Vietnam Journal of Science and Technology ***57*** *(5) (2019) 544-550*

doi:10.15625/2525-2518/57/5/13272

CHARACTERIZATION OF *TETRAGENOCOCCUS HALOPHILUS* FROM VIETNAMESE FISH MASH

Le Thanh Ha[1], Nguyen Nam Trinh[1], Le Thi My Chau[2], Nguyen Thi Minh Tu[1, *]

[1]*School of Biotechnology and Food Technology, Hanoi University of Science and Technology, 1 Dai Co Viet, Hai Ba Trung, Ha Noi*

[2]*School of Chemistry, Biology and Enviroment, Vinh University, 182 Le Duan street, Vinh city, Nghe An province*

[*]Email: *tu.nguyenthiminh@hust.edu.vn*

Received: 7 November 2018; Accepted for publication: 12 August 2019

Abstract. In this study, the intracellular aminopeptidase activities and volatile's profiles of seventeen *Tetragenococcus halophilus* strains, which were isolated previously from fish mash, were investigated. Among them, *Tetragenococcus halophilus* strains CH2-4 and CH6-2 exhibited the highest aminopeptidase activities of 2.1-2.2 U/mL toward Glutamic-pNA, while *T. halophilus* strains CH6-1 and V7-2 showed the highest aminopeptidase activities of 1.4-1.5 U/mL toward Leucine-pNA. The fish broth inoculated with *T. halophilus* strains CH2-4, CH6-2, CH6-1 and V7-2 for 14 days were treated to obtain the volatiles before being subjected to GC/MS analysis. A total of 19 volatile compounds, including esters (pentanoic acid, 2-ethylhexyl ester; pentanoic acid, 2-propenyl ester), alcohols (3-hexanol; 2-ethyl-1-hexanol; 2-furanmethanol), ketones, phenols, hydrocarbons and sulfur containing compounds (sulfurous acid, butyl 2-ethylhexyl ester and benzyl 2-chloroethyl sulfone) were identified. The groups of esters and alcohols were the most abundant based on the phenomena that 6 compounds were identified in each group. *T. halophilus* CH6-2 strains produced the highest amount of identified volatile compounds. Among 19 volatile compounds identified, 10 were detected in sample inoculated with strains CH6-2, while only 5 to 6 volatile compounds were observed in the samples inoculated with the other strains.

The strains *T. halophilus* CH6-2 could tolerate NaCl concentration of 25 %. The optimal condition for its growth was at 5-8 % of NaCl, pH of 6.5 to 7.5 and temperature of less than 37 °C.

Keywords: *Tetragenococcus halophilus,* aminopeptidase activity, fish sauce, volatile compound.

Classification numbers: 1.3.2, 1.4.4.

1. INTRODUCTION

Fish sauce, the popular seasoning in Viet Nam is produced through a natural fermentation process whereas indigenous microorganisms play an important role. The fish sauce is made by mixing fish and salt at ratio 3:1, and then the mixture is fermented for more than 12 months. For shortened fermentation time, improved flavor and aroma of fish sauce, as well as for a better

process control, many attempts have focused on using starter cultures like *Staphylococcus* [1], *Virgribacillus* [2], or mixture of *Bacillus* and *Lactobacillus* [3], and *Tetragenococcus* [4].

Tetragenococcus are the most common bacterium in high-salt fermented food products. This bacterium has been found in various food products, such as Japanese traditional fermented fish sauce (squid liver sauce) [5], Cambodian fish product [6], and fish *nukazuke* [7]. The predominant *Tetragenococcus* in the final stage of fish sauce fermentation when aroma, colour and flavor have developed was confirmed by metagenomic method, suggesting their role for flavor and aroma development [8]. The use of *Tetragenococcus halophilus* as starter culture for enzyme hydrolyzed fish protein could reduce the fermentation time to 6 months without affecting the quality of the fish sauce product [8, 9]. In our previous study, 17 strains of genus *Tetragenococcus halophilus* isolated from Cat Hai and Cua Hoi factories were screened [10]. All the 17 strains were able to use fish protein for their growth, produce oligopeptide by hydrolysis of fish protein and reduce pH by acid production. In this study, their intracellular aminopeptidase activities and volatile compound formation were investigated. Furthermore, the growth characteristics of selected strain with respect to pH, temperature and salt concentration were also studied.

2. MATERIALS AND METHODS

2.1. Materials

Seventeen *Tetragenococcus halophilus* strains isolated from Cat Hai and Cua Hoi factories from previous study [10] were stored at -20 °C in glycerol 25 %.

Chemicals for De Man, Rogosa and Sharp (MRS) medium of bacteriological grade were purchased from LAB (UK) and Merck (Germany). NaCl (China) for media was of analytical grade. Chemicals for analysis of analytical grade were purchased from Merck (Germany). Fresh small fish was purchased in Sam Son, Thanh Hoa province, stored in ice and transferred to the lab.

2.2. Method

2.2.1. Intracellular aminopeptidase activities assay

The studied strains were cultured in MRS broth [11] containing 10 % NaCl then incubated at 30 °C for 3-5 days under anaerobic condition. Cells were harvested by centrifuging at 10,000×g for 15 min at 4 °C. Afterward, 250 µl of 5 mg/ml lysozyme were added to the cells and the mixture was incubated at 37 °C for 1 h. Subsequently, the cells were washed twice with 0.2 M phosphate buffer (pH 7.5) and resuspended in 2 ml of the same buffer. The cells were disrupted using a sonicator for 2 min. Cell debris was removed by centrifugation at 10,000×g for 15 min at 4 °C. The supernatant was used to determine intracellular aminopeptidases activity using amino p-nitroanilide derivatives Glutamic-pNA and Leucine-pNA [12].

One ml of reaction mixture consisted of 0.1 ml of crude enzyme, 0.1 ml of substrates (Glu-pNA, Leu-pNA at concentration of 20 mM) and 0.8 ml of 0.2 M phosphate buffer (pH 7.5). The reaction mixture was incubated at 60 °C for 2 h and then 80 % acetic acid was added to stop the reaction. The release of p-nitroanilide was measured using a spectrophotometer (PDS 303S, Apel, Japan) at 405 nm. One unit of enzyme activity (U) was defined as the release of 1 nmole of p-nitroanilide per minute.

2.2.2 Formation of volatile compounds in fish broth

The four selected isolates were cultured on MRS agar containing 10 % NaCl supplemented with 0.5 % $CaCO_3$ and incubated at 30 °C for 48 h under anaerobic condition. One loopful of pure culture was inoculated to 50 ml of fish broth containing 25 % NaCl (FB25) and incubated at 30 °C for 14 days under anaerobic condition. The FB25 was prepared as previously reported [10]. After 14 days, the supernatant was collected by centrifuging at 10,000×g for 10 min at 4 °C. Volatile compounds were obtained using modified procedure described by Yuan-fan Yang [13]: distillation followed by extraction with dichloromethane. The GC-MS system used for analysis was Agilent Technologies 6890N with Agilent 5975 inert XL Mass selective Detector (Agilent Technologies Co., Ltd., Palo Alto, USA) operating at 70 eV in EI (electron ionization) mode. Substances were separated on a fused silica capillary column, HP-5MS flexible glass capillary gas chromatography column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies Co., Ltd., Palo Alto, USA).

2.2.3 Growth characteristics of selected T. halophilus

The selected strains were incubated in 10 mL MRS medium for 96 hours at either various pH from 6 to 8.5 keeping temperature of 35 °C and NaCl concentration of 7 %; or at various temperature from 25 to 40 °C keeping pH of 7 and NaCl concentration of 7 %; or at various salt concentration from 0 % to 25 % while keeping temperature of 35 °C and pH of 7. The growth of the strains was evaluated by OD at 600 nm.

3. RESULTS AND DISCUSSION

3.1. Intracellular aminopeptidase activities

Seventeen *T. halophilus* strains were cultured and then treated according to 2.2.1. The aminopeptidase activities were determined, and the results are presented in Figure 1.

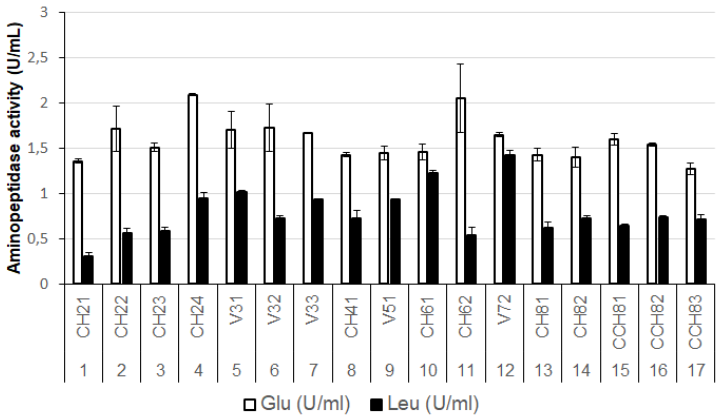

Figure 1. Aminopeptidase activities of 17 *T. halophilus* strains on various substrates. (Glu = Glutamic- pNA; Leu = Leucine-pNA).

All strains showed high intracellular aminopeptidase activities towards Glutamic-pNA and Leucine-pNA. Their aminopeptidase activities towards Glutamic-pNA varied from 1.3 U/mL to 2.2 U/mL, which was higher than those towards Leucine-pNA, which was from 0.3 U/mL to 1.5 U/mL (*Figure 1*). In this study, *T. halophilus* strains showed the opposite trend compared to *T. halophilus* strains from Thailand fish sauce Nampla, which had higher aminopeptidase activities towards Leucine than Glutamic acid [14].

The *T. halophilus* strains CH2-4 and CH6-2 possessed the highest aminopeptidase activity towards Glutamic-pNA, reaching 2.1-2.2 U/mL, which was as high as the highest one of strain MCD10-5-10 from Thailand fish sauce Nampla [14]. The strains V7-2 and V6-1 exhibited the highest aminopeptidase activity towards Leucine-pNA (around 1.3-1.4 U/mL), but still were about 1.7-fold lower than the highest aminopeptidase activity of MRC5-5-2 from Thailand (2.4 U/mL). Strains with high aminopeptidase activities were preferred since amino acids were suggested being precursor for volatile compound formation. Leucine is normally converted to carboxylic acids, such as 2-methylpropanoic acid and 3-methylpropanoic acid, which contributed to cheesy note [15]. In addition, leucine catabolism activity may result in 3-methylbutanal, subsequently, it is converted to 3-methylbutanol and 3-methylbutanoic acid via reduction and oxidation, respectively [15]. Intracellular aminopeptidase activities towards glutamic acid could be useful for flavor improvement since glutamic acid contributed to meaty flavor of fish sauce [16]. Thus, strains CH2-4, CH6-2, CH6-1 and V7-2 were selected for further volatile compounds analysis.

3.2. Formation of volatile compounds

The four selected strains CH2-4, CH6-2, CH6-1 and V7-2 were cultured and treated as described in 2.2.2. The volatile composition is presented in Table 1.

A total of 19 volatile compounds have been detected in 4 samples. They could be divided into groups of esters, alcohols, phenols, ketones, hydrocarbon and sulphur containing compounds. According to previously reported paper, volatile compounds formed by *T. halophilus* are from alcohol, aldehyde, ester and ketone groups [14]. However, in this study no volatile compounds of aldehyde group were detected. The important role in alcohols formation in fish broth containing 25 % NaCl by *T. halophilus* was concluded [14]. Similar phenomenon was also observed in this study, suggesting that alcohols are the most abundant volatile compound group which were detected. Among 4 investigated strains, the CH6-2 produced 10 volatile compounds among 19 identified components in total, followed by CH2-4 with 7 components. Strains V7-2 and CH6-1 produced only 5 volatile compounds each. The esters of pentanoic and butanoic acid, which were found having strong effect on fish sauce aroma [17], were detected in samples inoculated with CH6-2 and CH2-4 but not with CH6-1 and V7-2. Interestingly, strains CH6-2 produced three different esters of pentanoic and butanoic acid. The strain V7-2 produced the most alcohols volatile compounds among four strains, including active volatile compounds for fish sauce like 2-ethyl-1-hexanol, and 2-furanmethanol. Meanwhile, CH6-2 formed predominant derivatives of pentanol. Sulfur containing compounds were also found in samples inoculated with CH6-2 and CH2-4. Based on the volatile profiles, growth ability in 25% NaCl as well as the oligopeptides formation in a previous study [10], CH6-2 was selected for further study as starter culture. Further study is needed to evaluate the influence of volatile compounds on aroma and taste of fish sauce product.

Table 1. Volatile compounds of fish broth containing 25 % NaCl inoculated with various strains of *T. halophilus*.

STT	Volatile compounds	% peak area				
		KC	CH6-1	V7-2	CH6-2	CH2-4
Esters						
1	Pentanoic acid, 2-ethylhexyl ester	ND	ND	ND	ND	13.02
2	Pentanoic acid, 2-propenyl ester	2.65	ND	ND	2.39	ND
3	Pentanoic acid, 2,2,4-trimethyl-3-hydroxy-, isobutyl ester	ND	ND	ND	2.14	ND
4	Butanoic acid, 2,4-dibromo-, ethyl ester	ND	ND	ND	28.48	ND
5	2,2-Dimethyl-3-pentanol, chlorodifluoroacetate	2.17	1.68	2.67	1.61	11.56
6	4,4-Dimethyl-2-pentanol, chlorodifluoroacetate	ND	ND	ND	21.25	ND
Alcohols						
7	Cyclobutanemethanol, α-methyl-	ND	ND	3.27	ND	ND
8	Cyclopentanol, 1-methyl-	3.01	ND	ND	0.79	18.36
9	3-Hexanol	55.63	55.82	ND	0.99	0.88
10	1-Hexanol, 2-ethyl-	ND	ND	2.56	ND	ND
11	4-methyl-2,3-pentanediol	ND	ND	53.17	ND	ND
12	2-Furanmethanol, tetrahydro-5-methyl-, *trans*-	ND	ND	2.54	ND	ND
Phenols						
13	Phenol, 2-(phenylmethoxy)-	ND	ND	ND	ND	0.76
Ketones						
14	2-Heptanone, 4,6-dimethyl-	31.97	31.93	ND	ND	0.5
15	Ethyl trans-2-pentenoate	0.87	ND	ND	ND	ND
Hydrocarbons						
16	Hexane, 1-(3-butenyloxy)-	ND	2.57	ND	36.25	ND
Sulfur containing compounds						
17	Sulfurous acid, butyl 2-ethylhexyl ester	ND	ND	ND	ND	0.54
18	Sulfurous acid, butyl isohexyl ester	ND	ND	ND	1.61	ND
19	Benzyl 2-chloroethyl sulfone	ND	1.01	ND	0.66	ND

"ND" - Not detected

"KC"- Control sample without inoculating *T. halophilus*

3.3. Growth characteristics of selected strain *T. halophilus* CH6-2

The effect of pH, salt concentration, temperature and incubation time on growth of strains CH6-2 was conducted. The growth of the culture was evaluated by optical density at 600 nm. The result is presented in Figure 2.

The strain CH6-2 grew best at NaCl concentration of 5-8 %, which is similarly reported earlier for *T. halophilus* growing well in 5 to 10 % NaCl [18]. In a medium without NaCl, and at high NaCl concentration of 18 and 25 %, the growth could still be observed but it was very poorly observed. The OD600 at 25 % NaCl was reduced to 10 times compared to that at 6.5 %. The optimum pH for the growth of CH6-2 was 7 despite the fact that the growth could be observed in wide pH range from 6 to 8.5. However, at pH 6 the OD600 was reduced in half. Similar to results reported by Gurtler [18], in this experiment, the pH optimum for *T. halophilus*

was found in a range from 6.5 to 7.5. In this study, *T. halophilus* CH6-2 could tolerate temperature of 40 °C but 30-35 °C was for the best growth.

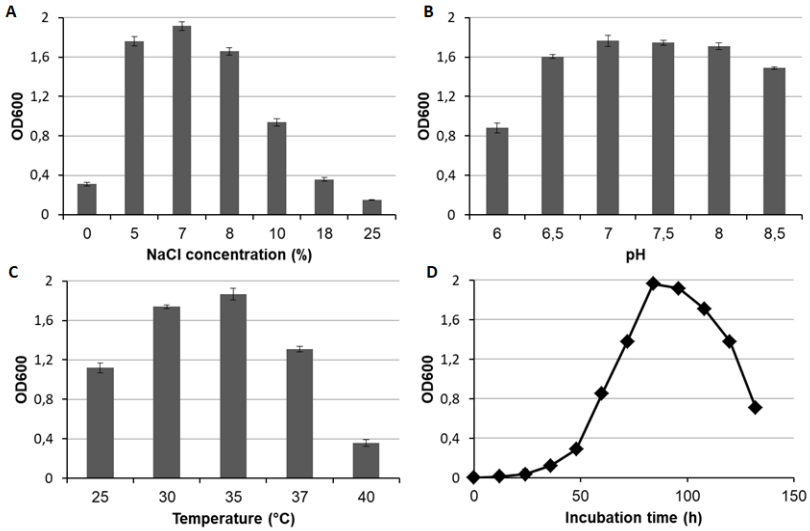

Figure 2. The influence of NaCl concentration (A), pH medium (B), incubation temperature (C) and incubation time (D) on the growth of *T. halophilus* CH6-2 (Cultivation on MRS medium).

4. CONCLUSIONS

Tetragenococcus halophilus in this study exhibited intracellular aminopeptidase activities towards glutamic acid and leucine. Volatile profiles confirmed the abundant formation of esters and alcohols by *T. halophilus*, with the CH6-2 strain forming the most volatile compounds, and in this study it is the most potential strain as starter culture for fish sauce fermentation. *T. halophilus* CH6-2 strain possessed similar growth characteritics to other *T. halophilus* in terms of dependence on pH, temperature and NaCl concentration.

Acknowledgment: The authors would like to thank Ministry of Education and Training for providing fund for project B2017-BKA-37 to pursue this research.

REFERENCES

1. Udomsil N., Rodtong S., Tanasupawat S., and Yongsawatdigul J. - Improvement of Fish Sauce Quality by Strain CMC5-3-1: A Novel Species of *Staphylococcus* sp. Journal of Food Science **80** (2015) M2015-M2022.
2. Yongsawatdigul J., Rodtong S., and Raksakulthai N. - Acceleration of Thai Fish Sauce Fermentation Using Proteinases and Bacterial Starter Cultures. Journal of Food Science **72** (2007) M382-M390.
3. Nguyễn T. V. A., Bùi T. T. H., Nguyễn Đ. T., and Nguyễn T. H. - Screening of halophilic lactic acid bacteria and *Bacillus* for applying to aroma development of fish sauce. Proceeding of National Conference on Biotechnology 2013 2 (2013) 54-59.
4. Udomsil N., Rodtong S., Choi Y. J., Hua Y., and Yongsawatdigul J. - Use of *Tetragenococcus halophilus* as a Starter Culture for Flavor Improvement in Fish Sauce Fermentation. Journal of Agricultural and Food Chemistry **59** (2011) 8401-8408.

5. Satomi, M., Kimura, B., Mizoi, M., Sato, T., and Fujii, T. - *Tetragenococcus muriaticus* sp. nov., a New Moderately Halophilic Lactic Acid Bacterium Isolated from Fermented Squid Liver Sauce. International Journal of Systematic and Evolutionary Microbiology **47** (1997) 832-836.
6. Chuon M. R., Shiomoto M., Koyanagi T., Sasaki T., Michihata T., Chan S., Mao S., and Enomoto T. - Microbial and chemical properties of Cambodian traditional fermented fish products, J. Sci. Food. Agric. **94** (2014) 1124-31.
7. Kuda T., Izawa Y., Ishii S., Takahashi H., Torido Y., and Kimura B. - Suppressive effect of *Tetragenococcus halophilus*, isolated from fish-nukazuke, on histamine accumulation in salted and fermented fish, Food Chemistry **130** (2012) 569-574.
8. Lee S. H., Jung J. Y., and Jeon C. O. - Bacterial community dynamics and metabolite changes in myeolchi-aekjeot, a Korean traditional fermented fish sauce, during fermentation, Int. J. Food. Microbiol. **203** (2015) 15-22.
9. Udomsil N., Chen S., Rodtong S., and Yongsawatdigul J. - Improvement of fish sauce quality by combined inoculation of *Tetragenococcus halophilus* MS33 and *Virgibacillus* sp. SK37, Food Control **73** (2017) 930-938.
10. Nga T. T., Tra D. X., and Ha L. T. - Isolation of halophilic lactic bacteria *Tetragenococcus halophilus* from Vietnamese fish sauce, Vietnam Journal of Science and Technology **55** (2017) 186-194.
11. Lee H. M. and Lee Y. - A differential medium for lactic acid-producing bacteria in a mixed culture. Letters in Applied Microbiology **46** (2008) 676-681.
12. Magboul A. A. A. and McSweeney P. L. H. - Purification and characterization of an aminopeptidase from *Lactobacillus curvatus* DPC2024, International Dairy Journal **9** (1999) 107-116.
13. Yang Y. F., Chen S. R., Ni H., and Ye X. Q. - Analysis of volatile components in a Chinese fish sauce, Fuzhou Yulu, by gas chromatography-mass spectrometry, Journal of Zhejiang University Science B **9** (2008) 977-981.
14. Udomsil N., Rodtong S., Tanasupawat S., and Yongsawatdigul J. - Proteinase-producing halophilic lactic acid bacteria isolated from fish sauce fermentation and their ability to produce volatile compounds, International Journal of Food Microbiology **141** (2010) 186-194.
15. Yvon M. and Rijnen L. - Cheese flavour formation by amino acid catabolism **11** (2001) 185-201.
16. Park J. N., Watanabe T., Endoh K. I., Watanabe K., and Abe H. - Taste-active components in a Vietnamese fish sauce, Fisheries Science **68** (2002) 913-920.
17. Jiang J., Zeng Q., and Zhu Z. - Analysis of Volatile Compounds in Traditional Chinese Fish Sauce (Yu Lu), Food Bioprocess Technology **4** (2011) 266-271.
18. Gürtler M., Gänzle M. G., Wolf G., and Hammes W. P. - Physiological Diversity Among Strains of *Tetragenococcus halophilus*, Systematic and Applied Microbiology **21** (1998) 107-112.