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Preparation of High GABA-Enriched Yeast Extract by Non-*Saccharomyces* Yeasts Isolated from Korean Traditional Fermented Soybean Product

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High GABA-enriched yeast extract, for various nutritionally and pharmaceutically important functional foods, was prepared using a novel isolate of *Debaryomyces hansenii* JBCC541. Under optimized conditions, GABA conversion rates are significantly enhanced up to 7.55 g/l by *D. hansenii* JBCC541, increasing their synthesis yield 40 times. The total amino acid content of the prepared yeast extract was 10733.86 mg/l (257.36 mg/g), consisting of alanine, lysine, glutamine, leucine, and valine as the primary amino acids. The GABA content was significantly enhanced up to 6790 mg/l (162.80 mg/g) in the presence of glutamic acid, with approximately 10-fold higher GABA production. Flavor amino acids were also highly enhanced, indicating that the prepared yeast extract might be useful for preparing various functional and sensuous foods. Our results were promising as a GABA-enriched yeast extract preparation tool ensuring a suitable food material level with the potential for functionally enhanced food industrial applications.

Keywords: **Gamma-aminobutyric acid, *Debaryomyces hansenii*, yeast extract, amino acids, food additives**

Introduction

γ-aminobutyric acid (GABA) is a non-protein amino acid that is distributed extensively in nature [1]. GABA is known as one of the major inhibitory neurotransmitters in the central nervous system of animals [2] and possesses many physiological functions such as a neurotransmitter, diuretic and tranquilizer effects, and induction of hypotension [3–5]. Due to various outstanding physiological functions of GABA, the needs extensively expanded in the area of pharmaceutical and functional foods industry. Although the GABA is usually found in natural products, but the GABA contents are commonly very low in natural foods and significantly differ depending on the origin [6]. Thus, the needs for a simple preparation on industrial scale or the preparation method for GABA enhanced foods are greatly increased [7, 8].

GABA is synthesized by glutamate decarboxylase (GAD) (EC 4.1.1.15), a pyridoxal 5'-phosphate dependent enzyme that catalyzes the irreversible α-decarboxylation of L-glutamate to GABA [9], and is widely distributed among eukaryotes and prokaryotes [10]. Until now, the fungal strain *Monascus purpureus* has been shown to produce GABA [11]. Lactic acid bacteria (LAB) is the most investigated microorganism in production of GABA [12], and it was isolated to show GABA synthesis abilities from many fermented foods, including cheeses [9], paocai [13], fresh milk [14] and kimchi [15]. In the case of yeasts, *Saccharomyces cerevisiae* MJ2 strain which was isolated from the surface of kiwi was found to produce GABA [16]. Recently, the non-*Saccharomyces* yeasts such as *Pichia amonala*, *P. jadinii*, *P. guilliermondii*, and *Candida utilis* were being isolated from

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marine as GABA producing yeasts [17]. However, very few yeast strains which can produce GABA have been isolated and characterized from food sources to our current knowledge.

The Korean traditional fermented soybean product, meju, is a major ingredient used to prepare many fermented Korean soybean foods such as doenjang (soybean paste), ganjang (soy sauce), and gochujang (paper paste). Due to various microorganisms including bacteria, fungi, and yeasts are known to be distributed in meju, and would be a good source for indigenous functional strains and microbial enzyme catalysts due to the amino acid rich environments of meju. Specifically, the non-*Saccharomyces* yeasts such as *Hansenular capsulata* S-13 [18], *C. versatilis*, *P. membranaefacieus* [19], and *Kluveromyces maxianus* var. *lactis* [20] are currently found throughout the meju fermentation and showed functionality of the highest killer toxin activities, the 5'-ribonucleotides as flavor enhancers and galacto-oligosaccharide (GalOS), respectively.

Traditionally, yeast extract has been known as bacterial or cell culture media due to its high growth affective active compounds obtained by extracting the cell contents [21]. However, it has focused attention as food additives or bioflavouring materials in the food industry since it contains rich valuable amino acids and vitamin B complex as also nutritional active suppliers [22]. Moreover, a great attraction also are being increased recently due to its rich natural pool in nucleic acids, consisting of mainly ribonucleic acid after autolysis and partial hydrolysis of RNA, ribonucleotides such as guanosine 5'-monophosphate (GMP) and inosine 5'-monophosphate (IMP), which substances are efficient flavour enhancers [23–25]. Two important chemical compounds responsible for flavour enhancement action in crude preparations of IMP and GMP are well known in the food industry and are now commercially available worldwide. The world market of flavour enhancers surpasses US$ 1.1 billion per year [26].

This study was initiated to develop novel functional food additive material with high GABA content with yeast strains which contained various nutritive and bioflavour ingredient by adopting whole-cell reaction of novel non-*Saccharomyces* yeast [27–29]. Thus, we tried: 1) isolation of optimal indigenous yeast strains for whole-cell reaction from naturally fermented Korean traditional starter block, meju, 2) identification by chemical and molecular approaches and finally, 3) optimization of the whole-cell reaction by using the selected yeast starters for optimal synthesis of GABA and examined GABA contents with other valuable ingredients of the prepared yeast extract.

Materials and Methods

Isolation of yeast-like strains

The meju samples used in this study as microbial yeast sources were manufactured by using traditional methods in the Sunchang province (Korea). One gram of sample was suspended with 0.85% of sodium chloride solution, and then decimally diluted and homogenized. The 100 μl of diluted samples were respectively inoculated on to the YM plate containing 25 unit/ml of Penicillin-streptomycin solution (Sigma, USA) and incubated at 29℃ for 24–48 h. The yeast-like isolates were cultured in the YM broth (Difco, USA) and also purified by successive streaking on YM agar to isolate single strains for further experiments.

Screening of GABA producing yeast by whole-cell reaction

To screen the high GABA producing yeast, a whole-cell reaction (WCR) was carried out [17]. In brief, each isolates were grown at 10 mL of YM broth at 29℃ for 24–48 h, and separated from broth by centrifugation at 8,000 ×*g* for 10 min at 4℃. After washing with distilled water for two times, the cells (20%, wet weight) were mixed with reactive solutions comprised of 10 g/l of MSG and 50 g/l of glucose and then incubated at 37℃ for 72 h. After the WCR, the reaction mixture was boiled at 80℃ for 15 min to discontinue the GABA producing activity. The obtained reaction mixture of WCR was centrifuged and the obtained supernatant was directly used for analyzing extracellular GABA. The resultant yeast cells were re-washed and suspended in sterile distilled water. The yeast suspension was lysed by bead beating (Precellys24, Bertin Technologies, France), at six times with 5,000 rpm for 20 s each time, in the presence of 0.5 mm Zirconia/silica beads (Biospec, USA). Lysed yeast suspension was centrifuged, and the supernatant fluid was obtained for analyzing intracellular GABA. The obtained intra- and extracellular supernatant fluids were filtered through the 0.45 μm cellulose membrane,

before analysis of GABA.

Identification by biochemical analysis

For biochemical analysis of yeast-like strains, the API 20C AUX kit (BioMerieux, USA) was used according to the manufacturer's instructions in order to assist with identification. The obtained results were interpreted by using the database (version 4.0) in the Apiweb (www.apiweb.biomerieux.com) software.

Identification by sequence analysis of 26S rDNA and IGS

Identification of the isolates was performed by sequence analysis of 26S rDNA region and intergenic spacer (IGS) regions. Colony PCR was conducted by using Taq DNA polymerase kit (GeneAll, Korea) obtained with crude templates, 25 pM of universal primer sets: 26S rDNA (NL1: 5'-GCATATCAATAAGCGGAGGAAAAG-3' and NL4: 5'-GGTCCGTGTTTCAAGACGG-3') and IGS (CNL12: 5'-CTGAACGCCTCTAAGTCAG-3' and CNS1: 5'-GAGACAAGCATATGACTACTG-3'). Amplification of both regions are conducted in a PCR thermal cycler (Bio-Rad, USA) under the following conditions: 26S rDNA regions (35 cycles of 94℃ for 1 min, 52℃ for 45 s, 72℃ for 1 min) and IGS regions (35 cycles of 94℃ for 40 s, 58℃ for 90 s, 72℃ for 2 min). Each PCR product was separated by gel electrophoresis and the bands were visualized by using the ChemiDoc XRS Imaging System (Bio-Rad) after staining with ethidium bromide. The confirmed PCR product was directly sequenced on DNA analyzer (ABI PRISM 3700, Applied Biosynthesis, USA) and analyzed by using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The multiple alignments and constructions of phylogenetic trees used the neighbor joining method [30] and CLUSTER W computer program. The phylogenetic tree was evaluated by bootstrap analysis through 1,000 replications [31, 32].

Analysis of GABA

The qualitative analysis of GABA was determined by using the thin-layer chromatography (DC-Alufolien 20 × 20 cm Kieselgel 60, Merck, Germany) after separation in a solvent mixture (*N*-butanol:acetic acid:water = 5:3:2) with 0.4% of ninhydrin for detection. After development, the plate was being dried for color detections on the hot plate at 80℃ for 5 min. For the quantitative analysis of GABA, a high performance liquid chromatography (Waters Co., USA) equipped with a reversed-phase Primesep 100 (4.6 × 50 mm, SIELC Technologies, USA) and evaporative light scattering detector (Alltech, USA) was used as quantitative analysis of GABA by using 10% of acetonitrile in water containing 0.1% of trifluoroacetic acid as the mobile phase. Analysis was performed at 37℃ with the isocratic flow rate of 1 ml/min. Before injection, all samples were filtered by the 0.45 μm membrane filter. The GABA production yield was also determined by measuring the total sum of intra- and extracellular GABA after WCR.

Optimization of whole-cell reaction for GABA production

For optimization of WCR, GABA production by selected yeasts was carried out. Unless otherwise stated, all reactions were performed in reactive solutions containing 20% of cells, 50 g/l of glucose and 10 g/l of MSG as substrates for WCR at 37℃ in a 20 ml test tube for 72 h. To find the effects of carbon source in the WCR for GABA, different carbon sources of maltose, sucrose, xylose, mannose and mannitol were investigated with 10 g/l of MSG. The effects of glucose addition were examined with concentrations of 0, 10, 30, 50 and 75 g/l under the same condition. Optimal concentrations of MSG and cell concentrations for maximum GABA production by WCR varied from 0 to 30 g/l and from 0 to 100%, respectively. In addition, the effects of temperature for GABA production were measured at 29, 37, 50 and 60℃, and the effects of temperature stability on yeast cells before the WCR was also examined at -20℃ and 4℃. To determine the optimal concentration of PLP added to WCR for maximum GABA production, the WCR was performed at the presence of 1 or 2 mM PLP or in the absence of PLP.

Preparation of the yeast extract with GABA

The selected yeast was inoculated in 10 ml YM broth and cultivated at 29℃ for 48 h at 600 rpm. Yeast culture was transferred in 250 ml of YM broth and total 1 L of media was cultivated. Subsequently, cells were spun down at 4℃, 5,000 ×*g* for 15 min, and the cell pellet was washed three times with distilled water. The WCR was performed as described above. After WCR, reacted solution was concentrated at 100℃ for 20 min with stirring every 5 min and centrifuged at 4℃, 5,000 ×*g* for 15 min. The resulting supernatants were stored at 4℃ until fur-

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ther analysis.

Amino acid composition of the yeast extract with GABA

Yeast extract containing GABA was analyzed by LC-MS-MS. For amino acid analysis with LC-MS analysis, 5 µl aliquots were injected on an UPLC system (Acquity Ultra Performance LC, Xevo TQ-S (QQQ), Waters). The LC-MS-MS was controlled by MassLynx software (version 4.1). The UPLC column was a IMTAKA Intrada Amino Acid (2 × 5 mm, 3 um). The mobile phase was acetonitrile:100 mM ammonium formate = 20:80 (v/v) (buffer A) and acetonitrile:THF:25 mM ammonium formate:formic acid = 9:75:16:0.3 (v/v/v/v) (buffer B, pH 8.0). The flow rate was 0.4 ml/min and make gradient from 0% to 100% buffer A for 10 min. The MS was equipped with an ESI interface operating at an ionization voltage of +3000 V and a source temperature of 380℃. The capillary voltage, con voltage and source offset were set at 3 kV, 30 kV and 30 V respectively. Quantification was performed using multiple reaction monitoring (MRM) [33]. The gas flow of desolvation, cone and nebulizer were set at 650 l/Hr, 150 l/Hr and 7 bar, respectively. For GABA analysis, Agilent 6410B Triple Quadrupole LC/MS (Agilent Technologies, Wilmington, USA) equipped with an ESI source was employed for the analysis. Glutamic acid and γ-amino butyric acid were purchased from Sigma Aldrich and used as reference standard. The 5 µl of the processed samples were injected into the HPLC system (1200 Series LC, Agilent Technologies, USA) fitted with Phenomenex Synergi Hydro-RP column (4 µm, 80 Å, 150 × 2 mm), maintained at 30℃. A mobile phase composed of 0.1% formic acid in distilled water (buffer A) and 0.1% formic acid in acetonitrile (buffer B) was used to separate the analysts and pumped into the ESI chamber at a flow rate of 0.5 ml/min for 20 min. Fragmentor voltage and collision voltage was set at 70 V. Detection of the ions was carried out in the MRM, by monitoring the transition pairs of m/z 147.9→83.9 (glutamic acid), 646.2→586.0 and 104→87 (γ-amino butyric acid). Data acquisition was performed with the MassHunter Software (Version B.04.00).

Statistical analysis

Data are expressed as the means values ± S.D. (n = 3). The statistical significance was determined by one-way analysis of variance (ANOVA) when using the IBM SPSS software version 21 (IBM SPSS Inc. USA), followed by ANOVA with the Duncan's test. Differences with $p < 0.05$ were considered statistically significant.

Results and Discussion

Screening of yeast-like strains for GABA production from meju

From a total of 643 yeast-like strains isolated from traditional fermented soybeans, meju, 103 strains displayed GABA producing activities by WCR while using the MSG as substrates. Then, ten GABA producing yeast-like strains, JBCC409, 440, 540, 541, 544, 555, 564, 578, 585, and 588 were firstly selected as high GABA producing strains, which showed GABA productivity (Fig. 1). The extracellular GABA showed all examined yeast-like strains, but only three yeast-like strains of JBCC541, 564 and 585 produced extracellular GABA. In comparison, other yeast-like strains of JBCC409, 440, 540, 544, 555, 578 and 588 showed various different ratios of the intra- and extracellular GABA contents after the WCR as: JBCC409, 31:96; JBCC440, 38:62; JBCC540, 54:46; JBCC544, 65:35; JBCC555, 40:60; JBCC578, 15:85; and JBCC588, 17:83. The selected JBCC541 yeast-like strains were shown to synthesis the highest GABA of 4.8641 g/l in extracellular GABA.

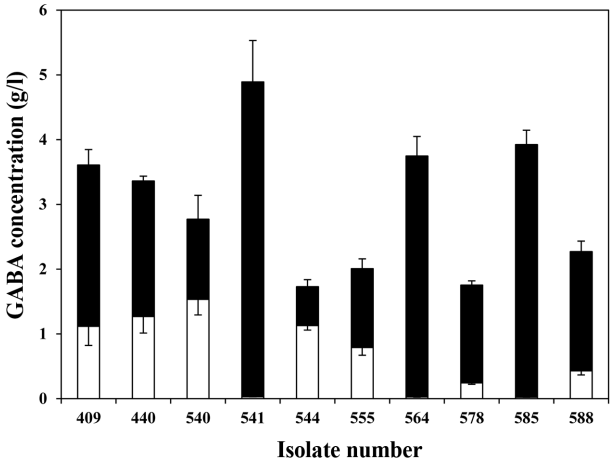

Fig. 1. Comparison of intracellular and extracellular γ-aminobutyric acid (GABA) content in 10 of screened yeast. Production of GABA in screened yeasts at 20% cells was examined at 37℃ for 72 h in conversion solution containing 1% monosodium glutamate and 5% glucose. Extracellular content (■), Intracellular content (□).

When we examined conversion ratios from MSG as substrates to GABA as products of WCR, the selected yeast-like strain of JBCC541 showed relatively high GABA conversion ratio with 99% at the extracellular. This GABA conversion capacity of WCR under our un-optimized WCR condition was higher than those of from previously reported yeast strains such as *Saccharomyces cerevisiae* (62.2 mg/l), *Candida* spp. (720.89 mg/l), *C. utilis* NBRC 10707 (653 mg/l), *Pichia* spp. (914.5 mg/l) and *P. anomala* ATCC 8168 (795 mg/l) (Masuda *et al*., 2008). Thus, we selected JBCC541 for further studies in this study.

Identification of high-level of GABA production yeast-like strains, JBCC541

When we examined the selected high level GABA producing yeast like strain by biochemical characters using API 20C AUX, the yeast like strain JBCC541 identified as *C. gilliermondii* with relatively low score of above 84% matching score. However, when the 26S rDNA sequences was examined, the selected JBCC541 strains showed high level identity of 99–100% with *Debaryomyces hansenii* (formally known as *C. famata*) and closely related to the sequences of *D. hansenii* species based on the evolutionary distance. Due to the low reliability, confused and inconsistency on the 26S rDNA sequences for identification of yeasts even though high similarity, we examined IGS of the selected JBCC541 and compared with other type strains. Since IGS recently has been employed for clear resolutions of confused and inconsistence identification results for yeast strains according to the diversity of length polymorphisms, and show successful identifications results of closely related yeast species [34]. The IGS sequences of JBCC541 were about 2,800 bp and showed 89–98% identity with *D. hansenii*, as indicated in Table 1. However, no matches were found with the type strains of *C. gilliermondii*. This results strongly suggested that the selected JBCC541 strain must belong to *D. hansenii*, naming *D. hansenii* JBCC541 for further study (KACC 93182P).

Table 1. Identities of the IGS regions of *Debaryomyces hansenii* (*Candida famata*) JBCC541 with closest known *D. hansenii* (*C. famata*) strains in the database.

Order	Database number*	Score	Identities (%)	Gaps
1	CBS 1961	508	382/431 (89)	18/431
2	CLIB 660	580	383/415 (92)	10/415
3	CBS 766	601	384/412 (93)	5/412
4	CBS 1795T	1572	880/894 (98)	1/894
5	CLIB 622	1572	880/894 (98)	1/894

*Database number the DDBJ/EMBL/GenBank database by Basic Local Alignment Search Tool (BLAST).

Optimal condition of whole-cell reactions for GABA conversions by *D. hansenii* JBCC541

To analyze the effects of carbon source on WCR, various carbon sources were used. The highest yield of GABA conversions was observed at glucose, showing about 2-folds of increased GABA production (Fig. 2A). It well agreed that, glucose was the appropriate carbon source addition for the GABA conversion among 10 types of carbon sources examined [35]. When we examined the optimal glucose concentrations ranging from 0–100 g/l, the GABA contents significantly increased to 10 g/l of glucose concentration (Fig. 2B). No significant differences were observed at above 10 g/l of glucose concentration on WCR. Previously, it has been reported that glucose addition could enhance the glutamate decarboxylase activity for WCR, and that GABA could not be produced without glucose addition even with glutamic acids as substrates due to unphosphorylated vitamin B6 in the absence of glucose [17]. However, we observed the GABA synthesis by WCR without glucose additions. This result could be caused by accumulation of glucose into yeast cell when culture in broth or according to different GAD activities from our isolated strain, JBCC541. To investigate the effects of different substrate concentrations on the conversion of GABA, different MSG concentrations with 10 g/l of glucose was examined. Additions of MSG to the whole-cell reactive solutions resulted in an increase of GABA production at a concentration dependent manner to 10 g/l of addition (Fig. 2C). Such result is in good agreement with GABA synthesis which is tended to decrease in yeast when the glutamic acids exceed by 20–50 g/l, due to strong regulations of GAD activities [35]. GAD catalyzes the irreversible α-decarboxylation of L-glutamate which produces GABA with PLP as the cofactor. As shown in Fig. 2D, the addition of PLP to WCR for GABA production significantly increases the GABA production yield at 1 mM. Previously, extracellular GABA contents were measured by *L. paracasei* NFRI 7415 in MRS medium which con-

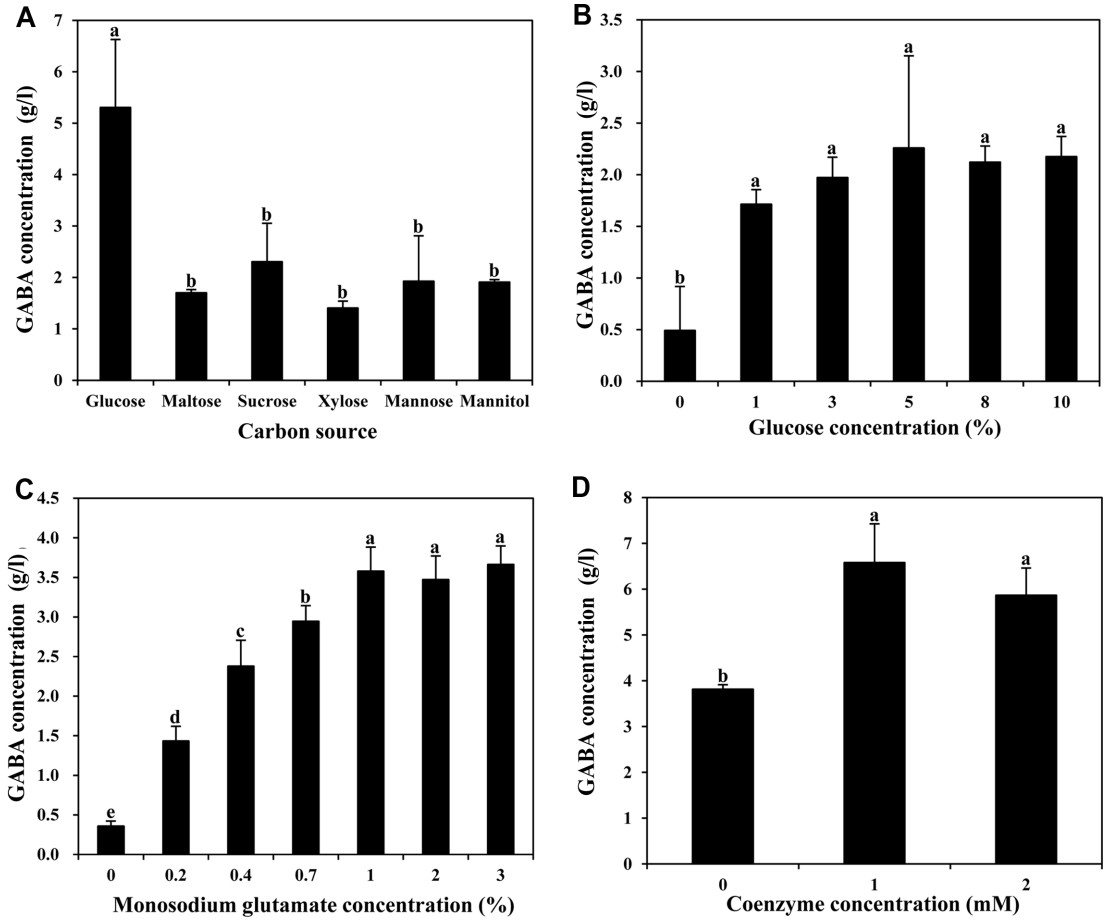

Fig. 2. Effect of different reactive solutions on extracellular GABA production by *D. hansenii* JBCC541. Type of carbon with 1% MSG concentration (A), glucose concentration with 1% MSG concentration (B), MSG concentration with 5% concentration (C), and PLP concentration with 1% MSG and 5% glucose concentration (D).

tains PLP at various concentrations, and showed that PLP additions effectively increases the extracellular GABA production [36]. The result is in good agreement with our results on the WCR when using JBCC541 cells. We also examined effects of cell concentration for WCR (Fig. 3A). The concentration of JBCC541 cells for the GABA production was also examined, and the optimal cell concentration was found to be 20% cell of JBCC541. The GABA concentration increased with increased cell concentrations and reached the highest GABA production at 20% cell concentration. However, cell loading of over 20% caused significant decreases of GABA production and it could be due to the cell lysis caused by endoglucanase or protease in yeast cells or by decreasing mass transfer rates of substrates into the cells [37]. The optimal reaction temperature of the WCR for GAD activity by JBCC541 cells was determined as 37℃ (Fig. 3B), and the results indicated high efficient conversions of MSG to GABA. The GAD activity of JBCC541 could be due to the decreased resistances of mass transfer on the cell surfaces, which covers the effect of cells and thus allows the synthesis rate to increase visibly. We also compared the whole cells for temperature stability of GAD activity by storing overnight at -20℃ and 4℃, and the frozen stored cells had no effects on WCR (Fig. 3C). However, when the JBCC541 cells were stored at 4℃, the GABA production activity on WCR was lower than the unstored JBCC541 cells. It seemed that the cells at 4℃ may cause an increased rate of enzyme inactivation.

Characterization of the yeast extract with GABA from *D. hansenii* JBCC541

Yeast extract were prepared in the presence or absence of glutamic acid by *D. hansenii* JBCC541 and

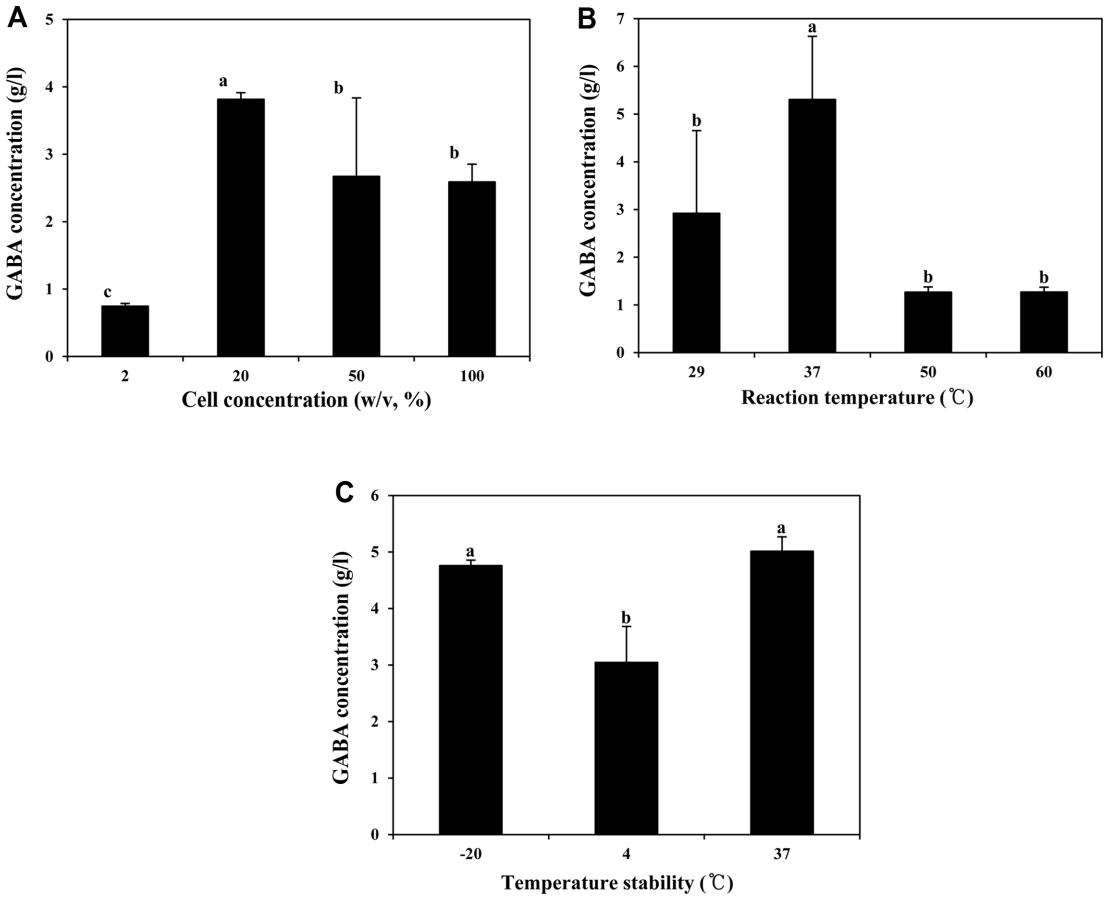

Fig. 3. Effect of different yeast concentration and temperature with reactive solutions on extracellular GABA production by *D. hansenii* JBCC541. Yeast cell concentration with 1% MSG and 5% glucose (A), reactive temperature with 1% MSG and 5% glucose concentration (B), temperature stability with 1% MSG and 5% glucose concentration (C).

the amino acid and GABA content were analyzed by GC-MS or LC-MS-MS as described above. As shown in Table 2, total amino acid content of the prepared yeast extract in the presence of glutamic acid as substrate was 10733.86 mg/l (257.36 mg/g) which are approximately four times increased content than the yeast extract prepared in the absence of glutamic acid of 2840.26 mg/l (86.45 mg/g). Major amino acids profile was also changed depending on the addition of glutamic acid. Compared to the amino acids profile of yeast extract prepared without glutamic acid, major amino acids of the amino acids profile of yeast extract prepared with glutamic acid were alanine, lysine, glutamine, leucine, and valine (Fig. 4). Without glutamic acid addition, GABA concentration was 530 mg/l (16.13 mg/g) in the prepared yeast extract, however, this GABA content greatly enhanced up to 6790 mg/l (162.80 mg/g) in the presence of glutamic acid, which approximately 10 times higher GABA production. Flavor amino acids (FAA) of the prepared yeast extract were also highly enhanced than the yeast extract pre-

Table 2. The amino acid composition of yeast extract by *Debaryomyces hansenii* JBCC541 after WCR and condensation process.

Amino acids	mg/l extract		mg/g protein	
	Control[d]	Yeast extract	Control	Yeast extract
Total amino acids[a]	2840.26	10733.86	86.45	257.36
Essential amino acids[b]	1385.86	1529.67	42.18	36.68
Flavour amino acids[c]	585.29	1519.43	17.81	36.43

[a]Sum of 19 amino acids content as described in Fig. 4.
[b]Valine, threonine, leucine, isoleucine, lysine, methionine, histidine, phenylalanine, and arginine.
[c]Glycine, alanine, aspartic acid, and glutamic acid.
[d]Whole cell reaction without monosodium glutamic acid.

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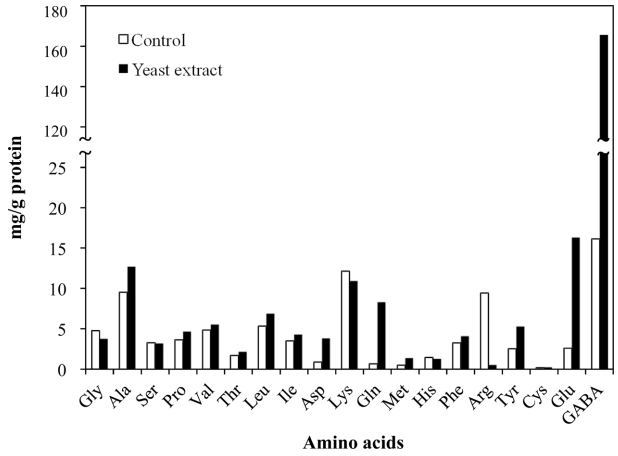

Fig. 4. The amino acid composition and GABA content of yeast extract by *D. hansenii* JBCC541 after whole cell reaction and condensation process.

pared without glutamic acid, indicating clearly that the prepared yeast extract might be useful for the preparation of the various functional foods by using this amino acids complex. Also, the essential amino acids such as valine, threonine, leucine, isoleucine, lysine, methionine, histidine, phenylalanine, and arginine account for about 40% of total amino acids. This amino acids composition is in good agreement with commercially available brewer's spent yeast extract which was prepared by mechanical disruption of cell wall [22].

The newly prepared yeast extract in this study by using novel non-*Saccharomyces* species, *D. hansenii* JBCC541, which isolated Korean traditional fermented foods showed very high content of GABA with other important amino acids with very simple process. This approach might be effective to obtain condensed yeast extract containing high-level GABA with valuable amino acid and could be a good source of application in food and dietary supplement industries as a protein and functional GABA rich ingredient.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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