한 국 균 학 회 지 The Korean Journal of Mycology Research Article

Screening of γ-Aminobutyric Acid (GABA)-Producing Wild Yeasts and their Microbiological Characteristics

Sang-Min Han[1], Sun-Jeong Jeon[2], Hyang-Burm Lee[2] and Jong-Soo Lee[1]*

[1]*Department of Biomedicinal Science and Biotechnology, Paichai University, Daejeon 35345, Korea*
[2]*Division of Food Technology, Biotechnology and Agrochemistry, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 61186, Korea*

ABSTRACT : From 182 non-pathogenic wild yeast isolates from flowers, *Pichia silvicola* UL6-1 and *Sporobolomyces carnicolor* 402-JB-1 were selected for potent γ-aminobutyric acid production and microbiological characteristics were investigated. *Pichia silvicola* UL6-1 formed ascospores and pseudomycelia. The strain was also halotolerant, growing well in 5% NaCl-containing yeast extract-peptone-dextrose (YPD) medium. *Sporobolomyces carnicolor* 402-JB-1 did not form ascospores or pseudomycelia and grew well on 10% glucose-yeast extract-peptone medium.

KEYWORDS : Gamma-aminobutyric acid, Microbiological characteristics, *Pichia silvicola* UL6-1, *Sporobolomyces carnicolor* 402-JB-1, Wild yeast

Introduction

Yeasts are heterotrophic and have relatively simple nutritional requirements. Besides fermented foods [1, 2], yeasts are distributed in natural habitats including flowers or plant debris in soils. Many species have been isolated from fermented foods and raw materials [3]. Recently, we isolated various wild yeast strains from flowers and soils [4-7] and their various phenotypes were investigated [8].

Gamma-aminobutyric acid (GABA) is a neurotransmitter in the central nervous system and is synthesized through decarboxylation by glutamate decarboxylase using the cofactor, pyridoxal-5-phosphate. GABA has hypotensive, tranquilizing, and diuretic effects, and is involved in the prevention of diabetes [9-12]. GABA is widely distributed in microorganisms, plants, and animals [13].

GABA production has been reported in yeasts such as *Saccharomyces cerevisiae* [14], *Debaryomyces hansenii*, and *Rhodotorula mucilaginosa* [15]. However, in these species, GABA production was relatively low and other associated physiological characteristics have not been studied.

This study was performed to obtain potent GABA-producing yeast strains for application to medicinal foods or agents, through screening of GABA production in non-pathogenic yeasts from wild flowers. Furthermore, microbiological characteristics of these strains were studied.

Kor. J. Mycol. 2016 June, **44**(2): 87-93
http://dx.doi.org/10.4489/KJM.2016.44.2.87
pISSN 0253-651X • eISSN 2383-5249

***Corresponding author**
E-mail: biotech8@pcu.ac.kr

Received February 29, 2016
Revised March 21, 2016
Accepted June 13, 2016

Materials and Methods

Strains and chemicals

Non-pathogenic yeast strains (182) were isolated from wild flowers in Korea and were used in this study [4, 7, 16-18].

Pyridoxal-5-phosphate and polyvinylidene fluoride membrane, used as a non-heating sterilization filter were purchased from Sigma-Aldrich (St. Louis, MO, USA) and thin-layer chromatography (TLC) plates were purchased from Merck KGaA (Darmstadt, Germany). Unless otherwise specified, all chemicals were of analytical grade.

Determination of GABA concentration by TLC

GABA content of cell-free extracts was determined by

method described by Holdiness [19] as follows. After incubating the yeast stains at 30°C for 48 hr, each cell-free extract was obtained by centrifugation at 9,000 × *g* for 10 min, with subsequent sonication of the cell pellets at 20 Hz for 5 min. Each cell-free extract was dissolved in distilled water and 20 μL was spotted on the TLC plate, which was then developed using the typical developing solvent, n-butanol-acetic acid-H_2O [4:1:1 (v/v/v)]. Developed TLC plates were dried at 60°C after spraying with ninhydrin solution (0.2%, w/v ethanol) for color development. The GABA spot was confirmed by comparison with a sample GABA standard spot.

Microbiological characteristics of the selected yeasts

The morphological and cultural characteristics of selected yeast strains were investigated according to Han et al. [20]. To assess ascospore formation, yeasts were cultured in yeast extract-peptone-dextrose (YPD) medium at 30°C for 24 hr and subsequently cultured for 5 days in ascospore-forming medium containing potassium acetate (1%), yeast extract (0.1%), and dextrose (0.05%). The strain was then observed using a microscope to assess ascospore formation. Yeast was successively cultured at 30°C for 7 days in YPD medium, yeast extract-malt extract medium, potato-dextrose medium, and glucose-peptone-yeast extract agar containing glucose (4%), peptone (0.5%), and yeast extract (0.5%). Pseudomycelium formation was determined by observing the shape of the cell in culture.

For examination of the detailed structure of selected yeasts by scanning electron microscopy (SEM) [20], selected yeasts were cultured in YPD medium and maintained in a 20% glycerol stock. The stock was diluted using a 0.05 M cacodylate buffer (pH 8.2). The diluted solution was centrifuged at 1,300 rpm for 1 min to obtain the yeast cell pellet, which was used for fixation. The strain was also cultured in potato-dextrose-broth (PDB) medium at a shaking speed of 150 rpm in the dark at 30°C for 48 hr. The sample was fixed with 2.5% paraformaldehyde-glutaraldehyde buffer with 0.05 M phosphate (pH 7.2) for 2 hr, washed with cacodylate buffer, post-fixed in 1% osmium tetroxide (in the same buffer) for 1 hr, and washed again with the same buffer. The sample was then dehydrated in graded ethanol followed by isoamyl acetate, and then dried under a fume hood. Finally, the samples were covered in gold using a sputter coater and observed with the Hitachi S4700 (Hitachi, Tokyo, Japan) field emission scanning electron microscope.

Results and Discussion

Screening of potent GABA-producing yeasts

GABA content of cell-free extracts from 182 wild yeast strains was investigated by TLC. Among the wild yeasts, *Kazachstania unispora* SY14-1 and *Metschnikowia reukaufii* SY20-7 from Seonyudo, *Nakazawaea holstii* 63-J-1 and *Pichia guilliermondii* 89-J-1 from Jeju island, and *Pichia scolyti* YJ14-2 and *Pichia silvicola* UL6-1 from Yokjido and Ulleungdo exhibited GABA production (Table 1).

Using a TLC plate, GABA production was also detected for *Sporobolomyces carnicolor* 73-D-3 and 374-CO-1 from Gyejoksan and Oseosan and *Sporobolomyces carnicolor* 402-JB-1 and *Sporobolomyces ruberrimus* 121-Z-3 from Baekamsan (Table 1).

Among aforementioned GABA-producing yeasts, we selected *Pichia silvicola* UL6-1 and *Sporobolomyces carnicolor* 402-JB-1 for enhanced production, assessed by stronger intensity on TLC plates.

Phylogenetic tree of *Pichia silvicola* UL6-1 and *Sporobolomyces carnicolor* 402-JB-1

The phylogenetic tree for the selected yeast strains is shown in Fig. 1. *Pichia silvicola* UL6-1 was closely related to *Nakazawaea holstii* 63-J-1 in this study, and *Sporobolomyces carnicolor* 402-JB-1 was closely related to *Sporobolomyces ruberrimus* 73-D-3 and 121-Z-3. The tree was generated by the neighbor-joining method, using MEGA v5.1.

Morphological and cultural characteristics of *Pichia silvicola* UL6-1 and *Sporobolomyces carnicolor* 402-JB-1

The morphological and cultural characteristics of *Pichia silvicola* UL6-1 and *Sporobolomyces carnicolor* 402-JB-1 are presented in Table 2.

Pichia silvicola UL6-1 was oval-shaped and employed a budding system for vegetative reproduction. The strain formed ascospores and pseudomycelia, and grew well in YPD medium, yeast extract-malt extract medium, potato-dextrose medium, and 5% NaCl-containing YPD medium.

Few studies on halophilic yeasts have been performed with the exception of *Zygosaccharomyces rouxii* from soybeans [21] and halotolerant protease-producing *Saccharomyces lipolytica* [22] and *Hansenula polymorpha* S-9 from traditional meju [21, 23]. It is known that halophilic microorganisms produce enzymes with advantages such as preventing microbial contamination in the enzyme industry and enhancing the flavor of salted foods during

Table 1. γ-Aminobutyric acid (GABA) content of wild yeasts from flowers in Korea

No.	Putative species	Isolated No.	GABA content	No.	Putative species	Isolated No.	GABA content
1	*Debaryomyces hansenii*	SY8-1	+[a]	21	*Rhodotorula glutinis*	YJ35-4	-
		SY8-2	+			YJ42-4	+
2	*Kazachstania servazzii*	SY14-3	-			YJ54-2	-
3	*Kazachstania unispora*	SY14-1	++	22	*Rhodotorula graminis*	YJ48-2	-
4	*Metschnikowia reukaufii*	SY20-1	-			YJ20-3	+
		SY20-7	++			YJ36-1	-
		SY32-1	+	23	*Rhodotorula minuta*	YJ27-1	-
		SY33-1	+	24	*Rhodotorula mucilaginosa*	YJ39-1	-
		SY33-7	+			YJ4-4	+
		SY38-1	-			YJ5-2	-
		SY38-2	+			YJ16-1	-
		SY44-6	+			YJ19-1	+
		SY44-7	-			YJ37-1	+
		SY46-5	+			YJ38-4	+
		SY46-7	+			YJ42-2	-
5	*Occultifur externus*	SY5-2	-			YJ46-1	+
6	*Pichia holstii*	SY20-2	+			YJ34-7	+
7	*Pichia scolyti*	SY3-1	+	25	*Rhodotorula nothofagi*	YJ1-1	-
		SY3-4	+			YJ22-2	-
8	*Rhodosporidium diobovatum*	SY4-2	-	26	*Rhodotorula slooffiae*	YJ10-3	+
		SY4-5	-	27	*Rhodotorula* sp.	YJ38-2	+
9	*Rhodotorula ingeniosa*	SY1-1	-	28	*Hanseniaspora uvarum*	UL19-1	+
10	*Rhodotorula minuta*	SY47-2	+	29	*Kuraishia capsulata*	UL40-2	+
		SY47-4	+	30	*Metschnikowia koreensis*	UL28-3	+
11	*Rhodotorula mucilaginosa*	SY7-3	-			UL32-1	-
12	*Rhodotorula slooffiae*	SY34-2	+			UL37-1	+
		SY34-4	+			UL38-2	+
		SY42-4	+			UL48-1	-
13	*Yarrowia lipolytica*	SY51-1	+	31	*Metschnikowia reukaufii*	UL22-1	-
		SY51-3	+			UL22-3	-
14	*Fibulobasidium inconspicuum*	YJ52-1	+			UL28-1	+
15	*Lodderomyces elongisporus*	YJ38-1	+			UL3-1	-
		YJ38-3	+			UL45-2	+
16	*Metschnikowia reukaufii*	YJ12-1	-	32	*Metschnikowia viticola*	UL19-3	+
		YJ13-1	+	33	*Meyerozyma caribbica*	UL10-1	+
		YJ30-1	-			UL5-1	+
		YJ35-2	+	34	*Pichia guilliermondii*	UL47-1	-
		YJ39-2	+	35	*Pichia mexicana*	UL26-1	+
		YJ42-3	-			UL29-4	-
		YJ45-3	+			UL16-2	+
		YJ51-2	+			UL7-3	+
17	*Meyerozyma guilliermondii*	YJ34-3	-			UL8-3	-
		YJ34-2	-	36	*Pichia scolyti*	UL23-2	+
18	*Pichia scolyti*	YJ14-2	++			UL25-2	-
19	*Pseudozyma* sp.	YJ37-2	-			UL33-1	-
20	*Rhodosporidium paludigenum*	YJ41-4	-			UL9-2	+

[a]Intensity of GABA content.

Table 1. γ-Aminobutyric acid (GABA) content of wild yeasts from flowers in Korea (Continued)

No.	Putative species	Isolated No.	GABA content	No.	Putative species	Isolated No.	GABA content
37	*Pichia silvicola*	UL6-1	+++	58	*Hannaella oryzae*	344-D-1	+
		UL25-3	+	59	*Pseudozyma aphidis*	72-D-2	-
38	*Pseudozyma hubeiensis*	UL29-2	-	60	*Rhodosporidium paludigenum*	91-D-2	+
39	*Rhodosporidium fluvialeno*	UL15-3	+			91-D-1	+
40	*Rhodotorula glutinis*	UL25-6	+	61	*Rhodotorula glutinis*	73-D-2	+
		UL26-3	+			344-D-2	+
		UL31-3	-	62	*Rhodotorula minuta*	73-D-1	+
		UL42-3	-	63	*Sporobolomyces ruberrimus*	73-D-3	++
41	*Rhodotorula graminis*	UL20-3	+	64	*Metschnikowia* sp.	379-CO-3	+
		UL9-3	+	65	*Meyerozyma guilliermondii*	371-CO-1	-
		UL6-4	+	66	*Pseudozyma rugulosa*	380-CO-1	+
		UL35-3	-	67	*Rhodotorula mucilaginosa*	380-CO-3	-
		UL36-4	-	68	*Rhodotorula nothofagi*	374-CO-2	-
42	*Rhodotorula mucilaginosa*	UL17-2	+			376-CO-2	-
		UL46-4	+	69	*Sporobolomyces carnicolor*	374-CO-1	++
43	*Sporidiobolus pararoseus*	UL42-5	+	70	*Metschnikowia koreensis*	DU4-1	+
44	*Sporobolomyces carnicolor*	UL32-3	+	71	*Metschnikowia* sp.	DU12-3	-
45	*Debaryomyces hansenii*	80-J-2	-	72	*Rhodotorula graminis*	DU12-2	-
		89-J-2	+	73	*Sporidiobolus pararoseus*	DU1-3-1	+
46	*Metschnikowia reukaufii*	67-J-3	+	74	*Sporobolomyces phaffii*	DU15-3	+
47	*Metschnikowia pulcherrima*	66-J-1	+	75	*Bullera japonica*	405-JB-1	-
48	*Nakazawaea holstii*	63-J-1	++	76	*Bulleromyces albus*	395-JB-2	+
		83-J-1	+			399-JB-2	+
49	*Pichia guilliermondii*	89-J-1	++	77	*Dioszegia takashimae*	407-JB-1	-
50	*Pseudozyma aphidis*	66-J-2	-			408-JB-2	-
		77-J-1	+	78	*Filobasidium floriforme*	394-JB-1	-
		64-J-1	-			396-JB-1	-
		94-J-3	-	79	*Hannaella oryzae*	401-JB-1	+
51	*Pseudozyma rugulosa*	84-J-1	+	80	*Metschnikowia* sp.	397-JB-2	-
		86-J-2	+			387-JB-1	-
		90-J-2	+			404-JB-1	-
52	*Pseudozyma* sp.	71-J-1	+	81	*Pseudozyma antarctica*	123-Z-4	+
53	*Rhodosporidium paludigenum*	86-J-1	+	82	*Pseudozyma aphidis*	121-Z-2	-
		89-J-3	-	83	*Rhodosporidium fluviale*	391-JB-3	-
		92-J-2	+			399-JB-1	-
		94-J-2	+	84	*Rhodotorula glutinis*	115-Z-1	-
54	*Sporobolomyces ruberrimus*	63-J-2	+			115-Z-4	+
		65-J-1	+	85	*Rhodotorula* sp.	409-JB-1	-
		70-J-1	+	86	*Sporidiobolus pararoseus*	405-JB-3	+
		87-J-1	+	87	*Sporobolomyces carnicolor*	402-JB-1	+++
		94-J-1	+			387-JB-3	+
		95-J-2	+	88	*Sporobolomyces oryzicola*	407-JB-2	-
55	*Starmerella bombicola*	80-J-1	-	89	*Sporobolomyces phaffii*	404-JB-2	+
56	*Debaryomyces hansenii*	72-D-4	+	90	*Sporobolomyces ruberrimus*	121-Z-1	+
57	*Filobasidium floriforme*	343-D-1	-			121-Z-3	++

[a]Intensity of GABA content.

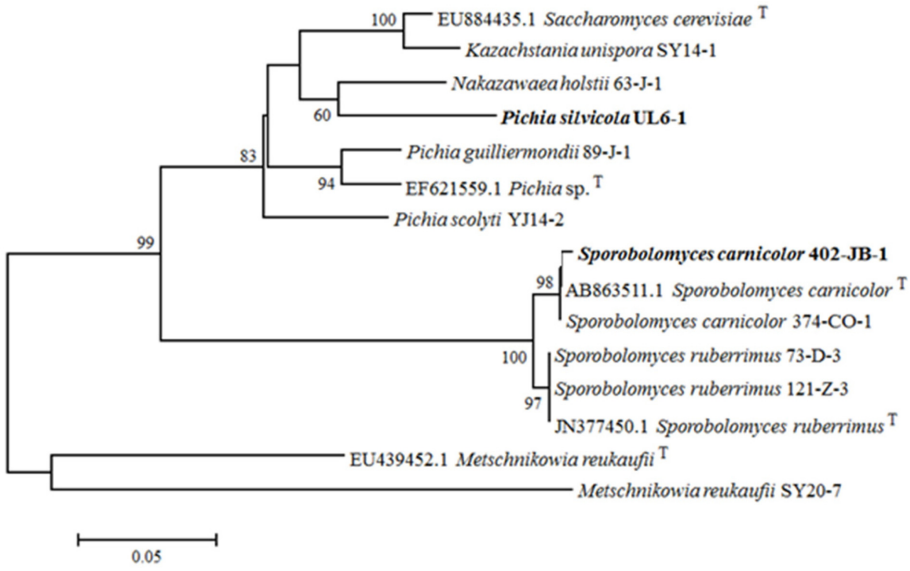

Fig. 1. Phylogenetic tree of *Pichia silvicola* UL6-1 and *Sporobolomyces carnicolor* 402-JB-1 based on the nucleotide sequences of large subunit 26S ribosomal DNA. T, Type strain.

Table 2. Microbiological characteristics of *Pichia silvicola* UL6-1 and *Sporobolomyces carnicolor* 402-JB-1

	Pichia silvicola UL6-1	*Sporobolomyces carnicolor* 402-JB-1
Morphological characteristics		
Shape	Oval	Oval
Vegetalle reproduction	B	B
Size (mm)	1.0 × 1.2 ~ 2.0 × 2.2	1.8 × 2.2 ~ 2.2 × 3.8
Ascospore	+	-
Pseudomycelium	+	-
Cultural and physiological characteristics		
Growth on YM	++	++
YPD	+++	+++
PD	++	+
Color on YPD	C	P
Growth on Vitamin-free medium	+	+
Growth on Glucose-YP (10%)	+	+
(50%)	-	-
Growth on NaCl-YPD (5%)	++	-
(20%)	-	-
Growth on temperature	25~30°C	25~30°C
pH	pH 4~8	pH 6~7

B, budding; YM, yeast extract-malt extract; C, cream; YPD, yeast extract-peptone-dextrose; PD, potato-dextrose; P, pink.

aging [21]. Therefore, *Pichia silvicola* UL6-1, identified in this study, should be very useful in preparing halotolerant enzymes or bioactive compounds for the food and medical industries.

Sporobolomyces carnicolor 402-JB-1 was oval-shaped and used a budding system for vegetative reproduction. Furthermore, the strain did not formed ascospores and pseudomycelia, and grew well in YPD medium and yeast extract-malt extract medium.

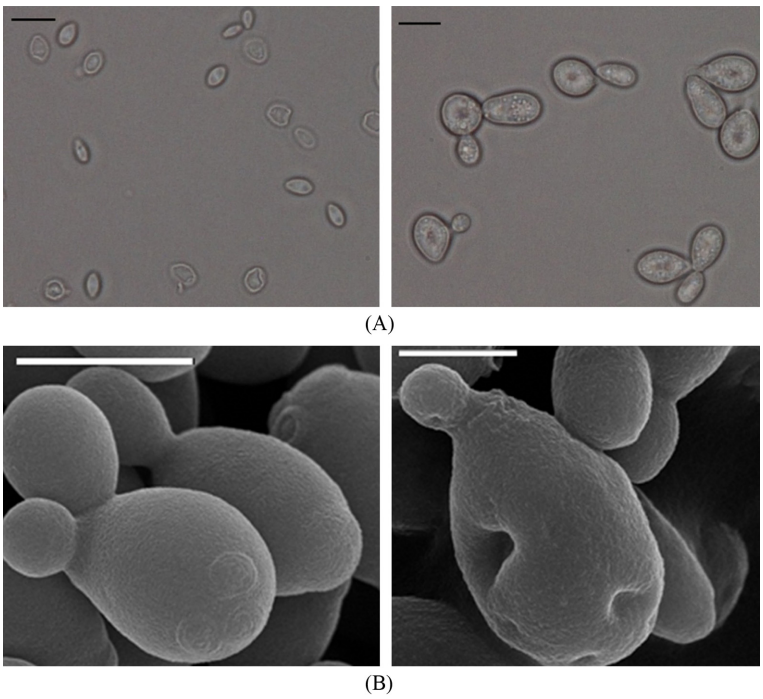

Fig. 2. Features of *Pichia silvicola* UL6-1 (left) and *Sporobolomyces carnicolor* 402-JB-1 (right) assessed by optical microscopy (A) and scanning scanning electron microscopy (B) (scale bar = 2 μm).

Structural characteristics

Fig. 2 shows features of *Pichia silvicola* UL6-1 and *Sporobolomyces carnicolor* 402-JB-1, during different media and cultural conditions, identified by optical microscopy and electron scanning microscopy (SEM). The typical shapes of vegetative cells of these strains were ellipsoidal to oval, commonly showing single cell forms and budding systems (Fig. 2).

Acknowledgements

This study was funded by Paichai University (2016), Republic of Korea.

REFERENCES

1. Yi SH, Ann YG, Choi JS, Lee JS. Development of peach fermented wine. Kor J Food Nutr 1996;9:409-12.
2. Lee TS, Lee SJ. Studies on the yeasts for the brewing of soy sauce (I): isolation, identification and classification of the yeasts in the soy sauce koji. J Kor Agric Chem Soc 1970;13:97-103.
3. Kim JH, Kim NM, Lee JS. Physiological characteristics and ethanol fermentation of thermotolerant yeast *Saccharomyces cerevisiae* OE-16 from traditional Meju. Kor J Food Nutr 1999;12:490-5.
4. Min JH, Ryu JJ, Kim HK, Lee JS. Isolation and identification of yeasts from wild flowers in Gyejoksan, Oseosan and Beakamsan of Korea. Kor J Mycol 2013;41:47-51.
5. Hyun SH, Min JH, Kim SA, Lee JS, Kim HK. Yeasts associated with fruits and blossoms collected from Hanbat arboretum, Daejeon, Korea. Kor J Mycol 2014;42:178-82.
6. Hyun SH, Lee JG, Park WJ, Kim HK, Lee JS. Isolation and diversity of yeasts from fruits and flowers of orchard in Sinammyeon of Yesan-gun, Chungcheongnam-do, Korea. Kor J Mycol 2014;42:21-7.
7. Hyun SH, Han SM, Lee JS. Isolation and physiological functionality of yeasts from wild flowers in Seonyudo of Gogunsanyeoldo, Jeollabuk-do, Korea. Kor J Mycol 2014;42:201-6.
8. Hyun SH, Han SM, Kim HK, Lee JS. Yeasts diversity of wild flowers in mountains of Korea and their physiological functionalities. Kor J Mycol 2015;43:137-41.
9. Hayakawa K, Kimura M, Kasaha K, Matsumoto K, Sansawa H, Yamori Y. Effect of a gamma-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar-Kyoto rats. Br J Nutr 2004; 92:411-7.
10. Inoue K, Shirai T, Ochiai H, Kasao M, Hayakawa K, Kimura M, Sansawa H. Blood-pressure-lowering effect of a novel fermented milk containing gamma-aminobutyric acid (GABA) in mild hypertensives. Eur J Clin Nutr 2003;57:490-5.
11. Jakobs C, Jaeken J, Gibson KM. Inherited disorders of GABA metabolism. J Inherit Metab Dis 1993;16:704-15.
12. Wong CG, Bottiglieri T, Snead OC 3rd. GABA, gamma-hydroxybutyric acid, and neurological disease. Ann Neurol 2003;54:3-12.
13. Ueno H. Enzymatic and structural aspects on glutamate decarboxylase. J Mol Catal B Enzym 2000;10:67-79.
14. Kim JH. Isolation and characterization of high GABA produ-

cing yeast isolated from fermented Bokbunja wine [dissertation]. Jeonju (Korea): University of Chonbuk; 2014.
15. Lee SH. Study on a gamma-aminobutyric acid (GABA) producing yeast isolated from Korean fermented soybean product, meju and optimal condition for GABA synthesis [dissertation]. Jeonju (Korea): University of Chonbuk; 2014.
16. Hyun SH, Min JH, Lee HB, Kim HK, Lee JS. Isolation and diversity of yeasts from wild flowers in Ulleungdo and Yokjido, Korea. Kor J Mycol 2014;42:28-33.
17. Hyun SH, Mun HY, Lee HB, Kim HK, Lee JS. Isolation of yeasts from wild flowers in Gyonggi-do province and Jeju island in Korea and the production of anti-gout xanthine oxidase inhibitor. Kor J Microbiol Biotechnol 2013;41:383-90.
18. Han SM, Hyun SH, Lee JS. Isolation and identification of yeasts from wild flowers in Deogyu mountain and their physiological functionalities. Kor J Mycol 2015;43:47-52.
19. Holdiness MR. Chromatographic analysis of glutamic acid decarboxylase in biological samples. J Chromatogr 1983;277:1-24.
20. Han SM, Hyun SH, Lee HB, Lee HW, Kim HK, Lee JS. Isolation and identification of yeasts from wild flowers collected around Jangseong lake in Jeollanam-do, Republic of Korea, and characterization of the unrecorded yeast *Bullera coprosmaensis*. Mycobiology 2015;43:266-71.
21. Jeong SC, Hyun KW, Kim JH, Lee JS. Isolation of halotolerant yeast and the production of extracellular protease. Kor J Biotechnol Bioeng 2001;16:158-62.
22. Kang KH, Bae IH, Lee CH. Studies on extracellular protease from *Saccharomyces lipolytica*: conditions of enzyme production. Kor J Appl Microbiol Biotechnol 1987;15:279-85.
23. Lee JS, Yi SH, Kwon SJ, Ahn C, Yoo JY. Enzyme activities and physiological functionality of yeasts from traditional Meju. Kor J Appl Microbiol Biotechnol 1997;25:448-53.