–1145.
(23) Son, H. S.; Hwang, G. S.; Park, W. M.; Hong, Y. S.; Lee, C. H. Metabolomic characterization of malolactic fermentation and fermentative behaviors of wine yeasts in grape wine. *J. Agric. Food Chem.* **2009**, *57*, 4801–4809.
(24) Wishart, D. Metabolomics: applications to food science and nutrition research. *Trends Food Sci. Technol.* **2008**, *19*, 482–493.
(25) Choi, H. S.; Kim, M. K.; Kim, M. K.; Park, H. S.; Song, G. S.; Lee, K. K.; Kim, T. Y.; Kim, J. G. An approach to increase vitamin D_2 level in *doenjang* (Fermented Soybean Paste) using mushrooms. *Food Sci. Biotechnol.* **2005**, *14*, 828–831.
(26) *Official methods of amino-type nitrogen analysis*; Korea Food & Drug Administration: Seoul, Korea, 2002; pp 9−15.
(27) Veselkov, K. A.; Lindon, J. C.; Ebbels, T. M. D.; Crockford, D.; Volynkin, V. V.; Holmes, E.; Davies, D. B.; Nicholson, J. K. Recursive segment-wise peak alignment of biological H^1 NMR spectra for improved metabolic biomarker recovery. *Anal. Chem.* **2009**, *81*, 56–66.
(28) Hotelling, H. The generalization of student's ratio. *Ann. Math. Stat.* **1931**, *2*, 360–378.
(29) Eriksson, L.; Trygg, J.; Wold, S. CV-ANOVA for significance testing of PLS and OPLS (R) models. *J. Chemom.* **2008**, *22*, 594–600.
(30) Lindgren, F.; Hansen, B.; Karcher, W.; Sjostrom, M.; Eriksson, L. Model validation by permutation tests: Applications to variable selection. *J. Chemom.* **1996**, *10*, 521–532.
(31) Knorr, D.; Heinz, V. Development of nonthermal methods for microbial control. In *Disinfection, sterilization, and preservation*; Block, S. S., Ed.; Lippincott Williams & Wilkins: Philadephia, 2001; pp 853−877.
(32) McClements, J. M. J.; Patterson, M. F.; Linton, M. The effect of growth stage and growth temperature on high hydrostatic pressure inactivation of some psychrotrophic bacteria in milk. *J. Food Protect.* **2001**, *64*, 514–522.
(33) Linton, M.; Patterson, M. F. High pressure processing of foods for microbiological safety and quality: (A short review). *Acta Microbiol. Immunol. Hung.* **2000**, *47*, 175–182.
(34) Farkas, D. F.; Hoover, D. G. High pressure processing. In: Kinetics of microbial inactivation of alternative food processing technologies. *J. Food Sci.* **2001**, *65*, 47–64.
(35) Arroyo, G.; Sanz, P. D.; Prestamo, G. Effect of high pressure on the reduction of microbial populations in vegetables. *J. Appl. Microbiol.* **1997**, *82*, 735–742.
(36) Eisenmenger, M. J.; Reyes-De-Corcuera, J. I. High pressure enhancement of enzymes: A review. *Enzyme Microb. Technol.* **2009**, *45*, 331–347.
(37) Mozhaev, V. V.; Lange, R.; Kudryashova, E. V.; Balny, C. Application of high hydrostatic pressure for increasing activity and stability of enzymes. *Biotechnol. Bioeng.* **1996**, *52*, 320–331.
(38) Mozhaev, V. V.; Bec, N.; Balny, C. Pressure effects on enzyme-reactions in mainly organic media-alpha-chymotrypsin in reversed micelles of aerosol OT in octane. *Biochem. Mol. Biol. Int.* **1994**, *34*, 191–199.
(39) Michels, P. C.; Clark, D. S. Pressure-enhanced activity and stability of a hyperthermophilic protease from a deep-sea methanogen. *Appl. Environ. Microb.* **1997**, *63*, 3985–3991.
(40) Pennisto, J. T. High hydrostatic pressure and enzymic activity-Inhibition of multimeric enzymes by dissociation. *Arch. Biochem. Biophys.* **1971**, *142*, 322–332.
(41) Morild, E. The theory of pressure effects on enzymes. *Adv. Protein Chem.* **1981**, *34*, 93–166.
(42) Kempf, B.; Bremer, E. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* **1998**, *170*, 319–330.
(43) Galinski, E. A.; Trüper, H. G. Microbial behaviour in salt-stressed ecosystems. *FEMS Microbiol. Rev.* **1994**, *15*, 95–108.
(44) Ventosa, A.; Nieto, J. J.; Oren, A. Biology of moderately halophilic aerobic bacteria. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 504–544.
(45) Brown, A. D. Microial water stress. *J. Bacteriol.* **1976**, *40*, 803–846.
(46) Csonka, L. N.; Epstein, W. Osmoregulation. In *Escherichia coli and salmonella. Cellular and molecular biology*; Neidhard, F. C., et al., Eds.; ASM Press: Washington, DC, 1996; pp 1210−1223.
(47) Miller, K. J.; Wood, J. M. Asmoadaptation by rhizosphere bacteria. *Annu. Rev. Microbiol.* **1996**, *50*, 101–136.
(48) Martins, L.; Huber, R.; Huber, H.; Stetter, K.; Da Costa, M.; Santos, H. Organic solutes in hyperthermophilic Archaea. *Appl. Environ. Microbiol.* **1997**, *63*, 896–902.
(49) Boch, j.; Kempf, B.; Bremer, E. Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously proved choline. *J. Bacteriol.* **1994**, *176*, 5364–5371.
(50) Whatmore, A. M.; Chudek, J. A.; Reed, R. H. The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. *J. Gen. Microbiol.* **1990**, *136*, 2527–2535.
(51) Whatmore, A. M.; Reed, R. H. Determination of turgor pressure in Bacillus-subtilis-A possible for K^+ in turgor regulation. *J. Gen. Microbiol.* **1990**, *136*, 2521–2526.
(52) Strom, A. R.; Kaasen, I. Erehalose metabolism in Escherichia-coli-Stress protection and stress regulation of gene-expression. *Mol. Microbiol.* **1993**, *8*, 205–210.
(53) Lamark, T.; Kaasen, I.; Eshoo, M. W.; Falkenberg, P.; McDougall, J.; Strom, A. R. DNA-sequence and analysis of the BET genes encoding the osmoregulatory choline glycine beteine pathway of *Escherichia coli*. *Mol. Microbiol.* **1991**, *5*, 1049–1064.
(54) Landfald, B.; Strom, A. R. Choline-glycine betaine pathway confers a high levels of osmotic tolerance in *Escherichia coli*. *J. Bacteriol.* **1986**, *165*, 849–855.
(55) Finkelstein, J. D.; Harris, B. J.; Kyle, W. E. Methionine metabolism in mammals: kinetic study of betaine-homocysteine methyltransferase. *Arch. Biochem. Biophys.* **1972**, *153*, 320–324.
(56) Millian, N. S.; Garrow, T. A. Human betaine-homocysteine methyltransferase is a zinc metalloenzyme. *Arch. Biochem. Biophys.* **1998**, *356*, 93–98.
(57) Wang, Z. X.; Zhuge, J.; Fang, H. Y.; Prior, B. A. Glycerol production by microbial fermentation: a review. *Biotechnol. Adv.* **2001**, *19*, 201–223.
(58) Vijaikishore, P.; Karanth, N. G. Glycerol production by immobilized cells of Pichia farinose. *Biotechnol. Lett.* **1986**, *8*, 257–260.
(59) Nieuwoudt, H.; Prior, B.; Pretorius, I.; Bauer, F. Glycerol and wine quality: fact and fiction. *Wynboer* **2002**, *9*, 96–101.
(60) Lubbers, S.; Verrer, C.; Voilley, A. The effect of glycerol on the perceived aroma of a model wine and a white wine. *Lebensm.-Wiss. Technol.* **2001**, *34*, 262–265.

Received for review May 25, 2010. Revised manuscript received July 4, 2010. Accepted July 05, 2010. This work received financial support from ARPC (Agricultural R&D Promotion Center, R0807381). The author acknowledges the Korea University grant for the research professorship of Dr. Y. S. Hong.