ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Ethyl Carbamate Degrading Enzyme from Yeast *Meyerozyma caribbica* strain SKa5: Purification and Biochemical Properties.

Jantaporn Thongekkaew[1]*, Tsutomu Fujii[2, 3], and Kazuo Masaki[4].

[1]Department of Biological Science, Faculty of Science, Ubon-Ratchathani University, Ubon-Ratchathani, 34190. Thailand.
[2]Quality and Evaluation Research Division, National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashihiroshima-shi, Hiroshima, 739-0046. Japan.
[3]Graduate School of Biosphere Sciences, Hiroshima University, 1-4-4 Kagamiyama, Higashihiroshima-shi, Hiroshima, 739-8528. Japan .
[4]Industrial Technology Center, Gifu Prefectural Government, 47 Kitaoyobi Hashimagun Kasamatsucho, Gifu, 501-6064. Japan.

ABSTRACT

Ethyl carbamate degrading enzyme from yeast which was isolated from spoilage fruit was studied. Based on ITS1-5.8S-ITS2 region and D1/D2 domains of the 26S subunit analysis, the selected yeast was identified as *Meyerozyma caribbica* and named as *M. caribbica* SKa5. The enzyme from cell extracted was purified by anion exchange chromatography. The molecular mass of the enzyme was approximately 55 kDa, as identified by SDS-PAGE. The optimum pH and temperature were 7.0-8.0 and 37°C, respectively. The enzyme was stable at pH range of 4.5–8.0 and at temperature range of 10-37°C. The enzyme also exhibited ethanol tolerance at concentration of 20% (v/v). In addition, the enzyme was not affected in the presence of Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, β–mercaptoethanol and EDTA. This favorable properties could become one of potential enzyme resources for removal of ethyl carbamate in alcoholic beverages and fermented foods industry.
Keyword: ethyl carbamate, ethyl carbamate degrading enzyme, *Meyerozyma caribbica*, alcoholic beverages, fermented food

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Ethyl carbamate (EC), also known as urethane, is the ethyl ester of carbamic acid. These precursor substances, e.g. urea, results from the degradation of arginine by yeasts, cyanate and citrulline react with ethanol to form EC in alcoholic beverages such as spirits, wine, beer, whiskey and sake. It also can be naturally formed in fermented foods such as bread, soy sauce and yogurt during the fermentation process or during storage [1-6]. EC was found to be carcinogenic and tetratogenic effect which can cause liver cancer, lung cancer, lymph cancer [7-9].

The amount of EC formed depends on the key factors of light exposure, elevated temperature and long-term storage. Recently, EC has been classified by the International Agency for Research on Cancer (IARC) as belonging to the 2A group of chemicals due to its genotoxity and carcinogenesis to human [10]. EC can be greatly reduced by fermentation strain selection, fermentation process improvement, and enzymatic decomposition. However, enzymatic decomposition method has been widely employed because of safety and pollution-free [11-14]. Acidic urease is one of enzyme which can degrade urea, the major precursor of EC [15-17]. However, EC, once formed in alcoholic beverages, could not be decomposed by urease. Another enzyme is ethyl carbamate degrading enzyme or urethanase, which have been successful in the removal of EC in Chinese rice wine [18]. Even ethyl carbamate degrading has been reported in several studies in order to reduce the hazard of ethyl carbamate [11, 18-23]. In view of the potential application of this enzyme, ethyl carbamate degrading enzyme-producing strains screening and study on enzymatic properties from various sources are desirable. The present study reports the purification and characterization of ethyl carbamate degrading enzyme from yeast strain identified as *Meyerozyma caribbica* SKa5 to investigate the potentially reduce the hazard of ethyl carbamate.

MATERIALS AND METHODS

Isolation of ethyl carbamate degrading enzyme-producing yeast

For isolation of ethyl carbamate degrading enzyme-producing yeast, twelve yeast strains isolated from soil and spoilage fruits were inoculated to medium containing 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 1% ethyl carbamate and 2% glucose. After incubating on a rotary shaker at 30°C for 4 days, growth rate was measured spectrophotometrically at 660 nm.

Molecular identification of selected yeast

Pure isolated yeast cells from 3 mL of 24-h culture were harvested by centrifugation and DNA extraction as described by Sambrook et al. (1989) [24]. Identification was carried out by sequencing the gene that encodes the 5.8S regions of ribosomal ribonucleic acid (rRNA) and the spacer regions ITS-1 and ITS-2 after PCR amplification. To do so, the universal primers ITS4 (5′ TCCTCCGCTTA-TTGATATGC 3′) and ITS5 (5′ GGAAGTAAAAGTCGTAACAAGG 3′) were used. The D1/D2 domains of the 26S subunit were also sequenced by using the primers NL1 (5′ GCATATCAATAAGCGGAGGAAAAG 3′) and NL4 (5′ GGTCCGTGTTTCAAGACGG 3'), according to the methodology described by Kurtzman and Robnett (1997) [25]; Esteve-Zarzoso et al. (1999) [26] and Leaw et al. (2006) [27]. The PCR product was purified by using QIA quick PCR Purification Kit and analyzed with 1.5% agarose gel electrophoresis. The PCR products were sequenced using the ITS4, ITS5, NL-1 and NL-4 primers using either Amersham Pharmacia ALF Express II or ABI 310 (capillary) automated DNA sequencer, following the manufacturer's instructions. For identification, the obtained sequences were compared with those of all known yeast species, available at the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) [28].

Preculture of microorganism selection

The selected yeast strain was grown in sterile ethyl carbamate medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 1% ethyl carbamate and 2%glucose) for 16 h at 30^{o}C was used as a preculture.

Production and Extraction of ethyl carbamate degrading enzyme

2000 ml of ethyl carbamate medium was inoculated with overnight preculture with initial OD_{660} at 0.1. After incubation under shaking (120 rpm) at 30°C for 3 days, the cell pellets were disrupted by multi bead shocker 50 times at intervals of 30 second then suspended in 0.1 M phosphate-buffered, pH 6.0. After centrifugation (12,000×g for 20 min), the supernatant were pooled and dialyzed for overnight against 20 mM phosphate-buffered, pH 6.0. After centrifugation (12,000×g for 20 min), the supernatant was used as the crude enzyme solution.

Enzyme activity assay

Ethyl carbamate degrading enzyme activity was assayed by measuring the amount of ammonia produce from ethyl carbamate. The standard reaction mixture (1000 μL) contained 950 μL of final concentration of 0.1% ethyl carbamate in 50 mM sodium phosphate buffer, pH 7.0 and 50 μL of enzyme. After incubation at 37°C for 90 min, the produced ammonia was determined spectrophotometrically at 630 nm. One unit of ethyl carbamate degrading enzyme activity is defined as the amount of enzyme necessary to liberate 1 μg of ammonia per minute.

Purification of ethyl carbamate degrading enzyme

A 65 ml of crude enzyme solution was loaded onto HiTrap Q HP column (Amersham Biosciences, Sweden) and eluted with linear gradient from 0 to 1 M NaCl in 20 mM sodium phosphate buffer, pH 6.0. The fractions were assayed for ethyl carbamate degrading enzyme activity and active fraction was further purified using HiTrap Q HP column with linear gradient from 0 to 0.5 M NaCl in 20 mM sodium phosphate buffer, pH 6.0. The fraction containing high activity was loaded onto HiTrap Q HP column and eluted with linear gradient from 0 to 0.5 M NaCl in 20 mM sodium phosphate buffer, pH 8.0. The fraction containing high activity was loaded onto HiTrap Q HP column and eluted with linear gradient from 0 to 0.4 M NaCl in 20 mM sodium phosphate buffer, pH 8.0. The fractions containing high activity was further investigated enzyme characterization. The relative molecular mass of purified ethyl carbamate degrading enzyme was estimated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis

The relative molecular mass of the purified ethyl carbamate degrading enzyme was estimated by SDS-PAGE using "multigel 4/20" (Daiichi Pure Chemicals, Japan) according to the method of Laemmli (1970) [29] and the proteins were stained with CBB Stain One (NACALAI TESQUE, INC, Japan). A Dual prestained SDS-PAGE Standards containing 10 sizes of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa was used for reference proteins.

Characterization of ethyl carbamate degrading enzyme

The optimum pH was determined by measuring the activity between pH 3.5 to 8.0 (50 mM) using citrate buffer (pH 3.5-5.5), sodium phosphate buffer (pH 6.0-8.0). Ethyl carbamate degrading enzyme activity was measured at 37°C under the assay condition. For determination of pH stability, the enzyme was preincubated in 50 mM of each buffer at 4°C for 16 h. The residual ethyl carbamate degrading enzyme activity was assayed under the assay condition.

The optimum temperature was determined by measuring the activity at different temperatures (25–60°C). For the determination of thermal stability, the enzyme was preincubated at 10–50°C for 16 h, and then, the activity was measured under the assay condition.

The effect of ethanol on ethyl carbamate degrading enzyme activity was studied. Reaction mixtures containing 0.1% ethyl carbamate in 50 mM phosphate buffer, pH 7.0, with various concentrations of ethanol (0-30%) were incubated at 37°C for 90 min and the residual activity was measured under the assay condition.

The effect of metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+}) and chemicals such as β–mercaptoethanol and EDTA on the enzyme activity was studied by incubating these ions at 5 or 10 mM final

ISSN: 0975-8585

concentrations in the standard reaction mixture. The residual ethyl carbamate degrading enzyme activity was assayed under the assay condition.

RESULTS

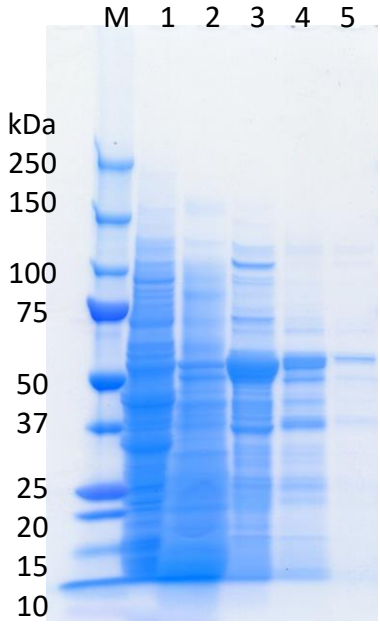
Isolation and identification of ethyl carbamate degrading enzyme-producing yeast

Twelve yeast strains isolated from soil and spoilage fruits were screened for ethyl carbamate degrading enzyme-producing yeast. After incubating on a rotary shaker at 30°C, cell growth rate was measured. Among them, a strain named SKa5 showed the highest growth rate then it was selected for further study. According to the BLAST analysis, the ITS nucleotide sequence of the isolated SKa5 is 100 % identical to that of *Meyerozyma caribbica*. Therefore, the strain is named as *M. caribbica* SKa5.

Purification and Characterization of ethyl carbamate degrading enzyme

Cell extracted of *M. caribbica* SKa5 was purified by ion exchange chromatography using HiTrap Q HP column. One peck was observed on the chromatogram. Active fraction was collected as purified ethyl carbamate degrading enzyme and used further enzyme characterization. The purity was confirmed on SDS–PAGE and its relative molecular mass was approximately 55 kDa (Fig. 1).

Figure 1: SDS-PAGE analysis of purified ethyl carbamate degrading enzyme from *M. caribbica* SKa5. Lane M: standard protein markers; Lane 1: crude protein; Lane 2: 1stHiTrap Q HP column; Lane 3: 2ndHiTrap Q HP column; Lane 4: 3rdHiTrap Q HP column; Lane 5: purified enzyme

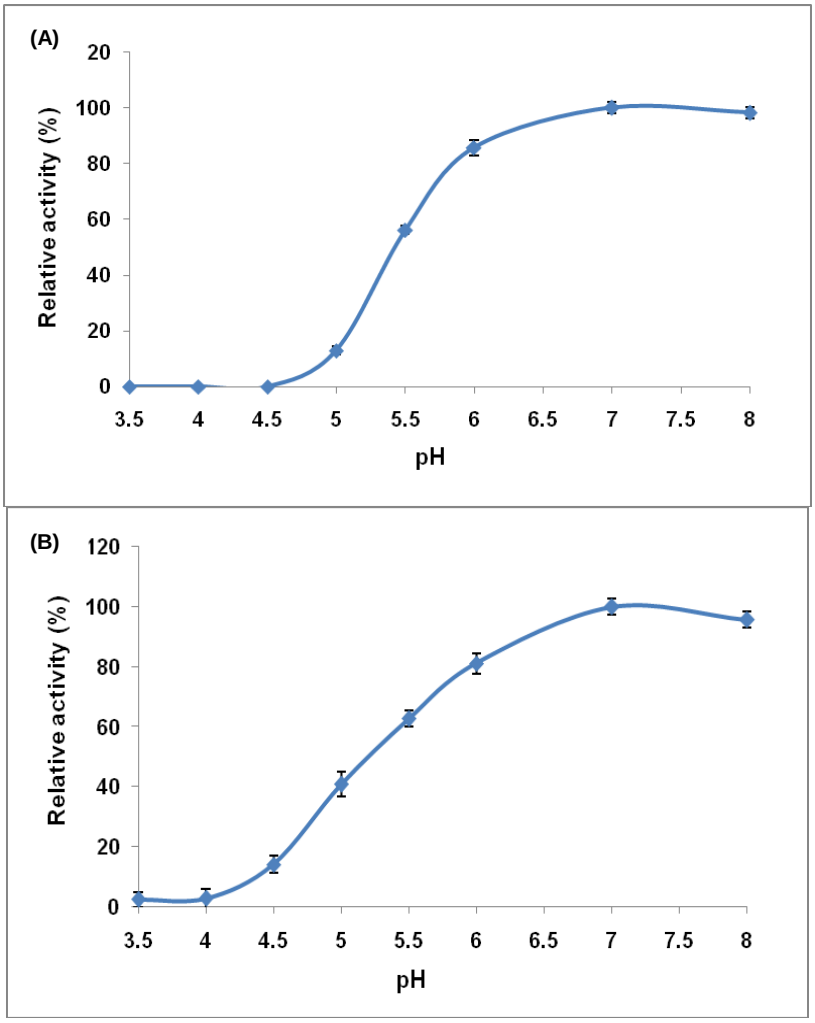


The biochemical properties of *M. caribbica* SKa5 ethyl carbamate degrading enzyme were investigated. The purified enzyme exhibited maximum activity at pH 7.0-8.0. Further decrease of pH at ranging of 5.5-6.0 resulted decline in enzyme activity and less than 20% relative activity was observed at pH 3.5-5.0 (Fig. 2A). The enzyme was found remarkably stable at pH range of 4.5-8.0 after 16 h of incubation (Fig. 2B).

The purified enzyme was active at temperature of 25-40°C which has the optimum temperature at 37°C (Fig. 3A). Thermostability profile of the enzyme revealed that the enzyme was stable at temperature from 10 up to 37°C for 16 h of pre-incubation period which retained activity more than 50%. But higher temperature up to 40°C causes denaturation of the enzyme (Fig. 3B).

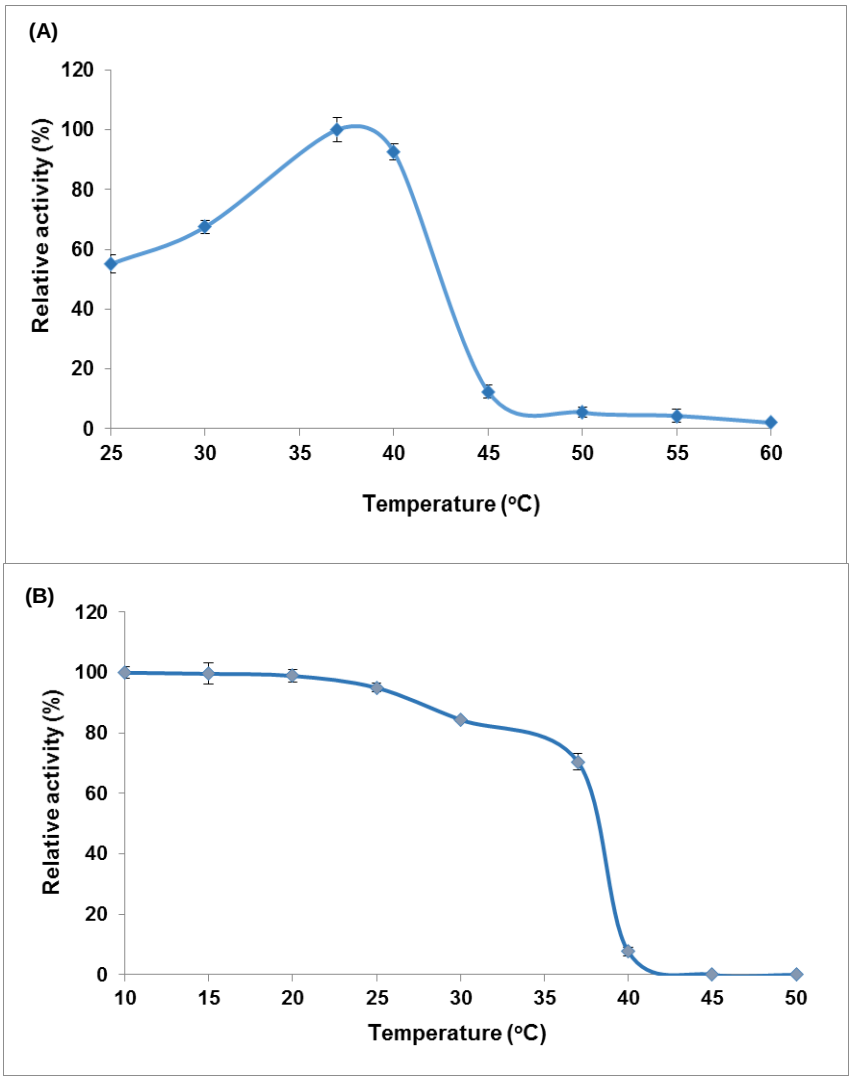
ISSN: 0975-8585

Figure 2: (A) Optimum pH profile of ethyl carbamate degrading enzyme from *M. caribbica* SKa5. pH profile was determined by incubating the enzyme at 37°C for 90 min at varying pHs 3.5 to 8.0 and (B) Stability of ethyl carbamate degrading enzyme from *M. caribbica* SKa5. The remaining activity of the enzyme was determined (at 37°C in 50 mM phosphate buffer (pH 7.0) for 90 min) after incubating at 4°C for 16 h at various pHs, from 3.5 to 8.



ISSN: 0975-8585

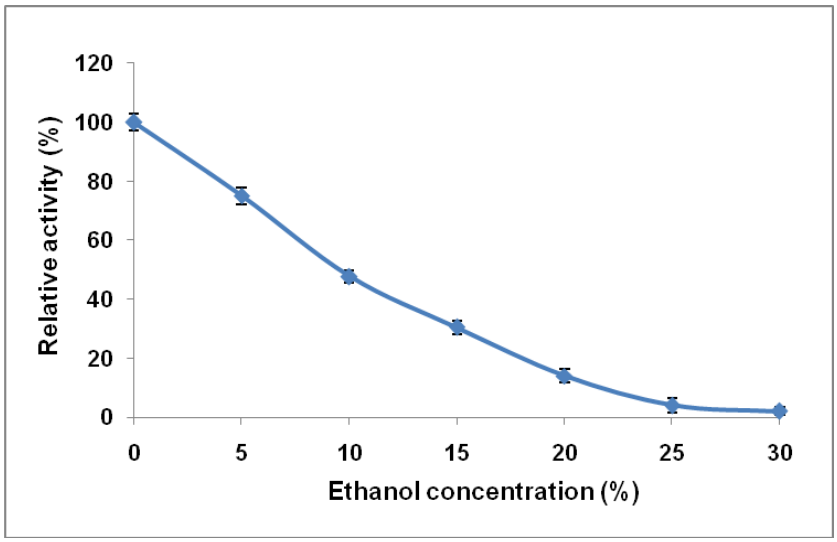
Figure 3: (A) Optimum temperature on the activity of ethyl carbamate degrading enzyme from *M. caribbica* SKa5. Temperature profile was determined by incubating the enzyme in 50mM phosphate buffer (pH 7.0) for 90 min at different temperatures (25 to 60°C) and (B) Thermalstability of ethyl carbamate degrading enzyme from *M. caribbica* SKa5. Thermal stability was carried out by incubating the enzyme in 50mM phosphate buffer (pH 7.0) at different temperatures (10–50°C) for 16 h before the remaining activity was assayed (at 37°C for 90 min)



The effect of various concentration of ethanol on enzyme activity was investigated. The result showed that the enzyme activity was decreased with the increased ethanol concentration (Fig. 4). However, the enzyme was tolerance at the concentration of ethanol up to 20% (v/v).

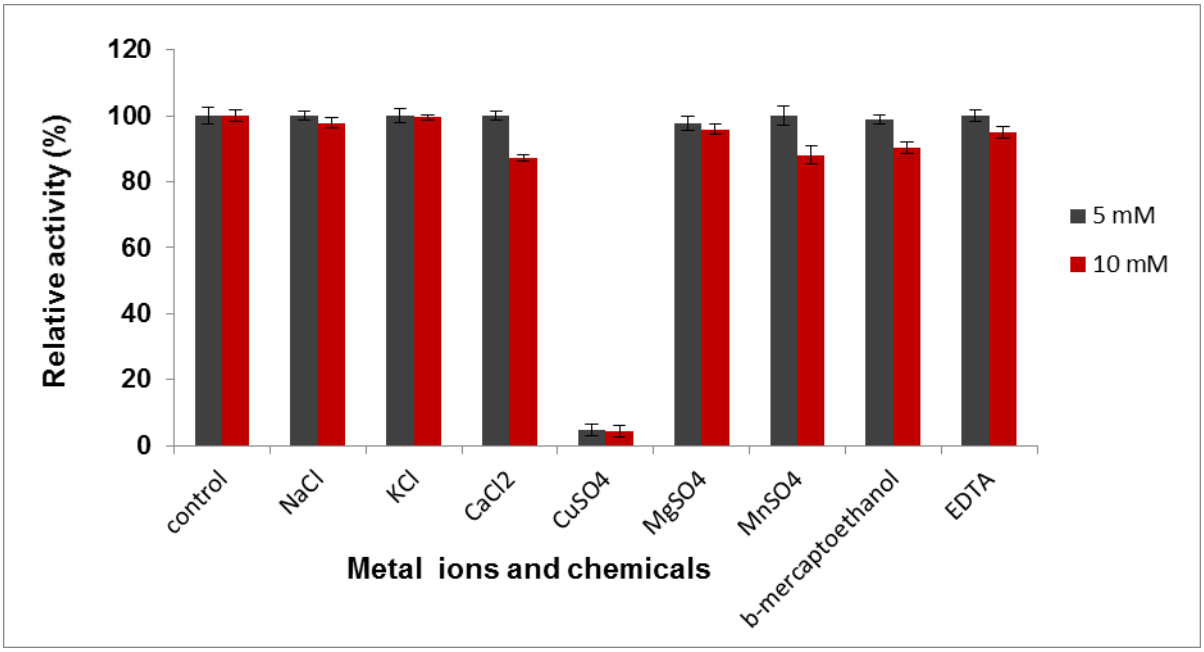
ISSN: 0975-8585

Figure 4: Effect of different %(v/v) of ethanol on ethyl carbamate degrading enzyme from *M. caribbica* SKa5. The effect of alcohol was determined by incubating the enzyme in 50 mM phosphate buffer (pH 7.0) for 30 min at different concentration of alcohol (0-30% (v/v)). The enzyme was assayed for the remaining activity under the assay condition.



The effect of various metal ions and chemical at concentration of 5 and 10 mM on the enzyme activity was also tested. The result showed that this enzyme was unaffected in the presence of Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, β–mercaptoethanol and EDTA but it was strongly inhibited in the presence of Cu^{2+} at both concentrations.

Figure 5 Effect of metal ions and chemicals on ethyl carbamate degrading enzyme from *M. caribbica* SKa5.



ISSN: 0975-8585

Table 1: Biochemical properties of ethyl carbamate degrading enzyme from *M. caribbica* SKa5 and other microorganisms

Strain	M_w(kDa)	Optimal pH	Optimal Temp. (°C)	Reference
Meyerozyma caribbica SKa5	55	7.0-8.0	37	This study
Bacillus lichenifomis sp. 103	ND	4.5	37	Zhao et al., 1991
Citrobacter sp.	ND	6.0-8.0	37	Kobashi et al., 1990
Penicillium variabile	13.7	6.0	50	Zhou et al., 2013
Klebsiella pneumonia	55	7.0	55	Bu et al., 2014
Micrococcus sp	ND	5.0	45	Mohapatra and Bapuji 1997

ND: not determined

DISCUSSIONS

Ethyl carbamate is genotoxic and a multisite carcinogen in animals and human. It occurs naturally in fermented foods and alcoholic beverages. Enzymatic removal of EC is an important way to eliminate its potential health damage to consumers. In this study, yeast namely *Meyerozyma caribbica* SKa5 was selected and characterized ethyl carbamate degrading enzyme. The cell extracted was purified by anion exchange chromatography. The molecular mass of the enzyme was approximately 55 kDa by SDS-PAGE. Comparing biochemical properties of *M. caribbica* SKa5 ethyl carbamate degrading enzyme with previous reports from other microorganism are summarized in Table 1. As shown in Table 1, the optimum pH of this enzyme was similar to some microorganism sources from previous studies such as *Citrobacter* sp. [11], *Penicillium variabile* [18] and *Klebsiella pneumonia* [30] but higher than that from *Bacillus lichenifomis* sp. 103 [20] and *Micrococcus* sp. [23]. At higher temperature up to 40°C causes denaturation of the enzyme. These properties were similar values for optimum temperature of ethyl carbamate degrading enzyme from *Citrobacter* sp. and *B. lichenifomis* sp. 103 (37°C). The ethanol tolerance of this enzyme (20% v/v) which is corresponding to the ethanol concentration of wine and sake is quite similar property to ethyl carbamate degrading enzyme from *B. lichenifomis* sp. 103. The enzyme was not affected in the presence of Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, β–mercaptoethanol and EDTA. No inhibit action of ethyl carbamate degrading enzyme activity by the chelating agent EDTA, indicating that divalent cations are not required for enzyme activation.

CONCLUSIONS

In this study, ethyl carbamate degrading enzyme producing yeast *Meyerozyma caribbica* SKa5 was screened, isolated and identified by molecular identification technique. The enzyme purification and biochemical properties of ethyl carbamate degrading enzyme produced by this strain were investigated. With good ethanol tolerance, this yeast resource can provide data on enzyme characteristics for the potential to remove ethyl carbamate in fermented food and alcoholic beverages which might be useful for applications in the field of food and beverage industry.

ACKNOWLEDGEMENTS

This research was supported financially by scientific cooperative research under New Core to Core Program **between Japan Society for the Promotion of Science (JSPS), National Research Council of Thailand (NRCT), Vietnam Ministry of Science and Technology (MOST), the National University of Laos, Beuth University of Applied Sciences and Brawijaya University.** We are gratefully acknowledged. We would like to thanks the Director of Brewing Technology Division, National Research Institute of Brewing, Japan for his kindly facilitate the laboratory to do collaborative research.

REFERENCES

[1] Conacher HBS, Page BD. Ethyl carbamate in alcoholic beverages: A Canadian case a history. Proceedings of Euro Food Tox II, European Society of Toxicology, Schwerzenbach, Switzerland, 1986, pp. 237–242.
[2] Dennis MJ, Howarth N, Key PE, Pointer M, Massey RC. Food Addit Contam 1989; 6: 383-389.
[3] Battaglia R, Conacher HBS, Page BD. Food Addit Contam 1990; 7: 477-496.
[4] Benson RW, Beland FA. Int J Toxicol 1997; 16, 521–544.
[5] Weber JV, Sharypov VI. Environ Chem Lett 2008; 7: 233–247.
[6] Thibon C, Marullo P, Claisse O, Cullin C, Dubourdieu D, Tominaga T.FEMS Yeast Res 2008; 8(7): 1076–1086.
[7] Schlatter J, Lutz WK. Food Chem Toxicol 1990; 28: 205–211.
[8] Zimmerli B, Schlatter J. Mutat Res 1991; 259: 325-350.
[9] Beland FA, Benson RW, Mellick PW, Kovatch RM, Roberts DW, Fang JL, Doerge DR. Food Chem Toxicol 2005; 43(1): 1–19.
[10] Summary and conclusions of the sixty-fourth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Rome, 8–17 February 2005. ftp://ftp.fao.org/es/esn/jecfa/jecfa64_summary.pdf
[11] Kobashi K, Takebe S, Sakai T. Chem Pharm Bull 1990; 38: 1326–1328.
[12] Bruno SNF, Vaitsman DS, Kunigami CN, Brasil MG. Food Chem 2007; 104(4): 1345–1352.
[13] Schehl B, Senn T, Lachenmeier DW, Rodicio R, Heinisch JJ. Appl Microbiol Biotechnol 2007; 74: 843–850.
[14] Jiao Z, Dong Y, Qihe Chen Q. Compr Rev Food Sci Food Saf 2014; 13: 611-626.
[15] Kobashi K, Takabe S. J Appl Toxicol 1988; l34: 73–74.
[16] Andrich L, Esti M, Moresi M.J. Agric. Food Chem 2010; 58: 6747–6753.
[17] Yang LQ, Wang SH, Tian YP. Appl Biochem Biotechnol 2010; 8: 8159–8160.
[18] Zhou N, Gu X, Tian Y. Appl Biochem Biotechnol 2013; 170: 718–728.
[19] Chun JZ, Lisa I, Kyoichi K. Chem Pharm Bull 1991; 39: 3303–3306.
[20] Zhao CJ, Imamura L, Kobashi K. Chem Pharm Bull 1991; 39: 3303–3306.
[21] Zhao CJ, Kobashi K. Biol Pharm Bull 1994; 17: 773–778.
[22] Zhao CJ, Kobashi K. Chem Pharm Bull 1994; 17: 773–778.
[23] Mohapatra BR, Bapuji M. Lett Appl Microbiol 1997; 25: 393–396.
[24] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a Laboratory manual. 2nd(Ed). Cold Spring Harbor Publishers Inc, New York, USA 1989.
[25] Kurtzman CP, Robnett CJ. J Clin Microbiol 1997; 35: 1216-1223.
[26] Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A. Int J Syst Evol Microbiol 1999; 49: 329-337.
[27] Leaw SN, Chang HC, Sun HF, Barton R, Bouchara JP, Chang TC. J Clin Microbiol 2006; 44: 693-699.
[28] Yarrow D. Methods for the isolation, maintenance and identification of yeasts. In: Kurtzman CP, Fell JW (eds) The yeasts, a taxonomic study, 4th ed. Elsevier, Amsterdam, The Netherlands, 1998.
[29] Laemmli UK. Nature 1970; 227: 680-685.
[30] Bu P, Chen J, Du G. Chin J Biotechnol 2014; 03.