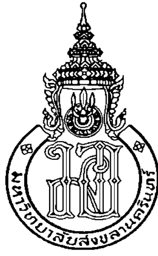

Histamine Degradation by Halophilic Archaea

Wanaporn Tapingkae

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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ชื่อวิทยานิพนธ์ การย่อยสลายฮีสตามีนโดยอาเคียที่ชอบเกลือ
ผู้เขียน นางสาววรรณพร ทะพิงค์แก
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บทคัดย่อ

ปริมาณฮีสตามีนที่สูงก่อให้เกิดผลเสียหายต่อทั้งคุณภาพและความปลอดภัยของน้ำปลา ดังนั้นการทดลองนี้จึงมีวัตถุประสงค์เพื่อคัดแยกเชื้ออาเคียที่ชอบเกลือที่มีความสามารถย่อยฮีสตามีนในสภาวะที่มีเกลือสูง ศึกษาเอนไซม์ที่เกี่ยวข้อง และศึกษาความเป็นไปได้ในการประยุกต์ใช้ในกระบวนการผลิตน้ำปลา จากเชื้ออาเคียที่ชอบเกลือ 156 สายพันธุ์ที่แยกได้จากผลิตภัณฑ์สัตว์น้ำหมักที่มีเกลือสูง พบว่าเชื้อ HDS3-1 ซึ่งแยกได้จากน้ำปลามีความสามารถย่อยฮีสตามีนสูงสุดเมื่อเลี้ยงในอาหารสูตร halophilic medium ที่ประกอบด้วยฮีสตามีน 5 มิลลิโมลาร์ เชื้อสายพันธุ์ HDS3-1 ไม่มีกิจกรรมของเอนไซม์ decarboxylase หรือมีความเป็นพิษต่อเซลล์ของ human colon adenocarcinoma (Caco-2), human liver hepatocarcinoma (HepG2) และ human larynx epithelial (HEp-2) กิจกรรมการย่อยฮีสตามีนของเชื้อสายพันธุ์ HDS3-1 เกิดขึ้นจากเอนไซม์ที่อยู่ภายในเซลล์และต้องการ 1-methoxy-5-methylphenazinium methylsulfate (PMS) ซึ่งเป็นตัวขนส่งอิเล็กตรอนและเร่งการย่อยฮีสตามีนอย่างจำเพาะ ผลการทดลองแสดงให้เห็นถึงความเกี่ยวข้องของกิจกรรมการย่อยฮีสตามีนของเชื้อ HDS3-1 และเอนไซม์ฮีสตามีน ดีไฮโดรจีเนส (histamine dehydrogenase) ที่ทนเกลือและทำงานได้ดีที่อุณหภูมิสูงภายใต้สภาวะที่เป็นกลาง

HDS3-1 และ HIS40-3^{T} เป็นจุลินทรีย์แกรมลบ และมีรูปร่างแท่ง เชื้อทั้งสองสายพันธุ์เจริญเติบโตได้ที่ช่วงอุณหภูมิ 20-60 องศาเซลเซียส (เหมาะสมที่ 37-40 องศาเซลเซียส) ความเข้มข้นเกลือที่ 1.7-5.1 โมลาร์ (เหมาะสมที่ 2.6-3.4 โมลาร์) และค่าความเป็นกรด-เบส 5.5-8.5 (เหมาะสมที่ 6.0-6.5) ลิปิดมีขั้วที่สำคัญของเชื้อทั้งสองสายพันธุ์เป็นอนุพันธุ์ชนิด $C_{20}C_{20}$ และ $C_{20}C_{25}$ ของฟอสฟาทิดิลกลีเซอรอล, ฟอสฟาทิดิลกลีเซอรอล ฟอสเฟตเมทิลเอสเทอร์, ฟอสฟาทิดิลกลีเซอรอล ซัลเฟต และ ไกลโคลิพิด 2 ชนิด องค์ประกอบ G+C ของ DNA เท่ากับ 64.0-65.4 โมลเปอร์เซนต์ ค่าความเหมือนของลำดับเบสในส่วนของ 16S rRNA และทั้ง DNA ระหว่างเชื้อทั้งสองสายพันธุ์เท่ากับ 99.7 และ 77.7 เปอร์เซนต์ ตามลำดับ โดยเปรียบเทียบจากลำดับเบสของสายพันธุ์ HIS40-3^{T} และ HDS3-1 จำนวนเท่ากับ 1,405 และ 1,353 ลำดับเบสตามลำดับ จากข้อมูลทางอนุกรมวิธาน สายพันธุ์ HIS40-3^{T} และ HDS3-1 ถูกจัดให้เป็นตัวแทนของสปีชีส์ใหม่ของ จีนัส

Natrinema ซึ่งชื่อ *Natrinema gari* sp. nov. ถูกเสนอ โดยสายพันธุ์หลัก คือ HIS40-3^T

เอนไซม์ฮีสตามีน ดีไฮโดจีเนสจากเชื้อ *Natrinema gari* HDS3-1 ถูกทำให้บริสุทธิ์ขึ้น 26.6 เท่า โดยการให้ความร้อน, ผ่านไอออนโครมาโทกราฟีชนิดลบ และเจลฟิลเตรชันโครมาโทกราฟี โดยได้ผลผลิตจากการแยกเท่ากับ 70.5 เปอร์เซนต์ น้ำหนักโมเลกุลของเอนไซม์เท่ากับ 127.5 กิโลดาลตัน เอนไซม์ประกอบด้วย 3 หน่วยย่อยซึ่งมีน้ำหนักโมเลกุลเท่ากับ 69.1 29.3 และ 27.7 กิโลดาลตัน ตามลำดับ เอนไซม์มีความสามารถในการออกซิไดซ์ไบโอเจนิคเอมีนหลายชนิด แต่สามารถทำปฏิกิริยากับฮีสตามีนได้มากที่สุด ความเข้มข้นของเกลือโซเดียมคลอไรด์ ค่าความเป็นกรด-เบส และอุณหภูมิที่เหมาะสมสำหรับการทำงานของเอนไซม์เท่ากับ 3.5-5.0 โมลาร์ 6.5-8.5 และ 40-60 องศาเซลเซียส ตามลำดับ เอนไซม์ทนความร้อนได้ที่อุณหภูมิสูงถึง 50 องศาเซลเซียส และที่ช่วงของค่าความเป็นกรด-เบสเท่ากับ 7.0-9.0 กิจกรรมของเอนไซม์ฮีสตามีน ดีไฮโดรจีเนสที่บริสุทธิ์ถูกยับยั้งด้วยสารประกอบกลุ่มคาร์บอนิล เอนไซม์มีความจำเพาะต่อฮิสตามีนโดยมีค่า V_{max} K_m และ k_{cat} เท่ากับ 2.5 ไมโครโมลต่อนาที 58.7 ไมโครโมลาร์ และ 5.4 ต่อวินาที ตามลำดับ

เซลล์ของเชื้อ *Nnm. gari* HDS3-1 ถูกตรึงบนวัสดุหลายชนิดด้วยวิธีที่แตกต่างกัน จากการศึกษาพบว่า ซีไลท์มีความสามารถในการตรึงเซลล์ได้สูงสุด โดยเซลล์ที่ถูกตรึงบนซีไลท์เหลือกิจกรรมของเอนไซม์ฮีสตามีน ดีไฮโดรจีเนสมากถึง 94 เปอร์เซนต์ เมื่อเทียบกับเซลล์ที่ไม่ถูกตรึง และคุณสมบัติในการทำงานของเซลล์ที่ถูกตรึงบนซีไลท์มีลักษณะที่เหมือนกับเซลล์ที่ไม่ได้ถูกตรึง ทั้งในส่วนของความเข้มข้นของเกลือโซเดียมคลอไรด์ที่เหมาะสม (4.0-5.0 โมลาร์) ค่าความเป็นกรด-เบสที่เหมาะสม (6.5-7.5) และอุณหภูมิที่เหมาะสม (40-55 องศาเซียลเซียส) อย่างไรก็ตาม กระบวนการตรึงเซลล์เพิ่มความคงตัวของเซลล์ต่อความเข้มข้นของเกลือโซเดียมคลอไรด์ที่เพิ่มขึ้น และอุณหภูมิการบ่มที่สูงขึ้น ทั้งเซลล์ที่ไม่ถูกตรึงและถูกตรึงมีความจำเพาะต่อการย่อยสลายฮีสตามีน เซลล์ที่ถูกตรึงสามารถนำกลับมาใช้สำหรับการย่อยสลายฮีสตามีนได้อย่างน้อย 7 รอบ โดยที่ความสามารถในการย่อยไม่เปลี่ยนแปลง เซลล์ที่ถูกตรึงมีแนวโน้มที่จะสามารถใช้ย่อยสลายฮีสตามีนในผลิตภัณฑ์ที่มีเกลือเป็นองค์ประกอบสูง เช่น น้ำปลาได้

เซลล์อิสระและเซลล์ที่ถูกตรึงบนซีไลท์ของเชื้อ *Nnm. gari* HDS3-1 ที่ระดับ 5 และ 10 เปอร์เซ็นต์ มีความสามารถในการลดปริมาณฮีสตามีนในน้ำปลาได้จริง จากการทดลองพบว่า น้ำปลาที่ผ่านการทำปฏิกิริยากับเซลล์ของเชื้อ *Nnm. gari* HDS3-1 ทั้งในรูปเซลล์อิสระและเซลล์ที่ถูกตรึง มีปริมาณฮีสตามีนต่ำกว่ากลุ่มควบคุมทั้ง 2 กลุ่ม การเติมเซลล์ที่ไม่ถูกตรึงหรือเซลล์ที่ถูกตรึงในระดับที่สูงกว่า จะมีผลทำให้เกิดการย่อยสลายฮีสตามีนสูงขึ้น เมื่อเปรียบเทียบระหว่างกลุ่มการทดลองพบว่าน้ำปลาที่เติมเซลล์ที่ถูกตรึงที่ระดับ 10 เปอร์เซนต์มีค่าการย่อยสลายฮีสตามีนสูงสุด น้ำปลาที่ได้จากทุกกลุ่มทดลองมีค่าความเป็นกรด-เบส ไนโตรเจนทั้งหมด ไนโตรเจนจาก

กรดอะมิโน ฟอร์มาลดีไฮด์ในโตรเจน และแอมโมเนียไนโตรเจน ไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ (*P*>0.05) จากการทดสอบทางประสาทสัมผัสโดยผู้เชี่ยวชาญและผู้บริโภคทั่วไปชี้ให้เห็นว่า สี กลิ่น และความชอบโดยรวมของตัวอย่างน้ำปลาจากทุกกลุ่มการทดลองไม่มีความแตกแตกต่างกันอย่างมีนัย สำคัญทางสถิติ (*P*>0.05) อย่างไรก็ตามน้ำปลาที่ผ่านการเติมเซลล์ที่ถูกตรึงทั้ง 2 ระดับมีแนวโน้มได้รับการยอมรับสูงกว่า จากผลการทดลองแสดงให้เห็นว่าเซลล์ของเชื้อ HDS3-1 ที่ถูกตรึงสามารถนำไปประยุกต์ใช้สำหรับย่อยสลายฮีสตามีนในน้ำปลาได้ โดยไม่มีผลเสียต่อลักษณะโดยรวมของผลิตภัณฑ์

Thesis Title Histamine Degradation by Halophilic Archaea

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ABSTRACT

The presence of high level of histamine is detrimental to the quality and safety of fish sauce. Therefore, this study aimed to screen for the ability of extremely halophilic archaea to degrade histamine in high salt condition, characterize the enzyme involved and study the feasibility to apply in fish sauce processing. Of 156 extremely halophilic archaea isolated from various salt-fermented fishery products, HDS3-1 from fish sauce exhibited the highest histamine degradation activity when cultured in halophilic medium containing 5 mM histamine (free-base). The strain neither exhibited decarboxylase activity toward all tested amino acids nor was toxic to human colon adenocarcinoma (Caco-2), human liver hepatocarcinoma (HepG2) and human larynx epithelial (HEp-2) cells. Based on histamine assay, histamine-degrading activity of HDS3-1 was located in the intracellular fraction, required the presence of 1-methoxy-5-methylphenazinium methylsulfate (PMS), an electron carrier, and selectively catalyzed histamine as a substrate. The results suggested a link of histamine-degrading activity of HDS3-1 to the presence of salt-tolerant and thermo-neutrophilic histamine dehydrogenase.

HDS3-1 and HIS40-3^T isolated from anchovy fish sauce (nam-pla) were gram-negative rod-shaped halophilic archaea. The two strains were able to grow at 20 to 60°C (optimum 37 to 40°C), at 1.7 to 5.1 M NaCl (optimum 2.6 to 3.4 M NaCl) and at pH 5.5 to 8.5 (optimum pH 6.0 to 6.5). The major polar lipids of the isolates were $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and two glycolipids. The DNA G+C contents were 64.0-65.4 mol%. Levels of 16S rRNA gene sequence similarity and DNA-DNA relatedness between the two strains were 99.7 and 77.7%, respectively. Based on these taxonomic data, strains HDS3-1 and HIS40-3^T are considered to represent a novel species of the genus *Natrinema*, for which the

name *Natrinema gari* nov. is proposed and the type strain is HIS40-3^T.

Histamine dehydrogenase from *Nnm. gari* HDS3-1 was purified to homogeneity by heat treatment (60°C, 15 min), HiTrap Q XL column anion exchange chromatography and Superose 12 10/300 gel filtration chromatography. Purity was increased to 26.6-fold with approximately 70.5% yield. The molecular mass of the enzyme was 127.5 kDa. The enzyme consisted of three different subunits having molecular masses of 69.1, 29.3 and 27.7 kDa. The enzyme readily oxidized various biogenic amines but not as high as histamine. The optimum NaCl concentration, pH and temperature of the purified histamine dehydrogenase were 3.5 to 5.0 M NaCl, pH 6.5 to 8.5 and 40 to 60°C, respectively. Enzyme was stable to heat treatment up to 50°C and over a pH range of 7.0 to 9.0. The purified histamine dehydrogenase activity was effectively inhibited by carbonyl reagents. The enzyme had high affinity and activity toward histamine with V_{max}, K_m and k_{cat} values of 2.5 µmol min^{-1}, 58.7 µM and 5.4 s^{-1}, respectively.

Whole cell of *Nnm. gari* HDS3-1 was immobilized on various matrices by different techniques. The bound whole cell on celite retained the histamine-degrading activity as high as 94% of the original activity of the free whole cell. The catalytic properties of the immobilized whole cell on the celite support were similar to the corresponding free whole cell including optimal NaCl concentration (4.0 to 5.0 M NaCl), optimal pH (pH 6.5 to 7.5) and optimal temperature (40 to 55°C). However, the stabilities of the histamine-degrading activity either in the presence of NaCl at elevated concentrations or incubation at elevated temperatures were significantly improved by the immobilized process. Both free and immobilized whole cell were highly specific toward histamine. The immobilized whole cell could be reused for the degradation of histamine up to 7 cycles without any significant loss in activity. The immobilized whole cell has the potential to be applied for the degradation of histamine in the products containing high salinity, like fish sauce.

Fish sauce added with free and immobilized whole cell of *Nnm. gari* HDS3-1 at the levels of 5% and 10% (w/v) showed lower histamine contents than those of the controls (Control-1 and Control-2). The higher free or immobilized whole cell added, the faster histamine degradation rate was observed. Among all treatments, fish sauce with 10% immobilized whole cell added exhibited the greatest histamine

degradation, particularly at the 1st day of incubation. Fish sauce from all treatments had similar pH, total nitrogen, amino nitrogen, formaldehyde nitrogen and ammonical nitrogen to those of the control (P>0.05). An acceptability test by fish sauce experts revealed that the samples were not significantly different in color, aroma and overall acceptance (P>0.05). However, fish sauce samples treated with immobilized whole cell at both levels tended to have higher acceptability than those of with and without whole cell added samples. The results implied that immobilized whole cell of *Nnm. gari* HDS3-1 can be applied to degrade histamine in the high salted condition without any adverse changes in the overall characteristics of the fish sauce.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The presence of high level of histamine is detrimental to the quality and safety of some Thai fermented fishery products especially those produced from scombroid fish species. Histamine intoxication or Scombroid poisoning is one of major health significances associated with ingestion of foods containing high histamine. Among a variety of fermented fishery products in Thailand, fish sauce is particularly important in its economic value for both local and international markets. With a concern on the toxicological effects, high content of histamine in commercial products becomes a major threat of exporting of these products, due to a more strenuous regulatory measure and inspection. Depending on the quality of fish used and uncontrolled outdoor fermentation process, fish sauce is often known to contain high level of histamine.

Due to its pharmaceutical processes such as allergy and inflammation, histamine has been considered as the most important biogenic amine. Allergy-like food poisoning generally occurs when food containing 500 ppm or more of histamine is consumed (Arnold and Brown, 1978). However, poisoning may be caused in some individuals even when histamine has not reached this level. Although the mechanism of food-based histamine allergies has not yet been determined, it is probably due to the synergistic action with other amines contained in food, such as putrescine and cadaverine (Hall *et al*., 1999; Sourkes and Missala, 1981; Bieganski *et al*., 1980). In the United States, the toxic level of histamine that poses a risk to health is set at 500 ppm, and the caution level is 50 ppm. The European Union has also set a level of 100-200 ppm for seafood, and Codex has also proposed regulations at this level (Lehane and Olley, 2000; Anonymous, 1998; Anonymous, 1991).

Histamine is heat stable and is not detectable through organoleptic analysis by even trained panelists (Arnold *et al*., 1980). Except for the gamma irradiation, no other food processing methods are available for histamine degradation

(Kim *et al*., 2004; Etkind *et al*., 1987). Therefore histamine, if present, is difficult to destroy and posts a risk of food intoxication.

It has long been known that certain microorganisms are useful to degrade histamine and some other amines biologically through the activities of histamine oxidase and/or histamine dehydrogenase (Bozkurt and Erkmen, 2002; Gardini *et al*., 2002; Dapkevicius *et al*., 2000; Siddiqui *et al*., 2000; Leuschner *et al*., 1998; Murooka *et al*., 1979; Umezu *et al*., 1979; Ienistea, 1971). Nevertheless, the applications of these microorganisms were restricted by unfavorable physiological conditions for growth and enzyme activity such as low oxygen concentration, low pH value, undesirable temperature and especially in the presence of NaCl. Application of fermenting microorganisms to degrade histamine in salt-fermented food is still limited. Anchovy fish sauce produced in Thailand has been favored by many ethnic groups as a superior product for the better flavor-enhancing quality. Given the importance of the product, histamine should be minimized in the products to ensure food safety. This study was mainly related to development of a biological method for removing histamine present in fish sauce by using a pure culture of selected halophilic archaeon and its enzyme.

1.2 Literature review

Histamine

Histamine (β-imidazolylethylamine) is one of the most important mediators involved in various physiological and pathological conditions, including neurotransmission and numerous brain functions, secretion of pituitary hormones, regulation of gastrointestinal and circulatory functions and inflammatory reactions (Leurs *et al*., 1995). It can be formed and degraded as a result of normal metabolic activities in animals, plants and microorganisms (Silla Santos, 1996; Halász *et al*., 1994). Figure 1 shows the major pathways for the formation of histamine. Histamine accumulation in foods requires the availability of precursor (histidine), the presence of microorganisms with histidine decarboxylases and favorable conditions for their growth and decarboxylase activity (Ten Brink *et al*., 1990).

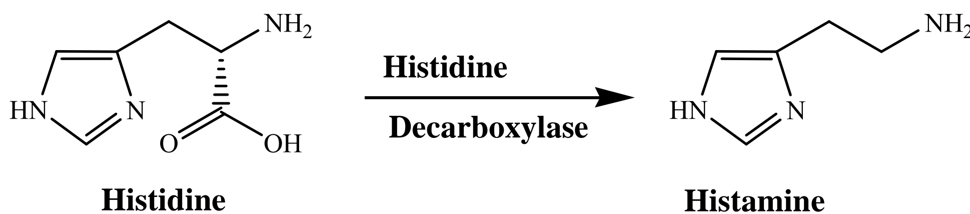


Figure 1. Metabolism pathway for the formation of histamine.
Source: Halász *et al*. (1994).

The presences of histamine in fermented foods do not usually represent any health hazard to individuals unless large amounts are ingested, or the natural mechanism for the catabolism of the histamine is inhibited or genetically deficient. Determination of the exact toxicity threshold of histamine in individuals is extremely difficult. The toxic dose is strongly dependent on the efficiency of the detoxification mechanisms of different individuals. Typical symptoms may be observed in certain individuals and include nausea, sweating, headache and hyper- or hypotension (Lehane and Olley, 2000). Therefore, the Food and Drug Administration (FDA) established an advisory level of 500 ppm to be hazardous to human health (FDA, 1998). Above this level, humans begin to experience the allergy-like symptoms of the disease. However, poisoning may be caused in some individuals even when histamine has not reached this level (Bartholomev *et al*., 1978).

As the highest biological activities among other amines, the production of histamine is of particular interest. High amount of histamine can be found in fermented foods derived from raw materials with high protein content. It can be found as a consequence of microbial activity in many kinds of food such as wine, cheeses, fermented meat (Suzzi and Gardini, 2003; Lehane and Olley, 2000; Shalaby, 1996; Silla Santos, 1996), fermented fish (Stute *et al*., 2002; Hernández-Herrero *et al*., 1999; Sato *et al*., 1995;Yatsunami and Echigo, 1992) and fermented vegetables (Tsai *et al*., 2005; Mah *et al*., 2002; Kalać *et al*., 2000; Chin *et al*., 1989; Okuzumi *et al*., 1981; Taylor *et al*., 1978). Histidine decarboxylase activity is widely distributed in the strains of genera *Bacillus* (Rodriguez-Jerez *et al*., 1994a), *Photobacterium* (Jørgensen

et al., 2000; Morii *et al*., 1988), as well as in genera of the family *Enterobacteriaceae*, such as *Citrobacter*, *Klebsiella*, *Escherichia*, *Proteus*, *Salmonella* and *Shigella* (Marino *et al*., 2000; Roig-Sagués *et al*., 1996; Edwards *et al*., 1987; Voigt and Eitenmiller, 1977) and *Micrococcaceae*, such as *Staphylococcus*, *Micrococcus* and *Kocuria* (Martuscelli *et al*., 2000; Rodriguez-Jerez *et al*., 1994b). Furthermore, many lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*, *Enterococcus*, *Carnobacterium*, *Pediococcus*, *Lactococcus* and *Leuconostoc* are able to decarboxylate histidine (Lonvaud-Funel, 2001; Bover-Cid and Holzapfel, 1999; de Llano *et al*., 1998; Maijala *et al*., 1995; Edwards and Sandine, 1981).

Fish sauce

Fish sauce is a traditional, fermented fish product commonly used as a condiment in the Southeast Asia. Apart from its unique pleasant flavor, it is an important source of nitrogen supplement in the diet in this region. Fish sauce contains nitrogen at 20 g/l, of which 16% is present as amino acids. Among Southeast Asian countries, Thailand is the largest producer, where 390 fish sauce factories are located. The annual production of fish sauce is approximately 459,700 kiloliters with the approximate values for domestic consumption and exports of 6,600 and 1,056 million Baht, respectively (Krung Thai Bank, 2005). Fish sauce can be produced from both marine and freshwater fish. However, it has been known that fish sauce produced from marine fish exhibits higher flavor as well as general acceptability than that from freshwater fish. This is possibly due to the higher fat content in the former than the latter. Among fish species, which can be used as raw materials for fish sauce production, Indian anchovy (*Stolephorus indicus*) has been recognized nationwide as the best raw material, which render the highest quality fish sauce (Saisithi, 1994). The fish are mixed with 30-35% (w/v) salt thoroughly to prevent spoilage, and subsequently placed in a fermentation tank or vat, with salt spread at the bottom. Due to the floatation of fish in the brine, processor has to ensure that fish has to be submersing under the liquid to avoid the spoilage. The salt-fish mixture is left in a concrete tank at the temperature range of 35-40°C and fermentation is allowed to proceed for 8-18 months. During fermentation, proteins are hydrolyzed, mainly as a

result of autolytic action by the digestive proteinases in fish (Orejana and Liston, 1981). However, bacterial enzymes were found to involve in hydrolysis (Saisithi *et al*., 1966). Uyenco *et al*. (1952) observed higher amino nitrogen content in *Patis* prepared from whole anchovies than that from gutted fish. Therefore, visceral enzymes possibly play a major role in protein hydrolysis. Trypsin in pyloric caeca and pepsin in the stomach are the major source of endopeptidases. Nevertheless, pepsin is unlikely to be important in fish sauce production since the pH of brine system is generally alkaline. The brownish liquid obtained is collected, filtrated and used as fish sauce concentrate. Saturated brine is added into the residue to extract the remained soluble matters. This liquid is mixed with the concentrate to produce first grade fish sauce. After first extraction process, the residue is re-extracted with saturated brine to obtain the second quality fish sauce (Saisithi, 1994). Total nitrogen (total N) content is used as an indicator to determine the grade and price of fish sauce in Thailand, with products containing over 20 g/L total N classified as Grade I and 15 to 20 g/L total N as Grade II (TISI, 1983).

Some researches were carried out to accelerate the solubilization process. Fish sauce production can be accelerated by lowering the pH and salt content to enhance the rapid autolysis, particularly caused by pepsin (Gildberg *et al*., 1984; Beddows and Ardesshir, 1979). Additionally, some proteinases were also added to increase the rate of hydrolysis (Beddows and Ardeshir, 1979). Nevertheless, it has been known that the longer fermentation period, the better aroma and flavor are obtainable in fish sauce (Brilantes, 1999). Flavor of fish sauce is attributable to a variety of compounds, including acids, alcohol, amine, nitrogen-containing compounds, lactone, carbonyl and sulfur containing compounds (Peralta *et al*., 1996; McIver *et al*., 1982). Short-chain volatile fatty (VFA) acids were found to be variable, depending on the type of fish sauce. Those products with less VFA were described as less cheesy and more ammonical than those with greater amounts of VFA (Dougan and Howard, 1975). Recently, Sanceda *et al*. (2001) reported that straight chain VFAs were developed from fat and certain branched VFAs were derived from the degradation of specific amino acids.

Histamine formation in fish sauce

Histamine is considered to be the most important biogenic amines detected in fish sauce. Variable levels of histamine, ranging from 20 to 757 ppm, were found in a few fish sauce samples as reported by Kirschbaum *et al*. (2000) and Sanceda *et al*. (1999). Brillantes and Samosorn (2001) examined the levels of histamine in 549 commercial samples of fish sauce. They found that histamine contents varied from 100 to 1,000 ppm and also noted that products from small-scale factories had relatively lower histamine contents than those from large-scale operations. Varying levels of histamine, ranging from 0 to 729 ppm were found in a total of 45 fish sauces analyzed: 20 from Thailand, 10 from Vietnam, 1 from Singapore, 1 from Hong Kong, 2 from Philippines and 11 were imported products of unknown origins (Stute *et al*., 2002).

The overall histamine formation in fish and subsequent fishery products is related to fish species, free histidine content of fish muscle, quality of the raw material, the presence of bacterial histidine decarboxylase, and environmental conditions to promote growth of histamine-forming bacteria (Lehane and Olley, 2000). Histamine formation has been mostly widely noticed in Scombroid fish species as well as those containing high levels of free histidine in their muscle. Endogenous production of decarboxylase in fish muscle is insignificant when compared with that of exogenous bacterial enzymes (Rawles *et al*., 1996). Generally, biogenic amine formation by bacteria is enhanced at elevated storage temperatures (Kim *et al*., 2000) as well as high temperature abuse of post-harvested fish. The level is related to the combination of time and temperature that fish are exposed to. Therefore, histamine and other biogenic amines can be accumulated in these fish as well as products made from abused or improperly handled fish.

The freshness of raw meterial (fish) was the most critical point to control histamine formation in fish sauce production. In Thailand, anchovy is most commonly used for fish sauce production. Unfortunately, it is regarded as by-catch species with little value, and often no attention is given to the quality of the fish. Often when fish is delivered to the manufacturing facility at the high ambient temperature, which is commonly over 30°C, fish has already entered the stage of

spoilage, increasing chances to generate histamine and other biogenic amines in the final fish sauce produced. Due to over-exploitation of these species in the Gulf of Thailand, the fishing fleet must travel farther distance, leading to the lower quality raw materials. In addition, histamine and biogenic amine formation during post-harvest handling is associated with safety concern of the fish sauce product.

Sanceda *et al*. (1999) found that fish sauce produced by spoiled sardine showed the higher histamine content than that made from fresh sardine. It is speculated that improper handling and storage of the fish before salting possibly accelerate bacterial spoilage and biogenic amine formation. Post mortem proteolysis induced by the high ambient temperature in the region prior to salting also can accelerate formation of biogenic amines by liberating additional free histidine from muscle protein, providing more substrates for the bacterial histidine decarboxylase. Histidine originally present in fish muscle as well as that released by post mortem autolysis can be subsequently decarboxylated to histamine by histamine-forming bacteria, such as *Morganella morganii*, which is reported as the most prolific bacterial histamine former and is often present in fish (Kim *et al*., 2000).

Yongsawatdigul *et al*. (2004) found that fish sauce produced from spoiled anchovy showed higher histamine content than that made from fresh anchovy. It is speculated that improper handling and storage of fish before salting possibly accelerate bacterial spoilage and histamine formation. Post mortem proteolysis induced by the high ambient temperature prior to salting also can accelerate formation of histamine by liberating additional free histidine from muscle protein, providing more substrates for the bacterial histidine decarboxylase. Histidine originally present in fish muscle as well as that released by post mortem autolysis can be subsequently decarboxylated to histamine by histamine-forming bacteria.

The bacteria responsible for histamine formation in salt-fermented products differ from those in non-salted products. However, only few halophilic histamine formers have been isolated (Kimura *et al*., 2000; Hernández-Herrero *et al*., 1999; Satomi *et al*., 1997; Sato *et al*., 1995; Okuzumi *et al*., 1994; Yatsunami and Echigo, 1991) and very little is known about the effects of factors such as pH, NaCl, glucose and growth stage on histamine formation by halophilic histamine formers. Hernández-Herrero *et al*. (1999) isolated halotolerant and halophilic histamine-

forming bacteria from ripening salted anchovies and the effect of NaCl on histamine formation has been studied in these bacteria.

Tetragenococcus muriaticus is a halophilic lactic acid bacterium that is phylogenically closely related to *Tetragenococcus halophilus*, a typical lactic acid fermenter in soy sauce (Röling and van Verse-veld, 1996; Sakaguchi, 1958) and in fish sauce (Ito *et al*., 1985). This bacterium can thrive at NaCl concentration as high as 25% (w/v) (Satomi *et al*., 1997). It has been isolated as a potent halophilic histamine former from Japanese traditional fermented fish sauce (squid liver sauce) and added into the genus *Tetragenococcus* as a new species (Satomi *et al*. 1997). Kobayashi *et al*. (2000) studied the distribution of this specie in other fermented products and found that it was widely distributed in mixed populations with *T. halophilus* in fermented products like Japanese fermented puffer fish ovaries. The same study also found that the ability of this organism to produce histamine was strain specific. Karnop (1988) isolated a halophilic bacterium as the main histamine former in semi-preserved anchovies and identified it as *Pediococcus halophilus*, which is *T. halophilus* according to the current classification (Anonymous, 1994). Thus, it is likely that histamine-forming strains of either *T. halophilus* or *T. muriaticus* are ubiquitous in salted food products and that they play important roles in histamine formation.

The presence of histamine in fish sauce does not pose any health threat compared with other fishery products because average uptake of fish sauce is relatively small, about 20 mL/person/day (NNB, 2000). However, it probably implies poor hygienic qualities of raw material and/or manufacturing processes. Furthermore, the histamine contents in most fish sauces are higher than 20 mg/100 g (200 ppm), the guideline established by the Australian Food Standards Code (ANZFA, 1998) and the Canadian Food Inspection Agency (CFIA, 2005) and 50 mg/100 g (500 ppm), the guideline by the U.S. Food and Drug Administration (FDA, 1998). In addition, most of the samples contained histamine of over 50 mg/100 mL, the maximum limit for fish sauce in Thailand (FIQD, 2000). Therefore, fish sauce produced from these countries may run into the risk of being rejected for export to the Australian and Canadian, thereby dangering the exporting status in the future.

Histamine prevention method in fish sauce

Histamine cannot be destroyed by any preparation methods, including freezing, canning or smoking (Etkind *et al*., 1987). Therefore, many researchers have investigated for the controlling of formation of histamine before and during the fermentation process. Taylor (1986) cites a number of studies on the effect of storage temperature on histamine formation in various types of fish. While all the studies agreed that histamine formation is negligible in fish stored at 0°C or below, other results were variable. Similarly, Rodtong *et al*. (2005) reported that Indian anchovy is another fish species susceptible to high level of histamine when subjected to temperature abuse. Ice and cold storage (15°C) can delay microbial growth, fish spoilage and histamine accumulation. They also proposed that levels of ≥10% (w/v) NaCl could inhibit histamine formation by *Morganella morganii*, *Proteus vulgaris* and *Enterobacter aerogenes*, which are the main histamine-forming bacteria in anchovy. Low-temperature storage (<10°C) effectively controls the growth of most histamine producing bacteria, which require a warm temperature for growth. However, bacteria that grow at refrigerated temperatures (psychrophile) can produce smaller amounts of histamine in fish stored at temperatures between 0 and 10°C (Stratton and Taylor, 1991; Klausen and Huss, 1987; Ritchie and Mackie, 1979). Regardless of the species involved, bacteria must grow to a large enough population for significant production of histamine. In fish subjected to elevated temperatures, even for short periods, a large population of bacteria is established. During subsequent refrigeration, although bacterial growth decreases, residual enzyme activity continues slowly and histamine levels continue to increase (Stratton and Taylor, 1991; Klausen and Huss, 1987).

Brillantes *et al*. (2002) also pointed that the delay and improper handlings such as insufficient icing, as well as, improper mixing of fish and salt through layering in the collection vessels could have caused the lower quality in fish sauce samples. These conditions could have also allowed the growth of histamine-forming bacteria causing higher histamine contents as confirmed by the sharp increase in histamine after 4 h and increased gradually during fermentation. These could be due to the presence of histidine decarboxylase that can continue to convert histidine to

histamine even after mixing of fish and salt. It has been reported that histidine decarboxylase already produced by bacteria that have ceased growing can still convert histidine to histamine (Baranowski *et al*., 1985; Kimata and Kawai, 1953). Moreover, halophilic and/or halotolerant histamine-forming bacteria may have also produced the enzyme during fermentation. Kimura *et al*. (2001) indicated that *T. muriaticus*, a halophilic lactic acid bacterium, isolated from fish sauce can produced histamine even at 20% (w/v) NaCl, indicating that NaCl could not prevent histamine formation by this bacterium. Similarly, Okuzumi *et al*. (1981) reported significant histamine production by halophilic (salt-loving) bacteria growing at 5°C (300-400 mg histamine/100 mL of mackerel homogenate). However, Sanceda *et al*. (1999) found that histamine content of fish sauce produced by spoiled fish added with histidine rose to a high level but decreased continuously with incubation time. They suggested that this decrease may due to the action of histamine-decomposing bacteria. Nevertheless, none of the work has been done on the mechanism of histamine-decomposing bacteria in fish sauce fermentation.

Histamine degradation

Many bacteria have been shown to convert primary amines via an oxidative deamination step into products that can be utilized either as a source of carbon and/or energy, as a source of nitrogen, or both (Hacisalihoglu *et al*., 1997; Levering *et al*., 1981). Two types of enzymes, amine dehydrogenase (ADH) and amine oxidase (AO), are normally implicated in this form of oxidation. ADHs catalyze the oxidative deamination of primary amines to their corresponding aldehyde and ammonia, but AOs generate hydrogen peroxide (H_2O_2) in addition to aldehyde and ammonia (Figure 2).

The potential role of microorganisms in food fermentations with histamine oxidases and histamine dehydrogenases activity has been investigated to prevent or reduce the accumulation of histamine in foods. Findings of these microorganisms are useful to degrade some amines biologically (Bozkurt and Erkmen, 2002; Gardini *et al*., 2002; Dapkevicius *et al*., 2000; Siddiqui *et al*., 2000; Leuschner *et al*., 1998; Murooka *et al*., 1979; Umezu *et al*., 1979; Ienistea, 1971).

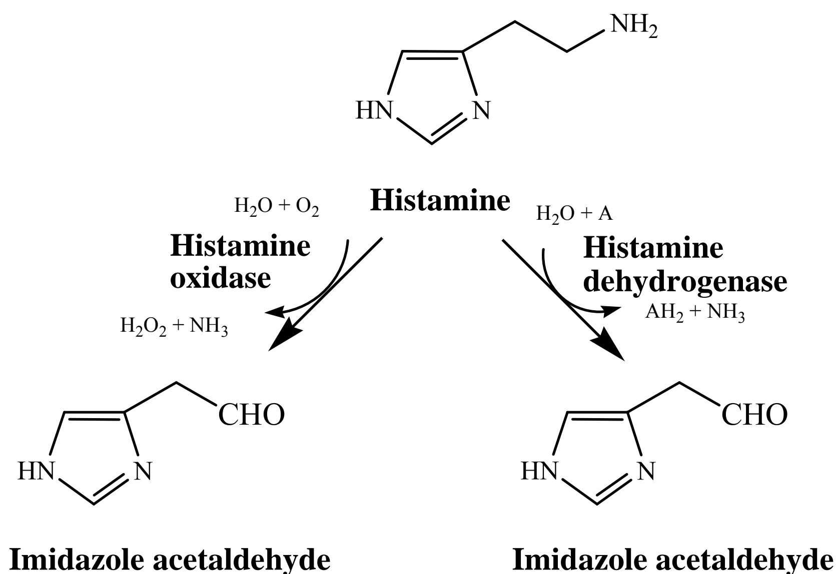


Figure 2. Degradation pathways of histamine. A and AH_2 are a two-electron acceptor (or two moles of one-electron acceptor) and its reduced form, respectively. For example; nicotinamide adenine dinucleotide phosphate NAD(P), ferricyanide, flavin adenine dinucleotide (FAD), flavin mono-nucleotide (FMN), cytochrome c, nitroblue tetrazolium (NBT) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS).

Source: Fujieda *et al*. (2005) and Sekiguchi *et al*. (2004).

Many studies have been carried out to evaluate the histamine-degrading activity of different microbial species isolated from fermented foods such as *Lactobacillus*, *Micrococcus* and *Staphylococcus* (Leuschner *et al*., 1998; Yamashita *et al*., 1993; Murooka *et al*., 1979; Umezu *et al*., 1979; Voigt and Eitenmiller, 1977; Ienistea, 1971). Nevertheless, it has been found that the *in vitro* degradation of histamine by microorganisms is not quantitatively reproducible with *in vivo* studies due to the more severe conditions and, in particular, to the low O_2 tension, pH and temperature.

Since the first report of methylamine dehydrogenase from *Pseudomonas* AM1 (Eady and Large, 1968), various ADHs have been reported so far

mostly in prokaryotes such as *Serratia marcescens* (Tabor and Kellogg, 1970), *Pseudomonas putida* (Shinagawa *et al*., 1988; Durham and Perry, 1978; Eady and Large, 1971), *Methylomonas* sp. (Matsumoto, 1978), *Paracoccus denitrifycans* (Takagi *et al*., 1999; Husain and Davidson, 1987), *Pseudomonas aeruginosa* (Hisano *et al*., 1990), *Citrobacter freundii* (Hisano *et al*., 1990), *Nocardioides simplex* (Siddiqui *et al*., 2000), *Alcaligenes faecalis* (Chistoserdov, 2001) and *Alcaligenes xylosoxidans* (Fujieda *et al*., 2004; Kondo *et al*., 2004).

However, high amount of biogenic amines can also be formed in salted foods, such as sardines salted and fermented with rice bran (Yatsunami and Echigo, 1992), fish sauce (Stute *et al*., 2002; Sato *et al*., 1995), salted anchovies (Hernández-Herrero *et al*., 1999), soy sauces (Chin *et al*., 1989), Jeotkals (Mah *et al*., 2002; Okuzumi *et al*., 1981), sauerkraut (Kalać *et al*., 2000; Taylor *et al*., 1978) and kimchi (Tsai *et al*., 2005). Thus, the applications of these microorganisms and/or enzymes might be restricted by unfavorable physiological conditions for enzyme activity such as low stabilities in the presence of high salt concentration. Since, none of studied microorganisms were salt tolerant, which probably will not appropriate for the salt-fermented products. Application of fermenting microorganisms to degrade histamine in salt-fermented food is still limited. Therefore, halophilic archaea should be considered as a source of enzyme production, which can degrade histamine and other biogenic amines in high salt content fermented foods.

Halophilic archaea

Classification

Archaea have long been considered as bacteria due to their prokaryotic morphology, circular genomes and gene organization in operons, but in 1977 Woese could clearly distinguish archaea as a third domain of life by applying rRNA phylogeny (Woese and Fox, 1977). Their status as a separate domain is further supported by their unique features such as a distinctive cell membrane containing phenyl side chains that are ether-linked to *sn*-glycerol 1-phosphate. Within the domain *Archaea* halophilic microorganisms occur in three families: the *Halobacteriaceae*, the

Methanospirillaceae and the *Methanosarcinaceae* (Figure 3). The *Methanospirilla-ceae* and the *Methanosarcinaceae* contain non-halophilic representatives as well as organisms that are adapted to seawater salinity and to hypersaline conditions. Some of these can grow at salt concentrations up to 300 g/L.

The order *Halobacteriales* with a single family, the *Halobacteriaceae*, consists entirely of halophiles. The most salt-requiring and salt-tolerant of all microorganisms are found in this family. They live in natural environments where the salt concentration is very high (as high as 5 M or 25% (w/v) NaCl) and grow optimally at 3.4-5.1 M (20-30%, w/v) NaCl (Table 1). These prokaryotes require salt for growth and will not grow at low salt concentrations. Their cell walls, ribosomes and enzymes are stabilized by Na^+. The halophilic archaea is strictly aerobic even high salt conditions with less availability of O_2 for respiration. They adapt to the high-salt environment by the development of purple membrane, actually patches of light-harvesting pigment in the plasma membrane. The pigment is bacteriorhodopsin contains 25% lipids and 75% protein. It reacts with light resulting in a proton gradient on the membrane just as in the case of the respiratory chain allowing the synthesis of ATP. Accordingly, the extreme halophiles can produce efficiently ATP by normal way (respiration) and adaptation one (bacteriorhodopsin).

As of February 2009, this family is divided into 27 genera by rRNA sequencing and other criteria (Euxéby, 2009); *Haladaptatus* (*Hap*.), *Halalkalicoccus* (*Hac*.), *Haloarcula* (*Har*.), *Halobacterium* (*Hbt*.), *Halobaculum* (*Hbl*.), *Halobiforma* (*Hbf*.), *Halococcus* (*Hcc*.), *Haloferax* (*Hfx*.), *Halogeometricum* (*Hgm*.), *Halomicrobium* (*Hmc*.), *Halopiger* (*Hpg*.), *Haloplanus* (*Hpn*.), *Haloquadratum* (*Hqr*.), *Halorhabdus* (*Hrd*.), *Halorubrum* (*Hrr*.), *Halosarcina* (*Hsn*.), *Halosimplex* (*Hsx*.), *Halostagnicola* (*Hst*.), *Haloterrigena* (*Htg*.), *Halovivax* (*Hvx*.), *Natrialba* (*Nab*.), *Natrinema* (*Nnm*.), *Natronobacterium* (*Nbt*.), *Natronococcus* (*Ncc*.), *Natronolimnobius* (*Nln*.), *Natronomonas* (*Nmn*.) and *Natronorubrum* (*Nrr*.). Figure 4 provides a list of the presently recognized genera and species in the *Halobacteriales.*

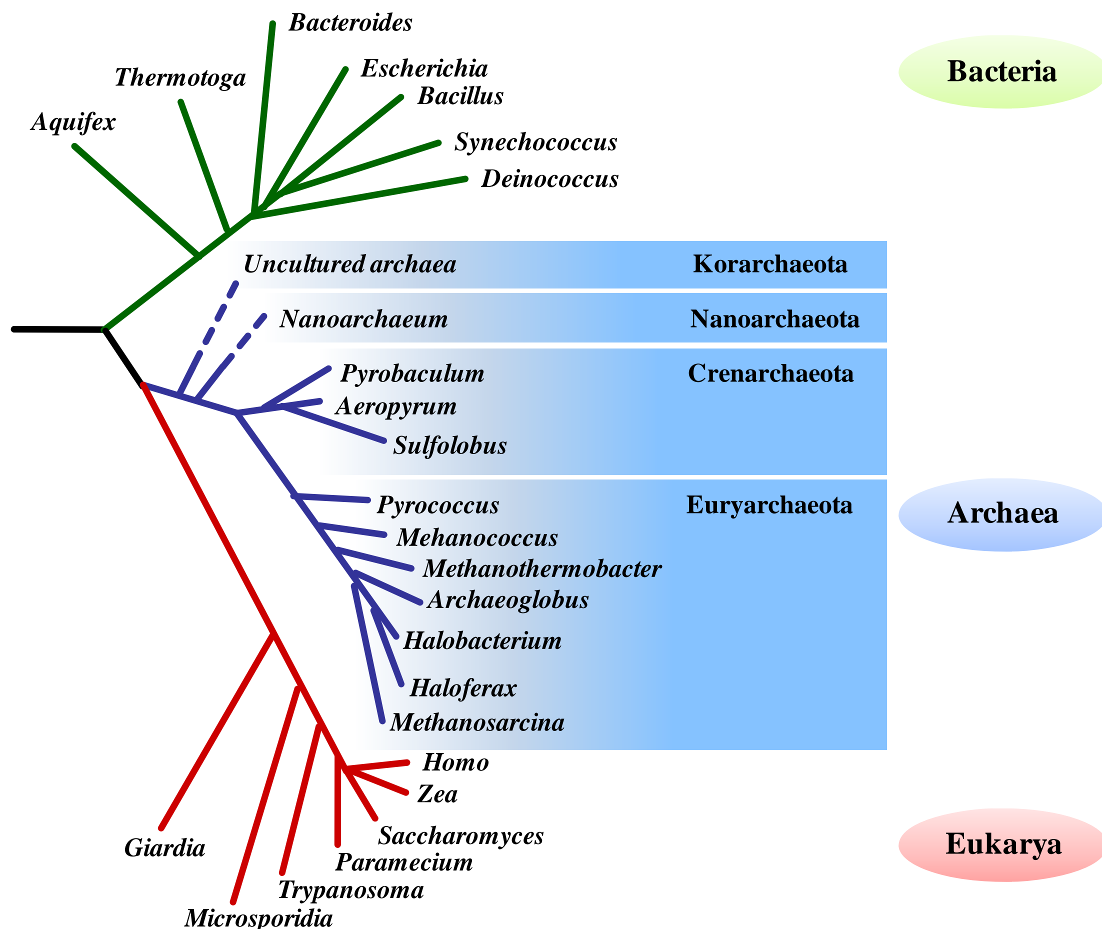


Figure 3. The rRNA tree of life. Molecular phylogeny clearly distinguishes archaea (blue) as a distinct domain of life independent from bacteria (green) and eukarya (red). Archaea comprise several phyla of which euryarchaeota (blue) are the most diverse group, including all methanogenes and halophiles but also thermophilic as well as psychrophilic species. Members of crenarchaeota are virtually all hyperthermophiles. Of the remaining phyla, korarchaeota and nanoarchaeota, no or only one species have been cultured so far, thus, their position in the tree is only indicated by dashed branches.

Source: Allers and Mevarech (2005).

Table 1. Ionic composition of the selected hypersaline environments

Ion	Concentration (g/L)			
	Great Salt Lake	Dead Sea	Typical Soda Lake	Seawater (for comparison)
Na^+	105	40	142	11
K^+	7	8	2	0.4
Mg^{2+}	11	44	<0.1	1.3
Ca^{2+}	0.3	17	<0.1	0.4
Cl^-	181	225	155	0.4
SO_4^{2-}	0.7	0.2	67	0.1
pH	7.7	6.1	11	8.1

Source: Madigan *et al*. (2000).

Classification of the species belonging to the family *Halobacteriaceae* is currently based on a polyphasic approach (Oren *et al*., 1997), which includes the evaluation of properties such as cell morphology, growth characteristics, chemotaxomic traits (notably the presence or absence of specific polar lipids) and nucleic acid sequence data. As with other living things, archaeal cells have an outer cell membrane that serves as a barrier between the cell and its environment. Within the membrane is the cytoplasm, where the living functions of the archaeon take place and where the DNA is located. Around the outside of nearly all archaeal cells is a cell wall, a semi-rigid layer that helps the cell maintained its shape and chemical equilibrium. All three of these regions may be distinguished in the cells of bacteria and most other living things, but when you take a closer look at each region, you find that the similarities are merely structural, not chemical.

In other words, archaea build the same structures as other organisms, but they build them from different chemical components. For instance, the cell walls of all bacteria contain the chemical peptidoglycan. Archaeal cell walls do not contain this compound, though some species contain a similar one. Likewise, archaea do not produce walls of cellulose (as do plants) or chitin (as do fungi). The cell wall of archaea is chemically distinct (Figure 5).

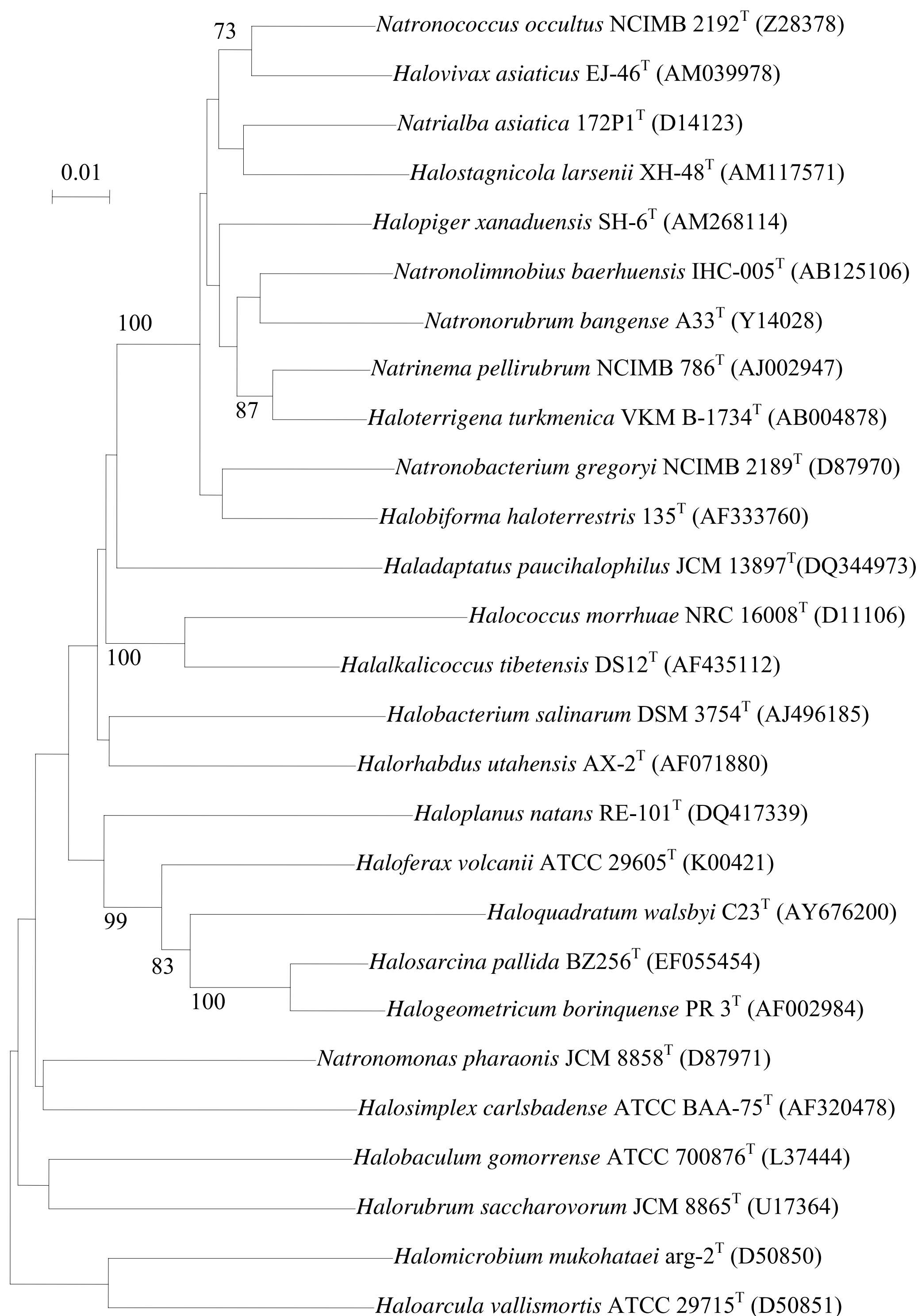


Figure 4. Phylogenetic trees showing genus belonging to the family *Halobacteriaceae*.

Source: Euxéby (2009).

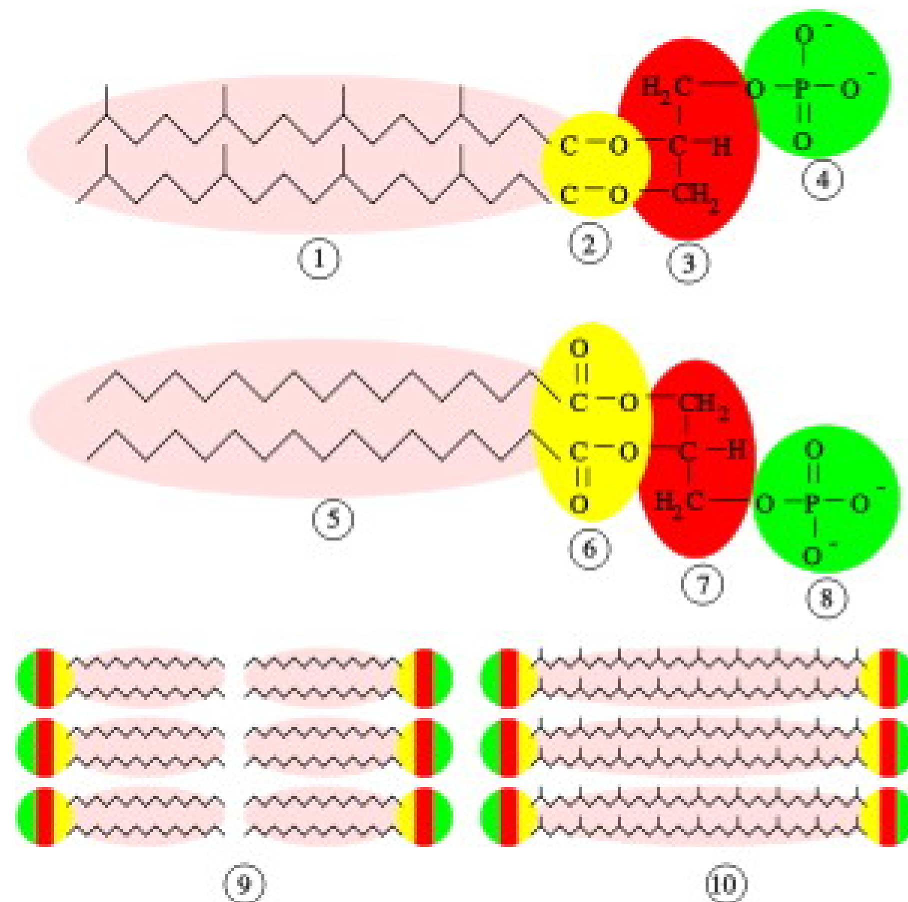


Figure 5. Membrane structures. **Top:** an archaeal phospholipid, 1 isoprene side chain, 2 ether linkage, 3 L-glycerol, 4 phosphate moieties. **Middle:** a bacterial and eukaryotic phospholipid: 5 fatty acid, 6 ester linkage, 7 D-glycerol, 8 phosphate moieties. **Bottom:** 9 lipid bilayer of bacteria and eukaryotes, 10 lipid monolayer of some archaea.

Source: Koga and Morii (2007).

Examination of the polar lipids present has proven extremely useful for the rapid characterization of isolates, as many genera have a distinctive polar lipid signature (Kamekura, 1998, 1999; Torreblanca *et al*., 1986). The *Halobacteriaceae* have archaeal-type lipids based on branched 2,3-di-*O*-phytanyl-*sn*-glycerol ($C_{20}C_{20}$) and sometimes also 2-*O*-sesterterpanyl-3-*O*-phytanyl-*sn*-glycerol ($C_{25}C_{20}$) chains, bound to glycerol by ether bonds. All known species contain the diether derivatives of

phosphatidylglycerol (PG) and methyl ester of phosphatidylglycerol methylophosphate (PGP-Me) (Figure 6). The glycolipids and sulfolipids have been found in different members of the *Halobacteriaceae* (Table 2).

In accordance with Recommendation 30b of the *International Code of Nomenclature of Bacteria* (Ksuhner, 1993), which calls for the development of minimal standards for describing new species, Oren *et al*. (1997) propose minimal standards for descriptions of new taxa of aerobic halophilic archaea (order *Halobacteriales).*

The purpose of this article is to provide microbiologists involved in the taxonomy of the aerobic halophilic archaea (order *Halobacteriales,* family *Halobacteriaceae;* also called halobacteria below) a framework for studying them. Adherence to the suggested minimum standards below should help stabilize the taxonomy of the *Halobacteriales.* With the consent and support of the Subcommittee on the Taxonomy of *Halobacteriaceae* Oren *et al*. (1997) have adopted a polyphasic view of halophile taxonomy as suggested by Murray *et al.* (1990). Oren *et al*. (1997) recommend that all future taxonomic publications on the *Halobacteriales* should contain data on phenetic, chemical and molecular properties. Oren *et al*. (1997) thus recognize that modern natural classification requires as complete a data set as possible, including phenotypic and genotypic information.

The current classification is based on the following three kinds of data: (i) phenotypic data, such as cell morphology, growth properties, etc. (Tindall, 1992); (ii) chemical data, especially the patterns of polar lipids present in the membranes (Tindall, 1992; Torreblanca *et al*., 1986) (differences in polar lipid patterns have been particularly important at the genus level); and (iii) 16S rRNA sequence information and DNA-DNA hybridization data. During the last few years a fairly complete database of 16S rRNA sequences of the type strains of the species in the *Halobacteriales* has become available and has enabled the construction of a phylogenetic tree (Kamekura *et al*., 1995; McGenity and Grant, 1995). This tree is supported by DNA-DNA hybridization data and by relationships derived from numerical taxonomy distance matrices based on phenotypic data.

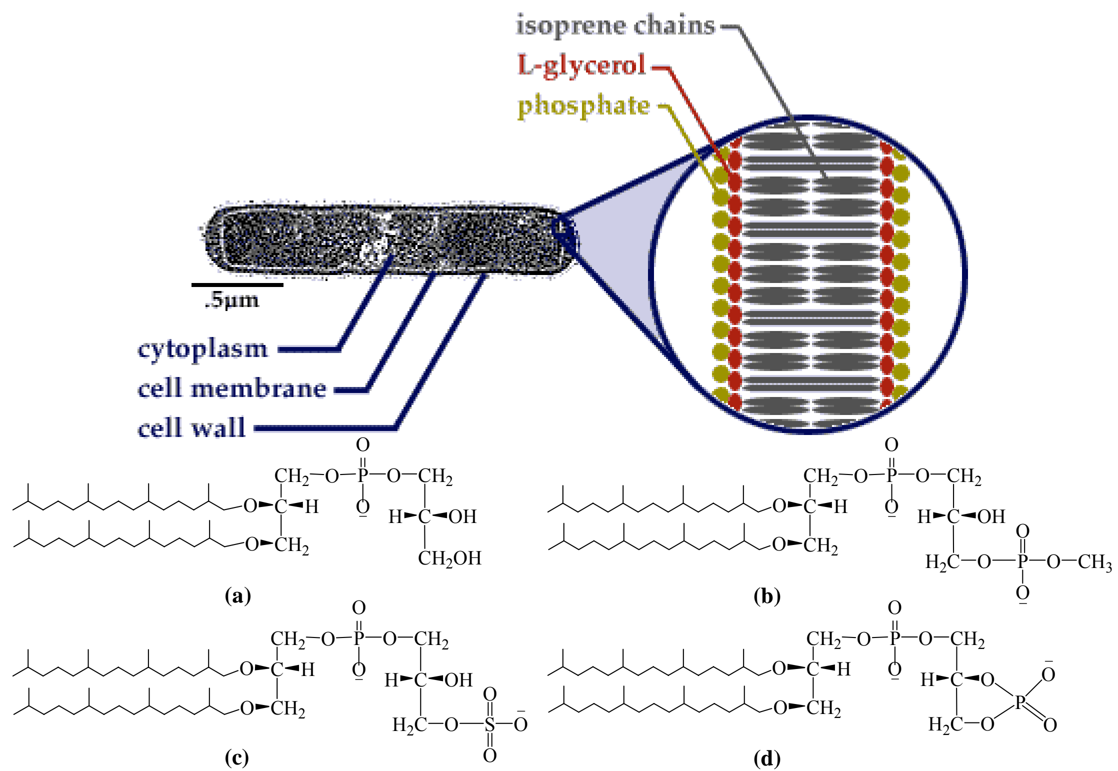


Figure 6. Basic archaeal structure: The three primary regions of an archaeal cell are the cytoplasm, cell membrane, and cell wall. Above, these three regions are labeled, with an enlargement at right of the cell membrane structure. Archaeal cell membranes are chemically different from all other living things, including a "backwards" glycerol molecule and isoprene derivatives in place of fatty acids. Structure of the phytyl diether derivatives of phosphatidylglycerol (a), phosphatidylglycerol phosphate-methyl ester (diether analog) (b), phosphatidylglycerol sulfate (diether analog) (c) and the cyclic phosphatidylglycerol phosphate (d) of extreme halophiles.

Source: Kates *et al*. (1993).

Table 2. Major glycolipids of different members of the *Halobacteriaceae*

Genus and species	Major glycolipids present; minor components in parentheses
Halobacterium	S-TGD-1, S-TeGD (TGD-1, TeGD)
Haloarcula (except *Haloarcula mukohataei*)	TGD-2 (DGD-2)
Haloferax	S-DGD-1
Halococcus	S-DGD-1, S-TGD
Halobaculum	S-DGD-1
Halorubrum	S-DGD-1, S-DGD-3 or S-DGD-5
Halogeometricum	Unidentified glycolipids
Natrialba asiatica	S_2-DGD-1
Natrinema	Unidentified glycolipids
Haloterrigena	Unidentified glycolipids
Natronobacterium	None; Small amounts of DGD-4 have been detected in an Indian isolate
Natronomonas	None
Natronococcus	None

Abbreviations: S-TGD-1, 3-HSO_3-Galρ-β-(1→6)-Manρ-α(1→2)l-Glcρ-α(1→1)-*sn*-glyceroldiether; S-TeGD, 3-HSO_3-Glcρ-β-(1→6)-[Gal*f*-α-(1→3)]-Manρ-α(1→2)-Glcρ-α(1→1)-*sn*-glyceroldiether; S-DGD-1, 6-HSO_3-Manρ-α(1→2)-Glcρ-α(1→1)-*sn*-glyceroldiether; S-DGD-3, 2-HSO_3-Manρ-α(1→4)-Glcρ-α(1→1)-*sn*-glycerol-diether; S-DGD-5, 2-HSO_3-Manρ-α(1→2)-Glcρ-α(1→1)-*sn*-glyceroldiether; S_2-DGD-1, 2,6-HSO_3-Manρ-α(1→2)-Glcρ-α(1→1)-*sn*-glyceroldiether; DGD-4, Glcρ-α(1→6)-Glcρ-α(1→1)-*sn*-glyceroldiether

Source: Kamekura and Kates (1999).

The subcommittee proposes that placement of a new taxon should preferentially be consistent with phylogeny, which is usually based on nucleic acid sequences. The phenetic differences within the group are relatively small because of the relatively conserved halobacterial phenotype. The sequences represented in rRNAs provide the only resource so far recognized for both discerning and testing phylogenetic associations that has the appropriate qualities of universality, genetic stability, and conservation of structure (Murray *et al*., 1990). However, differences in evolutionary rates in various groups of organisms prevent the use of phylogenetic parameters alone in delineating taxa. Therefore, the integrated use of phylogenetic and phenotypic characteristics, known as polyphasic taxonomy is necessary for the delineation of taxa at all levels from kingdom to genus. When workers are discriminating between closely related species of the same genus, DNA-DNA hybridization should be the method of choice, in accordance with the proposed molecular definition of species (Murray *et al*., 1990).

It is preferable to base descriptions of new species on characteristics of as many isolates as possible; these isolates should be obtained from different locations, and a diverse selection of them should be preserved as reference strains. For long-time preservation storage in liquid nitrogen, freeze-drying, or storage at -80°C on ceramic or plastic beads is recommended. Preservation methods for halobacteria have been described by Tindall (1992). One strain of each species, preferably the strain that most closely resembles the hypothetical median strain, should be designated the type strain. According to Rule 18a of the *Bacteriologial Code* (Ksuhner, 1993), all newly described bacterial taxa must be represented by a type culture deposited in a permanent culture collection.

In addition, descriptions of new taxa of *Halobacteriales* should be based on the following principles: (i) type strains of related taxa should be included for comparison, and it is recommended that in addition at least one species from each of the recognized genera (Figure 4) be used for comparative purposes in studies of new isolates; (ii) the characters that differentiate each new taxon from all previously described species should be adequately described, and it is recommended that the new taxon should be able to be identified and differentiated by methods available outside specialized laboratories; (iii) the methods used must be given in detail, or references

to readily available publications must be given; and (iv) the description of each new taxon should be published in a journal that has wide circulation, preferably the International Journal of Systematic Bacteriology, and when described elsewhere, the new taxon should be included as soon as possible on one of the lists that validate the publication of new names and new combinations previously effectively published outside the International Journal of Systematic Bacteriology that appear periodically in that journal. The subcommittee proposes the characters described below and listed in Table 3 and 4 as minimum features that should be contained in descriptions of new halobacterial taxa. However, we recognize that description of extraordinary microorganisms may require flexibility in the application of the tests described below. The proposal below specifies the minimal requirement for tests and does not in any way limit the extent of investigations beyond this.

Physiology and metabolism of halophilic archaea

Osmotic adaptation

Members of the *Halobacteriaceae* require 2-4 M (12-23%) NaCl for optimal growth and most halophilic archaea thrive up to the limit of saturation for sodium chloride (around 5.5 M (32%, w/v) NaCl), although growth of some species is rather slow at this salinity Haloarchaea are unable to grow below concentrations of 1.5 M (9%, w/v) NaCl, and *Halobacterium salinarum* has been shown to require large amounts of sodium. Sodium ions are, for example, needed, e.g. for cell wall integrity and many transport processes in *Halobacterium*, and cannot be replaced by other ions such as potassium (Madigan *et al*., 2000). In contrast to most halophiles that accumulate or synthesize intracellular organic compounds (compatible solutes) to withstand the osmotic pressure that accompanies life in hypersaline environments, *Halobacterium* produces no compatible solutes. Instead, it pumps large amounts of potassium into the cytoplasm (salt-in strategy) so that the intracellular K^+ concentration is considerable higher than the extracellular Na^+ concentration.

Table 3. Required minimal standards for description of halobacterial taxa

Importance	Minimal standards
Required	Colonial and cell morphology
	Motility
	Pigmentation
	Gram stain
	Salt concentrations required to prevent cell lysis
	Optimum NaCl and $MgCl_2$ concentrations for growth
	Range of salt concentrations enabling growth
	Temperature and pH ranges for growth
	Anaerobic growth in the presence of nitrate
	Reduction of nitrate to nitrite
	Formation of gas from nitrate
	Anaerobic growth in the presence of arginine
	Production of acids from a range of carbohydrates
	Ability to grow on a range of single carbon sources
	Catalase and oxidase activities
	Formation of indole
	Starch, gelatin, casein, Tween 80 hydrolysis
	Sensitivity to different antimicrobials
	novobiocin, bacitracin, anisomycin, aphidicolin, erythromycin, penicillin, ampicillin, rifampin, chloramphenicol, neomycin
	Characterization of polar lipids:
	Types of glycolipids present
	Presence or absence of phosphatidylglycerosulfate
	G+C content of DNA
	16S rRNA nucleotide sequence information
	DNA-DNA hybridization with related species (for descriptions of new species only)

Source: Oren *et al*. (1997).

Table 4. Recommended minimal standards for description of halobacterial taxa

Importance	Minimal standards
Recommended	Electron microscopy
	Anaerobic growth in the presence of
	Dimethyl sulfoxide (DMSO)
	Trimethylamine N-oxide (TMAO)
	Phosphatase activity
	Urease activity
	β-Galactosidase activity
	Lysine decarboxylase activity
	Ornithine decarboxylase activity
	Presence of glycoprotein in the cell envelope
	Presence of PHA
	Presence of plasmids
	Electrophoresis of cellular proteins

Source: Oren *et al*. (1997).

Thus, the inorganic potassium is employed as a compatible solute that keeps the cell in positive water balance and counteracts the tendency of the cell to become dehydrated at high osmotic pressure or ionic strength. However, under high ionic strength proteins tend to aggregate and often lose their activity, so that the complete intracellular machinery of haloarchaea requires adaptation to such an environment. It has been shown, for example, that the ribosomes of *Halobacterium* require high potassium levels for stability and halophilic enzymes exhibit highly polar surfaces in order to remain in solution (Figure 7) (Kennedy *et al*., 2001; Madigan *et al*., 2000). Therefore cytoplasmic proteins of halophiles reveal high ratios of acidic amino acids, mainly aspartate residues. Known proteins of haloalkaliphilic species have this typical amino acid distribution pattern, too, but haloalkaliphiles also produce a compatible solute, 2-sulfotrehalose, whose concentration increases with the salinity of the medium (Desmarais *et al*., 1997).

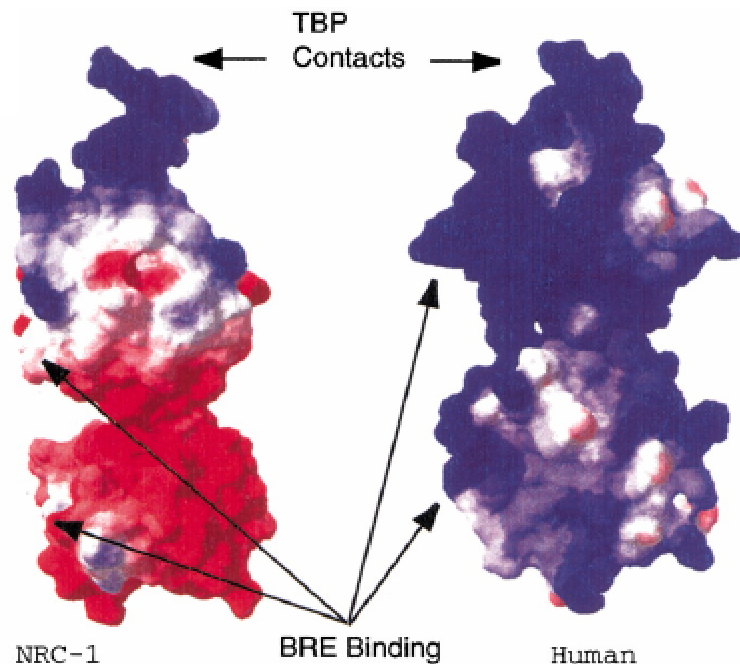


Figure 7. Surface charge comparison for a halophilic (NRC-1 TFBe) and a non-halophilic protein (Human transcription initiation factor TFIIB). Acidic character is indicated by red, basic character is indicated by blue, and neutral areas are indicated by white. The sites for BRE and TBP contacts are indicated within the structural models.

Source: Kennedy *et al*. (2001).

The cell wall of *Hbt. salinarum*, *Halobacterium volcanii* and *Haloarcula japonica* is composed of a glycoprotein (Csg) with exceptionally high contents of acidic amino acids (Nakamura *et al*., 1995; Sumper, 1993). In the extreme halophilic *Hbt. salinarum*, further negative surface charges are introduced by sulfate groups of N-linked saccharide units and a large N-terminally linked, sulphated sugar unit lacking in the more moderate halophile, *Hbt. volcanii* (Sumper, 1993). The negative charges of the acidic amino acids and sugar moieties are shielded by sodium ions, and are absolutely essential for maintaining cellular integrity (Madigan *et al*., 2000).

Nutritional demands, nutrient transport and sensing

Members of the *Halobacteriaceae* differ greatly in their nutritional demands. While simple growth requirements were first described for *Halobacterim mediterranei* and later for other species of the genera *Haloferax* and *Haloarcula*, *Hbt. salinarum* exhibits very complex nutritional demands. Although designed synthetic media for this widely studied species contain 10 to 21 amino acids, vitamin supplements (folate, biotin, thiamine), and sometimes also glycerol (Grey and Fitt, 1976; Oesterhelt and Krippahl, 1973), growth curves often do not show a typical exponential growth phase (Oren, 2002). There are also indications that even rich media based on yeast extract lacks some compounds to grow certain haloarchaeal strains, and it was reported that growth often improved when the medium is supplemented with a lysate of *Hbt. salinarum* cells. In contrast, the synthetic medium for *Hbt. volcanii* contains apart from simple carbon sources only the stimulatory vitamins thiamine and folate, inorganic salts as well as ammonium as nitrogen source (Kauri *et al*., 1990).

Most halophilic archaea preferentially use amino acids as carbon and energy source, but utilize also other compounds of hypersaline habitats such as glycerol and tricarboxylic acid (TCA) cycle intermediates that are excreted by *Dunaliella* and the cyanobacterium *Microcoleus chtonoplastes*, respectively (Oren, 2002). For example, the synthetic medium for *Hbt. volcanii* contains succinate and glycerol as carbon sources (Kauri *et al*., 1990). Sugars such as glucose, fructose, and sucrose are catabolized only by some haloarchaea, such as *Hfx. mediterranei* and *Halorubrum saccharovorum* (Altekar and Rangaswamy, 1992; Rawal *et al*., 1988). *Halobacterium* does not grow on sugars but growth of is stimulated by the addition of carbohydrates to the medium (Oren, 2002) and glucose can be transformed to gluconate (Sonawat *et al*., 1990). Oxidation of carbohydrates is often incomplete, and *Hrr. saccharovorum* was found to excrete acetate and pyruvate when grown on various sugars (Oren, 2002). Acetate can also be metabolized but it was found that acetate is used very poorly by haloarchaea. Degradation of fatty acids has not been reported yet but is likely since all genes for the fatty acid β-oxidation pathway are present in the halobacterial genomes. Many species of *Halobacteriaceae* produce

exoenzymes for the degradation of polymeric substances, e.g. the alkaline serine protease halolysin of *Halobacterium* (Kamekura *et al*., 1992) and an α-amylase of *Natronococcus* (Kobayashi *et al*., 1994). Finally, several haloarchaeal isolates have been described to degrade aliphatic hydrocarbons and aromatic compounds (Oren, 2002).

Ammonia and nitrate can be assimilated by some haloarchaea, e.g. by *Haloferax* species (Martinez-Espinosa *et al*., 2001; Kauri *et al*., 1990), but these ions are scarce in hypersaline environments and especially in soda lakes due to the lack of nitrifying organisms and high pH levels, respectively. Thus, amino acids are generally the preferred nitrogen source of most haloarchaea. In membrane vesicle studies it was shown, that *Hbt. salinarum* facilitates the uptake of several amino acids such as leucine, glutamate, and tyrosine, mostly dependent on sodium (Oren, 2002). *Halobacterium* further correlates intracellular pools of amino acids where glutamate and aspartate are most prominent with their rate of transport. Membrane transport systems for acetate and propionate have been studied in the haloalkaliphile *Natronococcus occultus* involving amongst others a sodium-dependent high affinity transporter. Glucose and fructose transport of *Hbt. volcanii* is also sodium-driven (Oren, 2002).

Hbt. salinarum is able to sense branched amino acids, methionine, cysteine, arginine and several peptides and to move toward attractant signals. Arginine chemotaxis is enabled by the cytoplasmic transducer Car, while membrane-bound BasT is involved in sensing of the remaining amino acids (Kokoeva *et al*., 2002). *Hbt. salinarum* contains several further transducers, the functions of some have been elucidated; HemAT/HtrVIII for oxygen-sensing (Hou *et al*., 2001), HtrI/HtrII for orange/blue light phototaxis (Hoff *et al*., 1997), and MpcT as proton motive force sensor (Koch and Oesterhelt, 2005). *Halobacterium* tranducers trigger a signalling pathway to the flagella motor which resembles bacterial signalling cascades and involves a typical bacterial-type two-component regulatory system (CheA/CheY). The *Halobacterium* motor is well understood on the functional level and, thus, a dynamic model could be established (Nutsch *et al*., 2003). However, the protein components of the archaeal motor remain to be identified on the genomic level.

Energy metabolism

Haloarchaea are aerobic chemoorganotrophs that degrade carbon sources such as amino acids, glycerol, and organic acids via the TCA cycle (Ghosh and Sonawat, 1998) and a respiratory electron transport chain (Schafer *et al.*, 1996). Due to the low solubility of oxygen in salt-saturated brines, molecular oxygen easily becomes a limiting factor for oxidative respiration though. Some halophiles are able to cope by the production of gas vesicles that enable floating of the cell towards the water surface. Furthermore, aerotaxis has been observed for *Halobacterium*, which is triggered by the oxygen sensor HemAT. However, many halophiles can also grow anaerobically by using alternative electron acceptors such as dimethylsulfoxide, triethylamine N-oxide, fumarate, or nitrate (Oren, 2002). While triethylamine N-oxide is often present in fish tissues as an osmotic solute, the ecological relevance of dimethylsulfoxide is unclear. Nitrate dissimilation might also be limited in hypersaline brines since it is unlikely to be regenerated by nitrification.

Halobacterium employs two further modes of energy conservation under anaerobic conditions. First, it is able of photophosphorylation by using the light-driven proton pump bacteriorhodopsin building up proton motive force for ATP generation. The retinal protein bacteriorhodopsin is one of the best studied proteins, and structurally and functionally resembles the rhodopsin of the eye. Since biosynthesis of the retinal moiety is oxygen-dependent, trace concentrations of oxygen are required for light-mediated ATP synthesis in *Halobacterium* though (Oesterhelt and Krippahl, 1983). As a second possibility to cover energy requirements when grown anaerobically, *Halobacterium* is able to ferment arginine via the arginine deiminase pathway (Ruepp and Soppa, 1996; Hartmann *et al.*, 1980). In this pathway, arginine is converted to ornithine and carbamoylphosphate, which is further split into carbon dioxide and ammonia with concomitant ATP production. While plasmidencoded enzymes for arginine fermentation are uncommon amongst haloarchaea (Oren, 2002), genes for bacteriorhodopsin and other retinal proteins (halorhodopsin, sensory rhodopsin) were found in several other *Halobacteriaceae*.

The application of halophilic archaea

As a result of adaptation to the hypersaline condition, many halophiles have evolved unique properties of considerable biotechnological and therefore, commercial interest. Many novel and unique properties of these organisms, such as enzymes that function in saturated salts and pigmentation etc. suggest that they have even greater potential for biotechnology. Halophiles are the most likely source of enzymes that not only are salt-tolerant but also thermotolerant. They constitute a heterogeneous group of microorganisms including species belonging to different genera.

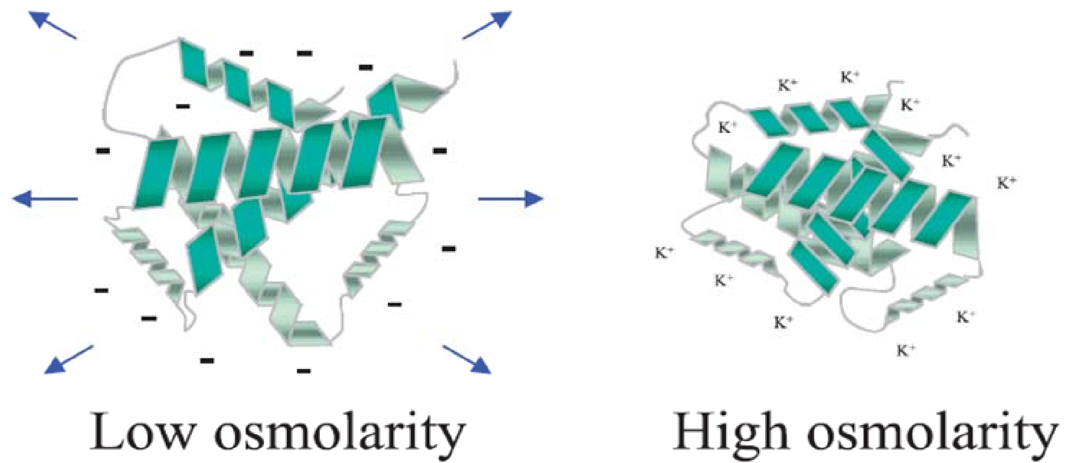
The isolation of halophiles able to produce extracellular enzymes will provide the possibility to have optimal activities at different salt concentrations. However, their biotechnological possibilities have not been extensively exploited. Over the past years a variety of enzymes such as pullulanases and xylanases as well as several lipolytic enzymes from non-halophilic microorganisms with different substrate specificities has been characterized and the corresponding genes have been cloned (Arpigny and Jaeger, 1999; Niehaus *et al*., 1999). However, enzymes produced by halophilic microorganisms have not been studied or described up to date. Screening and isolation of extracellular enzyme producers will provide insight on the possibility to use different halophiles as a source of extremophilic enzymes in biotechnological processes. Extracellular hydrolytic enzymes such as amylases, proteases, and lipases have quite diverse potential usages in different areas such as food industry, feed additive, biomedical sciences and chemical industries. Industrial processes are carried out under specific physical and chemical conditions which cannot always be adjusted to the optimal values required for the activity of the available enzymes. For that reason, it would be of great importance to have available enzymes showing optimal activities at different values of salt concentrations and temperature.

Halophiles produce a large variety of stable and unique biomolecules that may be useful for practical application. Halophilic microorganisms produce stable enzymes (including many hydrolytic enzymes such as DNAases, lipases, amylases, gelatinases and proteases) capable of functioning under conditions that lead

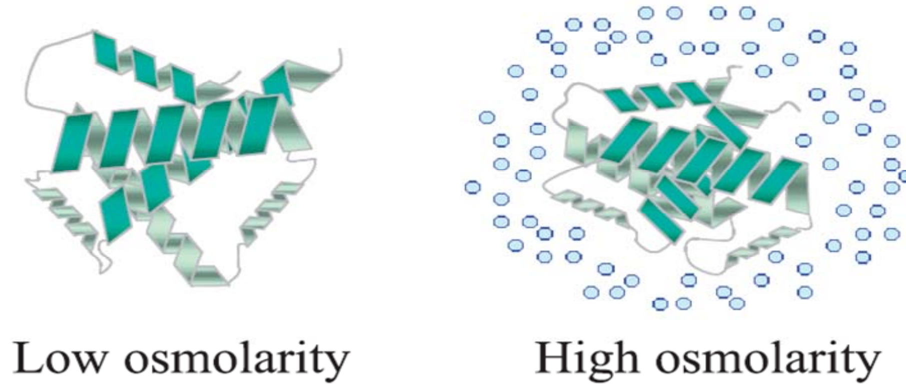
to precipitation or denaturation of most proteins. Halophilic proteins compete effectively with salts for hydration, a property that may result in resistance to other low-water-activity environments, such as in the presence of organic solvents. They accumulate salts such as NaCl or KCl up to concentrations that are isotonic with the environment. As a result, proteins from halophiles have to cope with very high salt concentrations (up to about 4 M KCl and over 5 M NaCl) (Haki and Rakshit, 2003; Oren, 2002; Eichler, 2001; Hough and Danson, 1999).

Halophilic proteins employ different adaptation mechanisms. Proteins from halophilic organisms have a biased amino acid composition in order to remain stable and active at high ionic strength. Halophilic proteins typically have an excess of acidic amino acids (i.e. glutamate and aspartate) on their surface (Madern *et al*., 2000; Mevarech *et al*., 2000; Da Costa *et al*., 1998; Danson and Hough, 1997), although such a high proportion of acidic amino acids is not present in the amylase from the thermophilic halophile *Halothermothrix orenii* (Mijts and Patel, 2002). Negative charges on the halophilic proteins bind significant amounts of hydrated ions, thus reducing their surface hydrophobicity and decreasing the tendency to aggregate at high salt concentration. Halophilic proteins are distinguished from their non-halophilic homologous proteins by exhibiting remarkable instability in solutions with low salt concentrations and by maintaining soluble and active conformations in high concentrations of salt, for example, up to 4 M NaCl (Madern *et al*., 2000; Mevarech *et al*., 2000; Da Costa *et al*., 1998; Danson and Hough, 1997; Eisenberg, 1995).

Halophiles respond to increases in osmotic pressure in different ways. The extremely halophilic archaea, the *Halobacteriaceae*, accumulate K^+, while other bacteria accumulate compatible solutes (e.g. glycine, betaine, sugars, polyols, amino acids and ectoines), which help them to maintain an environment isotonic with the growth medium (Da Costa *et al*., 1998; Danson and Hough, 1997). These substances also help to protect cells against stresses like high temperature, desiccation and freezing. Consequently, in surroundings with lower salt concentrations, the solubility of halophilic proteins is often very low (Madern *et al*., 2000; Mevarech *et al*., 2000; Da Costa *et al*., 1998; Danson and Hough, 1997) (Figure 8).



(A) Salting in- structures maintained by surface charges of acidic amino acids (aspartic and glutamic acid residues)



(B) Salting out- biomolecular structures maintained by cytoplasmic solutes (e.g. betaine)

Figure 8. A reminder on the molecular strategies adopted by halophiles.

Source: Kates *et al*. (1993).

Halophilic enzymes

Halophiles from the archaeal domain provide the main source of extremely halophilic enzymes. The potentials of halophiles and haloenzymes have been reviewed previously (Oren, 2002; Eichler, 2001; Madern *et al*., 2000; Hough and Danson, 1999; Sellek and Chaudhuri, 1999; Da Costa *et al*., 1998; Danson and Hough, 1997). The production of halophilic enzymes, such as xylanases, amylases, proteases and lipases, has been reported for some halophiles belonging to the genera *Acinetobacter*, *Haloferax*, *Halobacterium*, *Halorhabdus*, *Marinococcus*, *Micrococcus*, *Natronococcus*, *Bacillus*, *Halobacillus* and *Halothermothrix* (Oren, 2002; Eichler, 2001; Madern *et al*., 2000; Sellek and Chaudhuri, 1999; Da Costa *et al*., 1998; Danson and Hough, 1997; Adams *et al*., 1995). Table 5 shows some recent reports on these enzymes (Amoozegar *et al*., 2003; Perez-Pomares *et al*., 2003; Sanchez-Porro *et al*., 2003; Waino and Ingvorsen, 2003; Deutch, 2002; Mijts and Patel, 2002).

However, many of these enzymes have not been investigated in detail or application (Sellek and Chaudhuri, 1999). Although the halophilic enzymes can perform enzymatic functions identical to those of their non-halophilic counterparts, these enzymes have been shown to exhibit substantially different properties, especially, the requirement for high salt concentrations (1-4 M NaCl) for activity and stability and a high excess of acidic over basic amino residues (Mevarech *et al*., 2000). It is argued that the high negative surface charge of halophilic proteins makes them more soluble and renders them more flexible at high salt concentrations, conditions under which non-halophilic proteins tend to aggregate and become rigid. This high surface charge is neutralized mainly by tightly bound water dipoles (Madern *et al*., 2000; Mevarech *et al*., 2000; Da Costa *et al*., 1998; Danson and Hough, 1997). The requirement of high salt concentration for the stabilization of halophilic enzymes, on the other hand, is due to a low affinity binding of the salt to specific sites on the surface of the folded polypeptide, thus stabilizing the active conformation of the protein (Mevarech *et al*., 2000). The dependence of the stability and the catalytic properties of halobacterial proteins on salt concentration has been the subject of studies for many years.

Table 5. Production of extremophilic enzymes by halophiles

Halophiles	Halophilic enzymes	Optimum temperature (°C)	Optimum pH	Optimum/Stability	Ref.
Halothermothrix orenii	α-Amylase	65	7.5	Tolerates up to 25% NaCl	Mijts and Patel (2002)
Bacillus dipsosauri	α-Amylase	60	6.5	Stable up to 60°C	Deutch (2002)
Halobacillus sp. MA-2	Amylase	50	7.5-8.5	Maximum stable at 5% NaCl	Amoozegar *et al*.(2003)
Haloferax mediterranei	α-Amylase	50-60	7-8	Stable at 2-4 M NaCl Optimum at 3 M NaCl	Perez-Pomares *et al*. (2003)
Halobacterium bacterium CL8	Xylanase 1 Xylanase 2	60 65	6.0 6.0	Stable 7 min at 60°C Stable 192 min at 60°C	Wejse *et al*. (2003)
Halorhabdus utahensis	β-Xylanase β-Xylosidase	55 70-65	NA NA	Optimum at 5-15% NaCl Optimum at 5% NaCl	Waino and Ingvorsen (2003)
Pseudoalteromonas sp. strain CP76	Protease CPI	55	8.5	Tolerates at 0-4 M NaCl Optimum at 7.5% NaCl	Sanchez-Porro *et al*. (2003)

NA = not available

The property of low solubility of halophilic enzymes has been taken advantage of by applying them in aqueous/organic and non-aqueous media (Marhuenda-Egea and Bonete, 2002). Therefore, halophiles are likely to provide significant opportunities for histamine degradation in high salt-fermented foods. Uniquely, halophilic Archaea accumulate K^+ intracellularly for osmoregulation, requiring adaptation of intracellular and extracellular proteins for activity and stability in 4 M KCl and <5 M NaCl, respectively (Danson and Hough, 1997). Halophilic eubacteria and eukarya tend to accumulate compatible organic solutes such as sugars, amino acids, and ectoines (Ventosa and Nieto, 1995), so their intracellular enzymes are not halophilic. Out of all the halophilic enzymes isolated, malate dehydrogenase from *Haloarcula marismortui* has been studied in the most detail by Eisenberg *et al.* (1992). Their findings have been reviewed, in addition to other work on the halophilic adaptations of proteins (Danson and Hough, 1998; Danson and Hough, 1997; Eisenberg *et al.*, 1992). The main structural features of halophilic enzymes are described briefly below:

1. The high content (up to 20% of all residues) of aspartic and glutamic acid residues found in clusters on the enzyme surface. These attract hydrated counterions to the enzyme surface, reducing the surface tension at the protein-solvent interface, and hence preventing precipitation of the protein.
2. Extensive ion-pair networks have been observed. These increase in effectiveness with increasing salt concentrations. They result in part from the aforementioned increase in acidic residue content. They are also believed to responsible for the stability observed at high temperatures with halophilic malate dehydrogenase (Dym *et al.*, 1995) and dihydrolipoamide dehydrogenase (Danson and Hough, 1998).
3. Fewer strongly hydrophobic residues (tyrosine, tryptophan, phenylalanine) are found to compensate for the highly salting-out nature of the medium.

As a result of this adaptation, halophilic enzymes rapidly denature and dissociate at NaCl/KCl concentrations below ≈1 M and can be irreversible. This feature, rather than stability at high salt concentrations, has been proposed as the

defining feature of halophilicity (Bohm and Jaenicke, 1994). This distinguishes true halophilic enzymes from enzymes such as thermolysin, which despite being soluble and activated by >1 M salt, is still active and stable in the absence of salt (Inouye *et al*., 1998).

Whole cell immobilization

Immobilization of whole cell provides an alternative to fermentation and enzyme immobilization. Cell immobilization is the physical confinement or localization of intact cells to a certain defined region of space with preservation of some desired catalytic activity. The use of immobilized whole microbial cells and/or organelles eliminates the often tedious, time consuming, and expensive steps involved in isolation and purification of intracellular enzymes (Ohmiya *et al*., 1977). It also tends to enhance the stability of the enzyme by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation. The metabolically active cell immobilization is particularly preferred where co-factors are necessary for the catalytic reactions. Since co-factor regeneration machinery is an integral function of the cell, its external supply is uneconomical. Due to the potential advantages of immobilization over free cell system, immobilized cells have been used in a wide spectrum of applications, such as biodegradation of toxic compounds, such as phenol, pyridine, dibenxothiophene, quinoline and carbazole, production of ethanol and biosensors (Wang *et al*., 2007; Li *et al*., 2005; Wang *et al*., 2002; Wang *et al*., 2001; Lee *et al*., 1994; Dwyer *et al*., 1986).

Immobilization of whole cell has been shown to be a better alternative to immobilization of isolated enzymes (D'Souza, 1989; Mattiasson, 1988; Tampion and Tampion, 1987). Doing so avoids the lengthy and expensive operations of enzyme purification, preserves the enzyme in its natural environment thus protecting it from inactivation either during immobilization or its subsequent use in continuous system. The major limitations which may need to be addressed while using such cells are the diffusion of substrate and products through the cell wall, and unwanted side reactions due to the presence of other enzymes. The cells can be immobilized either in a viable or a nonviable form. Immobilized nonviable cell preparations, which are

normally obtained by permeabilizing the intact cells, for the expression of intracellular activity are useful for simple processes that require single-enzyme with no requirement for cofactor regeneration, like hydrolysis of sucrose or lactose (D’Souza, 1989; Mattiasson, 1988; Tampion and Tampion, 1987). On the other hand immobilized viable cells, which serve as ‘controlled catalytic biomass’, have opened new avenues for continuous fermentation on heterogeneous catalysis basis by serving as self-proliferating biocatalysts (Tanaka and Nakajima, 1990; D’Souza, 1989).

Most of the enzymes used at industrial scale are normally the extracellular enzymes produced by the microbes. This has been mainly due to their ease of isolation as crude enzymes from the fermentation broth. Moreover, the extracellular enzymes are more stable to external environmental perturbations compared to the intracellular enzymes. However, over 90% of the enzymes produced by a cell are intracellular (D’Souza, 1989). The economic exploitation of these, having a variety of biochemical potentials, has been limited in view of the high cost involved in their isolation. Also, compared to extracellular enzymes, the intracellular enzymes are more labile. Delicate and expensive separation methods are required to release the enzymes undamaged from the cell, and to isolate them. This increases the labor and the cost of the enzyme. These problems could now be obviated by the use of permeabilized cells as a source of enzyme. Permeabilization of the cells removes the barrier for the free diffusion of the substrate/product across the cell membrane, and also empties the cell of most of the small molecular weight cofactors, etc., thus minimizing the unwanted side reactions. Such permeabilized cells, which are often referred to as nonviable or nongrowing cells, can be exploited in an immobilized form as a very economical source of intracellular enzyme for simple bioconversions like hydrolysis, isomerization and oxidation reactions that do not need a cofactor-regeneration system (D’Souza, 1989). The decision to immobilize cells either in a viable or nonviable form is very important and depends on their ultimate application. Thus the permeabilized *Kluyveromyces fragilis* cells, which are nonviable, convert lactose in milk to glucose and galactose (Joshi *et al*., 1989), whereas the viable (nonpermeabilized) cells convert the lactose to ethanol and are useful in the complete desugaration of milk (Rao *et al*., 1988).

Techniques and supports for immobilization

A large number of techniques and supports are now available for the immobilization of cells on a variety of natural and synthetic supports. The choice of the support as well as the technique depends on the nature of the enzyme, nature of the substrate and its ultimate application. Therefore, it will not be possible to suggest any universal means of immobilization. It can only be said that the search must continue for matrices which provide facile, secure immobilization with good interaction with substrates, and which conform in shape, size, density and so on to the use for which they are intended. Care has to be taken to select the support materials as well as the reagents used for immobilization, which have GRAS status, particularly when their ultimate applications are in the food processing and pharmaceutical industries. Macromolecular, colloidal, viscous, sticky, dense or particulate food constituents or waste streams also limit the choice of reactor and support geometries. Commercial success has been achieved when support materials have been chosen for their flow properties, low cost, nontoxicity, maximum biocatalysts loading while retaining desirable flow characteristics, operational durability, ease of availability and ease of immobilization (Gekas and Lopeiz-Leiva, 1985).

Many methods namely adsorption, covalent bonding, crosslinking, entrapment and encapsulation are widely used for immobilization. These categories are commonly used in immobilized enzyme technology. However, due to the completely different size and environmental parameters of the cells, the relative importance of these methods is considerably different. The criteria imposed for cell immobilization technique usually determine the nature of the application.

Adsorption

The adsorption phenomenon is based on electrostatic interactions (van der Walls forces) between the charged support and microbial cell, the actual zeta potential on both of them plays a significant role in cell-support interactions. Unfortunately, the actual charge on support surfaces is still unknown and this limits the proper choice for microbial attachment. Along with charge on the cell surface, the

composition of cell wall carrier composition will also play a predominant role (Kolot, 1981). Carrier properties, other than zeta potential, will also greatly influence cell-support interaction. All glasses or ceramic supports are consisted of varying proportions of oxides of alumina, silica, magnesium, zirconium, etc. which result in bond formation between the cell and the support. These forces are very weak, but sufficiently large in number to enable reasonable binding.

Covalent bonding

The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (crosslinking) agent. For covalent linking, chemical modification of the surface is necessary. However, covalent binding is an extensively used technique for the immobilization of enzymes, though it is not a good technique for the immobilization of cells (D'Souza, 1989).

Entrapment

Entrapment has been extensively used for the immobilization of cells, but not for enzymes. The major limitation of this technique for the immobilization of enzymes is the possible slow leakage during continuous use in view of the small molecular size compared to the cells (D'Souza, 1989). The polymer matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photo cross-linkable resins, polyacrylamide, polyester, polystyrene and polyurethane. As a rule, the entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into surrounding medium while still allowing penetration of substrate.

Cross linking

Microbial cells can be immobilized by cross-linking each other with bi- or multifunctional reagents. Among these, glutaraldehyde which interacts with the

amino groups through a base reaction has been extensively used in view of its GRAS status, low cost, high efficiency and stability (Nakajima *et al*., 1993).

1.3 Objectives of study

1. To select the most effective halophilic archaea for histamine degradation.
2. To systematically identify the selected strain of halophilic archaea.
3. To purify and characterize the predominant enzyme involving in the histamine-degrading activity.
4. To immobilize the whole cell of the selected strain for histamine degradation.
5. To evaluate the potential use of selected strain or its enzyme for removing histamine in fish sauce.

CHAPTER 2

DEGRADATION OF HISTAMINE BY HALOPHILIC ARCHAEA ISOLATED FROM SALT-FERMENTED FISHERY PRODUCTS

2.1 Abstract

The presence of high level of histamine is detrimental to the quality and safety of fish sauce. Therefore, this study aimed to screen for the ability of extremely halophilic archaea to degrade histamine in high salt condition and characterize the enzyme involved. Of 156 extremely halophilic archaea isolated from various salt-fermented fishery products, HDS3-1 from fish sauce exhibited highest histamine degradation activity when cultured in halophilic medium containing 5 mM histamine (free-base), followed by HDS1-1, HPC1-2, and HIS40-3, respectively. Based on 16S rRNA gene sequence similarities, HDS3-1, HDS1-1, and HIS40-3 were classified as members of genus *Natrinema* while HPC1-2 was related most closely to species of the genus *Halobacterium*. These strains neither exhibited decarboxylase activity toward all tested amino acids nor were toxic to human colon adenocarcinoma (Caco2), human liver hepatocarcinoma (HepG2), and human larynx epithelial (HEp-2) cells. Based on histamine assay, histamine-degrading activity of HDS3-1 was located in the intracellular fraction, required the presence of 1-methoxy-5-methylphenazinium methylsulfate (PMS), an electron carrier, and selectively catalyzed histamine as a substrate. The pH, salt concentration, and temperature optima for the activity were at pH 6.5-8, 3.5-5 M NaCl, and 40-55°C. The activity retained full activity at pH 6.5-9, in the presence of NaCl above 2.5 M, and temperature lower than 50°C. The results suggested a link of histamine-degrading activity of HDS3-1 to the presence of salt-tolerant and thermo-neutrophilic histamine dehydrogenase.

2.2 Introduction

The presence of high levels of histamine is detrimental to the quality and safety of foods, particularly, fish sauce and other fermented fishery products from scombroid species which are important condiment of Thailand (Brillantes *et al*., 2002). Histamine in foods is mainly formed by histamine decarboxylase activity from several kinds of bacteria (Lehane and Olley, 2000). The Food and Drug Administration (FDA) established an advisory level of 500 ppm to be hazardous to human health (FDA, 1998). Histamine is heat stable and is not detectable through organoleptic analysis by even trained panelists (Arnold *et al*., 1980). Except for the gamma irradiation, no other food processing methods are available for histamine degradation (Kim *et al*., 2004). Therefore histamine, if present, is difficult to destroy and posts a risk of food intoxication.

Microorganisms and enzyme can provide a means of controlling histamine accumulation (Ienistea, 1971). The presence of histamine-degrading enzymes either histamine oxidases or histamine dehydrogenases has been reported in various higher organisms (Rinaldi *et al*., 1983; Isobe *et al*., 1980; Pionetti, 1974; McGowan and Muir, 1971) as well as in microorganisms (Bakke *et al*., 2005; Sekiguchi *et al*., 2004; Siddiqui *et al*., 2000; Choi *et al*., 1995; Yamashita *et al*., 1993; Underberg and Lembke, 1986; Murooka *et al*., 1979; Umezu *et al*., 1979; Suzuki *et al*., 1972; Ienistea, 1971). Therefore, the application of starter strains that possess histamine-degrading activity might be a way for decreasing the amount of histamine produced *in situ* (Kim *et al*., 2004; Gardini *et al*., 2002; Dapkevicius *et al*., 2000). Nevertheless, the applications of these microorganisms and enzymes have been restricted by unfavorable physiological conditions for growth and enzyme activity such as low oxygen concentration, low pH value, undesirable temperature, and especially in the high salinity. The extremely halophilic archaea, in particular, are well adapted to saturating NaCl concentrations (grow optimally above 3.4-5.1 mol/L or 20-30% NaCl). They have a number of novel molecular characteristics, especially for the enzymes that function in high salt concentration (3-4 M NaCl), such as lipase (Boutaiba *et al*., 2006), protease (Namwong *et al*., 2006), nitrate reductase (Martínez-Espinosa *et al*., 2005), β-galactosidase (Homes *et al*., 1997), and glucose

dehydrogenase (Bonete *et al*., 1996). Therefore, in this present study, extremely halophilic archaea isolated from several salt-fermented products were screened for the ability to degrade histamine in high salt condition.

2.3 Materials and Methods

Chemicals

Histamine dihydrochloride and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Sigma Chemical Company (St. Louis, MO). The other chemicals were of analytical grade.

Isolation and culture conditions

Extremely halophilic archaea were isolated from various salt-fermented fishery products made in Thailand. Fish sauce samples were collected during early, middle, and late stages of the fermentation from many factories, whereas other fermented fish samples were obtained from local markets. The samples were spread onto agar plates of halophilic medium (Appendix A-1) and incubated at 37°C for 7 days. Several colonies with different morphological characteristics were picked. A pure culture was obtained by repeated transfers of separate colonies on agar plates of the same medium. The single colony was inoculated into the halophilic liquid medium and incubated at 37°C in a shaker incubator (Sartorius, Certomat® BS-1, Goettingen, Germany) at 200 rpm for 7 days. Cells were harvested by centrifugation of cultured broth at 10,000 ×*g* at 4°C for 15 min. The pellet was washed twice with halophilic liquid medium. The obtained pellet was suspended in halophilic liquid medium containing 20% (v/v) glycerol and placed in a 2 mL cryotube. The stock cultures were kept in the -20°C freezer until used.

Screening of histamine-degrading halophilic archaea

The inoculum was prepared by inoculating 250 µL of stock culture into 5 mL of halophilic medium and incubated at 37°C in a shaker incubator at 200 rpm for 7 days. Inoculum was subsequently added at 5% (v/v) into halophilic liquid medium containing 5 mM of histamine (free-base) (Appendix A-2) and incubated at 37°C in a shaker incubator at 200 rpm for 7 days. The supernatant was obtained by centrifugation of cultured broth at 10,000 ×*g* at 4°C for 15 min. The histamine concentration of supernatant was measured by using AOAC (1995) (Appendix B-1). The histamine-degrading activity was expressed as the percentage degradation of histamine in the supernatant.

The histamine degradation activity was calculated by the following equation:

$$\text{Histamine degradation activity (\%)} = \frac{(\text{His}_{0\ \text{day}} - \text{His}_{7\ \text{days}})}{\text{His}_{0\ \text{day}}} \times 100$$

where: $\text{His}_{0\ \text{day}}$ is histamine content at the beginning of the inoculation period.
$\text{His}_{7\ \text{days}}$ is histamine content at the end of the inoculation period (7 days).

Test for amino acids decarboxylation

The inoculum of the selected strains was inoculated to halophilic liquid medium supplemented with histidine, tryptophan, lysine, ornithine, arginine, tyrosine, and phenylalanine at 1% (w/v) (Appendix A-3). The cultures were incubated at 37°C in a shaker incubator at 200 rpm for 7 and 14 days. The supernatant was collected by centrifugation at 10,000 ×*g* at 4°C for 15 min. Biogenic amines in supernatant were extracted and derivertized according to the procedure of Eerola *et al*. (1993) with a slight modification (Appendix B-2). In brief, the supernatant (480 µL) was added with 20 µL of 2 mg/mL of 1,7-diaminoheptane as the internal standard and 500 µL of 0.4 M perchloric acid. The mixture was mixed thoroughly by a vortex mixer for 5 min

and then centrifuged at a speed of 15,000 ×*g* at 25°C for 5 min. The obtained supernatant (300 μL) was mixed with 60 μL of 2 N NaOH and 90 μL of saturated sodium bicarbonate. A 600 μL of 10 mg/mL of dansyl chloride was added to each sample, mixed, and then incubated for 45 min at 40°C. Residual dansyl chloride was removed by adding 30 μL of 25% (v/v) ammonia and centrifuged at 3,500 ×*g* for 5 min. The supernatant was collected and filtered through a 0.45 μm membrane filter prior to analysis by a high performance liquid chromatography (HPLC). A Waters Separation Module 2690 was operated to give a flow rate of 1.5 mL min of the mobile phase of 0.1% (v/v) acetic acid (solvent A) and acetonitrile containing 0.1% (v/v) acetic acid (solvent B). Separation was achieved using a column of Hypersil BDS C18 (300 × 7.8 mm i.d.) set at 40°C. Samples (20 μL) were injected and a photo diode array (Model Waters 996), set at the wavelength of 254 nm, was used as the detector. Data were processed and analyzed using Millennium 32 software (Waters, Milford, MA, USA).

Cytotoxicity testing

The cytotoxicity of the selected strain was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) colorimetric assay (Plumb *et al*., 1989). The human colon adenocarcinoma (Caco2), human liver hepatocarcinoma (HepG2), and human larynx epithelial (HEp-2) cells were used as the target cell lines. This assay was a modified version of conventional direct and indirect contact tests conformed to the published standard methods (BS-EN30993-5 and ISO10993-5) (ISO, 1992). The detail of the MTT colorimetric assay is described clearly in Appendix B-3.

Identification of the selected strains

The physiological and biochemical characteristics of the selected strains were performed according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al*., 1997) (Appendix A-1). DNA was isolated and purified according to the method of Saito and Miura

(1963). The G+C content was determined by the method of Tamaoka and Komagata (1984) using a reverse-phase high performance liquid chromatography (HPLC). The 16S rRNA gene sequences consisting of more than 1,200 bp were PCR amplified with primers D30F (5'-ATTCCGGTTGATCCTGC-'3, positions 6-12 according to the *Escherichia coli* numbering system) and D56R (5'-CTTGTTACGACTT-'3, position 1492-1509). The amplified DNA fragment was separated by agarose gel electrophoresis and recovered by using GenElute Minus EtBr Spin Column (Sigma). The sequence was determined using BigDye Terminator Cycle Sequencing Ready Reaction kit (ver. 3.0: Applied Biosystems) in the ABI PRISM 310 Genetic analyzer (Applied Biosystems) with the following primers: D30F (5'-ATTCCGGTTGATCCTGC-'3, positions 6-12), D33R (5'-TCGCGCCTGCGCCC CGT-'3, positions 344-360), D34R (5'GGTCTCGCTCGTT GCCTG-'3, positions 1096-1113), and D56R (5'-CTTGTTACGACTT-'3, position 1492-1509). The sequence was compared to reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center for Biotechnology Information database using the blast search. The alignment was subjected to phylogenetic analysis with the neighbour-joining (Saitou and Nei, 1987). Confidence in the branching pattern was assessed by analysis of 1,000 bootstrap replicates (Felsenstien, 1985).

Localization of the histamine-degrading activity in HDS 3-1

The localization of the histamine-degrading activity was determined by using the method of Vyazmensky *et al*. (2000) with some modifications. Ten mL of cultured broth were prepared as previously described. The cell suspension was centrifuged at 15,000 ×*g* for 30 min and supernatant was collected and referred as to the extracellular fraction. To obtain membrane and intracellular fractions, the cells were washed twice with 4.5 M NaCl. The cells were resuspended in 50 mM Tris-HCl, pH 7.0 containing 3.5 M KCl at a ratio of 1:1 (w/v) and sonicated for a total of 2 min by a Vibra Cell VCX60 (Sonics and Materials Inc., USA). The sample was placed in ice and sonicated for 20s, followed by a 40s rest interval. The supernatant was collected by centrifugation at 15,000 ×*g* for 30 min. The obtained fraction was

referred as to the intracellular fraction. The membrane pellet was washed twice with 50 mM Tris-HCl buffer (pH 7.0) containing 3.5 M KCl and then extracted with 2% Triton X-100 in the same buffer at a ratio of 6 mg of Triton X-100 per mg of protein by stirring for 45 min at 4°C. The membrane associated fraction was collected by centrifugation at 15,000 ×*g* for 30 min at 4°C. All fractions were stored at 4°C until used.

Enzyme activity assay

Histamine-degrading activity was determined by measuring the decreased amount of histamine after reaction. The sample with an appropriate dilution (100 µL) was mixed with 0.9 mL of reaction mixture consisting of 55 mM Tris-HCl, pH 7.0, 5.0 M NaCl, 5.0 mM of histamine dihydrochloride (free-base), and 555 µM of 1-methoxy PMS. The reaction was incubated at 37°C for exactly 5, 10, 15, 20, 30, 60, and 90 min and immediately stopped the reaction by adding 1 mL of 0.1 N HCl. The concentrations of histamine were estimated by HPLC (Appendix B-2) and the fluorometric method of AOAC (1995) (Appendix B-1). A blank was run in the same manner, except the sample was added after addition of 0.1 N HCl. One unit of enzyme activity was defined as the amount of enzyme that catalyzed a reduction of 1 µmole of histamine per min under the specified conditions.

Protein determination

Protein concentration was determined by the method of Bradford (1976). Bovine serum albumin was used as a standard.

Characterization of the histamine-degrading activity of selected strain

Substrate preference

The substrate preference of the crude enzyme on various amines degradation including tryptamine, β-phenylethylamine, putrescine, cadaverine, tyramine, spermidine, and spermine was determined by replacing histamine in the reaction mixture. The reactions were determined at 45°C in an incubator shaker with a speed of 200 rpm for 1 h and immediately stopped by adding 1 mL of 0.4 M perchloric acid. The mixture was centrifuged at 10,000 ×*g* for 15 min. Blank was done in the same manner, except the crude enzyme was added after addition of 1 mL 0.4 M perchloric acid. The content of biogenic amines in reaction mixture and blank were extracted, derivatized according to the procedure of Eerola *et al*. (1993) with a slight modification, and determined by a HPLC as described above (Appendix B-2).

Effect of salt concentration on activity and stability

To remove salt, crude enzyme was dialyzed against 100 volumes of 50 mM Tris-HCl buffer, pH 7.0 at 4°C. Desalted sample was subjected to activity determination in the presence of NaCl at various concentrations (0-5 M) using the standard assay conditions. To study the effect of NaCl on stability, the desalted sample was dialyzed against 100 volumes of buffers containing different NaCl concentrations (0-5 M) and incubated at room temperature for 24 h. The remaining activity was assayed using the standard assay conditions.

Effect of pH on activity and stability

The activity was assayed at 37°C over the pH range of 4-9 (50 mM acetate buffer (pH 4.0-5.5); 50 mM phosphate buffer (pH 6.0-6.5); and 50 mM Tris-HCl buffer (pH 7.0-9.0)) in the presence of 4.5 M NaCl. To study the effect of pH on stability, enzyme activity was determined with the same buffers, but their concentrations were changed to 100 mM. The enzyme was mixed with buffer in an

equal volume in the presence of 4.5 M NaCl and incubated for 24 h at 30°C. The remaining activity was assayed using the standard assay conditions.

Effect of temperature on activity and stability

Enzyme activity was assayed at different temperatures in the range of 30-80°C as previously described. To study the effect of temperature on enzyme stability, the dialyzed enzyme in 50 mM Tris-HCl buffer, pH 7.0 was incubated at various temperatures ranging from 4-80°C for 1 h in the absence and presence of 4.5 M NaCl. Thereafter, the enzyme mixture was immediately cooled in an ice bath, and the remaining activity was assayed under the standard assay conditions. Relative activity of the enzyme was calculated, in comparison with that without heating.

Statistic analysis

All experiments were run in triplicate. A completely randomized design was used throughout this study. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows: SPSS Inc.).

2.4 Results and Discussion

Screening of histamine-degrading halophilic archaea

A total of 156 halophilic archaea strains were isolated from various salt-fermented fishery products made in Thailand. Of these strains, 60 strains exhibited histamine degradation activity when cultivated in halophilic medium containing histamine. HDS3-1 from 3-month fermented fish sauce exhibited the highest histamine-degrading activity followed by HDS1-1, HPC1-2, and HIS40-3, respectively (Figure 1). Therefore, these 4 strains were selected for further studies.

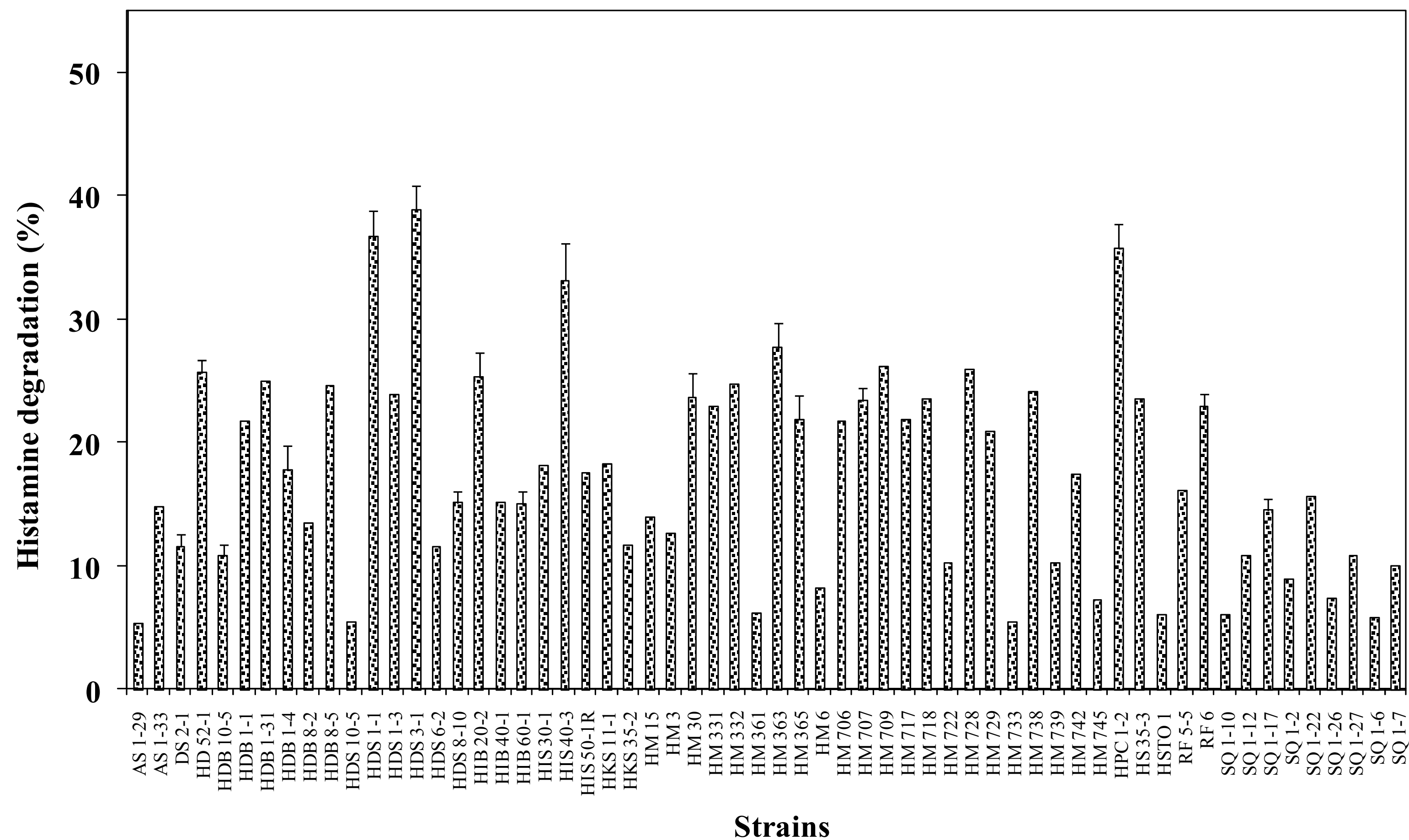


Figure 9. Strains exhibiting the potential to degrade histamine by growing in halophilic liquid medium containing 5 mM histamine (free-base) and incubated at 37°C for 7 days. Bars represent the standard deviation (n = 3).

The ability of microorganisms to degrade histamine has been mainly observed among members of *Lactobacillus*, *Micrococcus*, *Klebsiella*, and *Staphylococcus* (Gardini *et al*., 2002; Leuschner *et al*., 1998; Ishizuka *et al*., 1993; Yamashita *et al*., 1993). However, histamine-degrading activity from these microbial sources might decline rapidly due to high salt content (25% NaCl) and acidic pH (5.5) of fish sauce. Therefore, the addition of these microorganisms as a source of histamine-degrading enzyme might not be an effective measure to promote histamine degradation. To our knowledge there was only one study that demonstrated the histamine-degrading activity of *Virgibacillus halodenitrificans* SK33, moderately halophilic bacteria isolated from Thai fish sauce under the high salt condition (Yongsawatdigul *et al*., 2007). Although 50% of histamine level was reduced, the addition of this strain in fish sauce fermentation resulted in the increase in tyramine content almost 2 times higher than control without inoculation during the course of 4-month fermentation.

Identification of the selected strains

All selected strains showed characteristics of extremely halophilic archaea that required salt higher than 1.7 M NaCl to prevent cell from lysis and showed the maximum growth rates at 2.6-4.3 M NaCl. The basic characteristics of isolates were summarized in Table 6. Analysis of 16S rRNA sequences of these selected strains indicated that all isolates were in the phylogenetic branch of the *Halobacteriales*. Strain HPC1-2 was placed with the genus *Halobacterium*, the closest database relative of which was *Halobacterium salinarum* DSM 3754^T with 99.2% sequence similarity. Strain HPC1-2 has been recently designated *Halobacterium piscisalsi* (Yachai *et al*., 2008). On the other hand, strains HDS3-1, HDS1-1, and HIS40-3 were placed in a single cluster affiliated to the genus *Natrinema*, the closest database relative of which were *Natrinema pallidum* NCIMB 777^T with 99.1, 98.1, and 99.1% sequence similarity, respectively. Phenotypic, chemotaxonomic, and genetic studies suggest that strain HIS40-3 and HDS3-1 constitute a new species, for which the name *Natrinema gari* was proposed (Tapingkae *et al*., 2008). However, strain HDS1-1 is distinguished from strains HIS40-3 and HDS3-1 in some phenotypic

characteristics as indicated by the results in Table 6. In this point, it is not certain if strain HDS1-1 can be classified into the same species as strains HIS40-3 and HDS3-1 because of insufficient data such as other genotypic and chemotaxonomic characteristics. Based on the results, strain HDS1-1 would be another novel member of this genus. Further work on DNA-DNA homology, genotypic and chemotaxonomic characteristics are under investigation for the determination of the specie. The 16S rRNA gene sequences of strain HDS1-1 was deposited in the GenBank database under accession number AB289742 (Figure 10).

Amino acid decarboxylation

The amine producing capacity of these strains was tested in halophilic liquid medium supplemented with 1% (w/v) of the corresponding amino acids substrates. No formation of histamine, tryptamine, β-phenylethylamine, putrescine, cadaverine, tyramine, spermidine or spermine was detected at neither 7 nor 14 days of incubation (data not shown). The ability of microorganisms to decarboxylate amino acids is highly variable depending on species, strain, and environmental conditions (Coton *et al*., 1998). Due to the direct toxic effect of biogenic amines (Bardocz, 1995), it was important that no undesirable compounds especially biogenic amines are formed by the use of these selected microorganisms. Since, HDS3-1 was exhibited highest histamine-degrading activity among strains tested and was not formed any tested biogenic amines. Therefore, HDS3-1 was selected for further study.

Cytotoxicity test

The cytotoxic potential of strain HDS3-1 in Caco2, HepG2, and HEp-2 cells *in vitro* were assessed by measuring total cellular metabolic activity using the MTT assay (Table 7). The MTT assay has been applied successfully to detect toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. (Pedersen *et al*., 2002). From the cytotoxicity profiles, the whole cell extract of strain HDS3-1 was found to be nontoxic to Caco2, HepG2, and HEp-2 cells at all level tested.

Table 6. Some characteristics of strain HIS40-3, HDS1-1, HDS3-1 and HPC1-2

Characteristics	Strain			
	HIS40-3	HDS1-1	HDS3-1	HPC1-2
NaCl concentration (M) required to prevent cell lysis	> 1.7 M	> 2.1 M	> 1.7 M	> 1.7 M
NaCl optimum (M)	2.6-3.4	2.6-3.4	2.6-3.4	3.4-4.3
NaCl requirement (M)	≥ 1.7 M	≥ 2.1 M	≥ 1.7 M	≥ 2.6 M
Optimum temperature for growth (°C)	37-40°C	37-40°C	37-40°C	37-40°C
pH range for growth	5.5-8.5	5.5-8.5	5.5-8.5	5.0-8.0
Optimum pH for growth	6.0-6.5	6.5-7.0	6.0-6.5	7.0-7.5
Anaerobic growth in the presence of nitrate	-	-	-	+
Reduction of nitrate to nitrite	-	-	-	-
Catalase activity	w	w	w	w
Oxidase activity	+	-	+	+
Gelatin hydrolysis	+	-	+	+
Tween 80 hydrolysis	-	-	-	s
Anaerobic growth in the presence of DMSO*	+	+	+	+
DNA G + C content (mol%)	65.4	65.6	64.0	65.5

+, positive; -, negative; s, strong; w, weak

*DMSO (dimethylsulfoxide)

Localization of histamine-degrading activity from *Natrinema* sp. strain HDS3-1

To examine the cellular location of histamine-degrading activity of HDS3-1, extracellular, intracellular, and membrane-bound fractions were assayed in the presence and absence of 1-methoxy PMS. By using HPLC and AOAC methods, the histamine content in the reaction mixture decreased about one-third of its initial concentration within 30 min and retained constant until the end of 90 min incubation (Figure 11).

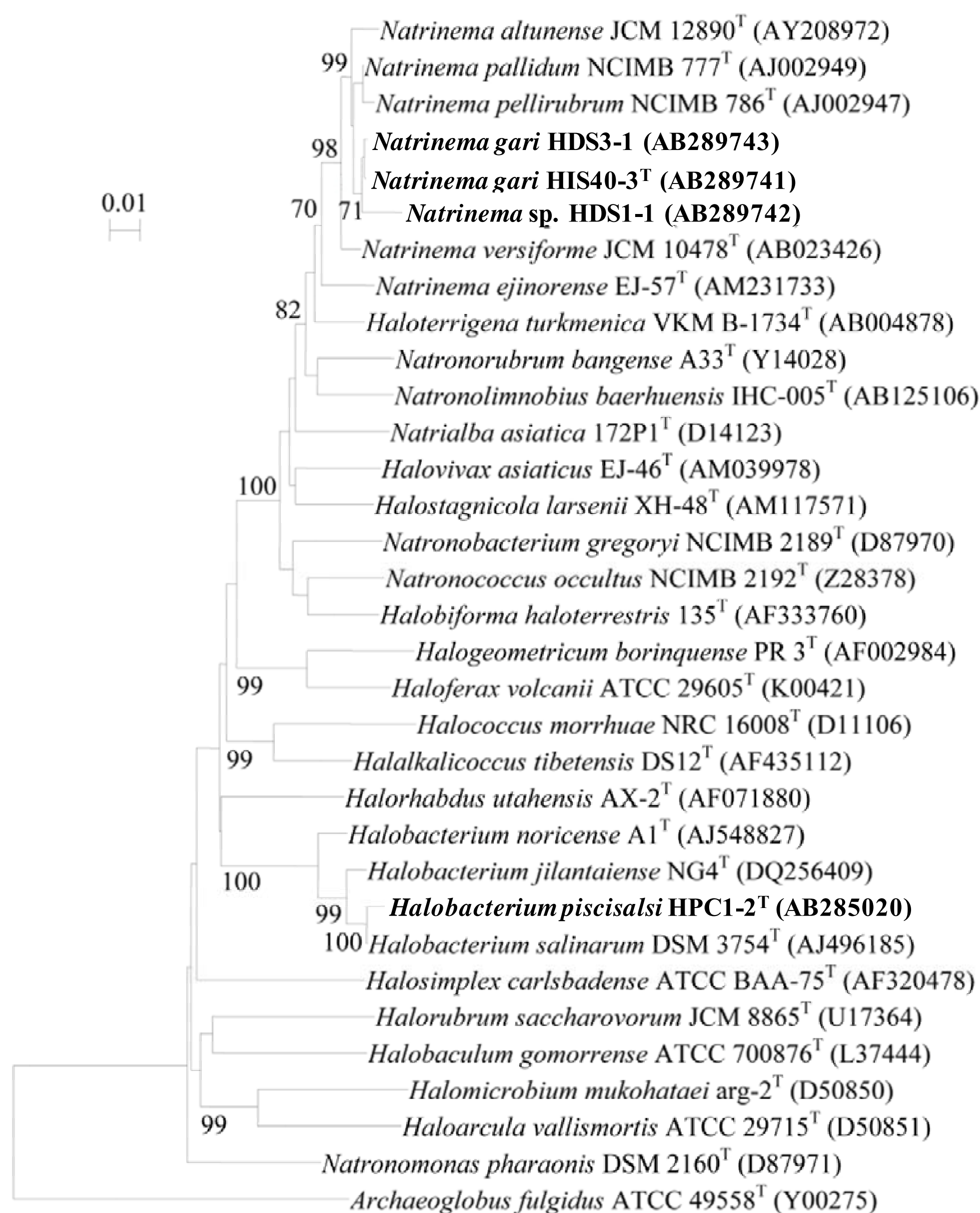


Figure 10. Phylogenetic tree showing the relationships between strain HIS40-3, HDS1-1, HDS 3-1, HPC1-2, and related bacterial species, based on 16S rRNA gene sequences. The branching pattern was generated by the neighbor-joining method. Bootstrap values higher than 70 out of 100 subreplicates are indicated at the respective bifurcations. The bar represents 0.01 substitutions per nucleotide.

Table 7. Percentage of cell line survival after treating with whole cell extract of strain HDS3-1 compared with control (cell line without whole cell extract of strain HDS3-1)

Target cell line	Whole cell extract of strain HDS3-1 (µg/mL)								IC_{50} (µg/mL)
	250	125	62.5	31.25	15.625	7.8125	3.906	1.953	
Caco-2	71±0.3	88±0.2	79±0.1	87±0.5	85±0.2	90±0.2	92±0.2	98±0.1	>250
HepG2	81±0.4	83±0.1	87±0.5	92±0.2	96±0.1	97±0.2	95±0.2	92±0.1	>250
HEp-2	56±0.2	57±0.2	63±0.3	61±0.1	69±0.1	74±0.3	84±0.2	99±0.9	>250

Abbreviation: human colon adenocarcinoma (Caco-2), human liver hepatocarcinoma (HepG2) and human larynx epithelial (HEp-2) cells.

The indication of toxicity has been evaluated in 2 ranges:

a) At % survival >50% will be evaluated for no toxicity
b) At % survival <50% will be evaluated for toxicity with IC_{50}

Histamine content did not decrease in the absence of 1-methoxy PMS, indicating that the intracellular fraction of HDS3-1 did not use molecular of oxygen as an electron acceptor. Since histamine-degrading activity was found in an intracellular fraction of HDS3-1 only in the presence of 1-methoxy PMS, the result suggested a link to the dehydrogenase activity. In other microorganisms, amine dehydrogenases were found to be localized in the various locations of the cells; the periplasmic space, membrane, as well as cytoplasmic space. Amine dehydrogenase is known to be enzyme that catalyzes the oxidative deamination of amines to its corresponding aldehydes plus ammonia. Various amine dehydrogenases have been reported so far mostly in prokaryotes such as *Methylomonas* sp. (Matsumoto, 1978), *Paracoccus denitrifycans* (Husain and Davidson, 1987), *Pseudomonas aeruginosa* and *Citrobacter freundii* (Hisano *et al*., 1990), *Alcaligenes faecalis* (Chistoserdov, 2001), *Nocardioides simplex* (Siddiqui *et al*., 2000), and *Alcaligenes xylosoxidans* (Kondo *et al*., 2004). Moreover, these enzymes were found to be both constitutive and inducible. Although many bacteria harbor the enzymes for primary amine deamination, the growth of bacteria with some primary amines as a sole source of carbon and energy has not been shown to be successful, due to the toxicity of the aldehydes generated during the oxidation processes.

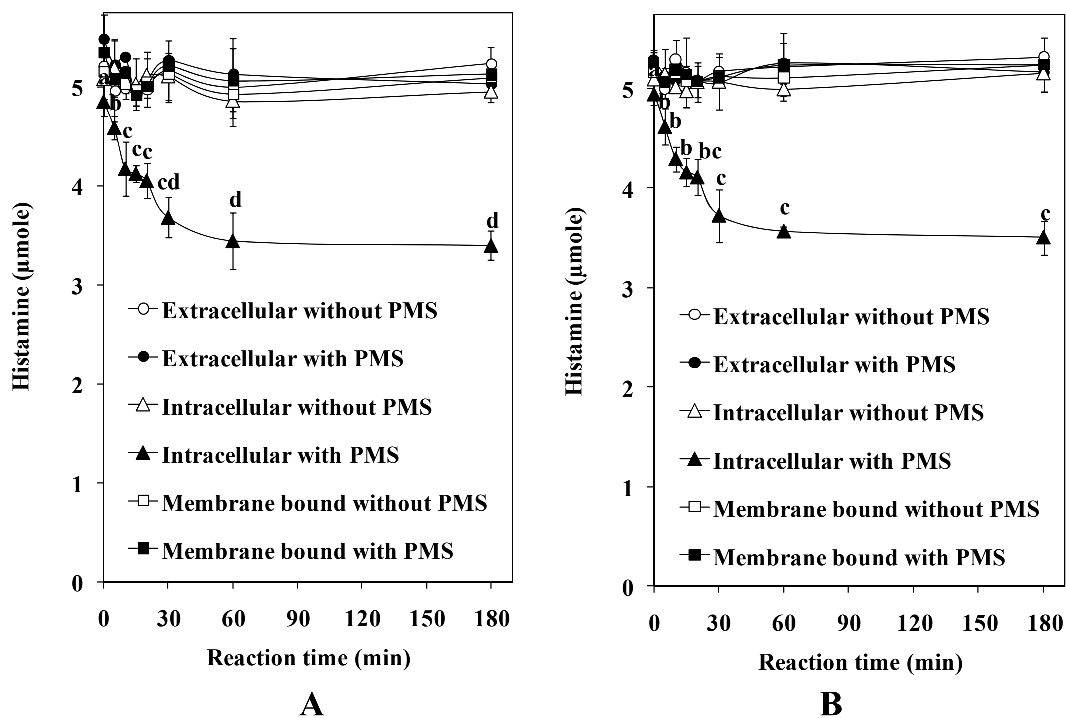


Figure 11. Time course of histamine-degrading activity from various cellular fractions of HDS3-1 measured by the HPLC (A) and AOAC fluorometric detection (B). The different letters in the same line denote the significant differences ($P<0.05$). Bars represent the standard deviation (n = 3). The degradation of histamine was analyzed for 180 min at pH 7.0 and 45°C in the presence of 4.5 M NaCl. The histamine-degrading activity for the intracellular fraction was 65.0 ± 12.8 Units/mL (38.4 ± 7.5 Units/mg).

Characterization of the histamine-degrading activity from the intracellular fraction of HDS3-1

Substrate preference

Figure 12 shows that the intracellular fraction of the selected strain preferably catalyzed histamine rather than other amines tested. Relative activities of 20% or less were observed for other biogenic amines. Many amine dehydrogenases found in various bacteria showed different specificity. The purified amine dehydrogenase from *Pseudomonas* AM1 was found to have a wide specificity, oxidizing many compounds that contain a primary amino group, notably ethanolamine and histamine (Eady and Large, 1968). Whereas, the purified histamine dehydrogenase from *Nocardioides simplex* IFO 12069 showed high activity toward histamine, but it still oxidized agmatine and putrescine at a rate of 30% compared to histamine (2000). Among 20 kinds of biogenic amines including histamine studied by Bakke *et al*. (2005), the purified recombinant histamine dehydrogenase from *Rhizobium* sp. 4-9 was very specific toward histamine and only oxidized agmatine and 1,3-diaminopropane with the rate of 10 and 13% of histamine, respectively. Fujieda *et al*. (2004) indicated that the clear difference between histamine dehydrogenase and other amine dehydrogenases is the substrate specificity. Histamine dehydrogenase is active almost exclusively toward histamine, while low activity is observed for other amines. Therefore, the histamine-degrading activity of HDS3-1 was thought to be mediated by histamine dehydrogenase.

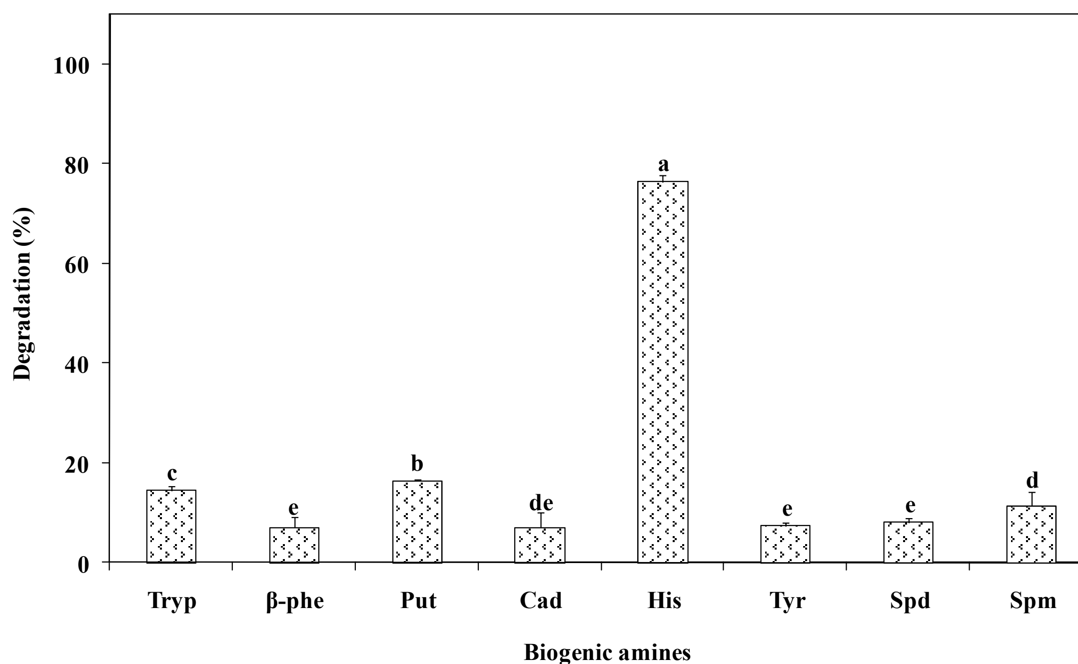


Figure 12. Biogenic amines degradation by an intracellular fraction of HDS3-1. Reaction was carried out in the presence of 4.5 M NaCl at 45°C, pH 7 under shaking condition (200 rpm) for 1 h. The different letters in the column denote the significant differences (*P*<0.05). Bars represent the standard deviation (n = 3). Tryp; tryptamine, β-phe; β-phenylethylamine, Put; putrescine, Cad; cadaverine, His; histamine, Tyr; tyramine, Spd; spermidine, and Spm; spermine. Bars represent the standard deviation (n = 3).

Effect of salt concentration on activity and stability

The highest histamine-degrading activity of the intracellular fraction from HDS3-1 was observed in the presence of NaCl at the concentration range of 3.5-5 M (Figure 13A). In the absence of salt, activity was less than 20% of that under optimum conditions. This result clearly indicated that the histamine-degrading activity was salt-dependent. The histamine-degrading activity was stable for at least 24 h when incubated in the presence of NaCl at the concentration higher than 2.5 M (Figure 13B). Less than 50% of the residual activity was detected at the NaCl concentrations below 1 M. In general, many halophilic enzymes require the presence of NaCl concentrations in the range of 1-4 M for optimum activity and stability (Mevarech *et al*., 2000). On the basis of salt requirement, it was also noticed that the histamine-degrading activity of HDS3-1 could be regained upon reestablishment of the high salt concentration, similar to several halophilic enzymes of various extreme halophiles (Boutaiba *et al*., 2006; Namwong *et al*., 2006; Martínez-Espinosa *et al*., 2005; Homes *et al*., 1997; Bonete *et al*., 1996). Halophilic proteins employ different adaptation mechanisms. Proteins from halophilic organisms have a biased amino acid composition in order to remain stable and active at high ionic strength. Halophilic proteins typically have an excess of acidic amino acids (i.e. glutamate and aspartate) on their surface (Boutaiba *et al*., 2006; Namwong *et al*., 2006; Martínez-Espinosa *et al*., 2005; Homes *et al*., 1997; Bonete *et al*., 1996). Negative charges on the halophilic proteins bind significant amounts of hydrated ions, thus reducing their surface hydrophobicity and decreasing the tendency to aggregate at high salt concentration. At the molecular level, this probably suggested the high flexibility of protein structure that allowed fast change from the fully active form in the presence of salt to an inactive form in the absence of salt.

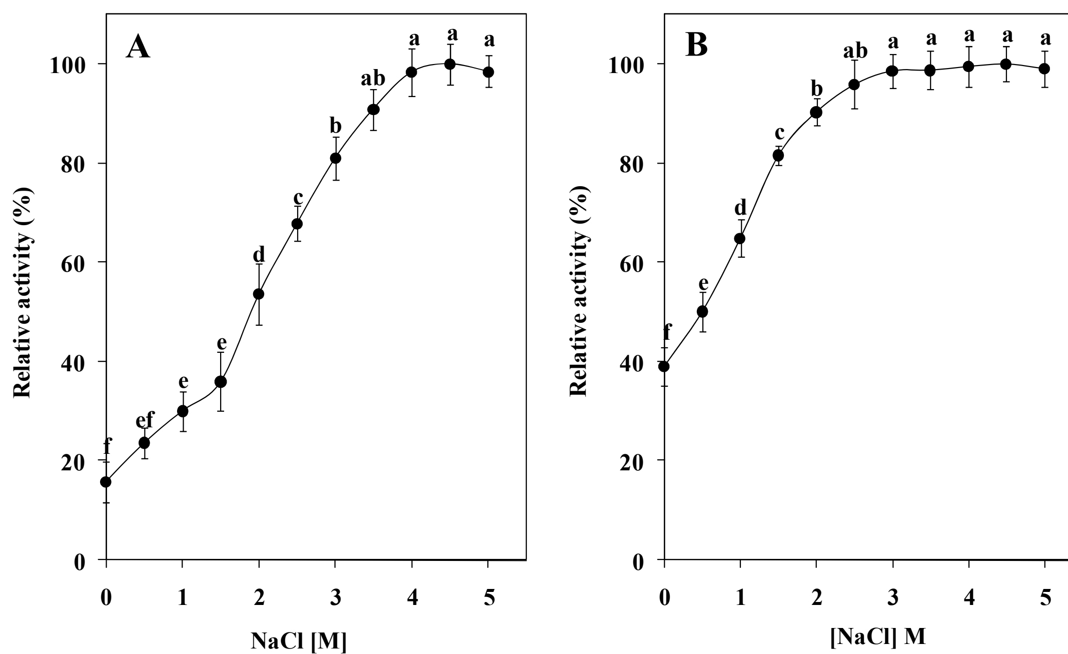


Figure 13. NaCl concentration profile (A) and stability (B) of histamine-degrading activity from HDS3-1. The different letters in the same line denote the significant differences ($P<0.05$). Bars represent the standard deviation (n = 3). (A) Residual activity was analyzed using histamine as a substrate for 30 min at pH 7.0 and 37°C at the various NaCl concentrations. (B) For the stability test, the enzyme was incubated at 30°C for 24 h in 50 mM Tris-HCl buffer (pH 7.0) contained NaCl at various concentrations. Residual activity was analyzed using histamine as a substrate for 30 min, pH 7.0 in the presence of 4.5 M NaCl at 37°C.

Effect of pH on activity and stability

The highest histamine-degrading activity was observed in the pH range of 6.5-8 (Figure 14A). At pH 4-4.5, no activity was detected but as the pH increased the activity increased rapidly until reaching the maximum. Above pH 8 the activity decreased, although at pH 9, it was still over 80% of highest activity. Nevertheless, the pH optimum of the histamine dehydrogenase from the selected strain was lower than those from *Alcaligenes xylosoxidans* (pH 8.0) (Kondo *et al*., 2004), *Nocardioides simplex* IFO 12069 (pH 8.5) (Siddiqui *et al*., 2005), and *Rhizobium* sp. 4-9 (pH 9.0) (Bakke *et al*., 2005). The histamine-degrading activity was stable for at least 24 h when incubated in the buffers at pH 6.5-9 (Figure 14B). However, the activity gradually decreased under acidic condition in which the residual activity was lower than 50% at pH below 5 which reflected a pH dependent stability of the enzyme. The stability of the enzyme over the wide range of pH was similar to aromatic amine dehydrogenase from *Alcaligenes xylosoxidans* (pH 5-12) (Kondo *et al*., 2004), methylamine dehydrogenases from *Methylobacterium extorgquens* AM1 (pH 2.6-10.6) (Eady *et al*., 1968), *Methylomonas* sp. (pH 4.4-10.6) (Matsumoto, 1978), and *Paracoccus denitrificans* (pH 3.0-10.5) (Husain and Davidson, 1987).

Effect of temperature on activity and stability

The highest histamine-degrading activity was observed at 40-55°C (Figure 15A). The enzyme retained more than 50% of the highest activity at 80°C which probably reflected the thermal stability of the enzyme. The enzyme activity was quite stable at 50°C, with residual activities of 95 and 80% for incubation with and without 4.5 M NaCl, respectively. However, at higher temperatures, the effect of NaCl was more marked as demonstrated by the divergence of the two profiles (Figure 15B). No activity was detected in the salt free preparation incubated at 100°C for 1 h while the preparation incubated with NaCl still had a residual activity of 22%. These results clearly show that the thermal stability of the histamine-degrading activity of HDS3-1 increased in the presence of NaCl, similar to most of the enzymes from the halophilic archaea.

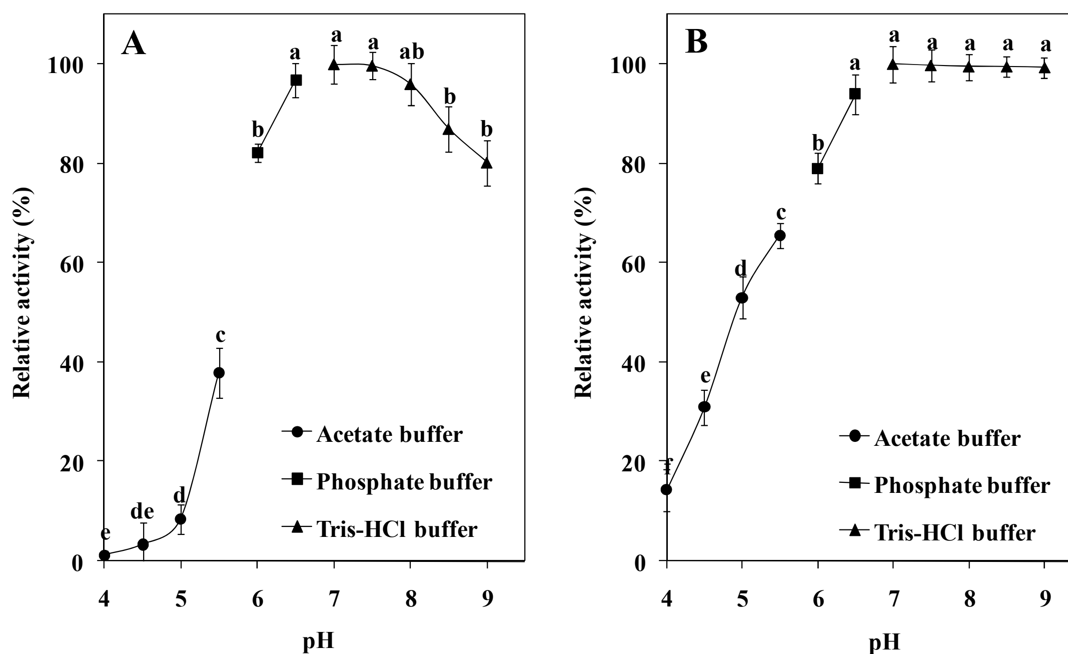


Figure 14. pH profile (A) and stability (B) of histamine-degrading activity of HDS3-1. The different letters in the same line denote the significant differences (*P*<0.05). Bars represent the standard deviation (n = 3). (A) Residual activity was analyzed using histamine as a substrate for 30 min at 37°C in the presence of 4.5 M NaCl at various pHs. (B) For the stability test, the enzyme was incubated at 30°C for 24 h in the desired pH buffer at various pH values in the presence of 4.5 M NaCl. Residual activity was analyzed using histamine as a substrate for 30 min at 37°C, pH 7.0 in the presence of 4.5 M NaCl.

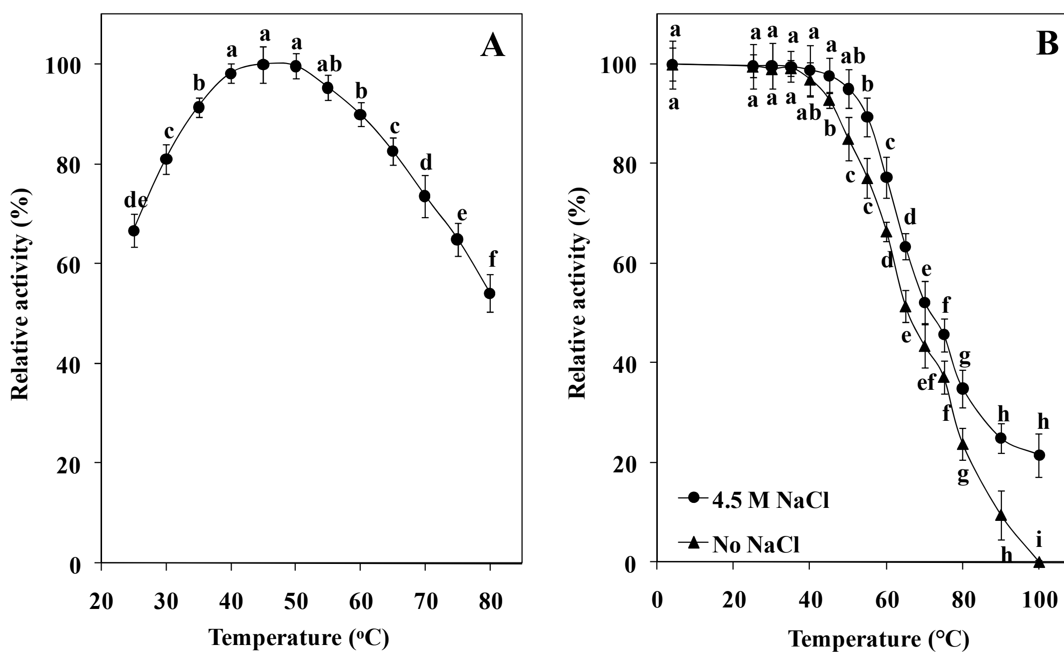


Figure 15. Temperature profile (A) and thermal stability (B) of histamine-degrading enzyme from HDS3-1. The different letters in the same line denote the significant differences (P<0.05). Bars represent the standard deviation (n = 3). (A) Residual activity was analyzed using histamine as a substrate for 30 min at pH 7.0 in the presence of 4.5 M NaCl at various temperatures. (B) For the stability test, the enzyme was incubated for 1 h at the temperatures indicated in 50 mM Tris-HCl buffer (pH 7.0) in the presence or absence of 4.5 M NaCl, and then cooled on ice. Residual activity was analyzed using histamine as a substrate for 30 min at pH 7.0 in the presence of 4.5 M NaCl at 45°C.

The effect of salt on the thermal stability of enzymes was variable in non-halophilic archaea. However, it was frequently found that thermal stability of both intracellular and extracellular enzymes of the halophilic archaeons could be improved by NaCl (Obón *et al*., 1996; Bhatnagar *et al*., 2005). Reversible refolding after high temperature treatment has been demonstrated for the β-lactamase of the moderately halophilic eubacterium *Chromohalobacter* by Tokunaga *et al*. (2004), who claimed that irreversible aggregation was prevented by the abundance of acidic amino acids, which in turn is a characteristic feature of halophilic enzymes (Madern *et al*., 2000).

2.5 Conclusion

Halophilic archaeon, strain HDS3-1, isolated from fish sauce exhibited the ability to degrade histamine in hypersaline condition. The histamine-degrading activity of HDS3-1 was located in the intracellular fraction, required the presence of an electron acceptor, and highly specific to histamine. The histamine-degrading activity was highest at pH 6.5-8, in the presence of NaCl at 3.5-5 M, and at 40-55°C. The activity was found to be stable pH 6.5-9, in the presence of NaCl above 2.5 M, and temperature lower than 50°C. The results suggested a link of histamine-degrading activity of HDS3-1 to the presence of salt-tolerant and thermo-neutrophilic histamine dehydrogenase.

CHAPTER 3

NATRINEMA GARI SP. NOV., A HALOPHILIC ARCHAEON ISOLATED FROM FISH SAUCE IN THAILAND

3.1 Abstract

Two Gram-negative rod-shaped halophilic archaea, designated strains HIS40-3^T and HDS3-1, were isolated from anchovy fish sauce (nam-pla) collected from two different locations in Thailand. The two strains were able to grow at 20-60°C (optimum 37-40°C), at 1.7-5.1 M NaCl (optimum 2.6-3.4 M NaCl) and at pH 5.5-8.5 (optimum pH 6.0-6.5). Hypotonic treatment with less than 1.7 M NaCl caused cell lysis. The major polar lipids of the isolates were $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, two glycolipids and one unidentified lipid. The DNA G+C contents were 64.0-65.4 mol%. In addition to phenotypic and chemotaxonomic characteristics, phylogenetic analysis based on 16S rRNA gene sequence similarities showed that strains HIS40-3^T and HDS3-1 were related most closely to species of the genus *Natrinema*. Level of 16S rRNA gene sequence similarity between strains HIS40-3^T and HDS3-1 and the type strains of recognized *Natrinema* species were 99.1-96.6%. The two novel strains could be distinguished from the recognized *Natrinema* species on the basis of low levels of DNA-DNA relatedness and differences in whole-cell protein patterns and phenotypic properties. Levels of 16S rRNA gene sequence similarity and DNA-DNA relatedness between the two strains were 99.7 and 77.7%, respectively, suggesting that they should be classified as representing a single species. Based on these taxonomic data, strains HIS40-3^T and HDS3-1 are considered to represent a novel species of the genus *Natrinema*, for which the name *Natrinema gari* sp. nov. is proposed. The type strain is HIS40-3^T (=BCC 24370^T =JCM 14663^T =PCU 303^T).

3.2 Introduction

Fish sauce (nam-pla) is a traditional fermented fish product commonly used as a condiment in the South-East Asia. Apart from its unique and pleasant flavor, it provides an important supplementary source of nitrogen in the diet of people in this region. Fish sauce contains nitrogen at 20 g/L, of which 16% is present as amino acids (Phithakpol *et al*., 1995). Fish sauce contains a high concentration of NaCl, allowing various halophilic microorganisms to thrive (Lopetcharat *et al*., 2001; Tanasupawat and Komagata, 2001). Nevertheless, just a few types of bacteria have been isolated from fish sauce samples and subjected to taxonomic study. In Thailand, *Halobacterium salinarum* and *Halococcus thailandensis* have been isolated from fish sauce samples: these are extremely halophilic archaea that grow optimally at 20-25% (w/v) NaCl (Namwong *et al*., 2007; Thongthai *et al*., 1992). Other bacteria isolated from fish sauce are moderately halophilic microorganisms that grew optimally at 3-15% (w/v) NaCl, such as *Tetragenococcus halophilus*, *Tetragenococcus muriaticus*, *Halobacillus thailandensis* and *Lentibacillus juripiscarius* (Namwong *et al*., 2005; Thongsanit *et al*., 2002; Chaiyanan *et al*., 1999). At the time of writing, the genus *Natrinema* comprises five recognized species. *Natrinema pellirubrum* and *Natrinema pallidum* were created by McGenity *et al*. (1998) as a result of reclassification of strains of *Halobacterium salinarum* and *Halobacterium halobium*, respectively. On the basis of 16S rRNA gene sequence analysis, phenotypic properties and polar lipid composition, *Natrinema versiforme* (Xin *et al*., 2000) and *Natrinema altunense* (Xu *et al*., 2005) were also included within the genus. *Natrinema ejinorense* has been described more recently (Castillo *et al*., 2006). Here, we describe the taxonomic properties of two extremely halophilic archaea, strains HIS40-3^{T} and HDS3-1, isolated from fermented fish sauce, and these are suggested to represent a novel species of the genus *Natrinema*.

3.3 Materials and Methods

Isolation procedure, strains and growth conditions

Strains HIS40-3^T and HDS3-1 were isolated from the anchovy fish sauce fermented for 40 days collected from Samut Prakarn Province and an anchovy fish sauce sample fermented for 3 months collected from Samut Songkram Province, central Thailand, respectively. Samples were plated on agar plates of halophilic medium (Appendix A-1) and incubated at 37°C for 1-2 weeks. Pure culture was obtained by repeated transfers of separate colonies on agar plates of the same medium. *Nnm. pallidum* JCM 8980^T, *Nnm. pellirubrum* JCM 10476^T and *Nnm. altunense* JCM 12890^T were used as reference strains in all tests of phenotypic characteristics. Unless otherwise stated, strains were grown in halophilic broth (with shaking at 200 rpm) or on agar plates of the halophilic medium and cultivated at 37°C for 1-2 weeks.

Morphological, cultural and physiological characteristics

Phenotypic characterization was carried out in accordance with the recommended minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al*., 1997). Colony and cell morphology were examined for the cells grown on agar plates at 37°C for 14 days (Appendix A-4). Growth at various temperatures (20-60°C) was examined. NaCl requirement was determined in the halophilic medium containing various NaCl concentrations (0-5.1 M). Similarly, the requirement of the strains for Mg^{2+} was tested in halophilic medium without $MgSO_4.7H_20$ but supplemented with 0-1.0 M $MgCl_2$. Growth was determined by measuring culture turbidity at 600 nm.

Biochemical characteristics

Anaerobic growth was tested on agar plates in the presence of L-arginine (1 g/L), nitrate (1 g/L), or dimethylsulfoxide (DMSO) (10 g/L) (Appendix A-

5, A-6 and A-7). Production of indole (Appendix A-8) and reduction of nitrate and nitrite (Appendix A-7) were also tested. Utilization of sugars, alcohols, amino acids and organic acids and acid production were determined in modified Leifson medium supplemented with 0.01% (w/v) yeast extract and 4.3 M NaCl, but with casitone and Tris/HCl omitted (Leifson, 1963) (Appendix A-9). Oxidase (Appendix A-10) and catalase (Appendix A-11) activities and hydrolysis of casein, gelatin, starch and Tween 80 (Appendix A-12) were determined according to the methods of Barrow and Feltham (1993). Casamino acids were omitted from the test medium for determination of hydrolysis of gelatin and casein.

Sensitivity to antimicrobial agents

Determination of the antibiotic susceptibility of the strains was tested according to the methods described by Stan-Lotter *et al.* (2002). The detail of the sensitivity to antimicrobial agents is described clearly in Appendix A-13.

Quinones and polar lipids analyses

Menaquinones were analyzed as described by Komagata and Suzuki (1987). Polar lipids were determined according to the method of Minnikin *et al.* (1984). The details of the quinines and polar lipids analyses are described clearly in Appendix A-14, A-15, and A-16.

Sequencing of 16S rRNA

DNA was isolated and purified according to the method of Saito and Miura (1963) (Appendix A-17). The 16S rRNA gene sequences of the strains HIS40-3^T and HDS3-1, comprising of 1405 and 1353 bp, respectively, were amplified by PCR with primers D30F (5'-ATTCCGGTTGATCCTGC-3', positions 6-12 according to the *Escherichia coli* numbering system) and D56R (5'-CTTGTTACGACTT-3', positions 1492-1509). The detail of the 16S rRNA analysis is described clearly in Appendix A-18. The amplified DNA fragment was separated by agarose gel

electrophoresis and was recovered by using GenElute Minus EtBr Spin Column (Sigma). The sequence was determined by using a BigDye Terminator Cycle Sequencing Ready Reaction kit (v. 3.0; Applied Biosystems) in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) with the following primers: D30F, D33R (5'-TCGCGCCTGCGCCCCGT-3', positions 344-360), D34R (5'GGTCTCGCTCGTTG CCTG-3', positions 1096-1113) and D56R. The sequence was compared to reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center for Biotechnology Information database using BLAST searches. The alignment was subjected to phylogenetic analysis with the neighbour-joining (Saitou and Nei, 1987), maximum-likelihood and maximum-parsimony methods, by using programs in the CLUSTAL_X and MEGA 4 packages (Tamura *et al*., 2007; Thompson *et al*., 1997). Confidence in the branching pattern was assessed by analysis of 1,000 bootstrap replicates (Felsenstein, 1985).

DNA G+C content and DNA-DNA hybridization

The G+C content was determined by the method of Tamaoka and Komagata (1984) by using a reversed-phase HPLC (Appendix A-17). DNA-DNA hybridization was determined as reported by Ezaki *et al*. (1989) and levels of relatedness were determined by the colorimetric method as reported by Tanasupawat *et al*. (2000) (Appendix A-19).

3.4 Results and Discussion

Phenotypic characteristics

Cells of strains HIS40-3^T and HDS3-1 were motile, Gram-negative rods (0.5-0.8 × 2.0-3.0 μm) (Figure 16). Colonies formed on agar plates were circular (1-2 mm in diameter), smooth, translucent and pale-orange pigmented. The two strains were able to grow over a wide range of NaCl concentrations, from 1.7 M (approximately 10%, w/v) to 5.1 M (approximately 30%, w/v). Hypotonic treatment with less than 1.7 M NaCl caused cell lysis.

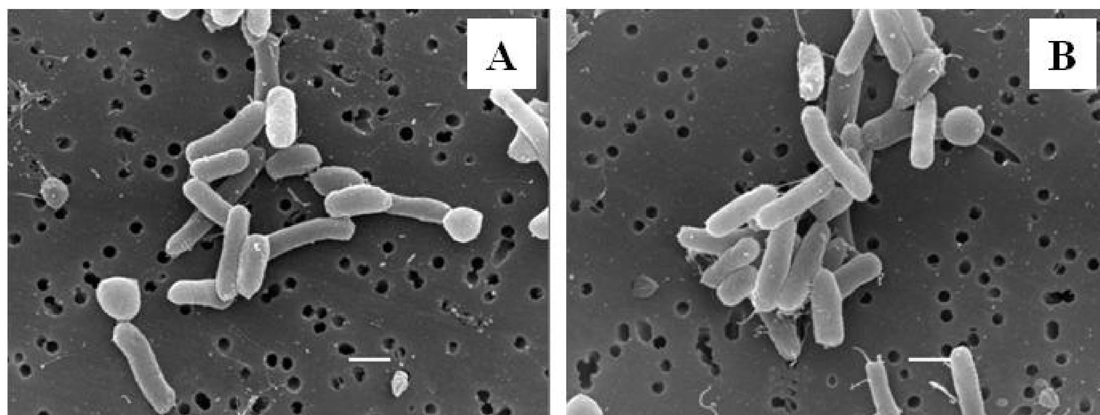


Figure 16. Scanning electron micrographs of cells of strain HIS40-3^T (A) and strain HDS3-1 (B) grown on halophilic medium at 37°C. Bars, 1µm.

Strains HIS40-3^T and HDS3-1 grew optimally in the presence of 2.6-3.4 M (15-20%, w/v) NaCl, similar to most halophilic archaea (Grant *et al.*, 2001). The strains grew over a wide range of $MgCl_2$ concentrations from 0-1.0 M and grew optimally at around 0.1-0.2 M. The two strains grew at 20-60°C (optimum 37-40°C) and at pH 5.5-8.5 (optimum pH 6.0-6.5). The two strains were positive for catalase and oxidase. Indole production from tryptophan was negative. Casein, starch and Tween 80 were not hydrolyzed. Gelatin was liquefied. The strains showed anaerobic growth in the presence of DMSO but not with nitrate or arginine. Nitrate was not reduced and gas formation was not observed.

Sensitivity to antimicrobial agents

The strains were sensitive to rifampicin (30 µg), bacitracin (10 µg) and novobiocin (5 µg). Strains HIS40-3^T and HDS3-1 utilized several carbohydrates. Among them, strong acid formation was observed only from glycerol for both strains and from arabinose for strain HIS40-3^T. There were some differences in the utilization of carbon sources between strains HIS40-3^T and HDS3-1. Some characteristics that distinguish strains HIS40-3^T and HDS3-1 from other members of the genus *Natrinema* are summarized in Table 8.

Table 8. Differential characteristics between strains HIS40-3^T and HDS3-1 and recognized *Natrinema* species

Strains: 1, strain HIS40-3^T; 2, strain HDS3-1; 3, *Nnm. pallidum* JCM 8980^T; 4, *Nnm. pellirubrum* JCM 10476^T; 5, *Nnm. altunense* JCM 12890^T (data in columns 1-5 from this study unless indicated); 6, *Nnm. versiforme* JCM 10478^T (data from Xin *et al*., 2000); 7, *Nnm. ejinorense* JCM 13890^T (Castillo *et al*., 2006). +, Positive; -, negative; +/-, doubtful; ND, no data available

Characteristic	1	2	3	4	5	6	7
Cell morphology	Rods	Rods	Rods	Rods	Rods	Pleomorphic	Pleomorphic
Motility	+	+	+	+	+	-	-
Pigmentation	Pale orange	Pale orange	Pale orange	Pale orange	Pale orange	Light-red	Light-red
NaCl concentration (M) required to prevent cell lysis	> 1.7	> 1.7	> 1.7	> 1.7	> 2.1	> 1.0	> 1.5
NaCl requirement (M)	≥ 1.7	≥ 1.7	≥ 1.7	≥ 1.7	≥ 2.1	≥ 1.5	≥ 1.8
NaCl optimum (M)	2.6-3.4	2.6-3.4	3.4-4.3	2.6-3.4	2.6-3.4	3.4-4.3	3.4
$MgCl_2$ optimum (M)	0.1-0.2	0.1-0.2	0.1-0.2	0.1-0.2	0.1-0.2	0.15	ND
Temperature for growth (°C)							
Range	20-60	20-60	25-60	20-60	20-60	20-53	25-50
Optimum	37-40	37-40	30-37	30-37	37-40	37-46	37
pH for growth							
Range	5.5-8.5	5.5-8.5	5.5-8.5	5.5-8.5	6.0-8.5	6.0-8.0	6.0-8.5
Optimum	6.0-6.5	6.0-6.5	7.0-7.5	7.0-7.5	6.5-7.0	6.5-7.0	7.0
Anaerobic growth in the presence of nitrate	-	-	+	-	+	+	-
Reduction of nitrate to nitrite	-	-	+	+	+	+	-
Gas formation from nitrate	-	-	-	-	-	+	+
Oxidase activity	+	+	+	-	-	+	+
Indole formation	-	-	-	-	-	+	-
Hydrolysis of:							
Gelatin	+	+	+	-	+	-	+
Starch	-	-	-	-	-	+/-	+
Tween 80	-	-	+	-	+	+	+
DNA G+C content (mol%)	65.4	64.0	63.9	62.9	65.6*	64.2	64.7

* Data from Xu *et al*. (2005).

From these results, newly isolated strains HIS40-3^T and HDS3-1 were classified as extremely halophilic aerobic archaea because (i) TLC of whole-organism methanolysates revealed that they contain ether-linked isoprenoid lipids, (ii) they could grow in the presence of antibiotics effective against eubacteria (ampicillin, kanamycin and chloramphenicol) but not in the presence of antibiotics effective against eukaryotes (pravastatin and anisomycin), (iii) they could grow aerobically only in the presence of high concentration of NaCl and lysed at low levels of salinity and (iv) they possessed bacterial rhodopsins (Sugiyama *et al*., 1994; Tateno *et al*., 1994) which have been found only in members of the order *Halobacteriales* (Grant and Larsen, 1989).

Quinones and polar lipids

Strains HIS40-3^T and HDS3-1 possessed two menaquinones, MK-8 (76.3 and 74.6%, respectively) and MK-8(H_2) (23.7 and 25.5%), which are commonly detected in species of the genus *Natrinema* (Xin *et al*., 2000; McGenity *et al*., 1998). Two-dimensional TLC revealed that the two novel strains possessed glycerol diether analogues of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP), phosphatidylglycerol sulfate (PGS), pigment (Pi), two glycolipids and one unidentified lipid as shown in Figure 17. The core lipids were $C_{20}C_{20}$ and $C_{20}C_{25}$ diether, as shown from the two PG and PGP spots. The presence of the two unidentified glycolipid spots shared by the two described *Natrinema* species would support assignment of strains HIS40-3^T and HDS3-1 to the genus *Natrinema*. These two glycolipids were also found in varying amounts in the reference stains *Nnm. pallidum* JCM 8980^T, *Nnm. pellirubrum* JCM 10476^T and *Nnm. altunense* JCM 12890^T (McGenity *et al*., 1998; Xu *et al*., 2005).

16S rRNA gene sequences and phylogenetic tree

The neighbour-joining phylogenetic tree constructed on the basis of 16S rRNA gene sequence data of the two new isolates and other representative *Natrinema* species is shown in Figure 18.

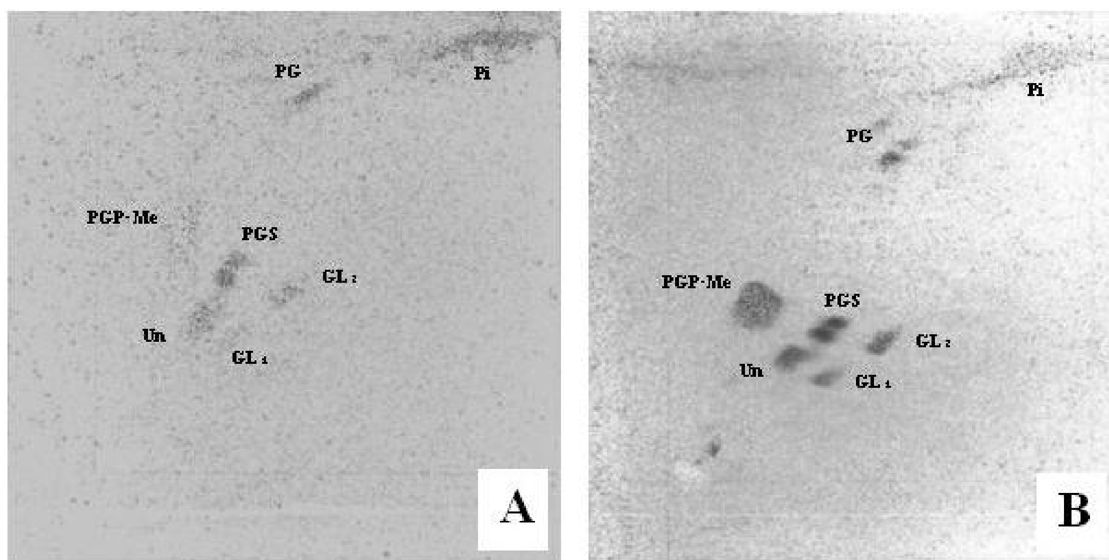


Figure 17. Two-dimensional TLC of polar lipids extracted from strain HIS40-3^T (A) and strain HDS3-1 (B). Separation of components was achieved by developing the plate with chloroform/methanol/water (65:25:4 by vol., horizontal direction) and then with chloroform/methanol/acetic acid/water (85:12:15:4 by vol., vertical direction). Spots were visualized by staining with anisaldehyde reagent and the plate was heated to 150°C for 10 min to show glycolipids and phospholipids. PG, Phosphatidyl-glycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; GL, unidentified glycolipid; Un, unknown lipid; Pi, pigment.

Strains HIS40-3^T and HDS3-1 formed a distinct cluster that fell within the genus *Natrinema*. 16S rRNA sequence similarity between the two novel strains was 99.7%, suggesting that they should be classified as representing a single species or as members of very closely related species. This is in accordance with the morphological and chemotaxonomic similarities detailed above.

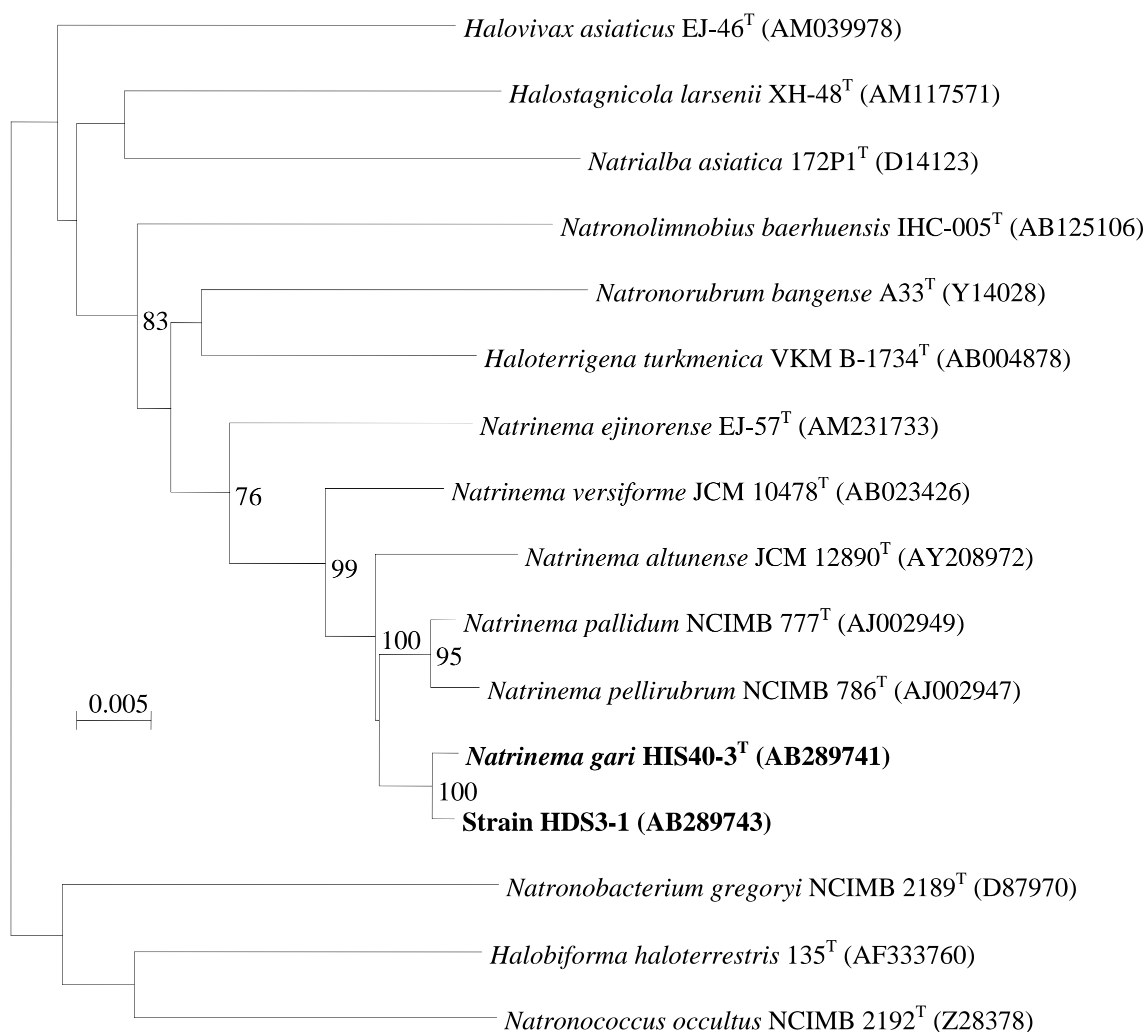


Figure 18. Phylogenetic trees showing the relationships between strains HIS40-3^T, HDS3-1 and related archaeal species based on 16S rRNA gene sequences. The branching pattern was generated according to the neighbour-joining method. Bootstrap values above 70%, based on 1000 replications, are shown at the nodes. Bar, 0.005 substitutions per 100 nucleotide positions.

The nearest neighbours of strains HIS40-3^{T} and HDS3-1 were *Nnm. pallidum* JCM 8980^{T} (99.1 and 99.1% 16S rRNA gene sequence similarity, respectively), *Nnm. pellirubrum* JCM 10476^{T} (98.7 and 98.7%), *Nnm. altunense* JCM 12890^{T} (98.5 and 98.5%), *Nnm. versiforme* JCM 10478^{T} (98.4 and 98.4%) and *Nnm. ejinorense* JCM 13890^{T} (96.6 and 96.7%).

DNA G+C content and DNA-DNA hybridization

The DNA G+C contents of strain HIS40-3^{T} and HDS3-1 were 65.4 and 64.0 mol%, respectively. In view of the unclear results of the methods described above, DNA-DNA hybridizations were carried out to clarify the taxonomic position of the strains. DNA-DNA homology values higher than 70% are generally accepted for a definition of species (Wayne *et al*., 1987). The DNA-DNA hybridization study revealed that strains HIS40-3^{T} and HDS3-1 were closely related, exhibiting levels of relatedness of 73.5-77.7% to each other; however, strains HIS40-3^{T} and HDS3-1 showed only low levels of DNA-DNA relatedness to *Nnm. pallidum* JCM 8980^{T} (40.5 and 42.5%, respectively), *Nnm. pellirubrum* JCM 10476^{T} (18.7 and 22.0%), *Nnm. altunense* JCM 12890^{T} (13.2 and 16.0%) and *Nnm. versiforme* JCM 10478^{T} (19.5 and 18.2%), indicating that these two novel strains are not members of any of these *Natrinema* species. Each value was obtained from two independent determinations.

Whole-cell proteins pattern

SDS-PAGE of whole-cell proteins can be used as a rapid method for distinguishing between bacterial species (Jackman, 1987). Strains HIS40-3^{T} and HDS3-1 had protein profiles that were very similar to each other (Figure 19). However, they did not resemble the profile of *Nnm. pallidum* JCM 8980^{T}.

This result differed from the 16S rRNA gene analysis and DNA-DNA hybridization results, which indicated that strains HIS40-3^{T} and HDS3-1 were most similar to *Nnm. pallidum* JCM 8980^{T}. Furthermore, the protein pattern of the novel strains was markedly different from those of the *Natrinema* species representatives.

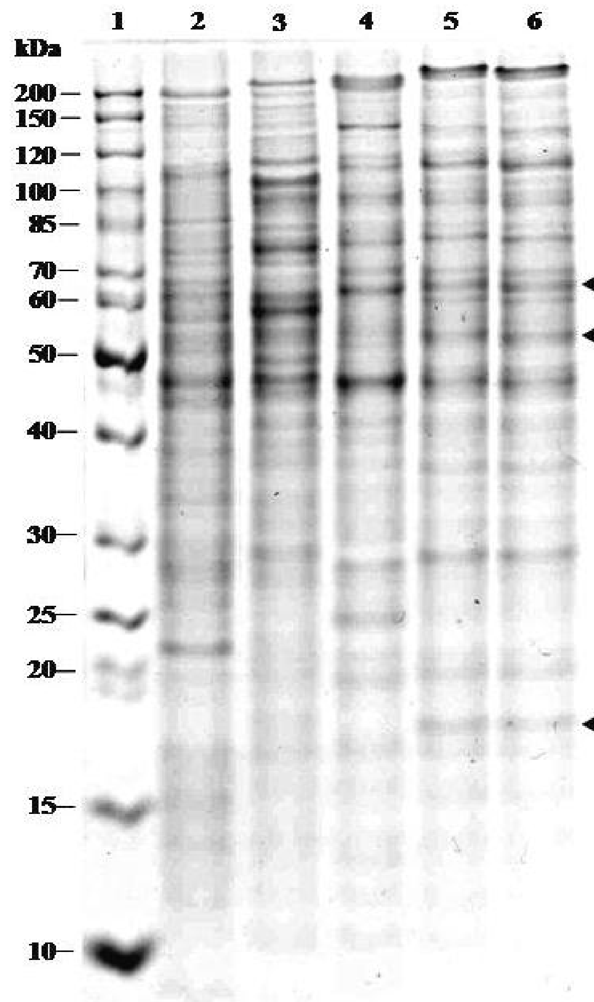


Figure 19. Whole-cell proteins extracted from strains HIS40-3^T, HDS3-1 and related strains of *Natrinema* species, following separation by SDS-PAGE (10%). Proteins were stained with Coomassie blue. Lanes: 1, molecular mass markers; 2, *Nnm. pallidum* JCM 8980^T; 3, *Nnm. pellirubrum* JCM 10476^T; 4, *Nnm. altunense* JCM 12890^T; 5, strain HIS40-3^T; 6, strain HDS3-1. Arrows indicated major differences in protein bands.

Based on morphology, whole-cell protein pattern, physiological and biochemical characteristics, G+C content, DNA-DNA hybridization and polar lipid content, strains HIS40-3^T and HDS3-1 were considered to represent the same species. They were similar to most haloarchaea, being sensitive to rifampicin, novobiocin and bacitracin, but resistant to kanamycin, tetracycline, streptomycin, neomycin and penicillin. However, they differed from all known *Natrinema* species in terms of their physiological and biochemical characteristics (Table 8). All *Natrinema* species tested were positive for nitrate reduction except strain HIS40-3^T, HDS3-1 and *Nnm. pallidum* JCM 8980^T. Hence, the differences, 16S rRNA gene sequences, DNA-DNA hybridization and protein profiles support our proposal of a novel species, *Natrinema gari* sp. nov.

3.5 Conclusion

On the basis of growth requirements, poor utilization of carbohydrates, antibiotic susceptibility, menaquinone content, overall phospholipid composition, DNA G+C contents and 16S rRNA gene sequence analysis, strains HIS40-3^T and HDS3-1 are considered to represent a single species of the genus *Natrinema*. However, they could be differentiated from recognized *Natrinema* species based on levels of DNA-DNA relatedness and differences in whole-cell protein patterns. The results of the present study thus suggest the strains HIS40-3^T and HDS3-1 represent a novel species of the genus *Natrinema*, for which the name *Natrinema gari* sp. nov. is proposed.

The description of *Natrinema gari* (ga´ri. L. gen. n. *gari* of a fish sauce, pertaining to the isolation of strains from fermented fish sauce) were summarized as follows: Cells are motile, Gram-negative rods, 0.5-0.8 × 2.0-3.0 µm in size. Colonies are pale orange, smooth, circular and elevated. Growth is chemo-organotrophic. Requires at least 1.7 M NaCl for growth (optimum 2.6-3.4 M NaCl). Growth occurs at 0-1.0 M $MgCl_2$ (optimum 0.1-0.2 M $MgCl_2$). The pH range for growth is 5.5-8.5 (optimum pH 6.0-6.5). The temperature range for growth is 20-60°C (optimum 37-40°C). Grows anaerobically in the presence of DMSO but not nitrate. Catalase- and oxidase-positive. Nitrate and nitrite are not reduced. Negative for

production of indole. Casein, starch and Tween 80 are not hydrolyzed. Gelatin is hydrolyzed. L-Arabinose, D-glucose and glycerol are utilized for growth. Does not utilize inulin, lactose, maltose, D-mannitol, D-mannose, melibiose, rhamnose, D-ribose, sorbitol, sucrose, D-xylose or citrate. Acid is produced from L-arabinose and glycerol. Susceptible to bacitracin (10 U), novobiocin (5 µg) and rifampicin (30 µg), but resistant to ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), neomycin (30 µg), streptomycin (10 µg) and tetracycline (30 µg). The predominant menaquinone is MK-8. Cells contain $C_{20}C_{20}$ and $C_{20}C_{25}$ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, two glycolipids and one unidentified lipid. The DNA G+C content of the type strain is 65.4 mol%.

The type strain, strain HIS40-3^T (= BCC 24370^T =JCM 14663^T =PCU 303^T), was isolated from fermented fish sauce in Thailand.

CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF HISTAMINE DEHYDROGENASE FROM *NATRINEMA GARI* HDS3-1

4.1 Abstract

Histamine dehydrogenase from *Natrinema gari* HDS3-1, a novel halophilic archaeon, was purified to homogeneity by heat treatment, anion-exchange chromatography and gel filtration chromatography. The purity was increased to 26.6-fold with approximately 70.5% yield. The molecular mass of the enzyme was 127.5 kDa when analyzed using Superose 12 10/300 gel filtration. The enzyme consisted of heterotrimeric subunits with three different molecular masses of 69.1, 29.3 and 27.7 kDa. The enzyme readily oxidized various biogenic amines but not as high as histamine. Activity increased continuously as NaCl concentration (0-5 M) increased. The optimum NaCl concentration, pH and temperature of the purified histamine dehydrogenase were 3.5-5.0 M NaCl, pH 6.5-8.5 and 40-60°C, respectively. Enzyme was stable to heat treatment up to 50°C, and over a pH range of 7.0-9.0. The residual histamine dehydrogenase activity fell to 50% when incubated at 80°C for 60 min with 4.5 M NaCl, and at 65°C when incubated without NaCl. The purified histamine dehydrogenase activity was effectively inhibited by carbonyl reagents. The enzyme had high affinity and activity toward histamine with V_{max}, K_m and k_{cat} values of 2.5 μmol min^{-1}, 57.1 μM and 5.3 s^{-1}, respectively.

4.2 Introduction

Nnm. gari HDS3-1 is a halophilic archaeon that posses histamine-degrading ability in the presence of high salt condition. Our studies indicated that histamine-degrading activity of *Nnm. gari* HDS3-1 was mainly located in the intracellular fraction and required the presence of 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS), an electron carrier. It preferably catalyzed histamine much more than other amines in which the pH, salt concentration and temperature optima for the activity were at pH 6.5-8, 3.5-5 M NaCl and 40-50°C. The activity retained full activity at pH 6.5-9, in the presence of NaCl above 2.5 M and temperature lower than 50°C. The results suggested a link of histamine-degrading activity of *Nnm. gari* HDS3-1 to the presence of salt-tolerant and thermo-neutrophilic histamine dehydrogenase.

Amine dehydrogenase is known to be enzyme that catalyzes the oxidative deamination of amines to its corresponding aldehydes plus ammonia. Since the first report of methylamine dehydrogenase from *Pseudomonas* AM1 (Eady and Large, 1968), various amine dehydrogenases have been reported mostly in prokaryotes such as *Serratia marcescens* (Tabor and Kellogg, 1970), *Pseudomonas putida* (Shinagawa *et al*., 1988; Durham and Perry, 1978; Eady and Large, 1971), *Methylomonas* sp. (Matsumoto, 1978), *Paracoccus denitrifycans* (Takagi *et al*., 1999; Husain and Davidson, 1987), *Pseudomonas aeruginosa* (Hisano *et al*., 1990), *Citrobacter freundii* (Hisano *et al*., 1990), *Nocardioides simplex* (Siddiqui *et al*., 2000), *Alcaligenes faecalis* (Chistoserdov, 2001), *Alcaligenes xylosoxidans* (Fujieda *et al*., 2004; Kondo *et al*., 2004) and *Rhizobium* sp. 4-9 (Sato *et al*. 2005). Focusing particularly on histamine oxidation, molecular and catalytic properties of histamine dehydrogenases from *Nocardioides simplex* (Siddiqui *et al*., 2000) and *Rhizobium* sp. 4-9 (Sato *et al*. 2005) were reported so far. Since no information of this enzyme in halophilic archaeon has been reported previously, the present study describes the purification and enzymatic properties of histamine dehydrogenase from *Nnm. gari* HDS3-1.

4.3 Materials and Methods

Chemicals

Histamine dihydrochloride, 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS), bovine serum albumin (BSA) and β-mercaptoethanol (BME) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HiTrap Q XL, Superose 12 and Vivaspin ultrafiltration devices (50,000 molecular weight cut off (MWCO)) were purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250, gel filtration standard and *N,N,N´,N´*-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Unstained protein ladder was purchased from Fermentas (Glen Burnie, MD, USA). The other chemicals were of analytical grade.

Culture and growth condition

Nnm. gari HDS3-1 was cultivated aerobically (200 rpm) at 37°C for 7 days in a halophilic liquid medium (Appendix A-1). After cultivation, the wet cell paste was harvested by centrifugation of cultured broth at 10,000 ×*g* at 4°C for 10 min. The cell pellet was washed twice with halophilic liquid medium. The obtained pellet was suspended in halophilic liquid medium containing 20% (v/v) glycerol and placed in a 2 mL cryotube. *Nnm. gari* HDS3-1 stock culture cells were kept in the -20°C freezer until used.

Enzyme preparation

The inoculum was prepared by inoculating 250 µL of stock culture, *Nnm. gari* HDS3-1, into 5 mL of halophilic liquid medium (Appendix A-1) and incubated at 37°C in a shaker incubator at 200 rpm for 7 days. After that, inoculum was added at 5% (v/v) into halophilic liquid medium containing 5 mM of histamine (free-base) (Appendix A-2) and incubated at 37°C in a shaker incubator at 200 rpm for 7 days. Cells were harvested by centrifugation of cultured broth at 10,000 ×*g* at

4°C for 15 min. The pellet was washed twice with with 4.5 M NaCl. The obtained pellet was suspended in 50 mM Tris-HCl, pH 8.0 referred to as standard buffer (SB) containing 4.5 M NaCl at a ratio of 1:1 (weight of the cell paste/volume of Tris-HCl buffer) and sonicated for a total of 2 min by a Vibra Cell VCX60 (Sonics and Materials Inc., USA). The supernatant was collected by centrifugation at 15,000 ×*g* for 30 min (4°C) and was referred to as crude enzyme extract.

Purification of histamine dehydrogenase

The obtained crude enzyme extract was heated rapidly to 60°C, maintained at this temperature for exactly 15 min and suddenly cooled in iced water. The resulting precipitate was discarded after centrifugation at 15,000 ×*g* at 4°C for 30 min. The supernatant was collected and dialyzed against SB for 12 h at 4°C.

All of the following purification steps were performed in the cold room using the ÄKTA explorer purification system (GE Healthcare). The dialyzed heat-treated sample was chromatographed on a HiTrap Q XL column (0.7 × 2.5 cm) (GE Healthcare), which was equilibrated with approximately five bed volumes of SB. The sample was loaded onto the column at a flow rate of 0.5 mL/min. The elution was performed with step-wise process using SB containing different NaCl concentrations: 0, 0.5, 1.0 and 2.0 M, respectively. Fractions of 0.5 mL were collected and those with histamine dehydrogenase activity were pooled and further purified by Superose 12 column. Absorbance at 280 nm (A_{280}) of each fraction was also measured.

Pooled fractions with histamine dehydrogenase activity from the HiTrap Q XL column were concentrated by Vivaspin ultrafiltration devices with 50,000 MWCO prior to size exclusion chromatography. The sample was applied to a Superose 12 10/300 GL column (1.0 × 30 cm) (GE Healthcare) previously equilibrated with approximately two bed volumes of SB containing 0.15 M NaCl. The sample was loaded onto the column and then eluted with the same buffer at a flow rate of 0.1 mL/min. Fractions of 0.5 mL were collected and subjected to A_{280} measurement.

Pooled fractions with histamine dehydrogenase activity from Superose 12 column were concentrated by Vivaspin ultrafiltration devices with 50,000 MWCO.

The sample was rechromatographed on the same HiTrap Q XL column, which was equilibrated with approximately five bed volumes of SB containing 0.15 M NaCl at a flow rate of 0.5 mL/min. The elution was performed using linear gradient of 0.15-2.0 M NaCl in SB at a flow rate of 0.5 mL/min. Fractions (0.5 ml) were collected and those with histamine dehydrogenase activity were pooled and used for further studies.

Histamine dehydrogenase activity assay

Histamine-degrading activity was determined by measuring the decreased amount of histamine after reaction. The sample with an appropriate dilution (100 µL) was mixed with 0.9 mL of reaction mixture consisting of 55 mM Tris-HCl, pH 7.0, 5.0 M NaCl, 5.0 mM of histamine dihydrochloride (free-base), and 555 µM of 1-methoxy PMS. The reaction was incubated at 37°C for exactly 5, 10, 15, 20, 30, 60, and 90 min and immediately stopped the reaction by adding 1 mL of 0.1 N HCl. The concentrations of histamine were estimated by HPLC (Appendix B-2) and the fluorometric method of AOAC (1995) (Appendix B-1). A blank was run in the same manner, except the sample was added after addition of 0.1 N HCl. One unit of enzyme activity was defined as the amount of enzyme that catalyzed a reduction of 1 µmole of histamine per min under the specified conditions.

Protein determination

Protein concentration was determined by the method of Bradford (1976). Bovine serum albumin was used as a standard. The detail of the protein determination method is described clearly in Appendix B-4.

Characterization of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1

Substrate preference

The preference of the purified enzyme on various amines including tryptamine, β-phenylethylamine, putrescine, cadaverine, tyramine, spermidine and spermine was determined by replacing histamine in the reaction mixture. The reactions were determined at 37°C for 30 min and immediately stopped by adding 1 mL of 0.4 M perchloric acid. Blank was done in the same manner except adding 1 mL of 0.4 M perchloric acid before the reaction mixture. Biogenic amines in supernatant were extracted and derivertized according to the procedure of Eerola *et al*. (1993) with a slight modification (Appendix B-2). In brief, the supernatant (480 µL) was added with 20 µL of 2 mg/mL of 1,7-diaminoheptane as the internal standard and 500 µL of 0.4 M perchloric acid. The mixture was mixed thoroughly by a vortex mixer for 5 min and then centrifuged at a speed of 15,000 ×*g* at 25°C for 5 min. The obtained supernatant (300 µL) was mixed with 60 µL of 2 N NaOH and 90 µL of saturated sodium bicarbonate. A 600 µL of 10 mg/mL of dansyl chloride was added to each sample, mixed, and then incubated for 45 min at 40°C. Residual dansyl chloride was removed by adding 30 µL of 25% (v/v) ammonia and centrifuged at 3,500 ×*g* for 5 min. The supernatant was collected and filtered through a 0.45 µm membrane filter prior to analysis by a high performance liquid chromatography (HPLC). A Waters Separation Module 2690 was operated to give a flow rate of 1.5 mL/min of the mobile phase of 0.1% (v/v) acetic acid (solvent A) and acetonitrile containing 0.1% (v/v) acetic acid (solvent B), as shown in a gradient elution program. Separation was achieved using a column of Hypersil BDS C18 (300 × 7.8 mm i.d.) set at 40°C. Samples (20 µL) were injected and a photo diode array (Model Waters 996), set at the wavelength of 254 nm, was used as the detector. Data were processed and analyzed using Millennium 32 software (Waters, Milford, MA, USA).

Effect of salt concentration on activity and stability

To remove salt, crude enzyme was dialyzed against 100 volumes of 50 mM Tris-HCl buffer, pH 7.0 at 4°C. Desalted sample was subjected to activity determination in the presence of NaCl at various concentrations (0-5 M) using the standard assay conditions. To study the effect of NaCl on stability, the desalted sample was dialyzed against 100 volumes of buffers containing different NaCl concentrations (0-5 M) and incubated at room temperature for 24 h. The remaining activity was assayed using the standard assay conditions.

Effect of pH on activity and stability

The activity was assayed at 37°C over the pH range of 4-9 (50 mM acetate buffer (pH 4.0-5.5); 50 mM phosphate buffer (pH 6.0-6.5); and 50 mM Tris-HCl buffer (pH 7.0-9.0)) in the presence of 4.5 M NaCl. To study the effect of pH on stability, enzyme activity was determined with the same buffers, but their concentrations were changed to 100 mM. The enzyme was mixed with buffer in an equal volume in the presence of 4.5 M NaCl and incubated for 24 h at 30°C. The remaining activity was assayed using the standard assay conditions.

Effect of temperature on activity and stability

Enzyme activity was assayed at different temperatures in the range of 30-80°C as previously described. To study the effect of temperature on enzyme stability, the dialyzed enzyme in 50 mM Tris-HCl buffer, pH 7.0 was incubated at various temperatures ranging from 4-80°C for 1 h in the absence and presence of 4.5 M NaCl. Thereafter, the enzyme mixture was immediately cooled in an ice bath, and the remaining activity was assayed under the standard assay conditions. Relative activity of the enzyme was calculated, in comparison with that without heating.

Effect of inhibitors on the purified histamine dehydrogenase activity

The effect of inhibitors on the purified histamine dehydrogenase activity was determined by incubating the purified enzyme with an equal volume of enzyme inhibitor solution to obtain the final concentration designated (1 mM ρ-chloromercuribenzoate (PCMB), 1 mM iodoacetate, 1 mM isoniazid, 0.5 mM semicarbazide, 0.5 mM hydroxylamine, 5 mM potassium cyanide (KCN), 1.3 µM cuprizone, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM tryptophan and 0.2 mM putrescine). The mixture was allowed to stand at room temperature (30°C) for 15 min. Thereafter, the remaining activity was measured and the percent inhibition was calculated. The control was conducted in the same manner except that deionized water was used instead of inhibitors.

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). Protein solutions were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer (0.125 M Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 2% BME; 0.002% bromophenol blue) and boiled for 10 min. The samples (15 µg) were loaded on the gel made of 4% stacking and 12.5% separating gels and subjected to electrophoresis at a constant current of 15 mA per gel using an ATTO AE-6530 Dual mini-slab system. After electrophoresis, the gels were stained with 0.125% Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 30% methanol and 7% acetic acid.

Native-PAGE was performed using 12.5% separating gels in a similar manner with SDS-PAGE, except that the sample was not heated and the addition of SDS and reducing agent was omitted (Appendix B-5).

Activity staining

After electrophoresis, gels were immersed in 100 mL of 1 mM of histamine dihydrochloride (free-base) for 30 min with constant agitation at 4°C-allow

the substrate to penetrate into the gels. The gels were then transferred to the standard assay reaction mixture containing 55 µM of dichlorophenolindophenol (DCPIP) act as electron acceptor and incubated at 37°C for 30 min with constant agitation to develop the activity zone. Development of blue zones on green background indicated histamine dehydrogenase activity (Eady and Large, 1968).

Determination of molecular weight

The molecular weight of the purified histamine dehydrogenase was determined using size exclusion chromatography on Superose 12 10/300 GL column (1.0 × 30 cm) (GE Healthcare), in the cold room using the ÄKTA explorer purification system (GE Healthcare). The protein solution was passed through the column equilibrated with approximately two bed volumes of SB containing 0.15 M NaCl at a flow rate of 0.1 mL/min and then eluted with the same buffer. A flow rate of 0.1 mL/min was maintained throughout. The histamine dehydrogenase separated on size exclusion chromatography was estimated for its molecular weight by plotting available partition coefficient (K_{av}) against the logarithm of molecular weight of the protein standards. The elution volume (V_e) was measured for each protein standard and the purified histamine dehydrogenase. Void volume (V_o) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standards used included thyroglobulin (bovine) (M_r 670,000), γ-globulin (bovine) (M_r 158,000), ovalbumin (chicken) (M_r 44,000), myoglobin (horse) (M_r 17,000) and vitamin B_{12} (M_r 1,350).

Kinetic studies

Purified histamine dehydrogenase was diluted with 4.5 M NaCl to obtain a concentration of 0.1 mg/mL. Then, the activity was assayed with 900 µL of different final concentrations of histamine ranging from 0.025-1.0 mM (in the presence of 500 µM 1-methoxy PMS) and 1-methoxy PMS ranging from 0.025-0.5 mM (in the presence of 5.0 mM histamine). The determinations were repeated twice and the V_{max}, K_m and k_{cat} values for the purified enzyme were evaluated by plotting the data on a Lineweaver-Burk double-reciprocal graph (Lineweaver and Burk, 1934).

Statistic analysis

All experiments were run in triplicate. A completely randomized design was used throughout this study. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows: SPSS Inc.).

4.4 Results and Discussion

Purification of histamine dehydrogenase from *Nnm. gari* HDS3-1

Purification of histamine dehydrogenase is summarized in Table 9. After heat treatment, total activity of approximately 92.9% was remained, while 7.1% of protein was removed. From the result, purity of 1.8-fold was obtained with heat treatment. After loading the dialyzed active heat treatment fraction onto anion exchanger, HiTrap Q XL column, the column was eluted using a 0-2.0 M NaCl segmented gradient. HiTrap Q XL separated the heat treatment fraction into three protein peaks (A_{280}), an activity peak was found after elution at 1.0 M NaCl (Figure 20). A large amount of proteins was removed with small loss in enzyme activity, leading to 6.6-fold purity. Fractions with activity were pooled. Specific activity increased to 36.8 units/mg protein.

Pooled active HiTrap Q XL fractions were further purified by a size exclusion chromatography using Seperose 12. The column separated histamine dehydrogenase from other proteins with higher molecular weight (Figure 21). Approximately 73.7% of activity was retained and purification fold of 18.4 was obtained. Therefore, pooled active fractions were rechromatographed on a second HiTrap Q XL chromatography. After loading onto the second HiTrap Q XL, the column was eluted by a linear gradient of 0.15-2.0 M NaCl (Figure 22). From the result, this step effectively separated histamine dehydrogenase from the contaminants. The 26.6-fold increase in purity with a yield of 70.5% was observed at this step.

Table 9. Purification of histamine dehydrogenase from *Nnm. gari* HDS3-1

Purification steps	Total activity (units*)	Total protein (mg**)	Specific activity (units/mg)	Purity (fold)	Yield (%)
Crude enzyme extract	5,011.0	900.2	5.6	1.0	100.0
Heat treatment	4,656.4	470.8	9.9	1.8	92.9
1st HiTrap Q XL	3,810.2	103.4	36.8	6.6	76.0
Seperose 12	3,694.2	36.1	102.5	18.4	73.7
2nd HiTrap Q XL	3,531.9	23.9	147.8	26.6	70.5

*The unit of enzyme activity is expressed as the µmoles of histamine degraded per h.

**Protein concentration was measured by Bradford method.

The purity of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 was evaluated by using native gel electrophoresis, followed by activity staining (Figure 23A and 23B). Protein pattern of intracellular fraction extracted from *Nnm. gari* HDS3-1 during purification process is also shown in Figure 24. Crude extract contained a variety of proteins with different molecular weight (lane 1). After heat-treatment, some proteins were slightly removed (lane 2). Those proteins disappeared with heating process were presumed to be heat labile proteins. After subjected to HiTrap Q XL column (lane 3), it was noted that most of the proteins disappeared and smaller bands with a wide range of MW were observed. After Superose 12 chromatography, proteins with MW of higher than 30 kDa and lower than 25 kDa were removed, indicated that a large amount of proteins was removed during purification (lane 3 and 4). After subsequently subjecting onto a 2nd HiTrap Q XL column (lane 5), only one single protein band appeared on native gel electrophoresis (Figure 23A.) The protein band position was identical to the activity band observed after activity staining (Figure 23B).

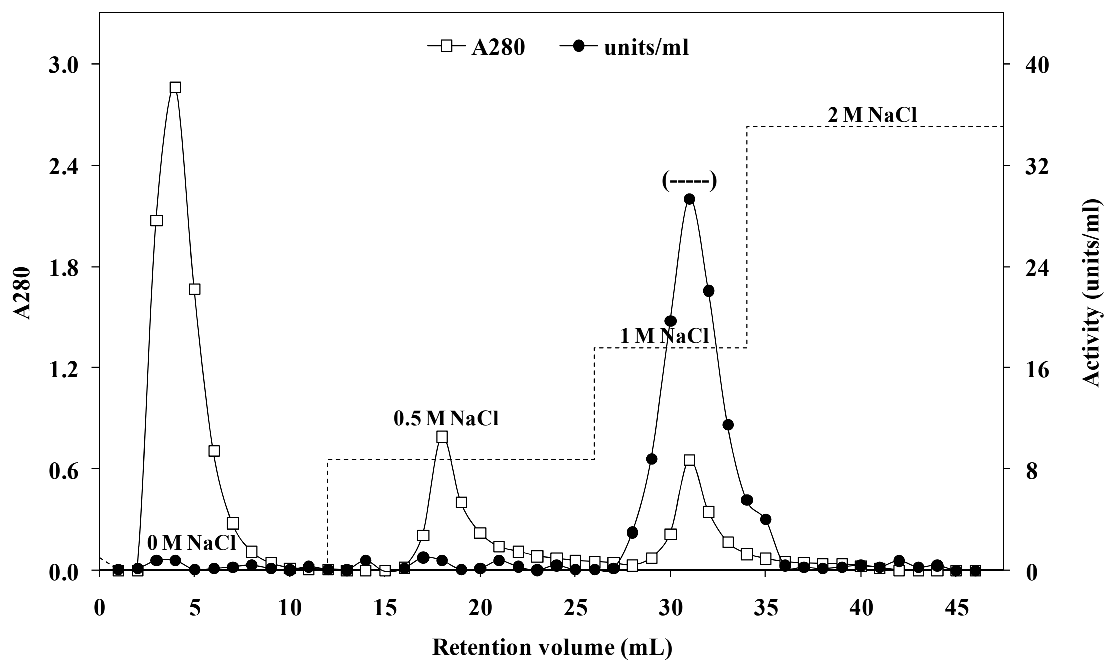


Figure 20. Elution profile of heat-treated *Nnm. gari* HDS3-1 histamine dehydrogenase on the 1st HiTrap Q XL column. Enzyme solution was dialyzed against SB and applied onto a HiTrap Q XL column. Elution was carried out with a step-wise gradient of 0-2.0 M NaCl in SB. Fractions of 0.5 mL were collected.

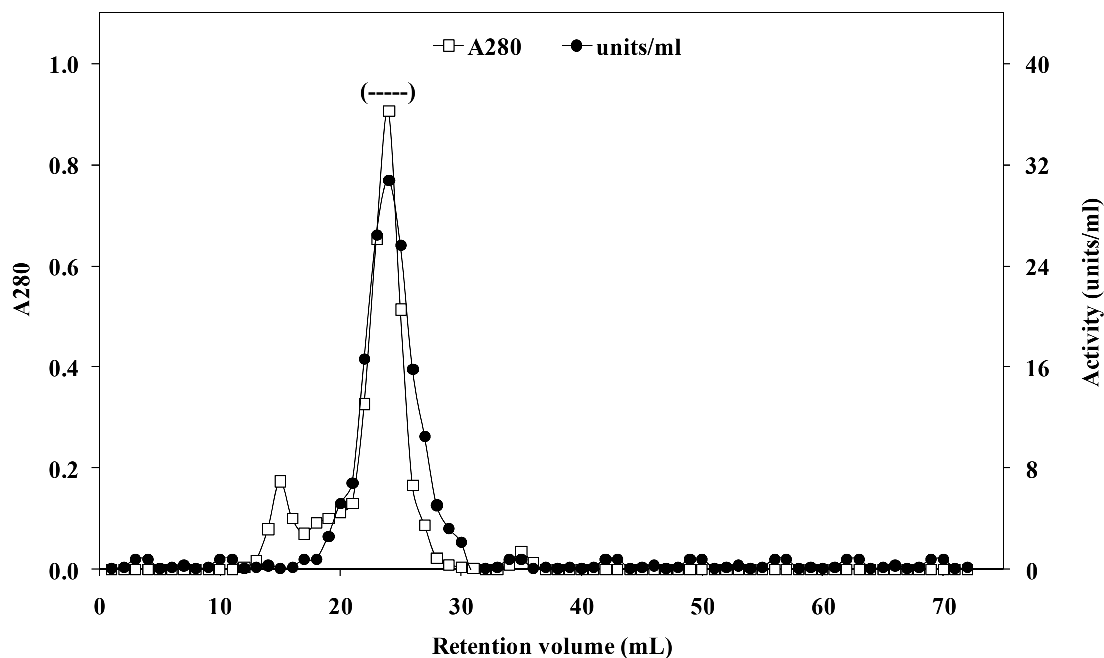


Figure 21. Elution profile of histamine dehydrogenase on Superose 12 column. Pooled fractions from the 1st HiTrap Q XL chromatography were concentrated by Vivaspin ultrafiltration devices with 50,000 MWCO and applied onto a Superose 12 column. Elution was carried out with 0.15 M NaCl in SB at a flow rate of 0.1 mL/min. Fractions of 0.5 mL were collected.

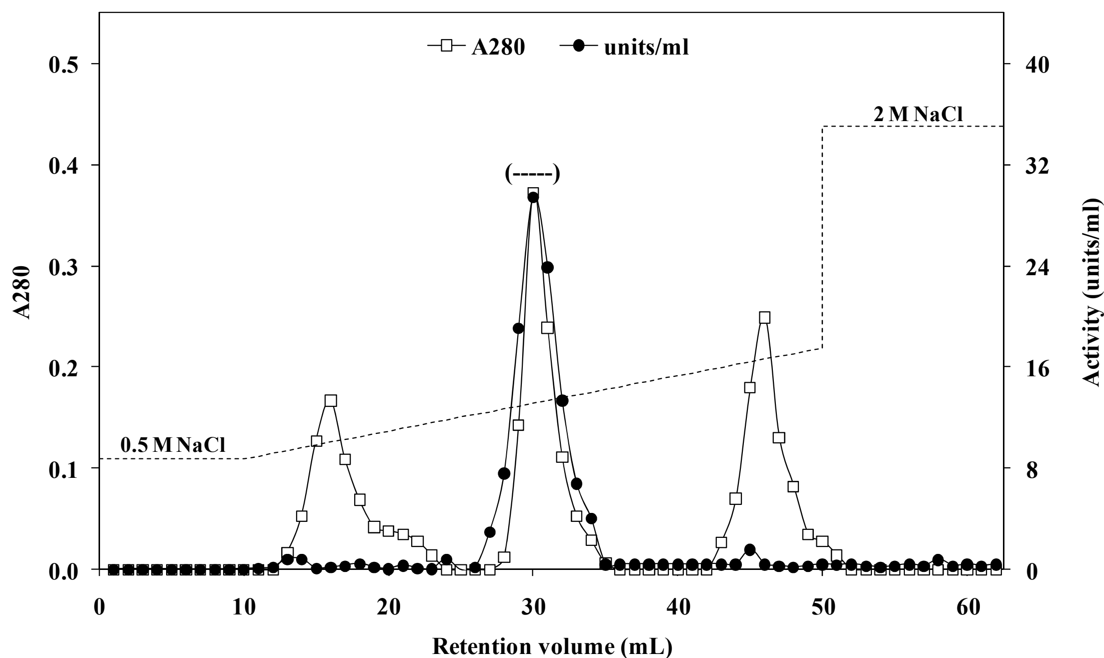


Figure 22. Elution profile of histamine dehydrogenase on the 2nd HiTrap Q XL column. Pooled fractions from Superose 12 chromatography were concentrated by Vivaspin ultrafiltration devices with 50,000 MWCO and applied onto a second HiTrap Q XL column. Elution was carried out with a linear gradient of 0.15-2.0 M NaCl in SB. Fractions of 0.5 mL were collected.

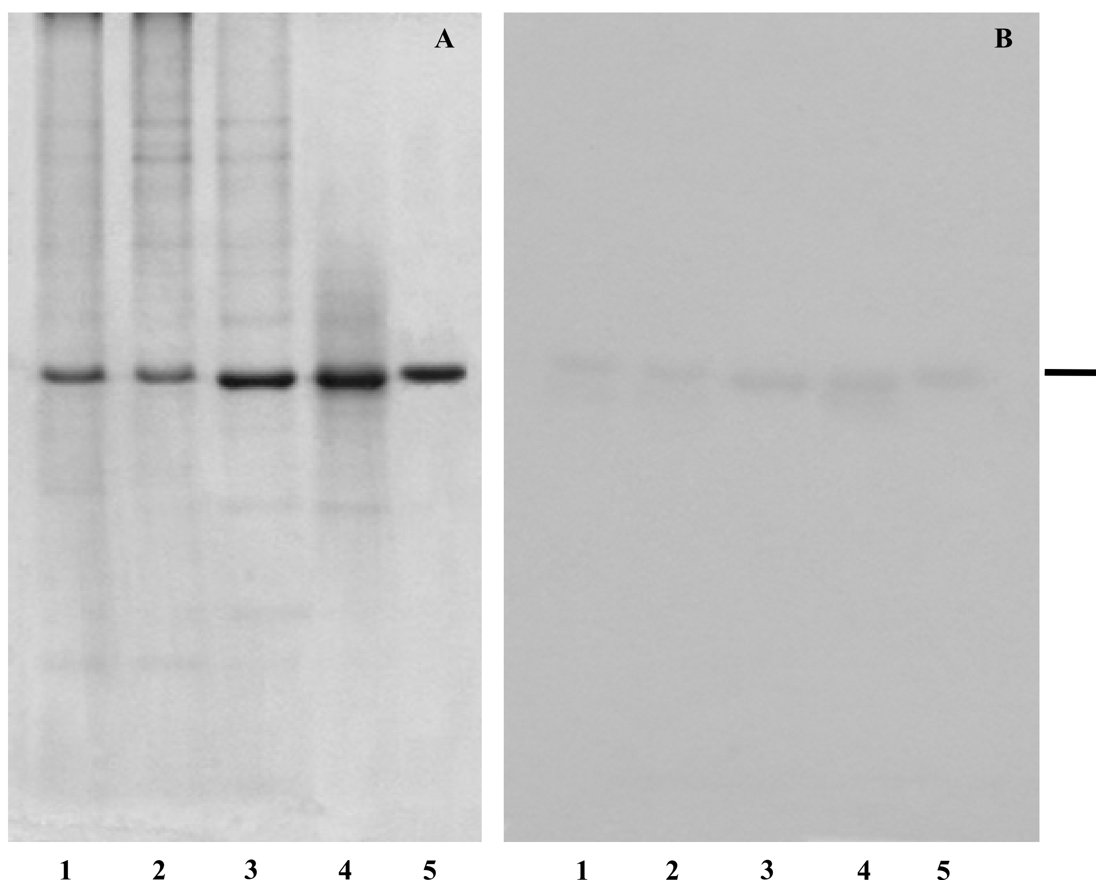


Figure 23. Protein pattern (**A**) and activity staining (B) of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 on native gel electrophoresis 12.5% followed by staining for histamine dehydrogenase activity at pH 8.0, 50°C. Lane 1, crude enzyme extract; lane 2, heat treatment; lane 3, 1st HiTrap Q XL fraction; lane 4, Seperose 12 fraction; lane 5, 2nd HiTrap Q XL fraction. Approximately same amount (15 µg) of protein on each lane was applied to 12.5% polyacrylamide gel and electrophoresis was carried out for 2 h at 15 mA per gel. After electrophoresis, the left-hand gel (A) was stained with Coomassie brilliant blue R-250 for protein and the right-hand gel (B) was stained for enzyme activity by the method described in Materials and Methods. The position to the active staining band of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 is indicated to the right.

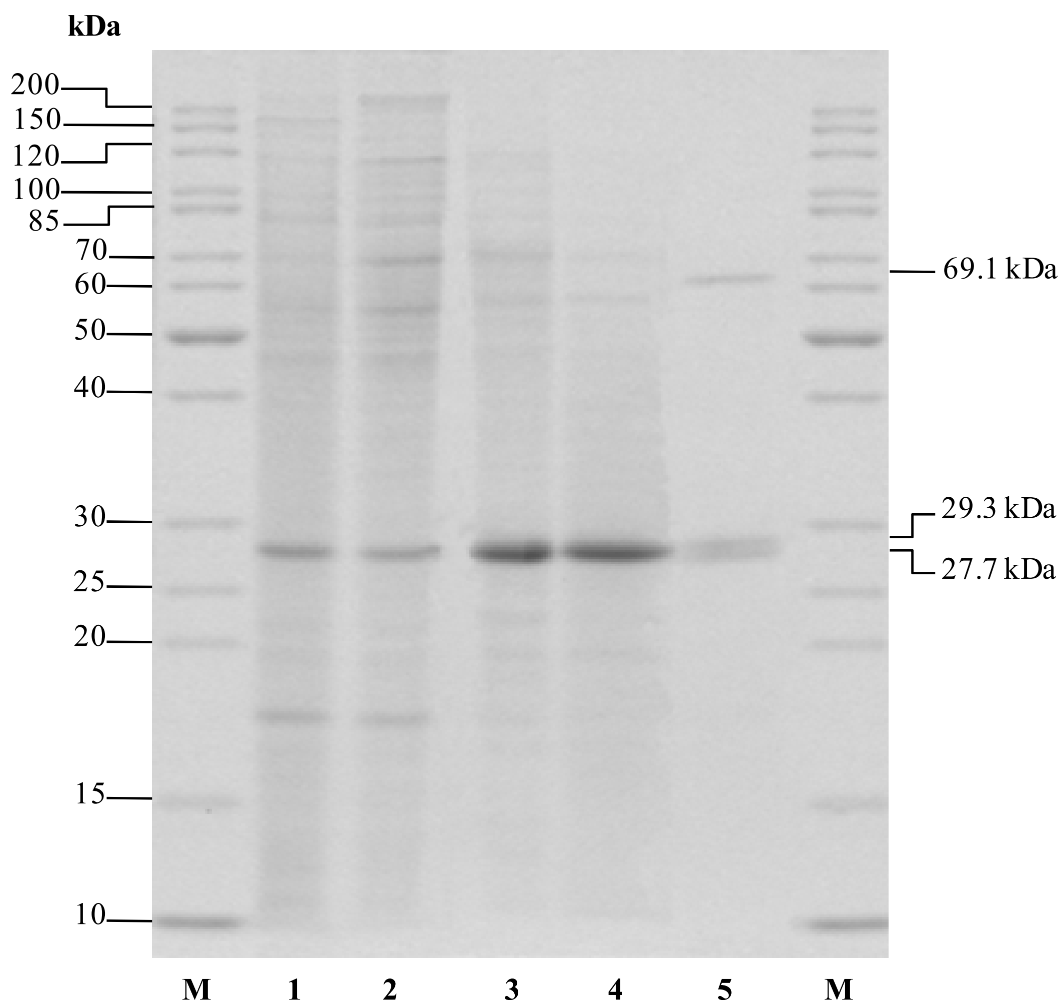


Figure 24. SDS-PAGE pattern (with reducing agent; 2% β-mercaptoethanol) of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1. M, molecular weight standard; lane 1, crude enzyme extract; lane 2, heat treatment; land 3, 1st HiTrap Q XL fraction; lane 4, Seperose 12 fraction; lane 5, 2nd HiTrap Q XL fraction. Approximately same amount (15 μg) of protein on each lane was applied to 12.5% polyacrylamide gel and electrophoresis was carried out for 2 h at 15 mA per gel. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 for protein. Positions of molecular mass markers are shown to the left. The positions of the three subunits of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 are indicated to the right.

Characterization of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1

Molecular mass and subunit structure

The molecular mass of the purified enzyme was determined to be 127.5 kDa by gel filtration chromatography on Superose 12 (Figure 25A). SDS-PAGE analysis showed that the purified enzyme had three different kinds of subunits, with the molecular masses of 69.1, 29.3 and 27.7 kDa, respectively (Figure 25B). Corresponded with the molecular mass of the native purified enzyme, these results showed that enzyme exhibited a heterotrimeric structure of subunits.

The molecular weight and subunit size of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 were not similar to all known histamine and/or amine dehydrogenases reported previously. For examples, histamine dehydrogenases from *Nocardioides simplex* IFO 12069 and *Rhizobium* sp. 4-9 were shown to have a molecular mass of about 150 kDa and consisted of two homogeneous subunits (Bakke *et al*., 2005; Siddiqui *et al*., 2000) Aromatic amine dehydrogenase from *Alcaligenes xylosoxidans* IFO13495 (95.5 kDa) consisted of heterotetrameric subunits ($\alpha_2\beta_2$) with two different molecular masses of 42.3 kDa and 15.2 kDa (Kondo *et al*., 2004). Amine dehydrogenases from *Pseudomonas* sp. (Shinagawa *et al*., 1988) and *Pseudomonas putida* (Adachi *et al*., 1998), aromatic amine dehydrogenase (Govindaraj *et al*., 1994) and methylamine dehydrogenase (Matsumoto *et al*., 1978) were also exhibited nonidentical subunit and different molecular masses.

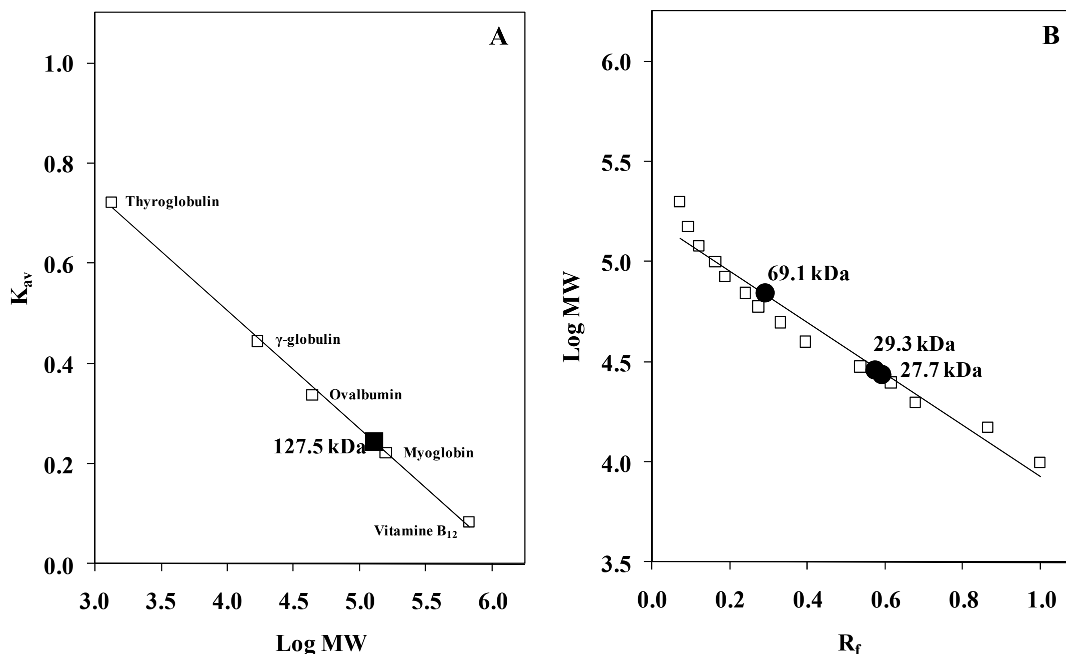


Figure 25. Calibration curve for the molecular weight determination on Superose 12 10/300 GL chromatography, in the cold room using the ÄKTA explorer purification system (GE Healthcare) (A) and the subunit molecular weight determination by SDS-PAGE with reducing agent (2% β-mercaptoethanol) (B) of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1. ■, histamine dehydrogeanse and ●, histamine dehydrogenase subunits.

Degrading of amines by purified histamine dehydrogenase from *Nnm. gari* HDS3-1

To investigate the substrate specificity of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1, an enzyme assay was performed using biogenic amines that are prominent in food. Figure 26 shows that purified histamine dehydrogenase from *Nnm. gari* HDS3-1 catalyze histamine much more efficiently compare to tryptamine, β-phenylethylamine, putrescine, cadaverine, tyramine, spermidine and spermine ($P<0.05$).

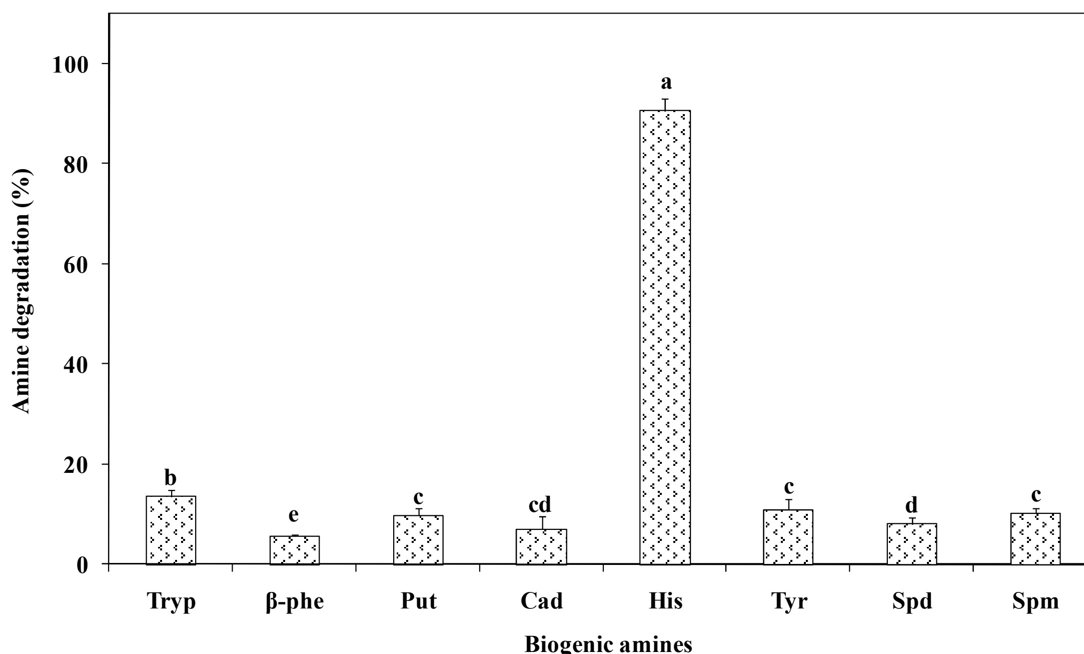


Figure 26. Biogenic amines degradation by the purified histamine dehydrogenase from *Nnm. gari* HDS3-1. Reaction was carried out in the presence of 4.5 M NaCl at 45°C, pH 7 under shaking condition (200 rpm) for 1 h. The different letters in the column denote the significant differences ($P<0.05$). Average ± S.D. from a triplicate determination. Tryp; tryptamine, β-phe; β-phenylethylamine, Put; putrescine, Cad; cadaverine, His; histamine, Tyr; tyramine, Spd; spermidine and Spm; spermine. The experiments were done in triplicate and the error bars represent the variation between the readings.

The substrate selectivity study of amine dehydrogenase was based on their activities in each amine. Many amine dehydrogenases have also been found in bacteria, and some of them have also been used for the detection of histamine. The purified amine dehydrogenase from *Pseudomonas* AM1 was found to have a wide specificity, oxidizing many compounds that contain a primary amino group, notably ethanolamine and histamine (Eady and Large, 1968). Whereas, the purified histamine dehydrogenase from *Nocardioides simplex* IFO 12069 showed high activity toward

histamine, but it still oxidized agmatine and putrescine at a rate of 30% compared to histamine (Siddiqui *et al*., 2000). Among 20 kinds of biogenic amines including histamine study by Bakke *et al*. (2005), the purified recombinant histamine dehydrogenase from *Rhizobium* sp. 4-9 was very specific toward histamine and only oxidized agmatine and 1,3-diaminopropane with the rate of 10 and 13% of histamine, respectively. Fujieda *et al*. (2004) indicated that the clear difference between histamine dehydrogenase and other amine dehydrogenase is the activity that almost exclusively toward histamine, while low activity to other amines.

Optimal salt concentration and salt stability

The maximal activity was attained in 3.5-5 M NaCl (Figure 27A), while more than 50% of the highest activity was obtained at 1.5 M NaCl. It was noted that in the absent of salt, the purified histamine dehydrogenase still retained 20% of its maximal activity. Figure 27B shows the effect of NaCl concentrations on the stability of the purified enzyme. The purified histamine dehydrogenase was stable for at least 24 h when incubated in the 50 mM Tris-HCl buffer pH 7.0 at around 3-5 M NaCl concentration. In the absent of salt, the enzyme activity was 3 times less stable than in the optimal NaCl concentration ($P<0.05$). Loss of activity might be due to the denaturation of enzymes caused by the “salting out” effect. NaCl at higher concentration possibly competed with the enzyme in water binding, resulting in a stronger protein-protein interaction, which was possibly associated with precipitation. This led to the loss in activity in the presence of high NaCl concentrations. The result showed that purified histamine dehydrogenase from *Nnm. gari* HDS3-1 was still active and able to degrade histamine in the reaction mixture containing high NaCl concentrations.

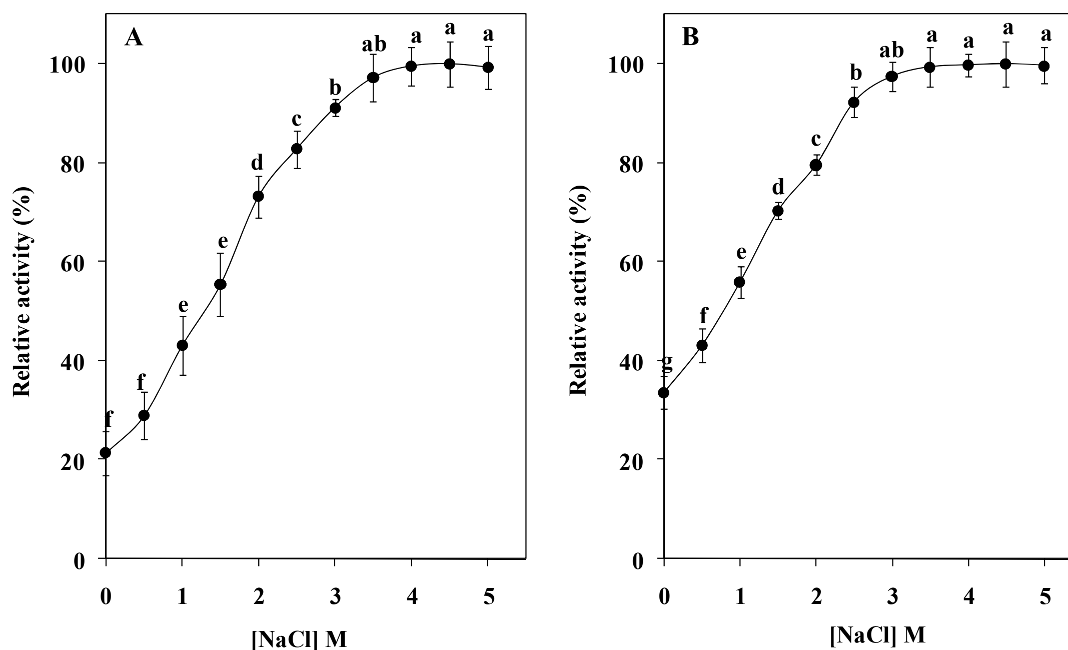


Figure 27. NaCl concentrations profile (A) and stability (B) of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1. The different letters in the same line denote the significant differences (P<0.05). Average ± S.D. from a triplicate determination. (A) Residual activity was analyzed using histamine as a substrate for 30 min at pH 7.0 and 45°C at the various NaCl concentrations. (B) For the stability test, the enzyme was incubated at 30°C for 24 h in 50 mM Tris-HCl buffer (pH 7.0) contained NaCl at various concentrations. Residual activity was analyzed using histamine as a substrate for 30 min, pH 7.0 in the presence of 4.5 M NaCl at 45°C.

Optimal pH and pH stability

The pH profile of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 is shown in Figure 28A. The activity was high in pH range of 6.5-8.0 but considerable loss of activity was observed at very acidic pH. No activity was found at pH ≤ 4. The sharp decrease in histamine degradation activity by purified histamine dehydrogenase at low and high pH might be attributed to irreversible denaturation.The purified histamine dehydrogenase from *Nnm. gari* HDS3-1 exhibited the maximal activity towards histamine at pH 6.5-8.5, similar to those from *Alcaligenes xylosoxidans* (pH 8.0) (Kondo *et al*., 2004) and *Alcaligenes faecalis* (pH 8.0) (Iwaki *et al*., 1983). The effect of pH on the stability of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 is depicted in Figure 28B. The enzyme was stable over a broad pH range (pH 6.5-8.0). Sharp decreases in activities were observed at slightly acidic pH for the enzyme, whereas, only slightly decrease in activity was found at alkaline pH. No activity was found after incubation at pH ≤ 5. From the result, the purified histamine dehydrogenase might undergo denaturation under acidic conditions, where the conformational change took place and enzyme could not bind to the substrate properly. The stability of the enzyme at the wide range of pH was comparable to the pH stability of aromatic amine dehydrogenase from *Alcaligenes xylosoxidans* (pH 5-12) (Kondo *et al*., 2004), methylamine dehydrogenases from *Methylobacterium extorgquens* AM1 (pH 2.6-10.6) (Eady and Large, 1968), *Methylomonas* sp. (pH 4.4-10.6) (Matsumoto, 1978) and *Paracoccus denitrificans* (pH 3.0-10.5) (Husain and Davidson, 1987).

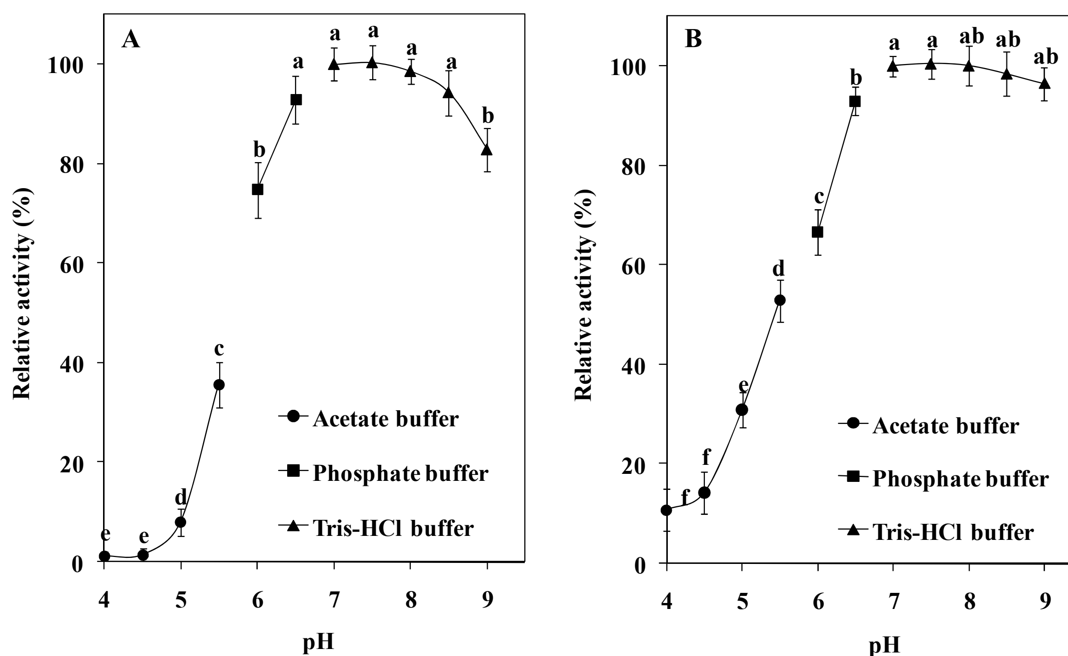


Figure 28. pH profile (A) and stability (B) of histamine-degrading activity of HDS3-1. The different letters in the same line denote the significant differences ($P<0.05$). Average ± S.D. from a triplicate determination. (A) Residual activity was analyzed using histamine as a substrate for 30 min at 45°C in the presence of 4.5 M NaCl at various pHs. (B) For the stability test, the enzyme was incubated at 30°C for 24 h in the desired pH buffer at various pH values in the presence of 4.5 M NaCl. Residual activity was analyzed using histamine as a substrate for 30 min at 45°C, pH 7.0 in the presence of 4.5 M NaCl.

Optimal temperature and thermal stability

The temperature profile of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 is presented in Figure 29A. The enzyme had optimal temperature at 40-60°C. An appreciable decrease in activity of enzyme was observed at temperatures above 60°C, presumably as a result of thermal inactivation. Enzyme was inactivated at high temperature, possibly due to the partial unfolding of the enzyme molecule. The residual enzyme activity of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 was examined with histamine at 45°C and pH 7.0 after heat treatment with and without 4.5 M NaCl at various temperatures for 1 h (Figure 29B). The enzyme was quite stable at 50°C, with residual activities of 95 and 85% for incubation with and without 4 M NaCl, respectively. Even at 70°C the residual activity after the incubation was above 50%, with only a minor influence of NaCl. However, at higher temperatures NaCl had a significant effect, as demonstrated by the divergence of the two profiles. For example, when the purified enzyme was incubated for 1 h at 80°C in the presence of NaCl, the residual activity was 40%, double the residual activity (20%) obtained without NaCl. The thermal stability of the enzyme was distinctively different from other histamine dehydrogenases. Similar results were observed with the thermal stability of the enzyme obtained from halophilic archaea (Bhatnagar *et al*., 2005; Obon *et al*., 1996; Manitz and Holldorf, 1993).

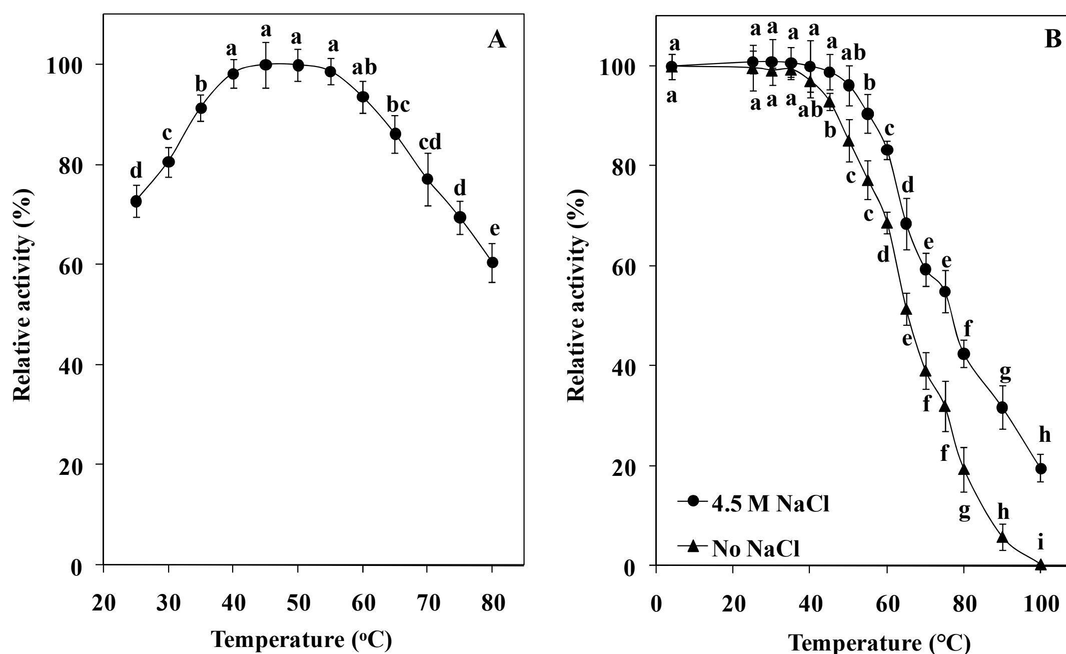


Figure 29. Temperature profile (A) and thermal stability (B) of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1. The different letters in the same line denote the significant differences (P<0.05). Average ± S.D. from a triplicate determination. (A) Residual activity was analyzed using histamine as a substrate for 30 min at pH 7.0 in the presence of 4.5 M NaCl at various temperatures. (B) For the stability test, the enzyme was incubated for 1 h at the temperatures indicated in 50 mM Tris-HCl buffer (pH 7.0) in the presence or absence of 4.5 M NaCl, and then cooled on ice. Residual activity was analyzed using histamine as a substrate for 30 min at pH 7.0 in the presence of 4.5 M NaCl at 45°C.

Electron acceptor

The availability of electron acceptors for histamine dehydrogenase activity was examined (Table 10). Purified histamine dehydrogenase required the presence of 1-methoxy PMS as an electron carrier for oxidation of histamine. Natural electron acceptor such as FAD^+ and riboflavin (vitamin B_2) could also be used, although the activities were only about 50 and 30% of that in the presence of 1-methoxy PMS. The results confirm not only the link to dehydrogenase activity but also the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 had very restricted electron acceptor specificity. It should be noted that the enzyme did not require NAD^+ and cytochrome c which were specific electron acceptors of other amine dehydrogenases. The purified histamine dehydrogenase from *Nnm. gari* HDS3-1 was probably related to histamine dehydrogenases from *Nocardioides simplex* and *Rhizobium* that are known to have an unusual covalently bound 6-S-cysteinyl flavin mononucleotide (FMN) and [4Fe-4S] cluster as redox cofactors (Fujieda *et al*., 2004; Bakke *et al*., 2005).

Effect of inhibitors

The enzyme activity was completely inhibited by isoniazid, semicarbazide and hydroxylamine which are common carbonyl agents (Table 11). These results suggest that the active site of the enzyme contains a carbonyl group. Most of the carbonyl reagents showed very strong inhibition on various amine dehydrogenase (Govindaraj *et al*., 1994; Shinagawa *et al*., 1988; Durham and Perry, 1978). The inhibition by hydroxylamine may be due to its binding directly to the ferric ion (Takemori *et al*., 1960). The means of inhibition by cuprizone may be similar to that of the other carbonyl agents since it has been shown that cuprizone can act as a carbonyl agent rather than a copper chelator (Lindstrom and Pettersson. 1974). In addition, it should be noted that this enzyme was inhibited by L-tryptophan. This characteristic was similar to histamine dehydrogenase from *Nocardioides simplex* and tryptophan tryptophylquinone-containing aromatic amine dehydrogenase (Govindaraj *et al*., 1994).

Table 10. Specificity of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1 for primary electron acceptors

Acceptor	Final concentration		Relative activity* (%)
None			0[d]
1-methoxy PMS	0.5	mM	100[a]
NAD (Nicotinamide adenine dinucleotide)	2	μM	2[d]
FAD (Flavin adenosine dinucleotide)	0.02	μM	53[b]
Cytochrome *c***	0.25	μM	0[d]
Sulfate ($MgSO_4.7H_2O$)	50	mM	1[d]
Iron II ($FeCl_2.4H_2O$)	0.25	mM	1[d]
Iron III ($FeCl_3$)	0.5	mM	0[d]
Nitrate (NO_3^-)	8	mM	1[d]
Nitrite (NO_2^-)	2.5	mM	1[d]
Riboflavin (Vitamin B_2)	0.5	mM	29[c]
Niacin (Vitamin B_3)	1.5	mM	0[d]
Pyridoxal (Vitamin B_6)	1	mM	1[d]
L-ascorbate (Vitamin C)	1	mM	0[d]
BHT (Butylhydroxytoluene)	1	mM	0[d]

* Relative activity is expressed as percentage of the reaction rate obtained for histamine. A rate of 100% corresponds to 126.94 units/mg protein. The different letters in the column denote the significant differences (P<0.05).

** Horse heart (Sigma, type VI).

Table 11. Effect of various inhibitors on the activity of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1*

Inhibitor	Final concentration	% Inhibition
Sulfhydryl agents		
ρ-Chloromercuribenzoate (PCMB)	1.00 mM	27[c]
Iodoacetate	1.00 mM	20[c]
Carbonyl agents		
Isoniazid	1.00 mM	100[a]
Semicarbazide	0.50 mM	100[a]
Hydroxylamine	0.50 mM	100[a]
Potassium cyanide (KCN)	5.00 mM	47[b]
Chelators		
Cuprizone	1.30 µM	100[a]
Ethylenediaminetetraacetic acid (EDTA)	1.00 mM	0[d]
Others		
Tryptophan	0.20 mM	92[a]
Putrescine	0.20 mM	25[c]

* Each enzyme solution was incubated with the same volume of inhibitor at 30°C for 15 min and the residual activity was measured under the standard assay conditions. The different letters in the column denote the significant differences ($P<0.05$).

Kinetic studies

The kinetic of the homogeneous preparation from *Nnm. gari* HDS3-1 for histamine oxidation were studies by using the multi-substrate reactions. The analysis of these reactions was performed by keeping the concentration of histamine constant and varying 1-methoxy PMS. Under these conditions, the enzyme behaves like a single-substrate enzyme and a plot of v by [S] gives apparent K_m and V_{max} constants for 1-methoxy PMS. Moreover, a set of these measurements was also performed at various fixed concentrations of histamine. These data can be used to work out what the mechanism of the reaction is. The kinetic constants, K_m, V_{max} and k_{cat}, were calculated by Lineweaver-Burk plots of enzyme activity *vs*. variable histamine (Figure 30A) and 1-methoxy PMS (Figure 30B) concentrations. The Michaelis constants, K_m, V_{max} and k_{cat} values, for histamine and 1-methoxy PMS were estimated to be 58.69 µM, 2.54 µmol/min and 5.43 (s^{-1}), and 69.77 µM, 3.08 µmol/min and 6.58 (s^{-1}), respectively. The purified histamine dehydrogenase from *Nnm. gari* HDS3-1 had lower K_m value for histamine (59 µM) than those of aromatic amine dehydrogenase from *Alcaligenes xylosoxidans* (171 µM) (Kondo *et al*., 2004), histamine dehydrogenase from *Nocardioides simplex* (75 µM) (Siddiqui *et al*., 2000), aromatic amine dehydrogenase from *Alcaligenes faecalis* (5.6 mM) (Hyun and Davidson, 1995) and methylamine dehydrogenase from *Paracoccus denitrificans* (1.5 mM) (Davidson, 1989) (Table 12). This result suggests that purified histamine dehydrogenase from *Nnm. gari* HDS3-1 has higher affinity to histamine, compared to those of other sources. Low K_m values were also found for histamine dehydrogenase from *Nocardioides simplex* (31 µM) (Limburg *et al*., 2005) and purified histamine dehydrogenase from *Rhizobium* sp. 4-9 (10 µM) (Sato *et al*., 2005). The purified histamine dehydrogenase from *Nnm. gari* HDS3-1 had a slightly lower turnover number (k_{cat}) (5.4 s^{-1}) than that of the purified histamine dehydrogenase from *Rhizobium* sp. 4-9 (8.3 s^{-1}) (Sato *et al*., 2005).

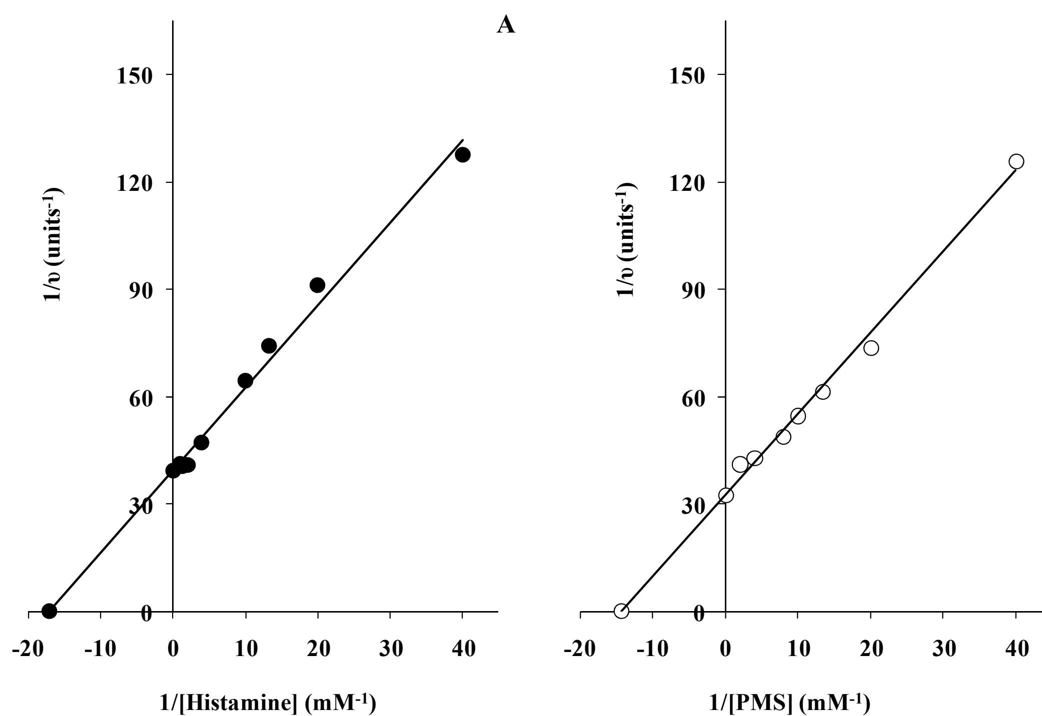


Figure 30. Kinetics of the reaction of the purified histamine dehydrogenase from *Nnm. gari* HDS3-1. (A) Double reciprocal plots of enzyme activity against histamine concentration in the presence of 500 μM 1-methoxy PMS. (B) Double reciprocal plots of enzyme activity against 1-methoxy PMS concentration in the presence of 5.0 mM histamine. The assays were determined at pH 7.0 and 45°C in the presence of 4.5 M NaCl. The final enzyme concentration for the assay was 0.1 mg/mL.

Table 12. Kinetic properties for histamine oxidation of amine dehydrogenase from various sources

Source	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Nnm. gari HDS3-1	58.69	5.43	92.44
A. xylosoxidans (Kondo *et al*., 2004)	171	ND*	ND
N. simplex (Siddiqui *et al*., 2000)	75	ND	ND
A. faecalis (Hyun and Davidson, 1995)	5,600	ND	ND
P. denitrificans (Davidson, 1989)	1,500	ND	ND
N. simplex (Limburg *et al*., 2005)	31	6.6	210
Rhizobium sp. 4-9 (Sato *et al*., 2005)	10	8.3	830

* ND, not determined.

4.5 Conclusion

Purified histamine dehydrogenase from *Natrinema gari* HDS3-1 had a molecular mass of about 127.5 kDa and was suggested to be a trimer of subunits that had molecular masses of 69.1, 29.3 and 27.7 kDa. The enzyme had a maximal activity at pH 6.5-8.5 and 40-60°C in the presence of 3.5-5.0 M NaCl. The enzyme was quite stable to the heat treatment and was also stable at high salt concentration up to 3.0-5.0 M. The enzyme was identified to be histamine dehydrogenase based on requirement of electron acceptor, 1-methoxy PMS, substrate specificity and inhibition by carbonyl agents. These characteristics suggest the unique molecular properties that are distinctive from other known histamine dehydrogenases.

CHAPTER 5

WHOLE CELL IMMOBILIZATION OF *NATRINEMA GARI* HDS3-1 FOR HISTAMINE DEGRADATION

5.1 Abstract

Whole cell of *Natrinema gari* HDS3-1, a novel halophilic archaeon, was immobilized on various matrices by different techniques. Among all matrices tested, celite showed the highest immobilization yield. The immobilized whole cell retained the histamine-degrading activity as high as 94% of the original activity detected in free whole cells. The catalytic properties of the immobilized whole cell on the celite support were similar to the corresponding free whole cell including optimal NaCl concentration (4.0-5.0 M), optimal pH (6.5-7.5), and optimal temperature (40-55°C). Histamine-degrading activity either in the presence of NaCl at elevated concentrations or incubation at elevated temperatures became more stable by the immobilization. Both free and immobilized whole cells were able to degrade histamine specifically. The immobilized whole cells could be reused for the degradation of histamine up to 7 cycles without any significant loss in activity. The immobilized whole cell has the potential to be applied for the degradation of histamine in the products containing high salt, like fish sauce.

5.2 Introduction

The presence of high level of histamine in seafoods and seafood products may cause the rejection for import and is also associated with the food poisoning. Histamine is heat stable and not detectable through organoleptic analysis by even trained panelists (Arnold *et al*., 1980). Except for the gamma irradiation, no other food processing methods are available for histamine degradation (Kim *et al*., 2004; Etkind *et al*., 1987).

Natrinema gari HDS3-1 is a novel halophilic archaeon that can reduce histamine in the culture broth during cultivation in the presence of 4.3 M NaCl. The histamine-degrading activity was mediated through the intracellular enzyme and needed an electron acceptor for its activity (Tapingkae, 2009). Due to the limitations of applying *Nnm. gari* HDS3-1 cells and its enzyme caused by the slow growth rate, low yield of enzyme, and the cost of cell or enzyme preparation, the immobilization of whole cells might provide the potential advantages over applications of free cell system and enzyme.

Cell immobilization is the physical confinement or localization of intact cells to a certain defined region of space with preservation of some desired catalytic activity (Karel *et al*., 1985). The use of immobilized whole microbial cells and/or organelles eliminates the often tedious, time consuming, and expensive steps involved in isolation and purification of intracellular enzymes (Ohmiya *et al*., 1977). Additionally, the stability of enzyme can be enhanced by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation (Karel *et al*., 1985). Furthermore, the metabolically active cell immobilization is particularly preferred where co-factor regeneration machinery is necessary for the catalytic reaction (Bernal *et al*., 2007).

Immobilization commonly is accomplished by using four techniques, physical entrapment within a porous matrix, attachment or adsorption to a pre-formed carrier, self-aggregation by flocculation and cell contained behind barrier (Pilkington *et al*., 1998). Nevertheless, a few critical parameters such as the cost of immobilization, mass transport limitations, applicability to a specific end-product, etc. should be taken into consideration. To maximize the use of whole cells of the *Nnm. gari* HDS3-1 for degradation of histamine in the presence of high salt concentration without cell disintegration and complex purification of the enzyme, the appropriate immobilization technique should be applied. The objectives of this study were to select the most effective immobilization method for the whole cell of *Nnm. gari* HDS3-1 and to study the properties of both free and immobilized whole cells.

5.3 Materials and Methods

Culture and growth condition for *Nnm. gari* HDS3-1

Nnm. gari HDS3-1 was grown on agar plates of halophilic medium (Appendix A-1). The inoculum was prepared by inoculating a loopful of cultures into 5 mL of halophilic broth and incubated at 37°C in a shaker incubator (Sartorius, Certomat® BS-1, Goettingen, Germany) at 200 rpm for 7 days. Cells were cultivated by inoculating 5% (v/v) of seed cultures into 200 mL of halophilic medium containing 500 ppm of histamine (free-base) (Appendix A-2) in 500-mL Erlenmeyer flask, and incubated at 37°C in a shaker incubator at 200 rpm for 7 days. Wet cells were harvested in the exponential phase (A_{600} ~ 5.0) by centrifugation at 15,000 ×*g* for 10 min. The pellet was washed twice with 4.3 M NaCl and resuspended in 50 mM Tris-HCl buffer, pH 7.0 containing 4.3 M NaCl.

Histamine-degrading activity assay and protein determination

The histamine-degrading activity was assayed using histamine as substrate. Unless otherwise stated, the free or immobilized whole cells (0.1 g) was added into 1 mL of standard assay mixture consisting of 50 mM Tris-HCl buffer, pH 7.0, 4.3 M NaCl, 500 µM 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) and 5 mM histamine dihydrochloride. The reaction mixture was incubated at 37°C for precisely 1 h under static condition unless otherwise stated. Enzymatic reaction was terminated by adding 1 mL of 0.1 N HCl. Blank was done in the same manner, except the free or immobilized whole cells was added after addition of 1 mL 0.1 N HCl. The histamine contents of reaction and blank were determined by the fluorometric method of AOAC (1995) (Appendix B-1). One unit of activity was defined as the degradation of 1 µmole histamine per h per g matrix under the specified conditions.

Protein was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard. The amount of bound protein was determined indirectly from the difference between the amount of protein

introduced into the coupling reaction mixture and the total amount of protein in both filtrate and washing solutions.

Immobilization of whole cell

Adsorption

Pig bone: The procedure of Negishi *et al*. (1989) was used with some modifications. Pig bone powders were washed with ethanol for five times, followed by distilled water. Five grams of washed pig bone were added to 10 mL of 50 mM Tris-HCl buffer, pH 7.0 containing 4.5 M NaCl (referred as standard buffer) and 1 g of wet cell paste. The mixture was agitated (100 rpm) for 1 h at 0°C. The immobilized whole cells were filtered using Whatman paper No.1, washed with a standard buffer until no protein was released, dried under vacuum for 3 h at room temperature and stored at 4°C until used.

Chitosan flakes: The procedure of Pereira *et al*. (2003) was applied with some modifications. Five grams of chitosan flakes were soaked in hexane under agitation conditions (100 rpm) for 1 h, and air dried. The soaked chitosan flakes were added to 10 mL of standard buffer containing 1 g of wet cell paste. The mixture was agitated (100 rpm) for 3 h at room temperature, and allowed to stand for 18 h at 4°C. The immobilized whole cell was filtered using Whatman paper No. 1, washed and stored as previously mentioned.

Celite: The procedure of Khare and Nakajima (2000) was used with some modifications. Celite 545 (5 g) was added to 10 mL of standard buffer containing 1 g of wet cell paste. The mixture was agitated (100 rpm) for 1 h at 0°C. The immobilized whole cells were filtered using Whatman paper No. 1, washed and stored as previously mentioned.

Entrapment

Agar: The procedure of Takeno *et al*. (2005) was used with some modifications. Standard buffer containing 4% (w/v) agar (solidifying agent, Difco Laboratories, Becton, Dickinson and Company, Sparks, MD, USA) was autoclaved at 121°C (15 psi) for 15 min. Warm agar solution (50°C) (2.5 mL) was mixed with 2.5 mL of standard buffer containing 1 g of wet cell paste at 60°C. This mixture was quickly poured into a plate to obtain the thickness of 8 mm and cooled at room temperature. The immobilized whole cells were cut into 8 mm cubes (~ 8 × 8 × 8 mm) and washed with standard buffer until no protein was released. The obtained immobilized whole cell was then stored at 4°C until used.

Ca^{2+}-Alginate beads: The procedure of Takeno *et al*. (2005) was used with some modifications. A 2.5 mL of alginate solution (3%, w/v) was mixed with 2.5 mL standard buffer containing 1 g whole cell. The slurry was added dropwise (diameter ~ 2 mm) into 100 mL of 0.15 M $CaCl_2$. After 30 min, the gel beads containing immobilized whole cells were washed and stored as described above.

Cross-linking

Alginate-chitosan beads: The procedure of Vidyasagar *et al*. (2006) was used with some modifications. A 2.5 mL of 3% (w/v) alginate solution was mixed with 2.5 mL of standard buffer containing 1 g of wet cell paste. The slurry was added dropwise (diameter ~ 2 mm) into 100 mL of 0.5% (w/v) chitosan dissolved in 3% (v/v) acetic acid solution containing 0.5% (w/v) $CaCl_2$. After 30 min, the gel beads containing immobilized whole cells were washed and stored as previously mentioned.

Cross-linked alginate beads: The procedure of Vidyasagar *et al*. (2006) was used with some modifications. A 2 mL of 3% (w/v) sodium alginate was mixed with 2 mL of standard buffer containing 1 g of wet cell paste. One mL of glutaraldehyde solution (0.5%, v/v) was added, followed by gentle mixing. The

mixture was kept for 90 min at 30°C. The slurry was added dropwise (diameter ~ 2 mm) into 100 mL of 0.05 M $CaCl_2$. After 30 min, the gel beads containing immobilized whole cells were washed and stored as previously mentioned.

Cross-linked chitosan beads: The procedure of Carrara and Rubiolo (1994) was used with some modifications. Chitosan (3 g) was dissolved in 2% (v/v) acetic acid with the continuous stirring for 90 min. The chitosan solution was filtered through cheesecloth to remove insoluble materials and was added dropwise (diameter ~ 4 mm) into 150 mL of 1.5% (w/v) sodium triphosphate solution with the gentle stirring. The chitosan beads were washed with the standard buffer until neutrality. The beads were activated with standard buffer containing 1% (v/v) glutaraldehyde for 24 h at room temperature. A 5 g of the activated chitosan beads was mixed with 10 mL of standard buffer containing 1 g of wet cell paste. The mixture was gently agitated (100 rpm) at room temperature overnight. The beads obtained were washed and stored as previously mentioned.

Eggshell: The procedure of Venkaiah and Kumar (1994) was used with some modifications. Small pieces of hen eggshell were boiled for 20 min, washed with acetone and dried in an oven at 60°C for 12 h. The pieces were crushed in a blender for 5 min and sieved through a wire mesh (100 mesh size). Ground eggshell (5 g) was mixed with 10 mL of standard buffer containing 1 g of wet cell paste. The mixture was gently agitated at 100 rpm and 2 mL of 6% (v/v) glutaraldehyde was slowly added during stirring. The mixture was incubated at 10°C for 12 h. The residues were obtained by centrifugation at 4°C, 6,000 ×*g* for 15 min. The immobilized whole cell was washed, dried, and stored as previously mentioned.

Selection of immobilization method

Whole cells immobilized by different techniques were subjected to determination of histamine-degrading activity, protein loading ratio, and immobilization yield, which were defined as follows:

Histamine-degrading activity (units/g matrix)

$$= \frac{\text{Activity of immobilized whole cell}}{\text{Amount of immobilized whole cell}}$$

Protein loading ratio (%)

$$= \frac{\text{Amount of protein bound (loaded)}}{\text{Amount of protein initial (introduced)}} \times 100\%$$

Immobilization yield (%)

$$= \frac{\text{Total activity of immobilized whole cell (units)}}{\text{Total activity of load free whole cell (units)}} \times 100\%$$

Immobilization method rendering the maximal immobilization yield was chosen for further study.

Effect of salt concentration on activity and stability of free and immobilized whole cells

To remove salt, 1 g of free and immobilized whole cells (~1×10^6 CFU) was dialyzed against 100 volumes of 50 mM Tris-HCl buffer, pH 7.0 at 4°C. Desalted samples were subjected to activity determination in the presence of NaCl at various concentrations (0 to 5 M) using the standard assay conditions. To study the effect of NaCl on stability, the desalted samples were dialyzed against 100 volumes of standard buffers containing different NaCl concentrations (0-5 M) at room temperature for 24 h. The remaining activity was assayed using the standard assay conditions.

Effect of pH on activity and stability of free and immobilized whole cells

The activity of free and immobilized whole cells were assayed at 37°C over the pH range of 4 to 9 (50 mM acetate buffer (pH 4.0-5.5); 50 mM phosphate buffer (pH 6.0-6.5); and 50 mM Tris-HCl buffer (pH 7.0-9.0)). To study the effect of pH on stability, free and immobilized whole cells were incubated in buffers at the

designated pH in the presence of 4.5 M NaCl for 24 h at 30°C. The remaining activity was assayed using the standard assay conditions.

Effect of temperature on activity and stability of free and immobilized whole cells

Enzyme activity was assayed at different temperatures in the range of 30-80°C as previously described. To study the effect of temperature on enzyme stability, the free and immobilized whole cells were subjected to heating at different temperatures ranging from 4-80°C for 1 h, in a temperature-controlled water bath (Memmert, Germany). Thereafter, the enzyme mixture was immediately cooled in an ice bath, and the remaining activity was assayed under the standard assay conditions. Relative activity of free and immobilized whole cells was calculated, in comparison with that without heating.

Operational stability of free and immobilized whole cells

The operational stability of the free and immobilized whole cells was assayed by using 1 g of free and immobilized whole cells (~1×10^6 CFU) in 10 mL of standard assay mixture containing histamine in successive batches. The samples were kept in an incubator shaker with a shaking speed of 200 rpm at 37°C for 1 h. Samples were taken and measured for the residual histamine content by using the fluorometric method of AOAC (1995). At the end of each batch, the free and immobilized whole cells were removed from the reaction medium and washed with 5 volumes of 50 mM Tris-HCl buffer (pH 7.0) containing 4.5 M NaCl to remove any substrate or product retained in the matrix. The free and immobilized whole cells were introduced into a fresh medium. Activities were estimated at the end of each cycle. The above-mentioned steps were repeated until the enzyme lost 50% of its total activity. The residual activity was calculated and expressed relatively to that found for the first cycle.

Substrate preference of free and immobilized whole cells

The substrate preference of free and immobilized whole cells was determined by measuring the degradation of biogenic amines including histamine, tryptamine, β-phenylethylamine, putrescine, cadaverine, tyramine, spermidine and spermine. Free and immobilized whole cells (~1×10^6 CFU) were added to 10 mL of assay mixture containing 50 mM Tris-HCl buffer (pH 7.0), 4.5 M NaCl, 500 µM 1-methoxy PMS and 5 mM of each biogenic amines. The reaction mixtures were incubated at 45°C in an incubator shaker with a speed of 200 rpm for 1 h and immediately stopped by adding 1 mL of 0.4 N perchloric acid. The mixture was centrifuged at 15,000 ×*g* for 10 min. The supernatant was subjected to analysis of biogenic amines. Blank was done in the same manner, except free or immobilized whole cells were added after addition of 1 mL 0.4 N perchloric acid.

Biogenic amines in supernatant were extracted and derivertized according to the procedure of Eerola *et al*. (1993) with a slight modification. The supernatant (480 µL) was added with 20 µL of 2 mg/mL of 1,7-diaminoheptane as the internal standard and 500 µL of 0.4 M perchloric acid. The mixtures were mixed thoroughly by a vortex mixer for 5 min and then centrifuged at a speed of 15,000 ×*g* at 25°C for 5 min. The obtained supernatant (300 µL) was mixed with 60 µL of 2 N NaOH and 90 µL of saturated sodium bicarbonate. A 600 µL of 10 mg/mL of dansyl chloride was added to each sample, mixed well, and then incubated for 45 min at 40°C. Residual dansyl chloride was removed by adding 30 µL of 25% (v/v) ammonia and centrifuged at 3,500 ×*g* for 5 min. The supernatant was collected and filtered through a 0.45 µm membrane filter prior to analysis by high performance liquid chromatography (HPLC). A Waters Separation Module 2690 was operated to give a flow rate of 1.5 mL/min of the mobile phase of 0.1% (v/v) acetic acid (solvent A) and acetonitrile containing 0.1% (v/v) acetic acid (solvent B). Separation was achieved using a column of Hypersil BDS C18 (300 × 7.8 mm i.d.) set at 40°C. Samples (20 µL) were injected and a photo diode array (Model Waters 996), set at the wavelength of 254 nm, was used as the detector. Data were processed and analyzed using Millennium 32 software (Waters, Milford, MA, USA).

Statistic analysis

All experiments were run in triplicate. A completely randomized design was used throughout this study. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows: SPSS Inc.).

5.4. Results and Discussion

Selection of the matrices for immobilization of *Nnm. gari* HDS3-1

Whole cells of *Nnm. gari* HDS3-1 was immobilized on various matrices by different methods (Table 13). The immobilized whole cells prepared by adsorption on celite had the highest immobilization activity (17.5 units/g matrix) and the highest immobilization yield (94.1%), followed by entrapment in agar (solidifying agent, Difco^{TM}). Celite presents a porous structure, which allows bacterial cells to be adhered to the surface. Celite consists of highly porous diatomaceous beads composed of silica (SiO_2) and some other inorganic oxides. Due to its chemical inertness and unique interconnected pore structure, celite is suitable for physical adsorption (Chang *et al*., 2007) and was selected as matrix for entrapment of *Nnm. gari* BCC24369 cells. Apart from immobilization yield, adsorption on the celite support is probably the most suitable immobilization procedure owing to simplicity, low cost, and good stability (Keshavarz *et al*., 1990).

The adsorption phenomenon is fundamentally based on weak attraction forces to an inert carrier that has not been chemically derived (Kolot, 1981). In the present study, pig bone and chitosan flakes were less effective than celite on whole cell adsorption. This is probably due to an unfavorable morphological and physical characteristic for binding to the external cell walls of the halophilic archaea which typically contain negatively charged glycoproteins and sulfate (Litchfield *et al*., 1999). Pig bone particles generally have irregular shapes and porous structures which are formed by collagen and hydroxyapatite ($Ca_5(PO_4)_3(OH)$) (Negishi *et al*., 1989).

Table 13. Immobilization of histamine-degrading enzyme from whole cell of *Nnm. gari* HDS3-1 on various matrices

Support	Total protein (mg)		Loading efficiency (%)	Unbound activity (units/mL)	Immobilized activity (units/g support)	Immobilization yield (%)
	Bound	Unbound				
Pig Bone	3.7 ± 0.1^{e}*	1.48 ± 0.1^{b}	71.6 ± 8.0^{d}	5.0 ± 1.2^{a}	10.9 ± 0.5^{b}	58.5 ± 2.6^{c}
Chitosan Flakes	3.6 ± 0.1^{f}	1.63 ± 0.1^{a}	68.7 ± 9.0^{d}	2.1 ± 0.9^{b}	10.8 ± 0.6^{b}	58.2 ± 3.5^{c}
Celite	5.0 ± 0.0^{b}	0.2 ± 0.0^{e}	96.3 ± 1.0^{b}	0.5 ± 0.1^{c}	17.5 ± 0.6^{a}	94.1 ± 3.4^{a}
Agar	5.2 ± 0.0^{a}	0.0 ± 0.0^{e}	100.0 ± 0.0^{a}	0.0 ± 0.0^{d}	15.5 ± 0.9^{b}	83.4 ± 4.7^{b}
Ca^{2+}-Alginate beads	5.2 ± 0.0^{a}	0.0 ± 0.0^{e}	100.0 ± 0.0^{a}	0.0 ± 0.0^{d}	7.7 ± 0.5^{c}	41.1 ± 2.5^{d}
Alginate-Chitosan beads	5.2 ± 0.0^{a}	0.0 ± 0.0^{e}	100.0 ± 0.0^{a}	0.0 ± 0.0^{d}	8.3 ± 0.5^{c}	44.5 ± 2.5^{d}
Cross-linked alginate beads	5.2 ± 0.0^{a}	0.0 ± 0.0^{e}	100.0 ± 0.0^{a}	0.0 ± 0.0^{d}	3.0 ± 0.6^{e}	16.2 ± 3.1^{e}
Cross-linked chitosan beads	4.2 ± 0.1^{d}	1.05 ± 0.1^{c}	79.8 ± 12.0^{cd}	1.4 ± 0.4^{b}	3.7 ± 1.2^{d}	19.6 ± 6.5^{e}
Eggshell	4.5 ± 0.0^{c}	0.69 ± 0.0^{d}	86.8 ± 4.0^{c}	0.4 ± 0.1^{c}	3.9 ± 0.7^{d}	20.8 ± 4.0^{e}

* Different superscripts in the same column indicate significant differences (P<0.05).

** Mean ± SD (n = 3).

Assays were done in the presence of 4.3 M NaCl at 37°C, pH 7.0 under static condition. 100% of the immobilized yield is equivalent to the histamine-degrading activity of 18.6 ± 1.2 units/g.

Since the OH^- can be replaced by fluoride, chloride and carbonate, it was more likely to have sterical hindrances and charge-charge repulsion with the external cell walls of the halophilic archaea. On the other hand, adsorbing ability of chitosan flakes was probably limited by the intrinsic crystalline structure though the adsorption can be readily caused by interaction between amino groups of chitosan and cells.

With the entrapment technique, the cells entrapped in 2% (w/v) agar exhibited maximum immobilization activity. Higher concentrations of agar in the gel decreased the histamine-degrading activity of immobilized cells (data not shown) probably due to smaller pore size in the blocks which might have interfered with the diffusion of substrate and product in the gel. The apparent activity observed in Ca^{2+}-alginate beads was 50% lower than that found in agar support. The decrease in activity might be due to the chemical instability of enzyme and matrices under high salt concentrations (Horitsu *et al*., 1990; Martinsen *et al*., 1989). To enhance the chemical resistance and mechanical strength of alginate and chitosan beads, the cross-linked alginate and chitosan beads were prepared using either chitosan or glutaraldehyde as a cross-linking agent. Although better bead characteristics were observed in which the beads did not dissolve easily in the presence of salt, immobilization via cross-linking on the alginate or chitosan beads did not significantly improve the immobilization activity of the whole cell ($P>0.05$). Moreover, the addition of glutaraldehyde also resulted in a significant decrease in histamine-degrading activity of the immobilized whole cells. Similar effect of glutaraldehyde was observed with eggshell matrix. This was probably attributed to some forms of toxicity or inhibition caused by glutaraldehyde as reported by Nighojkar *et al*. (2006), Ragnitz *et al*. (2001), and Bryjak *et al*. (1993).

Characterization of free and immobilized whole cells from *Nnm. gari* HDS3-1

Effect of salt

The optimum NaCl concentrations of histamine-degrading activities of free and immobilized whole cells were found at 4-5 M and 3-5 M, respectively

(Figure 31A). The decrease in NaCl concentration resulted in lowering enzyme activity. The lowest enzyme activities of free and immobilized whole cells were found in the absence of NaCl, in which 38.1 and 52.0% of activity was obtained, respectively. This result clearly indicates that the histamine-degrading activity of the whole cell was salt dependent. Figure 31B shows the effect of NaCl concentrations on the stability of the preparations. The histamine-degrading activity was stable for at least 24 h when incubated in 50 mM Tris-HCl buffer pH 7.0 in the presence of 3.5-5.0 M NaCl.

The enzyme activities of free and immobilized whole cells retained about 50% in the presence of 1.5 and 2.5 M NaCl, respectively. In the absence of salt, the enzyme of immobilized whole cell was more stable than that of free whole cell ($P<0.05$). The result indicated that the immobilization improved the stability of the cell to NaCl at sub-optimal level. Many halophilic enzymes require the presence of NaCl or KCl concentrations in the range of 1 to 4 M for optimum activity and stability (Mevarech *et al*., 2000). Upon exposure to low ionic strength, most of the halophilic or halotolerant enzymes were inactivated irreversibly (Holmes *et al*., 1997; Studdert *et al*., 1997). However, immobilized whole cell of *Nnm. gari* HDS3-1 regained their histamine-degrading activity upon reestablishment to high salt environment.

Effect of pH

The optimum pH values of the histamine-degrading activity of free and immobilized whole cells were 6.5-7.5 (Figure 32A). At pH 4, only 10% of activity was found for both preparations. As the pH increased, the activity increased rapidly, reaching a maximum at pH 7. The activity decreased at pH above 7 in which at pH 9 the relative activities of 69.0 and 71.0% were obtained for free and immobilized whole cells, respectively. The histamine-degrading activity was stable for at least 24 h when both of the preparations were incubated in the buffers at pH ranging from 6-9 (Figure 32B). However, the activity decreased markedly at pH below 6 and lower than 50% at pH 4. There were no significant differences in pH stability between the free and immobilized whole cells ($P>0.05$).

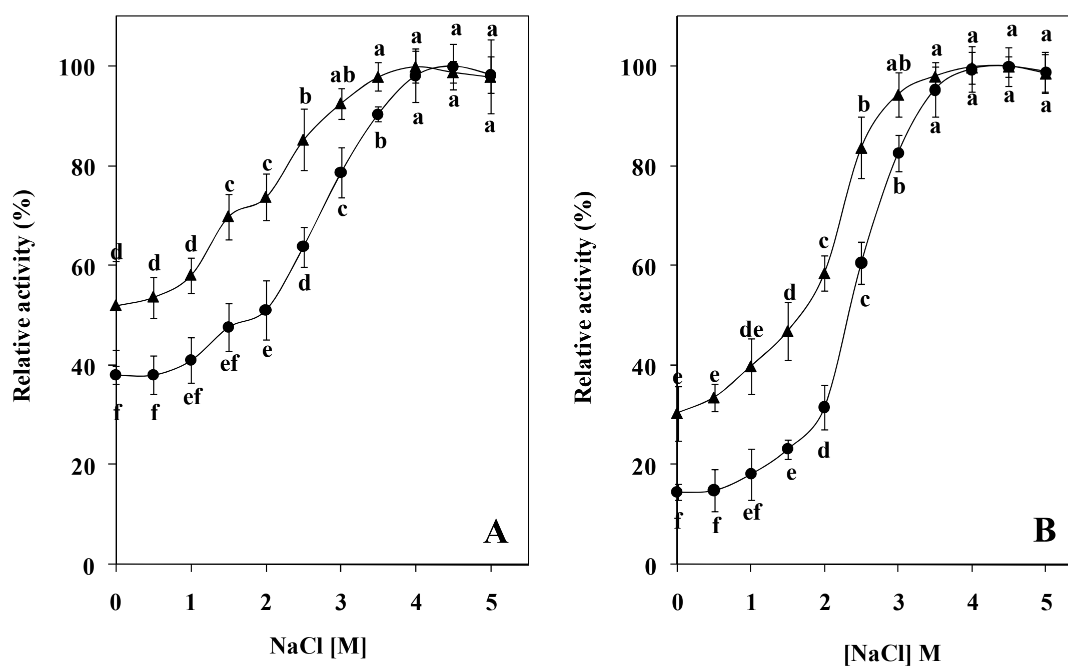


Figure 31. Effect of NaCl concentration on histamine-degrading activity (A) and stability (B) of free (●) and immobilized whole cells (▲) on celite support. Histamine-degrading activity was determined by incubating free and immobilized whole cells at 37°C in 50 mM Tris-HCl buffer, pH 7.0 in the presence of various salt concentrations (0-5 M NaCl) under static condition. Bars represent the standard deviation (n=3). The different letters in the same line denote the significant differences ($P<0.05$).

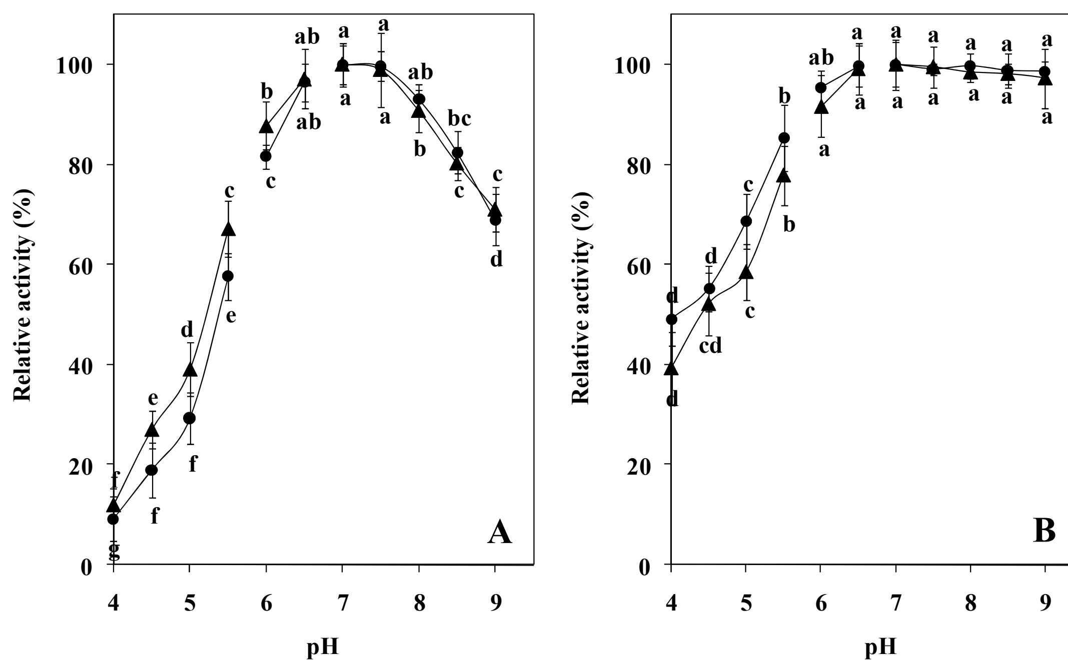


Figure 32. pH profile (A) and pH stability (B) of free (●) and immobilized whole cells (▲) on celite support. For the pH profile, histamine-degrading activity was determined by incubating free and immobilized whole cells at 37°C at various pH values in the presence of 4.5 M NaCl under static condition. For the pH stability, free and immobilized whole cells were incubated in buffers at the designated pH in the presence of 4.5 M NaCl for 24 h at 30°C. Bars represent the standard deviation (n = 3). The different letters in the same line denote the significant differences ($P<0.05$).

Effect of temperature

Figure 33A shows the effect of temperature on the histamine-degrading activity of free and immobilized whole cells. The maximum activity was observed at temperature around 40-60°C for both preparations. At 80°C, the preparations showed the relative activities of 50.1% and 65.8% for free and immobilized whole cells, respectively. The enzyme activities of free and immobilized whole cells retained over 90% after incubating at 4°C to 50°C for 1 h (Figure 33B). The activities of both cell types rapidly decreased after being incubated at temperature higher than 55°C for 1 h. However, the immobilized whole cells retained more activity than free whole cells when heated at temperature ranging from 65-80°C ($P<0.05$). The result indicated that immobilization slightly improved the stability of cell to heat treatment. The higher temperature profile observed in case of immobilized whole cell may be because of some conformational effects due to adsorption, which protects the cellular proteins against heat denaturation.

Operational stability of the free and immobilized whole cells

Both free and immobilized whole cells could be used up to 5 and 7 times, respectively without decreasing their activity (Figure 34). With increasing repetitions, the activity of immobilized whole cells gradually decreased but still remained over 90% at the 8th cycle ($P>0.05$). In contrast, the activity of free whole cell rapidly decreased after the 5th cycle and remained only 50% at the 8th cycle ($P<0.05$). At the 10th cycle, the activity of free and immobilized whole cells retained about 30% and 50%, respectively ($P<0.05$). The best immobilization yield of whole cell of *Nnm. gari* HDS3-1 on celite indicated that cell should have some specific groups that can attach to the SiO_2. However, its major drawback was also found in the immobilized whole cell by desorption from the support during utilization of the biocatalyst by the conditions used.

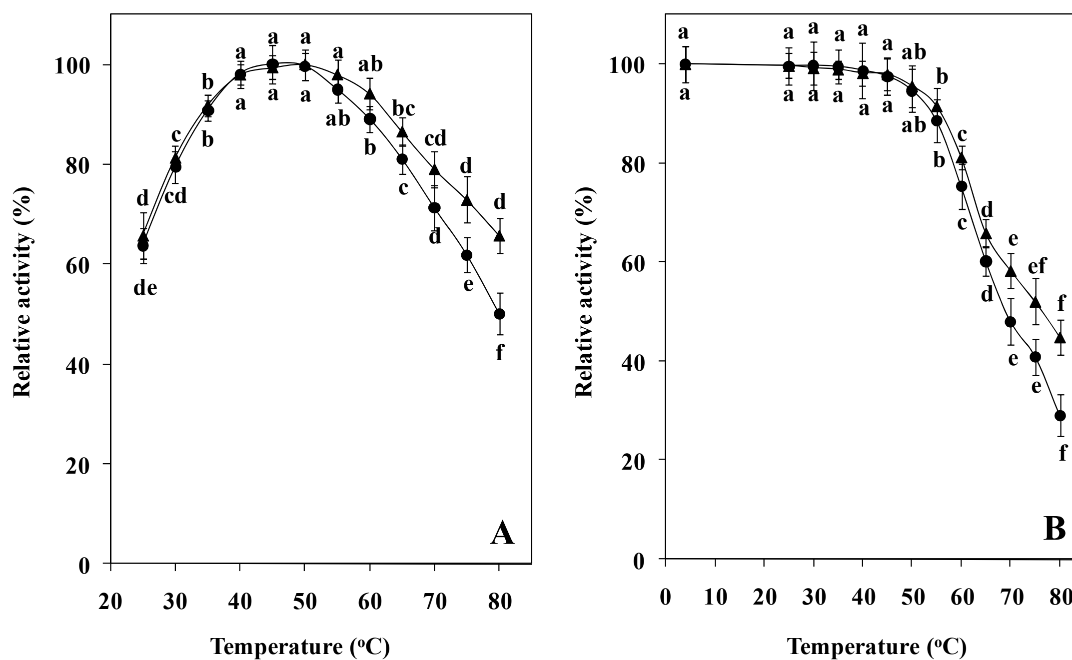


Figure 33. Temperature profile (A) and thermal stability (B) of free (●) and immobilized whole cells (▲) on celite support. For the temperature profile, histamine-degrading activity was determined by incubating free and immobilized whole cells at various temperatures, in 50 mM Tris-HCl buffer, pH 7.0 in the presence of 4.5 M NaCl under static condition. For the thermal stability, free and immobilized whole cells were incubated in 50 mM Tris-HCl buffer, pH 7.0 in the presence of 4.5 M NaCl for 24 h at the designated temperature. Bars represent the standard deviation (n=3). The different letters in the same line denote the significant differences (P<0.05).

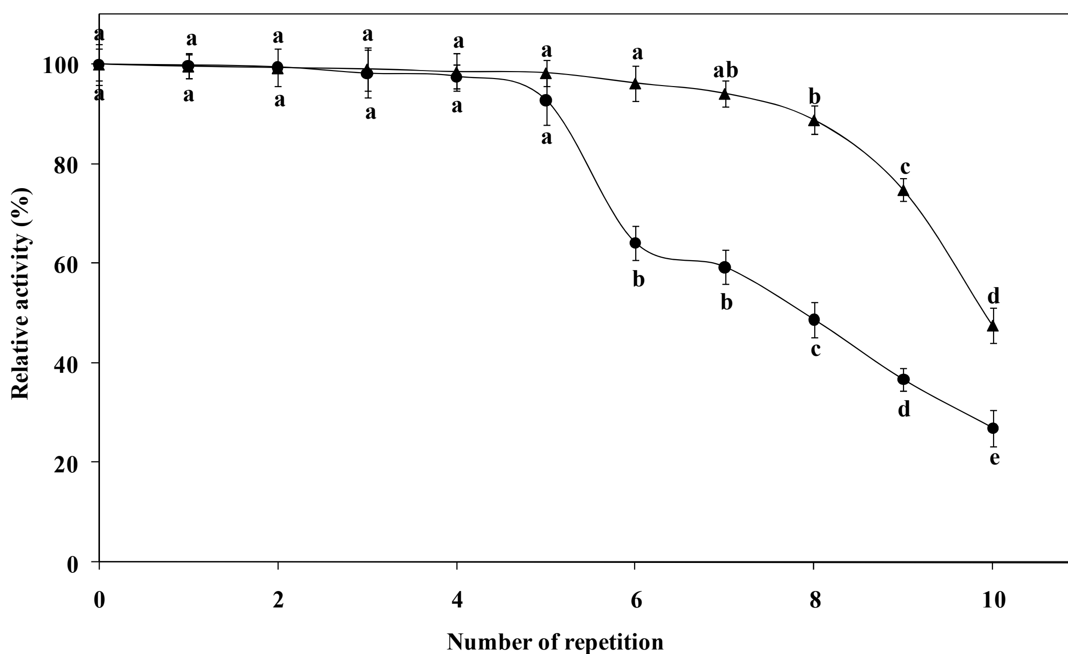


Figure 34. Effect of reusability on the histamine-degrading activity of free (●) and immobilized (▲) whole cells. 100% equivalent to 33.5 and 33.2 units/g for free and immobilized whole cells on celite support, respectively. Bars represent the standard deviation (n = 3). The different letters in the same line denote the significant differences (*P*<0.05).

Generally, adsorption is a simple physical process in which the forces involved in cell attachment are so weak; cells that are several micrometers across are not strongly adsorbed and are readily lost from the surface of the adsorbent. The less effectiveness in the retention of whole cell on celite compared with other immobilization was reported. Fernandes *et al.* (2002) indicated that whole cells of *Pseudomonas putida* immobilized on celite showed lower cell retention than those of microencapsulated in Ca-alginate or κ-carrageenan.

Substrate selectivity

Figure 35 shows that both of the preparations degraded histamine much more efficiently compared to tryptamine, β-phenylethylamine, putrescine, cadaverine, tyramine, spermidine, and spermine ($P<0.05$). About 80% of histamine was degraded after treated with either free or immobilized whole cells. However, putrescine and tryptamine were also degraded to some levels. Only small amounts of other biogenic amines determined were degraded. The purified amine dehydrogenase from *Pseudomonas* AM1 had a wide specificity and could oxidise many compounds that contain a primary amino group, notably ethanolamine and histamine (Eady and Large, 1968). Whereas the purified histamine dehydrogenase from *Nocardioides simplex* IFO 12069 showed high activity toward histamine, but it still oxidized agmatine and putrescine at a rate of 30%, compared to histamine (Siddiqui *et al*., 2000). Among 20 kinds of biogenic amines including histamine studied by Bakke *et al*. (2005), the purified recombinant histamine dehydrogenase from *Rhizobium* sp. 4-9 was very specific toward histamine and only oxidized agmatine and 1,3-diaminopropane with the rate of 10 and 13% of histamine, respectively. Fujieda *et al*. (2004) indicated that the difference between histamine dehydrogenase and other amine dehydrogenase were the specificity toward histamine with low activity to other amines of the latter. The results confirmed that the enzyme in whole cell was most likely histamine dehydrogenase. Due to the high efficacy in degrading histamine at high salt concentration, immobilized whole cells could be used to remove histamine in fish sauce which contained high salt (~25-30% NaCl).

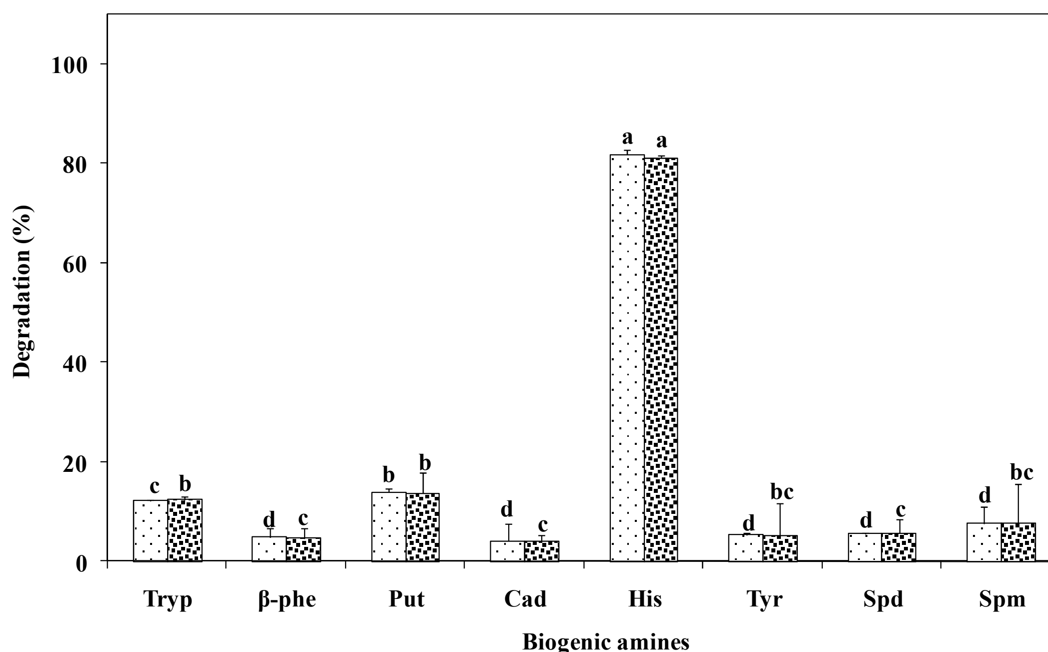


Figure 35. Substrate preference of free (▒) and immobilized (▓) whole cells on celite support. The assay was done in 50 mM Tris-HCl buffer, pH 7.0 in the presence of 4.5 M NaCl at 45°C, under shaking condition (200 rpm) for 1 h. Bars represent the standard deviation (n = 3). The different letters in the column denote the significant differences ($P<0.05$). Tryp: tryptamine; β-phe: β-phenylethylamine; Put: putrescine; Cad: cadaverine; His: histamine; Tyr: tyramine; Spd: spermidine and Spm: spermine.

5.5 Conclusion

Whole cell adsorption to celite provided the best compromise between biocatalyst retention and apparent activity in degradation of histamine. The immobilized whole cells of *Natrinema gari* HDS3-1 on the celite support was more promising than the free whole cells for histamine degradation in that they were stable to high salt, elevated temperatures, and reusable.

CHAPTER 6

EFFECTS OF FREE AND IMMOBILIZED WHOLE CELLS OF *NATRINEMA GARI* HDS3-1 ON THE HISTAMINE DEGRADATION AND CHARACTERISTICS OF FISH SAUCE

6.1 Abstract

Feasibility study of using free and immobilized whole cells of *Natrinema gari* HDS3-1 to degrade histamine in fish sauce was investigated. Free and immobilized whole cells were added at levels of 5% and 10% (w/v) to a fish sauce that was prior supplemented with 0.2% (w/v) riboflavin and adjusted to pH 7.0. The samples were then incubated at an ambient temperature (30°C) for 2 days. Fish sauce samples added with free and immobilized whole cells had lower histamine contents than those of the control (either without addition of free or immobilized whole cell) throughout the incubation period. The rate of histamine degradation was dependent on the amount of free and immobilized whole cells added. Among all treatments, fish sauce added with immobilized whole cell at 10% (w/v) exhibited the greatest histamine degradation, particularly at the 1st day of incubation. All treated samples had similar pH, total nitrogen, amino nitrogen, formaldehyde nitrogen, and ammonical nitrogen to those of the untreated sample (P>0.05). Except histamine, no changes in other biogenic amines were observed after treatment. Fish sauce treated with immobilized whole cell at both levels (5% and 10%, w/v) tended to gain higher acceptability than those with and without whole cells addition. The results implied that the immobilized whole cell of *Nnm. gari* HDS3-1 can be applied to degrade histamine in fish sauce without adverse effect or changes in the overall acceptability.

6.2 Introduction

In recent years, more concerns about food safety, together with the consumer's demand for safe and healthier products, have promoted studies of compounds with harmful effects on human health. The presence of high level of histamine is detrimental to the quality and safety of some Thai fermented fishery products especially those produced from scombroid fish species. Histamine intoxication or Scombroid poisoning is one of major health significances associated with ingestion of foods containing high histamine. Among a variety of fermented fishery products in Thailand, fish sauce is particularly important in its economic value for both local and international markets. With a concern on the toxicological effects, high content of histamine in commercial products becomes a major threat of exporting of these products, due to a more strenuous regulatory measure and inspection.

Depending on the quality of fish used and uncontrolled outdoor fermentation process, often fish sauce is known to contain high level of histamine. Due to its pharmaceutical processes such as allergy and inflammation, histamine has been considered as the most important biogenic amine. Allergy-like food poisoning generally occurs when food containing 1,000 ppm or more of histamine is consumed (Arnold and Brown, 1978). However, poisoning may be caused in some individuals even when histamine has not reached this level. Although the mechanism of food-based histamine allergies has not yet been determined, it is probably due to the synergistic action with other amines contained in food, such as putrescine and cadaverine (Hall *et al*., 1999; Sourkes and Missala, 1981; Bieganski *et al*., 1980). In the United States, the toxic level of histamine that poses a risk to health is set at 500 ppm, and the caution level is 50 ppm. The European Union has also set a level of 100-200 ppm for seafood, and Codex has also proposed regulations at this level (Lehane and Olley, 2000; Anonymous, 1998; Anonymous, 1991). Histamine is heat stable and is not detectable through organoleptic analysis by even trained panelists (Arnold *et al*., 1980). Except for the gamma irradiation, no other food processing methods are available for histamine degradation (Kim *et al*., 2004; Etkind *et al*., 1987). Therefore histamine, if present, is difficult to destroy and posts a risk of food intoxication.

Microorganisms and enzyme can provide a means of controlling histamine accumulation (Voigt and Eitenmiller, 1978; Ienista, 1971; McGowan and Muir, 1971). The presence of histamine-degrading enzymes either histamine oxidases or histamine dehydrogenases has been reported in various higher organisms as well as in microorganisms. Nevertheless, the applications of these microorganisms and enzymes have been restricted by unfavorable physiological conditions for growth and enzyme activity such as low oxygen concentration, low pH value, undesirable temperature and especially in the high salinity. In the course of screening useful microorganism and enzyme capable of degrading histamine in high salt condition, we found *Natrinema gari* HDS3-1 that catalyzed the oxidation of histamine specifically through the activity of histamine dehydrogenase. To study the feasibility of applying this microorganism, in this study the free and immobilized whole cells of the *Nnm. gari* HDS3-1 were used as source of intracellular enzyme for degradation of histamine in commercial fish sauce. The effects on the physico-chemical properties and acceptability of treated fish sauce were also investigated.

6.3 Materials and Methods

Chemicals

Histamine dihydrochloride was purchased from Sigma Chemical Company (St. Louis, MO). Riboflavin was purchased from Acros Organics (Geel, Belgium). The other chemicals were of analytical grade.

Preparation of free and immobilized whole cells of *Nnm. gari* HDS3-1

Nnm. gari HDS3-1 was grown on agar plates of halophilic medium (Appendix A-1) by incubating at 37°C for 1 week. The inoculum was prepared by inoculating a loopful of cultures into 5 mL of halophilic broth and incubated at 37°C in a shaker incubator at 200 rpm for 7 days. Cells were cultivated by inoculating 5% (v/v) of seed cultures into 200 mL of histamine broth in 500-mL Erlenmeyer flask, halophilic medium containing 5 mM histamine (free-base) (Appendix A-2), and

incubated at 37°C in a shaker incubator at 200 rpm for 7 days. Wet cell paste was harvested in the exponential phase (the optical density was about 5.0 at 600 nm) by centrifugation at 15,000 ×*g* for 30 min. The pellet was washed twice with 4.3 M NaCl, resuspended in 50 mM Tris-HCl buffer, pH 7.0 containing 4.3 M NaCl and referred as to "free whole cell ". For immobilization, the procedure of Khare and Nakajima (2000) was used with some modifications. Celite 545 (5 g) was added to 10 mL of 50 mM Tris-HCl buffer, pH 7.0 containing 4.3 M NaCl containing 1 g of wet cell paste. The mixture was agitated (100 rpm) for 1 h at 0°C. The immobilized whole cell were filtered, washed with the same buffer until no protein released, dried under freeze-drying and stored at 4°C until used.

Sample preparation and treatments

Commercial fish sauce sample collected at end of fermentation process (18th month) were obtained from the Thai Fish Sauce Factory (Squid Brand) Co., Ltd, Samutsongkram province. In this study, the experiment was carried out in 6 treatments including:

Control 1: Commercial fish sauce with no treatment

Control 2: Commercial fish sauce with prior supplementation of 0.2% (w/v) riboflavin (using as an electron acceptor for the enzyme activity) and adjusted pH to 7.0

WC5: Control 2 added with 5% (w/v) of free whole cell of *Nnm. gari* HDS3-1 (containing histamine dehydrogenase activity equal to 84.5 ± 3.0 units)

WC10: Control 2 added with 10% (w/v) of free whole cell of *Nnm. gari* HDS3-1 (containing histamine dehydrogenase activity equal to 169.0 ± 5.8 units)

IWC5: Control 2 added with 5% (w/v) of immobilized whole cell of *Nnm. gari* HDS3-1 (containing histamine dehydrogenase activity equal to 89.8 ± 3.5 units)

IWC10: Control 2 added with 10% (w/v) of immobilized whole cell of *Nnm. gari* HDS3-1 (containing histamine dehydrogenase activity equal to 179.5 ± 4.9 units)

The mixtures were transferred to the screw cab glass bottles. Incubation was carried out at ambient temperature (28-32°C) for 2 days. Samples were taken at each time interval (0, 1, and 2 days) and centrifuged for 15 min at 10,000 ×*g*. The liquid obtained was used for analysis.

Determination of histamine

Histamine was analyzed by AOAC (1995) (Appendix B-1). Fish sauce (5 mL) was homogenized in 25 mL of methanol for 2 min and the mixture was incubated in a water bath at 60°C for 15 min. After cooling to 25°C, the volume was adjusted to 50 mL with methanol and centrifuged at 3,500 ×*g* for 10 min. The methanol filtrate was collected and loaded onto an ion exchange column (200 × 7 mm) packed with Dowex 1-X8 (Sigma Chemical Co., St. Louis, MO, USA), which was converted to hydroxide form by 2 N NaOH. The sample eluents and the standard solutions were derivatized with *o*-phthaldialdehyde (OPA). The fluorescence intensity of the derivatized products was then measured using a RF-1501 spectrofluorophoto-meter (Shimadzu, Kyoto, Japan) at an excitation wavelength of 350 nm and emission wavelength of 444 nm. Histamine standard solutions ranging from 0.1 to 0.5 ppm were used to prepare a standard curve. Histamine contents in the samples were calculated from the standard curve.

Biogenic amine analysis

Determination of tryptamine, β-phenylethylamine, putrescine, cadaverine, tyramine, spermidine, spermine, and histamine were carried out by high-performance liquid chromatography (HPLC) by the method of Eerola *et al*. (1993) with a slight modification. The detail of the biogenic amine determination method is described clearly in Appendix B-2.

Nonenzymatic browning and pH

Nonenzymatic browning of samples was determined by measuring melanoidin pigment formation using the method of Hoyle and Merritt (1994) with a slight modification. Five mL of sample were centrifuged for 30 min at 10,000 ×*g*. The supernatant obtained was subjected to absorbance measurement at 420 nm. A_{420} was used as an index of browning intensity. Direct pH measurement was taken using a standard pH meter (Mettler Teledo 320, Switzerland).

Color evaluation

The color of the fish sauce samples was measured in the L^*, a^*, and b^* mode of CIE by a Minolta Colour Meter CR300 (Minolta Camera Ltd., Osaka, Japan). The system provides the values of three colour components: L^* (black-white component, luminosity), a^* (+red to -green component), and b^* (+yellow to -blue component). Samples (35 mL) were placed into a glass bottle (2 cm diameter). The sample was illuminated with D65-artificial daylight (10° standard angle) according to the procedure provided by the manufacturer. The color variations of each sample were compensated by recording the average of three readings taken on the surface of the sample.

Total nitrogen

Total nitrogen content of fish sauce samples was measured using the Kjeldahl method (AOAC, 1999). Total nitrogen content was expressed as g nitrogen/L.

Ammonia nitrogen, formaldehyde nitrogen, and amino nitrogen

Amino nitrogen, ammonia nitrogen, and formaldehyde nitrogen contents were determined as described by the Thai Industrial Standard (1983).

Formaldehyde nitrogen was determined by the titration method. One mL of sample was mixed with 9 mL of distilled water and titrated to pH 7.0 with 0.1 M NaOH. Ten mL of formaldehyde solution (38% v/v, pH 9.0) were then added to the neutralized samples. Titration was continued to pH 9.0 with 0.1 M NaOH. Formaldehyde nitrogen content was calculated as follows:

$$\text{Formaldehyde nitrogen content (g/L)} = \text{mL } (NaOH_{pH7\text{-}pH9}) \times 0.1 \times 14$$

To determine ammonia nitrogen, 50 mL of 10-fold diluted samples were placed in a Kjeldahl flask containing 100 mL of distilled water and 3 g of MgO. The mixture was distilled to release volatile nitrogen into 50 mL of 4% (w/v) boric acid containing methyl red-bromocresol green. The distillate was finally titrated with 0.1 M HCl until the end-point was obtained.

Ammonia nitrogen content was calculated as follows:

$$\text{Ammonia nitrogen content (g/L)} = 14 \times 0.2 \times Y$$

where, Y is the volume of HCl (mL)

Amino nitrogen content was calculated using the following formula:

Amino nitrogen content (g/L) = formaldehyde nitrogen content − ammonia nitrogen content

Acceptability testing

The fish sauce samples, obtained after 2 days of incubation, were evaluated for acceptance by an untrained 30-member panel according to the method of Chamber and Wolf (1996). The panelists were staffs of the Food Biotechnology Laboratory at the National Center for Genetic Engineering and Biotechnology (BIOTEC), of age ranging from 20 to 45 years. All panelists had sensorial acquaintance with fish sauce. Panelists were asked to give acceptance scores for four

attributes: colour, aroma, and overall acceptance using the nine-point hedonic scale. A nine-point hedonic scale, in which a score of 1 represented extreme dislike, 5 represented neither like nor dislike, and 9 represented like extremely, was used for evaluation. Samples were coded with three-digit random numbers and were presented to the panelists at ambient temperature. Fish sauce samples were served to each panelist at the same time in a random order. The ratings of each attribute were converted to numerical scores for further statistical analysis (Appendix B-6).

Statistic analysis

A completely randomized design was used throughout this study. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980). Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for Windows: SPSS Inc.).

6.4 Results and Discussion

Effect on histamine and other biogenic amines

Histamine contents in the samples added with free and immobilized whole cells of *Nnm. gari* HDS3-1 are shown in Figure 36. The initial histamine contents of Control 1 and Control 2 were approximately 870 ppm, exceeding both US and Thai standards which were set at 500 ppm (Anonymous, 1998; FIQD, 2000). High contents of histamine ranging from 100-1,000 ppm were frequently detected in commercial fish sauce made from anchovy (Brillantes and Samosorn, 2002; Kirschbaum *et al*., 2000; Sanceda *et al*., 1996). During incubation for 2 days, the histamine contents of both groups were remained at the same level. The results suggested that addition of riboflavin and adjusting pH to 7.0 did not affect the histamine content of fish sauce sample. Dramatic decreases in histamine content were found in fish sauce samples treated with free or immobilized whole cell of *Nnm. gari* HDS3-1 (P<0.05).

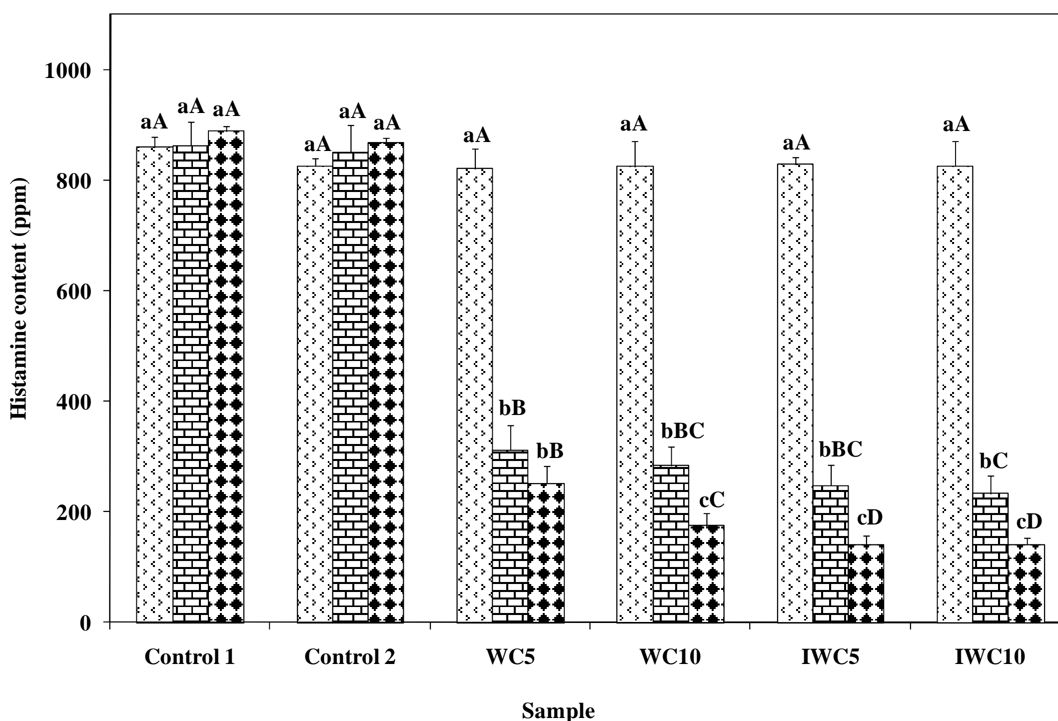


Figure 36. Changes in the histamine content of the untreated (Control 1) and treated (Control 2) fish sauce end-product samples added with different levels (5% and 10%, w/v) of free (WC) and immobilized (IWC) whole cells of *Nnm. gari* HDS3-1 incubated at an ambient temperature for 2 days. Average ± S.D. from a triplicate determination. Different letters (a, b, c) in the same treatment indicate significant differences (P<0.05). Different letters (A, B, C, D) in the same incubation time indicate significant differences (P<0.05). 0 day ( ), 1 day ( ), and 2 days ( ).

After incubation for 1 day, the histamine content of WC5, WC10, IWC5, and IWC10 were decreased by 63.4, 66.7, 70.8, and 72.7%, respectively when compared to Control 2. When incubated for 2 days, the histamine contents were successively decreased to 71.7, 78.8, 81.8, and 81.8%, respectively. After incubation for 2 days, the lowest content of histamine was found in IWC5 and IWC10 (P<0.05).

The biogenic amine contents in fish sauce sample at 2nd day of incubation are shown in Table 14 and Figure 37. Beside histamine, no changes in other biogenic amines, including tryptamine, β-phenylalanine, putrescine, cadaverine, and tyramine were observed (P>0.05). Yongsawatdigul *et al*. (2000) also reported a high level of histamine (14.14 to 78.30 mg/100 mL), tryptamine (1.24 to 5.80 mg/mL), putrescine (3.41 to 47.22 mg/100 mL), cadaverine (8.66 to 75.57 mg/100 mL), and tyramine (0.37 to 23.77 mg/100 mL) in anchovy fish sauce. As described in Chapter 5, the result reconfirmed the substrate preference of both free and immobilized whole cells of *Nnm. gari* HDS3-1 toward histamine. The results suggested that free and immobilized whole cells of *Nnm. gari* HDS3-1 could be applied to reduce histamine content in fish sauce.

Effect on physical properties of fish sauce

The pH, browning index of fish sauce, as measured by A_{420}, and the color values of fish sauces samples at the 2nd day are shown in Table 15. The pH value of untreated fish sauce end-product samples (Control 1) was approximately 5.70. The pH value of treated fish sauce end-product samples (Control 2) was adjusted to 7.0 to obtain the optimal pH of enzyme activity. After incubation with free and immobilized whole cells of *Nnm. gari* HDS3-1, pH values of fish sauce samples were not significantly different (P>0.05).

The browning index and L*, a*, b* values of fish sauce samples were significantly affected by the addition of 0.2% (w/v) riboflavin (P<0.05). The browning index of "Control 2" was increased about 4 times, while the L*, a*, b* values increased to 30.04, 0.83, and 0.80, respectively. Riboflavin (vitamin B_2) is yellow-orange in color thereby contributing to the color of fish sauce, besides the primary function as an electron acceptor for the histamine-degrading activity. Riboflavin is permitted in most countries for use as a coloring agent in several foods such as dressings, sherbet, beverages, instant desserts, etc. There is no usage restriction as long as the level conforms to Good Manufacturing Practices (GMP) (Chattopadhyay *et al*., 2008).

Table 14. Biogenic amine contents of the untreated (Control 1) and treated (Control 2) fish sauce end-product samples added with different levels (5% and 10%, w/v) of free (WC) and immobilized (IWC) whole cells of *Nnm. gari* HDS3-1 incubated at an ambient temperature for 2 days

Sample	Biogenic amine (µg/mL or ppm)							
	His	Tryp	β-phe	Put	Cad	Tyr	Spd	Spm
Control 1	869.4 ± 26.5 [a]	265.3 ± 7.6	269.0 ± 11.4	274.6 ± 16.3	82.5 ± 5.4	15.2 ± 0.3	ND	ND
Control 2	871.0 ± 32.4 [a]	266.5 ± 8.9	269.2 ± 8.9	274.8 ± 16.3	82.6 ± 4.9	15.2 ± 0.2	ND	ND
WC5	228.0 ± 3.1 [b]	266.3 ± 7.3	269.7 ± 11.0	274.8 ± 16.4	82.5 ± 5.6	15.2 ± 0.3	ND	ND
WC10	185.9 ± 1.4 [c]	265.2 ± 8.4	269.9 ± 9.7	275.1 ± 15.2	82.5 ± 4.7	15.2 ± 0.2	ND	ND
IWC5	134.8 ± 2.4 [d]	266.4 ± 7.4	270.4 ± 10.4	274.8 ± 15.2	82.6 ± 4.9	15.2 ± 0.2	ND	ND
IWC10	131.4 ± 2.1 [d]	265.7 ± 9.1	269.3 ± 10.3	274.6 ± 13.3	82.3 ± 4.7	15.2 ± 0.2	ND	ND

His; histamine, Tryp; tryptamine, β-phe; β-phenylethylamine, Put; putrescine, Cad; cadaverine, Tyr; tyramine, Spd; spermidine, and Spm; spermine. Average ± S.D. from a triplicate determination. Different letters (a, b, c, d) in the same column indicate significant differences ($P<0.05$). ND = not detected.

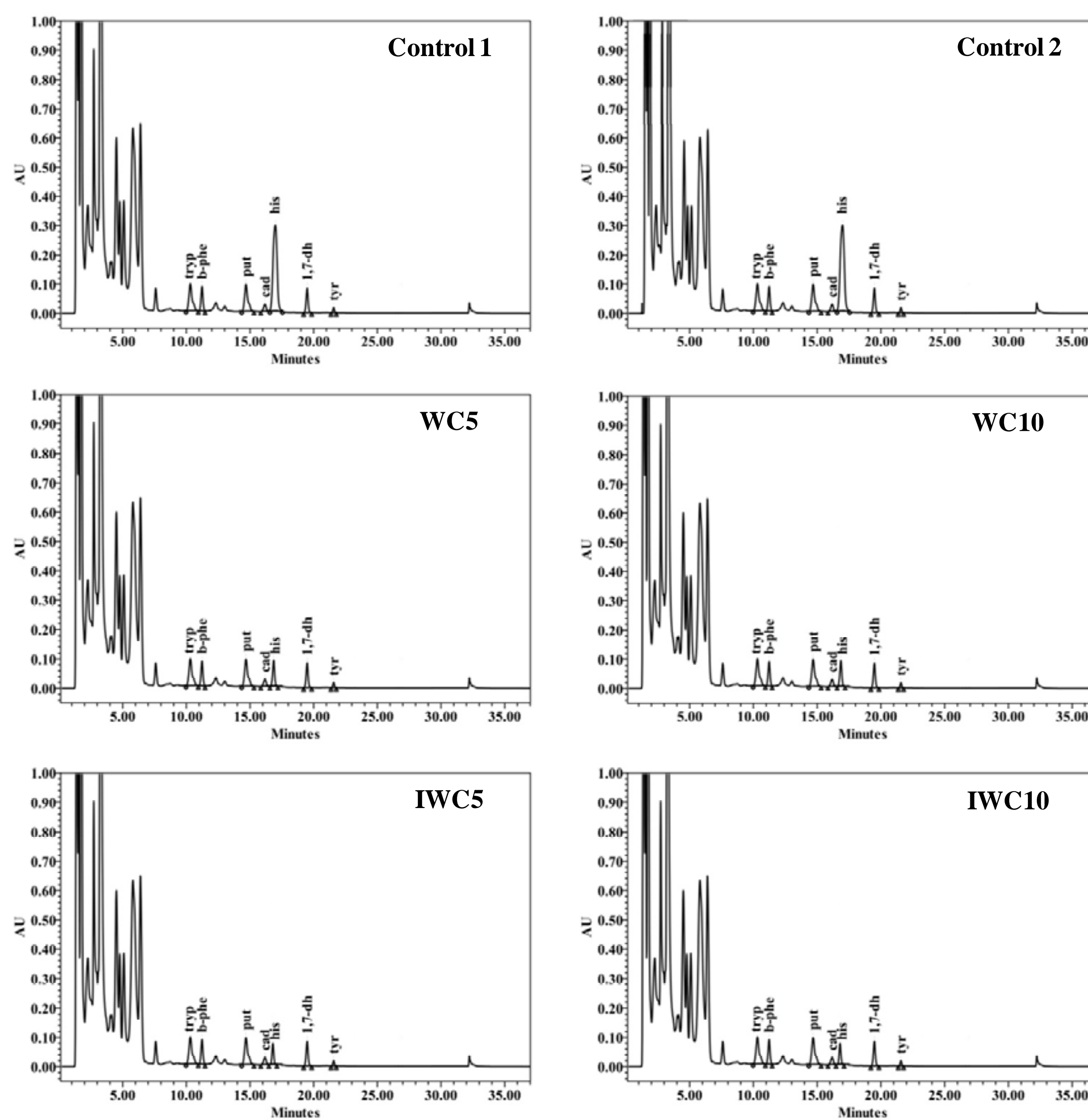


Figure 37. HPLC chromatograms of biogenic amines contents in the untreated (Control 1) and treated (Control 2) fish sauce end-products added with 5% and 10% (w/v) of free (WC) and immobilized (IWC) whole cells of *Nnm. gari* HDS3-1 incubated at an ambient temperature at the 2nd day.

Table 15. pH and color parameters of the untreated (Control 1) and treated (Control 2) fish sauce end-product samples added with different levels (5% and 10%, w/v) of free (WC) and immobilized (IWC) whole cells of *Nnm. gari* HDS3-1 incubated at an ambient temperature (2nd day)

Sample	pH	Browning intensity	L^* (lightness)	a^* (redness)	b^* (yellowness)
Control 1	5.70 ± 0.00 [c]	1.53 ± 0.05 [b]	27.04 ± 0.02 [b]	0.75 ± 0.02 [b]	0.54 ± 0.09 [b]
Control 2	6.95 ± 0.01 [ab]	5.88 ± 0.02 [a]	30.04 ± 0.12 [a]	0.83 ± 0.07 [ab]	0.80 ± 0.09 [a]
WC5	6.94 ± 0.01 [b]	6.00 ± 0.12 [a]	30.23 ± 0.19 [a]	0.88 ± 0.06 [a]	0.77 ± 0.09 [a]
WC10	6.95 ± 0.00 [ab]	5.91 ± 0.04 [a]	30.73 ± 0.28 [a]	0.88 ± 0.09 [a]	0.77 ± 0.12 [a]
IWC5	6.96 ± 0.01 [ab]	5.86 ± 0.05 [a]	30.64 ± 0.10 [a]	0.86 ± 0.05 [a]	0.89 ± 0.07 [a]
IWC10	6.96 ± 0.00 [a]	5.96 ± 0.01 [a]	30.41 ± 0.23 [a]	0.88 ± 0.05 [a]	0.81 ±0.12 [a]

Average ± S.D. from a triplicate determination. Different letters (a, b, c, d, e) in the same column indicate significant differences (P<0.05).

The Recommended Dietary Allowance (RDA) for riboflavin is varied from 1.4 to 3.2 mg, depending on age, gender, and other factors, although higher levels may support many functions (Flynn *et al*., 2003). There were no significantly different in the browning index and L^*, a^*, b^* values after treating with free and immobilized whole cells of *Nnm. gari* HDS3-1 (P>0.05). The results suggested that the addition of free and immobilized whole cells of *Nnm. gari* HDS3-1 did not affect the color of fish sauce samples including browning index and L^*, a^*, b^* values.

Nitrogen-containing constituents in fish sauce

No marked differences in the total nitrogen, formaldehyde nitrogen, ammonical nitrogen, and amino nitrogen were observed among treated samples and Control 1 after 2 day incubation at an ambient temperature (Table 16). The results from previous sections suggested that histamine in fish sauce samples was degraded by intracellular histamine dehydrogenase in whole cell of *Nnm. gari* HDS3-1.

Table 16. The total nitrogen, formaldehyde nitrogen, ammonical nitrogen, and amino nitrogen of the untreated (Control 1) and treated (Control 2) fish sauce end-product samples added with different levels (5% and 10%, w/v) of free (WC) and immobilized (IWC) whole cells of *Nnm. gari* HDS3-1 incubated at an ambient temperature (2nd day)

Sample	Total Nitrogen (g / L)	Formaldehyde nitrogen (g / L)	Ammonical nitrogen (g / L)	Amino nitrogen (g / L)
Control 1	27.84 ± 0.10	15.44 ± 0.00	3.04 ± 0.18	12.40 ± 0.08
Control 2	27.80 ± 0.46	15.46 ± 0.13	2.94 ± 0.14	12.52 ± 0.09
WC5	27.91± 0.17	15.38 ± 0.02	2.88 ± 0.11	12.49 ± 0.01
WC10	27.95 ± 0.17	15.50 ± 0.07	2.93 ± 0.10	12.57 ± 0.18
IWC5	28.19 ± 0.46	15.47 ± 0.15	2.88 ± 0.12	12.59 ± 0.13
IWC10	28.29 ± 0.39	15.46 ± 0.09	2.90 ± 0.13	12.56 ± 0.13

Average ± S.D. from a triplicate determination. Different letters (a, b, c) in the same column indicate significant differences ($P<0.05$).

Histamine dehydrogenase generally catalyzes the oxidative deamination of histamine to produce imidazole acetaldehyde and ammonia (Fujieda *et al*., 2005; Sekiguchi *et al*., 2004). By the action of histamine dehydrogenase activity, 650 ppm of histamine degradation could be stoichiometrically converted to ammonia at the equivalent amount of 0.082 g N/L. Ammonia was suggested as one of the key components of volatile bases giving ammoniacal notes (Dougan and Howard, 1975). However, there is no evidence that ammonia is the aroma-active component for ammoniacal notes in fish sauce (Lopetcharat *et al*., 2001). Due to the sensitivity of method used in this study, the increasing amount of ammonia generated from histamine in the reaction might not be accurately determined or too little to contribute to the ammonia nitrogen content of these fish sauce samples.

Acceptability of fish sauce

Figure 38 shows the acceptability of fish sauce added with free and immobilized whole cells of *Nnm. gari* HDS3-1 at different levels. The color acceptability of untreated fish sauce was significantly lower (P<0.05) when compared with other treatments. As indicated by browning index and other color values, the color of fish sauce after adding with 0.2% (w/v) riboflavin became more orange-red which is the good characteristic of fish sauce (Saisithi *et al*., 1994). Fish sauce added with immobilized whole cell at 10% (IWC10) had highest odor (P>0.05) and overall acceptability (P<0.05) scores, while the lowest score was found in fish sauce added with free whole cell at 10% (WC10). The lowest overall odor acceptability scores of fish sauce added with free whole cell might be the effect of a strong characteristic odor of halophilic bacteria (Warren, 2006).

Flavor and aroma of fish sauce exert a strong influence on consumers' choice of fish sauce (Thongthai *et al*., 1992). The odor of fish sauce generally had more impact on the overall acceptability than the colour. A number of reports revealed that volatile acids were the most abundant group of volatile compounds in fish sauce (Saisithi *et al*., 1966; Truong Van-Chom, 1952). Various volatile compounds, including acids, carbonyls, nitrogen-containing compounds, and sulfur-containing compounds, are formed during fermentation and believed to be responsible for the distinct aroma of fish sauce (Fukami *et al*., 2002; Peralta *et al*., 1996; Shimoda *et al*., 1996).

6.5 Conclusion

Addition of the free and immobilized whole cells of *Nnm. gari* HDS3-1 can decrease the histamine content present in fish sauce without undesirable changes. Riboflavin (GRAS) can be used as an electron acceptor. Additionally, the fish sauce treated with immobilized whole cell tended to have a better acceptability on the overall sensory characteristics. Therefore, immobilized whole cell of *Nnm. gari* HDS3-1 could be used for the histamine degradation in fish sauce with no detrimental effect on the quality.

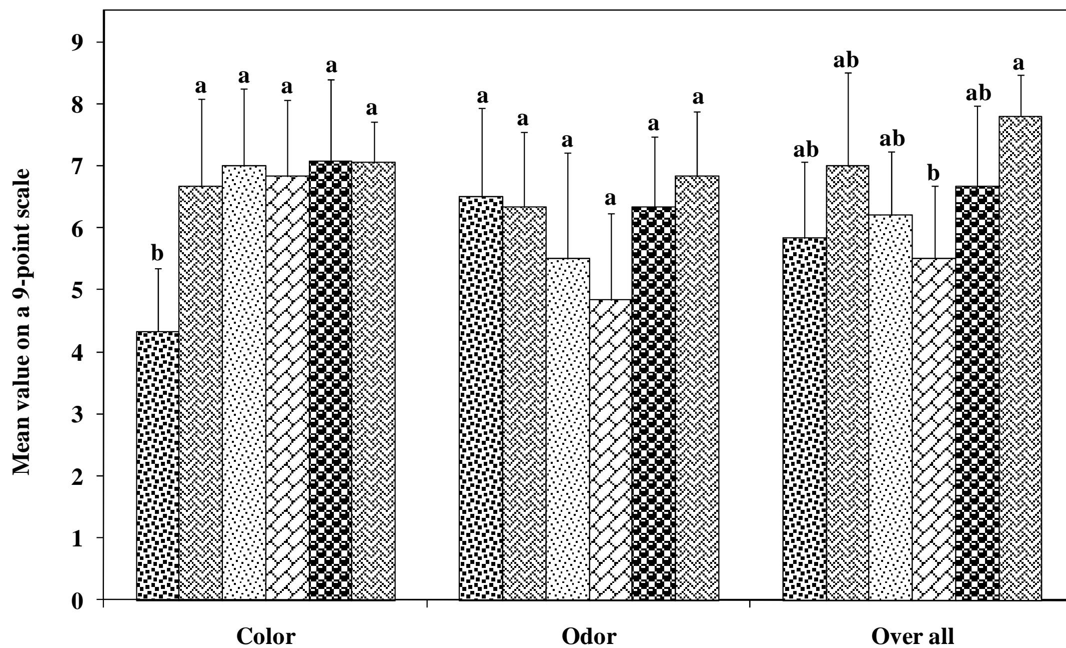


Figure 38. Acceptability of the untreated (Control 1) and treated (Control 2) fish sauce end-product samples added with different levels (5% and 10%) of free (WC) and immobilized (IWC) whole cells of *Nnm. gari* HDS3-1 incubated at an ambient temperature (2nd day). Mean value of consumer judgments' on a 9-point hedonic scale with 9 = extremely good. Average ± S.D. from a triplicate determination. Different letters (a, b, c) in the same parameter indicate significant differences ($P<0.05$). Control 1 ( ), Control 2 ( ), WC5 ( ), WC10 ( ), IWC5 ( ) and IWC10 ( ).

CHAPTER 7

SUMMARY AND FUTURE WORKS

7.1 Summary

Out of the 156 extremely halophilic archaea isolated from various salt-fermented fishery products. Strain HDS3-1, isolated from an anchovy fish sauce sample fermented for 3 months, exhibited the highest ability to degrade histamine in hypersaline condition. The histamine-degrading activity of strain HDS3-1 was located in an intracellular fraction, required the presence of 1-methoxy-5-methylphenazinium methylsulfate (PMS) as an electron acceptor and selectively catalyzed histamine as a substrate. The optimal salt concentration, pH and temperature for the activity were in the presence of NaCl at 3.5 to 5 M, at pH 6.5 to 8 and 40 to 55°C. The activity was found to be stable at the wide pH range of 6.5 to 9, in the presence of NaCl above 2.5 M and temperature lower than 50°C. NaCl increased the activity and prevented thermal inactivation of the intracellular fraction. The results suggested a link of histamine-degrading activity of strain HDS3-1 to the halophilic as well as thermo-neutrophilic histamine dehydrogenase activity.

On the basis of growth requirements, poor utilization of carbohydrates, antibiotic susceptibility, menaquinone content, overall phospholipid composition, DNA G+C contents and 16S rRNA gene sequence analysis, strains HDS3-1 and HIS40-3^T are considered to represent a single species of the genus *Natrinema.* However, they were differentiated from recognized *Natrinema* species based on levels of DNA-DNA relatedness and differences in whole-cell protein patterns. These results suggested that the strains HDS3-1 and HIS40-3^T represent a novel species of the genus, for which the name *Natrinema gari* sp. nov. is proposed.

The purified histamine dehydrogenase from *Natrinema gari* HDS3-1 had a molecular mass of about 127.5 kDa, formed by 3 different subunits (heterotrimeric) of a molecular mass of 69.1, 29.3 and 27.7 kDa. The purified enzyme had similar properties to the crude enzyme preparation (the intracellular fraction). The enzyme had a maximal activity at pH 6.5 to 8.5 and 40 to 60°C in the presence of

3.5 to 5.0 M NaCl. The enzyme was quite stable to the heat treatment and was also stable at high salt concentrations up to 3.5 to 5.0 M. The enzyme was identified to be histamine dehydrogenase based on substrate specificity and inhibitor study. The V_{max}, K_m and k_{cat} values of the enzyme toward histamine were at 2.5 µmol min^{-1}, 57.1 µM and 5.3 s^{-1}, respectively. These characteristics suggest that the enzymes could be an important biotechnological tool for the fish processing and food industries, in which high salt concentration is used.

Whole cell immobilization by means of adsorption to celite provided the best compromise between biocatalyst retention and apparent activity. The catalytic properties of the immobilized whole cell on the celite support were similar to the corresponding free whole cell including optimal NaCl concentration (4.0 to 5.0 M NaCl), optimal pH (pH 6.5 to 7.5) and optimal temperature (45 to 55°C). However, the stabilities of the histamine-degrading activity either in the presence of NaCl at elevated concentrations or incubation at elevated temperatures were significantly improved by the immobilized process. Both free and immobilized whole cells were highly specific toward histamine. The immobilized whole cell could be reused for the degradation of histamine up to 7 cycles without any significant loss in activity. The immobilized whole cell of *Nnm. gari* HDS3-1 on the celite support is more promising than the free whole cell on the histamine catalytic activity, stability to NaCl, stability at elevated temperatures and reusability.

The addition of the free and immobilized whole cells of *Nnm. gari* HDS3-1 can decrease the histamine content present in fish sauce without undesirable changes. Free and immobilized whole cells were added at levels of 5% and 10% (w/v), to a fish sauce that was prior supplemented with 0.2% (w/v) riboflavin and adjusted to pH 7.0. The samples were then incubated at an ambient temperature (30°C) for 2 days. Fish sauce samples added with free and immobilized whole cells had lower histamine contents than those of the control (either without addition of free or immobilized whole cell) throughout the incubation period. The rate of histamine degradation was dependent on the amount of free and immobilized whole cells added. Among all treatments, fish sauce added with immobilized whole cell at 10% (w/v) exhibited the greatest histamine degradation, particularly at the 1st day of incubation. All treated samples had similar pH, total nitrogen, amino nitrogen, formaldehyde

nitrogen and ammonical nitrogen to those of the untreated sample (P>0.05). Except histamine, no changes in other biogenic amines were observed after treatment. Fish sauce treated with immobilized whole cell at both levels (5% and 10%, w/v) tended to gain higher acceptability than those with and without whole cells addition.

7.2 Future works

1. Structural and molecular properties of histamine dehydrogenase from *Nnm. gari* HDS3-1.
2. Process optimization and application of immobilized whole cell of *Nnm. gari* HDS3-1 to degrade histamine in fish sauce.
3. Application of *Nnm. gari* HDS3-1 as a starter culture in fish sauce production.
4. Application of free and immobilized whole cells of *Nnm. gari* HDS3-1 to degrade histamine in other salt fermented food products.

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APPENDIX A

CULTURE MEDIA AND IDENTIFICATION METHODS

1. Halophilic medium

Yeast extract	5	g
Casamino acid	5	g
Sodium glutamate	1	g
Trisodium citrate	3	g
$MgSO_4.7H_2O$	20	g
KCl	2	g
NaCl	250	g
$FeCl_2.4H_2O$	36	mg
$MnCl_2.4H_2O$	0.36	mg
Agar	20	g
Distilled water	1,000	mL

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

2. Histamine medium

Halophilic medium		
Histamine	837	mg

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

3. Decarboxylase medium

Halophilic medium		
Histidine	1%	(w/v)
Tryptophan	1%	(w/v)
Lysine	1%	(w/v)
Ornithine	1%	(w/v)
Arginine	1%	(w/v)
Tyrosine	1%	(w/v)
Phenylalanine	1%	(w/v)

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

4. Gram staining

Gram staining was performed by using air-dried slides which were fixed and simultaneously desalted in 2% acetic acid for 5 min, dried before staining by standard procedures (Dussault, 1955). Thin smear of bacterial colony was prepared on a clean slide. Slide was fixed by passing through flame. The smear was covered with crystal violet for 30 sec, then rinsed with water and drained. Next, the smear was covered with iodine for 30 sec, then with rinsed water. Decolorized with ethanol 95% and washed with water, then it was counter stained about 30 s with safranin. Blot slide was dried and examined under oil immersion (1,000×). Colonial appearances were examined after incubated for 3-7 days.

5. Anaerobic growth in the presence of L-arginine

Anaerobic growth in the presence of L-arginine was determined by inoculating the archaea into on agar plates in the presence of L-arginine and incubated for 1-2 weeks. Cultures were incubated under an anaerobic condition at 37°C. Growth

was determined by measuring culture turbidity at 600 nm. A positive reaction for hydrolysis L-arginine was also shown by a colour change of the indicator to red.

L-arginine agar medium

Peptone	1	g
NaCl	250	g
K_2HPO_4	0.3	g
Phenol red, 1.0% (w/v)	1	mL
L(+)arginine hydrochloride	10	g
Agar	20	g
Distilled water	1,000	mL

Dissolve and adjust pH to 7.2 with NaOH and distribute into tubes or screw-capped (6 mm) bottles to a depth of about 16 mm (3.5 mL). Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

6. Anaerobic growth in the presence of nitrate and nitrate reduction

Anaerobic growth on agar plates in the presence of nitrate (1 g/L) and the nitrate reduction was observed by inoculating the cultures on nitrate broth under an anaerobic condition for 7 or 14 days. Cultures were incubated at 37°C. Growth was determined by measuring culture turbidity at 600 nm. After incubation, for nitrate reductase activity, 2 drops of sulphanilic acid solution and 3 drops of *N*,*N*-dimethyl-1-naphthylamine solution were added into peptone nitrate broth inoculating with the test microorganisms. If the nitrite is present a pink colour would develop within 5 min.

Nitrate broth

Beef extract	10	g
Peptone	10	g
NaCl	250	g
Potassium nitrate	3	g
Distilled water	1,000	mL

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

Nitrate reduction test reagent

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	mL

Dissolve by gentle heating in a fume hood.

***N*,*N*-dimethyl-1-napthylamine solution**

N,*N*-dimethyl-1-naphthylamine	0.5	g
5 N Acetic acid	100	mL

Dissolve by gentle heating in a fume hood.

7. Anaerobic growth in the presence of DMSO (dimethylsulfoxide)

Anaerobic growth in the presence of DMSO was determined by inoculating the archaea into on agar plates in the presence of DMSO (10 g/L) and incubated for 1-2 weeks. Cultures were incubated under an anaerobic condition at 37°C. Growth was determined by measuring culture turbidity at 600 nm.

DMSO medium

DMSO	10	g
Peptone	1	g
NaCl	250	g
K_2HPO_4	0.3	g
Agar	20	g

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

8. Indole test

Indole test was observed by inoculating the cultures on tyrptone broth for 7 days or 14 days. After incubation, 4 drops of Kovacs' reagent was added.

Tyrptone broth

Bactopeptone	10	g
NaCl	250	g
Distilled water	1,000	mL

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

Kovacs' reagent

ρ-Dimethylaminobenzaldehyde	5	g
Amyl alcohol	75	g
Conc. HCl	25	mL

Dissolve the aldehyde in the alcohol by gently warming in a water bath (about 50°C to 55°C). Protect from light and store at 4°C.

9. Acid production test

The acid production from carbon sources were performed in modified Leifson agar medium as described by Leifson (1963). The medium was supplemented with 1% (w/v) of the respective carbohydrates (L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inulin, lactose, *myo*-inositol, maltose, D-mannitol, mannose, melibiose, melizitose, raffinose, rhamnose, D-ribose, salicin, sucrose, D-sorbitol, D-trehalose and D-xylose) or 0.01% (w/v) of the respective amino acids (L-arginine, L-glutamic acid and L-serine). The broth was inoculated with 5% (v/v) of inoculum and incubated at 37°C for 14 days. Growth was measured at 660 nm. The positive results were shown by colour change of the indicator from red to yellow.

Modified Leifson medium

Yeast extract	0.1	g
Ammonium sulfate	0.5	g
NaCl	25	g
Phenol red (1%, w/v)	1	mL
Agar	20	g
Distilled water	950	mL

Dissolve and adjust pH to 7.5 with NaOH. Make up to 1 L with distilled water. Separately add with different carbon sources at a final concentration of 1% (w/v) or amino acids at a final concentration of 0.01% (w/v). Sterile by autoclaving at 110°C, 15 pounds/inch^2 pressure for 10 min.

10. Oxidase test

Oxidase test was determined by dropping small amount of 1% tetramethyl-*ρ*-phenylenediamine on sterile filter paper disc, the colonial appearance of dark-purple colour revealed the positive result.

11. Catalase test

Catalase test was performed by flooding the colonies with 3% (v/v) hydrogen peroxide (H_2O_2) then became the gas bubbles denoted a positive reaction.

12. Hydrolysis of casein, gelatin, starch and tween 80

The hydrolysis of casein, gelatin, starch and tyrosine were determined by inoculating the archaea into casein, gelatin, starch and tween 80 agar medium. The plates were incubated for 1-2 weeks as recommended by Barrow and Feltham (1993). Clear colourless zones after the plates were flooded with Lugol's iodine or 10% trichloroacetic acid indicated the hydrolysis of starch and gelatin, respectively. Clear zone indicate areas of the hydrolysis of casein and tween 80.

Casein agar medium

Halophilic agar medium (omitted casamino acid and reduced yeast extract to 1 g)

Skim milk	1%	(w/v)

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

Gelatin agar medium

Halophilic agar medium (omitted casamino acid and reduced yeast extract to 1 g)

Gelatin	10%	(w/v)

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

Starch agar medium

Halophilic agar medium (omitted casamino acid and reduced yeast extract to 1 g)

Starch	10%	(w/v)

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

Tween 80 agar medium

Halophilic agar medium (omitted casamino acid and reduced yeast extract to 1 g)

Tween 80	2	mL

Dissolve and adjust pH to 7.2 with NaOH. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

Lugol's iodine

KI	40	g
Distilled water	100	mL

10% Trichloroacetic acid

Trichloroacetic acid (TCA)	5	g
Distilled water	100	mL

Add conc. TCA into the distilled water.

13. Antibiotics test

The susceptibility of antibiotics was tested by spreading cell suspensions onto halophilic medium agar plates and then the antibiotic paper discs (6 mm in diameter) were applied on the medium. Zones of inhibition were measured following 14 days of incubation at 37°C. Sensitivity was considered as strong when the zone of inhibition extended more than 3 mm beyond the antibiotic disc (Stan-Lotter *et al*., 2002).

Antibiotic paper disc (6 mm Ø)

Gentamicin	10	µg
Neomycin	30	µg
Rifampicin	30	µg
Nalidixic acid	30	µg
Choramphenicol	30	µg
Bacitracin	10	µg
Kanamycin	30	µg
Streptomycin	10	µg
Tetracycline	30	µg
Ampicilin	10	µg
Novobiocin	5 and 30	µg

14. Dried cells preparation

Dried cells of the selected strain was prepared by inoculating 250 µL of stock culture into 5 mL of halophilic medium (Appendix A-1) and incubated at

37°C in a shaker incubator at 200 rpm for 7 days. After that, inoculum was added at 5% (v/v) into halophilic liquid medium containing 5 mM of histamine (free-base) (Appendix A-2) and incubated at 37°C in a shaker incubator at 200 rpm for 7 days. Wet cell paste was obtained by centrifugation of cultured broth at 10,000 ×*g* at 4°C for 10 min. The cells were washed twice with 25% (w/v) NaCl and centrifuged at 10,000 ×*g* (4°C) for 10 min. The obtained wet cell paste was freeze dried and kept at 4°C until used.

15. Quinones (Komagata and Suzuki, 1987)

Dried cells (100-500 mg) (Appendix A-13) were extracted with chloroform: MeOH (2:1) overnight. The suspension was then filtered and dried under rotary evaporator. The dried sample was Dissolve with a small amount of acetone and applied onto a silica gel thin layer chromatography (TLC) (Merck no. 1.05744). The applied TLC was then developed by 100% benzene and the band of menaquinone was detected by using a UV lamp (254 nm). The menaquinone band was scraped and Dissolve with HPLC acetone. The suspension was filtered and dried up with N_2 gas. The menaquinone sample was analyzed by HPLC.

16. Polar lipids

Dried cells (150-300 mg) (Appendix A-14) were added with 3 mL of MeOH: 0.3% NaCl aq. (100:10) and 3 mL of petroleum ether. The solution was mixed for 15 min. The lower layer was added with 1 mL of petroleum ether and then the solution was mixed for 2-5 min. The lower layer was added with chloroform: MeOH: water (90:100:30) and mixed for 1 h. The upper layer was transferred into another tube. The lower layer was extracted again with chloroform: MeOH: water (50:100:40) and the supernatant was transferred to the upper layer tube. The upper layer tube was added with 1.3 mL of chloroform and water. The final lower layer was dried with N_2 gas (<37°C). The polar lipid fraction was Dissolve with 60 µL of chloroform: MeOH (2:1) and applied to 3 plates of two-dimensional silica high

performance thin layer chromatography (HPTLC) no. 1.05633 and was developed with the following solvent systems.

The first solvent system was chloroform: MeOH: water (65:25:4). The second solvent system was chloroform: acetic acid: MeOH: water (40:7.5:6:2). HPTLC was sprayed with iodine until polar lipid appeared. Subsequently, the first plate was sprayed with Ninhydrin reagent and then heated at 110°C for 10 min. Dittmer and Lester reagent was sprayed onto the plate and then blue colour were detected on the spot containing phospholipids. The second plate was sprayed with Anisaldehyde reagent and then heated at 110°C for 10 min after spraying. Green-yellow and blue colors were detected on spot containing glycolipids and other lipids, respectively. The third plate was sprayed with Dragendroff's reagent and then orange color was detected on spot containing phosphatidyl chroline (Kämpfer and Kroppenstedt, 1996; Sasser *et al*., 1990).

Ninhydrin solution

Ninhydrin	0.5	g
1-Butanol saturated in water	100	mL

Dittmer and Lester reagent

Solution A

MoO_3	4.011	g
25 N H_2SO_4	100	mL

Dissolve 4.011 g of MoO_3 in 100 mL of 25 N H_2SO_4 by heating.

Solution B

Molybdenum powder	0.178	g
Solution A	50	mL

Add 0.178 g of molybdenum powder to 50 mL of solution A and boiled it for 15 min. Cool and remove the precipitate by decantation.

Note: Before spraying, mixed solution A (50 mL) plus solution B (50 mL) plus water (100 mL). The final solution was greenish yellow in color. If

too little water was used it will be blue; if too much, yellow. The spray was stable for months.

Anisaldehyde reagent

Ethanol	90	mL
H_2SO_4	5	mL
ρ-Anisaldehyde	5	mL
Acetic acid	1	mL

Dragendorff's reagent

Solution A

Basic bismuth nitrate	1.7	g
Acetic acid	20	mL
Distilled water	80	mL

Solution B

KI	40	g
Distilled water	100	mL

Before spraying, mixed solution A (10 mL) plus solution B (10 mL) plus acetic acid (10 mL).

17. DNA base composition

Chromosomal DNA was isolated from cells grown in halophilic agar plate for 7 days according to the method of Tamaoka (1994) and Saito and Miura (1963). Cells were harvested and suspended in 10 mL of saline-EDTA buffer pH 8.0. The cell suspension was with 1.0 mL of 10% (w/v) SDS and incubated at 50°C for 10 min. The phenol extraction was then carried out by adding an equal volume of phenol: chloroform (1:1) to the sample to remove protein and other debris. The upper layer of the mixture was collected after centrifugation at 12,000 ×*g* for 20 min. Chromosomal DNA was precipitated with two volumes of ice cold absolute ethanol. DNA was

Dissolve with 0.1× SSC and treated with RNase A, RNase T_1 and protease K solution at 37°C for 1 h to remove RNA and protein, respectively. Chromosomal DNA was stored in 0.1× SSC at 4°C.

The 10 μL of heated DNA (1 mg/mL) was hydrolyzed with 10 μL nuclease P_1 at 50°C for 1 h and with 10 μL of alkaline phosphatase at 37°C for 1 h. The DNA base composition of the selected strain was determined using the HPLC method of Tamaoka and Komagata (1984). An equimolar mixture of nucleotides (Yamasa Shoyu, Cholshi, Japan) was used as the quantitative standard.

Saline-EDTA buffer pH 8.0

NaCl	8.76	g
EDTA	37.22	g

Dissolve and adjust pH to 8.0 by adding 0.1 N HCl. Make up to 1 L and sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

10% (w/v) SDS

Sodium dodecyl sulphate	10	g
Distilled water	90	mL

Dissolve and make up to 100 mL with distilled water.

Phenol: Chloroform (1:1, v/v)

Crystalline phenol was liquidified in water bath at 65°C and mixed with chloroform in the ratio of 1:1 (v/v). The solution was stored in a light tight bottle.

20× SSC

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	950	mL

Dissolve and adjust pH to 7.0 with 1 N NaOH. Make up to 1 L with distilled water and sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

Note: To prepare 0.1× SSC and 0.2× SSC, the 20× SSC were diluted at 200 and 100 times, respectively before used.

0.15 M NaCl

NaCl	0.84	g

Dissolve with 100 mL distilled water and sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

RNase A solution

RNase A	20	mg
0.15 M NaCl	10	mL

Dissolve 20 mg of RNase A in 10 mL 0.15 M NaCl and heat at 95°C for 5-10 min. Kept in -20°C.

0.1 M Tris-HCl (pH 7.5)

Tris(hydroxymethyl)aminomethane	1.21	g
Distilled water	90	mL

Dissolve and adjust to pH 7.5 by adding 0.1 N HCl. Make to 100 mL with distilled water.

RNase T_1 solution

RNase T_1	80	µL
0.1 M Tris-HCl (pH 7.5)	10	mL

Mix 80 µL of RNase T_1 in 10 mL of 0.1 M Tris-HCl (pH 7.5) and heat at 95°C for 5 min. Keep in -20°C.

50 mM Tris-HCl (pH 7.5)

Tris(hydroxymethyl)aminomethane	605	mg
Distilled water	90	mL

Dissolve and adjust to pH 7.5 by adding 0.1 N HCl. Make to 100 mL with distilled water.

Proteinase K

Proteinase K (Sigma)	4	mg
50 mM Tris-HCl (pH 7.5)	1	mL

Use freshly prepared solution.

40 mM CH_3COONa + 12 mM $ZnSO_4$ (pH 5.3)

CH_3COONa	3.28	g
$ZnSO_4$	1.94	g
Distilled water	90	mL

Dissolve and adjust to pH 5.3 by adding 0.1 N HCl or 0.1 N NaOH. Make to 100 mL with distilled water.

Nuclease P_1 solution

Nuclease P_1	0.1	mg
40 mM CH_3COONa + 12 mM $ZnSO_4$ (pH 5.3)	1	mL

Dissolve and store at 4°C.

0.1 M Tris-HCl (pH 8.1)

Tris(hydroxymethyl)aminomethane	1.21	g
Distilled water	90	mL

Dissolve and adjust to pH 8.1 by adding 0.1 N HCl. Make to 100 mL with distilled water.

Alkaline phosphatase solution

Alkaline phosphatase	2.4	units
0.1 M Tris-HCl (pH 8.1)	1	mL

18. 16S rRNA analysis

18.1. 16S rRNA amplification by PCR

The PCR was performed in a total volume of 50 µL containing 1 µL of DNA sample, 0.25 µL of *Taq* DNA polymerase, 5 µL of 10× polymerase buffer, 4 µL of dNTP mixture, 2.5 µL of 10 µM forward and reverse primers of D30F (5'-ATTCCGGTTGATCCTGC-'3, positions 6 to 12 according to the *Escherichia coli* numbering system) and D56R (5'-CTTGTTACGACTT-'3, position 1492-1509), and 34.75 µL of deionized water. A DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) was used with a temperature profile of 3 min at 95°C (denaturing of DNA), 15 s at 55°C (primer annealing) and 1 min at 72°C (polymerization) and a final extension for 5 min at 72°C. The PCR amplified products were analyzed by running 5 µL of the reaction mixture on a 1% agarose gel in Tris-acetate EDTA buffer. Agarose gel was stained in an ethidium bromide solution and examined under UV-transilluminator (UVP Inc.) to visualize the amplified 16S rRNA band.

18.2. 16S rRNA sequencing

The amplified 16S rRNA was used as templates for sequencing with big dry terminator sequencing Kit (Perkin Elmer) and analyzed by the ABI377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in a DNA Thermal Cycler (Gene Amp® PCR System 2400; Perkin Elmer) with a temperature profile of 30 s at 50°C (primer annealing), and 4 min at 60°C (polymerization). Sequencing was carried out in both forward and reverse directions.

18.3. 16S rRNA sequence analysis and phylogenetic tree construction

Homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server http://www.ncbi.nlm.nih.gov/BLAST/ against previously reported sequences at the GenBank/EMBL/DDBJ databases. The sequence was multiply aligned with selected sequences obtained from the three main database by using the CLUSTAL W version 1.81. The alignment was manually verified and adjust prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining, maximum parsimony methods and maximum-likelihood method in the MEGA program version 2.1. The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses based on 1,000 resamplings. The values for sequence similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL X program. Gaps and ambiguous nucleotides were eliminated from the calculations.

5× Tris-acetate EDTA (TAE) buffer

Tris-base	5.4	g
Boric acid	2.75	g
Na_2-EDTA	0.47	g
Distilled water	100	mL

Dissolve and dilute 5× before used.

Agarose gel

Agarose	1.6	g
1× TAE buffer	100	mL

Dissolve and melt in microwave for 2-3 min.

19. DNA-DNA hybridization

DNA labeling probe with photobiotin was started by mixing 10 µL of purified DNA solution (1 mg/mL) with 15 µL of photobiotin solution (1 mg/mL) in an Eppendorf tube. The mixture was irradiated with sunlamp for 30 min on ice. After irradiation, the excess photobiotins were removed by the addition of 100 µL of 0.1 M Tris-HCl buffer pH 9.0 followed by 100 µL of n-butanol. The upper layer was removed. A 100 µL of n-butanol was added and mixed well and removed the upper layer. The biotinylated DNA solution was boiled for 15 min and immediately cooled in ice. The solution was sonicated for 3 min and Dissolve with hybridization solution.

DNA-DNA hybridization solution was performed by method of Ezaki *et al*. (1989). A 100 µL of a heat denatured DNA solution was added to microdilution wells (Nunc-Immuno^{TM} Plate: MaxiSorp^{TM} surface) and fixed by incubation at 37°C for 2 h. After that, the DNA solution was removed. 200 µL of a Prehybridization solution was added to microdilution wells. The microdilution plate was incubated at hybridization temperature (50°C) for 1-2 h. The Prehybridization solution was removed from the wells and replaced with 100 µL of a hybridization mixture containing biotinylated DNA. The microdilution plate was incubated at hybridization temperature (50°C) for 15-18 h.

After hybridization, the microdilution wells were washed 3 times with 200 µL of 0.2×SSC buffer. Then 200 µL of solution I was added to microdilution wells and incubated at 30°C for 10 min. Solution I was removed from the wells and replaced with 100 µL of solution II. The microdilution plate was incubated at 37°C for 30 min. After incubation, the microdilution plate was washed for 3 times with 200 µL of PBS. A 100 µL of solution III was added, and the plate was incubated at 37°C for 10 min. The enzyme reaction was stopped with 100 µL of 2 M H_2SO_4 (Verlander, 1992). The absorbance was measured at 450 nm with Microplate Reader (Microplate Manager® 4.0 Bio-Rad Laboratories, Inc.) and calculated for the value of percentage of DNA homology.

0.1 M Tris-HCl (pH 9.0)

Tris(hydroxymethyl)aminomethane	1.21	g
Distilled water	90	mL

Dissolve and adjust to pH 9.0 by adding 0.1 N HCl. Make to 100 mL with distilled water.

100× Denhardt solution

Bovine serum albumin	2%	(w/v)
Polyvinyl pyrrolidone	2%	(w/v)
Ficoll 400	2%	(w/v)
Distilled water	90	mL

Dissolve and adjust to 100 mL with distilled water.

100 mM Tris-HCl (pH 7.6)

Tris(hydroxymethyl)aminomethanein	12.1	g
Distilled water	950	mL

Dissolve and adjust to pH 7.6 with 0.1 N HCl. Make up to 1 L with distilled water.

10 mM Na_2-EDTA (pH 7.6)

Na_2-EDTA	0.47	g
Distilled water	950	mL

Dissolve and adjust to pH 7.6 with 0.1 N HCl. Make up to 1 L with distilled water.

TE buffer

10 mM Tris-HCl (pH 7.6)	100	mL
1 mM Na_2-EDTA (pH 7.6)	100	mL

Make up to 1 L with distilled water.

10 mg/mL Salmon sperm DNA

Salmon sperm DNA	10	mg
10 mM TE buffer pH 7.6	1	mL

Dissolve and boil for 10 min, immediately cool in ice and sonicate for 3 min.

Prehybridization solution

100× Denhardt solution	5	mL
10 mg/mL salmon sperm DNA	1	mL
20× SSC	10	mL
Formamide	50	mL
Distilled water	34	mL

All of ingredients were mixed and keep at 4°C.

Hybridization solution

Prehybridization solution	100	mL
Dextran sulfate	5	g

All of ingredients were mixed and keep at 4°C.

Phosphate-buffer saline (PBS)

NaCl	8	g
KCl	0.2	g
KH_2PO_4	0.12	g
Na_2HPO_4 (anhydrous)	0.91	g
Distilled water	950	mL

Dissolve and make to 1 L with distilled water. Sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

Solution I

Bovine serum albumin (Fraction V)	0.25	g
Titron X-100	50	µL
PBS	50	mL

All of ingredients were mixed and keep at 4°C.

Solution II

Streptavidin-POD	1	µL
Solution I	4	mL

Dissolve Strepavidin-POD in solution I before used. The solution II was freshly prepared.

Solution III

3,3',5,5'-Tetramethylbenzidine (TMB) (10 mg/mL in DMFO)	100	mL
0.3% H_2O_2	100	mL
0.4 M Citric acid + 0.2 M Na_2HPO_4 buffer pH 6.2 in 10% DMFO	100	mL

Solution III was freshly prepared.

2 M H_2SO_4

H_2SO_4	22	mL
Distilled water	178	mL

The solution was sterile by autoclaving at 121°C, 15 pounds/inch^2 pressure for 15 min.

Ethidium bromide solution (10 mg/mL)

Ethidium bromide	1	g
Distilled water	100	mL

Dissolve and store in light-tight container at room temperature.

Gel loading buffer

Bromophenol blue	0.025	g
Distilled water	17	mL
Glycerol	3	mL

Dissolve and store in light-tight container in refrigerator.

APPENDIX B

STANDARD ASSAY METHODS

1. Histamine determination by fluorometric method of AOAC

An AOAC official method is based on fluorescent measurement and recognized as the most suitable method for the determination of histamine contained in fish and fermented food. The method uses *o*-phthalaldehyde as a fluorescent reagent, which yields a fluorophore, and the intensity of the fluorophore is measured by a spectrofluorometer. However, to obtain derivatives from this fluorophore and histamine, impurities in the sample must be removed.

1.1. Equipments and Reagents

1.1.1. Ion exchange resin

Resin (Bio-Rad AG1-X8, 50-100 mesh, Dowex) was converted to -OH form by adding *ca.* 15 mL of 2N NaOH per g resin. The mixture was swirled and let stood for 30 min. The liquid was decanted and repeated with the additional base. The resin was washed 2 times with distilled water. The resin was freshly prepared weekly and stored under water.

The ion exchange column was prepared by placing a glass wool plug at the base of the chromatographic tube. The -OH form resin was added to the tube for 8 cm from bed. The distilled water was maintained above the top of resin bed all the times. The column was washed with 10 mL water before applying each extract.

1.1.2. 3.57 N Phosphoric acid

85% H_3PO_4	121.8	mL

Make up to 1 L with distilled water.

1.1.3. *o*-Phtalicdicarboxaldehyde (OPT)

o-Phtalicdicarboxaldehyde	0.1%	(w/v)
Absolute methanol (analytical grade)	100	mL

1.1.4. Histamine standard

The stock standard (4.5 mM) was prepared by accurately weighted 8.37 mg histamine.2HCl into 10 mL volumetric flask and Dissolve and dilute to volume with 0.1 N HCl. Store in refrigerator. Prepare fresh weekly.

The working standard solution (0.45 mM) was prepared by adding 1 mL stock solution into 10 mL volumetric flask and dilute to volume with 0.1 N Cl. Prepare fresh weekly. The calibration of histamine standard was made in the ranges of 50 - 500 µM (Figure 39).

1.2. Procedure

A. Extraction

A 1 mL of sample was added to 5 mL of 90% (v/v) methanol in 10-mL volumetric flask. The flask was swirled for 1 min and heated at 60ºC for 15 min in water bath. The sample was cooled to room temperature and made up to volume with 75% (v/v) methanol. The sample was mixed and centrifuged at 3,500 ×*g* for 10 min to obtain the supernatant.

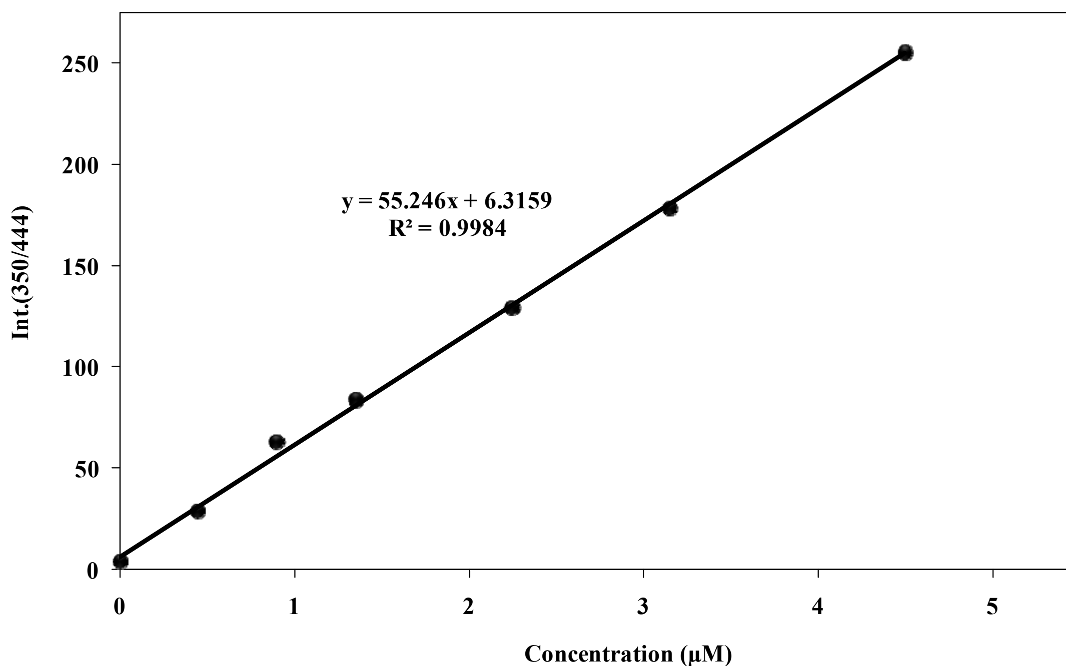


Figure 39. Standard curve of histamine using fluorometric method of AOAC (1995).

B. Column chromatography elution

A 5 mL of 1 N HCl was added to 50 mL volumetric flask. The 1 mL of obtained supernatant was placed into column. The supernatant was eluted into the volumetric flask until it was remained about 2 mm above resin. After that, the 4-5 column volumes of distilled water were added. The eluate was adjust to the volume with distilled water.

C. Derivatization and detection

The 1 mL of each diluted eluate and working standard was mixed with 2 mL of 0.1 N HCl. A 600 µL of 1 N NaOH was added and mixed. Within 5 min, 200 µL of OPT solution was added. After exactly 4 min, a 600 µL of 3.75 N H_2PO_4 was

added and mixed immediately. The fluorescence intensity of working standard solutions with water in reference cell was recorded using excitation wavelength of 350 nm and emission wavelength 444 nm within 1.5 h.

2. Biogenic amines determination by HPLC method

2.1. Reagents

0.4 M Perchloric acid

Perchloric acid	43	mL
Distilled water	1,900	mL

Perchloric acid was slowly added to distilled water, and the volume was made up to 1 L with distilled water.

2 N NaOH

NaOH	80	g
Distilled water	900	mL

Dissolve and make up to 1 L with distilled water.

Mixed standard biogenic amines

Cadaverine dihydrochloride	4.4	mg
Histamine dihydrochloride	4.2	mg
β-phenylethanolamine	3.4	mg
Putrescine dihydrochloride	4.7	mg
Spermidine trihydrochloride	3.5	mg
Spermine hydrochloride	4.5	mg
Tyramine hydrochloride	3.2	mg
Typtamine hydrochloride	3.2	Mg

Dissolve all amines in 5 mL volumetric flask and make up to the volume with distilled water.

A six-point standard curve for each amine was prepared from stock solutions and derivatization. Standard solutions, prepared in triplicate and containing all amines at 0.005, 0.010, 0.050, 0.100, 0.500 or 1.000 mg/mL.

2.2. Extraction

The sample (480 µL) was added with a 20 µL of 2 mg/mL of 1,7-diaminoheptane as the internal standard (IS) and extracted with a 500 µL of 0.4 M perchloric acid. The samples were extracted by a vortex mixer for 5 min. The supernatant was separated by centrifugation at 15,000 ×*g*, 25°C for 5 min.

2.3. Derivatization

The supernatant and mixed standards were derivatized by using the method of Eerola *et al*. (1993) with slight modifications. The supernatants (300 µL) were mixed with a 60 µl of 2 N NaOH and a 90 µl of saturated sodium bicarbonate. A 600 µL of dansyl chloride (10 mg/mL) was added and then incubated for 45 min at 40°C. The residual dansyl chloride was removed by adding 30 µl of 25% (v/v) ammonia and centrifuged at 3,500 ×*g* for 5 min. The supernatant was collected and filtered through a 0.45 µm membrane filter prior to analysis by high performance liquid chromatography (HPLC). A Waters Separation Module 2690 was operated to give a flow rate of 1.5 mL/min of the mobile phase of 0.1% (v/v) acetic acid (solvent A) and acetonitrile containing 0.1% (v/v) acetic acid (solvent B), as shown in a gradient elution programme (Table 17).

Table 17. Gradient elution program for biogenic amines analysis by HPLC

	Time (min)	Flow (mL/min)	%A	%B
1	0	1.5	55	45
2	15	1.5	40	60
3	25	1.5	10	90
4	30	1.5	10	90
5	32	1.5	55	45

Solvent A; 0.1% (v/v) acetic acid.

Solvent B; the mixture of acetonitrile with 0.1% (v/v) acetic acid.

Separation was achieved using a column of Hypersil BDS C18 (300 × 7.8 mm i.d.) set at 40°C. A sample, 20 µL, was injected and a photo diode array (Model Waters 996), set at the wavelength of 254 nm was used as the detector. Data were processed and analyzed using Millennium 32 software. The HPLC chromatogram of biogenic amines standard at 1.000 mg/mL was shown in Figure 40.

2.4 Standard curve and calculation

Calibration curves were created by plotting the concentration of each amine against the ratio of the standard peak area to that of the IS. Simple linear regression analysis was performed to calculate the slope and intercept. The correlation (r^2) for each amine was also determined (Figure 41). Biogenic amine concentrations in samples were then obtained by calculation of the ratio of sample peak area to IS and using the regression equations from the standard curves for each amine.

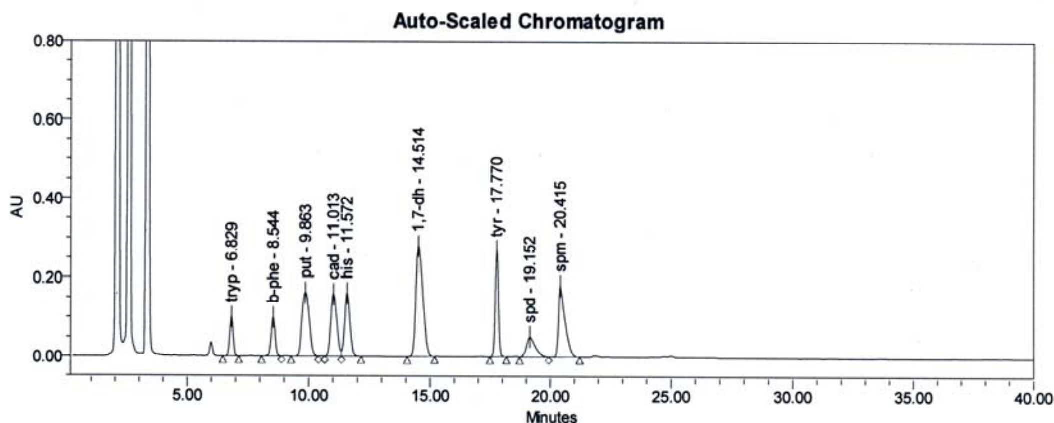


Figure 40. Biogenic amines standard chromatogram (1.000 mg/mL). Abbreviation: Tryp; tryptamine, β-phe; β-phenylethylamine, Put; putrescine, Cad; cadaverine, His; histamine, 1,7-dh; 1,7-diaminoheptane (internal standard), Tyr; tyramine, Spd; spermidine and Spm; spermine.

3. Cytotoxicity test

The MTT cell proliferation and viability assay is a safe, sensitive, *in vitro* assay for the measurement of cell proliferation or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. Cells are cultured in flat-bottomed, 96-well tissue culture plates. The cells are treated as per experimental design and incubation times are optimized for each cell type and system. The tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) is added to the wells and the cells are incubated. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals. Detergent is then added to the wells, solubilizing the crystals so the absorbance can be read using a spectrophotometer. Samples are read directly in the wells. The optimal wavelength for absorbance is 570 nm. The data is analyzed by plotting cell number versus absorbance, allowing quantitation of changes in cell proliferation. The rate of tetrazolium reduction is proportional to the rate of cell proliferation.

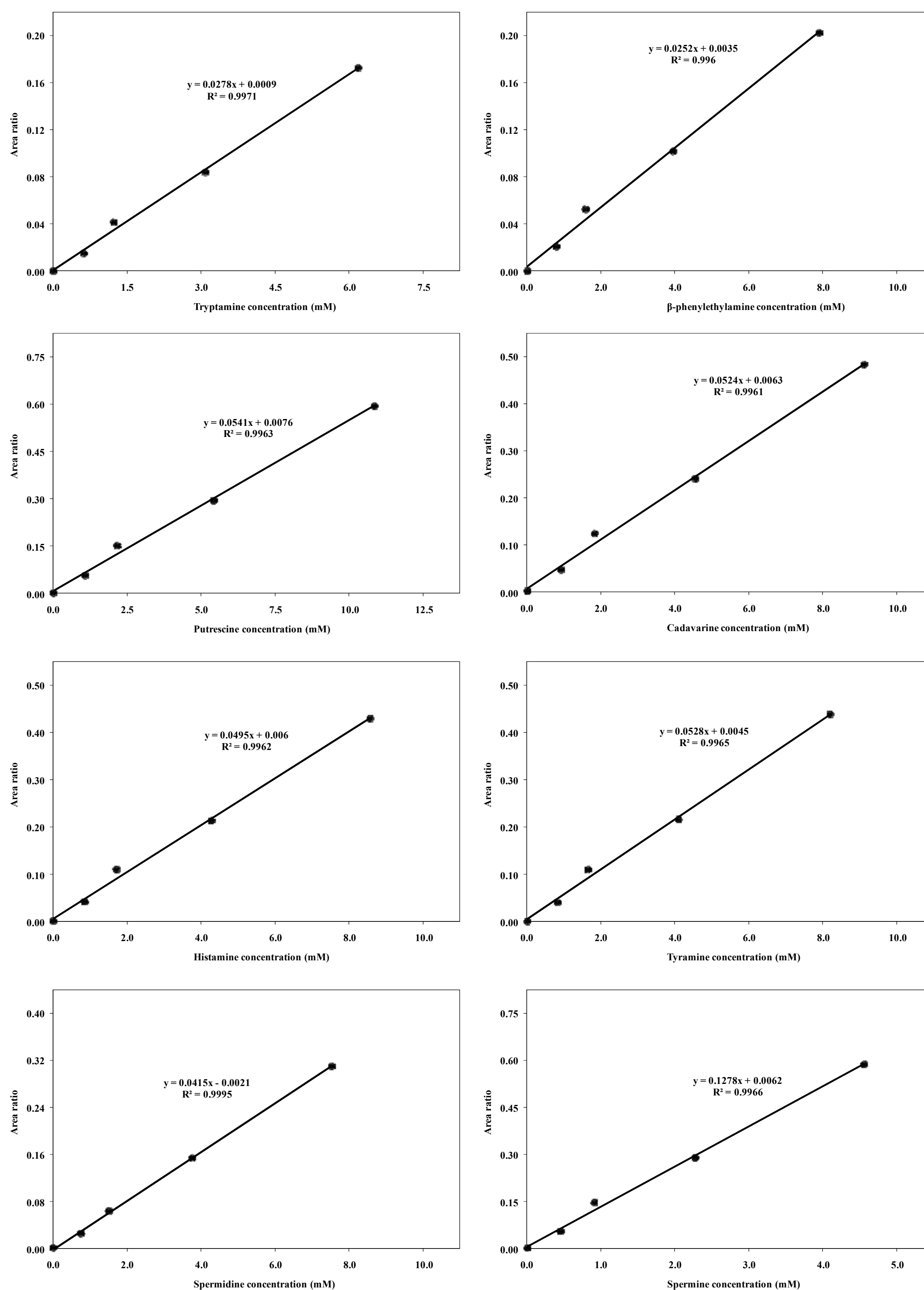


Figure 41. Standard curves of biogenic amines determined by HPLC.

3.1. Sample preparation

Whole cell extract of the HDS3-1 was prepared by inoculating 250 µL of stock culture into 5 mL of halophilic medium and incubated at 37°C in a shaker incubator at 200 rpm for 7 days. After that, inoculum was added at 5% (v/v) into halophilic liquid medium containing 5 mM of histamine (free-base) (Appendix A-2) and incubated at 37°C in a shaker incubator at 200 rpm for 7 days. Wet cell paste was obtained by centrifugation of cultured broth at 10,000 ×g at 4°C for 10 min. The wet cell paste was resuspended in 50 mM Tris-HCl, pH 7.0 containing 4.5 M NaCl at a ratio of 1:1 (w/v) and sonicated for a total of 2 min by a Vibra Cell VCX60 (Sonics and Materials Inc., USA). The sample was placed in ice and sonicated for 20s, followed by a 40s rest interval. The obtained suspension was freeze-dried and referred as to the whole cell extract of the HDS3-1.

The whole cell extract of the HDS3-1 was weighed and Dissolve in 0.1 N NaOH at 37°C for an hour and then centrifuged to collect supernatant and then adjust to give a stock concentration of 25 mg/mL. The sample extracts were serial diluted at a ratio of 1:2 in the respective growth medium of the cells to give the 8 final concentrations of 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.906 and 1.953 ug/mL.

3.2. Target cells

The target cells were included human colon adenocarcinoma (Caco2), human liver hepatocarcinoma (HepG2) and human larynx epithelial (HEp-2) cells. The target cells were grown in All 3 cell lines were grown in Minimum Essential Medium (MEM) and incubated at 37°C in a fully humidified, 5% CO_2 : air atmosphere.

3.3. MTT cytotoxicity test

The target cell lines of CaCo2, HepG2 and HEp-2 were seeded in a 96-well plate and incubated for 48 hours. The sample at 8 different concentrations were

added to the target cell lines and incubated for 24 hours. The test samples were removed from the cell cultures and the cells were reincubated for a further 24 hours in fresh medium and then tested with MTT assay. The optical density was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC_{50} for each toxin sample. A dose-response curve was derived from 8 concentrations in the test range using 4 wells per concentration. Results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells compared to controls.

4. Determination of protein

The protein concentration was measured by the method of Bradford (1976). Bovine serum albumin was used as a standard. This technique is simpler, faster and more sensitive than the Lowry method. Moreover, when compared with the Lowry method, it is subject to less interference by common reagents and nonprotein components of biological samples. The dye appears to bind most readily to arginyl and lysyl residues of proteins (but does not bind to the free amino acids). This assay is suitable for measuring between 10 and 100 µg protein.

4.1. Reagent

Bradford reagent

Coomassie blue G250	100	g
Ethanol 95%	50	g
85% phosphoric acid	900	ML

Dissolve coomassie blue in ethanol, slowly add phosphoric acid and make up to 1 L with distilled water. The reagent should be filtered through Whatman No. 1 filter paper and then store in an amber bottle at room temperature. It is stable for several weeks.

Standard Bovine Serum Albumin (BSA)

Bovine serum albumin	15	g
Distilled water	10	mL

Dissolve and adjust to the volume with distilled water. The solution can be stored frozen at -20°C.

4.2. Procedure

A 100 µL of sample containing protein between 15 and 150 µg was prepared. If the approximate sample concentration is unknown, a range of dilutions (1, 1/10, 1/100, 1/1000) should be assayed. Each sample was prepared in duplicates. For the calibration curve, the duplicate volumes of 10, 20, 30, 50 and 100 µL of 1.5 mg/mL γ-globulin standard solution into test tubes, and make each up to 100 µL with distilled water were prepared. A 100 µL of distilled water into an additional tube was provided as a reagent blank.

A 5 mL of Bradford reagent was added to each tube, and mixed well by gentle vortexing. A foaming should be avoided, which will lead to poor reproducibility. The A_{595} of the samples and standards against the reagent blank was measured between 2 min and 1 h after mixing. The 150-µg standard should give an A_{595} value of about 0.8 (Figure 42). The calibration curve should be constructed at each assay.

5. Polyacrylamide gel electrophoresis (PAGE) reagents

Monomer solution

Acrylamide	30%	(w/v)
Bisacrylamide	0.8%	(w/v)

Make up to 100 mL with deionized water.

Note: Acrylamide is a neurotoxin observes extreme caution to minimize skin contact and inhalation. The solution can be store up to 3 months at 4°C in the dark.

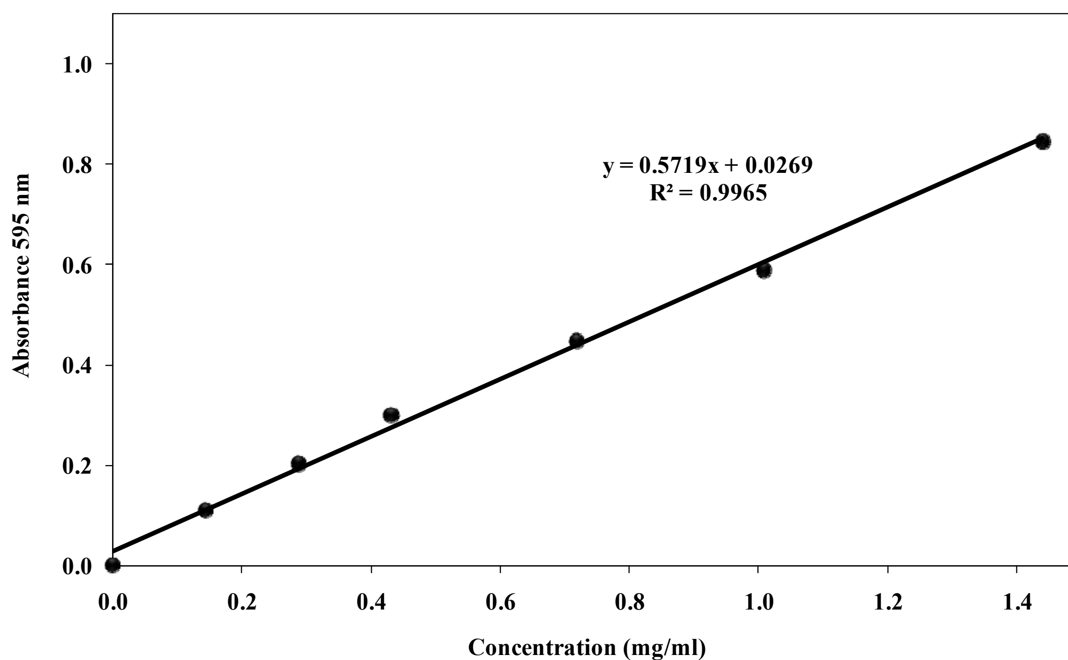


Figure 42. Standard curve of bovine serum albumin (BSA).

4× Resolving gel buffer

Tris(hydroxymethyl)aminomethane	18.15	g
Deionized water	90	mL

Dissolve and adjust the pH to 8.8 by using 0.1 N HCl. Make up to 100 mL with deionized water.

Note: The solution can be stored up to 3 months at 4°C in the dark.

4× Stacking gel buffer

Tris(hydroxymethyl)aminomethane	6	g
Deionized water	90	mL

Dissolve and adjust the pH to 6.8 by using 0.1 N HCl. Make up to 100 mL with deionized water.

Note: The solution can be stored up to 3 months at 4°C in the dark.

10× Tank buffer for SDS-PAGE

Tris(hydroxymethyl)aminomethane	30.28	g
Glycine	144.13	g
Sodium dodecyl sulfate	10	g
Distilled water	900	mL

Dissolve and make up to 1 L with distilled water.

Note: Dilute 10 times before use. The solution can be stored up to 1 month at room temperature. 10× Tank buffer for Native-PAGE was prepared in a similar manner, except that the addition of SDS was omitted.

10% Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulfate	10	g
Deionized water	90	mL

Dissolve and make to 100 mL with deionized water.

2× Sample buffer for SDS-PAGE

4× Stacking gel buffer	2.5	mL
Glycerol	2	mL
10% (w/v) SDS	4	mL
Broomphenol blue (2 mg/mL)	1	mL
β-mercaptoethanol	0.2	mL

Dissolve and make up to 10 mL with deionized water.

Note: The reagent should be filtered before use.

2× Sample buffer for Native-PAGE was prepared in a similar manner, except that the addition of SDS and β-mercaptoethanol were omitted.

12.5% Running gel for SDS-PAGE

Deionized water	4.1314	mL
4× Running gel buffer	3.25	mL
Monomer solution	5.4171	mL
10% (w/v) SDS	130	µL
10% (w/v) Ammonium persulfate	65	µL
TEMED	6.5	µL

Note: 12.5% Running gel for Native-PAGE was prepared in a similar manner, except that the addition of SDS was omitted.

4% Stacking gel for SDS-PAGE

Deionized water	3.053	mL
4× Stacking gel buffer	1.25	mL
Monomer solution	667	µL
10% (w/v) SDS	50	µL
10% (w/v) Ammonium persulfate	25	µL
TEMED	5	µL

Note: 4% Stacking gel for Native-PAGE was prepared in a similar manner, except that the addition of SDS was omitted.

Staining solution

Coomassie brilliant blue (R-250)	1.25	g
Ethanol	450	mL
Acetic acid	100	mL

Dissolve and make up to 1 L with distilled water.

Note: The reagent should be filtered before use. Store the solution in the dark.

Destaining solution

Methanol	300	mL
Acetic acid	100	mL

Dissolve and make up to 1 L with distilled water.

6. Sensory evaluation form

Fish sauce samples from Chapter 6, obtained after 2 days of incubation, were evaluated for acceptance by an untrained 30-member panel according to the method of Chamber and Wolf (1996). The panelists were staffs of the Food Biotechnology Laboratory at the National Center for Genetic Engineering and Biotechnology (BIOTEC), of age ranging from 20 to 45 years. All panelists had sensorial acquaintance with fish sauce. Panelists were asked to give acceptance scores for four attributes: colour, aroma and overall acceptance using the nine-point hedonic scale. A nine-point hedonic scale, in which a score of 1 represented extreme dislike, 5 represented neither like nor dislike and 9 represented like extremely, was used for evaluation. Samples were coded with three-digit random numbers and were presented to the panelists at ambient temperature. Fish sauce samples were served to each panelist at the same time in a random order.

For the assessment of aroma, each fish sample was absorbed on about half a strip (1 × 5 cm) of filter paper and panelists sniffed the samples. A line scale consists of a line, 10 cm long, drawn on the score sheet and labeled at the ends with the extremes of the scale, for example, absent and very strong, and perhaps also labeled in the middle. The assessor makes a mark on the line representing his/her perception of the intensity of the stimulus, and the distance of this mark from the origin is the measure of the intensity. The ratings of each attribute were converted to numerical scores for further statistical analysis.

Sensory evaluation form of fish sauce product was depicted as follows:

Sensory Evaluation Form of Fish Sauce Product

Date……………….…………..Product Code……………………………………………..…

Please evaluation each sample for degree of color, smell and overall ranking by crossing _×_ on the appropriate answer

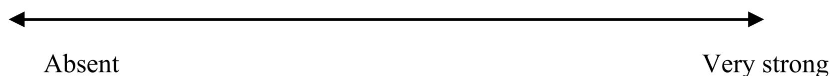
1. Color

_____ Like extremely
_____ Like very much
_____ Like moderately
_____ Like slightly
_____ Neither like nor dislike
_____ Dislike slightly
_____ Dislike moderately
_____ Dislike very much
_____ Extremely dislike

2. Smell

_____ Like extremely
_____ Like very much
_____ Like moderately
_____ Like slightly
_____ Neither like nor dislike
_____ Dislike slightly
_____ Dislike moderately
_____ Dislike very much
_____ Extremely dislike

3. Off-Odour / Unpleasant smell



Absent Very strong

4. Overall ranking

_____ Like extremely
_____ Like very much
_____ Like moderately
_____ Like slightly
_____ Neither like nor dislike
_____ Dislike slightly
_____ Dislike moderately
_____ Dike very much
_____ Extremely dislike

5. Acceptability

_____ Accept
_____ Not accept

7. Reasons of unacceptability of a product

……
……
…………………………………………

CURRICULUM VITAE

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Student ID 4883007

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Animal Science, Agricultural)	Chiang Mai University	2000
Master of Science (Animal Science, Agricultural)	Chiang Mai University	2003

Scholarship Awards during Enrolment

1. Ph.D. Student Research Scholarship by the Thailand Research Fund (TRF) under the Royal Golden Jubilee Ph.D. Program (PHD/0119/2548).
2. Student exchange program for the research training for 6 months at the University of Wisconsin-Madison (UW), according to the Memorandum of Understanding (MOU) between the TRF and the UW.
3. Award for participating as a speaker in the Pacific Fisheries Technologists 59th Annual Meeting "From Sea to Plate by the City of the Golden Gate" 3-6 February 2008. San Francisco, California, USA.

List of Publications and Proceedings

Publications

1. Tapingkae, W., Tanasupawat, S., Itoh, T., Parkin, K. L., Benjakul, S., Visessanguan, W. and Valyasevi, R. 2008. *Natrinema gari* sp. nov., a halophilic archaeon isolated from fish sauce in Thailand. Int. J. Syst. Evol. Microbiol. 58: 2378-2383.
2. Tapingkae, W., Tanasupawat, S., Parkin, K. L., Benjakul, S. and Visessanguan, W. 2009. Degradation of histamine by extremely halophilic archaea isolated from salt-fermented fishery products. Enzyme Microb. Technol. (In preparation)

3. Tapingkae, W., Parkin, K. L., Benjakul, S. and Visessanguan, W. 2009. Purification and characterization of histamine dehydrogenase from *Natrinema gari* BCC 24369. Food Chem. (In preparation)
4. Tapingkae, W., Parkin, K. L., Benjakul, S. and Visessanguan, W. 2009. Degradation of histamine by immobilized whole cell of *Natrinema gari* BCC 24369. Food Chem. (In preparation)

Proceedings

1. Tapingkae, W., Tanasupawat, S., Parkin, K. L., Benjakul, S. and Visessanguan, W. 2007. Degradation of histamine by extremely halophilic archaea isolated from salt-fermented fishery products. RGJ Seminar Series "Advanced Researches in Food Value Chain". Bangkok, Thailand.
2. Tapingkae, W., Tanasupawat, S., Parkin, K. L., Benjakul, S. and Visessanguan, W. 2008. Degradation of histamine by extremely halophilic archaea isolated from salt-fermented fishery products. 59th Pacific Fisheries Technologists Conference 2008. San Francisco, USA.
3. Tapingkae, W., Tanasupawat, S., Itoh, T., Parkin, K. L., Benjakul, S., Visessanguan, W. and Valyasevi, R. 2008. *Natrinema gari* sp. nov., a halophilic archaeon isolated from fish sauce in Thailand. 2nd BioAsia 2008 "Well Being for the Human Race". Bangkok, Thailand.
4. Tapingkae, W., Tanasupawat, S., Parkin, K. L., Benjakul, S. and Visessanguan, W. 2009. Degradation of histamine in fish sauce by free and immobilized whole cell of *Natrinema gari* BCC 24369. 60th Pacific Fisheries Technologists Conference 2009. Portland, Oregon, USA.