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Production of the Acetylcholinesterase Inhibitor from *Yarrowia lipolytica* S-3

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The acetylcholinesterase (AChE) inhibitor of *Yarrowia lipolytica* S-3 was maximally produced when it was incubated at 30°C for 36 h in an optimal medium containing 1% yeast extract, 2% peptone and 2% glucose, with an initial pH 6.0. The final AChE inhibitory activity under these conditions was an IC_{50} value of 64 mg/*ml*. After partial purification of the AChE inhibitor by means of systematic solvent extraction, the final IC_{50} value of the partially purified AChE inhibitor was 0.75 mg/*ml*. We prepared a test product by using the partially purified AChE inhibitor and then determined its stability for the development of a new antidementia commercial product. The test product was stable at room temperature for 15 weeks.

KEYWORDS : Acetylcholinesterase inhibitor, Antidementia, *Yarrowia lipolytica* S-3

Alzheimer's disease, which was first reported by Alios Alzheimer in 1907, is currently well understood in patients with a progressive neurodegenerative disease characterized by memory loss, language deterioration, impaired visuospatial skills, poor judgment, indifferent attitude, but preserved motor function (Alzheimer, 1907). Several neurotransmitters (acetylcholine (Ach), norepinephrine, and dopamine) and neuropeptides (somatostatin and the corticotrophin-releasing factor) are involved in Alzheimer's disease (Rossor *et al.*, 1980; Rossor, 1982). Deficits in ACh, norepinephrine, the corticotrophin-releasing factor and somatostatin are found in moderate-to-advanced cases of Alzheimer's disease AD (Davis *et al.*, 1999).

Acetylcholinesterase (AChE) is found primarily in the blood and neural synapses. Normally, AChE converts ACh into the inactive metabolites choline and acetate (Hasselmo, 1995). The role of AChE in rapidly clearing free ACh from the synapse is essential for proper muscle function (Yu and Dayan, 2005). AChE inhibitors are important because they reduce the activity of cholinergic neurons. They also reduce the rate at which ACh is broken down; hence, they increase the concentration of ACh in the brain (thereby compensating for the loss of ACh caused by the death of the cholinergic neurons). On the other hand, since a shortage of ACh in the brain has been associated with Alzheimer's disease, some drugs that inhibit AChE are used in the treatment of that disease (Eubanks *et al.*, 2006). The AChE inhibitor is used in the following FDA-approved drugs: Tacrine, Cognex, Aricept, Donepezil, Rivastigmine and Galantamine (Lahiri *et al.*, 2002).

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Note also that the useful physiological properties of yeast have led to its use in the field of biotechnology. Yeast is one of the most widely used model organisms in genetics and cell biology. However, scant information is available on bioactive compounds from yeasts other than the antihypertensive angiotensin I-converting enzyme inhibitor (Kim *et al.*, 2004) and the β-secretase inhibitor (Lee *et al.*, 2007). The study of new bioactive compounds from yeast is therefore needed.

In a previous paper (Lee, 2008), we reported the isolation and identification of the AChE inhibitor-producing *Yarrowia lipolytica* S-3. In this paper, we present the optimal conditions for the mass production of the AChE inhibitor from *Y. lipolytica* S-3 and report on the stability of a test product made by from a partially purified form of the AChE inhibitor.

Materials and Methods

Chemicals. Unless otherwise specified, all chemicals and solvents were of analytical grade. The following were purchased from the Sigma Chemical Co, USA: recombinant human Acetyl-cholinesterase (AChE, E.C. 3.1.1.7), Acetyl-thiocholine chloride (ATCh), and 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB). A VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used in the AChE activity.

Yeast strain and culture conditions. *Y. lipolytica* S-3, which was isolated from bread by our laboratory (Lee, 2008) was inoculated in YEPD medium containing 1.0% yeast extract, 2.0% peptone and 2.0% dextrose and cultured at 30°C for 48 hr.

Assay of the AChE inhibitory activity. The AChE inhibitory activity was measured spectrophotometrically by using the technique of Ellman *et al.* (1961). A mixture of 110 μl of an assay buffer (0.1 M sodium phosphate, pH 7.3), 30 μl of AChE (0.8 U/*ml*), 30 μl of a substrate (ATCh), 20 μl of DTNB, and 10 μl of a sample dissolved in the assay buffer was incubated for 60 min at 37°C. The reaction product 5-thio-2-nitrobenzoate, which was produced enzymatically was measured at 415 nm.

The inhibition ratio was obtained by the following equation: inhibition ratio (%) = $[1 - \{(S - S_0)/(C - C_0)\}] \times 100$, 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 control at zero time, S is the radiation of the 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 check the quenching effect of the samples, we added the sample solution to the reaction mixture C and investigated any reduction in the radiation of the sample. The IC_{50} value is defined as a the concentration of the AChE inhibitor that is required to inhibit 50% of the inhibitory (AChE) activity.

Optimal conditions for AChE inhibitor production. The optimal conditions for production of the AChE inhibitor from the S-3 strain were investigated in a range of 4.0~8.0 for the initial pH of the medium, 20~40°C for the culture temperature and 12~48 hr for the culture time.

Systematic solvent extraction and preparation of the test product. A culture broth of *Y. lipolytica* S-3 was fractionated stepwise with n-hexane, chloroform, ethyl acetate, butanol and water. We then investigated the AChE inhibitory activity of each fraction. An antidementia test product was prepared by using the partially purified AChE inhibitor (5%) of *Y. lipolytica* S-3. We also used vegetable fermented broth (50 brix, 1.0%), apple concentrates (72 brix, 3.0%) and caramel paste (70 brix, 1.0%) as additives for enhancing acceptability and functionality.

Table 1. Effects of media on production of the acetylcholinesterase inhibitor

Media[a]	Achetlycholinesterase inhibitory activity (%)
YEPD	69.3 ± 0.04[b]
YM	59.1 ± 0.03
PD	50.6 ± 0.04
SB	21.3 ± 0.02
ME	n.d
YE	40.0 ± 0.05

[a]*Y. lipolytica* S-3 was cultured in YEPD (Yeast extract 10.0 g, Peptone 20.0 g, Dextrose 20.0 g/*l*), YM (Yeast extract 3.0 g, Malt extract 3.0 g, Peptone 5.0 g, Dextrose 10.0 g/*l*), PD (Potato 200 g, Dextrose 20.0 g/*l*), SB (Glucose 40.0 g, Peptone 10.0 g/*l*), ME (Maltose 12.75 g, Glucose 2.75 g, Glycerol 2.35 g, Peptone 0.78 g/*l*) and YE (Peptone 5.0 g, Yeast extract 5.0 g, Beef extract 3.0 g, Sodium chloride 5.0 g/*l*) at 28°C for 48 h. After obtained cell-free extracts, its acetylcholinesterase inhibitory activities were assayed.
[b]Values show means ± SE from three experiments performed in triplicate.

Results and Discussion

Effect of media for on the extracellular AChE inhibitor production from *Y. lipolytica* S-3. The effects of media on the production of AChE inhibitor were showed Table 1. The maximal production can be obtained in a medium comprising yeast extract, -peptone and -dextrose (YEPD). Other media that yielded an AChE inhibitory activity rate of more than 50% include a medium of YM and PD media.

Effect of culture temperature and pH. To determine the optimum temperature for the production of the AChE

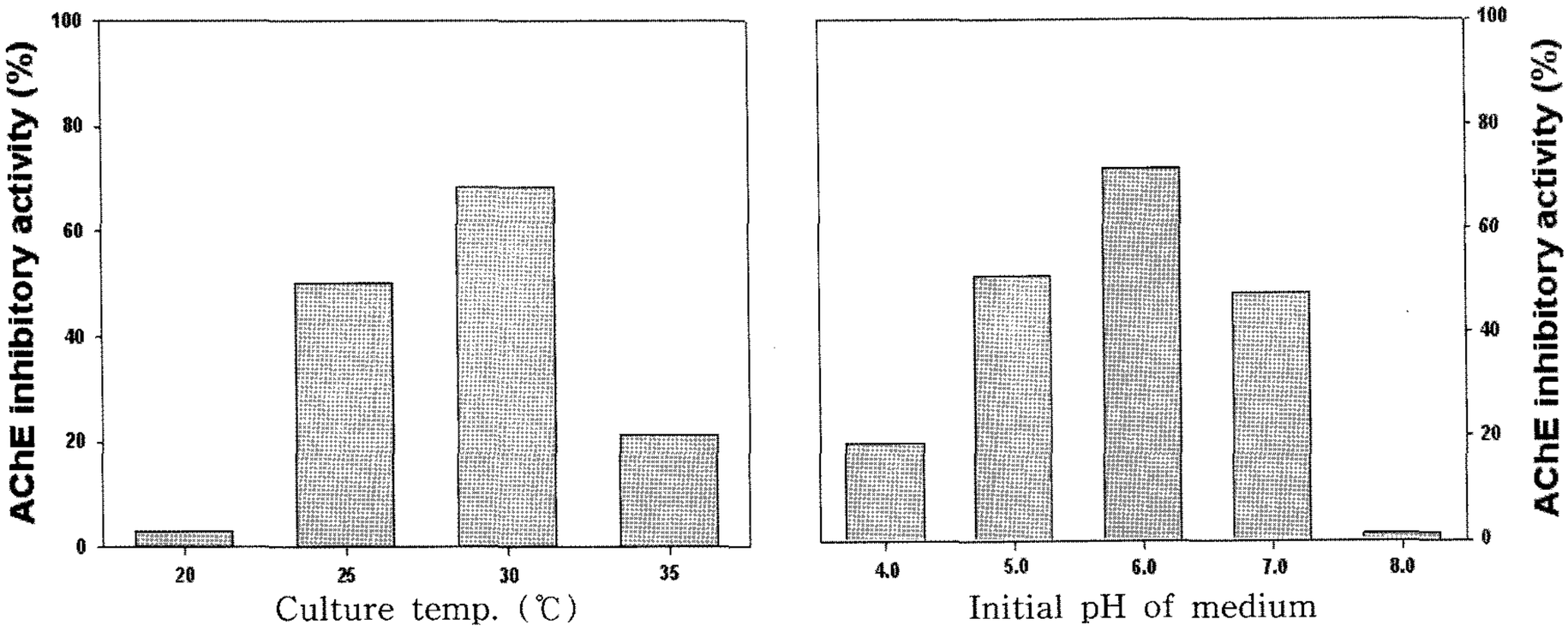

Fig. 1. Effects of culture temperature and initial pH of medium on production of acetylcholinesterase (AChE) inhibitor.

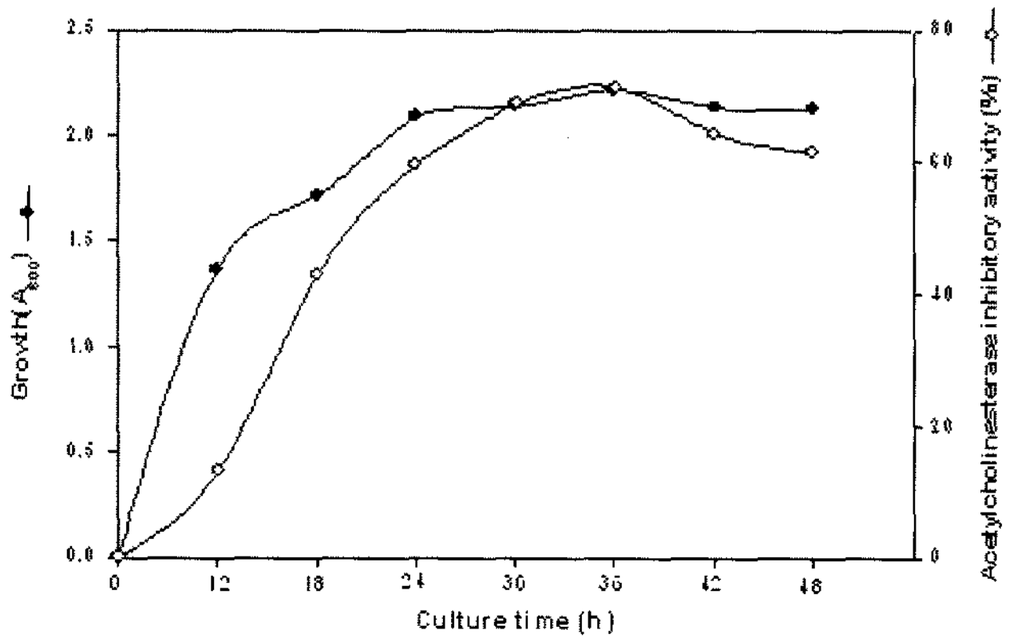

Fig. 2. Effects of culture time on production of the acetylcholinesterase inhibitor. *Yarrowia lipolytica* S-3 was cultured in YEPD medium containing 1.0% yeast extract, 2.0% peptone and 2.0% dextrose for indicated times.

inhibitor, we grew *Y. lipolytica* S-3 in a YEPD medium for 3 days at several temperatures and then measured the AChE inhibitory activity. As shown in Fig. 1, the optimal temperature for the AChE inhibitor production was 30°C.

The optimal initial pH of the medium for the AChE inhibitor production was also examined in a range of pH 4.0~8.0 (Fig. 1). The AChE inhibitor production reached a maximum at a pH of 6.0, but no AChE inhibitor was produced at below a pH 3.0 or above a pH 8.0.

Effect of culture time. The time course of the AChE inhibitor production was determined in a flask culture under optimal culture conditions for the AChE inhibitor production. As shown in Fig. 2, the AChE inhibitor production was increased as the cell growth increased, and the maximum AChE inhibitor production level was observed after 36 hr of cultivation.

From these results, we deduce that the optimal culture conditions for the production of the AChE inhibitor is a YEPD medium with an initial pH 6.0, a culture time of 36 hr and a culture temperature of 30°C.

Preparation and stability of the antidementia test product. A lyophilized cell-free extract of *Y. lipolytica* S-3 was produced by means of systematic solvent extraction. The ethyl acetate fraction showed the highest AChE inhibitory activity, namely 60.9% and the highest IC_{50} value namely 0.75 mg/*ml*. In addition, the AChE inhibitory activity was 55.3% for the chloroform fraction and 50.0% for the buthanol fraction (data are not shown). We prepared an antidementia test product by using the ethyl acetate extracts and we determined its stability while storing the product at room temperature and at 40°C for 15 weeks (Table 2).

We found that the pH, residual sugar and AChE inhibitory activity were not significantly changed during the 15 weeks. In addition, there was no significant increase in the viable cell counts though there was an increase of about 1.5 CFU/*ml* in the storage at 40°C for 15 weeks. The results suggest that the AChE inhibitor containing test product should be stable during the shelf-life of the product.

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Table 2. Changes of quality characteristics during storage of the antidemantia test product at room temperature and 40°C

	0	Room temp. (20°C)				40°C			
		2Wk[a]	4Wk	8Wk	15Wk	2Wk	4Wk	8Wk	15Wk
pH	3.8	3.9	3.9	3.8	3.8	4.0	4.1	4.2	4.1
Sugar (brix°)	17.5	17.5	16.8	16.2	16.0	17.0	15.2	12.3	12.5
Viable cell counts (Bacteria, CFU/*ml*)	1.0	1.2	1.5	1.8	1.5	1.5	1.8	2.1	2.1
Acetylcholinesterase inhibitory activity (%)	68.5 ± 0.7[b]	69.2 ± 0.5	69.0 ± 0.3	68.5 ± 0.4	67.7 ± 0.8	69.0 ± 0.2	68.7 ± 0.4	69.0 ± 0.3	68.5 ± 0.5

[a]Wk; week.
[b]Values show means ± SE from three experiments performed in triplicate.

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