Development of Biological Process for Histamine Degradation in Fish Sauce by Immobilized Whole Cells of *Natrinema gari* BCC 24369

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ชื่อวิทยานิพนธ์ การพัฒนากระบวนการทางชีวภาพสำหรับการย่อยสลายฮิสทามีนในน้ำปลา โดยใช้เซลล์ตรึงของเชื้อ *Natrinema gari* BCC 24369
ผู้เขียน นางสาวศิริพร ชัยแก้ว
สาขาวิชา วิทยาศาสตร์และเทคโนโลยีอาหาร
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บทคัดย่อ

ฮิสทามีนเป็นสารประกอบไบโอจินิกเอมีน (Biogenic amine) ที่ได้รับความสนใจมากที่สุดในผลิตภัณฑ์อาหารหมักจากสัตว์น้ำ โดยเฉพาะน้ำปลา เนื่องจากก่อให้เกิดอาการเป็นพิษที่เรียกว่า Scombroid fish (histamine) poisoning ซึ่งเป็นอาการแพ้ที่เกิดจากการบริโภคอาหารที่มีฮิสทามีนในปริมาณมากเกินไป ดังนั้น การมีฮิสทามีนในปริมาณที่สูงก่อให้เกิดผลเสียหายต่อทั้งคุณภาพและความปลอดภัยของน้ำปลา ในการศึกษาก่อนหน้านี้ พบว่า เซลล์อิสระและเซลล์ตรึงของเชื้ออาเคียที่ชอบเกลือสายพันธุ์ *Natrinema gari* BCC 24369 ที่มีกิจกรรมของเอนไซม์ฮิสทามีนดีไฮโดรจีเนส (Histamine dehydrogenase หรือ HADH) สามารถลดฮิสทามีนในสภาวะที่มีเกลือสูงและมีศักยภาพที่จะใช้กำจัดฮิสทามีนในน้ำปลาได้ อย่างไรก็ตาม การประยุกต์ใช้เซลล์และเซลล์ตรึงของเชื้อ *Nnm. gari* BCC 24369 ในระดับขยายขนาด ยังถูกจำกัดด้วยการเพาะเลี้ยงเชื้อที่ใช้เวลานาน ผลผลิตเอนไซม์ต่ำ จำเป็นต้องใช้ถังปฏิกรณ์ชีวภาพและเครื่องมือที่จำเพาะที่ใช้พลังงานสูง มีราคาสูง ซึ่งล้วนทำให้ต้นทุนโดยรวมของการผลิตเซลล์และเอนไซม์สูงขึ้น ดังนั้น ในการศึกษานี้ มีวัตถุประสงค์เพื่อปรับปรุงกระบวนการผลิต HADH และพัฒนากระบวนการทางชีวภาพเพื่อกำจัดฮิสทามีนในน้ำปลา โดยใช้เซลล์ตรึงของเชื้อ *Nnm. gari* BCC 24369

สำหรับการผลิตเอนไซม์อย่างง่ายและมีราคาถูก ในขั้นต้น ได้ทำการศึกษาสูตรอาหาร และสภาวะที่เหมาะสมเพื่อปรับปรุงประสิทธิภาพในการผลิต HADH ซึ่งเป็นเอนไซม์ที่อยู่ภายในเซลล์ ที่ผลิตจากเชื้อ *Nnm. gari* BCC 24369 จากนั้นได้ศึกษาการผลิต HADH ภายใต้สภาวะที่ไม่ผ่านการฆ่าเชื้อด้วยความร้อน และได้ทำการขยายขนาดการผลิตดังกล่าว ในถังโพลีโพรไพลีนขนาด 20 ลิตร จากผลการทดลอง อาหารเลี้ยงเชื้อสูตรที่เหมาะสมมีองค์ประกอบ คือ กรดคาซามิโน 15 กรัมต่อลิตร เกลือแมกนีเซียมซัลเฟต 75 กรัมต่อลิตร เกลือโซเดียมคลอไรด์ 273 กรัมต่อลิตร เฟอร์รัสคลอไรด์เตตระไฮเดรต 2.5 มิลลิกรัมต่อลิตร ยีสต์สกัด 10 กรัมต่อลิตร เกลือโซเดียมกลูตาเมต 5 กรัมต่อลิตร และเกลือโพแทสเซียมคลอไรด์ 5 กรัมต่อลิตร ค่าความเป็นกรดด่างเริ่มต้นของอาหารเลี้ยงเชื้อ และอุณหภูมิที่ใช้ในการบ่มเชื้อที่เหมาะสมคือ 7.5 และ 37 องศาเซลเซียส ตามลำดับ โดย HADH ที่ผลิตได้ภายใต้สภาวะที่เหมาะสมนี้ผลผลิตเพิ่มขึ้นเป็น 2.2 เท่า เมื่อเทียบกับการผลิตภายใต้สภาวะเดิมที่มีรายงานมาก่อนหน้านี้ เนื่องจากเชื้อ *Nnm. gari* BCC 24369 สามารถเจริญเติบโตได้ในสภาวะที่มีเกลือสูง ซึ่งยากต่อการปนเปื้อนของเชื้อจุลินทรีย์ชนิดอื่นๆ ทำให้สามารถขยายขนาดการผลิตได้ในสภาวะที่ไม่ผ่านการฆ่าเชื้อในระดับที่ใหญ่ขึ้น ทำให้ต้นทุนการผลิตมีราคาถูกลง และกระบวนการผลิตเป็นมิตรต่อสิ่งแวดล้อมมากขึ้น จากการศึกษาการผลิต HADH ในอาหารเลี้ยงเชื้อสูตรที่เหมาะสมข้างต้น โดยไม่ผ่านการฆ่าเชื้อด้วยความร้อนโดยสมบูรณ์ ในถังโพลีโพรไพลีนขนาด 20

ลิตร พบว่า ผลผลิตของ HADH ที่ได้จากการเพาะเลี้ยงเชื้อ *Nnm. gari* BCC 24369 ที่ระดับ 16 ลิตร ภายใต้สภาวะที่ไม่ผ่านการฆ่าเชื้อ เท่ากับ 858±12 ยูนิตต่อกรัมชีวมวล ซึ่งไม่แตกต่างอย่างมีนัยสำคัญกับสภาวะที่ผ่านการฆ่าเชื้อ (878±15 ยูนิตต่อกรัมชีวมวล, $p > 0.05$)

ได้ทำการศึกษาสภาวะที่เหมาะสมของการตรึงเซลล์ของเชื้อ *Nnm. gari* BCC 24369 บนไดอะตอม และศึกษาความเสถียรภาพในการใช้งานและประสิทธิภาพของเซลล์ตรึงสำหรับการย่อยสลายฮิสทามีนในสภาวะที่มีเกลือสูง โดยสภาวะที่เหมาะสมที่ใช้ในการตรึงเซลล์ประกอบด้วย เซลล์อิสระร้อยละ 10 โดยมวลต่อปริมาตร เกลือโซเดียมคลอไรด์ 4 โมลต่อลิตร ไดอะตอมร้อยละ 15 โดยมวลต่อปริมาตร ค่าความเป็นกรดด่างเริ่มต้นเท่ากับ 5.0 เขย่าที่ความเร็ว 100 รอบต่อนาที ที่อุณหภูมิ 4 องศาเซลเซียส เป็นเวลา 4 ชม โดยเซลล์ตรึงบนไดอะตอมภายใต้สภาวะที่เหมาะสมนี้ให้ค่ากิจกรรมการย่อยสลายฮิสทามีนสูงสุดเท่ากับ 35.3±0.8 ยูนิตต่อกรัมของตัวดูดซับ เซลล์ตรึงของเชื้อ *Nnm. gari* BCC 24369 ที่ถูกบรรจุในเครื่องปฏิกรณ์ชีวภาพแบบเบดนิ่ง (Fixed-bed bioreactor) สามารถลดฮิสทามีนในน้ำปลาได้อย่างมีประสิทธิผล และพบว่ามีเสถียรภาพในการใช้งานที่ดี ถึงแม้ว่าประสิทธิภาพการกำจัดฮิสทามีนของเซลล์ตรึงจะลดลงอย่างต่อเนื่อง แต่ยังคงเหลืออยู่มากกว่าร้อยละ 80 หลังการใช้งานในรอบที่ 4 และเหลืออยู่ประมาณร้อยละ 50 เมื่อผ่านการใช้งาน 9 รอบติดต่อกัน

ได้ทำการศึกษาประสิทธิภาพการกำจัดฮิสทามีนในน้ำปลา ของเซลล์ตรึงของเชื้อ *Nnm. gari* BCC 24369 ภายใต้สภาวะนิ่ง (Static condition) และสภาวะที่มีการไหลของน้ำปลาผ่านเบดนิ่ง (Fixed-bed flow process) ภายใต้สภาวะนิ่ง เซลล์ตรึงบนไดอะตอมมีประสิทธิภาพการย่อยสลายฮิสทามีนในน้ำปลา คิดเป็นร้อยละ 24±1 โดยมีสภาวะที่เหมาะสม ประกอบด้วย การใช้เซลล์ตรึงที่ความเข้มข้นร้อยละ 10 โดยมวลต่อปริมาตร ค่าความเป็นกรดด่างของน้ำปลาเท่ากับ 6.5 ความเข้มข้นไรโบฟลาวิน ร้อยละ 0.01 โดยมวลต่อปริมาตร ระยะเวลาในการบ่ม 2 ชั่วโมง การใช้เซลล์ตรึงของเชื้อ *Nnm. gari* BCC 24369 ร่วมกับเครื่องปฏิกรณ์ชีวภาพแบบเบดนิ่งมีประสิทธิภาพในการกำจัดฮิสทามีนสูงกว่าการใช้เซลล์ตรึงดังกล่าวภายใต้สภาวะนิ่งถึง 2 เท่า เมื่อทำการทดสอบในเครื่องปฏิกรณ์ชีวภาพแบบเบดนิ่งที่บรรจุด้วยเซลล์ตรึงของเชื้อ *Nnm. gari* BCC 24369 ที่มีการควบคุมการไหลเข้าของน้ำปลาอย่างต่อเนื่อง ในอัตราที่เหมาะสมเท่ากับ 0.5 มิลลิลิตรต่อนาที พบว่าปริมาณฮิสทามีนถูกกำจัดอย่างมีนัยสำคัญทางสถิติ คิดเป็น ร้อยละ 51±3 โดยไม่มีการเปลี่ยนแปลงปริมาณของไบโอจินิกเอมีนชนิดอื่น กรดอะมิโนอิสระ กรดอะมิโนทั้งหมด ค่าความเป็นกรดด่าง และปริมาณไนโตรเจนทั้งหมด จากการทดสอบทางประสาทสัมผัสโดยผู้บริโภคทั่วไปชี้ให้เห็นว่า สี กลิ่น กลิ่น-รส และความชอบโดยรวม ไม่มีความแตกแตกต่างกัน ในตัวอย่างน้ำปลาที่ผ่านและไม่ผ่านเครื่องปฏิกรณ์ชีวภาพแบบเบดนิ่งที่บรรจุด้วยเซลล์ตรึง ($p > 0.05$) จากการใช้งานเครื่องปฏิกรณ์ชีวภาพแบบเบดนิ่งที่บรรจุด้วยเซลล์ตรึงแบบต่อเนื่องนี้ ไม่เพียงแต่จะสามารถกำจัดฮิสทามีนได้อย่างมีประสิทธิภาพแล้ว ยังชะลอการเกิดสีเข้มของน้ำปลา โดยไม่ก่อให้เกิดผลเสียต่อคุณลักษณะพิเศษโดยรวมของน้ำปลาในระหว่างการเก็บรักษาเป็นเวลา 6 เดือน

Thesis Title Development of Biological Process for Histamine Degradation in Fish Sauce by Immobilized Whole Cells of *Natrinema gari* BCC 24369

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ABSTRACT

Histamine is a biogenic amine naturally present in some fermented fishery products, particularly, fish sauce. Scombroid fish (histamine) poisoning is one of major health significances associated with ingestion of foods containing high histamine. Therefore, the presence of high level of histamine is detrimental to the quality and safety of fish sauce. In previous study, *Natrinema gari* BCC 24369, a halophilic archaeon and its immobilized cells, having histamine dehydrogenase (HADH) activity can reduce histamine in high salt condition and have the potential to be applied for histamine removal in fish sauce. Nevertheless, the applications of *Nnm. gari* BCC 24369 and its immobilized whole cells at larger scale have been limited due to long cultivation time, low yield of enzyme, and requirement of specific equipments e.g. reactor with high energy consumption and high cost which increases the overall cost of the cell and enzyme production. Therefore, this study was mainly related to improvement of HADH production and development of a biological process for removing histamine in fish sauce by using immobilized whole cells of *Nnm. gari* BCC 24369.

Initially, the production of HADH, an intracellular enzyme produced by *Nnm. gari* BCC 24369 was optimized in order to improve the productivity and then production of HADH was investigated in optimized medium without sterilization in 20-l polypropylene tank to perform an economical and simple enzyme production. The optimal medium contained 15 g/l casamino acid, 75 g/l $MgSO_4.7H_2O$, 273 g/l NaCl, 2.5 mg/l $FeCl_2.4H_2O$, 10 g/l yeast extract, 5 g/l sodium glutamate and 5 g/l KCl. The optimal initial pH and incubation temperature were at 7.5 and 37°C, respectively. HADH production under optimal condition was 2.2-fold higher than those under un-

optimized condition. Owing to the ability of *Nnm. gari* BCC 24369 to grow at high salt conditions with difficulty contamination by other microorganisms, a more economical and eco-friendlier HADH production was scaled-up under completely non-sterile condition. At 16-l-scale batch cultivation of *Nnm. gari* BCC 24369, HADH productivity under non-sterile condition was 858±12 U/g cell biomass which was not significantly different from that under sterile condition (878±15 U/g cell biomass, $p > 0.05$).

The condition for whole cells immobilization of *Nnm. gari* BCC 24369 onto diatomite as the support was optimized and operational stability and efficiency of immobilized whole cells on histamine degradation at high salt condition was assessed. The optimal immobilization conditions composed of 10% (w/v) of free whole cell, 4 mol/l of NaCl, 15% (w/v) of diatomite, initial pH 5.0, agitation rate at 100 rpm and immobilization time of 4 h at 4°C. The diatomite-immobilized cells exhibited the highest histamine degrading activity of 35.3±0.8 U/g support. The immobilized whole cells of *Nnm. gari* BCC 24369 packed in fixed-bed bioreactor effectively removed histamine in fish sauce and found to have good operational stability. Although the histamine removal efficiency of immobilized whole cells gradually decreased but still remained over 80% at the 4^{th} cycle and retained about 50% after nine consecutive cycles.

The performance of immobilized cells of *Nnm. gari* BCC 24369 to remove histamine in fish sauce was assessed under static condition and fixed-bed flow process. Under static condition*, Nnm. gari* BCC 24369 cells immobilized on diatomite (10%, w/v) exhibited the optimal efficiency (24±1%) for histamine removal in fish sauce (pH 6.5) supplemented with riboflavin (0.01%, w/v) for 2 h incubation. The utilization of immobilized cells of *Nnm. gari* BCC 24369 in fixed-bed bioreactor was more promising than their utilization under static condition in that it exhibited 2-fold increase in histamine removal efficiency. In continuous fixed-bed bioreactor packed with immobilized cells under the optimal feeding flow rate of 0.5 ml/min, a significant amount of histamine (51±3%) was removed without changes on other biogenic amines contents, free and total amino acids composition, pH and total nitrogen content of fish sauce. An acceptability test also revealed that treated and untreated fish sauce samples were not significantly different in color, aroma, flavor

and overall acceptance ($p > 0.05$). Continuous fixed-bed bioreactor packed with immobilized cells not only efficiently removed histamine but also retarded color darkening without detrimental effect on the unique quality of fish sauce during 6 months storage.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

High levels of histamine in fermented fishery products are undesirable because they have adverse physiological effects on the human health. Among a variety of fermented fishery products in Thailand, fish sauce is particularly important in its economic value for both local and international markets. Depending on the quality of fish used, handling condition and uncontrolled fermentation process, fish sauce is often known to contain high level of histamine. With a concern on the toxicological effects, high content of histamine in commercial fish sauce becomes a major threat of export, due to a more strenuous regulatory measure and inspection. Histamine is regulated at a maximum level of 400 ppm by Codex and 500 ppm by Food and Drug Administration (FDA) (Codex, 2012; FDA, 1998). Fish sauce products often contain large quantities of histamine about 1000 ppm (Kuda and Miyawaki, 2010; Michihata *et al*. 2006; Brillantes and Samosorn, 2001). Generally, most companies deal with this problem by mixing portions of products high in histamine level with saturated brine and/or portions of products low in histamine level, in order to decrease or dilute the concentration of the hazard. However, the degree of dilution has been limited by total nitrogen content present in the final products in which total nitrogen is used as an indicator to determine the grade and price of fish sauce in Thailand, with products containing over 20 g/l classified as Grade I and 15 to 20 g/l as Grade II (TISI, 1983). Thus, this solution is not adequate to control the level of histamine in fish sauce products. Histamine is heat stable and undetectable through organoleptic analysis by even trained panelists (Arnold, 1980). Therefore, histamine, if present, is difficult to destroy and posts a risk of food intoxication.

Natrinema gari BCC 24369, a halophilic archaeon isolated from fish sauce, reduces histamine in the presence of high salt concentration up to 4.3 M NaCl (Tapingkae *et al*., 2008, 2010b). The histamine-degrading activity of *Nnm. gari* BCC 24369 is mediated through the presence of intracellular histamine dehydrogenase

(HADH) that catalyzes the oxidative deamination of histamine, resulting in the production of imidazole acetaldehyde and an ammonium ion (Zhou *et al*., 2014; Tapingkae *et al*., 2010b). This enzyme is a heterotrimer and its activity requires high levels of salt and co-factors. Due to ability to degrade histamine in high salt conditions, the cells of *Nnm. gari* BCC 24369 and its enzyme have the potential to be applied for histamine removal and determination of histamine content in products high in salt such as fish sauce.

Like other extremely halophilic bacteria, application of *Nnm. gari* BCC 24369 cells has been restricted by the slow growth rate, low yield of enzyme, high cost of cell and enzyme preparation, and production of off-odors and off-flavors in finished products (Robinson *et al*., 2005; Burns *et al*., 2004; Gram and Huss, 1996). To overcome these limitations, more economical and eco-friendlier process for HADH production with improved product yield should be developed. Owing to the ability of this archaeon to grow at high concentrations of NaCl, obvious economic advantage can be achieved by culturing under non-aseptic conditions instead of aseptic conditions. In addition, the immobilization of whole cells might provide potential advantages over applications of free whole cell and enzyme. Previously, Tapingkae *et al*. (2010a) successfully immobilized cells of *Nnm. gari* BCC 24369 on Celite® 545 in which celite-immobilized whole cells exhibited high activity retention, stability, and potential to be applied for histamine degradation in high salt condition (Tapingkae *et al*., 2010a). However, concern has been raised on safety and GRAS (Generally Recognized As Safe) status of Celite® 545 particularly when the ultimate application is for human consumption. Thus, this study focused on the use of low cost and high porosity, food grade support, diatomite instead of Celite® 545 with optimized condition for immobilization of whole cells *Nnm. gari* BCC 24369 in order to reduce the safety concern on support material, improve the immobilized activity, reduce the immobilization cost and thus increasing the potential use of immobilized *Nnm. gari* BCC 24369 for histamine degradation in food applications. 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. Therefore, this study was mainly related to development of a biological process for removing

histamine present in fish sauce by immobilized whole cells of *Nnm. gari* BCC 24369 onto diatomite.

1.2 Review of Literature

1.2.1 Histamine

Histamine (β-imidazolylethylamine) is the causative agent of scombroid poisoning, a food-borne chemical hazard (Hsu *et al*., 2009). High levels of histamine in foods, especially fermented fish products can have important vasoactive effects in humans (Mah *et al*., 2002; Lehane and Olley, 2000). Scombroid poisoning or histamine fish poisoning is a significant public health and safety concern and even a trade issue. Several outbreaks of the disease have been reported in many countries and histamine poisoning is one of the most frequently reported human illness associated with seafood throughout the world (Yesudhason *et al*., 2013). Figure 1 shows the major pathways for the formation of histamine. Histamine is produced from histidine via a one-step decarboxylation reaction catalyzed by histidine decarboxylase, which is found in many species of bacteria (Norval *et al*., 1989). 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). However, it is not the only route through which histidine is metabolised. The other route is its deamination into trans-urocanic acid (UCA) by histidine ammonia lyase (HAL), also known as histidase or histidinase (Lehane and Olley, 2000). Unlike histidine decarboxylase, HAL is found in the muscle and liver of the fish. It has a vast distribution in different species of bacteria as well (Baranowski, 1985; Shibatani *et al*., 1974). In fact, Baranowski (1985) recommended that UCA should be used as an alternative to histamine as a quality index of fish during storage at low temperature, since UCA has been detected as a predominant metabolite of histidine during incubation of different bacteria at low temperature.

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.

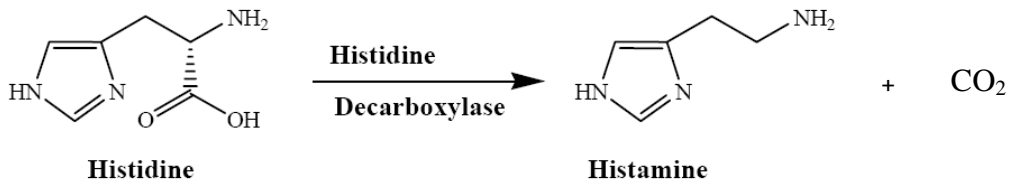

Figure 1. Metabolism pathway for the formation of histamine.
Source: Halász *et al*. (1994).

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).

1.2.2 Fish sauce

Fish sauce, *Nampla*, is a traditional fermented fish product commonly used as a condiment in the Southeast Asia. It is similar in organoleptic characteristics and method of production with other fish sauces from Cambodia, Indonesia, Malaysia, Myanmar, Philippines, Vietnam, and Japan. It is an important ingredient in most Thai dishes and it is a major export item for the country (Brillantes *et al*., 2002). 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. 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 intensity 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. Fish sauce is mainly produced from anchovies (*Stolephorus* spp.), mackerel (*Rastrelliger* spp.) and

herring (*Chupea* spp.) (Lopetcharat *et al*., 2001). Traditionally, the fish is produced by mixing with one part salt with two or three parts fish to prevent spoilage, and subsequently placed in a fermentation tank or vat, with salt spread at the bottom. Salt/fish ratio can be different in different regions. Due to the floatation of fish in the brine, processor has to ensure that fish has to be submersed 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 endogenous proteinases in fish muscle and digestive tract as well as proteinases produced by halophilic bacteria (Gildberg and Thongthai, 2001). The visceral enzymes possibly play a major role in protein hydrolysis. Trypsin and pepsin in digestive tract 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 the first extraction process, the residue is re-extracted with saturated brine to obtain the second quality fish sauce (Saisithi, 1994).

Chemical compositions of various fish sauce (i.e., nitrogen content, pH, amino acids, and volatile acids) have been reported broadly (Lopetcharat *et al*., 2001). Generally, as most of the polypeptide nitrogen decreases during the fermentation period, the amino acid content increases. The pH level drops due to the release of free amino acid from protein and large polypeptides. Total lipids decreased, but fatty acid composition did not change greatly during fermentation (Kim *et at*., 2004). Nutritionally, fish sauce is an important protein supplement, supplying as much as 7.5% of an individual's nitrogen intake (Amano, 1962). However, the high salt concentration of fish sauce limits its consumption.

The distribution of nitrogenous compounds in fish sauce varies according to the types of raw material used and methods of production. Total organic component ranges from 1.7 to 2.3%, of which 40-60% is in the form of free amino acids. Volatile nitrogen, mostly ammonia, comprises approximately 7-12% of the total nitrogen (Beddows *et al*., 1979; Uyenco *et al*., 1953). The amino acid profile of

various commercially produced fish sauces is shown in Table 1. Both bound and free amino acid contents contribute to the nutritional quality of fish sauce. The essential amino acids are usually fairly well preserved (Orejana, 1983; Beddows *et al*., 1976; Amano, 1962) and the high level of lysine in fish sauce compensates for low level of this amino acid in rice (Jansen and Howe, 1964).

The volatile compounds contributing to flavor of fish sauce are produced by non-enzymatic reactions of various components and enzymatic reactions by endogenous enzymes of fish origin and those of microorganisms surviving during fermentation (Fukami *et al*., 2004). Fermentation process normally takes a long time to ensure the solubilization as well as the flavor and color development of fish sauce. Fish sauce production can be accelerated by some proteinases to increase 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 (Brillantes, 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 (McIver *et al*., 1982).

1.2.3 Scombroid fish

Histamine formation has been mostly widely noticed in Scombroid fish species as well as those containing high levels of free histidine in their muscle which are often implicated in scombroid poisoning incidents when not properly processed and stored (Lehane and Olley, 2000). Scombroid fish belonging to the families Scombridae such as tuna (*Thunnus* spp.) and mackerel (*Scomber Japonicus*) and Scomberesocidae such as saury, are most commonly associated with histamine poisoning, but non-scombroid species (e.g., mahi-mahi (*Coryphaena* spp.), sardines (*Sardina pilchardus*), pilchards (*Sardina pilchardus*), anchovies (*Engraulis* spp.), herring (*Clupea* spp.), marlin (*Istiophoridae*) and bluefish (*Pomatomus saltratrix*) (Hwang *et al*., 1997; Taylor, 1986), Western Australian salmon (*Arripis truttaceus*), sockeye salmon (*Oncorhynchus nerka*), amberjack (*Seriola* spp.) Cape yellowtail (*Seriola lalandii*) (Gessner *et al*., 1996; Muller *et al*., 1992; Smart, 1992; Lange, 1988), and swordfish (*Xiphias gladius*) (Chang *et al*., 2008) can also be involved. These species are characterized by having relatively high levels of histidine in their

Table 1. Amino acid profile of various commercial fish sauces

Amino acids (mg/ml)	Fish sauce			
	Thai[1]	Malaysian[2]	Philippines[3]	Vietnam[4]
Alanine	5.38	1.53	6.74	4.20
Arginine	0.00	0.00	0.16	2.00
Aspartic acid	5.52	1.10	6.59	2.40
Cysteine	0.00	0.42	0.90	0.25
Glutamic acid	15.80	1.78	14.48	4.00
Glycine	4.14	0.44	5.61	2.40
Histidine*	2.55	1.66	2.82	0.30
Isoleucine*	3.86	0.98	4.01	4.00
Leucine*	4.65	1.64	5.72	4.00
Lysine*	7.34	0.40	6.02	4.00
Methionine*	1.94	0.48	2.98	0.80
Phenylalanine*	2.78	0.00	3.02	1.50
Proline	5.41	0.26	4.37	0.50
Serine	1.84	0.16	2.97	0.80
Threonine*	2.79	0.70	4.90	2.00
Tyrosine	0.42	0.32	0.53	0.80
Valine*	4.29	4.29	5.87	3.00

*Essential amino acid.

Source: [1]Tongthai and Okada (1981); [2]Beddows *et al*. (1976); [3]Orejana (1983); [4]Subba Rao (1967).

flesh (Taylor, 1986). Histidine levels vary from 1 g/kg in herring to as much as 15 g/kg in tuna (Ijomah *et al*., 1992). Fresh fish contains negligible quantities of histamine, usually, 0.1 mg/100 g (Frank *et al*., 1981). Histamine can be produced rapidly by bacterial decarboxylases in scombroid fish and other fish that have relatively high free histidine levels in their muscles when alive (Love, 1980). This occurs before post mortem proteolysis liberates additional histidine from muscle protein, and explains why histamine can reach high concentrations without the

formation of organoleptic (sensory) spoilage indicators (Sapin-Jaloustre and Sapin-Jaloustre, 1957).

1.2.4 Histamine formation in fish sauce

Histamine is considered to be the most important biogenic amines detected in fish sauce. Due to the types of fish and uncontrolled fermentation process used to produce fish sauce, often fish sauces are known to contain high levels of histamine (Dissaraphong *et al*., 2006). The overall histamine formation in fish and subsequent fishery products is related to fish species, free histidine content of fish muscle, the presence of bacterial histidine decarboxylase and environmental conditions to promote growth of histamine-forming bacteria (Lehane and Olley, 2000). Endogenous production of decarboxylase in fish muscle is insignificant when compared with that of exogenous bacterial enzymes. Generally, biogenic amine formation by bacteria is enhanced at elevated storage temperatures (Kim *et al*., 2004) 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. Histamine is a chemical that causes severe illness and is regulated in fish sauce at a maximum of 200 ppm by Canada and 500 ppm by the USA (Brillantes *et al*., 2002; FDA, 1998). Variable levels of histamine, ranging from 100 to 1,000 ppm, were found in fish sauce samples (Brillantes and Samosorn, 2001; Kirschbaum *et al*., 2000; Sanceda *et al*., 1999). Brillantes and Samosorn (2001) noted that products from small-scale factories had relatively lower histamine contents than those from large-scale operations.

The freshness of raw material (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*., 2004).

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.

Tetragenococcus muriaticus is a halophilic lactic acid bacterium that is phylogenically closely related to *Tetragenococcus halophilus*, a typical lactic acid fermenter in soy sauce (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). Korean salted and fermented seafoods include the bacteria *Achromobacter*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Halobacterium*, *Leuconostoc*, *Micrococcus*, *Pediococcus*, *Pseudomonas*, *Staphylococcus*, *Sarcina*, and the yeasts Saccharomyces and Torulopsis (Mah *et al*., 2003; Um and Lee, 1996; Mheen, 1993).

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. 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 40 mg/100 g (400 ppm), the guideline established by Codex Alimentarius (Codex, 2012). Therefore, fish sauce produced from some countries may run into the risk of being rejected for export, thereby dangering the exporting status in the future.

1.2.5 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 $\geq$ 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 produce 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 bacteria growing at 5°C (300-400 mg histamine/100 ml of mackerel homogenate).

1.2.6 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). 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*., 1979a, b; Voigt and Eitenmiller, 1977; Ienistea, 1971).

Two types of histamine degrading enzymes, HADH and histamine oxidase (HO) are normally implicated in this form of oxidation. HADH catalyzes the oxidative deamination of histamine to imidazole acytaldehyde and ammonia, but histamine oxidase generates hydrogen peroxide (H_2O_2) in addition to imidazole acytaldehyde and ammonia (Figure 2). The presence of histamine-degrading enzymes either HOs or HADHs has been reported in some microorganisms such as *Rhizobium* sp., *Nocardioides simplex* IFO 12069 and *Arthrobacter crystallopoietes* KAIT-B-007 (Bakke *et al*., 2005; Sekiguchi *et al*., 2004; Siddiqui *et al*., 2000). However, high amount of histamine can also be formed in salted foods, such as salted and fermented fish sauce (Stute *et al*., 2002; Sato *et al*., 1995), salted anchovies (Hernández-Herrero *et al*., 1999), soy sauces (Chin *et al*., 1989) and other salted and fermented fish products (Mah *et al*., 2002; Okuzumi *et al*., 1981). The applications of these microorganisms and/or enzymes to degrade histamine in salt-fermented food might be restricted by unfavorable physiological conditions for enzyme activity such as low stabilities in the presence of high salt concentration. Therefore, halophilic archaea should be considered as a source of enzyme production, which can degrade histamine in high salt content fermented foods.

1.2.7 Halophilic archaea

Halophilic archaea of the order *Halobacteriales* are found in hypersaline environments. 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. These prokaryotes require salt for growth and will not grow at low salt concentrations. They habitat salt lakes such as the Great Salt Lake, Utah, the Dead Sea, and other hypersaline water bodies such as the crystallizer ponds of solar salterns in which sea water is evaporated for the production of salt. Additional habitats in which these halophilic archaea often develop include salted fish and hides

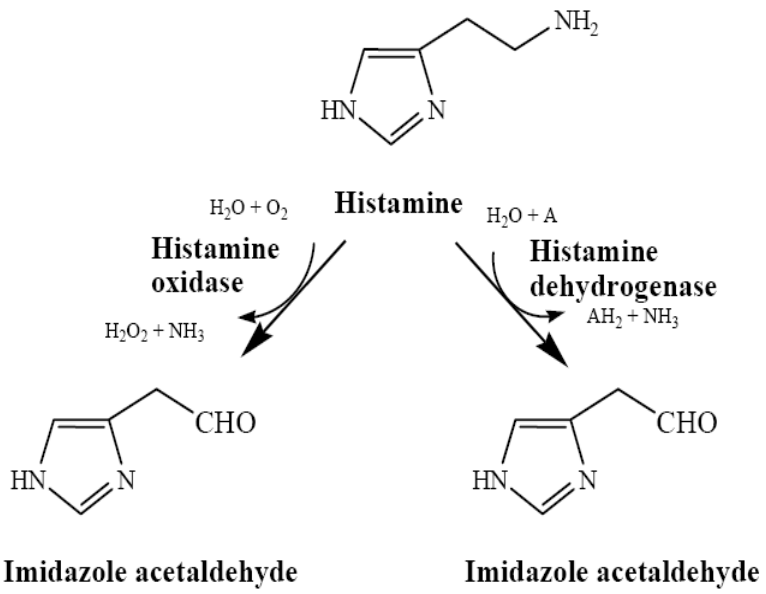

Figure 2. Degradation pathways of histamine. A and AH_2 are a two-electron acceptor (or two moles of one-electron acceptor) and its reduced form, respectively. For example; nicotinamide adenine dinucleotide phosphate NAD (P), ferricyanide, flavin adenine dinucleotide (FAD), flavin mono-nucleotide (FMN),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

Source: Zhou *et al*. (2014); Fujieda *et al*. (2004); Sekiguchi *et al*. (2004).

preserved by treatment with salt. They also occur in certain fermented food products in which molar concentrations of NaCl are added as part of the manufacturing process, such as Thai fish sauce (Oren, 2006).

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 3).

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 membranes of extremely halophilic archaea are characterized by the abundance of a diacidic phospholipid, archaetidylglycerol methylphosphate (PGP-Me), which accounts for 50-80 mol% of the polar lipids, and by the absence of phospholipids with choline, ethanolamine, inositol, and serine head groups. Because of its abundance in extreme archaeal halophiles, the diacidic phospholipid PGP-Me can be expected to play a major role in determining the properties of their membranes. It is believed that it contributes to membrane stability in hypersaline environments (Tenchov *et al*., 2006). The extreme halophiles also contain archaetidylglycerol (PG), and some strains have minor amounts of sulfated PG (Figure 4).

Halophilic archaea maintain an osmotic balance of their cytoplasm with the hypersaline environment by accumulating high concentrations of salt. Their cell walls, ribosomes and enzymes are stabilized by Na^+. 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, halophilic or halotolerant eubacteria are characterized by a much greater metabolic diversity. Their intracellular salt concentration is low, and they maintain an osmotic balance of their cytoplasm with the external medium by accumulating high concentrations of various organic osmotic solutes (e.g. glycine,

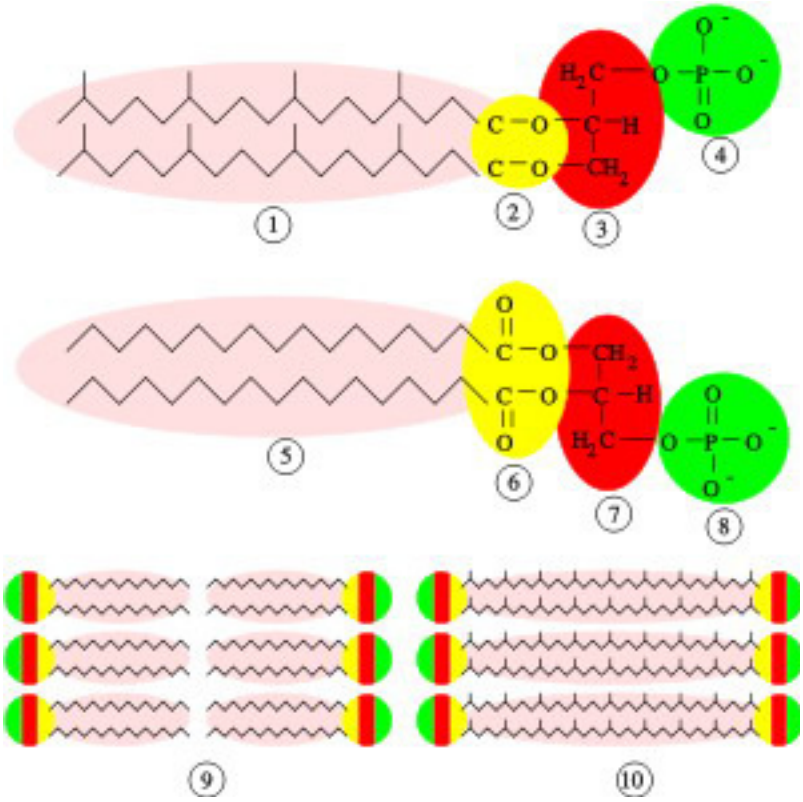

Figure 3. Membrane structures. Top: an archaeal phospholipid, 1 isoprene side chain, 2 ether linkage, 3 L-glycerol, 4 phosphate moieties. Middle: bacterial and eukaryotic phospholipid: 5 fatty acid, 6 ester linkage, 7 D-glycerol, 8 phosphate moieties. Bottom: 9 lipid bilayer of bacteria and eukaryotes,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

Source: Koga and Morii (2007).

betaine, sugars, polyols, amino acids, and ectoines), thus their intracellular enzymes have no special salt tolerance (Margesin and Schinner, 2001). 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).

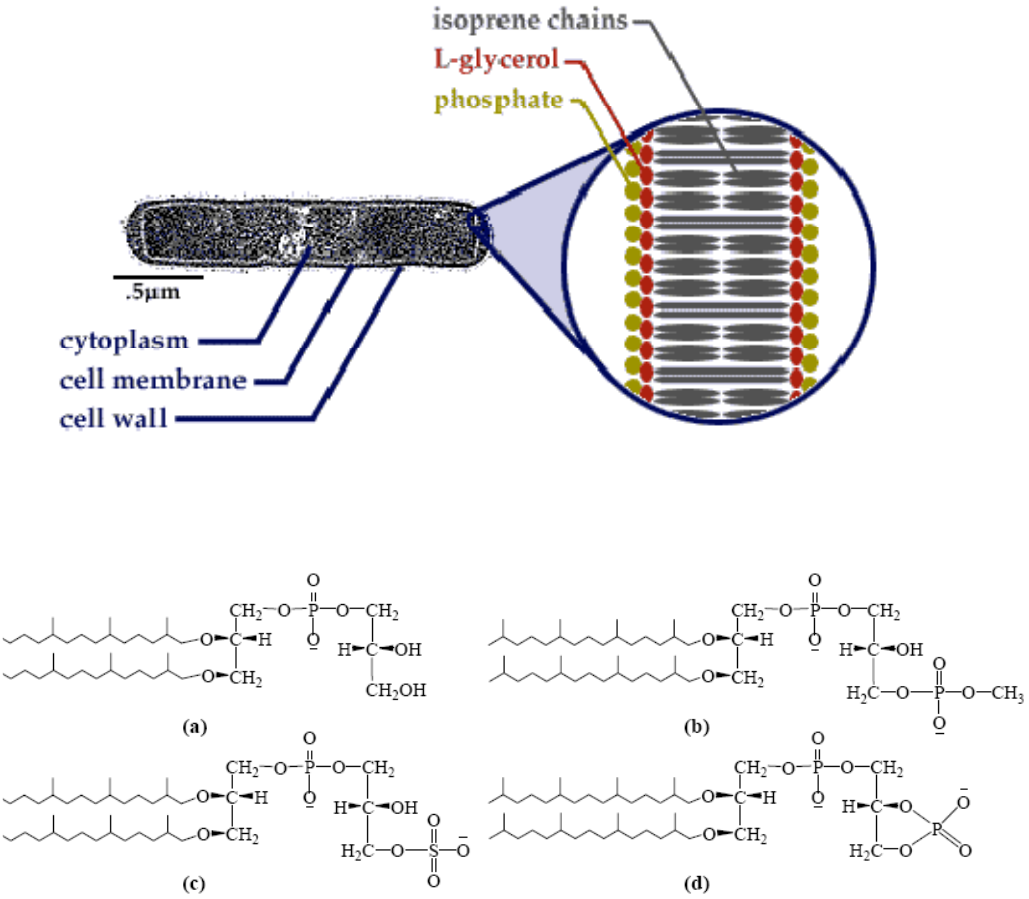

Figure 4. Basic archaeal structure: The three primary regions of an archaeal cell are the cytoplasm, cell membrane, and cell wall. Above, these three regions are labeled, with an enlargement at right of the cell membrane structure. Archaeal cell membranes are chemically different from all other living 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).

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.

1.2.8 Halophilic enzymes

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 and histamine-degrading enzymes such as HADH) 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. The extremely halophilic archaea, the *Halobacteriaceae*, 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 adapatation mechanisms. Proteins from halophilic organisms have a distinct amino acid composition in order to remain stable and active at high salt ionic strength. They typically have an excess of acidic amino acids such as 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 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, lipases and HADH has been reported for some halophiles belonging to the genera *Acinetobacter*, *Haloferax*, *Halobacterium*, *Halorhabdus*, *Marinococcus*, *Micrococcus*, *Natronococcus*, *Bacillus*, *Halobacillus, Halothermothrix* and *Natrinema* (Tapingkae *et al*., 2010a; 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). 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 (1-5 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). Halophilic nzymes with optimal activity at high-salt concentrations are useful for many harsh industrial processes, where concentrated salt solutions otherwise inhibits many enzymatic conversions (Amoozegar *et al*. 2003; Hutcheon *et al*., 2005; Oren *et al*., 2005).

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). With these advantages, the extremely halophilic archaea isolated from several salt-fermented products were screened for the ability to degrade histamine in high salt condition (Tapingkae *et al*., 2010a).

1.2.9 *Natrinema gari* BCC 24369, a halophilic archaeon

Nnm. gari BCC 24369, a halophilic archaeon, a novel species of the genus *Natrinema*, exhibited the ability to degrade histamine in hypersaline condition. Histamine degrading activity of *Nnm. gari* BCC 24369 was located in the intracellular fraction and required the presence of an electron carrier suggesting a link to the activity of dehydrogenase. The optimum NaCl concentrations of histamine-degrading activities of the cells were found at 3.5-5 M. The decrease in NaCl concentration resulted in lowered enzyme activity which indicated that the histamine-degrading activity of the whole cells was salt dependent. Thus, the applications of *Nnm. gari* BCC 24369 provides the potential advantages over applications of non-halophilic microganisms for the degradation of histamine under the high salt conditions (Tapingkae *et al*. 2010a, b).

Natrinema gari BCC 24369 was isolated from an anchovy fish sauce sample fermented for 3 months. Cells are motile, Gram-negative rods, 0.5-0.8 x 2.0-3.0 μm in size. Colonies are pale orange, smooth, circular and elevated. Growth is chemo-organotrophic. *Nnm. gari* BCC 24369 showed characteristics of extremely halophilic archaea that required salt at concentrations higher than 1.7 M NaCl to prevent cell from lysis. This strain was able to grow at 1.7-5.1 M NaCl (optimum 2.6-3.4 M NaCl). 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). Growth occurs at 0-1.0 M $MgCl_2$ (optimum 0.1-0.2 M $MgCl_2$). 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. *Nnm. gari* BCC 24369 did not produce biogenic amines including histamine, tryptamine, -β-phenylethylamine, putrescine, cadaverine, tyramine, spermidine or spermine which

showed the toxic effects. Based on in vitro cytotoxicity assay, the treatment with whole cell extract of *Nnm. gari* BCC 24369 to all target cell lines tested including human colon adenocarcinoma (Caco2), human liver hepatocarcinoma (HepG2), and human larynx epithelial (HEp-2) cells resulted in dose-dependent inhibitions of the cell growth with the IC_{50} values higher than 250 µg/ml. The neighbour-joining phylogenetic tree constructed on the basis of 16S rRNA gene sequence data of the *Nnm. gari* BCC 24369 and other representative *Natrinema* species is shown in Figure 5 (Tapingkae *et al*., 2008, 2010a).

1.2.10 Histamine dehydrogenase (HADH)

HADH in microorganisms such as prokaryotes has been rarely reported. Siddiqui *et al*. (2000) found a HADH in *N. simplex* IFO 12069. The enzyme had a molecular mass of 170 kDa and was suggested to be a dimer of subunits that had a molecular mass of 84 kDa. The K_m and V_{max} values for histamine were 0.075 mM and 4.76 μmol/min/mg, respectively. HADH from *N. simplex* belongs to the family of soluble iron-sulfur flavoproteins having one [4Fe-4S] cluster and one 6-S-cysteinyl flavin mononucleotide and and one adenine diphosphate (ADP), while the function of ADP remains unclear per monomer (Tsutsumi *et al*., 2009; Limburg *et al*., 2005; Fujieda *et al*., 2004). The application of HADH from these microorganisms and the cloning of this enzyme to detect histamine in food have been reported (Bakke *et al*., 2005; Sato *et al*., 2005; Fujieda *et al*., 2004; Takagi and Shikata, 2004). Nevertheless, the applications of these microorganisms and the enzyme 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.

Nnm. gari BCC 24369 can produce HADH which catalyzes the oxidative deamination of histamine to imidazole acetaldehyde and ammonia (Zhou *et al*., 2014), has a potential to degrade histamine under high salt condition with high catalytic activity and narrow substrate specificity. Based on histamine assay, histamine-degrading activity of *Nnm. gari* BCC 24369 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

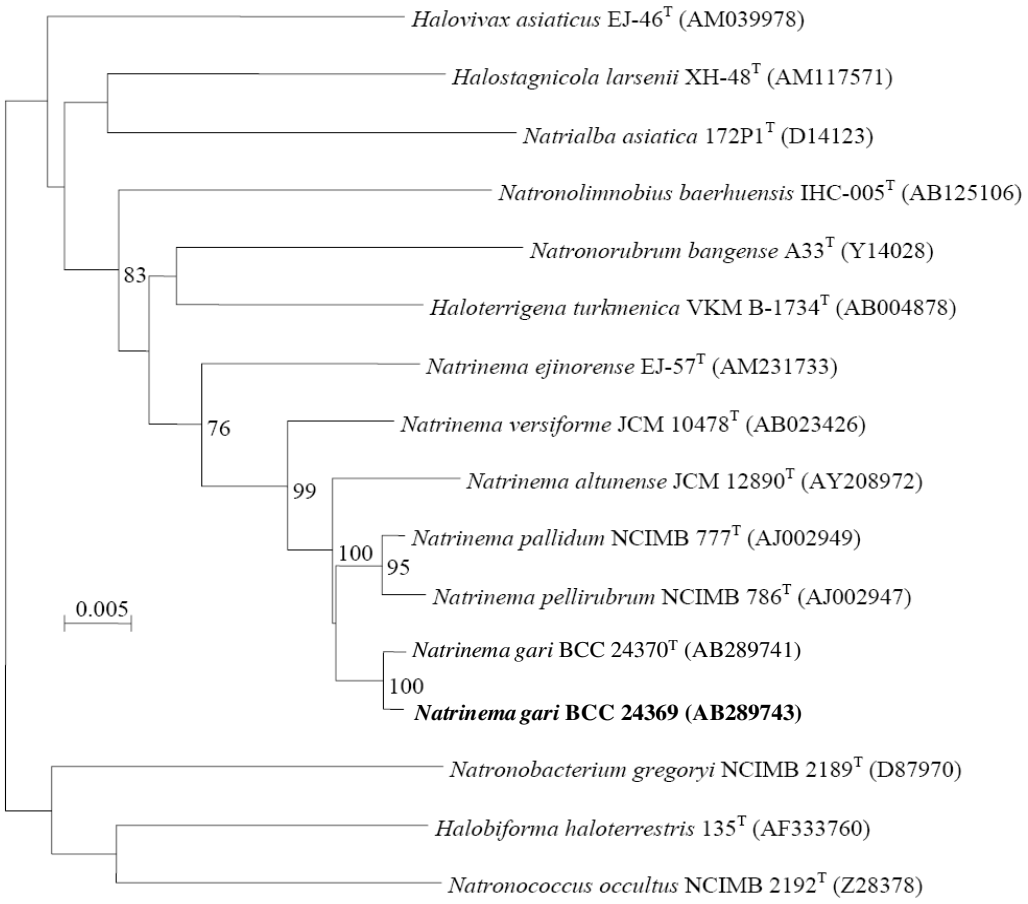

Figure 5. Phylogenetic tree strains *Nnm. gari* BCC 24369 and related archaeal species based on 16S rRNA gene sequences. The branching pattern was generated according to the neighbour-joining method. Bootstrap values above 70%, based on 1000 replications, are shown at nodes. Bar, 0.5 substitutions per 100 nucleotide positions.

Source: Tapingkae *et al*. (2008).

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 at pH 6.5-9, in the presence of NaCl above 2.5 M, and at temperature lower than 50°C. These results suggested a link of histamine-degrading activity of *Nnm. gari* BCC 24369 to the presence of salt tolerant and thermo-neutrophilic HADH. The enzyme consisted of heterotrimeric subunits with three different molecular masses of 49.0, 24.7 and 23.9 kDa (Zhou *et al*., 2014). The enzyme had high affinity and activity toward histamine with V_{max}, K_m

and k_{cat} values of 2.5 μmol/min, 57.1 μM and 5.3 1/s, respectively (Tapingkae, 2009; Tapingkae *et al*., 2010a).

However, the applications of HADH by *Nnm. gari* BCC 24369 to remove histamine on the industrial scale have been limited by low yield of enzyme, the slow growth rate and the cost of cell or enzyme preparation (Tapingkae *et al*., 2010b). The culture conditions had a remarkable impact on cell growth and enzyme production. Halophilic archaea are grown generally in complex media containing high concentration of nitrogen source such as yeast extract and casamino acids (Oren, 2006). The different members of the *Halobacteriales* grow in a variety of media (Table 2). Thus, optimization of medium composition might enhance the production of HADH by *Nnm. gari* BCC 24369.

1.2.11 Statistical optimization

The optimal design of the culture media is a very important aspect in the field of biotechnology, especially for maximizing the microbial metabolites production and minimizing the production cost (Bezbaruah *et al*., 1994). Statistical experimental design techniques are useful tools for screening for nutrients with significant impact on growth rate and/or enzyme production as they can provide statistical models, which support in understanding the interactions among the process parameters at varying levels. Furthermore, calculations of the optimal level of each parameter for a given target can be performed (Manikandan *et al*., 2009).

It is known that designing an appropriate fermentation conditions, besides of a productive strain construction, is crucial for optimization of the microbial fermentation processes (Kennedy and Krouse, 1999). Process optimization may involve the study of many physiological and chemical parameters, including media composition. Statistical optimization methodologies have various advantages of being rapid and reliable in short listing of nutrients at varying concentrations leading to significant reduction in the total number of experiments (Akolkar *et al*., 2009). Several statistical and non-statistical methods are available for optimization experiments (Montgomery, 2002; Felse and Panda, 1999).

Table 2. A selection of media used for the cultivation of neutrophilic and alkaliphilic members of the order *Halobacteriales*

	Medium no.				
Ingredient (g/l)*	1	2	3	4	5
NaCl	250	200	175	125	125
$MgSO_4 \cdot 7H_2O$	20	20	-	-	-
$MgCl_2 \cdot 6H_2O$	-	-	20	50	160
KCl	2	2	-	-	-
K_2SO_4	-	5	5	5	-
$CaCl_2 \cdot 2H_2O$	0.1	0.1	0.1	0.13	0.1
$Na_3Citrate \cdot 3H_2O$	3	3	-	-	-
Na glutamate	-	1	-	-	-
Na_2CO_3[a]	-	-	-	-	-
Yeast extract	10	5	5	5	1
Casein hydrolysate	7.5	5	-	-	1
Tryptone	-	-	-	5	-
Starch	-	-	-	-	2
$FeCl_3 \cdot 4H_2O$ (mg/l)	36	36	-	-	-
$MnCl_2 \cdot 4H_2O$ (mg/l)	0.36	0.36	-	-	-
Final pH (to be adjusted with NaOH or HCl)	7.2-7.4	7.0-7.4	6.8	6.8	6.8

*For solid media, 20g agar/liter should be added. [a]Sterilized separately.

Media no. 1 and 2 are suitable for the growth of *Halobacterium* and other neutrophilic extremely halophilic representatives of the group. Medium no. 3 may be used for species that require less salt, such as many *Halorubrum* species. Types that require high concentrations of magnesium can be grown in medium no. 4 (*Haloferax volcanii*) or 5 (*Halorubrum sodomense*, *Halobaculum gomorrense*).

Source: Oren *et al*. (1995); Tindall and Collins (1986); Rodriguez-Valera *et al*. (1983); Mullakhanbhai and Larsen (1975); Payne *et al*. (1960).

One-factor-at-a-time design was a traditional method for optimization. The disadvantages of this method are that it is time consuming because they require a large number of experiments to determine the optimal content of each factor one at a time (He *et al*., 2009). Moreover, the potential interaction effects among factors are ignored (Fu *et al*., 2009). However, it is useful to select the best experimental treatment with a certain factor in the preliminary stage of optimization. Statistical designs, such as Plackett-Burman design and central composite design, overcome the disadvantages of one factor-at-a-time design by reducing the number of the tests while giving meaningful results (Fu *et al*., 2009). Thus, the statistical designs have been efficiently used for enhancing the yield of various bioprocesses (Bas and Boyaci, 2007). Some researchers combined advantages of both methods to optimize bioprocess by applying one-factor-at-a-time design followed by statistical designs of the Plackett-Burman design and central composite design (Badhan *et al*., 2007; Mao *et al*., 2005)

Plackett and Burman's statistical method (Plackett and Burman, 1946) is one of such approaches involving a two level fractional factorial saturated design that used only k+1 treatment combination to estimate to main effect of k factors independently (assuming that all interactions are negligible). Saturated designs are used in early stages of experimentation to screen out unimportant factors from among a large number of possible factors. In full factorial designs, the number of factors increases exponentially leading to an unmanageable number of experiments (Hunter, 1985). Hence, fractional factorial design like Plackett and Burman is an alternative and more efficient approach applied for initial screening and selection of most significant culture variables. Central composite design (CCD), a well-established, widely used statistical technique for determining the influence of key factors by a small number of experiments can be used for further optimization regarding the optimum level of each variable along with its interactions with other variables and their effects on product yield. (Dean and Voss, 2006; Pardeep and Satyanarayana, 2006).

In case of halophilic archaea, further biotechnological advances are often hampered by low growth rates in pure culture with generation times ranging from 1.5 h for *Haloterrigena turkmenica* (Robinson *et al*., 2005) to up to 1-2 days

reported for *Haloquadratum walsbyi* (Burns *et al*., 2004). Finally, improvements of haloarchaeal growth media were noted in early days of haloarchaeal research (Rodriguez-Valera, 1995; Kauri et al., 1990; Gouchnour and Kushner, 1969). However, to our knowledge, there were a few reports on statistical optimization of growth media and culture conditions for the microbial metabolites and biomass production by halophilic archaea. Statistical optimization has successfully been applied to increase yields of some haloarchaeal products such as xylanase production by halophilic eubacterium, strain SX15, extracellular protease production by *Halobacterium* sp. SP1(1), exopolymer by *Halobacterium* sp. SM5 and biomass production by *Halobacterium salinarum* VKMM 013 (Akolkar *et al*., 2009; Manikandan *et al*., 2009; Lungmann *et al*., 2007; Wejse, *et al*., 2003). Due to the improvement of product yield, reduction of development time and overall process costs (Kammoun *et al*., 2008; Pan *et al*., 2008; Ren *et al*., 2008), statistical experimental designs might provide the potential advantages over the application of traditional method for optimization.

1.2.12 Whole cells immobilization

The technology of immobilized cells can be applied in biological treatment to enhance the efficiency and effectiveness of biodegradation (Dursun and Tepe, 2005). Immobilized cells systems have found significant applications in pharmaceutical, food biotransformations, and processes for commodity and specialty chemical products (Tope *et al*., 2001). Cell immobilization has been defined as physical confinement or localization of viable microbial cells to a certain defined region of space in such a way as to limit their free migration and hydrodynamic characteristic which differ from those of the surrounding environment while retaining their catalytic activities for repeated and continuous use (Amim *et al*., 2010; Covizzi *et al*., 2007; Freeman and Lilly, 1998; Dervakos and Webb, 1991). The main advantages in the use of immobilized cells in comparison with free cells include their prolonged and repeated use, protection of cells against toxic substances, and changes in environment factors (e.g., temperature, pH), reduction the risk of contamination and eliminate the costly processes of cell recovery (Moriwaki *et al*., 2014; Dursun and Tepe, 2005). The cell immobilization emerged as an alternative for enzyme

immobilization (Woodward, 1988; Parascandola and Scardi, 1980; Cheetham *et al*., 1979). Immobilization of cells containing specific enzymes has further advantages such as time consuming, elimination of long and expensive procedures for enzymes separation, isolation and purification and it is vital to expand their application by enabling easy separation and purification of products from reaction mixtures and efficient recovery of catalyst (Stolarzewicz *et al*., 2011). It also tends to enhance the stability of the enzyme by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation. 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 (i.e., phenol, pyridine, dibenxothiophene, quinoline and carbazole), production of organic compounds and development of biosensors (Wang *et al*., 2007; Li *et al*., 2006; Tziotzios *et al*., 2005; Wang *et al*., 2001, 2002; Lee *et al*., 1994).

Immobilization of whole cells has been shown to be a better alternative to immobilization of isolated enzymes (D'Souza, 1989; Mattiasson, 1983; Tampion and Tampion, 1987). The microbial enzymes are generally unstable when isolated from their natural environment and also are easily denatured under working conditions. The direct immobilization of microbial cells that has a particular activity can minimize or even eliminate these problems (Moriwaki *et al*., 2014). 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; Tampion and Tampion, 1987; Mattiasson, 1983). On the other hand, the 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 non-growing 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.

1.2.13 Supports and matrices for cell immobilization

For food application, supports and matrices for cell immobilization ideally should meet the main following criteria (Mitropoulou *et al*., 2013; Klein and Wagner, 1983).

1. Be generally recognized as safe for food and pharmaceutical bioprocess applications
2. Be generally available in adequate quantities with consistent quality and acceptable price
3. Be easy and simple to handle and regenerate in the immobilization procedure

4. Have large specific surface
5. Not change the final food product quality (e.g., off-flavor or off-odor formations)
6. Be environmentally safe to dispose of, and/or be capable of recycling
7. Be resistant to microbial degradation
8. Retain chemical and thermal stability under bioprocess and storage conditions
9. Not reduce the desired biocatalyst activity of the cells
10. Not react with the substrates, nutrients or products
11. Have functional groups for cross-linking
12. Retain their physical integrity and be insoluble under the bioprocess reaction conditions

1.2.14 Techniques for cell immobilization

The immobilization techniques can be divided into following four major categories based on the physical mechanism employed, namely entrapment, adsorption or attachment, self-aggregation by flocculation (natural) or with artificially induces cross-linking agents, mechanical containment behind a barrier or encapsulation (Figure 6). These categories are commonly used in immobilized enzyme technology. 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.

1.2.14.1 Entrapment

Entrapment is an irreversible method, where immobilized cells are entrapped in a support matrix or inside fibers. This technique creates a protective barrier around the immobilized microbes, ensuring their prolonged viability during not only processing but also storage in polymers (Górecka and Jastrzębska, 2011). The matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, polyacrylamide, polyester, polystyrene and polyurethane (Ramakrishna and Prakasham, 1999; Lopez *et al*., 1997). Entrapment of

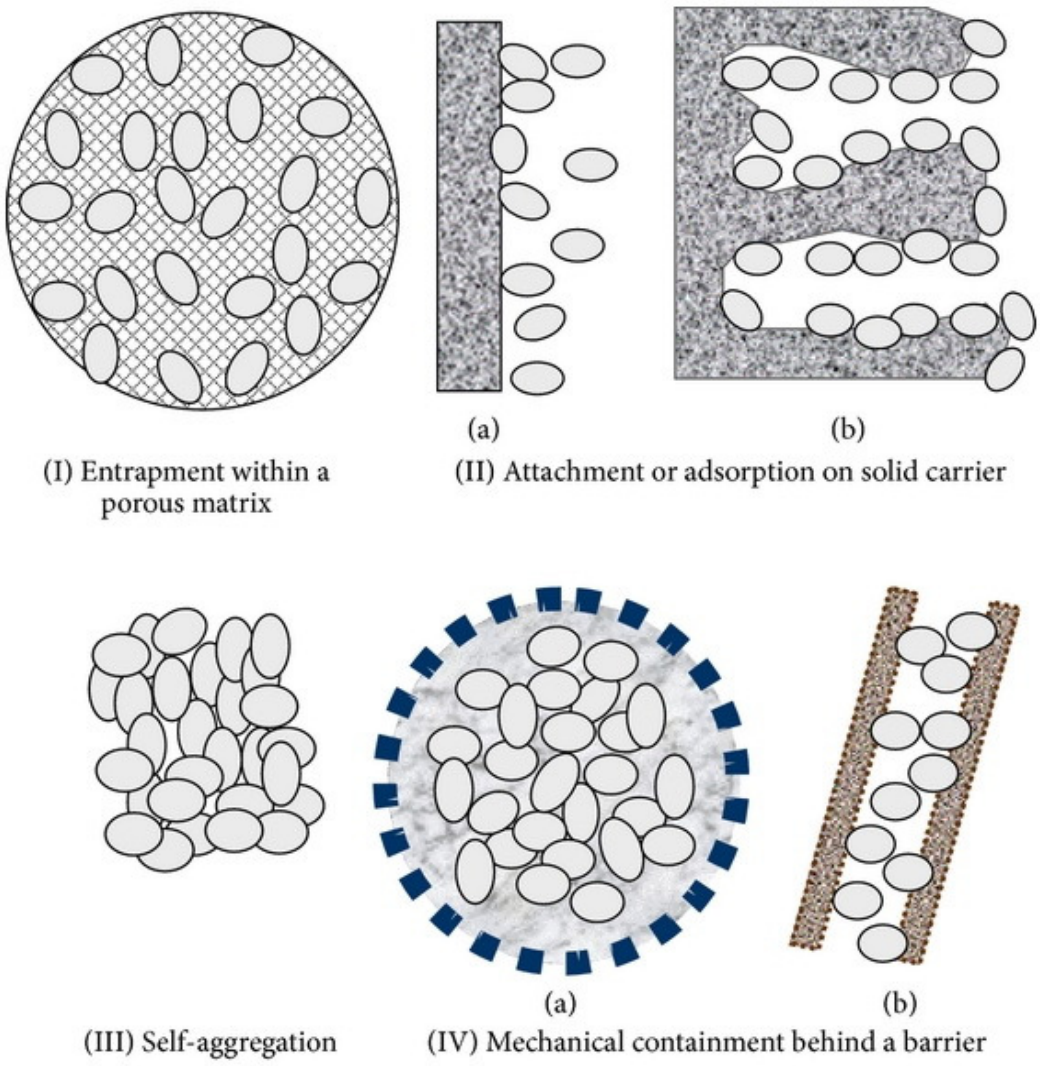

Figure 6. Basic methods for whole cell immobilization.
Source: Nedović *et al*. (2001).

the microorganisms in porous polymer carrier was often used to capture the microorganisms from the suspended solution and then obtain the immobilized cells. 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 (Akin, 1987). Entrapment allows high mechanical strength, but contains some disadvantages, such as cell leakage, costs of immobilization, diffusion limitations, and deactivation during immobilization and

abrasion of support material during usage (Akin, 1987). Another disadvantage is low loading capacity as microorganisms have to be incorporated into the support matrix (Stolarzewicz *et al*., 2011; Gao *et al*., 2010; Song *et al*., 2005; Krekeler *et al*., 1991).

1.2.14.2 Adsorption

Immobilization of cells through Immobilization by adsorption relies on the inherent tendencies of the cells to adhere to particular surfaces, or to flocculate, or to settle in the pores of the framework. This technique is based on the physical interaction between the microorganism and the carrier surfaces, while frequently reversible is simple, cheap and effective (Akin, 1987). The immobilization of microorganisms on properly chosen carriers stimulates microbial metabolism, protects cells from unfavorable agents, and preserves their physiological activity (Kozlyak *et al*., 1993; Nikovskaya, 1989). Immobilization of cells through adsorption perhaps is the simplest of all the techniques (D'Souza, 2002). Adsorption is based on weak forces, however, still enabling an efficient binding process. Usually in bonds formation, several forces are involved: van der Waals forces, ionic and hydrophobic interactions and hydrogen bonds. Both electrostatic and hydrophobic interactions govern the cell-support adhesion, which is the key step in controlling the cell immobilization on the support (Górecka and Jastrzębska, 2011; Hsu *et al*., 2004)

The basic advantage is the reversibility of binding which also helps in economic recovery of the support (D'Souza, 2002). There are many factors (such as the age and the physiological state of cells) that influence of the sorption of microbial cells. The surface structures of bacterial cells, superficial charges and hydrophobicity also play an important role in the cell adherent to solid surfaces (Oulahal *et al*., 2008; Chae *et al*., 2006). The composition of the reaction, its pH, and environment conditions considerably influence the adsorption of cells by changing their electrokinetic potential (Kilonzo and Bergougnou, 2012; Stanley, 1983). The nature of adsorbents is also important. Organic polymeric adsorbents (chitosan, polyurethane and polyvinyl resins) are chemically stable and show a great variety of surface properties and pore structures, whereas inorganic adsorbents (diatomite, clay, anthracite, porous glass, activated charcoal and ceramics) are resistant to biological degradation are affordable, and can be easily regenerated (Akin, 1987). Generally,

adsorption is a simple physical process in which the forces involved in cell attachment are so weak. Cells that are not strongly adsorbed are readily desorbed from the support during the repeated use (Tapingkae *et al*., 2010b).

1.2.14.3 Covalent bonding or cross linking

The mechanism involved in this method is based on covalent bond formation between activated inorganic support and cell in the presence of a binding (cross-linking) agent (Akin, 1987). For covalent linking, chemical modification of the surface is necessary. Cell walls contain functional groups such as -NH_2, -OH and -COOH that lend themselves to covalent coupling. These groups can be used advantageously to immobilize cells by cross-linking them to solid support. Cross-linking reduces cell washout and improves the mechanical strength of biocatalyst, yet usually does not create a significant diffusion barrier (Akin, 1987). Covalent attachment and cross-linking are effective and durable to enzymes, but it is rarely applied for immobilization of cells. It is caused mainly by the fact that agents used for covalent bonds formation are usually cytotoxic and it is difficult to find conditions when cells can be immobilized without any damage (Ramakrishna and Prakasham, 1999).

1.2.14.4 Encapsulation

Encapsulation is another irreversible immobilization method, similar to entrapment. In this process, cells are restricted by the membrane walls (usually in a form of a capsule), but free-floating within the core space (Górecka and Jastrzębska, 2011). The membrane itself is semi-permeable, allowing for free flow of substrates and nutrients and keeping the biocatalyst inside. The factor determining this phenomenon is the proper size of membrane, attuned to the size of core material. This limited access to the microcapsule interior is one of the main advantages of microencapsulation, for it protects the cells from the harsh environmental conditions. As most immobilization method, it prevents biocatalyst leakage, increasing the process efficiency as a result (Park and Chang, 2000). However, even though in encapsulation, high cell loading can be achieved, but the capsules are still very weak

(Song *et al*., 2005). The diffusion limitation is one of the inevitable drawbacks associated with encapsulation method (Lozinsky and Plieva, 1998).

1.2.15 Whole cells immobilization of *Natrinema gari* BCC 24369

Tapingkae *et al*. (2010b) investigated that the whole cells of *Nnm. gari* BCC 24369 was immobilized on various matrices by different techniques including adsorption, entrapment and cross-linking). They found that adsorption was the most effective technique for immobilization of *Nnm. gari* BCC 24369. Among all carriers tested (Celite® 545, pig bone and chitosan flakes), Celite® 545 showed the highest immobilization yield. The immobilized whole cells retained the histamine-degrading activity as high as 94% of the original activity of the free whole cells. 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 immobilization. The immobilized whole cells of *Nnm. gari* BCC 24369 were highly specific toward histamine among other amines. 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 cells of *Nnm. gari* BCC 24369 has the potential to be applied for the degradation of histamine in the products containing high salinity, like fish sauce. Celite® 545 presents a porous structure, which allows bacterial cells to be adhered to the surface. As an inert material, celite consists of highly porous diatomaceous beads composed of silica (SiO_2) and some other inorganic oxides. Gekas and Lopeiz-Leiva (1985) reported that commercial success has been achieved when carrier materials have been chosen for their flow properties, low cost, non-toxicity, maximum biocatalysts loading while retaining desirable flow characteristics, operational durability, ease of availability and ease of immobilization. However, it has no report to prove that Celite® 545 can be used to contact with food or safe for human health. Thus, care has to be taken to select the carrier 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.

1.2.16 Diatomite

Diatomite, or diatomaceous earth, is natural soft, very fine-grained and siliceous sedimentation. It consists of fossilized remains of diatoms including 87-91% silicon dioxide (SiO_2), with significant quantities of alumina (Al_2O_3) and ferric oxide (Fe_2O_3) (Wang *et al*., 2012; Ibrahim and Selim, 2011). It has a particle size ranging from less than 1 μm to more than 1 mm, but is typically from 10 to 200 μm. Scanning electron micrographs of diatomite are shown in Figure 7. The diatoms skeletons are highly porous, light in weight, and chemically stable and inert (Wang *et al*., 2012). The physical properties of natural and processed diatomite that provide unique commercial value in a broad spectrum of market end-uses include ornate fine structure, low bulk density, and high porosity and surface area.

Commercial diatomite product grades are affected by the size, shape, overall arrangement and proportions of the various types of frustules (particularly the effect on filtration rate and clarity, and absorptive capacity), and content of silica and various impurities, such as, certain minerals and chemicals (especially the form of iron), clay, sand, and organics (Inglethorpe, 1992). There are additional specialized application specifications; brightness/whiteness, absorptive capacity, and abrasiveness. A major influence on the grade is the extent of processing including naturally milled and dried, simple calcined (to 1,000°C) and flux-calcined (to 1,200°C with the addition of a flux, usually up to 10% of sodium compounds, such as, soda ash, salt, or sodium hydroxide) before the calcining step. Added fluxing agent sinters the diatomite particles and increases the particle size, thereby allowing increased flow rate during liquid filtration. When advantageous, calcining removes organics, reduces surface area (fuses fine structure) of particles and sinters them into small clusters, increases particle hardness, and oxidizes iron (changing the color of crude feed to pink); but also disadvantageously produces free crystalline silica known as free silica. Flux-calcining further affects the physical and chemical properties and makes a white product, believed to be colored by the conversion of iron to complex sodium-aluminum-iron silicates rather than to the oxide. Calcinated and flux-calcinated diatomite could be used successfully for filtration purposes (Founie, 2007).

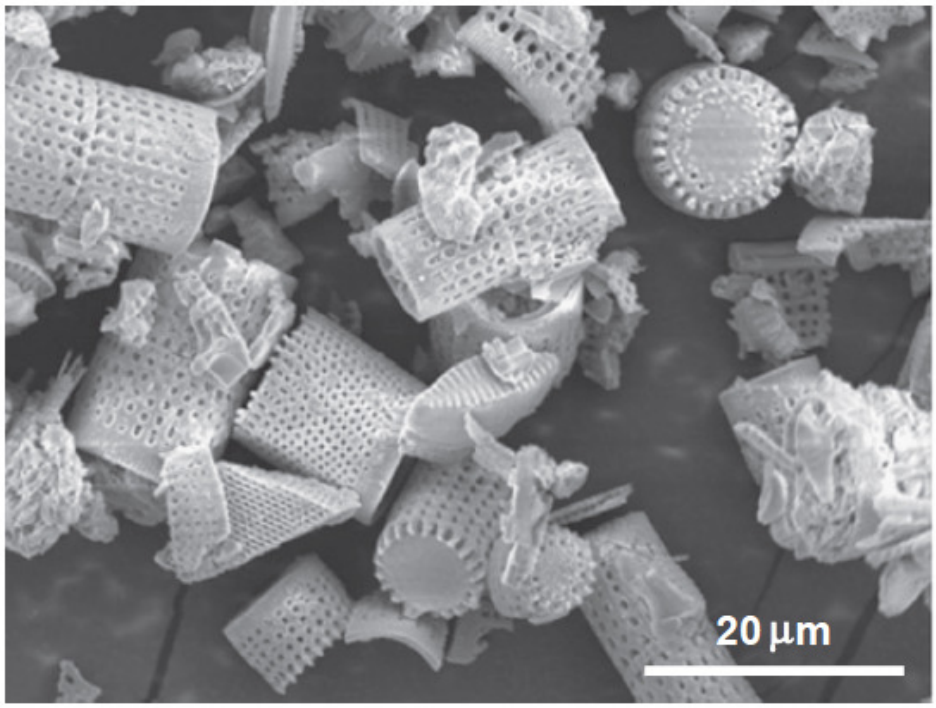

Figure 7. Scanning electron micrographs of diatomite.
Source: Palomino *et al*. (2011).

The purpose of heat treatment for production of calcined diatomite is to remove organics and further harden the exoskeletons of the diatoms in order to create a better filtering agent (Founie, 2007). This process causes the amorphous silica that makes up the exoskeleton of the diatom to turn in to crystalline silica (Flynn *et al*., 1991; Jahr, 1981). Quartz, cristobalite, and tridymite are the three most common crystalline forms of free silica. Calcining is a benefit if the diatomite is to be used as a filtering aid, however crystalline silica can be toxic to humans and animals when inhaled. Calcined is not used for animal feed and is not food grade. Natural diatomaceous earth known as biogenic silica is non-calcined meaning that it has not been treated at a high temperature (IARC, WHO, 1997). The amorphous silica remaining in its natural state is not considered harmful to human health and has been registered in many countries as a food additive (IARC, WHO, 1997; Anon, 1991). Un-calcined diatomite products containing less than 1% crystalline Silica (6 mg/m^3) is not classifiable as to carcinogenicity to humans (OSHA, 1999). Thus, food-

grade diatomite products are required to contain less than 1% crystalline silica. In contrast, calcined diatomite products generally contain over 70% crystalline silica (predominantly cristobalite and quartz), and flux calcined diatomite products may contain up to 75% crystalline silica which persist in the lungs, culminating in the development of chronic silicosis, emphysema, obstructive airways disease and lymph node fibrosis (de la Hoz *et al*., 2004; IARC, WHO, 1997; Rafnsson and Gunnarsdóttir, 1997; Absher, 1993).

Major producer of diatomite is Celite Corporation (Lompoc, CA and Quincy, WA) (Lemons, 1997). With respect to Celite, the finest grade is Filter-Cel®, a natural diatomite, which has been selectively quarried, dried, milled and air classified. Three calcined grades of diatomite include Celite® 505, Standard Super-Cel® and Celite® 512. The flux-calcined grades include Hyflo-Super-Cel®, Celite® 501, Celite® 503, Celite® 545, Celite® 540 and Celite® 535 (Hendricks, 2006).

Due to specific properties of natural diatomite (mild abrasiveness, high absorptive capacity, insulating ability, relative inertness and high brightness), diatomite has extensively been applied in filtration, insulation, absorption, mineral fillers, drug delivery and a fine abrasive in diverse industries. Thus, diatomite is used as filter-aid in the processing of liquid food stuffs such as beer, wine, whisky, fruit juices, vegetable juices and cleaning drinking water (Ibrahim and Selim, 2011; Inglethorpe, 1992).

Diatomite can be used as a microbial carrier. The possible mechanism is that diatomite particles have a strong capacity to adsorb microbial cells on the surfaces due to their high specific surface area (Wang *et al*., 2012). The porous cells of diatomite pellets can provide a home both for microbes and for oxygen, water, and nutrients to help sustain the life of the augmented colonies of microbes introduced in the pellets. Moreover, diatomite provided a kind of microenvironment around the microorganisms, in which the local environment was less aggressive than that in the extreme reaction and thus microorganisms could maintain and prolong their activities (Wang *et al*., 2012). Immobilization of *Pseudomonas* species on diatomite resulted in an increased efficiency of removing 3,5,6-trichloro-2-pyridinol from industrial wastewater (Feng *et al*., 1997). Immobilization of *Actinobacillus succinogenes* for the production of succinic acid was reported by Corona-González *et al*. (2014). The

oxidation rate of ferrous ions by diatomite supported *Thiobacillus ferrooxidans* was increased by about 20% (10-15 h shorter than that of free cells) (Yoshishige *et al*., 1999). Diatomite is also used as a carrier of non-pathogenic microbes inside a system for in situ bioremediation of contaminated soil and ground water (Hunt, 1996) and as a protective vehicle to protect *Bacillus sphaericus* from the high pH environment applied for self-healing concrete (Wang *et al*., 2012).

1.2.17 Immobilized whole cells bioreactor

The most popular immobilized cell bioreactors employed for various applications include continuous stirrer bioreactor, packed bed bioreactor, fluidized bed bioreactor and air-lift bioreactor (Figure 8). Bioreactors with immobilized cells have shorter reaction times, higher productivity and operational stability of the cells, as well as easier downstream processing (Genisheva *et al*., 2014). The immobilized cell bioreactor technology provides a cost effective means for the treatment of existing environmental waste problems, such as contaminated groundwater, or for the eradication of pollutants at their point of origin (Seignez *et al*., 1993; Hallas *et al.*, 1992; Friday and Portier, 1991; Heitkamp *et al*., 1990; O'Reilly and Crawford, 1989). This technology generally involves the colonization of a specialized microorganism onto inorganic biocarriers as fixed films and the utilization of these colonized surfaces in controllable reaction vessels or bioreactors. However, when dealing with immobilized cell systems it is of a big importance to choose the proper reactor type. This decision depends on the type of immobilization and type of support used, as well as on mass transfer requirements and conditions of the process (Genisheva *et al*., 2014).

Over the conventional type free-culture bioreactors, the immobilization cell bioreactors has the following advantages like continuous reactor operation at any desired liquid throughput without risk of cell washout, improved system productivity, less susceptibility to hydraulic and process upsets, reduced sludge production, protection of cells from toxic substrates, enhanced gas-liquid mass transfer rate, plug flow operation by maintaining the immobilized cells as a stationary phase (Sokol and Korpal, 2004; González *et al*., 2001a; Sa and Boaventura, 2001). Moreover, the potential main advantages claimed for the uses of immobilized viable cells include the

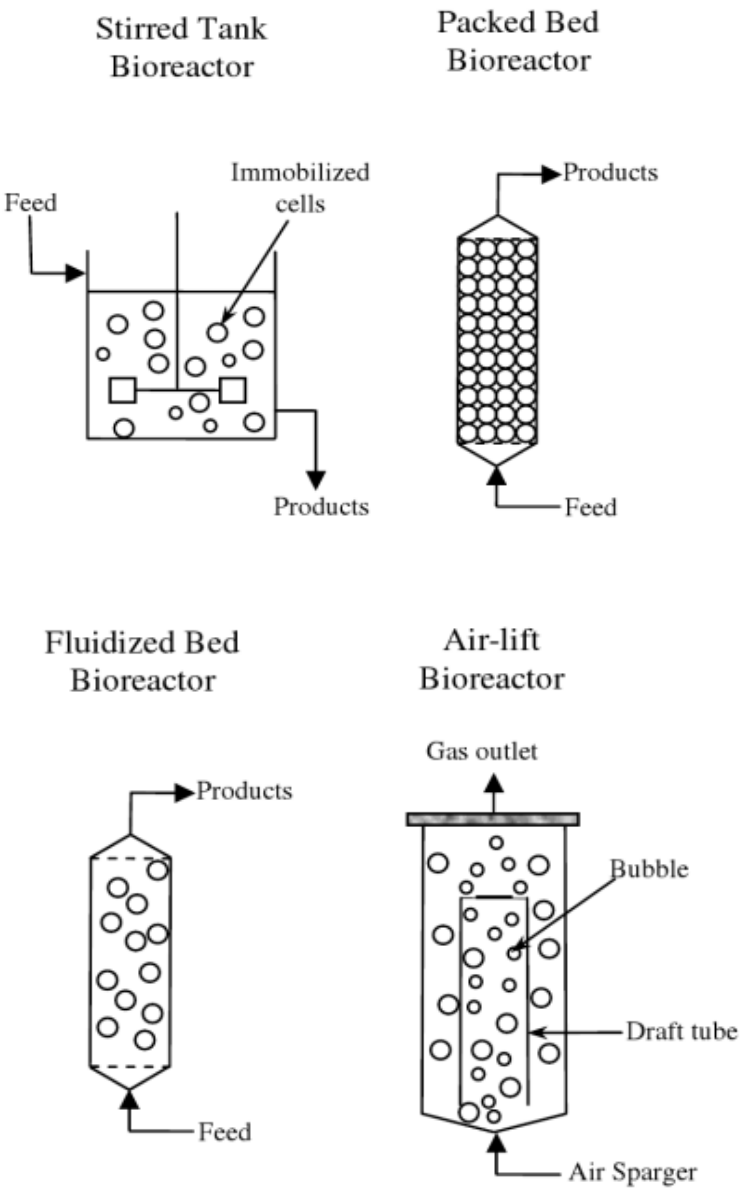

Figure 8. Various types of immobilized cell bioreactor.
Source: Zhu (2007).

easy separation and reuse of the cells, high cell concentrations, flexibility in bioreactor design and operation as well as the stabilization of several cell functions (Dervakos and Webb, 1991). Full exploitation of these potential advantages will strongly depend on the wise selection of a set of processing parameters allowing for high productivity combined with extended operational stability. This set includes:

immobilization method, mode of operation (e.g., repeated batch vs. continuous), aeration and mixing, bioreactor configuration, medium composition (including feeding of substrates, precursors, or additional nutrients), temperature, pH and, whenever required, *in situ* product and/or excess biomass removal. Understanding of the interrelationship between each of these parameters, cell physiology, productivity, and operational stability will allow rational, systematic design and evaluation of new processes (Freeman and Lilly, 1998). Rational approaches are required for new process design and operations to achieve good process stability as well as a significant reduction of production cost (Zhu, 2007).

1.2.17.1 Continuous stirrer tank bioreactor

Continuous stirrer tank bioreactors generally consist of a tank containing a stirrer. The tank is normally fitted with fixed baffles which improve the stirring efficiency. The substrate is continuously pumped into the reactor at the same time as the product is removed. Continuous stirrer tank bioreactor is an easily constructed, versatile and cheap reactor, which allows simple catalyst charging and replacement (Chaplin and Bucke, 1990). Its well-mixed nature permits straightforward control over the temperature and pH of the reaction, and the supply or removal of gases. Continuous stirrer tank bioreactors tend to be rather large as they need to be efficiently mixed. An ideal continuous stirrer tank bioreactor has complete back-mixing resulting in a minimization of the substrate concentration, and a maximization of the product concentration, relative to the final conversion, at every point within the reactor, the effectiveness factor being uniform throughout (Chaplin and Bucke, 1990). For the immobilized cells, high agitation is required to reduce the mass transfer limitation of both substrate and product. However, this may result in high shear forces which can cause damage to both the support and the cells (Zhu, 2007).

1.2.17.2 Packed or fixed bed bioreactor

The bioreactor system that can provide extremely high productivity within a compact size is the packed bed bioreactor or fixed bed bioreactor. Packed

bed bioreactor consists of a column packed with immobilized cells through which the substrate solution flows. It is operated in the plug-flow mode, with a minimum of back mixing. For industrial applications, upward flow is generally preferred over downward flow because it does not compress the immobilized bed in the column as downward flow does (Riitonen *et al*., 2013). By selecting an appropriate flow rate, it is possible to achieve a very high reaction rate or even complete conversion of the substrate in a single pass of the column. Shear forces are low in packed bed bioreactors because they are only caused by the fluid-phase velocity (Zhu, 2007). For industrial use of immobilized cells, packed bed bioreactors are widely used because of their simplicity and better operational control. Lee *et al*. (2003) also reported that packed bed bioreactor is cost effective with simple setup and easy operation. Similarly, Deront *et al*. (1998) and Beg *et al*. (1996) reported that biocatalysts attached to the supporting surfaces in packed bed bioreactor allow high volumetric loadings and to provide a long retention time without the need to separate or recycle the bed. Due to the potential advantages of packed bed bioreactors with immobilized whole cells, packed bed bioreactors have been used in a wide spectrum of applications, such as biodegradation of toxic compounds, such as phenol, cadmium, hydrogen sulfide, hexavalent chromium; Cr(VI) and synthetic dyes and production of biohydrogen and bioethanol (Kathiravan *et al*., 2010; Saeed *et al*., 2009; Vijayaraghavan *et al*., 2008; Wu and Yu, 2008; Cotrino *et al*., 2007; Wu *et al*., 2007; Tziotzios *et al*., 2005; Zhao *et al*., 2003). Although there are a number of advantages for using immobilized microorganisms, diffusion limitations and the compaction of the loading material due to liquid flow in packed bed bioreactor are the main disadvantages of these processes (Tepe and Dursun, 2008; Ramakrishna *et al*., 1988). These compaction effects are significantly greater when soft gel immobilized beads or particles of relatively small size are employed. The bed is gradually compressed resulting in decreased liquid flow rate and hence productivity (Ramakrishna *et al*., 1988). However, fixed-bed bioreactors are still the first choice in the industrial sector, mainly due to the simplicity of the design. Other bioreactor types provide better mixing, but high mechanical stresses and shear rates, large power inputs, investment cost, and difficulties in scale up are still the main issues constraining their wider application (Verica *et al*., 2013).

1.2.17.3 Fluidized bed bioreactor

In the fluidized bed bioreactor, the immobilized cells are maintained in motion by continuous flow of the feed solution. The fluidized bed bioreactor provides conditions that are intermediate to those of stirrer tank bioreactor and packed bed bioreactor. Mixing in the fluidized bed is better than packed bed and the shear rate is lower compared to the stirrer tank bioreactor. The advantages of this bioreactor compared to stirred tank are lower risk of contamination due to the absence of a shaft seal and higher productivity because of less back-mixing. In contrast to packed bed, it can better accommodate a third phase of gas flow and facilitates multiphase mixing, which reduces the mass transfer limitation and substrate or product inhibition (Zhu, 2007).

The fluidized bed bioreactor has been applied in waste treatment such as the quinoline removal in wastewater by immobilized whole cells of *Burkholderia pickettii*, the biodegradation of phenolic industrial wastewater by immobilized cells of *Pseudomonas putida* in calcium-alginate gel beads hardened with Al^{3+} and biotreatment of petroleum-contaminated water by immobilized *Rhodococcus* cells on hydrophobized sawdust support (Kuyukina *et al*., 2009; González *et al*., 2001b; Han *et al*., 2001) The information provided includes the particle density, terminal velocities, drag coefficient, minimum fluidization velocities for beads of various size, and bed expansion characteristics. Useful engineering data will be useful for designing fluidized bed bioreactors (Zhu, 2007). However, fluidized bed bioreactor was shown to exhibit operational and scale-up difficulties.

1.2.17.4 Airlift bioreactor

The air-lift bioreactor can be regarded as a special variation of the fluidized bed bioreactor. It usually contains an internal loop gas draft tube inside the column. Gas sparging induces the liquid upflow with the suspended particles in the inner draft tube. Subsequently, gas escapes from the top of the bioreactor and the liquid with the suspended particles is led through the gas-free downcomer. An external loop system may replace the inner draft tube for the recirculation of liquid in some air-lift bioreactors (Zhu, 2007).

Compared to the common fluidized bed bioreactor, the main advantage of the airlift bioreactor is the improved fluidization characteristics. The particles containing the immobilized cells are more easily fluidized in loop reactor systems and can be kept in suspension due to the circulation of the liquid phase even when working with high liquid-phase and gas-phase velocities. The undesired washout by bed expansion in the fluidized bed bioreactor can be avoided. The gas flow derived mixing in the airlift bioreactor results in a low shear rate and excellent liquid-solid and gas-liquid mass transfer (Zhu, 2007).

Kermanshahi pour *et al*. (2005) carried out the biodegradation of petroleum hydrocarbons in airlift bioreactor using an immobilized cell of microbial consortium from the indigenous microorganisms, which exist in diesel fuel contaminated soil. Zhang *et al*. (1997) developed an immobilized-cell-film airlift bioreactor with immobilization of the yeast cells on cotton cloth sheets by attachment. They found that enzyme productivity and production stability in the immobilized-cell system increased when compared to free suspension cultures in stirred-tank and airlift bioreactors. Similarly, Srinivasulu *et al*. (2002) reported that neomycin production by alginate immobilized *Streptomyces marinensis* cells in an airlift reactor was increased two and a half times as compared to the free cells in an airlift reactor.

1.3 Objectives of study

1. To optimize the culture conditions for HADH production by *Natrinema gari* BCC 24369
2. To study the feasibility of *Nnm. gari* BCC 24369 to produce HADH under non-sterile condition and the possibility of scaling-up fermentation process
3. To optimize the condition for whole cell immobilization of *Nnm. gari* BCC 24369 using diatomite as the support
4. To evaluate the potential use of immobilized whole cells of *Nnm. gari* BCC 24369 for removing histamine in fish sauce
5. To follow up the quality of fish sauce after treated with immobilized whole cells of *Nnm. gari* BCC 24369 packed in fixed-bed bioreactor during 6 months storage

CHAPTER 2

OPTIMIZATION AND SCALING UP OF HISTAMINE DEHYDROGENASE PRODUCTION BY *NATRINEMA GARI* BCC 24369 IN NON-STERILE CONDITION

2.1 Abstract

Statistic experimental designs were performed to optimize the production of histamine dehydrogenase (HADH) by *Natrinema gari* BCC 24369. The most important factors influencing HADH production as identified by Plackett-Burman design (PBD) were casamino acid, $MgSO_4.7H_2O$, NaCl, and $FeCl_2.4H_2O$. Central composite design (CCD) was employed to identify their optimal values that would yield maximum HADH production. The analysis indicated that the optimal medium was composed of 15 g/l casamino acid, 75 g/l $MgSO_4.7H_2O$, 273 g/l NaCl, 2.5 mg/l $FeCl_2.4H_2O$, 10 g/l yeast extract, 5 g/l sodium glutamate and 5 g/l KCl. Based on one-factor-at-a-time (OFAT) method, the optimum initial pH of culture medium and incubation temperature for enzyme production were at 7.5 and 37°C, respectively. The production of HADH under optimal condition was about 2.2-fold higher than that of un-optimized condition. Owing to the high salt tolerance of *Nnm. gari* BCC 24369, a more economical and eco-friendlier HADH production was scaled-up under completely non-sterile condition. At 16-l-scale batch cultivation of *Nnm. gari* BCC 24369, HADH productivity under non-sterile condition was 858±12 U/g cell biomass which was comparable to that under sterile condition (878±15 U/g cell biomass). These results demonstrate the feasibility and simplicity of commercial production of HADH production using *Nnm. gari* BCC 24369.

2.2 Introduction

Histamine dehydrogenase (HADH) from halophilic archeaon, *Nnm. gari* BCC 24369 catalyzes the oxidative deamination of histamine, resulting in the production of imidazole acetaldehyde and an ammonium ion (Zhou *et al*., 2014, Tapingkae *et al*., 2010b). This enzyme is a heterotrimer and its activity requires high levels of salt and co-factors. Due to ability to degrade histamine in high salt conditions, HADH can present various interesting applications in removal of histamine in products high in salt and development of enzyme-based biosensor and test kit for histamine determination (Köse *et al*., 2011; Tapingkae *et al*., 2010a, b; Sato *et al*., 2005; Takagi and Shikata, 2004). However, like other extremely halophilic bacteria, utilization of *Nnm. gari* BCC 24369 and its enzyme are not feasible at larger scale due to various reasons like cost of the chemicals, longer cultivation time and requirement of specific equipments with high energy requirements which increases the overall cost of the enzyme production (Tapingkae *et al*., 2010a; Robinson *et al*., 2005; Burns *et al*., 2004). To overcome these limitations, more economical and eco-friendlier process for HADH production with improved product yield should be developed. Owing to the ability of this archaeon to grow at high concentrations of NaCl, obvious economic advantage can be achieved by culturing under non-aseptic conditions instead of aseptic conditions.

Optimization of the culture media is an important strategy for maximizing the microbial metabolites production at the minimal production cost (Bezbaruah *et al*., 1994). Statistical experimental design techniques are useful tools for nutrients optimization with respect to growth and product yield and provide statistical models (Charyulu and Gnanamani, 2010). Plackett and Burman design (PBD) is a well known and widely used statistical technique for screening and selection of most significant culture variables (Plackett and Burman, 1946), while the central composite design (CCD) provides important information regarding the optimum level of each variable along with its interactions with other variables and their effects on product yield (Pardeep and Satyanarayana, 2006).

During the last decades, cultivation medium for enhancing enzyme and/or cell mass production by halophilic archaea have been optimized (He *et al.*, 2009; Manikandan *et al*., 2009; Vidyasagar *et al*., 2006; Wejse *et al*., 2003). Among various medium components, NaCl was the key factor having extreme influence on cells growth and their enzyme production (Vidyasagar *et al*., 2006; Wejse *et al*., 2003). Moreover, organic components as complex nitrogen and carbon sources were also found to be the important variables affecting cell growth and halophilic enzyme production (Siroosi *et al*., 2014; Manikandan *et al*., 2009; Vidyasagar *et al*., 2006). The commonly used organic components are casamino acid, yeast extract and tryptone (Oren, 2006). In addition to medium components, initial pH of cultivation medium and incubation temperature were found to play an important role in enzyme production by halophilic archaea and were also optimized (Vidyasagar *et al*., 2006).

The objectives of this study were to optimize the culture conditions for HADH production by *Nnm. gari* BCC 24369 using sequential statistical experimental design and to study the feasibility of the cells for enzyme production under non-sterile condition. To achieve the optimum conditions, the present study was carried out in three stages. Firstly, the PBD was applied to address the most significant medium components which affected HADH production. Secondly, CCD was employed to determine the optimal concentration of each significant medium component. Finally, optimal pH and incubation temperature was studied to maximize the HADH activity based on one-factor-at-a-time (OFAT) method. In order to perform an economical and simple enzyme production, due to the potential advantage of high salt tolerance of *Nnm. gari* BCC 24369, the production of HADH was investigated in optimized medium without sterilization in 20-l polypropylene tank.

2.3 Materials and Methods

2.3.1 Chemicals

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

2.3.2 Cultures and conditions

Natrinema gari BCC 24369 was obtained from National Center for Genetic Engineering and Biotechnology (BIOTEC) in Pathum Thani, Thailand. *Nnm. gari* BCC 24369 was grown on an agar plate of halophilic medium (Appendix A-1). The seed culture was prepared by inoculating a loopful of culture into 5 ml of halophilic liquid medium and incubating at 37^{o}C in a shaker incubator (Sartorius, Certomat® BS-1, Göttingen, Germany) at 200 rpm for 7 days. For optimization experiments, cells were cultivated by inoculating 5% (v/v) of seed culture into 400 ml of various medium in 1-l Erlenmeyer flask as design matrix in Table 3 and 4, and incubated at 37^{o}C in shaker incubator (200 rpm) for 7 days.

2.3.3 HADH preparation

Cell pellet was harvested by centrifugation of culture broth at 15,000×*g* for 10 min at 4°C and washed twice with 2 M NaCl. The cells were resuspended in 50 mM Tris-HCl buffer, pH 7.0 containing 2 M NaCl at a ratio of 1:1. The cell suspension was sonicated for 20 s, followed by a 40 s rest interval with a total of 2 min sonication by a Vibra Cell VCX60 (Sonics and Materials Inc., Newtown, CT, USA). The supernatant was collected by centrifugation at 15,000×g for 15 min at 4°C and referred to as crude enzyme extract.

2.3.4 Assay for HADH activity

HADH 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 standard assay reaction mixture consisting of 50 mM Tris-HCl, pH 7.0,

2 M NaCl, 5.0 mM of histamine dihydrochloride (free-base) and 555 μM of 1-methoxy PMS using as electron acceptor. The reaction was incubated at 50°C for exactly 1 h without shaking and terminated by adding 1 ml of 0.1 M HCl. The concentration of histamine was determined by the fluorometric method of AOAC (2005a) (Appendix B-1). A blank was run in the same manner, except the sample was added after addition of 0.1 M HCl. HADH activity was expressed in term of total activity in crude enzyme extract and the ratio of total HADH activity to total cell biomass. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed a reduction of 1 nmol of histamine per h under the specified conditions.

2.3.5 Determination of cell biomass concentration

The cell biomass concentration was measured by dry cell weight method. Cells were collected by centrifuging 10 ml of culture broth at 15,000×*g* for 10 min at 4°C. To remove residual broth and prevent the cell lysis, the cell pellet was washed twice with 10 ml of 2 M NaCl and centrifuged at 15,000×g for 10 min at 4°C. After washing, NaCl solution (2 M) was added to make the final volume of 1 ml, and dried in a pre-weighed aluminum pan at 105°C using a moisture analyzer (Sartorius model MA30-000V3, MettlerToledo, Germany) until constant weight was obtained. The net biomass weight of cells was estimated by subtracting with the dry weight of 1 ml of 2 M NaCl solution determined by the same method as described.

2.3.6 Optimization procedure and experimental design

2.3.6.1 Screening for the medium components for HADH production

Effect of ten factors, including histamine, yeast extract, casamino acid, sodium glutamate, trisodium citrate, $MgSO_4.7H_2O$, KCl, NaCl, $FeCl_2.4H_2O$ and $MnCl_2.4H_2O$ on HADH activity by *Nnm. gari* BCC 24369 was evaluated by PBD. This design gave an output of 13 experimental runs (1 run at center point) with 10 independent variables which was generated by Design Expert (Table 3). All the experiments were performed in triplicate and the average of total HADH activity was used as the responses (dependent variables). The response obtained in these 13 experiments was subjected to statistical analysis. The main effect of each variable on

total HADH activity calculated as the difference between the average of measurements made at the high value (+) and at the low value (-) of that factor. The components having confidence level greater than 95% were the most significant and were taken up for further optimization studies. Insignificant components were kept at the constant level (Natarajan and Rajendran, 2012). Effect of each variable on total HADH activity was evaluated by the ratio of the difference between the average response in the higher level of a given component and its lower level to the number of trial N. The effect of the given component on the response factor S_i, is

$$\text{Effect} = 2[\sum S_i^{+} - \sum S_i^{-}] / N \quad (1)$$

where S_i^{+} is the response parameter of an assembly in the screening design that contains the higher quantity of a given component, S_i^{-} is the response parameter of an assembly in the screening design that contains the lower quantity of a given component and 'i' varies from 1-10.

2.3.6.2 Optimization of the screened medium components for HADH production

CCD was employed to determine the optimum level of significant factors identified by PBD. A 30-run experiment generated by Design Expert was carried out with 6 trials at the center point (Table 4). All experiments were conducted in triplicate. The mean value of total HADH activity was taken as the response. A multiple regression analysis was applied to the data obtained. The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response, β_0 is the offset term, β_i is the linear offset, β_{ii} is the squared offset and β_{ij} is the interaction effect, while X_i and X_j denote the coded values of the variables.

All experiments were conducted in triplicate and their mean values were reported. The developed regression model was evaluated by analyzing the values of regression coefficients, analysis of variance (ANOVA), *p*- and *F*-values. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2. The statistical software package Design-Expert was used to identify the experimental design as well as to generate a regression model to predict

the optimum medium composition for HADH production. A final experiment was conducted to validate the CCD model developed.

2.3.6.3 Effect of initial pH and incubation temperature on HADH production

To study the effect of pH, samples of the optimized medium were prepared by adjusting pH ranging from 5.0 to 9.0 with 0.1 N or 1.0 N NaOH and 0.1 N or 1.0 N HCl before sterilization. Cells were cultivated by inoculating 5% (v/v) of seed culture into 400 ml of various medium in an 1-l Erlenmeyer flask. The cultivation was incubated aerobically (200 rpm) at 37°C for 7 days. To find the optimum temperature for the production of HADH, the cultivation was performed in the optimized medium at various incubation temperatures ranging from 20 to 50°C and incubated in a shaker incubator (200 rpm) for 7 days. The initial pH of culture medium and incubation temperature rendering the maximal enzyme activity were chosen for further study.

2.3.6.4 Time course of HADH production

HADH and cell biomass productions by *Nmm. gari* BCC 24369 were compared in un-optimized and optimized media adjusted to initial pH value of 7.5. Cultivation was conducted in a shaker incubator (200 rpm) at 37°C up to 9 days. Samples were daily taken to determine HADH activity and cell biomass.

2.3.7 HADH production under sterile and non-sterile conditions

Cells were cultivated by inoculating 5% (v/v) of seed culture into 400 ml of optimal medium in an 1-l Erlenmeyer flask. The sterile medium was done by autoclaving at 121°C and 15 pounds/inch^2 pressure for 15 min whereas non-sterile medium was prepared by dissolving the ingredients in distilled water, without any heat treatment, and adjusting to pH 7.5 using 0.1 N or 1.0 N NaOH. Neither the medium nor the flask was autoclaved. The flasks were incubated in a shaker incubator (200 rpm) at 37°C for 9 days. Samples were daily taken to determine the HADH activity and cell biomass.

2.3.8 Scaling up of HADH production

Schematic drawing of cultivation of *Nnm. gari* BCC 24369 in a 20-l polypropylene tank (Thermo Fisher Scientific Inc., Waltham, MA, USA) is shown in Figure 9. For non-sterile cultivation, the tank was filled with 16 l of culture medium and then was inoculated with 5% (v/v) of 7 days culture. The inoculum was prepared in an 1-l Erlenmeyer flask in a similar medium. The reactor tank was operated in batch mode for 9 days. The pH value was maintained at 7.5 due to the buffer properties of the medium. The temperature was controlled in the range of 28 to 32°C. Foaming was regulated by the addition of vegetable oil (0.03%, w/v) into the medium. The effect of aeration rate on growth and HADH productions was studied at 4.5 and 9 l/min. Air were supplied to the culture using an air pump equipped with an air flow meter. The circular air stone with dimension of 5 cm was used as an air generator in the tank. The supplied air composition was the same as atmospheric air composition. Samples (50 ml) were daily taken from the tank using plastic disposable syringe for determinations of enzyme activity, cell biomass and dissolved oxygen (DO) (AOAC, 2006, Appendix B-2). Colony and cell morphology were examined for the cells grown on halophilic agar plates at 37°C for 7 days. In contrast, for sterile cultivation the tank was filled with 16 l of culture medium and then sterilized by autoclaving at 121°C for 15 min, prior to inoculation.

2.3.9 Data analysis and software

Design-Expert (STAT-EASE Inc., Minneapolis, MN, USA) was used for the experimental designs and statistic analysis of the experimental data. 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., Chicago, IL, USA).

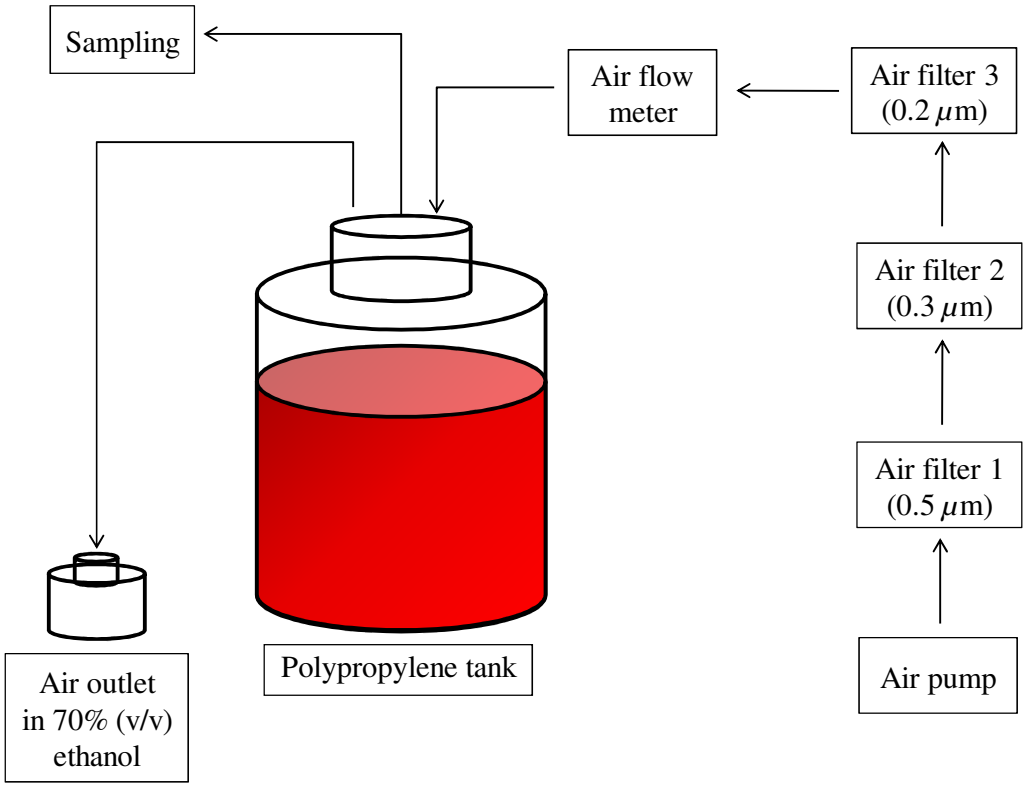

Figure 9. Schematic drawing of cultivation of *Nnm. gari* BCC 24369 in 20-l polypropylene tank.

2.4 Results and Discussion

2.4.1 Evaluation of medium components by PBD

A 10-factor-13-run (including 1 center point) PBD was used, and the response for each run is present in Table 3. In order to determine the influence of the significant factor, a standardized Pareto chart (Figure 10) was employed. Among the ten factors studied, casamino acid, $MgSO_4.7H_2O$, and NaCl had positively significant effect whereas $FeCl_2.4H_2O$ had negatively significant effect on HADH activity in the range of evaluated levels in PBD ($p < 0.05$). Among four significant factors, casamino acid was found to be the most important factor for HADH production by *Nnm. gari* BCC 24369 ($p < 0.01$). It has been reported that the partially hydrolyzed casein in the form of casamino acids had highly effect on enzyme production which was used as

Table 3. Plackett-Burman design for screening of significant factors

Run no.	Variables*										Total HADH activity (U)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	
1	500	10	1	5	5	50	0	225	5	0.5	78.0±25.5
2	0	10	10	0	5	50	5	225	5	0	445.0±24.6
3	500	1	10	5	0	50	5	275	5	0	675.8±36.2
4	0	10	1	5	5	5	5	275	50	0	107.5±15.4
5	0	1	10	0	5	50	0	275	50	0.5	277.5±18.2
6	0	1	1	5	0	50	5	225	50	0.5	87.5±6.1
7	500	1	1	0	5	5	5	275	5	0.5	133.4±5.7
8	500	10	1	0	0	50	0	275	50	0	156.5±6.3
9	500	10	10	0	0	5	5	225	50	0.5	189.0±17.4
10	0	10	10	5	0	5	0	275	5	0.5	457.8±25.1
11	500	1	10	5	5	5	0	225	50	0	128.5±15.2
12	0	1	1	0	0	5	0	225	5	0	0.0±0.0
13	250	5.5	5.5	2.5	2.5	27.5	2.5	250	27.5	0.25	163.0±7.5

*X_1: Histamine (g/l); X_2: Yeast extract (g/l); X_3: Casamino acid (g/l); X_4: Sodium glutamate (g/l); X_5: Trisodium citrate (g/l); X_6: $MgSO_4.7H_2O$ (g/l); X_7: KCl (g/l); X_8: NaCl (g/l); X_9: $FeCl_24H_2O$ (mg/l); X_{10}: $MnCl_2.4H_2O$ (mg/l)

nitrogen, vitamin, carbon and amino acid in microbiological culture media for haloarchaeal growth (Charlebios *et al*., 1987; Bernheimer *et al*., 1944). Patel *et al*. (2005) investigated that among the organic nitrogen sources including soya peptone, tryptone, caseitone, gelatin, casamino acid, peptone and yeast extract, casamino acid was the most important factor affecting protease production by haloalkaliphilic *Bacillus* sp. As an essential inorganic ion, magnesium had been reported to be an essential element for red halophiles which it was required for division of the cells (Manikandan *et al*., 2009; Javor, 1984). It was also found to be significant factor affecting HBF-3 production by *Halomonas* sp. V3á and growth of *Halobacterium salinarum* VKMM 013 (He *et al*., 2009; Manikandan *et al*., 2009). Based on high

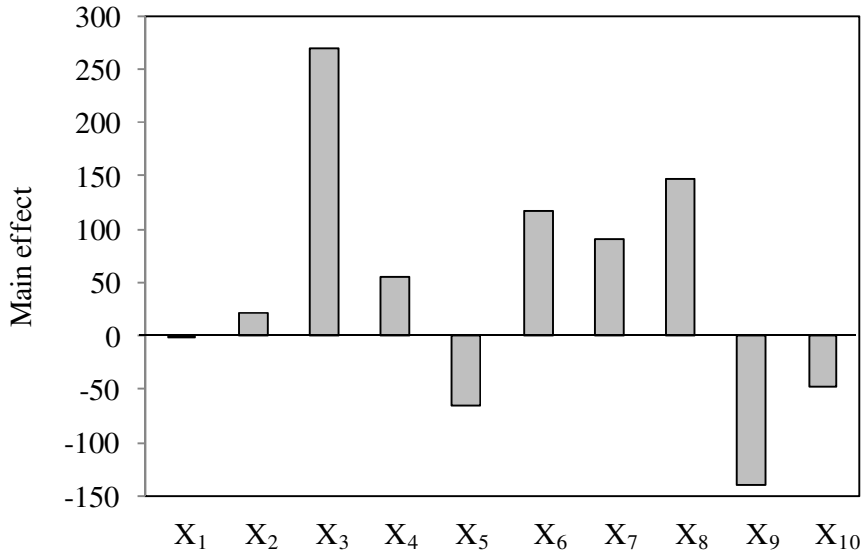

Figure 10. Main effects of the medium compositions on HADH production to the Plackett-Burman experimental results (X_1, Histamine; X_2, Yeast extract; X_3, Casamino acid; X_4, Sodium glutamate; X_5, Trisodium citrate; X_6, $MgSO_4.7H_2O$; X_7, KCl; X_8, NaCl; X_9, $FeCl_24H_2O$; X_{10}, $MnCl_2.4H_2O$).

salinity environments, previous studies have shown that sodium chloride is the key factor having extreme influence on cell growth which influence to their enzyme activity by halophilic microorganisms (Saum and Müller, 2008; Vidyasagar *et al*., 2006; Onraedt *et al*., 2005). For the metal element, iron is an essential nutrient for many red halophilic bacteria (Brown and Gibbons, 1955). The addition of adequate concentrations of iron in the medium could stimulate the growth of *Nnm. gari* BCC 24369 as well as HADH production. At high concentration, iron might become toxic to *Nnm. gari* BCC 24369 by forming toxic compounds and reactive free radicals within the cells as reported for many microorganisms (Nies, 1999). Thus, these four factors were selected for further optimization to evaluate their optimal levels in a wider range of their levels. Other six factors including yeast extract, sodium glutamate, KCl, histamine, trisodium citrate and $MnCl_2.4H_2O$ had no significant effect on HADH production ($p > 0.05$). Hence, no further optimization of these factors was carried out. Yeast extract, sodium glutamate and KCl had positive effect

on HADH production so they were set at their higher level, while histamine, trisodium citrate and $MnCl_2.4H_2O$ had negative effect on HADH production, so they were set at their lower level.

2.4.2 Optimization of the screened medium components by CCD

Among 30 experiments, the conditions used in experiment no. 8 demonstrated the highest HADH activity (Table 4). Fitting of the data with various models indicated that the production of HADH by *Nnm. gari* BCC 24369 was the most suitably described with quadratic polynomial model with significant terms given as follows:

$$Y = -55304.83 + 292.65A + 42.45B + 370.23C + 1503.33D - 0.14AB - 0.49AC - 4.75AD - 0.03BC - 1.66BD - 4.59CD - 3.83A^2 - 0.24B^2 - 0.63C^2 - 14.54D^2 \quad (3)$$

where Y is the response value of enzyme activity, and A, B, C and D are the coded values of casamino acid, $MgSO_4.7H_2O$, NaCl and $FeCl_2.4H_2O$, respectively.

The quadratic polynomial model was highly significant and sufficient to represent the actual relationship between the response and significant parameters. An *F*-test was statistically valid with very low probability value ($p < 0.05$) (Table 5). The coefficient (R^2) of model indicated 99.73% of variability in the response could be explained by this model. The R^2 value of 0.9973 was reasonably agreed with the adjusted R^2 value of 0.9948. The model *p*-value of 0.0001 indicated that the model terms were highly significant. Low "*Prob* > *F*" value indicated that model terms were significant. In this case, except the interaction terms of $MgSO_4.7H_2O$ and NaCl (BC), all model terms were significant. The value of adequate precision (signal to noise ratio) was high compared to the desirable value (desired > 4), which indicated that this model could be used to navigate the design space. The *p*-values for the model (< 0.0001) and for lack of fit (> 0.05) suggested that the obtained experimental data was a good fit with the model (Reddy *et al*., 2008). This confirmed that the accuracy and general ability of the quadratic model was good with the reasonable analysis of the associated response trends.

Based on the regression equation (3), the optimal levels of the four significant variables were casamino acid 15 g/l, $MgSO_4.7H_2O$ 75 g/l, NaCl 273 g/l

Table 4. CCD design and the experimental data

Run no.	Variable level				Total HADH activity (U)	
	Casamino (g/l)	$MgSO_4.7H_2O$ (g/l)	NaCl (g/l)	$FeCl_2.4H_2O$ (mg/l)	Predict-ed	Experimen-al
1	5	25	255	2.5	470	442±14
2	15	25	255	2.5	1,228	1,228±44
3	5	75	255	2.5	819	824±15
4	15	75	255	2.5	1,508	1,505±37
5	5	25	285	2.5	1,004	990±14
6	15	25	285	2.5	1,615	1,622±35
7	5	75	285	2.5	1,313	1,328±42
8	15	75	285	2.5	1,855	1,829±47
9	5	25	255	7.5	1,085	1,091±32
10	15	25	255	7.5	1,605	1,600±42
11	5	75	255	7.5	1,019	1,021±40
12	15	75	255	7.5	1,470	1,464±52
13	5	25	285	7.5	931	944±21
14	15	25	285	7.5	1,304	1,279±26
15	5	75	285	7.5	825	804±45
16	15	75	285	7.5	1,129	1,167±29
17	0	50	270	5	761	766±55
18	20	50	270	5	1,823	1,828±33
19	10	0	270	5	992	1,010±64
20	10	100	270	5	1,167	1,160±51
21	10	50	240	5	1,016	1,025±43
22	10	50	300	5	1,209	1,210±54
23	10	50	270	0	1,367	1,384±56
24	10	50	270	10	1,256	1,250±45
25	10	50	270	5	1,675	1,647±33
26	10	50	270	5	1,675	1,681±65
27	10	50	270	5	1,675	1,714±57
28	10	50	270	5	1,675	1,633±45
29	10	50	270	5	1,675	1,695±38
30	10	50	270	5	1,675	1,681±51

Table 5. Analysis of variance (ANOVA) for the quadratic model* for HADH activity

Source	Sum of squares	*F*-value	*P*-value>*F*
Model	$3.72x10^6$	394.18	<0.0001
A- Casamino acid	$1.69x10^6$	2513.17	<0.0001**
B- $MgSO_4.7H_2O$	$4.56x10^4$	67.64	<0.0001**
C- NaCl	$5.59x10^4$	82.90	<0.0001**
D- $FeCl_2.4H_2O$	$1.85x10^4$	27.42	0.0001**
AB	$4.69x10^3$	6.96	0.0186**
AC	$2.16x10^4$	32.06	<0.0001**
AD	$5.64x10^4$	83.69	<0.0001**
BC	$1.60x10^3$	2.37	0.1442
BD	$1.73x10^5$	256.14	<0.0001**
CD	$4.73x10^5$	702.29	<0.0001**
A^2	$2.52x10^5$	373.26	<0.0001**
B^2	$6.08x10^5$	902.20	<0.0001**
C^2	$5.43x10^5$	806.43	<0.0001**
D^2	$2.27x10^5$	336.22	<0.0001**
Residual	$1.01\ x10^4$		
Lack of fit	$5.57\ x10^3$	0.61	0.7615
Error	$4.54\ x10^3$		
Total	$3.73\ x10^6$		

*R^2 = 0.9793; adj. R^2 =0.9948; CV = 2.01%; adequate precision ratio = 75.47. AB, AC, AD, BC, BD and CD represent the interaction effect of the variables; A^2, B^2, C^2 and D^2 are the squared effects of the variables.

** The values of "*P*-value > *F*" less than 0.05 indicate the variables are significant.

and $FeCl_2.4H_2O$ 2.5 mg/l corresponding to the maximum HADH production of 1,852 U. The verification of the results using the optimized culture medium was accomplished. The maximum HADH production obtained experimentally was found to be 1,847±34 U which was obviously in close agreement with the model prediction.

2.4.3 Effect of initial pH of culture medium and incubation temperature on HADH production

The maximum HADH production by *Nnm. gari* BCC 24369 was observed at pH 7.5 (Figure 11a). HADH production decreased at pH below 6.5 or above 8.5. In general, initial pH value of culture medium is known to be important to cell growth and has a remarkable impact on metabolite production (Kivistö *et al*., 2010). Extremely halophilic archaea are known as neutrophilic and alkaliphilic microorganisms (Oren, 2006). A pH of 6 approximately coincides with the lower boundary of the range of pH values that support growth of halophilic archaea. In this study, the optimal initial pH of culture medium for HADH production by *Nnm. gari* BCC 24369 was similar to that of medium reported for other halophilic enzyme production (Giridhar and Chandra, 2010; Manikandan *et. al*, 2009; Prakash *et al*., 2009; Vidyasagar *et al*. 2006). HADH was highly produced in the wide temperature range of 28-50°C (Figure 11b). However, the maximum HADH production was observed at 37°C. It has been reported that halophilic archaea have a wide range of temperature preferences depending upon nature of adaptation and salt requirements (Wejse *et al*., 2003). Most halophilic archaea and their enzyme production have rather high temperature optima, in the range between 35 and 50°C (Vidyasagar *et al*., 2006; Shand and Perez, 1999; Lillo and Rodriguez-Valera, 1990; Rodriguez-Valera *et al*., 1980).

2.4.4 Time course of HADH production

HADH production by *Nnm. gari* BCC 24369 was growth associated with the maximal production at the early stationary growth phase (Figure 12). After 7 days incubation, the productions of HADH and cell biomass in the optimized medium increased by 2.2-fold and 2-fold, respectively when compared to un-optimized medium. The similar characteristics in HADH and cell biomass productions were noted during cultivation in optimized and un-optimized media. HADH production was low in the beginning of exponential growth phase and sharply increased during the exponential phase until reaching the maximum in the early stationary phase. Then, HADH production decreased throughout the stationary phase.

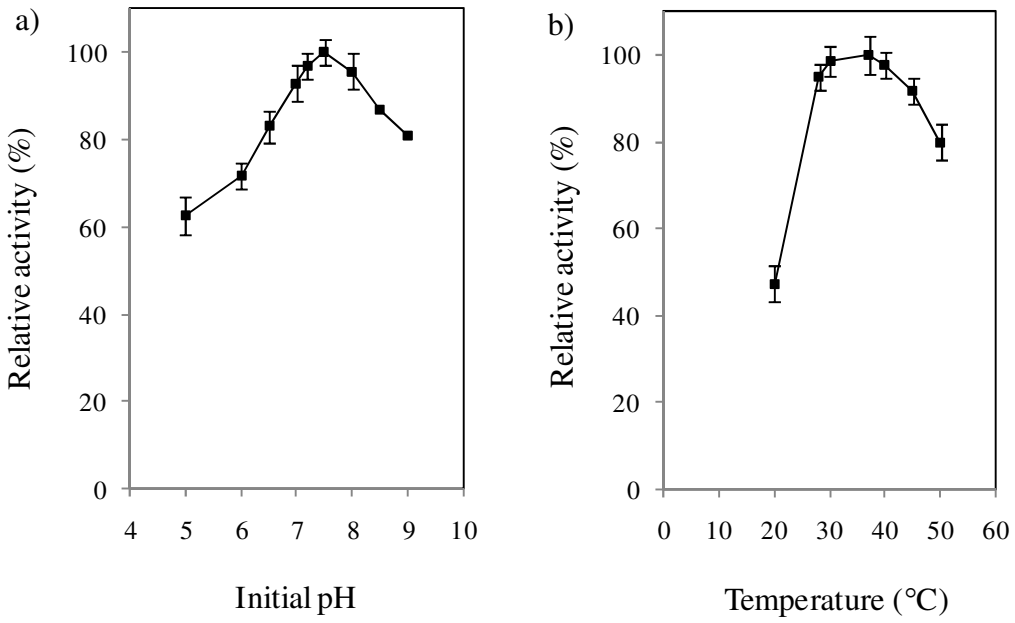

Figure 11. Effect of initial pH of culture medium and incubation temperature on HADH production by *Nnm. gari* BCC 24369 in 400 ml shake flask culture after 7 days incubation. Bars represent the standard deviation (n = 3).

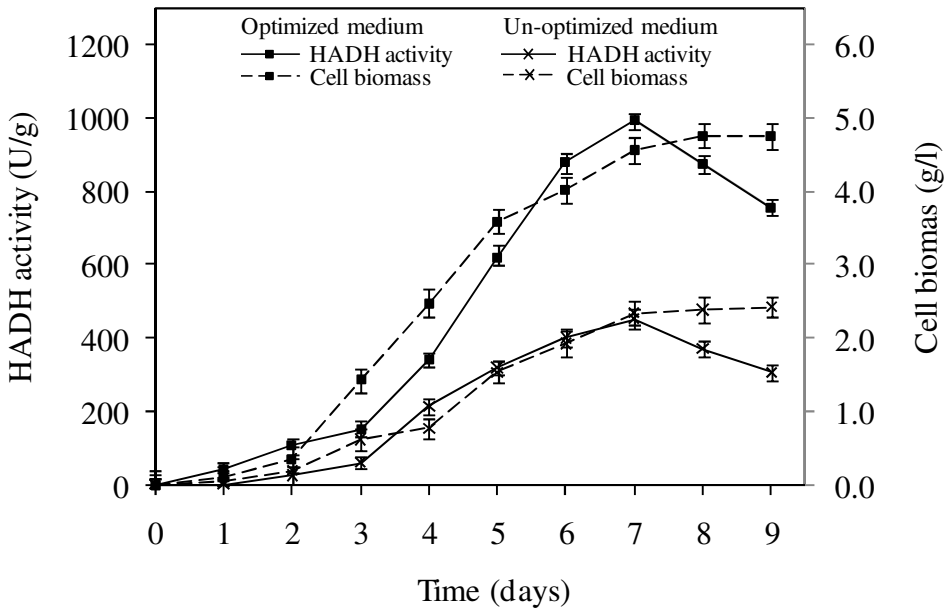

Figure 12. Time course of HADH and cell biomass productions by *Nnm. gari* BCC 24369 in optimized and un-optimized medium in 400 ml shake flask culture. Bars represent the standard deviation (n = 3).

2.4.5 HADH production under sterile and non-sterile conditions

The cells cultured under sterile and non-sterile conditions exhibited similar production profiles of HADH and cell biomass ($p > 0.05$, Figure 13). HADH and cell biomass productions were low in the beginning of exponential phase and rapidly increased during exponential phase until reaching the maximum in the early stationary phase (after 7 days). Then, HADH production gradually decreased ($p < 0.05$) whereas cell biomass production remained at maximum level ($p > 0.05$) throughout the stationary phase. The results show the ability of *Nnm. gari* BCC 24369 to grow and produce HADH under non-sterile condition. The high salt concentration (>25% w/v) was enough to inhibit the development of non-halophiles and/or their enzyme activities, thus allowing cultivations under relatively non-sterile conditions (Quillaguamán *et al*., 2005). Practically, non-sterilization means lower equipment requirement and energy consumption, the omission of the sterilization equipments and the decrease of labor cost (Metsoviti *et al*., 2013; Qin *et al*., 2009; Oren, 2006). Therefore, non-sterile cultivation of *Nnm. gari* BCC 24369 has the potential to be applied for industrial production of low-cost-high-volume HADH to compete with expensive traditional options.

2.4.6 Scaling up of HADH production

HADH production by *Nnm. gari* BCC 24369 in a 20-l polypropylene tank under sterile and non-sterile conditions supplied with different air flow rates are shown in Figure 14. After 7 days incubation, the highest enzyme and cell biomass productions were achieved under both non-sterile and sterile conditions ($p > 0.05$) with the air flow rate of 9 l/min. In contrast, the lowest enzyme and cell biomass production were observed in the culture under non-sterile condition with air flow rate 4.5 l/min. The remarkable changes in HADH and cell biomass productions by *Nnm. gari* BCC 24369 at different air flow rates revealed a significant effect of dissolved oxygen (DO) on growth. The DO concentration profiles were different between the air flow rates of 4.5 and 9 l/min which were observed after 4 days of incubation. At the air flow rate of 4.5 l/min, the DO concentration decreased in the early exponential growth phase suggesting insufficient of oxygen for utilization by the cells during cell

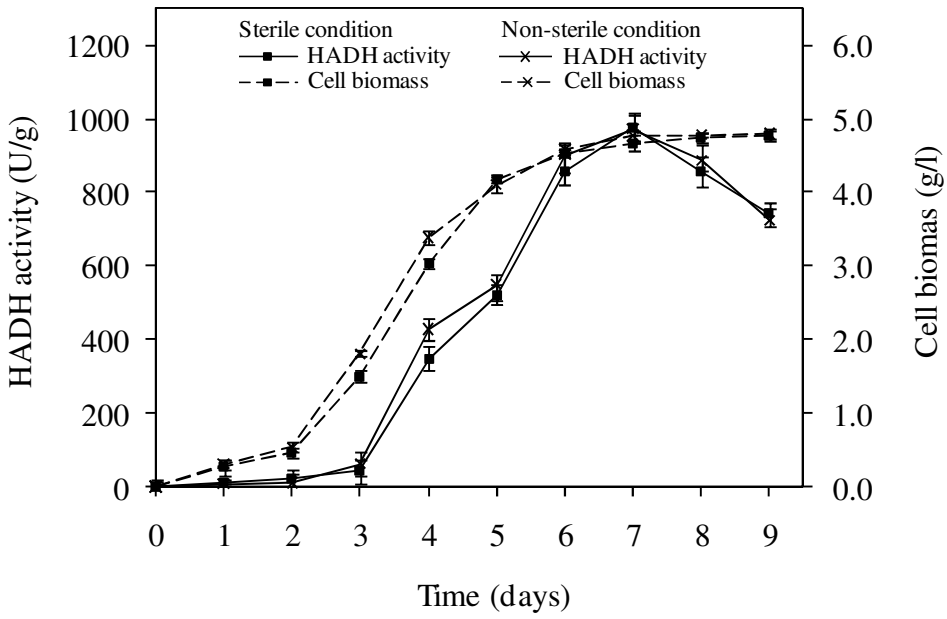

Figure 13. HADH and cell biomass productions by *Nnm. gari* BCC 24369 in optimized medium in 400 ml shake flask culture under sterile and non-sterile conditions. Bars represent the standard deviation (n = 3).

development. In contrast, no change in DO over the entire fermentation period was observed at air flow rate of 9 l/min. Comparing the HADH production obtained in 7 days incubation between air flow rates of 4.5 and 9 l/min, the strong influence of aeration rate on HADH production and strain growth is clearly demonstrated. The improvement of HADH activity was noticed when air flow rate increased from 4.5 to 9 l/min. These results indicated that oxygen was a critically limiting factor causing the early interruption of the culture development at low air flow rate of 4.5 l/min. In this condition, oxygen was most likely the rate-limiting substrate for the microbial growth, maintenance and HADH production (Radchenkova *et al*., 2014).

Aeration rate is the most important parameter implied on the design, operation and scale up of bioreactors (Garcia-Ochoa *et al*., 2000). The aeration rate is crucial in influencing the availability of nutrient and dissolved oxygen and controlling the rate of metabolite release from the cells (Fenice *et al*., 2012). Due to its low solubility in salt-saturated brines, oxygen may easily become a limiting factor for development of halophilic Archaea (Oren, 2006). Therefore, it is important to ensure

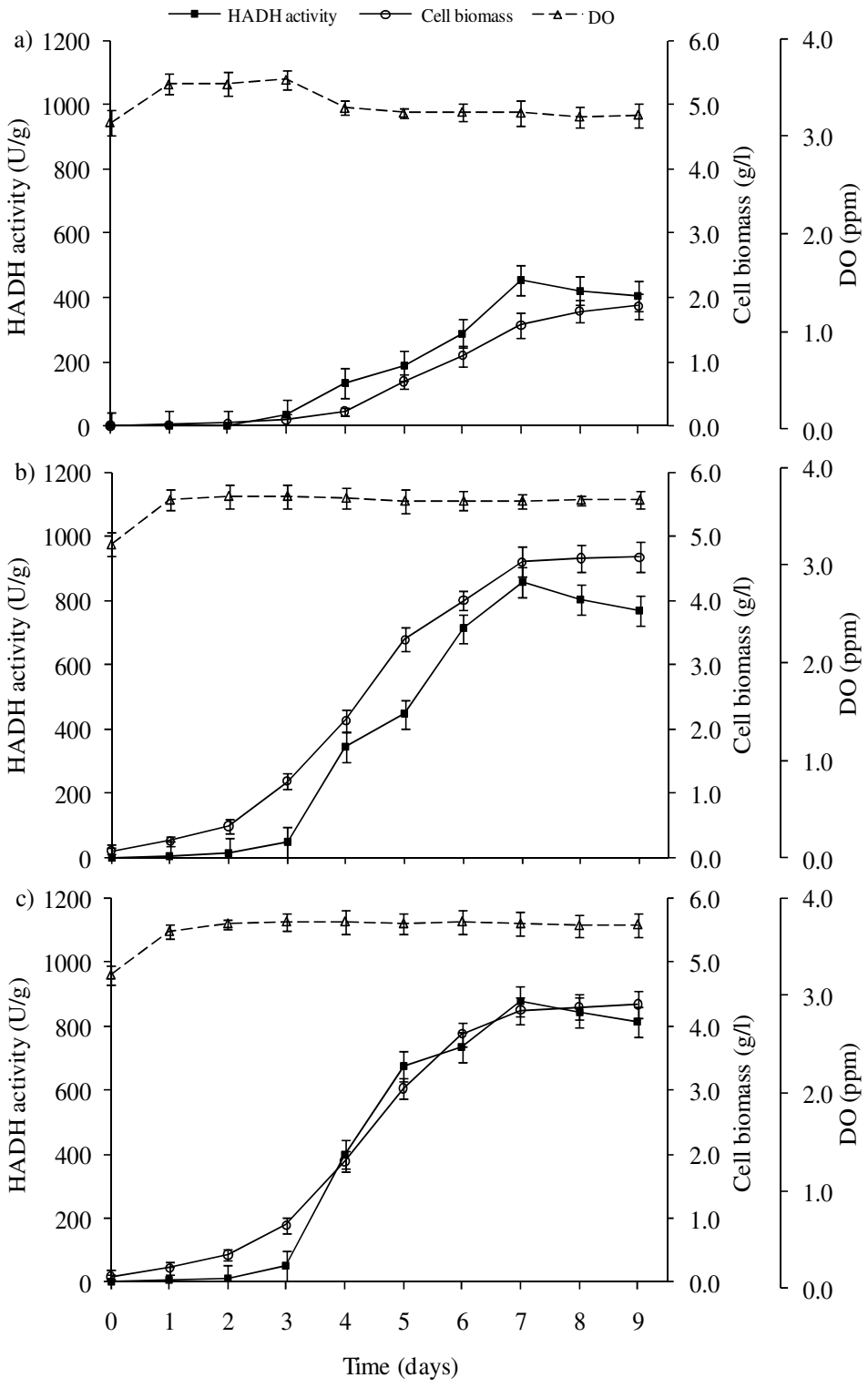

Figure 14. HADH productions by *Nnm. gari* BCC 24369 in 20-l polypropylene tank under non-sterile conditions with air flow rates of 4.5 (a) and 9 l/min (b), and sterile condition with air flow rate of 9 l/min (c). Bars represent the standard deviation (n = 3).

an adequate DO concentration in the culture broth (Garcia-Ochoa and Gomez, 2009). In this study, at air flow rate of 9 l/min, the abundance of DO concentration was existed in the culture medium and was enough for cell growth. Wejse *et al.* (2003) also investigated that an increase in oxygen transfer rate from 130 to 170 rpm affected approximately 10% increase in xylanase production by halophilic eubacterium, strain SX15.

Under air flow condition of 9 l/min, HADH production by *Nnm. gari* BCC 24369 under sterile and non-sterile conditions were not significantly different (878±15 and 858±12 U/g cell biomass, respectively, $p > 0.05$, Table 6) in which they were corresponding to 92±2 and 88±3% of the product yields at flask scales under sterile and non-sterile conditions, respectively. A decreasing in product yield upon scaling up fermentation process is a general observation (Hollinshead *et al*., 2014; Nguyen *et al*., 2014; Kim *et al*. 2002; Bylund *et al*., 2000; George *et al*. 1998). However, this scale-up strategy presents an easy operation system which provides an alternate design of the bioreactor corresponding to cost-effective enzyme production with low investment and operating costs. Therefore, this strategy had the potential for scaling up fermentation process for HADH production by *Nnm. gari* BCC 24369 in the larger scale.

For cell morphology, under both sterile and non-sterile conditions, colonies formed on halophilic agar plates were circular (1-2 mm in diameter), smooth, translucent and pale-orange pigmented (data not shown). Except *Nnm. gari* BCC 24369, other microorganism was not observed in the halophilic agar plate. Colony formation on agar plates was not significantly different between cultivation under sterile and non-sterile conditions. The colonies formed on agar plates were specifically morphological characteristic of *Nnm. gari* BCC 24369 (Tapingkae *et al*., 2010b). Thus, these results show the ability of *Nnm. gari* BCC 24369 to produce HADH under completely non-sterile condition containing high concentration of salt (~25%) without contamination. Asker and Ohta (2002) noticed that contamination of the culture of *Hfx. alexandrinus* under non-sterile condition was precluded at NaCl concentrations ranging from 20 to 25% (w/v). At lower NaCl concentrations, the culture was contaminated with a white growth of other halo-tolerated microorganisms.

Table 6. Summary of HADH and cell biomass productions by *Nnm. gari* BCC 24369 at 1-l flask and 20-l polypropylene tank (with air flow rate of 9 l/min) scales under sterile and non-sterile conditions after 7 days of cultivation

Conditions	1-l Flask		20-l Polypropylene tank		
	HADH activity (U/g)	Cell biomass (g/l)	HADH activity (U/g)	Cell biomass (g/l)	HADH Yield (%)
Sterile	955 ± 18^{aA}	4.7 ± 0.5^{aA}	878 ± 15^{aB}	4.3 ± 0.5^{aA}	92 ± 2^{a}
Non-sterile	976 ± 20^{aA}	4.8 ± 0.3^{aA}	858 ± 12^{aB}	4.6 ± 0.3^{aA}	88 ± 3^{a}

Mean ± S.D. from a triplicate determination. Different letters (A,B) in the same response (HADH activity) indicate significant differences ($p < 0.05$).

2.5 Conclusion

The experimental designs presented in this study effectively defined optimal media composition, which supported HADH production by *Nnm. gari* BCC 24369. With optimized condition, HADH activity increased 2.2-fold as compared to un-optimized condition. The high-salt tolerance of *Nnm. gari* BCC 24369 enables its cultivation under non-sterile and thus, cost-reducing conditions. Scale-up of HADH production by *Nnm. gari* BCC 24369 from 1-l shake flask to 20-l polypropylene tank was successfully performed both under sterile and non-sterile conditions based on high aeration rate. Thus, *Nnm. gari* BCC 24369 have distinct advantages in biotechnological processes as cultivation is relatively easy, risk of contamination is minimal and culture size can be scaled-up.

CHAPTER 3

WHOLE CELL IMMOBILIZATION OF *NATRINEMA GARI* BCC 24369 HAVING HISTAMINE DEHYROGENASE ON DIATOMITE FOR HISTAMINE DEGRADATION

3.1 Abstract

This study aimed to immobilize *Natrinema gari* BCC 24369, an extremely halophilic archaeon onto diatomite and to determine the ability of the immobilized cells to degrade histamine at high concentration of salt. Based on Plackett-Burman design (PBD) experiment, the concentrations of cell and NaCl were found to be the key parameters for histamine-degrading activity of immobilized cells. Subsequent investigation by central composite design (CCD) suggested 10% (w/v) of free whole cell, 4 M of NaCl, 15% (w/v) of diatomite, initial pH 5.0, agitation rate at 100 rpm and immobilization time of 4 h as optimal conditions for whole cell immobilization. The immobilized cells exhibited the highest histamine degrading activity of 35.3±0.8 U/g support, which was close to the model predicted value of 34.8 U/g support. In a fixed-bed bioreactor, the immobilized cells of *Nnm. gari* BCC 24369 on diatomite effectively removed histamine in fish sauce and were found to have good operational stability. Although the histamine removal efficiency of immobilized whole cells gradually decreased but still remained over 80% at the 4th cycle and retained about 50% after nine consecutive cycles.

3.2 Introduction

Whole cell immobilization techniques have gained attention and are being successfully applied in the biotechnological processes (Sidira *et al*., 2014; Liouni *et al*., 2008; Reddy *et al*., 2008). These are mainly due to the numerous advantages including enhanced fermentation productivity, feasibility of continuous processing and stability, recovery and recycling of the cells (Xi and Xu, 2005; Stewart and Russel, 1986; Margaritis and Merchant, 1984). Moreover, the use of immobilized whole cells eliminates the tedious, time-consuming, and expensive steps involved in isolation and purification of intracellular enzymes (Ohmiya *et al*., 1977). Proper selection of immobilization techniques is needed to maximize the advantages of immobilization (Ramakrishna and Prakasham, 1999). The methods commonly used for immobilizing cells are adsorption, covalent binding, entrapment in gels, and cross-linking using various types of support. Adsorption is the elementary and probably the simplest method of reversible immobilization (Kregiel, 2014). It is most commonly used for attachment of cells in various biotechnological processes, in particular processing of food for human consumption where chemical safety is of concern (Kourkoutas *et al*., 2004).

Natrinema gari BCC 24369, a halophilic archaeon isolated from fish sauce, reduces histamine in the presence of high salt concentration up to 4.3 M NaCl (Tapingkae *et al*., 2008; 2010b). The histamine-degrading activity of *Nnm. gari* BCC 24369 is mediated through the presence of intracellular histamine dehydrogenase (HADH) that catalyzes the oxidative deamination of histamine, resulting in the production of imidazole acetaldehyde and an ammonium ion (Zhou *et al*., 2014; Tapingkae *et al*., 2010b). This enzyme is a heterotrimer and its activity requires high levels of salt and co-factors. Like other extremely halophilic bacteria, application of *Nnm. gari* BCC 24369 cells has been restricted by the slow growth rate, low yield, high cost of cell preparation and production of off-odors and off-flavors in finished product (Tapingkae *et al*., 2010a; Gram and Huss, 1996). Therefore, the immobilization of whole cells might provide potential advantages over applications of free whole cell and enzyme. Previously, Tapingkae *et al*. (2010a) successfully immobilized cells of *Nnm. gari* BCC 24369 on Celite® 545 in which celite-

immobilized whole cell exhibited high activity retention, stability, and potential to be applied for histamine degradation in high salt condition. However, concern has been raised on safety and GRAS (Generally Recognized As Safe) status of Celite® 545 particularly when the ultimate application is for human consumption. In this present study, food grade diatomite which is widely accepted for being used as a filtering aid in food was used as support for immobilization of whole cells *Nnm. gari* BCC 24369. Food grade diatomite is a light-weight and porous sedimentary rock, which possibly allows the cells to encapsulate within silica matrixes. Due to its low cost, and readily availability and highly developed porous structure, diatomite has found a range of commercial applications, including uses in filtration, insulation, absorption, mineral fillers, drug delivery and a fine abrasive in diverse industries (Aw *et al*., 2012; Yuan *et al*., 2010; Zhang and Wang, 2006; Murathan and Benli, 2005). Diatomite has been successfully applied as cell carriers in several biological processes, such as degradation of 3,4-dichloroaniline in wastewater, water treatment, and production of succinic acid (Corona-González *et al*., 2014; Portier *et al*., 2006; Livingston and Willacy, 1991; Portier and Miller, 1991).

Statistical experimental design techniques especially Plackett-Burman design (PBD) and response surface methodology (RSM) are widely used to optimize conditions for immobilization processes (Ebrahimi *et al*., 2010; Hung *et al*., 2008; Chang *et al*., 2007). By accounting the interaction effects of several variables in optimization, PBD is preliminarily used for screening of the significant factors (Plackett and Burman, 1946). The optimum levels of significant factors can be further optimized by using RSM (Fu *et al*., 2009; Dean and Voss, 2006). The objectives of this study were to optimize the condition for whole cell immobilization of *Nnm. gari* BCC 24369 using diatomite as the support and to evaluate the operational stability and efficiency of immobilized whole cells on histamine degradation in fish sauce.

3.3 Materials and methods

3.3.1 Chemicals

Histamine dihydrochloride and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Food grade riboflavin was purchased from EMD Chemicals Inc. (New Jersey, USA). Food grade diatomite with the average particle size of 50 μm and containing less than 1% of crystalline silica (OSHA, 1999) was obtained from Cernic International Co., Ltd. (Nakhon Pathom, Thailand).

3.3.2 Organism and culture conditions

Natrinema gari BCC 24369 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 optimized medium (pH 7.5) containing 15 g/l casamino acid, 75 g/l $MgSO_4.7H_2O$, 273 g/l NaCl, 2.5 mg/l $FeCl_24H_2O$, 10 g/l yeast extract, 5 g/l sodium glutamate and 5 g/l KCl in 500-ml Erlenmeyer flask and incubated at 37°C in a shaker incubator at 200 rpm for 7 days.

3.3.3 Immobilization method

Cells were harvested by centrifugation of cultured broth at 10,000×*g* for 10 min at 4°C. The pellet was washed twice with 2 M NaCl. For the immobilization studies, the experiments were taken place in 250-ml Erlenmeyer flask with the working volume of 100 ml. Cell pellet at different concentrations was resuspended in phosphate-citrate buffer containing NaCl and diatomite at different concentrations (Table 7). The mixtures were continuously stirred on a magnetic stirrer (Cole Parmer, 04644 Series, Vernon Hills, IL, USA) at various speeds at 4°C up to 7 h. The immobilized whole cell onto diatomite were filtered, washed 3 times with the same buffer used during immobilization, dried at 40°C for 6h and kept at 4°C until

used. The immobilization yield was calculated as the percentage ratio of the total activity of immobilization whole cells to the total activity of free whole cells.

3.3.4 Histamine-degrading activity assay

Histamine-degrading activity was determined by measuring the decreased amount of histamine after reaction. The dried immobilized whole cell (0.1 g) was added into 1 ml of standard assay mixture consisting of 50 mM Tris-HCl buffer, pH 7.0, 2 M NaCl, 500 μM 1-methoxy PMS and 5 mM histamine dihydrochloride. The reaction mixture was incubated at 37°C for 1 h. Enzymatic reaction was terminated by adding 1 ml of 0.2 M HCl. Blank was done in the same manner, except the immobilized whole cell was added after addition of 1 ml of 0.2 M HCl. The histamine contents of reaction and blank were determined by the fluorometric method of AOAC (2005a). One unit (U) of activity was defined as the amount of enzyme necessary to degrade 1 μmol of histamine per h per g diatomite under the specified condition.

3.3.5 Screening of significant variables by PBD

Effect of six parameters, including the concentrations of diatomite, free whole cell of *Nnm. gari* BCC 24369 and NaCl, stirred speed, initial pH and immobilization time, on histamine-degrading activity was evaluated by PBD. This design gave an output of 13 experimental runs (1 run at center point) with 6 independent variables which was generated by Design Expert (Table 7). All the experiments were performed in triplicate and the average of histamine degrading activity of immobilized whole cells was used as the responses (dependent variables). The response obtained in these 13 experiments was subjected to statistical analysis. The main effect of each variable was calculated as the difference between the average of measurements made at the high value (+) and at the low value (-) of that factor. The components having confidence level greater than 95% were the most significant and were taken up for further optimization studies. Insignificant components were kept at the constant level (Natarajan and Rajendran, 2012). Effect of each variable on the histamine-degrading activity was evaluated by the ratio of the difference between the

average response in the higher level of a given component and its lower level to the number of trial N. The effect of the given component on the response factor S_i, is

$$\text{Effect} = 2[\Sigma S_i^{+} - \Sigma S_i^{+}]/N \quad (4)$$

where S_i^{+} is the response parameter of an assembly in the screening design that contains the higher quantity of a given component, S_i^{-} is the response parameter of an assembly in the screening design that contains the lower quantity of a given component and 'i' varies from 1-6.

3.3.6 Optimization of screened factors by CCD

CCD was applied to determine the optimum level of the significant factors identified by PBD. A 13-run experiment generated by Design Expert were carried out with 5 trials at the center point (Table 8). All experiments were conducted in triplicate. The mean value of histamine-degrading activity (U/g support) was taken as the response. A multiple regression analysis was applied to the data obtained. The behavior of the system was explained by the following quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (5)$$

where Y is the predicted response, β_0 is the offset term, β_i is the linear offset, β_{ii} is the squared offset and β_{ij} is the interaction effect, while X_i and X_j denote the coded values of the variables. All experiments were conducted in triplicate and their mean values were reported. The developed regression model was evaluated by analyzing the values of regression coefficients, analysis of variance (ANOVA), *p*- and *F*-values. The quality of fit of the polynomial model equation was expressed by the coefficient of determination, R^2. The statistical software package Design-Expert was used to identify the experimental design as well as to generate a regression model to predict the optimum immobilization conditions on histamine degrading activity. A final experiment was conducted to validate the CCD model developed.

3.3.7 Scanning electron microscopy (SEM) of diatomite and immobilized whole cells of *Nnm. gari* BCC 24369 on diatomite

For SEM analysis, diatomite and immobilized whole cells of *Nnm. gari* BCC 24369 were rinsed twice 0.1 M phosphate buffer containing 4 M NaCl (pH 5.0).

The wet samples were then fixed for 2 h by incubating in the same buffer containing 2.5% glutaraldehyde, followed by overnight at 4^{o}C. The samples were washed twice with 0.1 M phosphate buffer (pH 5.0) for 10 min per each and once with distilled water for 10 min. The samples were dehydrated with a graded series of ethanol (30, 50, 70, 95 and 100%) for 10 min per each. These were then dried using critical point dryer (Balzers, CPD 020, Balzers Union Ltd., Liechtenstain), mounted on aluminum stubs, and sputter-coated using sputter coater (Balzers, SCD 040, Balzers Union Ltd., Liechtenstain) with a gold layer and used for scanning. Samples were scanned in scanning electron microscope (JEOL, JSM-5410LV, JEOL Ltd., Japan) with an accelerating voltage of 15 kV.

3.3.8 Operational stability and efficiency of immobilized whole cells on histamine degradation in fish sauce

The reusability and efficiency of immobilized of *Nnm. gari* cells were assessed by carrying out repeated degradation of histamine in fish sauce in a fixed-bed bioreactor packed with immobilized whole cells. A glass column with a diameter of 2.5 cm and a height of 23 cm was packed with 100 g of immobilized whole cells to obtain a bed height of 20 cm. Commercial fish sauce with known histamine concentration (600 ppm) was supplemented with 10 mg% (w/v) riboflavin using as electron acceptor and adjusted to pH 6.5. The supplemented fish sauce (1 l) was continuously pumped upward from the bottom into the column at an influent flow rate of 0.5 ml/min using a peristaltic pump. Operational temperature of a fixed-bed bioreactor was controlled at an ambient temperature (28-30°C). The holding volume of column and time for each cycle were set at 100 ml and 100 min, respectively. The effluent fish sauce samples were collected from the top of column. The effluent fish sauce from each cycle (100 ml) was taken and analyzed for histamine contents by the fluorometric method of AOAC (2005a). The histamine removal efficiency in each cycle was calculated and expressed relatively to that of the first cycle (100%).

3.3.9 Statistical analysis

Design-Expert (STAT-EASE Inc., Minneapolis, MN, USA) was used for the experimental designs and statistic analysis of the experimental data. 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., Chicago, IL, USA).

3.4 Results and Discussion

3.4.1 Screening of significant variables by PBD

A 6-factor-13-run (including 1 center point) PBD was used, and the response for each run is present in Table 7. In order to determine the influence of the most important factors, a standardized Pareto chart (Figure 15) was employed. Among all factors studied, free whole cell and NaCl concentrations had positively significant effects on immobilized histamine-degrading activity in the range of evaluated levels in PBD ($p < 0.05$). Immobilization by adsorption relies on the inherent tendencies of the cells to adhere to particular surfaces, or to flocculate, or to settle in the pores of the framework (Akin, 2007). Such immobilization is usually achieved by keeping the support material and the actively growing cells in contact for a specific length of time. Cell loading of the support and adhesion behavior are limiting factors (Klein and Wagner, 1983) which are influenced by the physical and chemical parameters of the adsorption matrix, the microorganisms which should be immobilized especially the outer surface of their cell wall and finally, the composition and the fluid conditions of the surrounding liquid phase (Klein and Ziehr, 1990). For extremely halophilic microorganisms, the negative surface charges of the acidic amino acids and sugar moieties on their cell wall are shielded by sodium ions in environment which are absolutely essential for maintaining cellular integrity (Madigan *et al*., 2000). These might be an advantage of NaCl on stability of *Nnm. gari* BCC 24369 cells and their adsorption on diatomite during immobilization process. Therefore, optimal levels of whole cell and NaCl concentrations were

Table 7. Plackett-Burman experimental design and the experimental data

Run no.	Variables						Immobilized activity (U/g support)
	X_1 (%)	X_2 (%)	X_3 (M)	X_4 (rpm)	X_5	X_6 (h)	
1	5	5	2	100	5	1	19.1±0.6
2	5	15	5	100	7	7	28.3±4.8
3	5	5	2	200	5	7	15.0±2.4
4	5	5	5	100	7	7	22.6±1.4
5	15	5	5	200	5	7	21.8±0.9
6	15	15	2	200	7	7	27.6±0.6
7	15	15	2	100	5	7	29.5±0.1
8	15	15	5	100	5	1	29.7±0.2
9	15	5	5	200	7	1	24.0±0.2
10	5	15	2	200	7	1	22.9±0.9
11	15	5	2	100	7	1	16.4±0.1
12	5	15	5	200	5	1	27.7±0.4
13	10	10	3.5	150	6	4	25.4±2.8

X_1 = Diatomite concentration; X_2 = Free whole cell concentration; X_3 = NaCl concentration; X_4 = Stirred speed; X_5 = Initial pH; X_6 = Immobilization time

evaluated in a wider range by CCD. Other variables including diatomite concentration, stirred speed, immobilization time and initial pH had no significant effect on histamine-degrading activity ($p > 0.05$). Hence, no further optimization of these factors was carried out. Initial pH and stirred speed had negative effects for histamine-degrading activity. Thus, they were set at their lower levels. Diatomite concentration had positive effect on activity. As a result, it was set at its higher level and immobilization time was set at its center level.

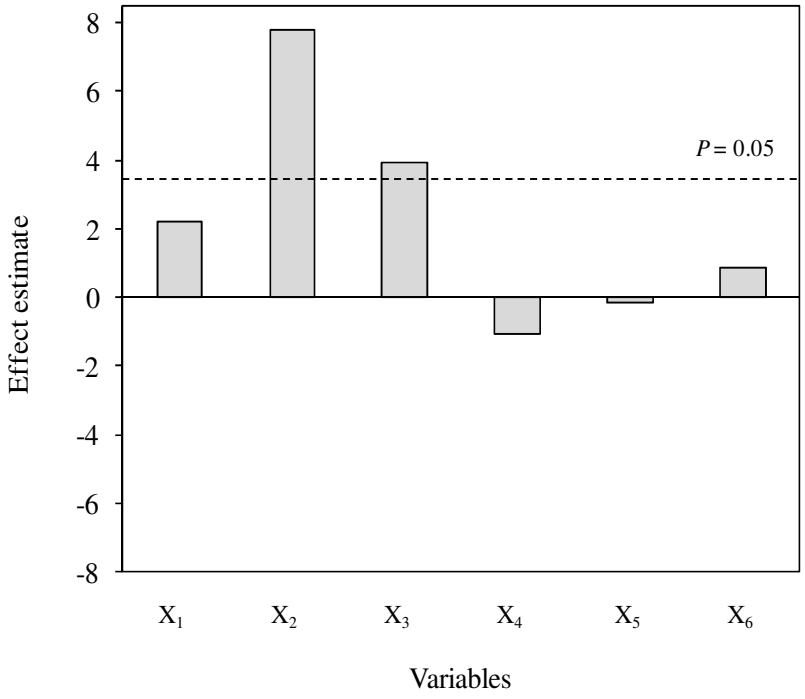

Figure 15. Pareto chart of standardized effects on immobilized histamine-degrading activity (X_1 = Diatomite concentration; X_2 = Free whole cell concentration; X_3 = NaCl concentration; X_4 = Stirred speed; X_5 = Initial pH; X_6 = Immobilization time).

3.4.2 Optimization by response surface methodology

Among the 13 experiments, the conditions used in experiment no. 1 showed the highest immobilized activity ($p < 0.05$, Table 8). Fitting of the data with various models indicated that immobilization efficiency of whole cells of *Nnm. gari* BCC 24369 on diatomite was the most suitably described with quadratic polynomial model with significant terms given as follows:

$$Y = 52.86 - 0.35A - 6.00B + 0.16AB - 0.029A^2 + 0.38B^2 \quad (6)$$

where Y is the predicted response for histamine-degrading activity (U/g support), A and B are the coded values for free whole cell and NaCl concentrations, respectively.

The quadratic polynomial model was highly significant and sufficient to represent the actual relationship between the response and significant parameters. An *F*-test was statistically valid with very low probability value ($p < 0.05$) (Table 9).

Table 8. CCD design with the predicted and experimental data

Experiment no.	Variables		Immobilized activity (U/g support)	
	Free whole cell (%, w/v)	NaCl (M)	Experimental	Predicted
1	10.0	4.0	35.3±0.8	34.8
2	20.0	4.0	28.7±1.7	29.1
3	10.0	5.0	33.7±0.3	33.4
4	20.0	5.0	28.7±0.3	29.8
5	7.93	4.5	34.2±0.8	34.7
6	22.07	4.5	28.0±0.8	27.6
7	15.0	3.79	32.5±0.6	32.9
8	15.0	5.21	33.0±0.1	32.6
9	15.0	4.5	32.1±0.2	32.6
10	15.0	4.5	33.2±2.0	32.6
11	15.0	4.5	32.2±0.4	32.6
12	15.0	4.5	32.2±0.4	32.6
13	15.0	4.5	32.3±0.1	32.6

Mean ± S.D. from triplicate determinations.

The determined coefficient (R^2) of model was 0.95, which indicated that 95% of variability in the response could be explained by this model. The R^2 value of 0.95 was reasonably agreed with the adjusted R^2 value of 0.92. The model *p*-value of 0.0001 (desired < 0.05) indicated that the model terms were significant. Low "*Prob* > *F*" value indicated that model terms were significant. In this case, two model terms of the second-order terms (A^2) and the first-order term of free whole cell concentration (A) were significant. The coefficient of variation of 1.94% (desired < 30%) indicated a good precision and reliability of the experiments carried out. The adequate precision measured the signal-to-noise ratio of 17.7 (desired > 4), suggesting an adequate signal. This confirmed that the accuracy and general ability of the quadratic model was good with the reasonable analysis of the associated response trends.

Three-dimensional response surface curves were plotted to study the interaction between the two selected factors and to determine the optimum values for

Table 9. Analysis of variance (ANOVA) for the quadratic model* for histamine-degrading activity

Source	Sum of squares	*F*-value	*P*-value Prob > *F*
Model	56.57	29.34	0.0001
X_2- Free whole cell	51.86	134.49	<0.0001
X_3- NaCl	0.100	0.26	0.6268
$X_2 X_3$	0.64	1.66	0.2386
X_2^2	3.72	9.65	0.0172
X_3^2	0.061	0.16	0.7023
Residual	2.70		
Lack of fit	1.88	3.06	0.1544
Error	0.82		
Total	59.27		

*R^2 = 0.95; adj. R^2 =0.92; CV = 1.94%; adequate precision ratio = 17.7. $X_2 X_3$ represents the interaction effect of the variables; X_2^2 and X_3^2 are the squared effects of the variables. The values of "Prob > *F*" less than 0.05 indicate the variables are significant.

maximum immobilized activity (Figure 16). The immobilized activity was affected by the concentrations of free whole cell and NaCl. There is no significant interaction between the immobilization parameters studied. In the range of study, the immobilized activity increased with increasing concentration of free whole cell from 8 to 10% (v/w) and decreased upon further increasing from 10 to 22% (w/v). Similarly, immobilized activity increased upon increasing concentration of NaCl from 3.8 to 4 M and slowly decreased when NaCl increased from 4 to 5 M. The optimal levels of the two variables determined by Design Expert were 10% (w/v) of free whole cell and 4 M of NaCl, corresponding to the predicted maximum immobilized activity of 34.8 U/g support. Histamine degrading activity of 35.3±0.8 U/g support and immobilization yield of 95.1±3% were obtained under the optimal condition (n = 3). Verification experiments confirmed the validity of the predicted model. As a result, the model from CCD was considered to be accurate and reliable for predicting the

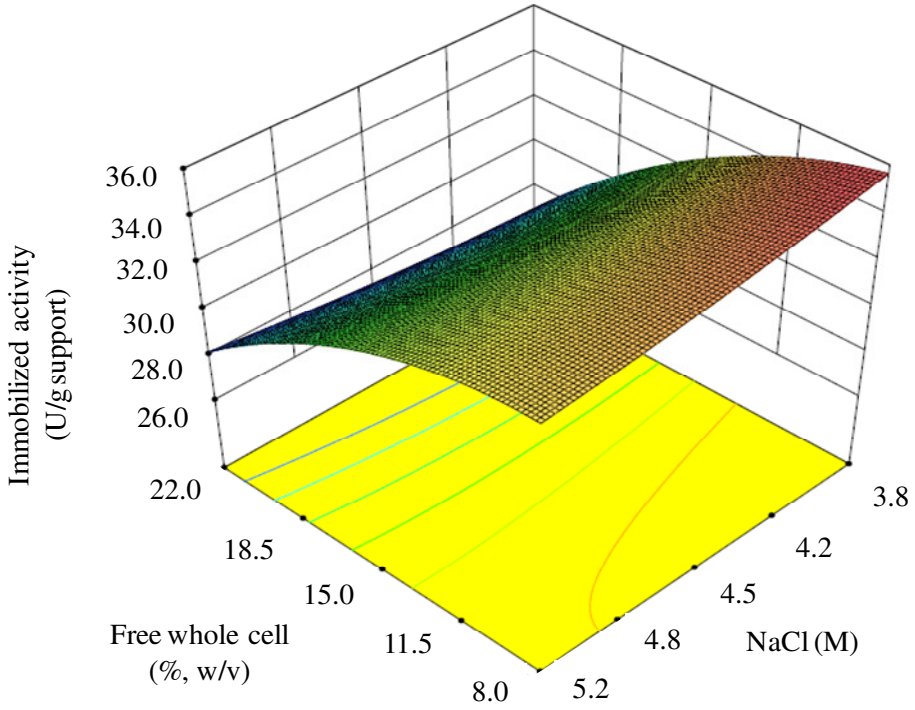

Figure 16. Response surface plot of the combined effects of free whole cell and NaCl concentrations on the histamine-degrading activity.

histamine degrading activity for whole cell immobilization of *Nnm. gari* BCC 24369 by adsorption on diatomite. The histamine-degrading activity of immobilized whole cells on diatomite increased about 2-fold as compared with immobilized whole cells on Celite® 545 (17.5±0.6 U/g support) (Tapingkae *et al*., 2010a). Similarly, Portier and Miller (1991) successfully immobilized microbial populations on a porous diatomite carrier to reduce toxic concentration of volatile organics, pesticides, petroleum aliphatics and aromatics from industrial effluent. Diatomite contains silanol groups (Si-OH) that are able to form hydrogen bonds on surface and therefore provides colonization potential, surface and structural integrity for the cell attachment (Lim *et al*., 2014; Yuan *et al*., 2004; Portier and Miller, 1991). The other characteristics that made diatomite suitable for immobilization are low density, high porosity, low thermal conductivity, high melting point and chemical inertness (Lopez *et al*., 2005). In this work, experimental designs were as a whole proved to be

adequate for the design and optimization of immobilized cell production for industrial scale.

3.4.3 SEM of diatomite and immobilized whole cells of *Nnm. gari* BCC 24369

Figure 17a shows the morphology of the support material, diatomite. Diatom shell is characteristic of macroporous and/or mesoporous structure and excellent thermal and mechanical stabilities (Zhang and Wang, 2006; Murathan and Benli, 2005; Yuan *et al*., 2004). The surfaces and structures are very rough and constructed with many holes and cavities, suggesting that this support is porous enough to provide the space for cell growth, protect cells and allow easy cells entrance during the immobilization step. Electron microscopic examination of whole cells immobilized on diatomite (Figure 17b), confirmed that some of cells were firmly adsorbed onto the surface of the support and also infiltrated into the pores of diatomite structures.

3.4.4 Operational stability and efficiency of immobilized whole cells on histamine degradation in fish sauce

The operational stability of immobilized whole cells was assessed by repeated using the immobilized cells for a total number of ten cycles and the results obtained are summarized in Figure 18. With increasing repetitions, the histamine removal efficiency of immobilized whole cells gradually decreased but still remained over 80% at the 4th cycle. The immobilized cells were found to have good operational utility because even after nine consecutive cycles, it retained about 50% of its initial activity in bound form. Thus, the immobilized cells will improve the operating cost effectiveness of cell. The immobilized whole cells of *Nnm. gari* BCC 24369 on diatomite packed in a fixed-bed bioreactor could be able to reduce histamine in fish sauce by 50% (294±13 ppm) when compared to that of control (600 ppm). Loss in activity upon reusing immobilized preparation is a general observation. A decrease in histamine removal efficiency of immobilized cells may be caused by desorption of cells from the support during the repeated cycles. Although simple physical adsorption presents lower effectiveness in terms of cell retention, this method offers

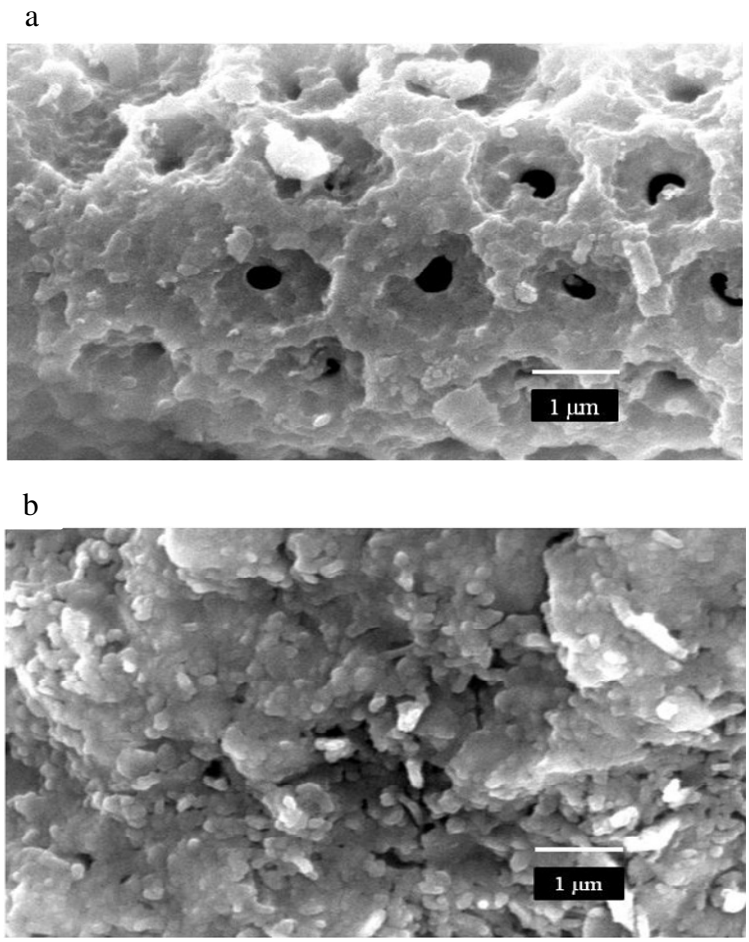

Figure 17. Scanning electron micrographs of diatomite (a) and immobilized whole cells of *Nnm. gari* BCC 24369 on diatomite (b).

several advantages such as simplicity, low cost, and lesser concern about safety and GRAS status of chemicals involved in immobilization process. In addition, the most impressed advantage of immobilized cells which is of practical utility for commercial purposes is that they can be separated from the soluble reaction products and reactants so that cost of process can be significantly reduced during repeated use.

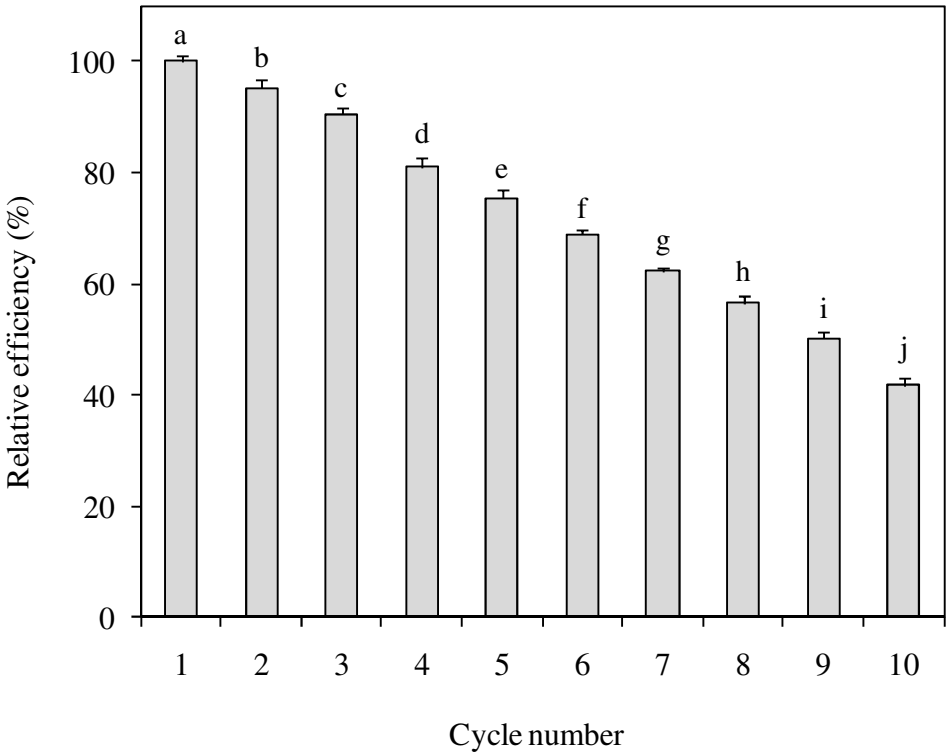

Figure 18. Operational stability and efficiency of immobilized whole cells of *Nnm. gari* BCC 24369 on histamine degradation in fish sauce. Bars represent the standard deviation (n = 3). Different letters on the bar denote significant differences ($p < 0.05$).

3.5 Conclusion

Statistical experimental designs based on PBD and CCD were found to be useful to optimize the condition for whole cell immobilization of *Nnm. gari* BCC 24369 onto diatomite. The optimum immobilization conditions were 10% (w/v) of free whole cell, 4 M of NaCl, 15% (w/v) of diatomite, initial pH 5.0, stirred speed at 100 rpm and immobilization time of 4 h. In an upward fixed-bed flow bioreactor packed with immobilized cells, a significant amount of histamine in fish sauce was removed (50%). Immobilization of *Nnm. gari* BCC 24369 cells onto diatomite offers several advantages, including the maintaining histamine-degrading activity, operational stability and reduced downstream processing costs of cell recovery and recycle.

CHAPTER 4

FIXED-BED DEGRADATION OF HISTAMINE IN FISH SAUCE BY IMMOBILIZED WHOLE CELLS OF *NATRINEMA GARI* BCC 24369

4.1 Abstract

Fish sauce often contains high level of histamine which becomes a major threat of exporting due to a more strenuous regulatory measure and inspection and is also associated with the food poisoning. Therefore, this study aimed to apply the ability of immobilized whole cells of *Natrinema gari* BCC 24369 onto diatomite to degrade histamine in fish sauce. Under static condition, *Nnm. gari* BCC 24369 cells immobilized on diatomite (10%, w/v) exhibited the optimal efficiency (24±1%) for histamine removal in fish sauce (pH 6.5) supplemented with riboflavin (0.01%, w/v) for 2 h incubation. The fixed-bed bioreactor was performed to assess the performance of immobilized cells of *Nnm. gari* BCC 24369 to degrade histamine in fish sauce. The operation of fixed-bed bioreactor in continuous mode was more promising than its operation under batch mode with reducing the operating time. In continuous fixed bed bioreactor under the optimal feeding flow rate of 0.5 ml/min, a significant amount of histamine (51±3%) was removed without adverse effects on quality and organoleptic properties of fish sauce. Continuous fixed-bed bioreactor packed with immobilized cells not only efficiently removed histamine but also retarded color darkening without detrimental effect on the unique quality of fish sauce during 6 months storage.

4.2 Introduction

High levels of histamine present in fish sauce are undesirable because they have adverse physiological effects on the human health. With a concern on the toxicological effects, high content of histamine in commercial fish sauce becomes a major threat of export, due to a more strenuous regulatory measure and inspection. Histamine is regulated in fish sauce at a maximum of 400 ppm by Codex (Codex, 2012). Histamine is heat stable and not detectable organoleptically by even trained panelists (Arnold *et al*., 1980). The emerging methods for controlling the histamine formation in food products are such as modified atmosphere packaging, irradiation, high hydrostatic pressure, the addition of food additives and preservatives, and the using of histamine-negative starter cultures (Chong *et al*., 2011). However, the mechanism underlying those methods is mainly the inhibition of the growth of histamine-producing bacteria and histidine decarboxylase activities. The attempts to remove histamine in foods without causing detrimental effects on quality and safety remain an emerging niche for fish sauce manufactures. (Zaman *et al*., 2014).

Natrinema gari BCC 24369, a halophilic archaeon isolated from fish sauce, reduces histamine in the presence of high salt concentration up to 4.3 M NaCl (Tapingkae *et al*., 2008, 2010b). The histamine-degrading activity of *Nnm. gari* BCC 24369 is mediated through the presence of intracellular histamine dehydrogenase that catalyzes the oxidative deamination of histamine, resulting in the production of imidazole acetaldehyde and an ammonium ion (Zhou *et al*., 2014; Tapingkae *et al*., 2010b). The immobilized cells of *Nnm. gari* BCC 24369 were more promising than the free whole cells and enzyme in which they exhibited high activity retention, stability, and thus increasing the potential application for histamine degradation in products high in salt such as fish sauce (Tapingkae *et al*., 2010a). To enhance the potential use of immobilized whole cells for food applications, this present study focused on food grade support, diatomite for immobilization of whole cells of *Nnm. gari* BCC 24369 to degrade histamine at high concentrations of salt. Due to its low cost, and readily availability and highly developed porous structure, diatomite has been applied as cell carriers in several biological processes (Corona-González *et al*., 2014; Portier *et al*., 2006; Portier and Miller, 1991).

The application of immobilized whole cells is frequently carried out in a fixed-bed flow process consisting of bioreactor packed with immobilized cells which is perfused by a liquid flow (Chen *et al*., 2014; Zhu, 2007). Due to simplicity of design and operation, fixed-bed bioreactor can be relatively easily scaled up to an industrial scale (Wu and Yu, 2008). For industrial applications, upward flow is generally preferred over downward flow because it does not compress the immobilized bed in the bioreactor as downward flow does (Riitonen *et al*., 2013). Therefore, the upward fixed-bed flow bioreactors with immobilized cells have been used in wide spectrum of applications, such as biodegradation, biosorption, biodecolorization and bioproduction (Mona *et al*., 2013; Saratale *et al*., 2011; Chen *et al.,* 2010; Vijayaraghavan *et al*., 2008; Wu and Yu, 2008). The main operating parameter in fixed-bed bioreactor is the liquid flow rate which influences residence time for interaction between the solute and the stationary phase in the bioreactor (Vu and Seidel-Morgenstern, 2011; Wu and Yu, 2008; Obradovic *et al*., 2004). Other parameters including initial solute concentration, operating temperature and bed length were also investigated for performance evaluation of fixed-bed bioreactor packed with immobilized cells (Chen *et al*., 2010, 2014).

The main objective of this study is to assess the ability of immobilized whole cells of *Nnm. gari* BCC 24369 onto food grade diatomite for histamine degradation in fish sauce. Firstly, the effect of operational conditions on the degradation capacity of immobilized cells was investigated under static conditions. Secondly, the performance of fixed-bed flow bioreactor packed with immobilized cells of *Nnm. gari* BCC 24369 was examined under batch and continuous operations. Thirdly, the physicochemical characteristics and sensory acceptability of fish sauce after treated with fixed-bed bioreactor were carried out to evaluate the quality of the final fish sauce. Finally, the long-term stability of fish sauce after treatment with a fixed-bed bioreactor was evaluated during storage at ambient temperature (28-32°C) for 6 months.

4.3 Materials and Methods

4.3.1 Chemicals

Histamine dihydrochloride, 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Food grade riboflavin was purchased from EMD Chemicals Inc. (New Jersey, USA). Food grade diatomite with the average particle size of 50 μm was obtained from Cernic International Co., Ltd. (Nakhon Pathom, Thailand). Commercial fish sauce was obtained from the Thai Fish Sauce Factory (Squid Brand) Co., Ltd. (Samutsongkram, Thailand). Commercial fish sauce added with histamine to obtain the final histamine concentration of 600 ppm was used through this study.

4.3.2 Cultures and conditions

Nnm. gari BCC 24369 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 (Sartorius, Certomat® BS-1, Göttingen, Germany) at 200 rpm for 7 days. Cells were cultivated by inoculating 5% (v/v) of seed cultures into 200 ml of optimized medium (pH 7.5) containing 15 g/l casamino acid, 75 g/l $MgSO_4.7H_2O$, 273 g/l NaCl, 2.5 mg/l $FeCl_24H_2O$, 10 g/l yeast extract, 5 g/l sodium glutamate and 5 g/l KCl in 500-ml Erlenmeyer flask and incubated at 37°C in a shaker incubator at 200 rpm for 7 days.

4.3.3 Immobilization method

Cells were harvested by centrifugation of cultured broth at 10,000×*g* for 10 min at 4°C. The pellet was washed twice with 2 M NaCl. After washing, cell pellet (10 g) was resuspended in 0.1 M phosphate-citrate buffer (pH 5) containing 4 M NaCl to obtain the final volume of 50 ml. The cell suspension was added into 50 ml of 0.1 M phosphate-citrate buffer (pH 5) containing 4 M NaCl and diatomite (15 g) in 250-ml Erlenmeyer flask. The mixture was continuously stirred on a magnetic stirrer (Cole Parmer, 04644 Series, Vernon Hills, IL, USA) with speed of 100 rpm at

4°C for 4 h. The immobilized whole cells were filtered, washed and kept at 4°C until used.

4.3.4 Histamine degradation in fish sauce by immobilized cells of *Nnm. gari* BCC 24369 under static condition

To evaluate the effect of operational and environmental conditions on the histamine removal efficiency of immobilized whole cells, the experiments were carried out at different concentrations of immobilized cell (0-20%, w/v), packing in nylon tea bags with size 60x60 mm and riboflavin (0-10 mg%, w/v, using as electron acceptor for histamine dehydrogenase activity), agitation speeds (0-150 rpm), pH values of fish sauce (5-8), and incubation time (0-24 h). Histamine degradation performance of immobilized *Nnm. gari* BCC 24369 on diatomite was studied in 250-ml Erlenmeyer flask containing 100 ml of fish sauce, except for the experiments investigating the effect of incubation time which was studied in 2-l Erlenmeyer flask containing 1 l of fish sauce. The operating temperature was controlled at an ambient temperature. All experiments were performed in triplicates. Controls (without immobilized whole cells) were always included. Histamine removal efficiency (%) is the percentage ratio of the removal of histamine concentration to initial histamine concentration in fish sauce.

4.3.5 Histamine degradation in fish sauce by immobilized whole cells packed in a fixed-bed bioreactor

For fixed-bed bioreactor preparation, a glass column used as bioreactor with a diameter of 2.5 cm and a height of 23 cm was packed with the immobilized whole cells (100 g) to obtain a particular bed height of 20 cm. The column was connected to the flow line of a peristaltic pump as shown in Figure 19. Operational temperature of a fixed-bed bioreactor was controlled at an ambient temperature. Fish sauce (pH 6.5) was supplemented with riboflavin (10 mg%, w/v) and pumped upward from the bottom into the column. The performance of immobilized in fixed bed bioreactor for histamine degradation was evaluated under both batch and continuous operations. For batch operation, the supplemented fish sauce (100 ml) was pumped

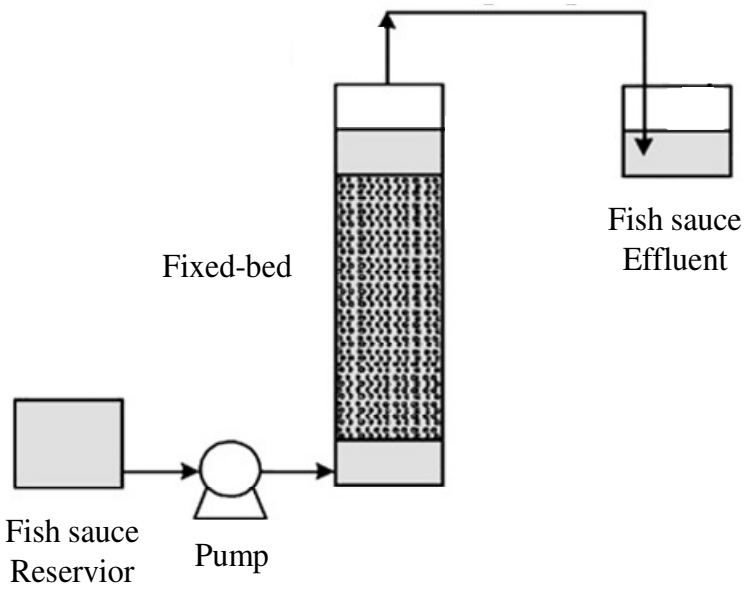

Figure 19. Schematic description of an upward fixed-bed flow column packed with immobilized whole cell of *Nnm. gari* BCC 24369 for histamine degradation in fish sauce.

into column at flow rate of 2 ml/min. The residence time of fish sauce in a fixed-bed bioreactor was set at 1 h per cycle. The effluent fish sauce samples were collected from the top of column. The column was washed thoroughly with 2 M NaCl (100 ml) by controlling flow rate at 2 ml/min, before use in the next cycle. The column was reused up to 10 cycles. Continuous operation was run in the same manner except no washing with 2 M NaCl. The fish sauce effluent from each cycle was analyzed and subsequently pooled for further determination of residual histamine content. To study the optimum influent flow rate of fish sauce under continuous mode, the supplemented fish sauce (1 l) was continuously pumped into the column at various flow rates (0.1-2 ml/min). The fish sauce effluent from each cycle was analyzed and subsequently pooled for further determination of residual histamine content, biogenic amine profile, free and total amino acids profile, pH value, total nitrogen content, color and sensory evaluation.

4.3.6 Scanning electron microscopy (SEM) of diatomite and immobilized whole cells of *Nnm. gari* BCC 24369 on diatomite

For SEM analysis, diatomite and immobilized whole cells of *Nnm. gari* BCC 24369 were rinsed twice 0.1 M phosphate buffer containing 4 M NaCl (pH 5.0). The wet samples were then fixed for 2 h by incubating in the same buffer containing 2.5% glutaraldehyde, followed by overnight at 4^{o}C. The samples were washed twice with 0.1 M phosphate buffer (pH 5.0) for 10 min per each and once with distilled water for 10 min. The samples were dehydrated with a graded series of ethanol (30, 50, 70, 95 and 100%) for 10 min per each. These were then dried using critical point dryer (Balzers, CPD 020, Balzers Union Ltd., Liechtenstain), mounted on aluminum stubs, and sputter-coated using sputter coater (Balzers, SCD 040, Balzers Union Ltd., Liechtenstain) with a gold layer and used for scanning. Samples were scanned in scanning electron microscope (JEOL, JSM-5410LV, JEOL Ltd., Japan) with an accelerating voltage of 15 kV.

4.3.7 Storage stability of fish sauce after treated with immobilized whole cells of *Nnm. gari* BCC 24369 packed in a fixed-bed bioreactor

The effect of immobilized cell treatment on the quality of fish sauce was evaluated during prolonged storage at ambient temperature (28-32^{o}C). The fish sauce before and after treatment were stored in screw cab glass bottles for 6 months. The samples were taken once monthly for determination of free and total amino acids contents, pH value, total nitrogen content and color evaluation. Experiments were carried out in triplicate.

4.3.8 Determination of histamine in fish sauce

Histamine was analyzed by AOAC (2005a) (Appendix B-1). The fish sauce sample (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 measured using the RF-1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan) at an excitation wavelength of 350 nm and emission wavelength of 444 nm. The histamine removal efficiency in each cycle and pooled fish sauce was calculated as the percentage removal of histamine in fish sauce.

4.3.9 Determination of biogenic amines

Tryptamine, β-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, spermine were extracted from fish sauce samples and derivatized 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-3.

4.3.10 Determinations of free and total amino acids

The seventeen amino acids; alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine were determined by the method described by Aristoy and Toldrá (1991) with some modifications (Appendix B-4).

4.3.11 Determinations of total nitrogen content and pH value

Total nitrogen content of fish sauce samples was measured using the Kjeldahl method (AOAC, 2005b). Total nitrogen content was expressed as g nitrogen/l. The pH value was measured by pH meter (Mettler Toledo, Gießen, Germany).

4.3.12 Color evaluation

The color of the fish sauce samples after treated by fixed-bed bioreactor packed with immobilized whole cells of *Nnm. gari* BCC 24369 was measured in the *L** (black-white component, luminosity), *a** (+red to -green component) and *b** (+yellow to -blue component) mode of CIE by UV/VIS/NIR

spectrophotometer (Lambda 950, Perkin Elmer Ltd., Waltham, MA, USA). Chroma (*c*), hue angle (*h*), browning index (*BI*) and color difference index (ΔE) were calculated was calculated from L^*, a^* and b^* color coordinates. Chroma represents color saturation which varies from dull (low value) to vivid color (high value) and hue angle is a qualitative indicator of the chromatic nature of color and defined as a color wheel with red-purple at an angle of 0°, yellow at 90°, bluish-green at 180°, and blue at 270° (McGuire, 1992). The ΔE was used to compare color changes in samples between at initial storage and each storage interval. The values for the above were computed using the following equations (Nasar-Abbas *et al*., 2008; Palou *et al*., 1999; Gonnet, 1993):

$$c = \sqrt{(\Delta a^*)^2 + (\Delta b^*)^2} \quad (7)$$

$$h = \arctan (b^*/ a^*) \quad (8)$$

$$BI = [100 (x - 0.310)] / 0.172 \quad (9)$$

$$x = (a^* + 1.75L^*) / (5.645 L^* + a^* - 3.012 b^*) \quad (10)$$

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (11)$$

4.3.13 Sensory evaluation

Fish sauce samples were evaluated for acceptance by an untrained 30-member panel according to the method of Chambers 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: color, aroma, flavor and overall acceptance using the nine-point hedonic scale. A nine-point hedonic scale was used for evaluation in which a score of 1 = dislike extremely, 5 = neither like nor dislike and 9 = like extremely. For color, fish sauce samples (50 ml) were contained in commercial glass bottles. 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 (Sanceda *et al*., 1994). For the taste, half a teaspoon of each sample was given to the subjects, one after the other, and they rinsed their mouths between samples by room temperature drinking water (Sanceda *et al*., 1994). Samples were coded with three-digit random

numbers and were presented to the panelists at ambient temperature. The ratings of each attribute were converted to numerical scores for further statistical analysis.

4.3.14 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance and mean comparison was carried out using Duncan's Multiple Range Test (Steel and Torrie, 1980). All data were analyzed by one-way analysis of variance (ANOVA) using the statistical Package for Social Sciences (SPSS for Windows: SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 Histamine removal efficiency of diatomite-immobilized cells under static conditions

The effect of various physicochemical conditions including agitation, immobilized cell and riboflavin concentrations, pH of fish sauce, and incubation time on the degradation of histamine by using *Nnm. gari* BCC 24369 cells immobilized on diatomite is shown in Figure 20. Histamine removal efficiency of immobilized cells under shaking conditions was lower than that under non-shaking condition ($p < 0.05$, Figure 20a). A similar decrease in efficiency of immobilized whole cells under shaking conditions was reported (Daâssi *et al*., 2013; Saratale *et al*., 2011). Under the shaking conditions, cells that are not strongly adsorbed are readily desorbed from the support during utilization, associated with loss in enzyme activity. Thus, static conditions were adopted to investigate the histamine degradation in the following experiments.

The optimum concentration of immobilized whole cell was observed at 10% (w/v, Figure 20b). As immobilized cell concentration increased, histamine removal efficiency gradually increased until reaching the maximum. Above 10% (w/v) of the immobilized whole cell, no changes in histamine removal efficiency were noticed.

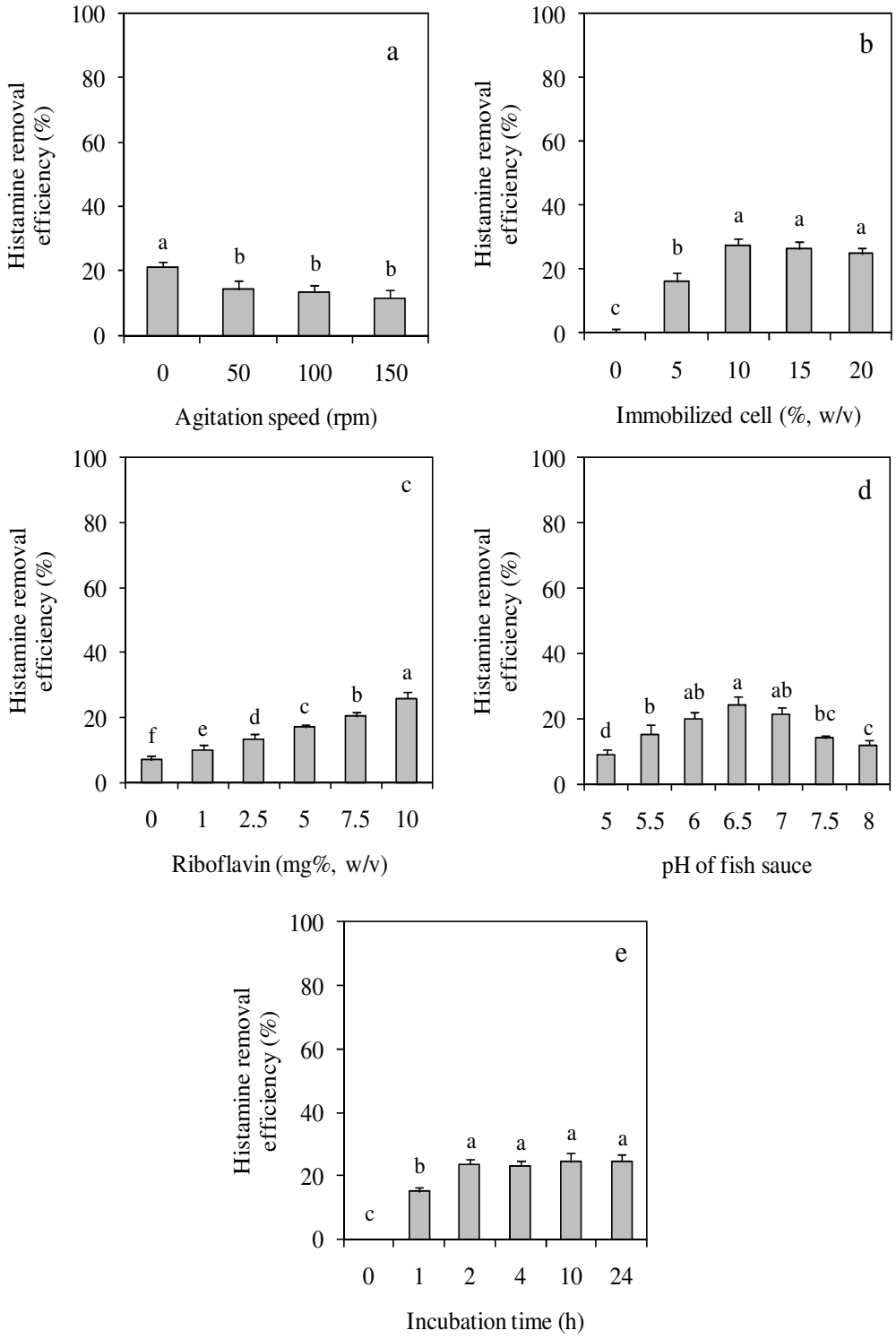

Figure 20. Effect of agitation (a), immobilized cells (b), riboflavin (c), pH of fish sauce (d) and incubation time (e) on histamine degradation. Bars represent the standard deviation (n = 3). Different letters indicate significant differences ($p < 0.05$).

For the riboflavin, the highest histamine removal efficiency was found at the concentration of 10 mg% (w/v, Figure 20c) which was the maximum solubility of riboflavin in fish sauce. The histamine removal efficiency increased with an increase of riboflavin concentration until reaching the maximum. In the absence of riboflavin, the immobilized whole cells exhibited little histamine removal efficiency of 7%. This result might be caused by the natural existence of riboflavin (vitamin B_2) in fish sauce (0.002 mg%, w/v) which was available to use as electron acceptor for activity of histamine dehydrogenase (Öhrvik *et al*., 2012; Thongthai and Gildberg, 2005). Besides the primary function as an electron acceptor for the histamine-degrading activity, yellow-orange color of riboflavin also contributed to the color of fish sauce. 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).

The optimum pH of fish sauce was in the range of 6.0-7.0 (Figure 20d). However, the maximum histamine removal efficiency was observed at pH 6.5 which was selected for further study. Generally, pH in the range of 5.0-6.5 is typical for traditional fish sauce (Codex, 2012). The time required for equilibrium degradation of histamine by *Nnm. gari* BCC 24369 immobilized on diatomite was 2 h (Figure 20e). Overall, the optimum conditions for histamine degradation in fish sauce by immobilized cells were 10% (w/v) immobilized cell of *Nnm. gari* BCC 24369, 10% mg (w/v) riboflavin, pH of fish sauce 6.5 and 2 h static incubation time in which the immobilized cells exhibited the maximum histamine removal efficiency of 24±1%.

4.4.2 Histamine removal efficiency of diatomite-immobilized cells packed in fixed-bed bioreactor

The maximum histamine removal efficiency of 60% was achieved after the first cycle and gradually decreased to 30% after the 2nd and 4th cycles under batch and continuous operations, respectively, and afterward (Figure 21, $p < 0.05$). After the 10th cycle, the efficiency remained about 10% in both operations ($p > 0.05$).

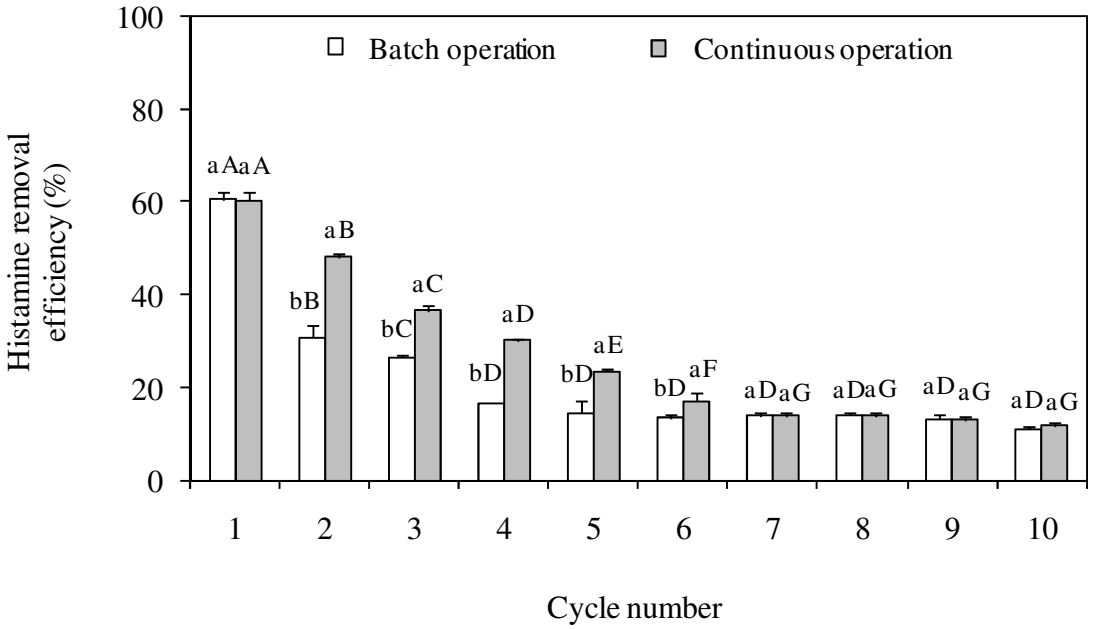

Figure 21. Histamine removal efficiency of immobilized whole cells of *Nnm. gari* BCC 24369 packed in fixed-bed bioreactor under batch and continuous operations with influent flow rate 2 ml/min. Bars represent the standard deviation (n = 3). Different letters (a-b) in the same cycle number indicate significant differences ($p < 0.05$). Different letters (A-G) in the same operation indicate significant differences ($p < 0.05$).

However, histamine content of pooled effluent fish sauce (1 l) obtained from 10th consecutive cycles was not significantly different between both cases in which it decreased by 40±3 and 44±2%, respectively (361±18 and 339±16 ppm, respectively, $p > 0.05$), when compared to that of control (600 ppm). Fixed-bed bioreactor packed with immobilized whole cells was more promising than static condition in that it exhibited 2-fold increase in histamine removal efficiency. The immobilized whole cells in fixed-bed bioreactor had high opportunity to remove histamine due to increased reaction surface area, which provided more binding site between immobilized whole cells in the bioreactor and histamine in fish sauce. The decreased histamine removal efficiency of immobilized whole cells during utilization was probably due to the major drawback of desorption of immobilized whole cells from diatomite support. Electron microscopic examination of whole cells immobilized on

diatomite showed that whole cells were firmly attached onto the diatomite surface before treatment (Figure 22a) and confirmed that some of cells released from diatomite at the 1st cycle after treatment (Figure 22b) and subsequently released for further repeated cycles (Figure 22c-e). Generally, adsorption is a simple physical process in which the forces involved in cell attachment are weak, cells that are several micrometers across are not strongly adsorbed and are readily lost from the surface of the adsorbent (Kharrat *et al*., 2011). However, fixed-bed bioreactor with continuous operation was more promising than batch operation in which it could reduce the operating time with omitting the salt-washing step.

The influent flow rate of fish sauce significantly affected histamine removal efficiency of diatomite-immobilized cells of *Nnm. gari* BCC 24369 packed in a continuous fixed-bed bioreactor (Figure 23). The optimal flow rate of fish sauce for maximum histamine removal efficiency of immobilized cells in the bioreactor was 0.5 ml/min with the retention time of 33 h. The efficiency in each cycle decreased with an increase in flow rate from 0.5 to 2 ml/min ($p < 0.05$). The maximum histamine removal efficiency of 60% was achieved after the first cycle in all flow conditions. With increasing repetitions, at flow rates of 0.1 and 0.2 ml/min, the efficiencies gradually decreased but still remained around 50% at the 5^{th} cycle ($p < 0.05$). In contrast, at flow rate of 2 ml/min, the efficiency rapidly decreased after the first cycle and was only 20% at the 5^{th} cycle ($p < 0.05$). At the 10th cycle, the efficiencies retained about 25, 25 and 10% under flow rates of 0.1, 0.5 and 2.0 ml/min, respectively ($p < 0.05$). Histamine content of pooled fish sauce obtained from 10^{th} consecutive cycles was not significantly different between flow conditions of 0.1 and 0.5 ml/min in which it effectively reduced by 51±3 and 51±2%, respectively (291±19 and 294±13 ppm, $p > 0.05$) when compared to that of control (600 ppm). In contrast, with higher flow rate of 2 ml/min, the reduction of histamine was lower ($p < 0.05$). At lower flow rates (0.1 and 0.5 ml/min), the residence time in the bioreactor was longer, and histamine had sufficient time to interact with the immobilized cells in the bioreactor. Similar observations were reported by Bagai and Madamwar (1997), and Wu and Yu (2008). Cells of *Nnm. gari* BCC 24369 were only passively attached to the surfaces of diatomite through weak interactions, an appropriate flow rate could

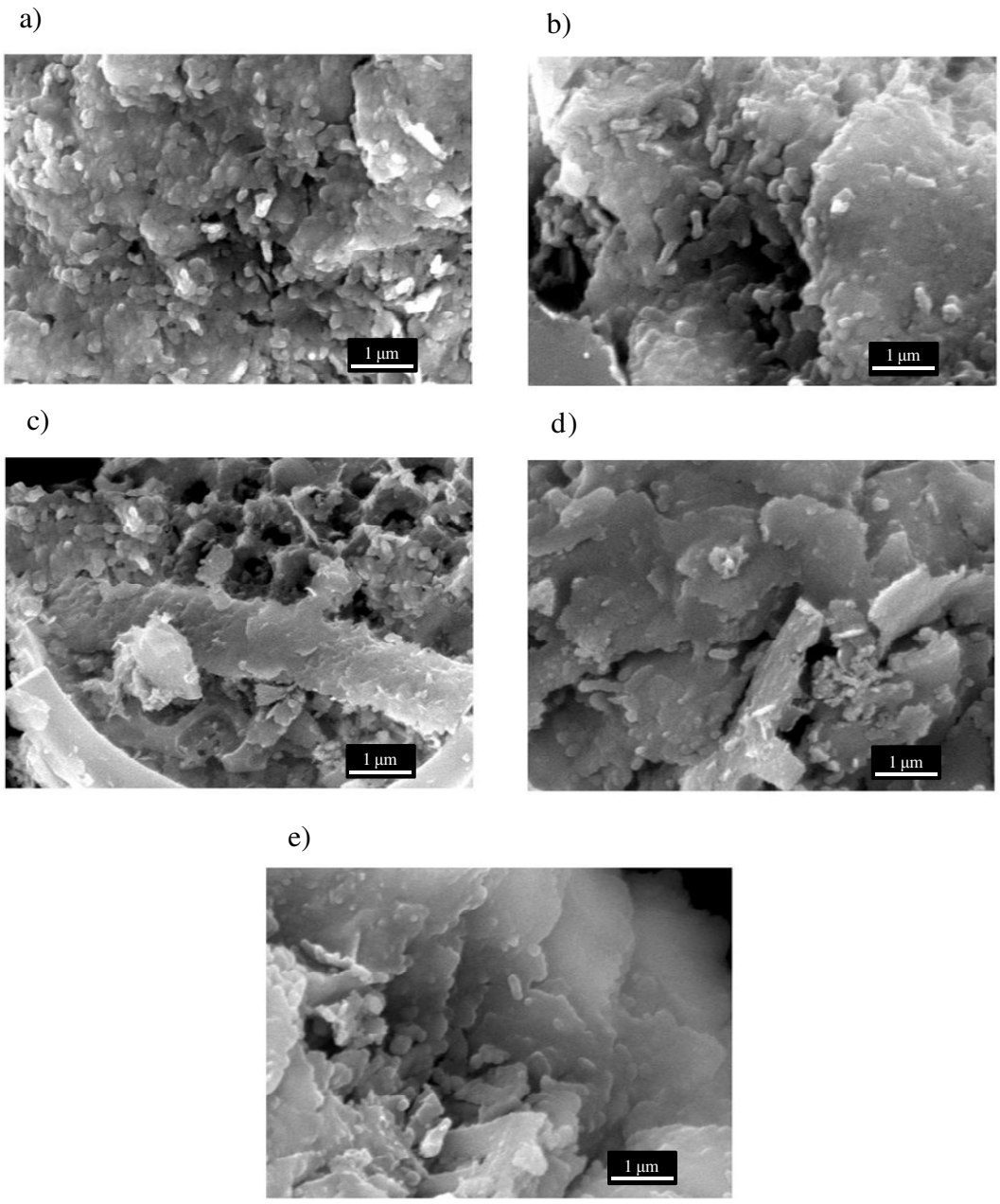

Figure 22. Scanning electron micrographs of immobilized cells of *Nnm. gari* BCC 24369 on diatomite before (a) and after treatments at the 1st (b), 3rd (c), 6th (d) and 10th (e) cycles.

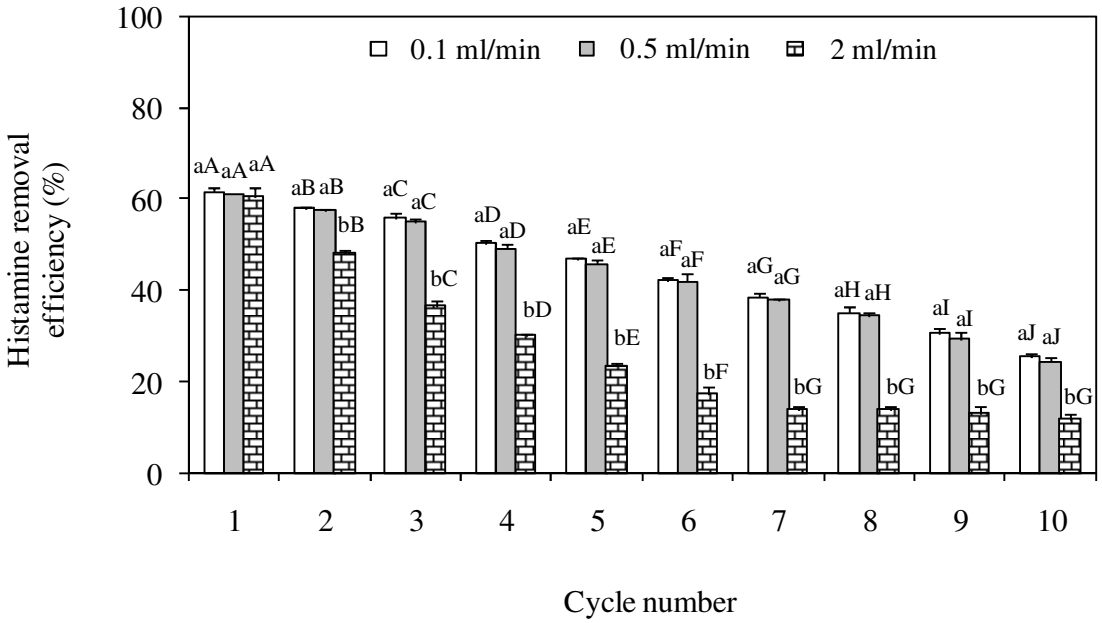

Figure 23. Histamine removal efficiency of a fixed-bed bioreactor packed with immobilized whole cells of *Nnm. gari* BCC 24369 with influent flow rates of 0.1, 0.5 and 2.0 ml/min. Bars represent the standard deviation (n = 3). Different letters (a-c) in the cycle number indicate significant differences ($p < 0.05$). Different letters (A-J) in the same flow rate indicate significant differences ($p < 0.05$).

provide sufficient driving force to allow the cells to attach onto diatomite surface. Since, no significant differences in histamine removal efficiency immobilized cells at low flow rates (0.1 and 0.5 ml/min) were observed, the operation of the continuous fixed-bed bioreactor with influent flow rate of 0.5 ml/min was more promising than its operation with flow rate of 0.1 ml/min with reducing time and energy consumptions which might provide potential advantages for further scaling up.

4.4.2.1 Biogenic amine profile

After treatment, except histamine, no changes in other biogenic amines were observed ($p > 0.05$, Figure 24). Tapingkae *et al*. (2010a) also investigated that histamine dehydrogenase from either free or immobilized whole cells of *Nnm. gari* BCC 24369 specifically catalyzed histamine with low activity to other amines.

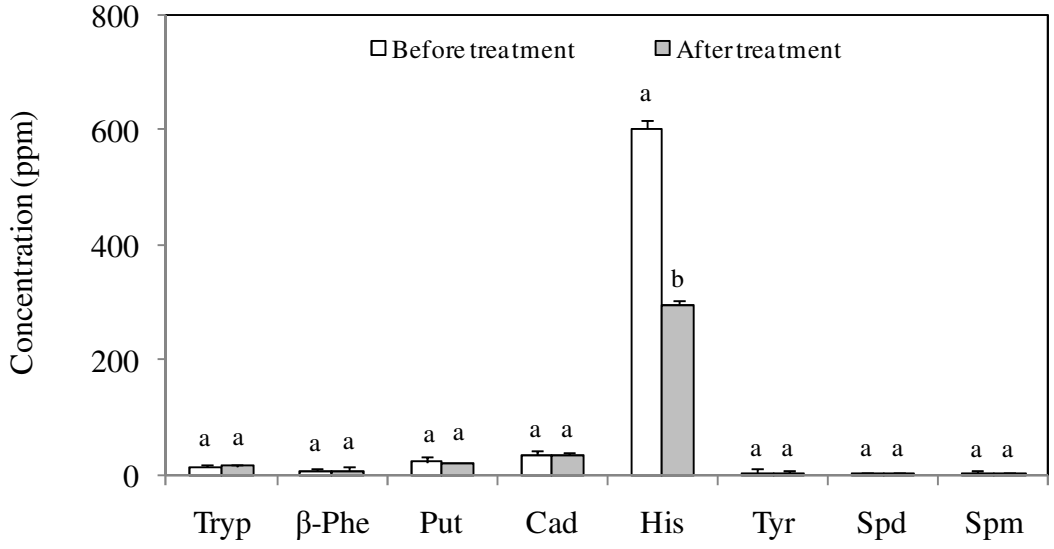

Figure 24. Biogenic amine contents of fish sauce before and after treatments with a fixed-bed bioreactor packed with immobilized whole cells of *Nnm. gari* BCC 24369. Tryp: tryptamine; β-phe: β-phenylethylamine; Put: putrescine; Cad: cadaverine; His: histamine; Tyr: tyramine; Spd: spermidine; Spm: spermine. Mean ± S.D. from a triplicate determination. Different letters in the same parameter indicate significant differences ($p < 0.05$).

Among 20 kinds of biogenic amines 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%, respectively. The results confirmed the substrate preference of immobilized whole cells of *Nnm. gari* BCC 24369 toward histamine in fish sauce.

4.4.2.2 Total and free amino acids profile

No marked differences were observed in total amino acids of fish sauce after treatment with a fixed-bed bioreactor packed with immobilized whole cells of *Nnm. gari* BCC 24369 ($p > 0.05$, Table 10). For free amino acids, no changes in other amino acids were observed except aspartic acid. It has been reported that glutamic acid, lysine, leucine, valine, alanine and/or glycine are the taste-determining

Table 10. Total and free amino acids profile of fish sauce before and after treatments with a fixed-bed bioreactor packed with immobilized whole cells of *Nnm. gari* BCC 24369

Amino acids	Total amino acid (mg/ml)		Free amino acid (mg/ml)	
	Before treatment	After treatment	Before treatment	After treatment
Alanine	9.52±0.00[a]	8.31±1.25[a]	8.94±0.63[a]	7.83±0.74[a]
Arginine	0.43±0.14[a]	0.30±0.04[a]	0.12±0.04[a]	0.14±0.02[a]
Aspartic acid	11.42±0.61[a]	10.29±0.64[a]	6.58±0.21[a]	6.25±0.04[b]
Cysteine	0	0	0.43±0.04[a]	0.39±0.14[a]
Glutamic acid	19.02±1.23[a]	17.18±1.04[a]	9.72±0.23[a]	9.43±0.34[a]
Glycine	6.34±0.23[a]	5.92±0.48[a]	3.24±0.14[a]	2.68±0.54[a]
Histidine	4.37±0.24[a]	3.94±0.43[a]	2.48±0.27[a]	2.14±0.42[a]
Isoleucine	4.51±0.04[a]	4.29±0.24[a]	4.13±0.13[a]	4.04±0.52[a]
Leucine	5.53±0.44[a]	5.02±0.24[a]	5.13±0.42[a]	4.21±0.65[a]
Lysine	11.91±0.29[a]	11.04±0.81[a]	9.64±0.51[a]	9.77±0.40[a]
Methionine	2.89±0.14[a]	2.64±0.31[a]	2.61±0.32[a]	2.14±0.34[a]
Phenylalanine	4.03±0.34[a]	3.88±0.33[a]	3.82±0.24[a]	3.80±0.07[a]
Proline	5.02±0.13[a]	4.52±0.54[a]	2.34±0.42[a]	1.74±0.34[a]
Serine	2.75±0.13[a]	2.41±0.40[a]	2.23±0.24[a]	1.94±0.14[a]
Threonine	8.11±1.21[a]	6.74±1.33[a]	7.81±0.47[a]	6.63±0.82[a]
Tyrosine	11.81±0.32[a]	11.54±0.64[a]	0.53±0.04[a]	0.44±0.14[a]
Valine	7.44±0.31[a]	6.73±1.04[a]	6.42±0.13[a]	6.37±0.24[a]
Total	115.10±6.12[a]	104.75±7.81[a]	76.17±4.22[a]	69.94±4.36[a]

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

amino acids in both modern Italian and Asian fish sauces (Smriga *et al*., 2010; Yoshida, 1998). The flavor of Thai fish sauce, for instance, is thought to arise in part from glutamic acid, histidine and proline (Jones, 1961). Moreover, the contribution of amino acids to the aroma of fish sauce has also been reported by Lopetcharat *et al*. (2001). The typical aroma of glutamic acid is meaty. Glycine, alanine, serine and threonine taste sweet while valine, phenylalanine and histidine give a bitter taste (Liu, 1989). The results imply that taste and aroma of fish sauce were not affected after treatment with a fixed-bed bioreactor packed with immobilized whole cells.

4.4.2.3 pH and total nitrogen content

No marked differences were observed in pH and total nitrogen content of fish sauce after treatment with fixed-bed bioreactor packed with immobilized whole cells of *Nnm. gari* BCC 24369 ($p > 0.05$, Table 11). Total nitrogen content is one of the most important quality factors for fish sauce. High quality fish sauce must have 15 g/l or higher total nitrogen content, based on the Kjeldahl method (Lopetcharat and Park, 2002; Lopetcharat *et al*., 2001). Moreover, total nitrogen content is used as an indicator to determine the grade and price of fish sauce in Thailand, with products containing over 20 g/l classified as Grade I and 15 to 20 g/l as Grade II (TISI, 1993).

4.4.2.4 Color of fish sauce

For the color of fish sauce, L^* (lightness) was slightly increased whereas the a^* (redness) and b^* (yellowness) values were decreased ($p < 0.05$, Table 11) after treatment. The total color difference (ΔE) from instrumental color measurement was more than 2.3, suggesting visually noticeable difference in color (Sharma and Trussell, 1997). In this present study, the color of fish sauce was initially intense red-brown but turned to be light red-brown after treatment with a fixed-bed bioreactor packed with immobilized whole cells. It was observed that a significant amount of brown compounds (melanoidins) could be initially adsorbed on the diatomite, thereby significantly decreasing the color of fish sauce (Lee *et al*., 1997; Leahy and Reineccius, 1989).

Table 11. pH, total nitrogen contents and color parameters (L^*, a^*, b^* values and ΔE) of fish sauce before and after treatments with a fixed-bed bioreactor packed with immobilized whole cells of *Nnm. gari* BCC 24369

Samples	pH	Total nitrogen (g/l)	Color parameters			
			L^*	a^*	b^*	ΔE
Before treatment	6.4±0.1[a]	21.5±0.0[a]	70.9±0.7[b]	13.6±0.3[a]	68.1±0.6[a]	10.0±0.5
After treatment	6.3±0.2[a]	21.6±0.2[a]	75.2±0.3[a]	8.9±0.1[b]	60.4±0.4[b]	

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

4.4.2.5 Sensory evaluation

Sensory analyses of fish sauces before and after treatment with fixed-bed bioreactor packed with immobilized whole cells of *Nnm. gari* BCC 24369 showed that there were no significant differences in liking scores on overall characteristics of fish sauce samples ($p > 0.05$, Figure 25). Although total color difference from instrumental color measurement indicated visually noticeable difference in color of fish sauce before and after treatment, the differences in color were not detectable by visual analysis of panelists. The results indicate that treatment with immobilized whole cells of *Nnm. gari* BCC 24369 did not contribute to the changes in the organoleptic properties of fish sauce.

4.4.3 Storage stability of fish sauce after treatment

4.4.3.1 Total and free amino acids profile

The decrease in total and free lysine was observed in treated fish sauce during storage whereas the decrease in total and free lysine and leucine was found in untreated fish sauce ($p < 0.05$, Figure 26). The loss in lysine and leucine might be caused by the decomposition via non-enzymatic browning reaction. This reaction comprises the condensation reaction between free amino groups, such as amino acids,

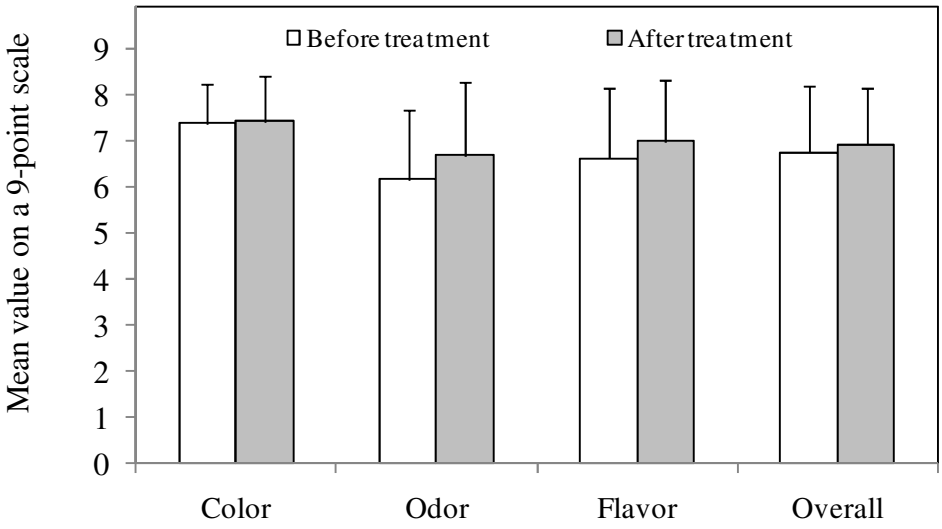

Figure 25. Color, odor, flavor and overall liking scores of fish sauce before and after treatments with a fixed-bed bioreactor packed with immobilized whole cells of *Nnm. gari* BCC 24369. Mean value of consumer judgments on a 9-point hedonic scale with 9 = extremely good.

amines and any nitrogenous compound with reducing carbonyl compounds, including reducing sugars, oxidation products, such as aldehydes and carbohydrate derivatives, such as glucose-6-phosphate (Lopetcharat *et al*., 2001; Kawashima and Yamanaka, 1996). Since, lysine contains two amino groups, it has the highest reactivity in Maillard reaction (Kwak and Lim, 2004; Monti *et al*., 2000). In addition, Kwak and Lim (2004) reported that basic amino acids, such as arginine, leucine, isoleucine, phenylalanine and valine had the higher reactivity in Maillard reaction than acidic amino acids, such as aspartic acid and glutamic acid. Generally, the flavor of fish sauce is thought to arise in part from glutamic acid, histidine and proline (Jones, 1961). The typical aroma of glutamic acid is meaty. Glycine, alanine, serine and threonine taste sweet while valine, phenylalanine and histidine give a bitter taste (Liu, 1989). The results indicated that treated fish sauce had no changes in aroma and flavor during storage.

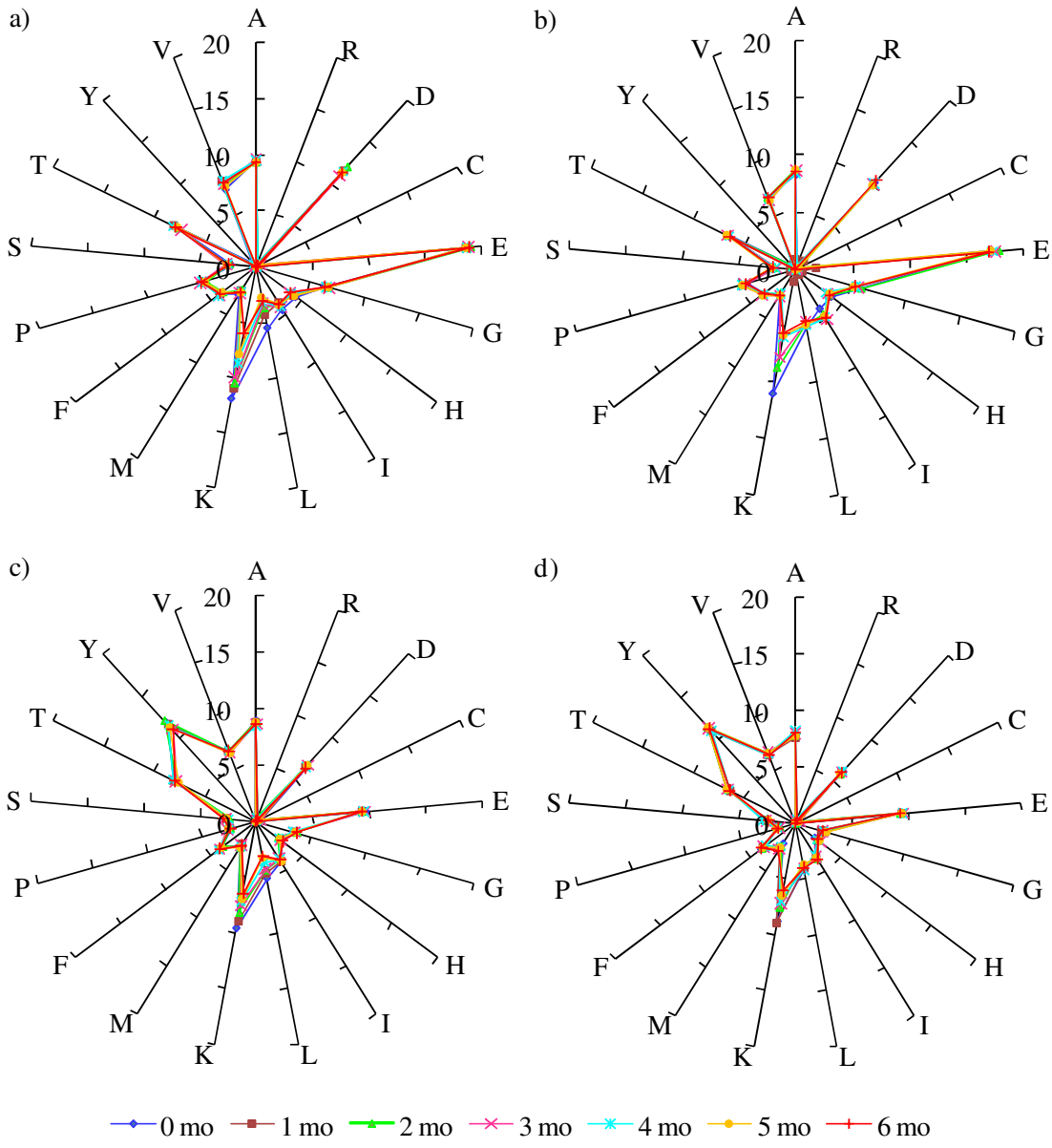

Figure 26. Total amino acids (mg/ml) of fish sauce before (a) and after (b) treatments, and free amino acids (mg/ml) of fish sauce before (c) and after (d) treatments during storage for 6 months. A: alanine; R: arginine; D: aspartic acid; C: cysteine; E: glutamic acid; G: glycine; H: histidine; I: isoleucine; L: leucine; K: lysine; M: methionine; F: phenylalanine; P: proline; S: serine; T: threonine; Y: tyrosine; V: valine.

4.4.3.2 pH and total nitrogen content

No marked differences in pH and total nitrogen between treated and untreated fish sauce were observed along with storage time ($p > 0.05$, Table 12). The pH of both samples was between 6.2-6.4 throughout storage time ($p > 0.05$). Total nitrogen of both samples was also constant during storage, all around 20.8-21.6 g/l ($p > 0.05$). Total nitrogen in fish sauce products is mainly from amino acids (80%) and other non-protein nitrogen compounds, such as nucleotide, small peptides, ammonia and urea (Zaman *et al*., 2011; Pérez-Villareal and Pozo, 1992). These components contribute to the specific aroma and flavor (Shahidi, 1994; Finne, 1992). The total nitrogen content is an objective index used to classify the quality of Thai fish sauce (Lopetcharat *et al*., 2001). High quality Thai fish sauce must have a total nitrogen content of 20 g/l based on the Kjedahl method (TISI, 1983).

4.4.3.3 Color of fish sauce

The immobilized cell treatment affected the color of fish sauce during storage in which it reduced color darkening. In treated fish sauce, the L^* and h values was lower and a^*, b^*, c, BI and ΔE values was higher than untreated fish sauce at the same period of time ($p < 0.05$, Figure 27). Although, changes in all color characteristics showed similar trends in both samples, the increase in BI and ΔE of untreated fish sauce was faster than those of treated fish sauce with the passage of time ($p < 0.05$). Browning increased rapidly in untreated fish sauce. At the end of storage, the browning of untreated fish sauce was increased about 2-fold whereas the browning of treated sample was increased only 1.4-fold (data not shown). The ΔE of untreated fish sauce also sharply increased and reached the level of visually noticeable difference in color (2.3) at the first month while in treated fish sauce, ΔE reached this level after 2 months.

Table 12. pH and total nitrogen contents of fish sauce before and after treatments with a fixed-bed bioreactor during storage for 6 months

Storage time (months)	pH		Total nitrogen (g/l)	
	Before treatment	After treatment	Before treatment	After treatment
0	6.4±0.1	6.3±0.2	21.5±0.0	21.6±0.2
1	6.3±0.2	6.4±0.2	21.4±0.3	20.8±0.8
2	6.2±0.2	6.2±0.2	21.6±0.4	21.2±0.6
3	6.4±0.2	6.4±0.1	21.0±0.5	21.6±0.2
4	6.2±0.1	6.3±0.3	20.7±0.8	21.1±0.6
5	6.2±0.3	6.3±0.2	20.8±0.9	21.0±0.5
6	6.0±0.3	6.4±0.1	20.9±0.6	20.8±0.7

Mean ± S.D. from a triplicate determination.

The increase in browning of both samples was in agreement with a decrease in the L^* and h values and increase in a^*, b^* and c values, indicating the formation of dark pigment as the storage time increased (Nasar-Abbas *et al*., 2008; Klomklao *et al*., 2006). Generally, fish sauce products became darker with melanoidin produced by non-enzymatic browning reaction, especially Maillard reaction during storage (Chavasit *et al*., 2003; Lee *et al*., 1997). Indices of L^* and h are particularly useful for tracking color change and used as indicators for customer acceptability of fish sauce products (Wrolstad *et al*., 2005). In this study, although L^* and h of treated fish sauce dramatically decreased but still higher than untreated fish sauce (64.7 and 74.5, respectively) and customer's unacceptable levels (50.0 and 64.5, respectively, unpublished data). The results indicated that a fixed-bed bioreactor with immobilized cell not only effectively removed histamine but also retarded color darkening in fish sauce during storage.

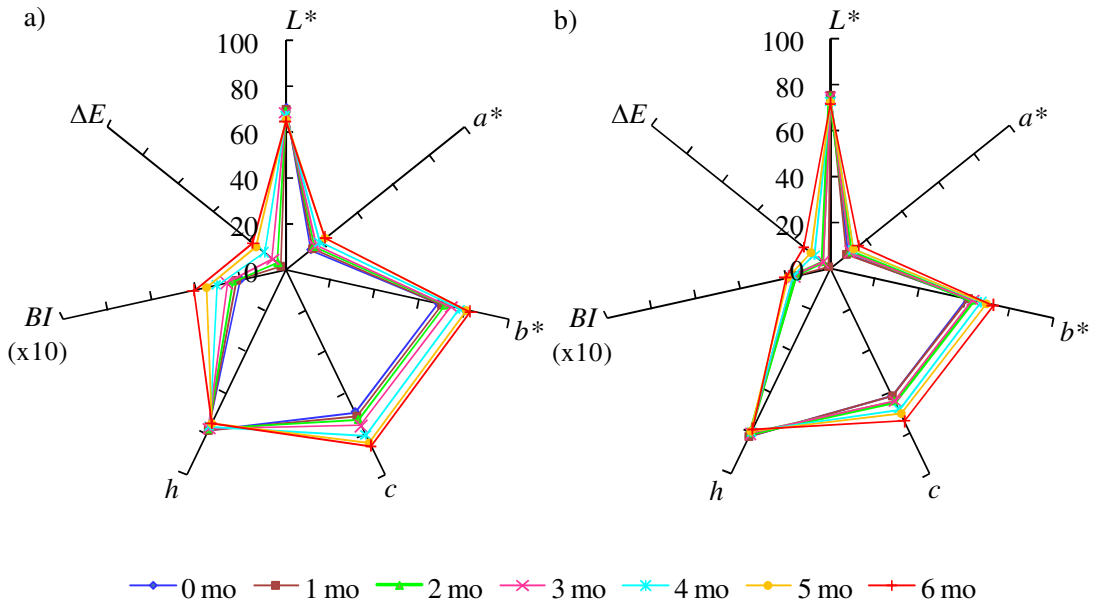

Figure 27. Color parameters (L^*, a^*, b^*, c, h, BI and ΔE) of fish sauce before (a) and after (b) treatments during storage for 6 months.

4.5 Conclusion

This work demonstrated the feasibility of applying immobilized cells of *Nnm. gari* BCC 24369 onto diatom for histamine degradation in fish sauce. Diatomite-immobilized cells successfully removed histamine in fish sauce both under static condition and under fixed bed flow process. The immobilized cells packed in continuous fixed-bed bioreactor exhibited the highest efficiency to remove histamine in fish sauce in which histamine content was reduced by 51% when compared to that of control (600 ppm). A fixed-bed bioreactor packed with immobilized cells of *Nnm. gari* BCC 24369 not only removed histamine but also minimized color darkening without detrimental effect on the unique quality of fish sauce during 6 months storage.

CHAPTER 5

SUMMARY AND FUTURE WORKS

5.1 Summary

1. The experimental designs effectively defined optimal media composition, which supported histamine dehydrogenase (HADH) production by *Natrinema gari* BCC 24369. According to the analysis of Plackett-Burman design (PBD) and central composite design (CCD), the optimal medium for histamine dehydrogenase production composed of 15 g/l of casamino acid, 75 g/l of $MgSO_4.7H_2O$, 273 g/l of NaCl, 2.5 mg/l of $FeCl_2.4H_2O$, 10 g/l of yeast extract, 5 g/l of sodium glutamate and 5 g/l of KCl. Based on one-factor-at-a-time (OFAT) method, the optimum initial pH of culture medium and incubation temperature for enzyme production were at 7.0-7.5 and 28-40°C, respectively. With optimized condition, HADH activity increased 2.2-fold as compared to un-optimized condition. The high-salt tolerance of *Nnm. gari* BCC 24369 enabled its cultivation and then led to HADH production under non-sterile and thus, cost-reducing conditions. Scale-up of HADH production by *Nnm. gari* BCC 24369 from 1-l shake flask to 20-l polypropylene tank was successfully performed both under sterile and non-sterile conditions based on high aeration rate. Thus, *Nnm. gari* BCC 24369 have distinct advantages in biotechnological processes as cultivation is relatively easy, risk of contamination is minimal and culture size can be scaled-up.

2. Food grade diatomite is a suitable and effective support for the immobilization of *Nnm. gari* BCC 24369 cells for degradation of histamine in anchovy fish sauce. The conditions for whole cells immobilization of *Nnm. gari* BCC 24369 onto diatomite support were optimized. PBD analysis and subsequent investigation by CCD suggested 10% (w/v) of free whole cell, 4 M of NaCl, 15% (w/v) of diatomite, initial pH 5.0, agitation rate at 100 rpm and immobilization time of 4 h as optimal conditions for whole cell immobilization. Immobilization of *Nnm. gari* BCC 24369 cells onto diatomite offers several advantages, including the maintaining histamine-degrading activity, operational stability and reduced downstream processing costs of cell recovery and recycle. Diatomite-immobilized cells can be

applied in practical application toward degradation of histamine in the products containing high concentration of salt, since the immobilized-cell system is effective and the diatomite material itself is non-toxic, available and inexpensive.

3. Diatomite-immobilized cells successfully removed histamine in fish sauce both under static condition and under fixed bed flow process. The immobilized cells packed in continuous fixed-bed flow column exhibited the highest efficiency to remove histamine in fish sauce in which histamine content was reduced by 51% when compared to that of control (600 ppm). A fixed-bed flow column packed with immobilized cells of *Nnm. gari* BCC 24369 not only removed histamine but also minimized color darkening without detrimental effect on the unique quality of fish sauce during 6 months storage. A fixed-bed flow column packed with immobilized cells of *Nnm. gari* BCC 24369 would be potentially useful for the large scale applications to remove histamine in fish sauce without any adverse changes in the overall characteristics of the product.

5.2 Future works

1. For industrial application, practicality of the developed fixed bed degradation process should be further investigated in real commercial fish sauce processing.

2. The operational and storage stability of diatomite-immobilized cells of *Nnm. gari* BCC 24369 should be further improved for longer usage.

3. The complete gene and protein sequences of HADH from *Nnm. gari* BCC 24369 should be further studied in order to better understand the physicochemical properties of the enzyme and thus to facilitate the overproduction of the enzyme for industrial uses.

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APPENDICES

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
$FeCl_4.4H_2O$	3.6	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. 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 7 days.

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*- Phthalaldehyde (OPT)

o- Phthalaldehyde	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 HCl. Prepare fresh weekly. The calibration of histamine standard was made in the ranges of 50 - 500 nM (Figure 28).

1.2 Procedure

1.2.1 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.

1.2.2 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 adjusted to the volume with distilled water.

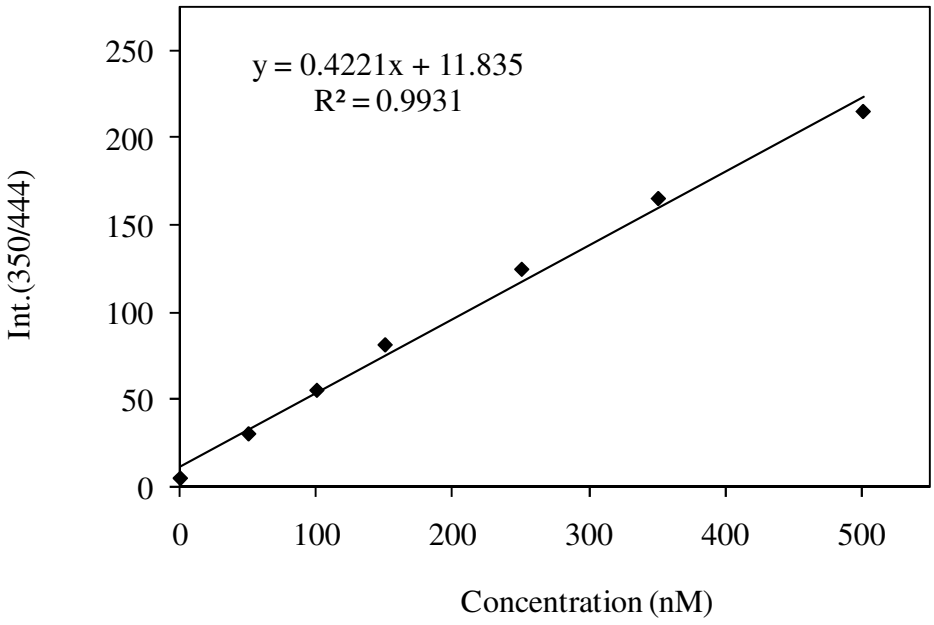

Figure 28. Standard curve of histamine using fluorometric method of AOAC (2005).

1.2.3 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. Dissolved oxygen determination by titrimetric method of AOAC

2.1 Reagents

2.1.1 Alkaline iodide-sodium azide

NaOH	500	g
NaI	135	g

Distilled water	950	ml
NaN_3	10	g

Dissolve NaOH and NaI in distilled water, dilute to 950 ml and cool. Slowly, with stirring, add solution of 10 g NaN_3 in 40 ml distilled water, and the volume was made up to 1 l with distilled water. Diluted and acidified solution must not give color with starch indicator. Stored in dark bottle with rubber stopper.

2.1.2 Manganese sulfate

$MnSO_4.H_2O$	364	g
Distilled water	900	ml

$MnSO_4.H_2O$ was slowly added to distilled water, and the volume was made up to 1 l with distilled water.

2.1.3 0.00208 M Potassium biiodate standard

$KH(IO_3)_2$	0.8125	g
Distilled water	900	ml

$KH(IO_3)_2$ standard was added to distilled water, and the volume was made up to 1 l with distilled water.

2.1.4 Potassium fluoride

$KF.2H_2O$	40	g
Distilled water	100	ml

2.1.5 0.025 M Sodium thiosulfate standard

$Na_2S_2O_3.5H_2O$	6.25	g
NaOH	0.25	g
Distilled water	900	ml

$Na_2S_2O_3.5H_2O$ and NaOH was added to distilled water, and the volume was made up to 1 l with distilled water.

2.1.6 Starch indicator

Starch	3	g
Boiling distilled water	500	ml

Disperse starch with few ml H_2O. Pour into 500 ml boiling water, boil few minutes, and let settle overnight.

2.2 Determination

Add 2.0 ml $MnSO_4$ solution and 2.0 ml alkaline I-NaN_3 solution to specimen in 250 ml BOD bottle, replace stopper, excluding air bubbles, and invert several times to mix. Let floc settle and repeat mixing. (Water with high chloride concentration requires 10 min contact with precipitate.) After floc has settled, leaving>/ 100 ml clear supernatant, remove stopper and add 2.0 ml H_2SO_4 down neck of bottle. Restopper and mix by inversion until I_2 is uniformly distributed. Immediately titrate 203 mL (3 ml is allowance for added reagents) with 0.025 M $Na_2S_2O_3$ to pale straw yellow. Add 1-2 ml starch indicator and titrate to disappearance of blue. Disregard reappearance of blue.

Dissolved O_2 mg/l (ppm) = (ml of 0.025 M $Na_2S_2O_3$ x 0.2/200) x 1000

3. Biogenic amines determination by HPLC method

3.1 Reagents

3.1.1 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.

3.1.2 2 N NaOH

NaOH	80	g
Distilled water	900	ml

Dissolve and make up to 1 l with distilled water.

3.1.3 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.

3.2 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.

3.3 Derivatization and detection

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 13).

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 500 mg/l was shown in Figure 29.

3.4 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 30). 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.

Table 13. Gradient elution program for biogenic amine 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.

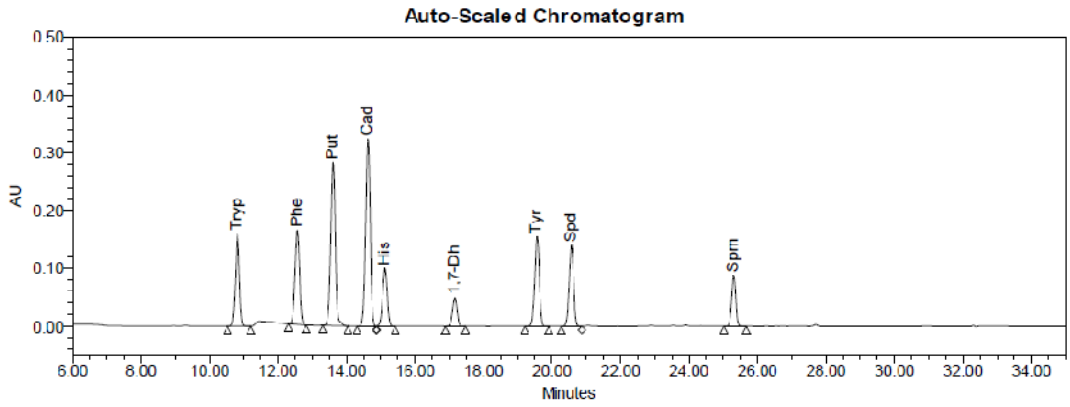

Figure 29. Biogenic amines standard chromatogram (500 mg/l). Abbreviation: Tryp; tryptamine, β-phe; β-phenylethylamine, Put; putrescine, Cad; cadaverine, His; histamine, 1,7-dh; 1,7- diaminoheptane (internal standard), Tyr; tyramine, Spd; spermidine and Spm; spermine.

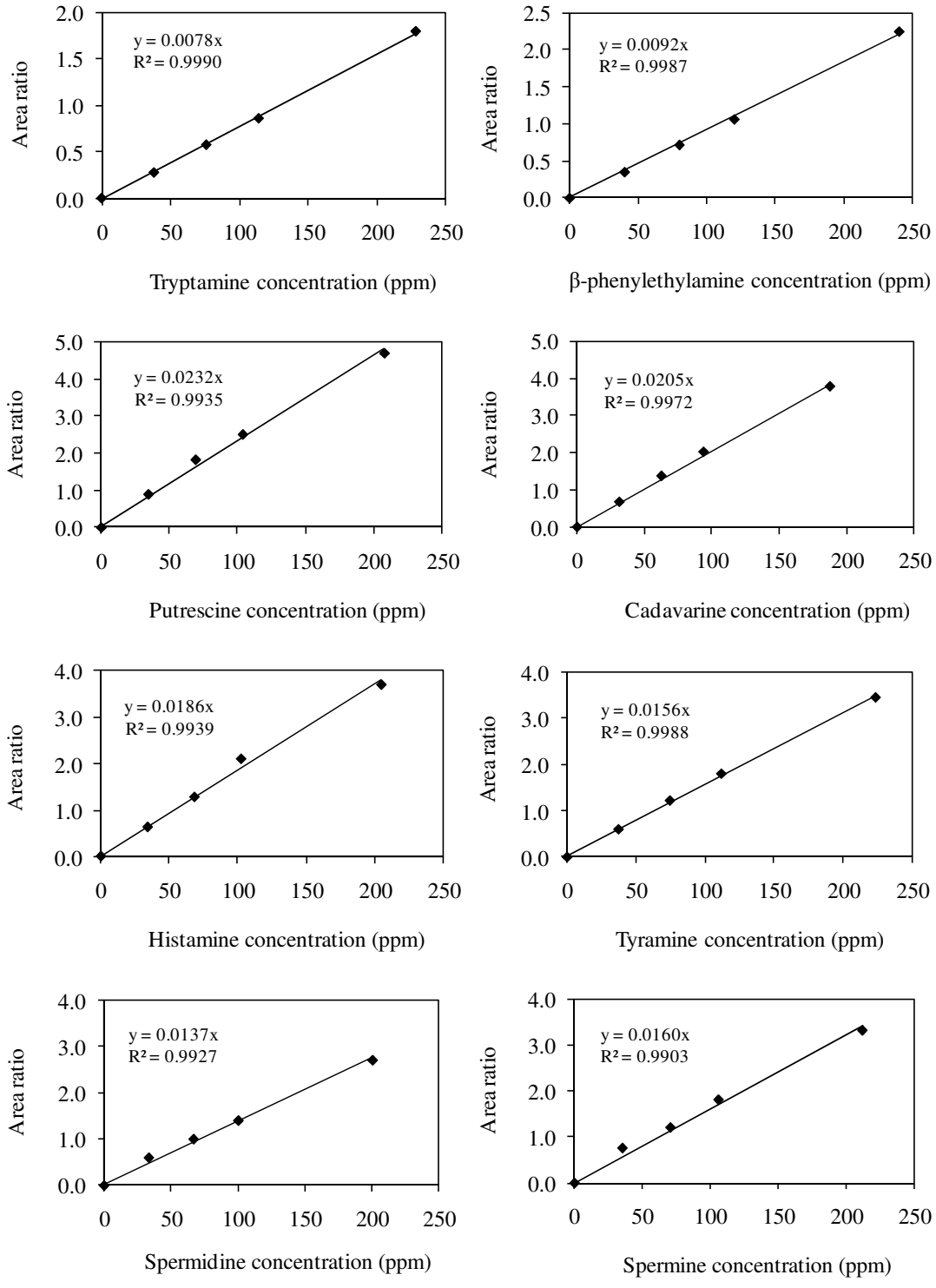

Figure 30. Standard curves of biogenic amines determined by HPLC.

4. Total and free amino acids determination by HPLC method

The amino acids were determined by HPLC according to the Waters AccQ.Tag method (Millipore Co-Operative, Milford, MA, USA). The AccQ.Tag method is based on a derivatizing reagent developed specifically for amino acid analysis. Waters AccQ.Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, or AQC) is an N-hydroxysuccinimide-activated hetocyclic carbamate, a new class of amine-derivatizing compounds. The AccQ.Fluor reagent reacts rapidly with primary and secondary amino acids to yield highly stable fluorescent derivatized amino acids that fluoresce strongly at 395 nm.

4.1 Reagents

4.1.1 2.5 mM α-aminobutyric acid (ABAA)

α-aminobutyric acid	6.45	mg
0.1 N HCl	24	ml

α-aminobutyric acid was slowly added to 0.1 N HCl, and the volume was made up to 25 ml with 0.1 N HCl.

4.1.2 Amino acid standard solution

The standard mixture contained a 2.5 mM concentration of each of amino acids.

Alanine	2.22	mg
Arginine	4.36	mg
Aspatate	3.33	mg
Cysteine	3.00	mg
Glutamate	3.78	mg
Glycine	1.88	mg
Histidine	3.88	mg
Isoleucine	3.28	mg
Leucine	3.28	mg
Lysine	3.66	mg

Methionine	3.73	mg
Phenylalanine	4.13	mg
Proline	2.88	mg
Serline	2.63	mg
Threonine	2.98	mg
Tyrosine	4.53	mg
Valine	2.93	mg

All amino acids were in 0.1 N HCl (10 ml).

4.2 Extraction

For free amino acid, the fish sauce sample (100 μl) was added with a 900 μl of 5% (w/v) trichloroacetic acid. For total amino acids, 100 μl of fish sauce sample was hydrolyzed with 5 ml of 6 N HCl for 16 h at 110ºC. Each sample was centrifuged at 12,000 *g* for 15 min. The supernatant (100 μl) was collected and added with 2.5 mM ABAA (20 μl) as the internal standard (IS) and Milli-Q water (880 μl). The mixture was filtered through a 0.45 μm membrane filter prior to derivertization. For the amino acid standard, the mixed standard amino acids (40 μl) was added with 2.5 mM ABAA (20 μl) as the internal standard (IS) and Milli-Q water (940 μl) prior to filtration and derivatization.

4.3 Derivatization and detection

The filtrate and mixed standards were derivatized by using the method of Hughes *et al*. (2002). The filtrate (10 μl) was added with Waters AccQ Fluor Borate buffer (70 μl) and AccQ Fluor^{TM} Reagent (20 μl). The mixture was incubated at 55°C for 10 min. Blank was run in the same manner except addition of Waters AccQ Fluor Borate buffer (10 μl) instead of sample. The amino acids were analyzed by HPLC. A Waters Separation Module 2690 was operated to give a flow rate of 1.0 ml/min of the mobile phase of AccQTag eluent A pH 5.02 (solvent A), Acetonitrile HPLC grade (solvent B) and Milli-Q water (solvent C), as shown in a gradient elution programme (Table 14).

Table 14. Gradient elution program for amino acid analysis by HPLC

	Time (min)	%A	%B	%C
1	0	100	0	0
2	0.5	99	1	0
3	21.0	95	5	0
4	22.0	91	9	0
5	32.5	83	17	0
6	38.0	0	60	40
7	46.0	100	0	0
8	55.0	100	0	0

Solvent A; AccQTag eluent A pH 5.02.

Solvent B; Acetonitrile (HPLC grade).

Solvent C; Milli-Q water.

Separation was achieved using a column of AccQ Tag C18 (3.9 x 150 mm i.d.) set at 37°C. A sample, 5 μl, was injected and fluorescent detector (Water 2475, USA), was set at excitation wavelength of 250 nm and Emission wavelength of 395 nm. Data were processed and analyzed using Millennium 32 software.

4.4 Calculation

Amino acid was identified by comparing the retention times between the sample chromatogram with that of the standard chromatogram. Quantification of the individual amino acids is calculated using a ratio of the sample to internal standard peak areas.

$$D_{Rfx} = [A_{SD} \times C_{IS\text{-}SD}] / [C_{SD} \times A_{IS\text{-}SD}]$$

$$C_X = [A_X \times C_{IS}] / [A_{IS} \times D_{Rfx}]$$

D_{Rfx} is the detector response factor for each amino acid.

A_{SD} is the peak area of each amino acid in standard solution.

C_{SD} is the concentration of each amino acid in standard solution.

$C_{IS\text{-}SD}$ is the concentration of ABAA in standard solution.

$A_{IS\text{-}SD}$ is the peak area of ABAA in standard solution.

C_{IS} is the concentration of ABAA in sample.

A_{IS} is the peak area of ABAA in sample.

C_x is the concentration of each amino acid in sample.

A_x is the peak area of each amino acid in sample.

5. Sensory evaluation form

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. Odor

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

3. Flavor

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

4. Overall ranking

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

5. Acceptability

_____ Accept
_____ Not accept

6. Reasons of unacceptability of a product

.....,.....
.....
.....

VITAE

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Research Award, National Research Council of Thailand (NRCT), 2012.

Student exchange program for the research training for 6 months at the University of California, Davis, CA, USA, 2014.

List of Publication and Proceedings

Publications

Chaikaew, S., Maeno, Y., Visessanguan, W., Ogura, K., Sugino, G., Lee, S. H. and Ishikawa, K. 2012. Application of thermophilic enzymes and water jet system to cassava pulp. Bioresource Technology. 126: 87-91.

Zhou, D., Visessanguan, W., Chaikaew, S., Benjakul, S., Oda, K. and Wlodawer, A. 2014. Crystallization and preliminary crystallographic analysis of histamine dehydrogenase from *Natrinema gari* BCC 24369. Acta Crystallographica. F. 70: 942-945.

Chaikaew, S., Tapingkae, W., Tanasupawat, S., Benjakul, S. and Visessanguan, W. Whole cell immobilization of *Natrinema gari* BCC 24369 for biological degradation of histamine in fish sauce. International Journal of Food Microbiology (Submitted).

Chaikaew, S., Powtongsook, S., Boonpayung, S., Tapingkae, W., Benjakul, S. and Visessanguan, W. Optimization and scaling up of histamine dehydrogenase production by *Natrinema gari* BCC 24369 in non-sterile condition. Process Biochemistry. (In preparation).

Chaikaew, S., Tepkasikul, P., Benjakul, S., Young, G. M. and Visessanguan, W. Histamine degradation in fish sauce by fixed-bed flow column packed with immobilized whole cells of *Natrinema gari* BCC 24369. Fisheries Science (In preparation).

Proceedings

Chaikaew, S., Maeno, Y., Ogura, K., Sugino, G., Lee, S. H. and Ishikawa, K. 2011. Application of thermophilic enzymes and water jet system to cassava pulp. The 3rd Thai-Japan Bioplastics and Biobased Materials Symposium 2011. Bangkok, Thailand.

Chaikaew, S., Visessanguan, W., Benjakul, S., Kruenate, J. and Kongniam, S. 2012. Immobilization of whole cells of *Natrinema gari* BCC 24369 for biodegradation of histamine in fish sauce. The 5th International Conference on IFIB-2012 Industrial Bioprocesses 2012. Taipei, Taiwan.

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Thai Patent

Wonnop Visessanguan, Wanaporn Tapingkae, Siriporn Chaikaew, Sootawat Benjakul and Somboon Tanasupawat. 2009. Biological Degradation of Histamine by Halophilic Archaea (ID: 0901005189).