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Isolation, Identification and Characterization of a Antidementia Acetylcholinesterase Inhibitor-Producing *Yarrowia lipolytica* S-3

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This report describes the isolation and identification of a potent acetylcholinesterase (AChE) inhibitor–producing yeasts. Of 731 species of yeast strain, the S-3 strain was selected as a potent producer of AChE inhibitor. The selected S-3 strain was investigated for its microbiological characteristics. The S-3 strain was found to be short-oval yeast that did not form an ascospore. The strain formed a pseudomycelium and grew in yeast malt medium containing 50% glucose and 10% ethanol. Finally, the S-3 strain was identified by its physiological characteristics and 26S ribosomal DNA sequences as *Yarrowia lipolytica* S-3.

KEYWORDS : Antidementia acetylcholinesterase inhibitor, *Yarrowia lipolytica* S-3, 26S rDNA sequences

Introduction

Characteristics of Alzheimer's disease (AD) include memory loss, impaired visuospatial skills, poor judgment, and indifferent attitude; however, motor function is preserved [1]. The reversible characteristic of vascular dementia, which accounts for 15% of dementia cases, is not observed with AD [2-5].

Several neurotransmitters (acetylcholine, norepinephrine, and dopamine) and neuropeptides (somatostatin and corticotrophin-releasing factor) are known to be involved in AD [3].

Acetylcholine (ACh) is converts by acetylcholinesterase (AChE) into choline and acetate [6]. AD has been associated with a shortage of ACh in the brain; therefore, some drugs that inhibit AChE are used in the treatment of that disease [3]. However, Tacrine, Cognex, Aricept, Donepezil, Rivastigmine, and Galantamine are the only Food and Drug Administration (FDA)-approved therapeutic drugs that contain AChE inhibitor [7]. The most common AChE inhibitors are designed to bind to the active site of AChE [8]. AChE inhibitors reduce the rate at which ACh is broken down thereby increasing the concentration of ACh in the brain. AChE inhibitors appear to moderate symptoms without altering the course of the underlying dementing process.

Discovery of the useful physiological properties of yeast has led to their use in the field of biotechnology. Yeasts are also used as model organisms in the fields of genetics and cell biology. Several yeasts, particularly *Saccharomyces cerevisiae*, have been used extensively in the fields of genetics, cell biology, and biotechnology. In addition, many proteins with importance in human biology were first discovered through study of their homologs in yeast; these include cell cycle proteins, signaling proteins, and protein-processing enzymes. Despite recent development of some bioactive compounds from yeasts [9-11], little is known about bioactive compounds from yeasts were. Therefore, further study on bioactive compounds from edible yeast is needed.

The objective of this study is to isolate and identify a potent antidementia AChE inhibitor-producing yeast for use in development of a AChE inhibitor with potential for use in the drug or functional food industries.

Materials and Methods

Microorganisms and chemicals. A total of 580 kinds of yeast isolated from natural sources [12] were used in this study. In addition, 153 kinds of yeast were also

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obtained from the Korea Culture Center of Microorganism (Seoul, Korea), Korea Agricultural Culture Collection (Suwon, Korea), and Korea Collection for Types Cultures (Daejeon, Korea).

Unless otherwise specified, all chemicals and solvents were of analytical grade. Recombinant human AChE (E.C. 3.1.1.7), acetylthiocholine chloride and 5,5'-dithiobis (2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Screening and identification of potent AChE inhibitor-producing yeast. Following inoculation in YE medium (0.5% peptone, 0.5% yeast extract, 0.3% beef extract, and 0.5% sodium chloride), yeast were cultured at 28°C for 48 hr. The mixture was centrifuged at 15,000 ×g for 15 min, and cells and supernatant were obtained. Measurement of AChE inhibitory activity of the supernatant was performed for selection of extracellular AChE inhibitor-producing yeast. Following suspension in distilled water, cells were washed, re-suspended in distilled water, and then disrupted using a glass bead. The mixture was then centrifuged again at 15,000 ×g (10 min, 4°C), followed by measurement of AChE inhibitory activities of the supernatant for selection of the intracellular AChE inhibitor-producing yeast.

The microbiological characteristics of the selected strain, S-3, were investigated according to the taxonomy and identification of microorganisms [12, 13]. The S-3 strain was incubated a yeast extract, peptone, dextrose (YEPD) agar substrate for 72 hr at 28°C. Then using an API 20C AUX (Bio Merieux SA, Marcy-L'Etoile, France), in accordance with the manufacturer's instructions, we conducted an assimilation of the carbon compounds. To investigate tolerance of the selected strain S-3, to salt, sugar, and ethanol, we cultured the strain in yeast malt (YM) broth containing various concentrations of NaCl, KCl, glucose, and ethanol. Growth was determined as absorbance at 660 nm [12].

In addition, to examine resistance to heavy metals and chemicals, the strains were streaked on a YM agar substrate containing various heavy metals (400 ppm to 1,200 ppm) and chemicals (10 ppm to 1,000 ppm). We then evaluated growth for 3~5 days at 30°C [14].

Yeast identification software apiwebTM (https://apiweb.Biomerieux.com) was used for evaluation of result. Taxonomic study [12, 15] of the morphological characteristics, assimilation of carbon sources, and homology of 26S ribosomal DNA sequence were employed for identification of the selected yeast.

Assay of AChE inhibitory activity. The methods of Ellman *et al.* [16, 17] were employed for spectrophotometric measurement of AChE inhibitory activity. A mixture containing 110 μL of 0.1 M sodium phosphate buffer (pH 7.3), 30 μL of AChE (0.8 U/mL), 30 μL of 20 mM substrate (acetylthiocholine chloride), 20 μL of 20 mM 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), and 10 μL of the sample dissolved in a sodium phosphate buffer was incubated for 60 min at 37°C. The enzymatically-produced reaction product, 5-thio-2-nitrobenzoate, was measured at 415 nm. The following equation was used to determine the inhibition ratio inhibition (%) = $[1 - \{(S - S_0)/(C - C_0)\}] \times 100$, where C is the radiation of a control (enzyme, assay buffer, DTNB, and substrate) after 60 min of incubation, C_0 is the radiation of the control at zero time, S is the radiation of tested samples (enzyme, sample solution, DTNB, and substrate) after 60 min of incubation, and S_0 is the radiation of the tested samples at zero time. All data represent the mean of duplicated experiments. To evaluate the quenching effect of the samples, we added the sample solution to reaction mixture C followed by investigation of any sample-induced reduction of radiation. The IC_{50} value is defined as the concentration of AChE inhibitor, required to inhibit 50% of the inhibitory (AChE) activity.

Sequencing of the D1/D2 domain of the large subunit (26S) ribosomal DNA. The Exgene™ Cell SV mini-kit (Cell DNA Isolation Mini-Kit; Geneall Biotechnology, Co. Ltd., Seoul, Korea) was used, according to the manufacturer's instructions, for extraction of DNA from yeast cells.

The sequencing of the D1/D2 domain of the large subunit (26S) ribosomal DNA was performed for major groups of isolated cells. Basically, the analysis was performed using the method described by van der Aa Kühle *et al.* [18] and van der Aa Kühle and Jespersen [19]. Primers used for amplification of the D1/D2 domain: NL-1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL-4 (5'-GGT CCG TGT TTC AAG ACG G-3'). Reactions were performed in an automatic thermal cycler (GeneAmp PCR Ayatem 2400; Perkin-Elmer, Norwalk, CT, USA) under the following conditions: the initial denaturation was conducted at 94°C for 3 min; 32 cycles at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min; the final extension was conducted at 72°C for 7 min, with the temperature held at 4°C. A gel purification kit (Amersham Biosciences AB, Uppsala, Sweden) was used for purification of the amplified products. Direct sequencing of the purified PCR products was performed with a CEQ 2000 dye terminator cycle sequencing kit (P/N 608000; Beckman Coulter Inc., Fullerton, CA, USA) in an automated sequencer (CEQ 2000 DNA Analysis System; Bechman Coulter Inc.), in accordance with the manufacturer's instructions. Cycle sequencing was performed in an automated thermal cycler (GeneAmp® PCR System 9700; Perkin Elmer) using the external primers NL-1 and NL-4. Using BLAST algorithm, sequences were compared with sequences reported in GeneBank. Finally, the sequences

were reported to GenBank.

Results and Discussion

Screening of AChE inhibitor-producing yeasts. In order to select a potent AChE inhibitor-producing yeast, we tested supernatant and cell-free extracts from 733 kinds of yeasts for determination of their AChE inhibitory activities (Table 1).

Of the yeasts tested, extracellular AChE inhibitory activity of the S-3 strain was the highest, 37.4%. The highest intracellular AChE inhibitory activity was observed in the 24-1 strain (26.5%). However, in the majority of the yeasts, including *Candida albicans*, *Debaryomyces hansenii*, *Kluyveromyces fragilis*, *Pichia anomala*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae* KCTC 7919, KCTC 7904, KCTC 7245, *Torulopsis sphaerica*, *Cryptococcus laurentii* var. *laurentii*, *Saccharomycodes* sp., and *Zygosaccharomyces rouxii*, AChE activity was either not detected or less than 5% in cell-free extracts and culture broths. Thus, we ultimately selected the S-3 strain as a new AChE inhibitor-producing yeast.

AChE inhibitory activity of the S-3 strain was higher them that of mushroom, *Umbilicaria esculenta* (22.4%) [20]; however, it was lower than those of natural products including Job's Tears (55.1%) [21], *Sorghum bicolor* (63.4%) [22] and walnut (72.6%) [23].

Table 2. Microbiological characteristics of the selected yeast, S-3

Shape: short-oval
Vegetative reproduction: budding
Ascospore: −, Pseudomycelium: +
Urease activity: +, Assimilation of nitrate: +
Growth in vitamine-free medium: +
Growth temperature (°C) and pH: 15~35°C, pH 4.0~8.0
Resistance: NaCl 9%, KCl 16%, glucose 50%, ethanol 10%, Hg^{2+} 1,200 ppm, Cu^{2+} 800 ppm, cycloheximide 1,000 ppm, propionic acid 0.1%.
Production of amylase (−), protease (+), lipase (+), citric acid (+,+), isocitric acid (+,+)
G + C content: 48.5 mol%

Microbiological characteristics of the selected strain, S-3. Table 2 shows a summary of the morphological and physiological characteristics of the S-3 strain. The S-3 strain is a short oval-shaped yeast that does not form as an ascospore; it prossesses pseudomycelium and urease

Table 1. Acetylcholinesterase inhibitory activities of the secondary screened yeasts

Strains		Cell-free extract	Culture broth
Cryptococcus albidus var. *albidus*	KCTC 17538	n.d	10.2
Issatchenkia orientalis	KCTC 7213	3.2	5.0
Candida krusei			
Lipomyces starkeyi	KCTC 17343	n.d	20.5
Pichia jadinii	KCTC 17564	n.d	17.1
Pichia membranifaciens	KCTC 7628	n.d	8.7
Rhodotorula glutinis var. *glutinis*	KCTC 7948	16.7	16.8
Debaryomyces sp.	KACC30052	5.4	8.6
Zygosaccharomyces mellis	KCCM 50160	n.d	20.9
No. 13-1[a]		23.1	n.d
No. 14-1		10.3	n.d
No. 17-1		15.7	n.d
No. 22-4		15.8	n.d
No. 24-1		26.5	n.d
No. 8-2		17.2	n.d
No. 8-3		14.0	n.d
No. D6		10.9	n.d
No. G2		n.d	16.0
No. NO3		15.6	20.1
No. OE4		n.d	10.5
No. OE21		7.5	n.d
No. S-3		n.d	37.4
No. S4K5		12.4	n.d
No. S-6		n.d	6.4
No. S-8		n.d	8.0
No. SP		n.d	13.7

Values are presented as percentage.
n.d, not detected.
[a]Isolated (unidentified) strains.

Table 3. Assimilation of the selected strain, S-3

Carbon compounds[a]	Assimilation[b]
None	−
Glucose	+
Glycerol	+
2-Keto-D-gluconate	−
L-Arabinose	−
D-Xylose	−
Adonitol	−
Xylitol	−
D-Galactose	−
Inositol	−
D-Sorbitol	−
α-Methyl-D-glucoside	−
N-Acetyl-D-glucosamine	+
D-Cellobiose	−
D-Lactose (bovine origin)	−
D-Maltose	−
D-Saccharose (sucrose)	−
D-Trehalose	−
D-Melezitose	−
D-Raffinose	−
Hyphae/Pseudo-Hyphae	−

[a]Assimilation of carbon compounds was determined by use of the API 20C AUX kit.
[b]+, positive assimilation; −, negative assimilation.

activity, and can assimilate KNO_3; it has a G + C content of 48.5 mol%.

To assist in identification of the strain, the API 20C AUX kit was used to determine assimilation of carbon compounds (Table 3). We observed assimilation of D-glucose, glycerol, and N-acetyl-glucosamine by the S-3 strain.

The S-3 strain grew well in YM medium containing 8% NaCl and 15% KCl. The *Yarrowia ipolytica* S-3 also grows in a YM medium containing 50% glucose (Table 2). The S-3 strains showed growth inhibition at ethanol concentrations above 10% (v/v). In addition, tolerance to divalent heavy metals Hg^{2+} (1,200 ppm) and Cu^{2+} (800 ppm) and 100 ppm of cycloheximide and 0.1% propionic acid was also observed.

Sequencing of the D1/D2 domains of the 26S rDNA and identification of the S-3 strain. The identical D1/D2 domain region sequences confirm the conspecificity of the strain. Results of a BLAST search of sequences in the GenBank (accession No. JQ 740739) using the D1/D2 domain region sequences of the strain as queries showed that the closest matches are the corresponding sequences of the ATCC 18942 strain of the species *Y. lipolytica* (a 100% match). Fig. 1 shows the phylogenetic tree of the S-3 strain using the nucleotide sequence from the 26S rDNA gene of the S-3 strain.

Based on microbiological characteristics, assimilation, and the 26S rDNA sequences, the S-3 strain was identified as the S-3 strain of the *Y. lipolytica* species. Several mushrooms and phytochemicals are known to produce eukaryotic AChE inhibitors that act as antidementia substance [20, 24]. However, this is the first report on the

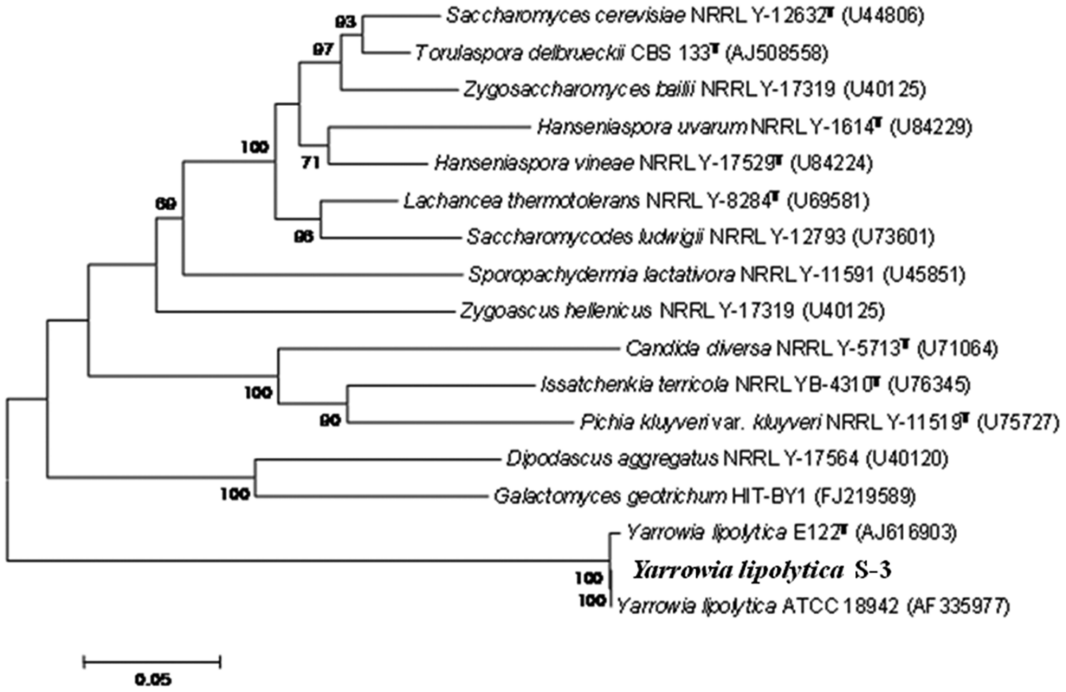

Fig. 1. Neighbor-joining tree showing the phylogenetic position of S-3 and other related taxa based on the 26S rRNA gene sequence.

AChE inhibitor-producing yeast, *Yarrowia lipolytica* S-3.

In our previous study [17], maximal production of the AChE inhibitor of *Y. lipolyica* S-3 was observed with incubation at 30°C for 36 hr in the optimal medium containing 1% yeast extract, 2% peptone, 2% glucose and initial pH 6.0; under the above conditions, the final AChE inhibitory activity was 6.4×10^4 μg/mL.

The results described above demonstrate the potential for use of *Y. lipolytica* S-3, a novel AChE inhibitor-producing strain, as an antidementia nutraceutical.

References

1. Alzheimer A. Üeber eine eigenartige Erkrankung der Hirnrinde. Allg. Z. Psychiatr Psychisch-Gerichtliche Med 1907;64:146-8.
2. Dugue M, Neugroschl J, Sewell M. Marin D. Review of dementia. Mt Sinai J Med 2003;70:45-53.
3. Eubanks LM, Rogers CJ, Beuscher AE 4th, Koob GF, Olson AJ, Dickerson TJ, Janda KD. A molecular link between the active component of marijuana and Alzheimer's disease pathology. Mol Pharm 2006;3:773-7.
4. Kaye WH, Sitaram N, Weingartner H, Ebert MH, Smallberg S, Gillin JC. Modest facilitation of memory in dementia with combined lecithin and anticholinesterase treatment. Biol Psychiatry 1982;17:275-80.
5. Khachaturian ZS. Diagnosis of Alzheimer's disease. Arch Neurol 1985;42:1097-105.
6. Hasselmo ME. Neuromodulation and cortical function: modeling the physiological basis of behavior. Behav Brain Res 1995;67:1-27.
7. Brenner GM, Stevens CW. Pharmacology. 2nd ed. Philadelphia, PA: Saunders Elsevier; 2006.
8. Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L, Silman I. Atomic structure of acetylcholinesterase from Torpedo californica: a prototypic acetylcholine-binding protein. Science 1991;253:872-9.
9. Kim JH, Lee DH, Jeong SC, Chung KS, Lee JS. Characterization of antihypertensive angiotensin I-converting enzyme inhibitor from *Saccharomyces cerevisiae*. J Microbiol Biotechnol 2004;14:1318-23.
10. Jeong SC, Lee DH, Lee JS. Production and characterization of an anti-angiogenic agent from *Saccharomyces cerevisiae* K-7. J Microbiol Biotechnol 2006;16:1904-11.
11. Lee DH, Lee DH, Lee JS. Characterization of a new antidementia β-secretase inhibitory peptide from *Saccharomyces cerevisiae*. Enzyme Microb Technol 2007;42:83-8.
12. Lee JS, Yi SH, Kwon SJ, Ahn C, Yoo JY. Isolation, identification and cultural conditions of yeasts from traditional Meju. Korean J Appl Microbiol Biotechnol 1997;25:435-41.
13. Hasegawa T. Taxonomy and identification of microorganisms. Tokyo: Hakhae Publishing Center; 1984. p. 153-254.
14. Sohn HY, Seu JH. Screening and characterization of thermotolerant alcohol-producing yeast. J Microbiol Biotechnol 1994;4:215-21.
15. Kreger-Van Rij. The yeasts: a taxonomic study. 3rd ed. Amsterdam: Elsevier Science; 1984.
16. Ellman GL, Courtney KD, Andres V Jr, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7:88-95.
17. Lee DH, Lee JS, Yi SH, Lee JS. Production of the acetylcholinesterase inhibitor from *Yarrowia lipolytica* S-3. Mycobiology 2008;36:102-5.
18. van der Aa Kühle A, Jesperen L, Glover RL, Diawara B, Jakobsen M. Identification and characterization of *Saccharomyces cerevisiae* strains isolated from West African sorghum beer. Yeast 2001;18:1069-79.
19. van der Aa Kühle A, Jespersen L. The taxonomic position of *Saccharomyces boulardii* as evaluated by sequence analysis of the D1/D2 domain of 26S rDNA, the ITS1-5.8S rDNA-ITS2 region and the mitochondrial cytochrome-c oxidase II gene. Syst Appl Microbiol 2003;26:564-71.
20. Lee JS, Min GH, Lee JS. Nutritional and physicochemical characteristics of the antidementia acetylcholinesterase-inhibiting methanol extracts from *Umbilicaria esculenta*. Mycobiology 2009;37:203-6.
21. Seo DS, Jang JH, Kim NM, Lee JS. Optimal extraction condition and characterization of antidementia acetylcholinesterase inhibitor from Job's tears (*Coix lachrymajobi* L.). Korean J Med Crop Sci 2009;17:434-8.
22. Song JE, Song JH, Cho SM, Min GH, Lee JS. Nutritional characteristics and physiological functionality of antidementia acetylcholinesterase inhibitor-containing methanol extract from *Sorghum bicolor*. Korean J Food Nutr 2010;23:226-32.
23. Lee EN, Song JH, Lee JS. Screening of a potent antidementia acetylcholinesterase inhibitor-containing fruits and optimal extraction conditions. Korean J Food Nutr 2010;23:318-23.
24. Jang CH, Eun JS, Park HW, Seo SM, Yang JH, Leem KH, Oh SH, Oh CH, Baek NI, Kim DK. An acetylcholinesterase inhibitor from the leaves of *Securinega suffruticosa*. Korean J Pharmacogn 2003;34:14-7.