Carotenoid Production by Halophilic Archaea and Its Applications

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ชื่อวิทยานิพนธ์ การผลิตแคโรทีนอยด์จากอาเคียที่ชอบเกลือและการประยุกต์ใช้

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บทคัดย่อ

ได้ทำการคัดแยกอาเคียที่ชอบเกลือที่สามารถสังเคราะห์แคโรทีนอยด์ จำนวน 124 สายพันธุ์จากผลิตภัณฑ์สัตว์น้ำหมักที่มีเกลือสูง สายพันธุ์ HM3 สามารถผลิตแคโรทีนอยด์ได้สูงสุด รองลงมาได้แก่ สายพันธุ์ AS133 HM322 และ HPC1-2 ตามลำดับ จากการศึกษาลักษณะทางฟีโนไทป์และอนุกรมวิธานเคมี พบว่า เชื้อทั้ง 4 สายพันธุ์สามารถเคลื่อนที่ได้ มีรูปร่างหลายแบบ ต้องการเกลืออย่างน้อย 2.1 โมลาร์สำหรับการเจริญ จัดอยู่ในจีนัส *Halobacterium* จากการศึกษาเปรียบเทียบความคล้ายคลึงของลำดับนิวคลีโอไทด์ของ 16S rDNA พบว่า เชื้อทั้ง 4 สายพันธุ์มีความใกล้เคียงกับ *Halobacterium salinarum* แคโรทีนอยด์ที่สกัดจากเชื้อที่คัดเลือกทั้งหมดนี้มีสเปกตรัมการดูดกลืนแสงและรูปแบบแคโรทีนอยด์ที่แยกได้โดยโครมาโทกราฟีเหมือนกัน แคโรทีนอยด์หลักที่แยกได้มีมวลของไอออนหลักเท่ากับ 740 m/z และมีรูปแบบการแตกตัวของไอออนที่เหมือนกัน ซึ่งสอดคล้องกับแบคเทอริโอรูเบอรินและอนุพันธ์ต่างๆ ของแบคเทอริโอรูเบอริน จากการศึกษาองค์ประกอบอาหารสำคัญที่มีผลต่อการเจริญและการผลิตแคโรทีนอยด์ของเชื้อ *Hbt. salinarum* HM3 โดยการประยุกต์ใช้วิธีการทางสถิติด้วยวิธี Plackett-Burman และ central composite design พบว่าการเลี้ยงในอาหารเลี้ยงเชื้อที่มีสารสกัดเนื้อ (Meat extract) 10 กรัมต่อลิตร และกลูโคส 10 กรัมต่อลิตร ที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 7 วัน สามารถผลิตแคโรทีนอยด์คิดเป็น 1.5 เท่าเมื่อเทียบกับอาหารสูตรดั้งเดิม

ได้ทำการพิสูจน์เอกลักษณ์ของอาเคียที่ชอบเกลือสายพันธุ์ HPC1-2 ซึ่งแยกได้จากปลาร้าเปรียบเทียบกับอาเคียสายพันธุ์อื่นๆ ที่มีความคล้ายคลึงมากที่สุดในจีนัส *Halobacterium* จากการศึกษาความคล้ายคลึงของลำดับนิวคลีโอไทด์ของ 16S rDNA แสดงให้เห็นว่า HPC1-2 มีความคล้ายคลึงร้อยละ 99.2 กับ *Hbt. salinarum* DSM 3754 และร้อยละ 97.8 กับ *Halobacterium jilantaiense* JCM 13558 อย่างไรก็ตาม จากการทำ ดีเอนเอ-ดีเอนเอไฮบริไดเซชั่น พบว่ามีความคล้ายคลึงทางดีเอ็นเอที่ต่ำ แสดงให้เห็นว่า สายพันธุ์ HPC1-2 แตกต่างจากเชื้อทั้งสอง และยังมีลักษณะทางสรีรวิทยาและชีวเคมีบางประการที่แตกต่างไป ดังนั้น สายพันธุ์ HPC1-2 จึงจัดเป็นตัวแทนของสายพันธุ์ใหม่ของ จีนัส *Halobacterium* มีชื่อว่า *Halobacterium piscisalsi* sp. nov.

ได้ทำการศึกษากิจกรรมการต้านปฏิกิริยาออกซิเดชั่นของแบคเทอริโอรูเบอรินจากเชื้อ *Hbt. salinarum* HM3 โดยวัดกิจกรรมการต้านอนุมูลอิสระชนิดต่างๆ ความสามารถในการลดซิงเกลทออกซิเจน และการป้องกันการเสียหายของพลาสมิดดีเอ็นเอ เปรียบเทียบกับแอสตาแซนทีน เบต้า-แคโรทีน ไลโคพีน และ ลูทีน พบว่า แบคเทอริโอรูเบอรินมีความสามารถในการต้านอนุมูลอิสระของ DPPH และ ABTS มากที่สุด โดยความเข้มข้นน้อยที่สุดที่ทำให้อนุมูลอิสระทั้งสองชนิดลดลงร้อยละ 50 (EC_{50}) มีค่าเท่ากับ 3.88 และ 15.80 มิลลิโมลาร์ ตามลำดับ แบคเทอริโอรูเบอรินมีความสามารถในการรีดิวซ์เฟอริกให้เป็นเฟอรัส และมีความสามารถในการลดซิงเกลทออกซิเจนสูงกว่าแคโรทีนอยด์ชนิดอื่น ๆ ที่ทำการศึกษา โดยมีค่าคงที่ของการลดลงซิงเกลทออกซิเจนเท่ากับ 2.48×10^{10}, 1.18×10^{10}, 1.49×10^{10}, 2.15×10^{10} และ 1.24×10^{10} M^{-1} s^{-1} สำหรับแบคเทอริโอรูเบอริน แอสตาแซนทีน เบต้า-แคโรทีน ไลโคพีน และ ลูทีน ตามลำดับ นอกจากนี้แบคเทอริโอรูเบอรินยังสามารถช่วยป้องกันการเสียหายพลาสมิดดีเอ็นเอจากอนุมูลอิสระไฮดรอกซิลที่เกิดจากปฏิกิริยาเฟนตอน

ได้ทำการศึกษาความคงตัวต่อความร้อนและแสงของแบคเทอริโอรูเบอรินจากเชื้อ *Hbt. salinarum* HM3 เมื่อละลายในน้ำมันถั่วเหลือง ปริมาณและกิจกรรมการต้านอนุมูลอิสระของแบคเทอริโอรูเบอรินลดลงประมาณร้อยละ 20 และ 40 ตามลำดับ หลังจากการให้ความร้อนที่อุณหภูมิ 90 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง ในสภาวะที่สัมผัสกับแสงที่ความเข้ม 5,000 ลักซ์ ที่อุณหภูมิ 20 องศาเซลเซียส เป็นเวลา 24 ชั่วโมง ปริมาณและกิจกรรมการจับอนุมูลอิสระของแบคเทอริโอรูเบอรินลดลงร้อยละ 10 และ 20 ตามลำดับ การเติมแบคเทอริโอรูเบอรินเป็นสีผสมในผลิตภัณฑ์เนื้อปลาบด ที่ความเข้มข้น 100 และ 200 มิลลิกรัมต่อกิโลกรัม ทำให้เจลมีสีชมพูส้ม ซึ่งมีค่า L* a* b* อยู่ในช่วง 51.2-67.6, 15.7-31.7 และ 15.7-23.4 ตามลำดับ โดยแบคเทอริโอรูเบอรินที่เติมทั้งสองระดับไม่มีผลต่อค่าความแข็งแรงและความยืดหยุ่นของเนื้อปลาบด (P>0.05) เมื่อเก็บรักษาผลิตภัณฑ์เนื้อปลาบดที่อุณหภูมิตู้เย็น ภายใต้การให้แสงที่ความเข้ม 2,500 ลักซ์ เป็นเวลา 7 วัน พบว่า ตัวอย่างที่เติมแบคเทอริโอรูเบอริน มีค่าการเปลี่ยนแปลงสี (ΔE^*_{ab}) และ ค่า Thiobarbituric acid reactive substances (TBARS) น้อยกว่ากลุ่มควบคุมที่ไม่ได้เติมแบคเทอริโอรูเบอริน จากผลการทดลองชี้ให้เห็นว่าแบคเทอริโอรูเบอรินมีความคงตัวที่ดีต่อความร้อนและแสง นอกเหนือจากคุณสมบัติการให้สีแล้ว แบคเทอริโอรูเบอรินยังมีคุณสมบัติต้านการเกิดออกซิเดชั่น ซึ่งช่วยลดการเกิดออกซิเดชั่นของไขมันระหว่างการเก็บรักษาของผลิตภัณฑ์เนื้อปลาบด

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ABSTRACT

Total 124 extremely halophilic archaea that possessed carotenoid producing ability were isolated from salt fermented foods in Thailand. The isolated strain HM3 exhibited the highest carotenoid-producing ability followed by AS133, HM322, and HPC1-2, respectively. On the basis of their phenotypic and chemotaxonomic characteristics, all 4 strains were motile, pleomorphic, required at least 2.1 M NaCl for growth and were assigned to the genus *Halobacterium*. Based on the 16S rDNA sequence similarity values, they were tentatively identified as *Halobacterium salinarum*. Total carotenoid extracts from all selected strains exhibited the same absorption spectra and chromatographic carotenoid profiles. All fractions showed the same parent ion mass at 740 m/z and the fragmentation pattern related to bacterioruberin. Application of a statistical approach using Plackett-Burman and central composite designs show that meat extract (10 g/L) and glucose (10 g/L) were important components affecting the growth and carotenoid production by *Hbt. salinarum* HM3. The organism produced carotenoid at 9.24 mg/L when cultivated in the optimized medium at 37 °C for 7 days with 1.5-fold increase compared to the original medium.

Of 4 selected strains, HPC1-2 isolated from *pla-ra* was subjected to further identification in comparison with other most closely related species of the genus *Halobacterium*. Based on 16S rDNA gene sequence similarity, strain HPC1-2 was related most closely to *Hbt. salinarum* DSM 3754 (99.2%) and *Hbt. jilantaiense* JCM 13558 (97.8%). However, low levels of DNA relatedness by DNA-DNA hybridization suggested that strain HPC1-2 was genotypically different from these closely related type strains. Strain HPC1-2 could also be differentiated based on physiological and biochemical characteristics. Therefore, strain HPC1-2 is considered

to represent a novel species of the genus *Halobacterium*, for which the name *Halobacterium piscisalsi* sp. nov. is proposed.

The antioxidant activities of bacterioruberin from *Hbt. salinarum* HM3 were assessed *in vitro* with respect to radical scavenging, singlet oxygen quenching abilities and plasmid relaxation assay compared with astaxanthin, β-carotene, lycopene, and lutein. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-Azinobis-3-ethylbenzo thizoline-6-sulphonate (ABTS) radical scavenging activity of bacterioruberin were highest among tested carotenoids with the effective concentration for 50% scavenging (EC_{50}) of 3.88 and 15.80 mM, respectively. The reducing power of bacterioruberin was 3.27. Singlet oxygen quenching ability of bacterioruberin was higher than other tested carotenoids, with the quenching rate constants (k_q) of 2.48×10^{10}, 1.18×10^{10}, 1.49×10^{10}, 2.15×10^{10} and 1.24×10^{10} M^{-1} s^{-1} for bacterioruberin, astaxanthin, β-carotene, lycopene, lutein, respectively. Bacterioruberin also exhibited protective effect on plasmid DNA damage by the attack of hydroxyl radical ($OH^{\bullet}$) generated from the Fenton reaction.

Stabilities of bacterioruberin from *Hbt. salinarum* HM3 upon heating and light exposure were investigated in soybean oil. Approximately 20% of the content and 40% of the DPPH radical scavenging activity of bacterioruberin decreased after heating at 90 °C for up to 24 h. Upon exposure to light at the intensity of 5,000 lux at 20 °C for 24 h, reductions in both content and DPPH radical scavenging activity of bacterioruberin were approximately 10 and 20%, respectively. Incorporation of bacterioruberin into surimi at the concentrations of 100 and 200 mg/kg resulted in orange pink color with the L*, a*, and b* values in the ranges of 51.2-67.6, 15.7-31.7, and 15.7-23.4, respectively. At the concentrations of bacterioruberin added, no differences in the breaking force and deformation of surimi gels were observed (P>0.05). During storage at the refrigeration temperatures with the light exposure at 2,500 lux for 7 days, the color differences (ΔE^*_{ab}) and thiobarbituric acid reactive substances (TBARS) values of surimi gels added with bacterioruberin had lower ΔE^*_{ab} and TBARS values than control with no bacterioruberin added. From the results, bacterioruberin exhibited relatively good stability upon heating at elevated treatments and light exposure. In addition to its color, bacterioruberin also exerted the antioxidative activity resulting in lower oxidation of lipids in surimi gels during storage.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Carotenoids are yellow to orange-red pigments that are present in a wide variety of bacteria, algae, fungi and plants. Carotenoids are currently being produced for animal and human consumption. Carotenoids are used as pigments to color the skin or egg yolks in poultry, to color the flesh of fish grown under aquaculture conditions, and to color the shells of crustaceans (Wilska-Jeszka, 1997). Carotenoids are also widely used as colorants in food for human consumption and also as constituents in vitamins and dietary supplements. There are increasing awareness and opportunities for the expanded use of carotenoids for vitamin and dietary supplement formulations. Convincing evidence has been seen in a number of epidemiological studies and direct clinical studies that suggest a central role for carotenoids as a means of reducing chronic diseases (van Poppel and Goldbohm, 1995). The most commercialized carotenoids are astaxanthin, β-carotene, and canthaxanthin (Marz, 2008).

With the rising global concern to avoid the undesirable effects of synthetic food colorants such as allergy, hypersensitivity, intolerance and childhood hyperactivity (Pollock and Warner, 1990), there is increasing interest in the biological production of carotenoids. There are several commercial operations currently used to produce carotenoids; *Dunaliella salina* - β-carotene (Venkatesh *et al*., 2005), *Phaffia rhodozyma* - astaxanthin) (Jacobson *et al*., 2002), *Haematococcus pluvialis* - astaxanthin (Boussiba, 2000), *Chlorella zofingiensis* - astaxanthin and canthaxanthin (Pelah *et al*., 2003). Due to the ease of contamination and difficulty of extracting the pigments, the new source of carotenoids production would be obtained. The red extremely halophilic bacteria (non-photosynthetic bacteria) produce derivatives of acyclic C_{50} bacterioruberin and ketocarotenoids such as 3-hydroxy echinenone or trans-astaxanthin, and canthaxanthin (Calo *et al*., 1995; Asker and Ohta, 1999; May *et al*.,

2004). The extremely halophilic bacteria can be grown under non-aseptic condition, and their carotenoids can be extracted directly from the cells without any mechanical disintegration (Asker *et al*., 2002). Therefore, the study on production of carotenoids from halophilic bacteria would be an approach to obtain the alternative commercial source of carotenoid.

1.2 Objectives of study

1. To isolate and screen of halophilic bacteria from salt fermented foods for carotenoids production.
2. To systematically identify the selected strain of halophilic archaea.
3. To characterize the carotenoids from selected strains.
4. To optimize the culture conditions for growth and carotenoid production.
5. To evaluate the potential applications of carotenoids as an antioxidant and food colorant in surimi-based products.

1.3 Literature review

Global market for carotenoids

The worldwide carotenoids market is estimated to be $766 million in 2007 according Marz (2008) (Table 1). With an estimated average annual growth rate (AAGR) of 2.3 %, this market is expected to reach $919 million by 2015. The feed market accounts for the largest application segment, valued at $462 million in 2007 and with an AAGR of 2.2% during the 6-year forecast period; this market is expected to reach $527 million by 2015. In recent years, carotenoids have gained outstanding importance in the nutraceutical fields as many studies confirm their antioxidative properties and the effects on the health status of elder people. Even though pharmaceutical constitutes less than 15% of the total market in 2007, it will increase its share to nearly 19% of the total as this market grows at an AAGR of 7% during the forecast period. The most important types of carotenoids are astaxanthin with 28% of total sales and β-carotene with a similar share. The third largest carotenoid is canthaxanthin with 19% of total sales. All other carotenoids show sales volumes in the range of $10 million to $80 millions.

Europe is the largest market for carotenoids with sales totaling nearly 45% of the worldwide market in 1999, mainly because coloring of food and feed are more important than other parts in the world. The U.S. market, with 19% of the total is relatively small. This is mainly because the coloring of fish and poultry but also of other food products with carotenoids is of subordinate importance. However, the use of carotenoids as antioxidants and natural cell protectants is prominent there and a significant amount of the typical carotenoids such as β-carotene and lycopene is large.

Table 1. Global market for carotenoids by application segments, 2007-2015

	2007 (M $)	2015 (M $)	2007-2015 AAGR %
Food	209	235	2.0
Feed	462	527	2.2
Pharmaceutical	115	173	7.0
Total	786	935	2.9

Source: Marz (2008).

According to Frigstad (2003), an expanding carotenoids market is witnessing a face-off between synthetic and natural carotenoids as customers become increasingly conscious about food content. With Europeans' diet beginning to include greater amounts of fortified foods, the natural segment has begun to gain on the synthetic one. While cheaper synthetic imports from China and India are threatening the profits of European manufacturers, natural carotenoids are likely to generate greater revenues as they are nearly four times more expensive than their synthetic equivalents. Increasing promotion of natural carotenoids and their growing demand in the developing dietary supplements market are driving the natural segment further. The five main carotenoids covered in this research are β-carotene, astaxanthin, canthaxanthin, lycopene, and lutein.

Commercial sources of carotenoids

The production of carotenoids from biological sources has been an area of intensive investigation. Because of the inherent biosynthetic capacity of a variety of different organisms, there has been a considerable effort to develop systems to produce carotenoids commercially from biological hosts. The advantages and disadvantages of biological production of carotenoids compared to traditional chemical syntheses were reviewed.

Chemical and biological synthesis of carotenoids

There are several advantages of chemical synthesis for carotenoid production. Chemical synthetic technology has been developed for many carotenoids. The syntheses produce carotenoids of exceptional purity and consistency, and the overall costs of production of these carotenoids are quite low. There are also several disadvantages for production of carotenoids by chemical synthesis. The synthesis of certain carotenoids is very complex. Knowledge and technology developed for the synthesis of one carotenoid may be applicable to the synthesis of other carotenoids, but often the synthesis of a new carotenoid requires the development of a new chemical route. Finally, some stereoisomers may not be active as the naturally occurring carotenoid isomers, may not be desired by the consuming public, or may have undesired side effects.

On the other hand, there are several distinct advantages in the biological production of carotenoids. With the natural productions of over 600 different carotenoids, there are a wide ranges of biosynthetic routes. Because of the characteristics of the biosynthesis of carotenoids, the knowledge of the biosynthesis of one carotenoid is applicable to other carotenoids. With biological production, only the naturally occurring stereoisomers are produced. However, there are also disadvantages in that mixtures of carotenoids are often produced in biological systems and may require further processing and purification. Due to these inherent biochemical and physiological constraints, as a consequence, the overall cost of carotenoid production is higher than that of a chemical synthetic process.

Biotechnological production of carotenoid

There are several commercial operations currently used to produce carotenoids.

Dunaliella species

Certain strains of the unicellular green alga *Dunaliella*, especially *Dunaliella salina* and *Dunaliella bardawil*, may accumulate large amounts of β-carotene under suitable conditions. Such cells then obtain a bright orange-red color. *Dunaliella* strain from Chile was reported to have high carotene content (Gomez *et al*., 1999). It accounted for about 20% of the total carotenoids. The β-carotene is accumulated in globules, located in the interthylacoid space within the cell's single chloroplast. The widely accepted hypothesis suggests that the carotene globules protect the cells against injury by high light intensity under limiting growth conditions by acting as a screen to absorb excess radiation (Ben-Amotz, 1999) (Figure 1). A 38 kDa protein was found associated with the globules, and may serve to stabilize the structure of the globules (Katz *et al*., 1995). The oily globules contain a mixture of the all-trans and the 9-cis isomers of β-carotene (Figure 2), together with minor amounts of other mono-cis and di-cis stereoisomers (Ben-Amotz, 1999). The biochemical pathway of phytoene of *Dunaliella bardawil* has been largely elucidated (Figure 2). The branching point for the formation of the all-trans and the 9-cis isomers of β-carotene was suggested to occur early in the pathway, probably at the level of phytoene (Ebenezer and Pattenden, 1993).

Xanthophyllomyces dendrorhous

X. dendrorhous is the teleomorphic state of *Phaffia rhodozyma* (Golubev, 1995). It is a red/pink-pigmented yeast, which was isolated from tree exudates in mountainous regions of Japan and Alaska in the late 1960s by Phaff *et al*. (1972). This heterobasidiomycete is capable of both fermenting sugars and producing carotenoids (Miller *et al*., 1976). The latter serve as antioxidants to protect *X. dendrorhous* from oxidative damage caused by active oxygen species (Schroeder and Johnson, 1995).

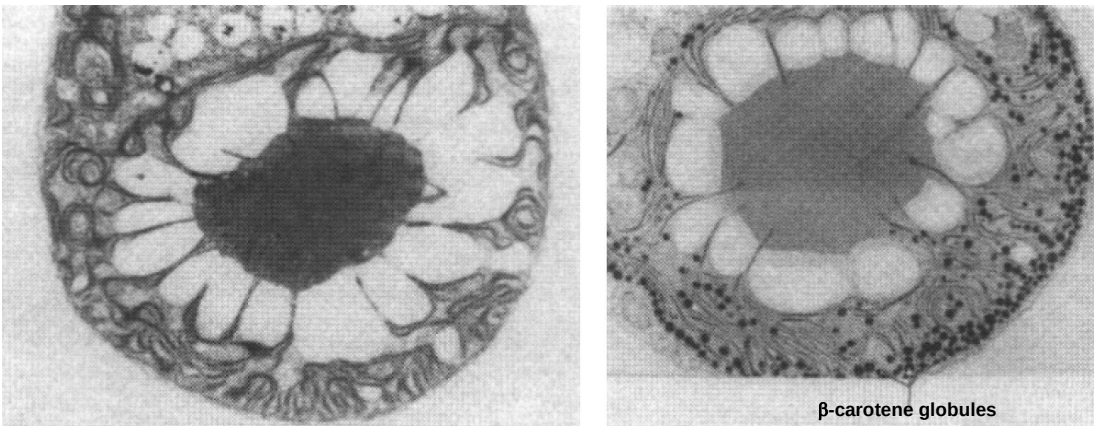

Figure 1. Electron micrographs of sections of the chloroplast from *Dunaliella bardawil* grown under low light (left panel) and high light (right panel).

Source: Katz *et al*. (1995).

Astaxanthin, an optically active compound, is the principal carotenoid produced by *X. dendrorhous*. It represents about 85% of the total carotenoid content (Andrewes *et al*., 1976). While most organisms known to produce astaxanthin synthesize the (3S,3PS)-isomer, *X. dendrorhous* produces the opposite isomer having the (3R,3PR)-configuration (Andrewes and Starr, 1976). Astaxanthin is formed via the mevalonate pathway, which starts at acetyl CoA and proceeds via mevalonate to isopentenyl pyrophosphate (IPP), the general precursor of all isoprenoids. Subsequently eight molecules of IPP are condensed to form the colorless carotenoid phytoene. Via four dehydrogenation and two cyclization reactions phytoene is converted into β-carotene. Finally β-carotene is oxidized to yield astaxanthin (Figure 3) (Andrewes *et al*., 1976). The existence of a monocyclic carotenoid-biosynthetic pathway in *X. dendrorhous* was proposed by An *et al*. (1999).

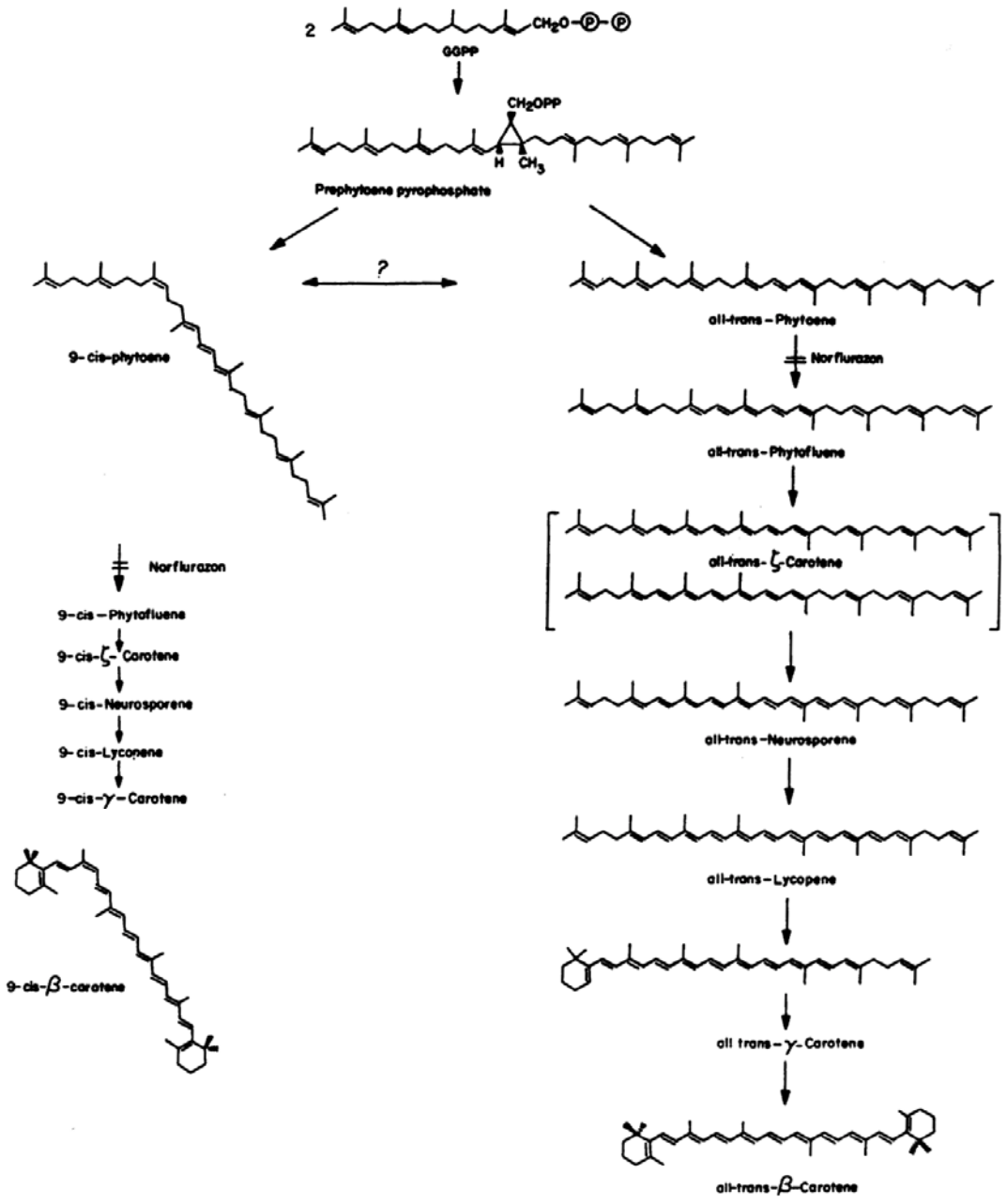

Figure 2. Suggested route of formation of the all-trans and 9-cis isomers of β-carotene and phytoene in *Dunaliella bardawil*.

Source: Oren (2002).

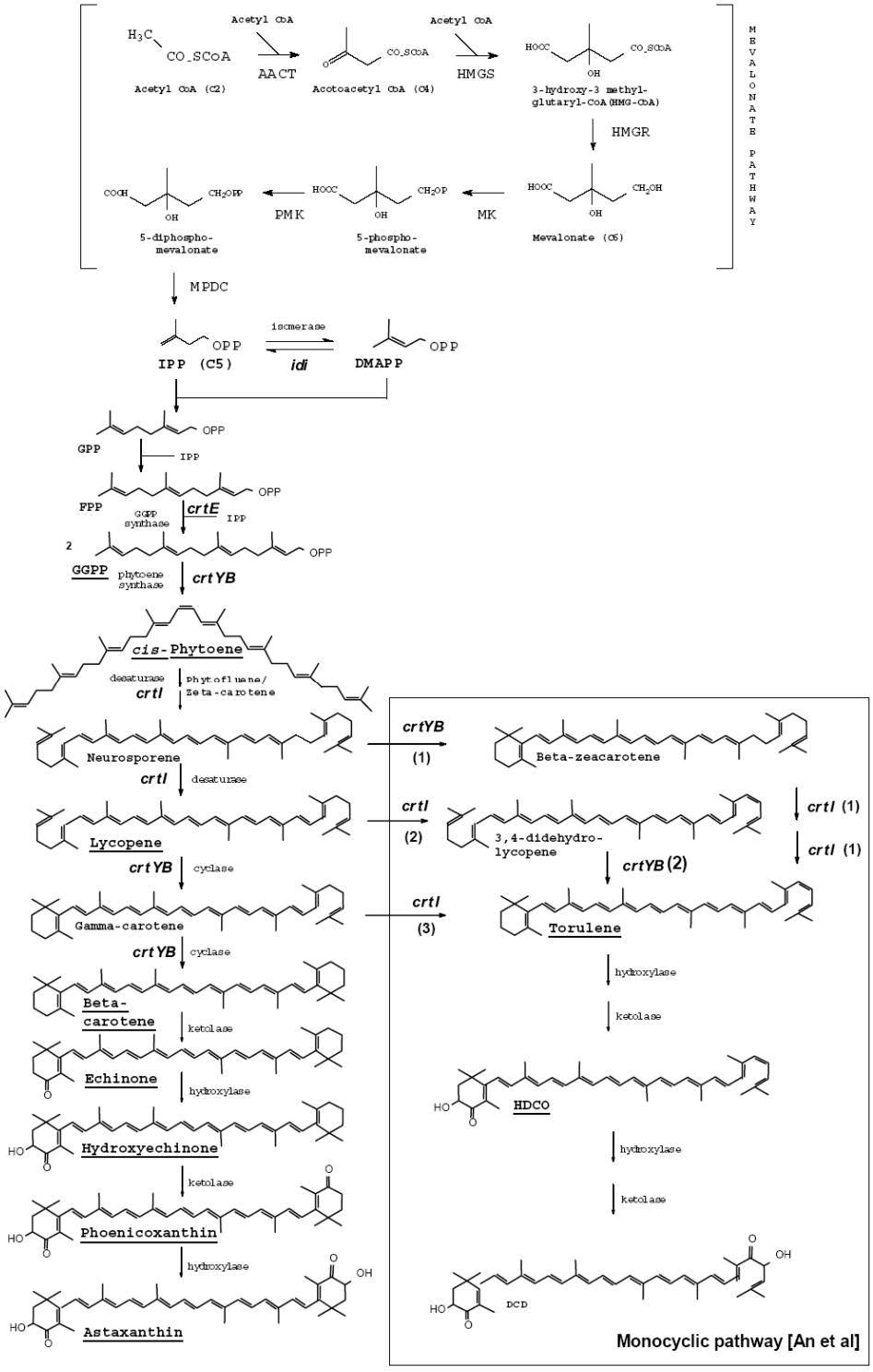

Figure 3. Dicyclic and monocyclic carotenoid-biosynthetic pathways in *X. dendrorhous* as proposed by Andrewes *et al*. (1976) and An *et al*. (1999) (boxed area).

Source: Visser *et al*. (2003).

Haematococcus pluvialis

The single-celled alga *H. pluvialis* has also been extensively studied as a host to produce astaxanthin. Technology has been developed to take advantage of the physiology of this alga. Under growing conditions this alga does not produce astaxanthin. However, when the culture is subjected to stress in which nutrients are eliminated from the growth medium, then the alga produces and accumulates astaxanthin. The levels of astaxanthin can be very high, and there are reports of astaxanthin accumulation of greater than 4% per dry mass (Boussiba *et al*., 2000). Under most conditions this level of astaxanthin synthesis and accumulation occurs only after several weeks of growth. Methods to reduce the time required to produce astaxanthin are currently under extensive study by several groups (Zlotnik *et al*., 1993) and pilot scale production is under way at several sites (García-Malea López *et al*., 2006). As with *Phaffia*, cells of *H. pluvialis* have been found to be a suitable delivery vehicle for astaxanthin for aquaculture and no further purification of the astaxanthin is required (Domínguez-Bocanegra *et al*., 2004).

Chlorococcum

The alga *Chlorococcum* is another promising commercial source of ketocarotenoids due to its relative fast growth rate, ease of cultivation in outdoor systems, and high tolerance to extreme pH and high temperature (Zhang and Lee, 1999). In the alga *Chlorococcum*, astaxanthin is synthesized from β-carotene through various pathways which are different from other astaxanthin producing microorganisms (Liu and Lee, 1999). The investigations for the carotenoid composition and biosynthetic pathway of astaxanthin in the alga *Chlorococcum* are important for the optimization of astaxanthin production.

It is generally agreed that β-carotene serves as a precursor of ketocarotenoids. Astaxanthin was synthesized from β-carotene by two hydroxylation reactions, at C-3 and 30, and two steps of direct oxidation to ketone groups at C-4 and 40 (Rise *et al*., 1994). The order of the reactions and the intermediate products in astaxanthin biosynthesis are different for various astaxanthin-producing microorganisms. The possible intermediates in the astaxanthin biosynthesis pathway contained β-carotene, echinenone, β-cryptoxanthin, canthaxanthin, zeaxanthin, 3-

hydroxyechinenone, 30-hydroxy-echinenone, adonirubin, and adonixanthin (Fraser *et al*., 1997). In the alga *Chlorella zofingiensis*, the hydroxylation step in astaxanthin biosynthesis might take place before oxygenation and astaxanthin (about 70%) is produced via the oxygenation of zeaxanthin while canthaxanthin (about 30%) might be the last stage of the oxygenation of β-carotene (Rise *et al*., 1994). In the green alga *Chlorococcum* (Liu and Lee, 1999), astaxanthin is synthesized from β-carotene by various pathways which are different from other astaxanthin-producing microorganisms. Liu and Lee (1999) postulated five possible pathways leading to the biosynthesis of astaxanthin but could not confirm which was the main biosynthetic pathway. All possible pathways started with the introduction of a keto group at C-4 or a hydroxyl group at C-3, followed by random addition of other keto and hydroxyl groups at both sides of the β-end groups. The alga *Chlorococcum* cells could accumulate a large amount of adonixanthin and canthaxanthin as well as astaxanthin. The conversion of β-carotene to adonixanthin or canthaxanthin was a very rapid reaction. Astaxanthins (free and esters), adonixanthins (free and esters), and canthaxanthin were the main ketocarotenoids in the *Chlorococcum* cells. On the one hand, canthaxanthin was present in the *Chlorococcum* cells, indicating that astaxanthin was synthesized from β-carotene via canthaxanthin (Figure 4).

Functions of carotenoids

Epidemiological evidence and experimental results suggest that dietary carotenoids inhibit onset of many diseases like arteriosclerosis, cataracts, age related macular degeneration, multiple sclerosis and most importantly cancer, all of which are mainly initiated by free radicals (Stahl and Sies 1996; Henneckens 1997). The two major theories behind the protective effect of carotenoids are provitamin-A and antioxidant. However, not all carotenoids can be converted to vitamin A.

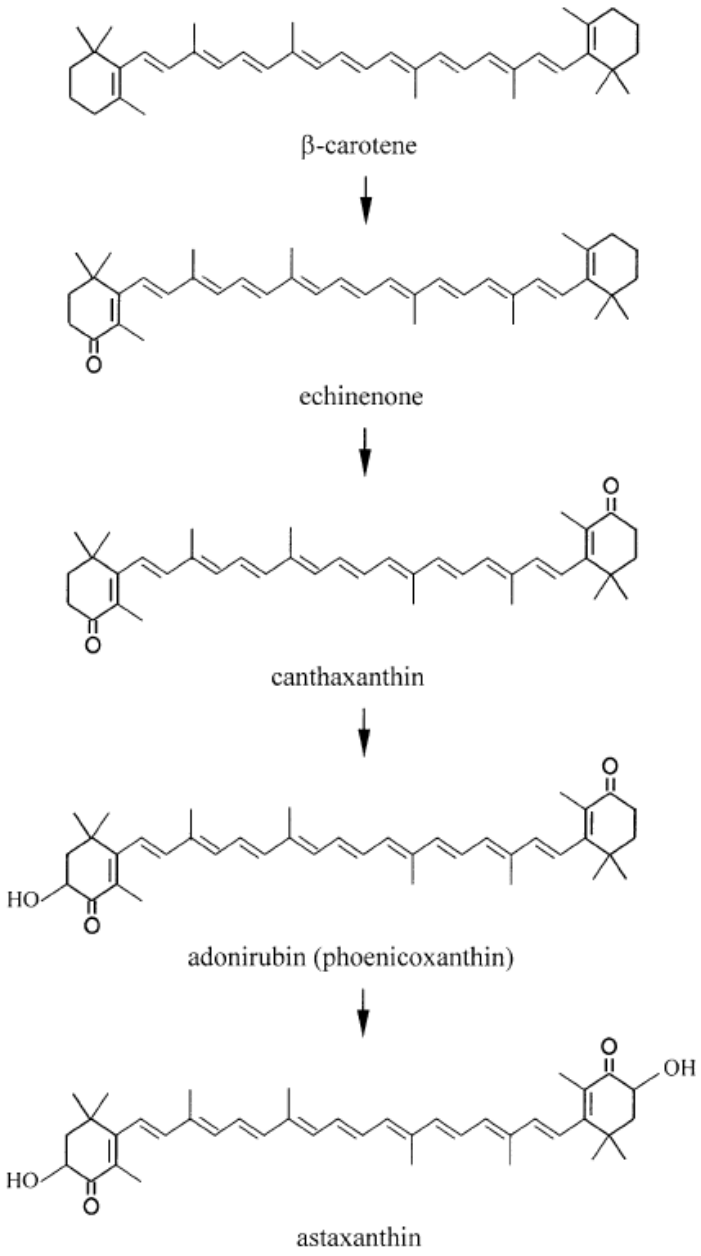

Figure 4. Postulated pathway for the biosynthesis of astaxanthin in the algae *Chlorococcum.*

Source: Yuan *et al*. (2002).

Canthaxanthin, a carotenoid with no provitamin-A activity, has been proposed to reduce the risk of cancer (Mayne and Parker 1989). Therefore, it must be concluded that anticancer effect can be partly attributable to their antioxidant nature. The second theory is that carotenoids have the ability to act as chain breaking antioxidants and thus protect cells against photo-oxidation. The ability of carotenoids to quench singlet oxygen is well known (Foote and Denny, 1968; Oliveros, *et al*., 1994) and reactions with radical species have also been studied (Burton and Ingold, 1984; Böhm *et al*., 1995).

Provitamin-A

The conversion of all trans β-carotene to retinol in the intestinal mucosal cells is the results of the action of several enzymes. β-carotene 15-15' dioxygenase cleaves the carotene molecule at central double bond that directly forms in two molecules of retinaldehyde. One molecule of retinaldehyde is subsequently reduced to retinol by retinal reductase enzyme, and other molecule is oxidized to retinoic acid. Alternatively, enzymatic cleavage of carotene occurs at the 8', 10' and 12' double bonds, which may indirectly produce retinoic acid and retinal in low concentrations (Hinds *et al*., 1997).

Antioxidant

There are several mechanisms by which carotenoids can act as antioxidants. These mechanisms can be summarized in the abilities of these molecules to act either as photoprotective agents against the harmful effects of light and oxygen or as compounds reactive against chemical species generated within cells and able to induce oxidative damage. The mechanism of its role as an antioxidant is described briefly here.

Reaction with singlet oxygen

Carotenoids act as antioxidants by physical quenching or reacting with a variety of free radicals. The benefit of physical quenching is that carotenoids may act as antioxidants without losing its own structure. Quenching of $^1O_2$ mainly leads to energy dissipation as heat, whereas the reactions between carotenoids and free radicals, such as lipid oxidation, lead to electron transfer or further radical reactions. The reviews in details of the antioxidant role of carotenoids as $^1O_2$ quenchers see Edge *et al*. (1997).

Reaction with oxygen radicals

Reactive oxygen gets generated in the cell as a result of various metabolic processes or following exposure to Xenobiotics. Reactive oxygen is reported to cleave DNA, peroxidize lipids, alter enzyme activity, deploymerize polysaccharides and kill cells. Carotenoids are known to quench these radicals and prevent degree of nuclear and cellular damage.

Reaction with free radicals

Free radicals are by-products of metabolic process and originate from environmental pollutants such as nitrogen dioxide and ozone in polluted air, heavy metals, and halogenated hydrocarbons, ionizing radiation and cigarette smoke. Free radicals can damage both structure and function of cell membranes, nucleic acids and electron dense regions of proteins. Peroxy radical, owing to its selectivity of reaction and ability to diffuse in biological system are potentially more dangerous than many other types of radicals and is reported to cause heart disease, cancer and process of aging (Edge *et al*., 1997). Carotenoids also have ability to quench these free radicals. Unlike the quenching of singlet oxygen, which leads to energy dissipation as heat, the reaction of a carotenoid with a free radical will lead to electron transfer or possibly addition reactions (Edge *et al*., 1997).

Halophilic archaea

Classification

Archaea have long been considered as bacteria due to their prokaryotic morphology, circular genomes and gene organization in operons, but in 1977 archaea were clearly distinguished as the third domain of life by applying rRNA phylogeny (Woese and Fox, 1977). Their status as a separate domain is further supported by their unique features such as a distinctive cell membrane containing phenyl side chains that are ether-linked to *sn*-glycerol 1-phosphate. Within the domain *Archaea* halophilic microorganisms occur in three families: the *Halobacteriaceae*, the *Methanospirillaceae* and the *Methanosarcinaceae* (Figure 5). The *Methanospirillaceae* and the *Methanosarcinaceae* contain non-halophilic representatives as well as organisms that are adapted to seawater salinity and to hypersaline conditions. Some of these can grow at salt concentrations up to 300 g/L.

The order *Halobacteriales* with a single family, the *Halobacteriaceae*, consists entirely of halophiles. The most salt-requiring and salt-tolerant of all microorganisms are found in this family. They live in natural environments where the salt concentration is very high (as high as 5 M or 25% (w/v) NaCl) and grow optimally at 3.4-5.1 M (20-30%, w/v) NaCl (Table 2). These prokaryotes require salt for growth and will not grow at low salt concentrations. Their cell walls, ribosomes and enzymes are stabilized by Na^+. The halophilic archaea is strictly aerobic even high salt conditions with less availability of O_2 for respiration. They adapt to the high-salt environment by the development of purple membrane, actually patches of light-harvesting pigment in the plasma membrane. The pigment is bacteriorhodopsin contains 25% lipids and 75% protein. It reacts with light resulting in a proton gradient on the membrane just as in the case of the respiratory chain allowing the synthesis of ATP. Accordingly, the extreme halophiles can produce efficiently ATP by normal way (respiration) and adaptation one (bacteriorhodopsin).

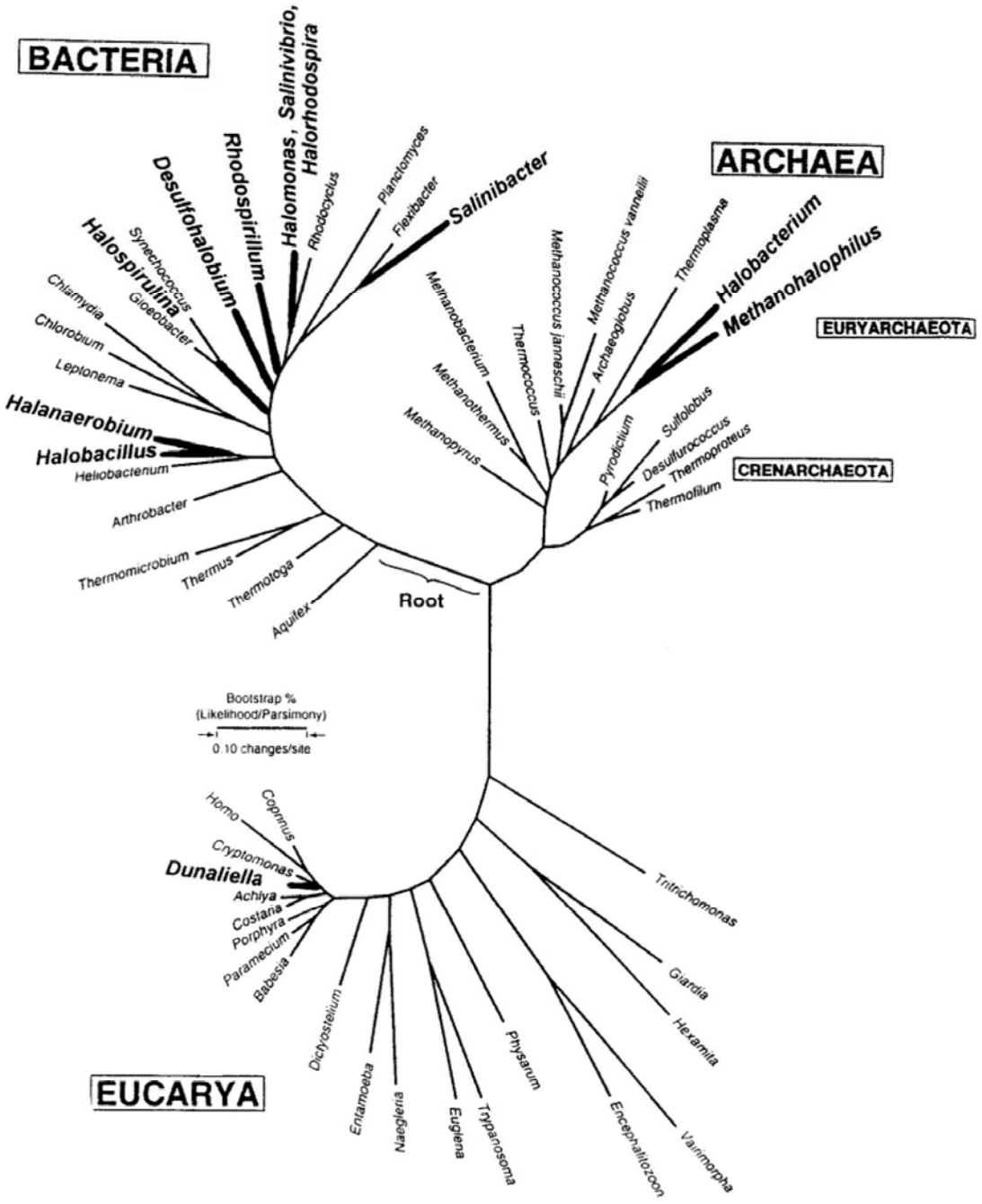

Figure 5. The universal phylogenic tree base on small subunit rRNA sequence comparisons. Those branches in which microorganisms have been identified are highlighted.

Source: Oren (2002).

Table 2. Ionic composition of selected hypersaline environments

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

Source: Madigan and Oren (1999).

As of May 2009 this family is divided into 27 genera by rRNA sequencing and other criteria (Euzéby, 2009); *Haladaptatus* (*Hap*.), *Halalkalicoccus* (*Hac*.), *Haloarcula* (*Har*.), *Halobacterium* (*Hbt*.), *Halobaculum* (*Hbl*.), *Halobiforma* (*Hbf*.), *Halococcus* (*Hcc*.), *Haloferax* (*Hfx*.), *Halogeometricum* (*Hgm*.), *Halomicrobium* (*Hmc*.), *Halopiger* (*Hpg*.), *Haloplanus* (*Hpn*.), *Haloquadratum* (*Hqr*.), *Halorhabdus* (*Hrd*.), *Halorubrum* (*Hrr*.), *Halosarcina* (*Hsn*.), *Halosimplex* (*Hsx*.), *Halostagnicola* (*Hst*.), *Haloterrigena* (*Htg*.), *Halovivax* (*Hvx*.), *Natrialba* (*Nab*.), *Natrinema* (*Nnm*.), *Natronobacterium* (*Nbt*.), *Natronococcus* (*Ncc*.), *Natronolimnobius* (*Nln*.), *Natronomonas* (*Nmn*.) and *Natronorubrum* (*Nrr*.). Figure 6 provides a list of the presently recognized genera and species in the *Halobacteriales.* 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.

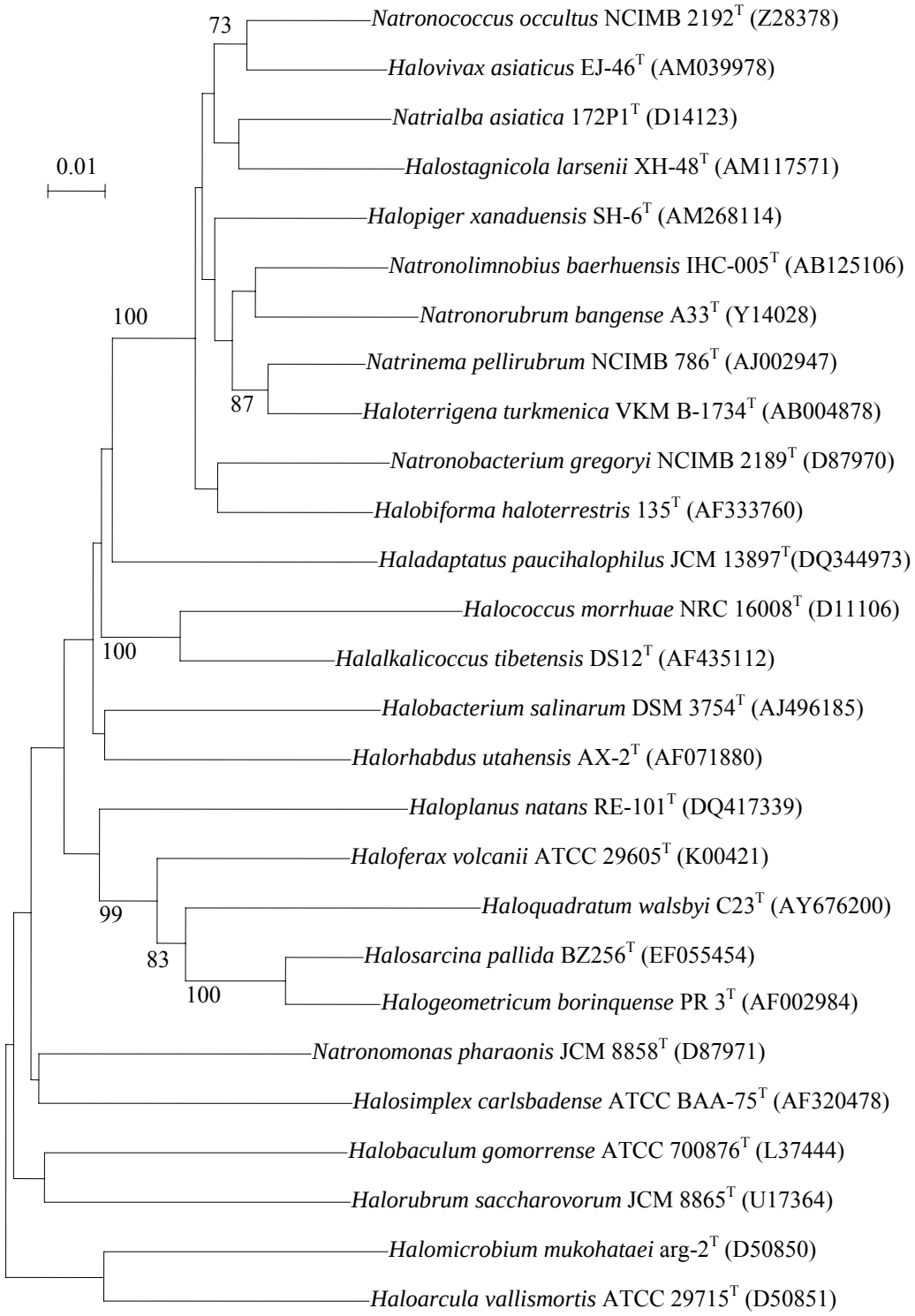

Figure 6. Phylogenetic trees showing genus belonging to the family *Halobacteriaceae*.

Source: Euzéby (2009).

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

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

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

newly described bacterial taxa must be represented by a type culture deposited in a permanent culture collection.

In addition, descriptions of new taxa of *Halobacteriales* should be based on the following principles: (i) type strains of related taxa should be included for comparison, and it is recommended that in addition at least one species from each of the recognized genera be used for comparative purposes in studies of new isolates; (ii) the characters that differentiate each new taxon from all previously described species should be adequately described, and it is recommended that the new taxon should be able to be identified and differentiated by methods available outside specialized laboratories; (iii) the methods used must be given in detail, or references to readily available publications must be given; and (iv) the description of each new taxon should be published in a journal that has wide circulation, preferably the International Journal of Systematic Bacteriology, and when described elsewhere, the new taxon should be included as soon as possible on one of the lists that validate the publication of new names and new combinations previously effectively published outside the International Journal of Systematic Bacteriology that appear periodically in that journal. The subcommittee proposes the characters described below and listed in Table 3 and 4 as minimum features that should be contained in descriptions of new halobacterial taxa. However, we recognize that description of extraordinary microorganisms may require flexibility in the application of the tests described below. The proposal below specifies the minimal requirement for tests and does not in any way limit the extent of investigations beyond this.

Oren *et al*. (1997) recommend that all future taxonomic publications on the *Halobacteriales* should contain data on phenetic, chemical and molecular properties. Oren *et al*. (1997) thus recognize that modern natural classification requires as complete a data set as possible, including phenotypic and genotypic information.

Table 3. Required minimal standards for description of halobacterial taxa

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

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

Table 4. Recommended minimal standards for description of halobacterial taxa

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

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

Cellular structures of halophilic archaea

Cell walls

The shape of the cells of halophilic *Archaea* is determined, as in other microorganisms, by the properties of their cell wall. Species of the family *Halobacteriaceae* appear in a variety of shapes. In addition to rods (*Halobacterium* and others) and spheres (*Halococcus*, *Natronococcus*) there are flat pleomorphic species such as *Haloferax*, the shape of which has been compared with potato chips (Englert *et al*., 1992). A yet uncultured representative of the family appears as extremely thin perfect squares or rectangles, while others such as *Haloarcula japonica* may show triangular cells. Four morphological types were identified: 1, Single, rod-shaped cells with a thin (10 nm) wall; 2, Irregular clusters of tightly packed cells in a common fibrillar capsule, the thickness of which depended on

culture conditions; 3, Single rods with a thickened (up to 50 nm) cell wall of fibrillar material, with nucleoids more compact than in normal cells; 4, Rounded cells with a three-layered capsule, consisting of an internal loose 50 nm thick fibrillar layer, an intermediate homogenous layer of 100 nm thickness, and an external 25 to 50 nm thick fibrillar layer (Kostrikina *et al*., 1991).

While the coccoid genera *Halococcus* and *Natronococcus* possess thick rigid cell walls that do not depend on high salt for structural stability, the other members of the *Halobacteriaceae* have a cell wall (often referred to as an S-layer) that consists of subunits of a large glycoprotein. Absence of "normal" wall components such as D-amino acids or teichoic acid in the *Halobacterium* cell wall had been noted long before the domain *Archaea* was recognized (Kushner and Onishi, 1968). The glycoprotein is essential for maintaining the rod shape of *Halobacterium* and the flat disk- or triangular shape of *Haloferax* and *Haloarcula* species. Considerable structural information on these wall glycoproteins has been obtained. The mature cell wall glycoproteins of *Halobacterium salinarum*, *Haloferax volcanii*, and *Haloarcula japonica* contain 818, 794, and 828 amino acids, respectively, with molecular masses of 86,538, 81,732 and 87,166 Da. SDS-PAA gel electrophoresis gives much higher apparent molecular masses (200, 170 and 170 kDa, respectively). Substitution of the proteins with sulfated sugar groups, as well as their modification with diphytanylglyceryl phosphate reduces their electrophoretic mobility (Kikuchi *et al*., 1999). This *Halobacterium* glycoprotein requires high NaCl concentrations for structural stability. When suspended in low salt solutions, the wall protein denatures, and this leads to lysis and cell death (Soo-Hoo and Brown, 1967). Lysis of *Halobacterium salinarum* in hypotonic solutions is not due to the build-up of osmotic pressure (Mohr and Larsen, 1963).

The primary structure of the protein backbone and the mode of its glycosylation vary among different species of halophilic *Archaea*. The glycosylation pattern of the Haloferax *volcanii* glycoprotein involves both *N*- and *O*-glycosidic bonds [glucosyl-(1→2)-galactose disaccharides *O*-linked to threonine residues] in a pattern that differs from that of *Halobacterium salinarum* (Sumper *et al*., 1990). The S-layer protein of *Haloferax volcanii* has 7 N-glycosylation sites, as compared to 12 in *Halobacterium salinarum* (including the sub NH_2-terminal negatively charged

repeating unit saccharide which is absent in *Haloferax volcanii*). The N-linked oligosaccharides of *Haloferax volcanii* are β-(1→4)-linked repeating glucose residues attached via asparaginyl-glucose linkages (Mengele and Sumper, 1992) (Figure 7). The replacement of charged sugar units (glucuronic acid) in *Halobacterium salinarum* by neutral sugars (glucose) in *Haloferax volcanii* results in a difference in charge density of the cell wall proteins of the two species (Figure 7). This difference may be related to the different salt requirement of the two species (Mengele and Sumper, 1992); *Haloferax volcanii* was described as a moderate halophile with high magnesium tolerance (Mullakhanbhai and Larsen, 1975).

The biosynthesis of the major *Haloferax volcanii* S-layer glycoprotein involves a maturation step following translocation of the protein across the membrane. Evidence for the processing step was obtained from an increase in its apparent molecular mass. This increase was unaffected by inhibition of protein synthesis and unrelated to glycosylation. Maturation also led to an increase in hydrophobicity (Eichler, 2001). This maturation process probably involves isoprenylation of the protein. A procedure for the preparation of inverted membrane vesicles of *Haloferax volcanii* was recently developed. It may prove useful in the study of the biosynthesis of the S-layer glycoprotein of *Haloferax volcanii*, its translocation and its glycosylation (Ring and Eichler, 2001).

The chemical analyses of the *Halococcus* cell wall identified glycine, glutamate, glucosamine and galactosamine among the components (Reistad, 1971). A more thorough analysis of the wall structure (Figure 8) has confirmed the presence of glucosamine and galactosamine derivatives in the sugar backbone. Sulfate groups are linked to hydroxyl groups in positions 2 and/or 3 of uronic acids, galactose and galactosamine residues. Glucose, galactose, galacturonic acid and all amino sugars are glycosidically linked to the cell wall polymer. Part of the glucose, the galactose, and to a lesser extent also the mannose residues possess more than two glycosidic linkages, and these residues represent possible branching points. Glycine residues may play a role in connecting glycan strands through peptide linkages between the amine groups of glucosamine and the carboxyl group of an uronic acid or gulosaminuronic acid (Schleifer *et al*., 1982).

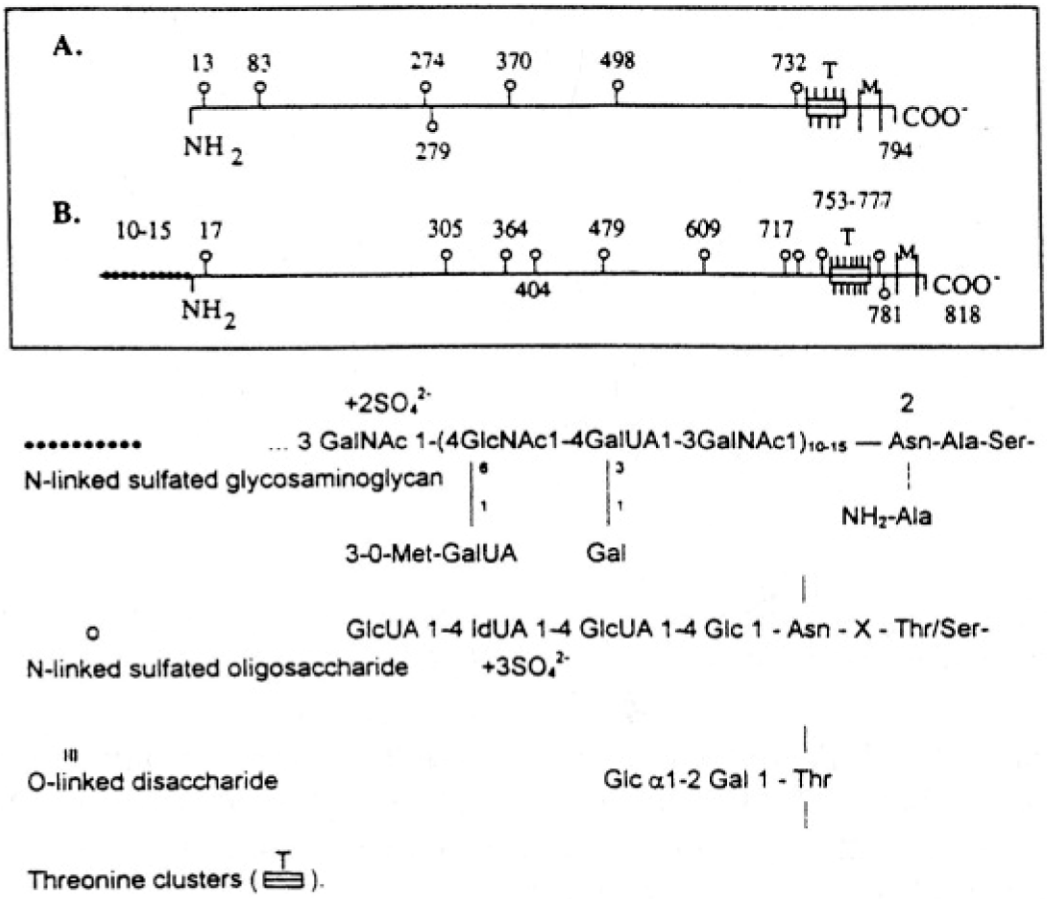

Figure 7. Model of the S-layer glycoprotein of *Haloferax volcanii* (A) and *Halobacterium salinarum* (B) and the glycosylation sites of the latter.

Source: Gilboa-Garber *et al*. (1998)

The alkaliphilic coccoid *Natronococcus occultus* also has a thick cell wall that retains its shape in the absence of salt. Its structure differs greatly from that of the cell wall polymer of *Halococcus*. It consists of repeating units of a poly(L-glutamine) glycoconjugate (Niemetz *et al*., 1997). Figure 9 presents a model of this structure.

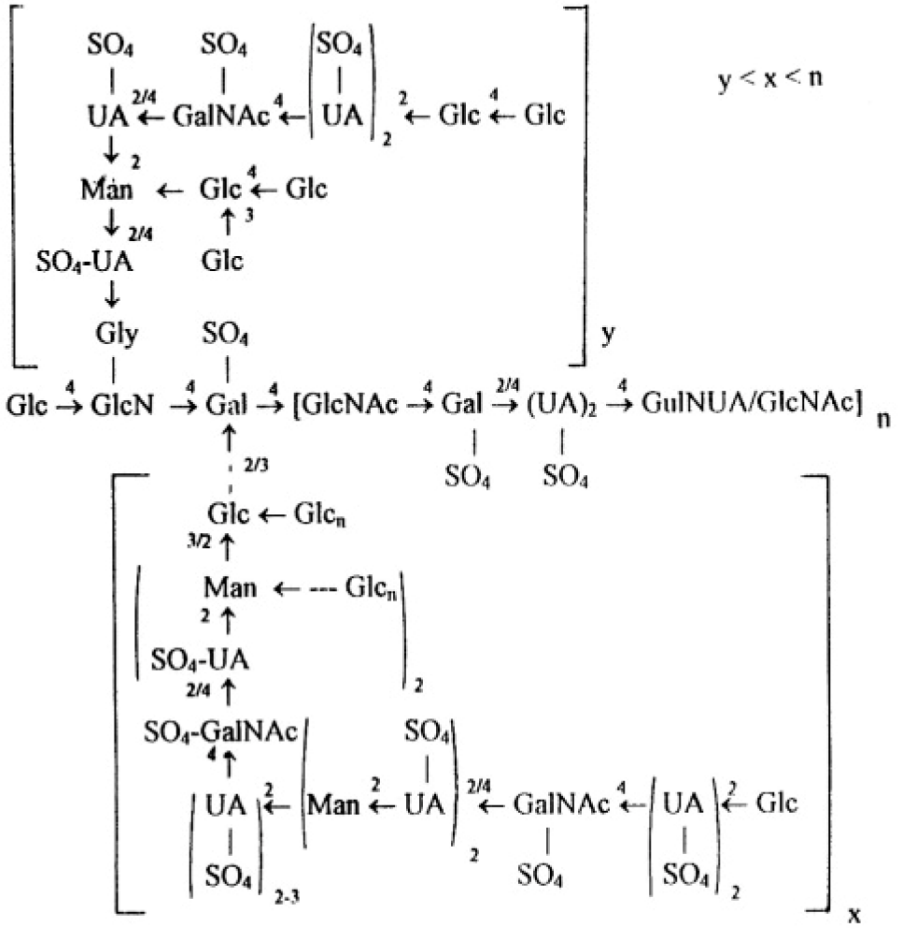

Figure 8. The tentative structure of the cell wall polymer of *Halococcus morrhuae*. Gal = galactose; GalNAc = N-acetylgalactosamine; Glc = glucose; GlcNAc = N-acetylglucosamine; Gly = glycine; GulNUA = N-acetylglucosaminuronic acid; Man = mannose; UA = uronic acid.

Source: Schleifer *et al*. (1982).

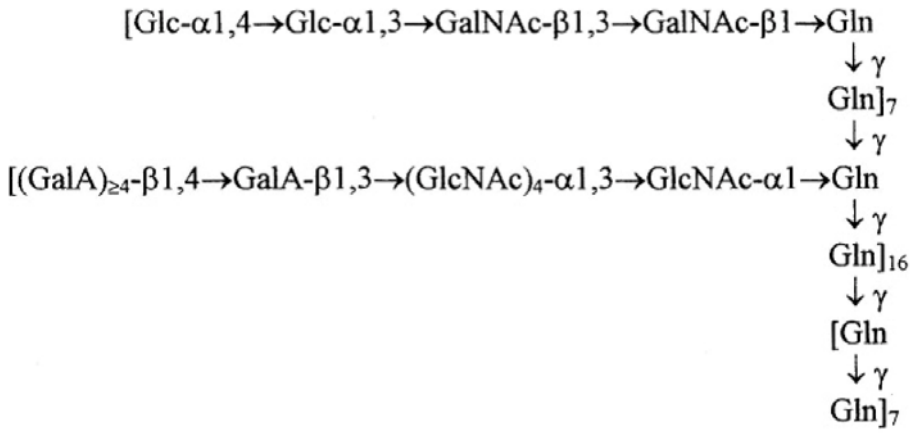

Figure 9. The tentative structure of the cell wall polymer of *Natronococcus occultus*. GalA = galctonic acid; GalNAc = N-acetylgalactosamine; Glc = glucose; GlcNAc = N-acetylglucosamine; Gln = glutamine.

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

Cytoplasmic membrane and its lipids

The cytoplasmic membrane is composed of lipids and proteins. It contains all the functions needed for respiratory electron transport, inward and outward transport of ions, nutrients, and other compounds, sensors that provide information about the extracellular environment and their transducers, and many other components. In addition, the retinal ion pumps bacteriorhodopsin and halorhodopsin are present in the membranes of many halophilic Archaea. The bacteriorhodopsin proton pump is sometimes organized in differentiated patches within the membrane.

The members of the *Halobacteriaceae* have archaeal-type lipids based on branched 20-carbon (phytanyl) and sometimes also 25-carbon (sesterterpanyl) chains, bound to glycerol by ether bonds. One- or two-dimensional thin-layer chromatography is a useful technique for the rapid characterization of the lipids present in halophilic archaeal isolates (Oren *et al*., 1996). Details on the lipid composition of halophilic *Archaea,* the biosynthetic pathways involved in their synthesis, and the analytical methods used in their characterization are found in several reviews (Kamekura and Kates, 1999; Kates, 1996).

The diether core lipid that forms the basis for most polar lipid structures present in the family *Halobacteriaceae* is 2,3-di-*O*-phytanyl-*sn*-glycerol (C_{20},C_{20}). Certain species, however, contain in addition the asymmetric 2-*O*-sesterterpanyl-3-*O*-phytanyl*sn*-glycerol (C_{25},C_{20}) in different amounts. A thin layer chromatographic procedure has been developed to separate the (C_{20},C_{20}) and (C_{25},C_{20}) lipid species (Ross *et al*., 1981). The (C_{25},C_{20}) core lipid is found in many of the alkaliphilic types, as well as in the neutrophilic *Natrialba asiatica* (Kamekura and Dyall-Smith, 1995), and in the genera *Natrinema* (McGenity *et al*., 1998) and *Halococcus.* C_{25},C_{20} core lipids were also detected in *Halobacterium halobium* IAM13167, which may be a *Halobacterium salinarum* strain (Morita *et al*., 1998). The lipids are expected to form 'zip' type bilayer membranes as exemplified in Figure 10 (De Rosa *et al*., 1983).

A great variety of polar lipids, including phospholipids, sulfolipids, and glycolipids, is encountered in the different representatives of the *Halobacteriaceae.* All known species contain the diether derivatives of PG and Me-PGP (Figure 11a and b). The presence of the methyl ester group in the Me-PGP structure was recognized only relatively recently (Kates, 1996). Phosphatidylglycerosulfate (PGS, Figure 11c) is present in many neutrophilic species (Hancock and Kates, 1973). Its absence in certain genera (*Haloferax, Natrialba, Halobaculum, Halococcus, Halogeometricum*) is a useful diagnostic feature in the classification and identification of the *Halobacteriaceae.* The alkaliphilic members characterized thus far all lack PGS. PG, Me-PGP, and PGS are the only phospholipids in most neutrophilic representatives of the *Halobacteriales.* Additional unidentified phospholipids have been detected in the genus *Natrinema* (McGenity *et al*., 1998). In *Natronococcus occultus* a phospholipid with a cyclic phosphate group has been identified: 2,3-di-*O*-phytanyl-*sn*-glycero-lphosphoryl-3'-*sn*-glycerol-1,2-cyclic phosphate (Figure 11d). The glycolipids and sulfolipids have been found in different members of the *Halobacteriaceae* (Table 5).

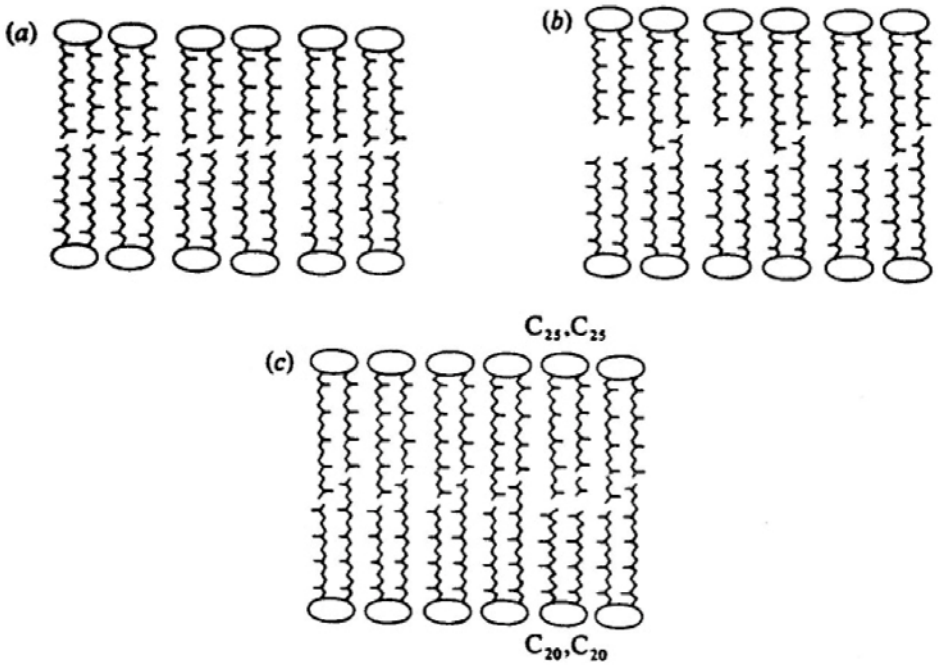

Figure 10. Halophile membrane structures. (a) Bilayer membrane structure formed by C_{20},C_{20} diether lipids, (b) "Zip" type 1 membrane formed by C_{20},C_{25} and C_{20},C_{20} diethers lipids (c) "Zip" type 2 membrane formed by C_{25},C_{25} and C_{20},C_{20} diethers lipids.

Source: De Rosa *et al*. (1983).

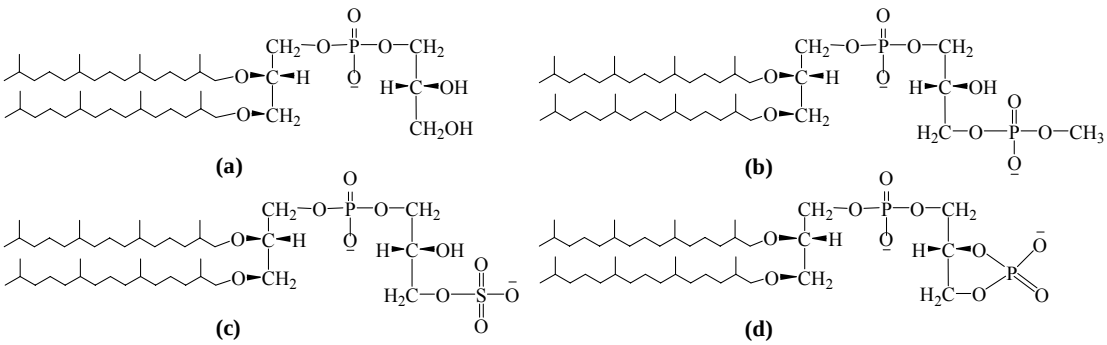

Figure 11. Structure of the phytyl diether derivatives of phosphatidylglycerol (PG) (a), the methyl ester of phosphatidylglycerophosphate (Me-PGP) (b), phosphatidylglycerosulphate (PGS) (c), and the cyclic PGP of *Natronococcus occultus* (d).

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

Table 5. Major glycolipids of different members of the *Halobacteriaceae*

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

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

Source: Kamekura and Kates (1999).

Physiology and metabolism of halophilic archaea

Osmotic adaptation

Members of the *Halobacteriaceae* require 2-4 M (12-23%) NaCl for optimal growth and most halophilic archaea thrive up to the limit of saturation for sodium chloride (around 5.5 M (32%, w/v) NaCl), although growth of some species is rather slow at this salinity Haloarchaea are unable to grow below concentrations of 1.5 M (9%, w/v) NaCl, and *Halobacterium salinarum* has been shown to require large amounts of sodium. Sodium ions are, for example, needed, e.g. for cell wall integrity and many transport processes in *Halobacterium*, and cannot be replaced by other ions such as potassium (Madigan and Oren, 1999). In contrast to most halophiles that accumulate or synthesize intracellular organic compounds (compatible solutes) to withstand the osmotic pressure that accompanies life in hypersaline environments, *Halobacterium* produces no compatible solutes. Instead, it pumps large amounts of potassium into the cytoplasm (salt-in strategy) so that the intracellular K^+ concentration is considerable higher than the extracellular Na^+ concentration.

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

The cell wall of *Hbt. salinarum*, *Hbt. volcanii* and *Haloarcula japonica* is composed of a glycoprotein with exceptionally high contents of acidic amino acids (Nakamura *et al*., 1995). In the extreme halophilic *Hbt. salinarum*,

further negative surface charges are introduced by sulfate groups of N-linked saccharide units and a large N-terminally linked, sulphated sugar unit lacking in the more moderate halophile, *Hbt. volcanii*. The negative charges of the acidic amino acids and sugar moieties are shielded by sodium ions, and are absolutely essential for maintaining cellular integrity (Madigan *et al*., 2000).

Nutritional demands, nutrient transport and sensing

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

Most halophilic archaea preferentially use amino acids as carbon and energy source, but utilize also other compounds of hypersaline habitats such as glycerol and tricarboxylic acid (TCA) cycle intermediates that are excreted by *Dunaliella* and the cyanobacterium *Microcoleus chtonoplastes*, respectively (Oren, 2002). For example, the synthetic medium for *Hbt. volcanii* contains succinate and glycerol as carbon sources (Kauri *et al*., 1990). Sugars such as glucose, fructose, and sucrose are catabolized only by some haloarchaea, such as *Hfx. mediterranei* and *Halorubrum saccharovorum* (Altekar and Rangaswamy, 1992). *Halobacterium* does not grow on sugars but growth of is stimulated by the addition of carbohydrates to the medium (Oren, 2002) and glucose can be transformed to gluconate (Sonawat *et al*., 1990). Oxidation of carbohydrates is often incomplete, and *Hrr. saccharovorum* was found to excrete acetate and pyruvate when grown on various sugars (Oren, 2002).

Acetate can also be metabolized but it was found that acetate is used very poorly by haloarchaea. Degradation of fatty acids has not been reported yet but is likely since all genes for the fatty acid β-oxidation pathway are present in the halobacterial genomes. Many species of *Halobacteriaceae* produce exoenzymes for the degradation of polymeric substances, e.g. the alkaline serine protease halolysin of *Halobacterium* (Kamekura *et al*., 1992) and an α-amylase of *Natronococcus* (Kobayashi *et al*., 1994). Finally, several haloarchaeal isolates have been described to degrade aliphatic hydrocarbons and aromatic compounds (Oren, 2002).

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

Hbt. salinarum is able to sense branched amino acids, methionine, cysteine, arginine and several peptides and to move toward attractant signals. Arginine chemotaxis is enabled by the cytoplasmic transducer Car, while membrane-bound BasT is involved in sensing of the remaining amino acids (Kokoeva *et al*., 2002). *Hbt. salinarum* contains several further transducers, the functions of some have been elucidated; HemAT/HtrVIII for oxygen-sensing (Hou *et al*., 2000), HtrI/HtrII for orange/blue light phototaxis (Hoff *et al*., 1997), and MpcT as proton motive force sensor. *Halobacterium* tranducers trigger a signalling pathway to the flagella motor which resembles bacterial signaling cascades and involves a typical bacterial-type two-component regulatory system (CheA/CheY). The *Halobacterium* motor is well understood on the functional level and, thus, a dynamic model could be established.

However, the protein components of the archaeal motor remain to be identified on the genomic level.

Energy metabolism

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

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

Carotenoids production of halophilic archaea

Most of halophilic archaea in the family of Halobacteriaceae are brightly red-orange due to a large amount of a red membrane consisting of carotenoid pigments. Hypersaline lakes and saltern crystallizer ponds approaching NaCl saturation are generally colored red due to the presence of archaeal carotenoids (Oren and Rodríguez-Valera, 2001). The main carotenoids of the halophilic Archaea are C-50 carotenoids, mainly bacterioruberin and its derivatives including monoanhydrobacterioruberin and bisanhydrobacterioruberin (Kelly *et al*., 1970; Kushwaha *et al*., 1975).

Several other derivatives have been found in minor amounts, such as the dodecaene C_{50} carotenoids 3',4'-dihydromonoanhydrobacterioruberin, haloxanthin (a 3',4'-dihydromonoanhydrobacterioruberin derivative with a peroxide end group), and 3',4'-epoxymonoanhydrobacterioruberin, identified in *Haloferax volcanii* (Rønnekleiv *et al*., 1995). The content of bacterioruberin pigments in the biomass has been used to monitor the density of halophilic archaeal communities in the Dead Sea (Oren and Gurevich, 1995). It was suggested that bacterioruberin is synthesized by addition of C_5 isoprene units to each end of the lycopene chain, followed by introduction of four hydroxyl groups. Evidence for such a pathway was obtained from inhibitor studies in which nicotine was used to inhibit the formation of bacterioruberin (Kushwaha and Kates, 1979). The presence of multiple genes for several of the steps in *Halobacterium* NRC-1 carotenoid production suggests that there may be more than one biosynthetic pathway (DasSarma *et al*., 2001). Lycopene is a precursor of carotene, but the amounts of lycopene and in the cells were not correlated. There was also no consistent correlation between the amounts of and carotenoids, implying that their biochemical pathways may be independent to a large extent (Kushwaha and Kates, 1979). Retinal, the prosthetic group of bacteriorhodopsin, halorhodopsin, and the sensory rhodopsins, is synthesized via lycopene and β-carotene (Sumper *et al*., 1976).

Other minor carotenoid compounds identified in halophilic Archaea are lycopersene, cis- and trans-phytoene, cis- and trans-phytofluene, and β-carotene. The low concentrations of these compounds suggest that they may serve as precursors

for the synthesis of retinal, lycopene, and the C_{50} pigments of the bacterioruberin group (Tindall, 1992). A halophilic archaeon isolated from a seawater evaporation pond near Alexandria, Egypt, produces considerable amounts of the C_{40} ketocarotenoid canthaxanthin (Asker and Ohta, 1999). As much as 0.34 mg of canthaxanthin was found per mg total carotenoids. In addition, β-carotene was present (0.03 mg per mg carotenoids) (Asker and Ohta, 1999), as are 3-hydroxyechinenone, cis-astaxanthin, lycopene, trisanhydrobacterio-ruberin, monoanhydrobacterioruberin, and bacterioruberin isomers. Calo *et al*. (1995) stated that the carotenoids 3-hydroxyechinenone and trans-astaxanthin occur in *Halobacterium salinarum*, *Haloarcula hispanica*, and *Haloferax mediterranei*; in *Halobacterium salinarum* 24% of the pigment on a per weight basis was 3-hydroxyechinenone, and 11% was identified as trans-astaxanthin.

Bacterioruberin pigments of *Halobacterium* and other members of the Halobacteriaceae protected against damage by high intensities of light in the visible and ultraviolet range of the spectrum, and reinforcement of the cell membrane. White mutants lacking bacterioruberins can easily be isolated from species such as *Halobacterium salinarum*. In the dark such mutants grow as well as the red wild type. However, when incubated at high light intensities approaching those of full sunlight, the white mutants are outcompeted by the pigmented parent strain, demonstrating the role of the carotenoid pigments in protecting the cells against light damage (Dundas and Larsen, 1963). Even more dramatic differences between the survival of the red wild type and the white mutants were obtained when photosensitizers such as toluidine blue or phenosafranine were added: the white variety was rapidly killed, but the carotenoid-rich cells showed excellent survival (Dundas and Larsen, 1963). When *Haloferax mediterranei* cells grown at 300 g/L (weakly pigmented) and 150 g/L NaCl (strongly pigmented) were exposed to strong illumination, the pigmented cells grew normally, while in the weakly pigmented cells growth ceased and part of the cells lysed. Nutrient-starved cells, however, were only little affected by high light intensities, independent of their bacterioruberin content (Rodriguez-Valera *et al*., 1982). The carotenoid pigments of *Halobacterium salinarum* were also claimed to protect the cells against UV radiation and aid in photoreactivation (Shahmohammadi *et al*., 1998). Bacterioruberin (which contains 13 conjugated double bonds) scavenges

hydroxyl radicals much more efficiently than β-carotene (which contains 11 conjugated double bonds). Therefore, the highly conjugated double bonds of bacterioruberin act as a very effective oxidation protector. In addition, it was also demonstrate that bacterioruberin confers resistance against oxidative DNA-damaging agents such as ionizing radiation and hydrogen peroxide *in vivo*.

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CHAPTER 2

CAROTENOID PRODUCTION BY EXTREMELY HALOPHILIC ARCHAEA ISOLATED FROM SALT-FERMENTED FOOD

2.1 Abstract

One hundred and twenty-four red extremely halophilic archaea were isolated from salt fermented foods in Thailand. Strain HM3 exhibited the highest carotenoid-producing ability followed by AS1-33, HM322, and HPC1-2, respectively. On the basis of their phenotypic and chemotaxonomic characteristics, all 4 strains were motile, pleomorphic, required at least 2.1 M NaCl for growth and were assigned to the genus *Halobacterium*. Based on the 16S rDNA sequence similarity values, they were tentatively identified as *Halobacterium salinarum*. Total carotenoid extracts from all selected strains exhibited the same absorption spectra and chromatographic carotenoid profiles with four main peaks. All fractions showed the same parent ion mass at 740 m/z and the fragmentation pattern related to bacterioruberin and its derivatives. Meat extract and glucose were identified as important components affecting the growth and carotenoid production by *Hbt. salinarum* HM3. Applying a statistical approach using Plackett-Burman and central composite designs was found to be very effective in optimizing the medium components with overall 1.5-fold increase in carotenoid production.

2.2 Introduction

Carotenoids are naturally occurring pigments synthesized as hydrocarbons (carotene; e.g., lycopene, α-carotene, and β-carotene) or their oxygenated derivatives (xanthophylls; e.g., lutein, α-cryptoxanthin and β-cryptoxanthin, zeaxanthin, canthaxanthin and astaxanthin) by plants and microorganisms. Carotenoids have received considerable attention because of their interesting pigment properties and, more importantly, their potential beneficial effects

on human health (Armstrong 1994, 1997; Sandmann 2001). In recent years, the interest in production of natural carotenoids by microbial fermentation has been increased. Carotenogenic microbes such as *Dunaliella salina*, *Xanthophyllomyces dendrorhous*, *Haematococcus pluvialis*, and *Blakeslea trispora* have been investigated for large-scale production (Jacobson *et al*., 2000; Mehta *et al*., 2003; Olaizola 2000; Raja *et al*., 2007). Microbial production of carotenoids is an environmental friendly method compared to chemical methods for the production of carotenoids and able to meet the increasing demand of natural carotenoids.

The extremely halophilic archaea had the unique features including: (i) the extremely high NaCl concentration used in the growth medium, which is useful to prevent contamination by other organisms. Because of this, no sterilization procedures are required (Asker and Ohta, 2002); (ii) NaCl concentration below 15% induces cell lysis (Asker and Ohta, 2002), so no cell disrupting devices are required as cells lyse spontaneously in freshwater; and (iii) sfurthermore, the procedures for extraction and purification of carotenoids seem to be simpler than those from other sources. In Thailand, sea salts were produced by evaporating sea water and used in various kinds of fermented food, especially, fish sauce, fermented shrimp (*ka-pi*), fermented crab (*poo-kem*) and fermented fish (*pla-ra*). These products are rich in various nutrients, particularly amino acids and peptides, and contain a high concentration of NaCl, which allow various halophilic microorganisms to thrive (Lopetcharat *et al*., 2001; Tanasupawat and Komagata, 2001). So, the salt-fermented food should be the good source for halophilic archaea. Therefore, the purposes of this present work are to study the carotenoid production by extremely halophilic archaea from salt fermented foods.

2.3 Materials and Methods

Isolation of extremely halophilic archaea

Extremely halophilic archaea were isolated from various kinds of salt-fermented foods including fish sauce, *ka-pi*, *poo-kem*, and *pla-ra* collected from local markets in Thailand. Samples were plated on agar plates of halophilic medium

comprising of (L^{-1}) 250 g NaCl, 5 g casamino acids, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4.7H_2O$, 0.036 g $FeCl_4.4H_2O$, 0.00036 g $MnCl_2.4H_2O$, 20 g agar (pH 7.2) and incubated at 200 rpm, 37 °C for 1-2 weeks. A pure culture was obtained by repeated transfers of separate colonies on agar plates of the same medium.

Cultivation and growth determination

One loop-full of isolated strains from slant agar were inoculated in 5 ml of the halophilic liquid medium in a 10 ml test tube and incubated on a rotary shaker (Memmert, BE500, Germany) at 200 rpm for 7 days at 37 °C. The cultures were centrifuged 8,000 × *g* for 10 min at 4 °C. The cell pellet was washed and adjusted an absorbance at 600 nm (A_{600}) to 1.0 with a sterile 25% (w/v) NaCl. Five percent of the cell suspensions were inoculated into 100 mL of the halophilic liquid medium in 250 ml Erlenmeyer flasks. The flasks were incubated in a rotary shaker (200 rpm) at 37 °C for 7 days. The archaea growth was determined by dry cell weight method (Asker and Otha, 1999)

Carotenoid extraction and analysis

The extraction of carotenoid was performed as described by Asker and Ohta (1999). Five ml of culture broth were centrifuged at 8,000 × *g* for 10 min at 4 °C. The harvested cells were resuspended in distilled water. Spontaneously, cellular lysis occurred, and then the pigments were extracted with methanol and transferred to hexane. The carotenoid extracts were scanned in the wavelength region of 300-600 nm using a spectrophotometer (Helios-alpha, Unicam, England). The total carotenoid content was estimated by measuring the absorbance at 490 nm (A_{490}). An average extinction coefficient ($E_{1cm}^{1\%}$) of 2500 was used to calculate the total carotenoid content (Kelly *et al*., 1970).

Identification of isolated strains

Morphological, cultural and physiological characteristics

Phenotypic tests were performed in accordance with the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al*., 1997). Growth at various temperatures (20-60 °C) was examined. NaCl requirement was determined in the above medium containing various NaCl concentrations (0-5.1 M). Similarly, the requirement of the strains for Mg^{2+} was tested in halophilic medium supplemented with 0-1.0 M $MgSO_4$. $7H_2O$. Growth was determined by measuring culture turbidity at 600 nm. Cell motility and morphology were examined by phase contrast and transmission electron microscopy of liquid cultures grown for 7 days. Gram staining was carried out as described by Dussault (1955). Colony morphology was observed by growth on agar plates of the halophilic medium after incubation at 37 °C for 7 days.

Biochemical characteristics

Anaerobic growth was tested on agar plates in the presence of nitrate (1 g/L), L-arginine (1 g/L), or DMSO (10 g/L). Catalase and oxidase activities and the hydrolysis of casein, gelatin, starch, and Tween 80 were tested according to the methods of Barrow and Feltham (1993). Casamino acids were omitted from the test medium for determination of hydrolysis of casein and gelatin. Additional enzyme activities were determined by using API test kits (API ZYM and API 20E) at 37 °C as recommended by the manufacturer (bioMérieux). Utilization of sugars, alcohols, amino acids and organic acids, and acid production from various substrates were determined in modified Leifson medium supplemented with 0.01% (w/v) yeast extract and 4.3 M NaCl, but lacking casitone and Tris-HCl (Leifson, 1963). Nitrate reduction, H_2S formation, and indole production were tested as described by Oren *et al*. (1997). Tests for DNase, urease, methyl red, the Voges-Proskauer reaction, lysine and ornithine decarboxylases were performed as described by Gerhardt *et al*. (1981). Determination of antibiotic susceptibility was tested according to the methods of Stan-Lotter *et al*. (2002). The susceptibility of antibiotics was tested by spreading cell suspensions onto halophilic medium agar plates and then the antibiotic paper discs (6

mm in diameter) were applied on the medium. Zones of inhibition were measured following 14 days of incubation at 37°C. Sensitivity was considered as strong when the zone of inhibition extended more than 3 mm beyond the antibiotic disc with the following antibiotics: gentamicin (10 µg), neomycin (30 µg), rifampicin (30 µg), nalidixic acid (30 µg), choramphenicol (30 µg), bacitracin (10 µg), kanamycin (30 µg), streptomycin (10 µg), tetracycline (30 µg), ampicilin (10 µg), and novobiocin (5 and 30 µg).

Sequencing of 16S rRNA

DNA was isolated and purified according to the method of Saito and Miura (1963). The 16S rRNA gene sequence was PCR-amplified with primers D30F (5′-ATTCCGGTTGAT CCTGC-3′; positions 6-12 according to the *Escherichia coli* numbering system) and D56R (5′-CTTGTTACGACTT-3′; positions 1492-1509). The amplified DNA fragment was separated by agarose gel electrophoresis and was recovered by using a GenElute Minus EtBr Spin Column (Sigma). The sequence was determined by using the BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.0 (Applied Biosystems) in an ABI PRISM 310 genetic analyser (Applied Biosystems) with the following primers: D30F, D33R (5′-TCGCGCCTGCGCCCCGT-3′; positions 344-360), D34R (5′-GGTCTCGCTCGTTGCCTG-3′; positions 1096-1113), and D56R. The sequence was compared with reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center for Biotechnology Information database by using BLAST searches. The alignment was subjected to phylogenetic analysis with the neighbour-joining method (Saitou and Nei, 1987) by using programs in the CLUSTAL_X (Thompson *et al*., 1997). Confidence in the branching pattern was assessed by analysis of 1,000 bootstrap replicates.

Instrumentation and HPLC analysis

Total carotenoid extracts were separated with an Inertsil ODS-3 reversed-phase column (250 mm × 4.6 mm, 5 μm) (GL Sciences Inc., Japan). A Water 2960 liquid chromatograph system (Waters, Milford, USA) consisting of a quaternary pump, an autosampler and a photodiode array detector Water 996 were used. The mobile phase was a gradient of A (Acetonitrile:water, 9:1 v/v) and B (Ethyl acetate) (0 min:100% A, 25 min:100% B, 35 min:100% A). The flow rate was 1.0 ml/min. The column temperature set at 25 °C, and the sample injection volume was 20 μL. The UV detection wavelength was set at 490 nm, and absorption spectra of compounds in the HPLC eluent were recorded between 300 and 800 nm. Spectral fine structure was expressed as % III/II and Z-peak intensities as % D_B/D_{II} (Ke *et al*., 1970).

Mass spectroscopy

The predominant fractions of carotenoids were collected from HPLC. The mass spectra were measured with a mass spectrometer (Hitachi M-80B, Ibaragi, Japan) EI mode. The temperature of the ion-chamber was 240 °C, the temperature of the direct heater was 280 °C, and the ionization was 70 eV.

Optimization of growth and carotenoid production

Effect of cultural conditions

The optimal cultural conditions for growth and carotenoid production was carried out by inoculating the strain HM3 in 50 mL halophilic broth in a 250 mL Erlenmeyer flask and incubating on a rotary shaker at 200 rpm for 7 days at 37 °C. Various parameters including NaCl, pH, temperature, and cultivation time were optimized using the halophilic medium. The NaCl concentrations were varied between 0 and 30 % (w/v). The pH values of media were adjusted into a range of 4.5-8.5 by using either 1N HCl or 1N NaOH. The halophilic media were incubated at different temperatures of 15-50 °C. The incubation time was studied at 0-14 days. Growth and carotenoid productions were determined as described previously.

Screening of carbon and nitrogen sources

Plackett-Burman design was used to identify important carbon and nitrogen sources that previously found to affect growth and carotenoid production of the strain HM3 (data not shown). The medium was prepared by substitution of casamino acid and yeast extract in the halophilic medium with the tested medium components. Total of twelve components including carbon and nitrogen sources were selected for the study with each variable being represented at two levels, high (+) and low (-) (Table 6). Growth and carotenoid production were determined as described previously.

Optimization of screened components

Response surface methodology using a five-level Central Composite Design (CCD) was used to optimize the screened components for enhanced growth and carotenoid production. A total of 13 trials were employed with five replicates at the centre point and an axial point located at a specified distance from the design centre in each direction on each axis. The codes and actual values of the variables at various levels are given in Table 7. The model was represented by the quadratic equation. The statistical analysis of the results was performed with the aid of statistical software package, Design Expert (version 7.0.b1.1, State-Ease, Inc, Minneapolis, MN, USA).

Carotenoid production under non-aseptic condition

The optimized medium was used to compare the effect of medium sterilization on the growth and carotenoid production. The non-aseptic medium was prepared by dissolving the ingredients in distilled water and adjusting to pH 7.0 using 1 M NaOH. The heat-aseptic medium was prepared with the same manner, followed by autoclaving for 10 min at 110 °C.

Table 6. Variables representing medium components used in Plackett-Burman design

Variables	Medium components	Nitrogen content (% v/v)	+ Values (g/L)	- Values (g/L)
X_1	Glucose	-	10	1
X_2	Glycerol	-	10	1
X_3	Xylose	-	10	1
X_4	Beef extract	10.3 ± 0.1	9.7	1
X_5	Casamino acids	9.2 ± 0.1	10.8	1
X_6	Casitone	9.2 ± 0.1	10.8	1
X_7	Gelysate peptone	14.7 ± 0.1	6.8	1
X_8	Meat extract	10.0 ± 0.1	10	1
X_9	Peptone	9.6 ± 0.2	10.4	1
X_{10}	Tryptone	9.6 ± 0.3	10.4	1
X_{11}	Yeast extract	9.8 ± 0.1	10.2	1

Statistic analysis

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

Table 7. Central composite design matrix for growth and carotenoid production

Run	Variables / levels			
	Meat extract		Glucose	
	Coded value	Actual value (g/L)	Coded value	Actual value (g/L)
1	1	10	-1	5
2	0	7.5	0	7.5
3	0	7.5	0	7.5
4	0	7.5	+∝	11.04
5	0	7.5	0	7.5
6	0	7.5	0	7.5
7	-1	5	1	10
8	0	7.5	0	7.5
9	1	10	1	10
10	+∝	11.04	0	7.5
11	-1	5	-1	5
12	-∝	3.96	0	7.5
13	0	7.5	-∝	3.96

∝ is the axial spacing and the signs '+' and '-' represent the positive and negative directions, respectively. Value of ∝ is 1.4.

2.4 Results and Discussion

Isolation and screening of carotenoid-producing halophilic bacteria

One hundred and twenty four red extremely halophilic archaea were isolated from salt-fermented foods. The carotenoid productions of isolated strains were in the range of 3.92- 6.60 mg/L (Table 8). Strains HM3 produced the highest amount of carotenoid at 6.60 ± 0.06 mg/L, followed by AS1-33, HM322 and HPC1-2 at 6.48 ± 0.11, 6.08 ± 0.11, and 5.92 ± 0.11 mg/L, respectively.

Table 8. Carotenoid production (mg/L) by the extremely halophilic archaea isolated from salt-fermented foods in Thailand

Strain	Carotenoid production	Strain	Carotenoid production	Strain	Carotenoid production
AS1-27	5.60 ± 0.45	AS5-47	4.40 ± 0.11	HM334	4.16 ± 0.79
AS1-29	5.40 ± 0.40	AS6-12	5.60 ± 0.45	HM340	4.04 ± 0.96
AS1-33	**6.48 ± 0.11**	AS6-16	4.20 ± 0.06	HM345	4.48 ± 0.57
AS2-15	4.92 ± 0.28	AS6-17	4.00 ± 0.00	HM360	4.60 ± 0.51
AS2-16	5.52 ± 0.45	AS6-22	4.88 ± 0.23	HM361	4.48 ± 0.57
AS2-8	4.68 ± 0.17	AS6-35	4.88 ± 0.23	HM363	4.28 ± 0.74
AS3-18	5.20 ± 0.34	AS6-7	5.40 ± 0.40	HM364	4.08 ± 0.91
AS3-20	4.72 ± 0.23	AS6-8	4.56 ± 0.11	HM365	4.12 ± 0.85
AS3-21	4.28 ± 0.40	DS2-1	4.24 ± 0.11	HM366	3.96 ± 0.96
AS3-23	5.40 ± 0.40	HCC8978	4.24 ± 0.11	HM4	4.96 ± 0.23
AS3-24	4.64 ± 0.23	HD52-1	4.08 ± 0.00	HM6	5.16 ± 0.06
AS3-25	4.48 ± 0.11	HDB10-5	4.48 ± 0.23	HM706	5.16 ± 0.06
AS3-30	4.52 ± 0.17	HDB1-1	4.16 ± 0.11	HM707	4.28 ± 0.74
AS3-31	4.88 ± 0.23	HDB1-11	4.24 ± 0.11	HM709	4.76 ± 0.40
AS3-34	4.76 ± 0.17	HDB1-31	4.20 ± 0.06	HM711	4.80 ± 0.34
AS3-35	4.76 ± 0.17	HDB1-4	4.08 ± 0.00	HM712	5.12 ± 0.11
AS3-37	5.32 ± 0.40	HDB26-2	4.12 ± 0.06	HM717	5.12 ± 0.11
AS3-41	5.00 ± 0.28	HDB5-2	4.60 ± 0.28	HM718	4.12 ± 0.85
AS3-43	4.72 ± 0.23	HDB8-2	4.56 ± 0.23	HM719	4.60 ± 0.51
AS3-5	5.40 ± 0.40	HDB8-5	4.00 ± 0.00	HM721	4.52 ± 0.51
AS3-6	5.00 ± 0.28	HDS10-5	4.28 ± 0.17	HM722	5.48 ± 0.17
AS3-7	5.44 ± 0.45	HDS1-1	4.20 ± 0.06	HM723	4.08 ± 0.91
AS3-9	4.80 ± 0.23	HDS1-3	4.28 ± 0.17	HM724	3.92 ± 1.02
AS4-1	4.20 ± 0.06	HDS3-1	4.60 ± 0.28	HM725	5.28 ± 0.00
AS4-11	5.08 ± 0.28	HDS6-2	4.16 ± 0.11	HM728	4.92 ± 0.28
AS4-15	5.52 ± 0.45	HDS8-10	4.16 ± 0.11	HM729	4.48 ± 0.57
AS4-19	5.08 ± 0.28	HIB20-2	4.04 ± 0.06	HM730	3.96 ± 0.96
AS4-2	4.72 ± 0.23	HIB40-1	4.36 ± 0.17	HM733	5.48 ± 0.17
AS4-22	5.40 ± 0.40	HIB60-1	4.24 ± 0.11	HM734	4.08 ± 0.91
AS4-31	4.68 ± 0.17	HIS30-1	4.24 ± 0.11	HM738	4.40 ± 0.68
AS4-37	4.60 ± 0.17	HIS40-3	4.52 ± 0.28	HM739	4.44 ± 0.62
AS4-41	5.68 ± 0.45	HIS50-1R	4.56 ± 0.23	HM741	4.40 ± 0.68
AS4-52	4.84 ± 0.28	HKS11-1	4.28 ± 0.17	HM742	4.20 ± 0.85
AS5-12	5.20 ± 0.34	HKS200-2	4.60 ± 0.28	HM743	4.60 ± 0.51
AS5-23	4.68 ± 0.17	HKS35-2	4.60 ± 0.28	HM745	4.24 ± 0.79
AS5-27	4.88 ± 0.23	HM15	4.84 ± 0.28	HM746	4.24 ± 0.79
AS5-34	4.88 ± 0.23	HM21	5.24 ± 0.06	**HPC1-2**	**5.92 ± 0.11**
AS5-36	4.52 ± 0.17	**HM3**	**6.60 ± 0.06**	HS35-3	4.04 ± 0.06
AS5-38	4.32 ± 0.11	HM30	5.00 ± 0.17	RF5-5	4.36 ± 0.17
AS5-41	4.60 ± 0.17	HM331	4.96 ± 0.23	RF6	4.00 ± 0.00
AS5-42	4.48 ± 0.11	**HM332**	**6.08 ± 0.11**		
AS5-45	4.60 ± 0.17	HM334	4.72 ± 0.45		

Mean ± SD (standard deviation) from three determinations

Strain HM3 and HM322 were isolated from *poo-kem* in Nakhonsawan province, Thailand. Strain AS1-33 was isolated from fish sauce, while strain HPC1-2 was isolated from *pla-ra* in Singburi province, Thailand. The carotenoid production by extremely halophilic archaea was also reported. Calo *et al*. (1995) reported that the carotenoids were produced by *Hbt. salinarum*, *Haloarcula hispanica*, and *Haloferax mediterranei* at 2.40, 1.35, and 0.7 mg/L. In addition, *Hfx. alexandrinus* produced carotenoids under optimized condition at 6.45 mg/L. The results suggested that all selected strains produced high content of carotenoids even in the un-optimized cultural conditions.

Identification of the selected strains

Phylogenetic tree constructed by the neighbor-joining method indicated the relationship between strain AS1-33, HM3, HM332, HPC1-2 and other related archaea found in the GenBank database (Figure 12). All selected strains were affiliated to the genus *Halobacterium* which closest to *Hbt. salinarum* DSM 3754^T. The sequence similarity values of strain HM3, AS1-33, HM332, and HPC1-2 compared to *Hbt. salinarum* DSM 3754^T were 99.8, 99.5, 99.3, and 99.2%, respectively. The phenotypic and chemotaxonomic studies of all selected strains compared with *Hbt. salinarum* DSM 3754^T are shown in Table 9. All of selected strains grew optimally at NaCl of 3.4-4.3 M indicating that all strains were an extremely halophilic archaea. Furthermore, strain AS1-33, HM322, and HM3 required NaCl more than 2.1 M to prevent their cell lysis while strain HPC1-2 required NaCl more than 1.7 M. Moreover, strain HPC1-2 was distinguished from other strains in some phenotypic and biochemical characteristics as indicated by the results in Table 9. Based on the results, strain HPC1-2 would be another novel member of this genus. Based on the highest carotenoid production, strain HM3 was selected to optimize their growth and carotenoid production.

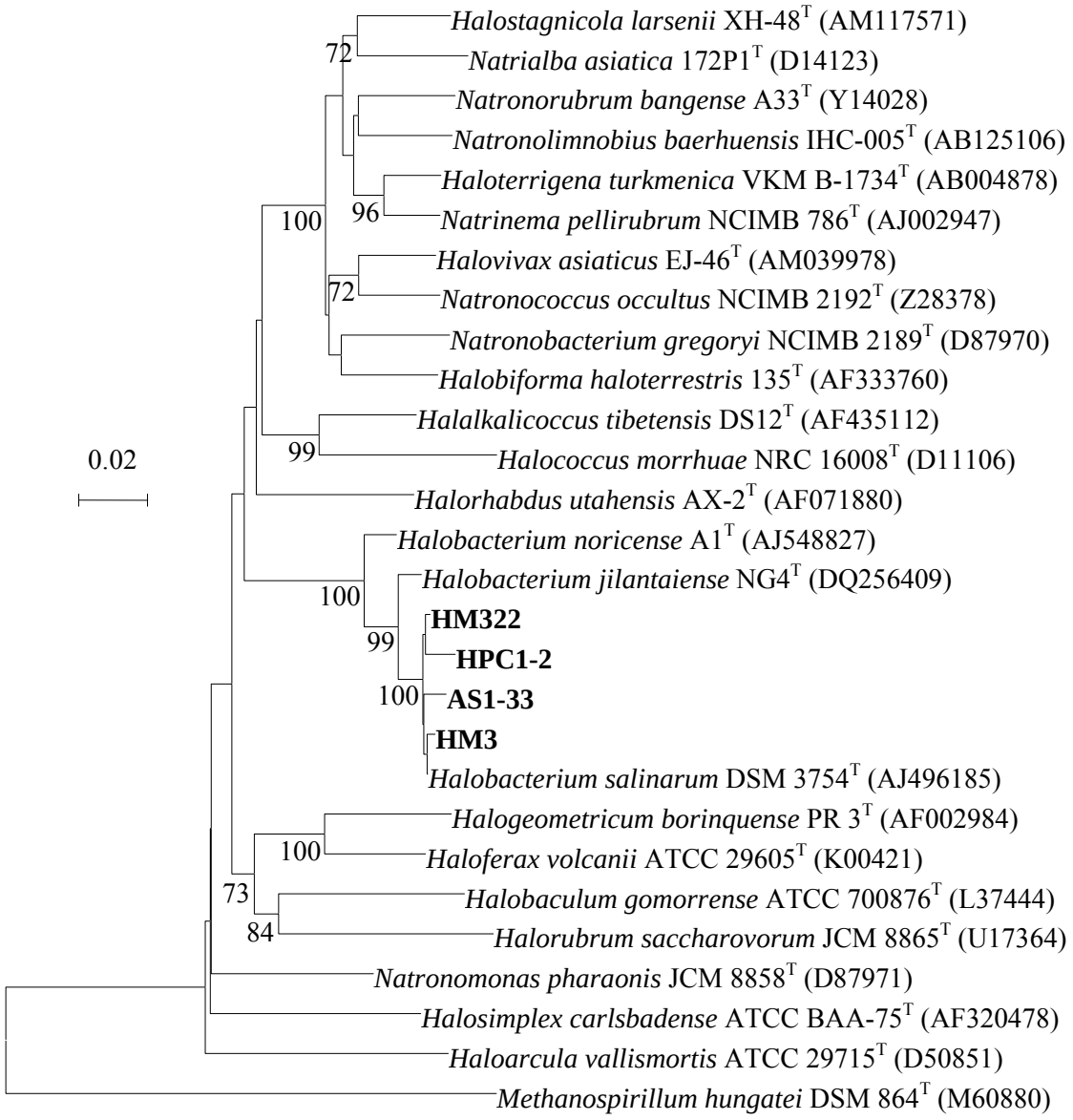

Figure 12. Phylogenetic tree showing the relationships between strain AS1-33, HM3, HM322, HPC1-2 and related archaeal species based on 16S rRNA gene sequences. The branching pattern was generated according to the neighbour-joining method. Bootstrap percentages above 70 %, based on 1000 replications, are shown at the nodes. Bar, 0.01 substitutions per 100 nucleotide positions.

Table 9. Differential characteristics between strains AS1-33, HM322, HM3, HPC1-2 and *Hbt. salinarum* DSM 3754^T

Characteristic	HM3	AS1-33	HM322	HPC1-2	DSM 3754^T
Cell morphology	Rod	Rod	Rod	Rods	Rod
Pigmentation	Red	Red	Red	Red	Red
pH ranges for growth	5.0-8.0	5.0-8.5	5.0-8.0	5.0-8.0	4.5-8.5
Optimum NaCl concentrations for growth (M)	3.4-4.3	3.4-4.3	3.4-4.3	3.4-4.3	3.4-4.3
Optimum Mg^{2+} concentrations for growth (M)	0.4-0.5	0.4-0.5	0.5-0.6	0.5-0.6	0.5-0.6
NaCl concentrations required to prevent cell lysis (M)	> 2.1	> 2.1	> 2.1	> 1.7	> 1.7
Hydrolysis of:					
Casein	+	+	-	+	+
Tween 80	+	+	-	+	-
Acid production of acids from:					
Glucose	+	-	-	-	-
Glycerol	+	+	-	+	+
Xylose	+	-	-	-	+
Utilization of carbon sources:					
Citrate	-	-	-	+	+
Glucose	+	-	-	-	-
Glycerol	+	-	-	-	-
Inulin	-	+	-	-	+
Lactose	-	-	-	+	-
Maltose	-	-	-	-	+
Melizitose	-	-	-	+	-
D-Xylose	+	-	-	-	+
Esterase (C_4)	-	-	-	+	-
Esterase lipase (C_8)	-	-	-	+	-
Lipase (C_{14})	-	-	-	+	-
G + C content of DNA	65.6	63.8	61.2	65.5	67.1-71.2*

+, Positive; -, negative; *Data from Grant (2001).

Carotenoid production

Carotenoid extracts from all selected strains gave an identical absorption spectrum, with typical characteristics of three fingered and two *cis* maxima (Figure 13) which corresponded to bacterioruberin. Britton (1995) described that bacterioruberin and its derivatives exhibited the characteristic spectral peaks of red carotenoids at nearly identical absorption maxima at 467, 493, and 527 nm for three fingered peaks and at 370 and 385 nm for two *cis* peaks. Upon a separation by HPLC, total carotenoid extracts from all selected strains exhibited typical chromatographic carotenoid profile with four major peaks and two minor peaks (Figure 14). The UV-Visible spectra of four major peaks indicated the differences at *cis* peaks around at 387-388 nm. The intensities of *cis* peak ($\%D_B/D_{II}$) were 15, 20, and 79 for peak no. 2, 3, and 4, respectively. Rønnekleiv and Liaaen-Jensen (1992) showed that the natural geometrical isomers of bacterioruberin can be separated by using HPLC. The intensities of *cis* peak ($\%D_B/D_{II}$) were 13, 19, and 78 for 5Z, 9Z, and 13Z-bacterioruberin, respectively ((Figure 15). The mass spectra of four main peaks are shown in Figure 16. The parental ion peak was found at m/z 740 which corresponded to the molecular weight of bacterioruberin. In addition, fragment ions appearing in their mass spectrum showed the losses of m/z 18 (H_20), m/z 92 (toluene), m/z 106 (xylene), and m/z 158 (dimethyldihydronaphthalene) (Figure 17). From the results, it can be concluded that the main pigment of strain HM3 and other selected strains was (all-trans)- bacterioruberin, accompanied by three *cis* isomers (probably 5Z, 9Z, and 13Z, respectively). Kelly *et al*. (1970) suggested that bacterioruberin is a characteristic carotenoid obtained from halophilic bacteria. Kushwaha *et al*. (1975) found that various strains of halophilic archaea including *Hbt. salinarum*, *Halobacterium saccharovorum*, and *Halobacterium vallismortis*, and other haloarchaea produced bacterioruberin and monoanhydrobacterioruberin. Furthermore, bacterioruberin have also been isolated from a highly radiation-resistant bacterium *Rubrobacter radiotolerans* (Saito *et al*., 1994), a psychrotrophic bacterium, *Micrococcus roseus* (Strand *et al*., 1997), and a psychrotrophic bacterium *Arthrobacter agilis* (Fong *et al*., 2001).

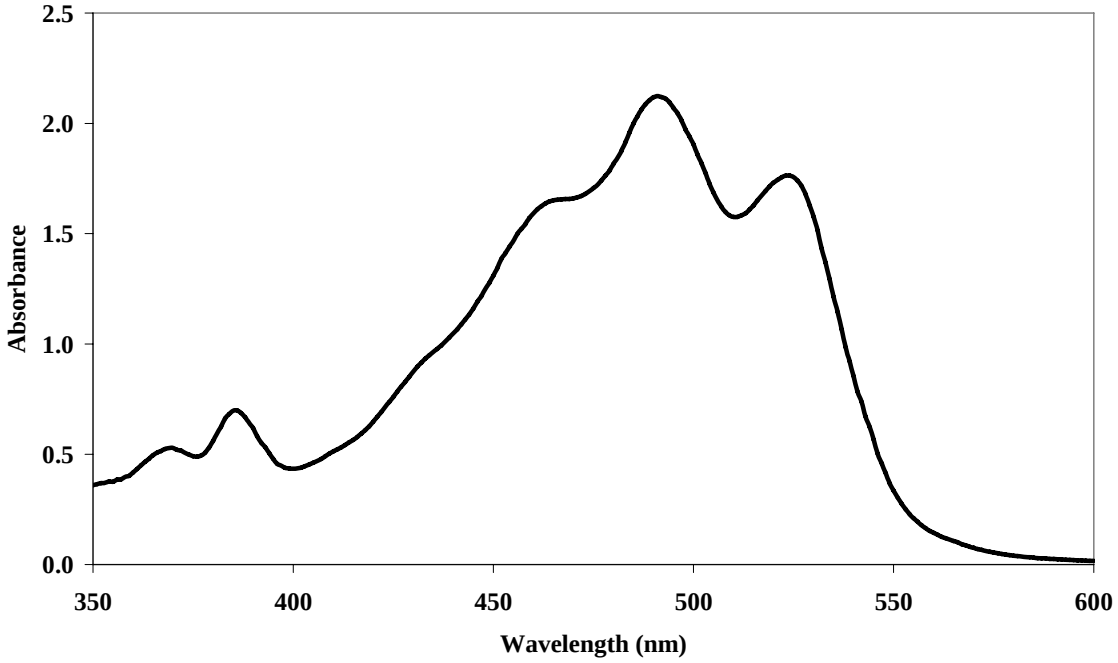

Figure 13. Typical absorption spectra of carotenoids produced by selected strain.

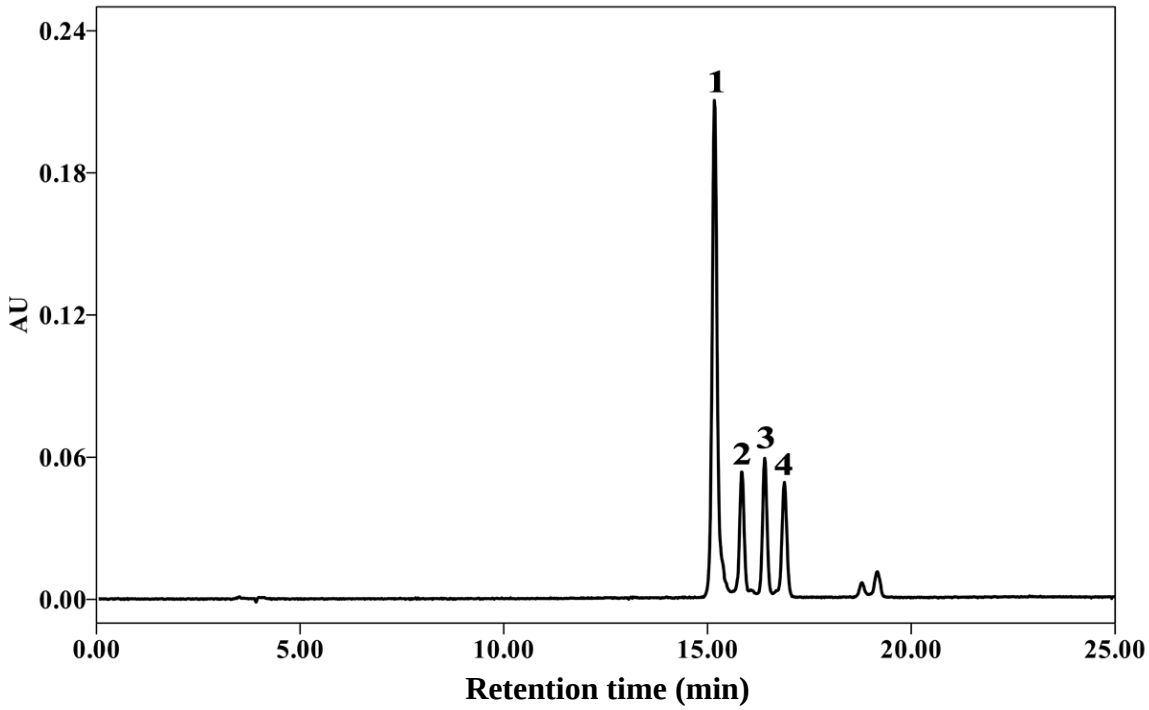

Figure 14. HPLC chromatogram of carotenoids produced by selected strains separated by HPLC using an Inertsil ODS-3 C_{18} reversed-phase column (4.6 mm × 250 mm).

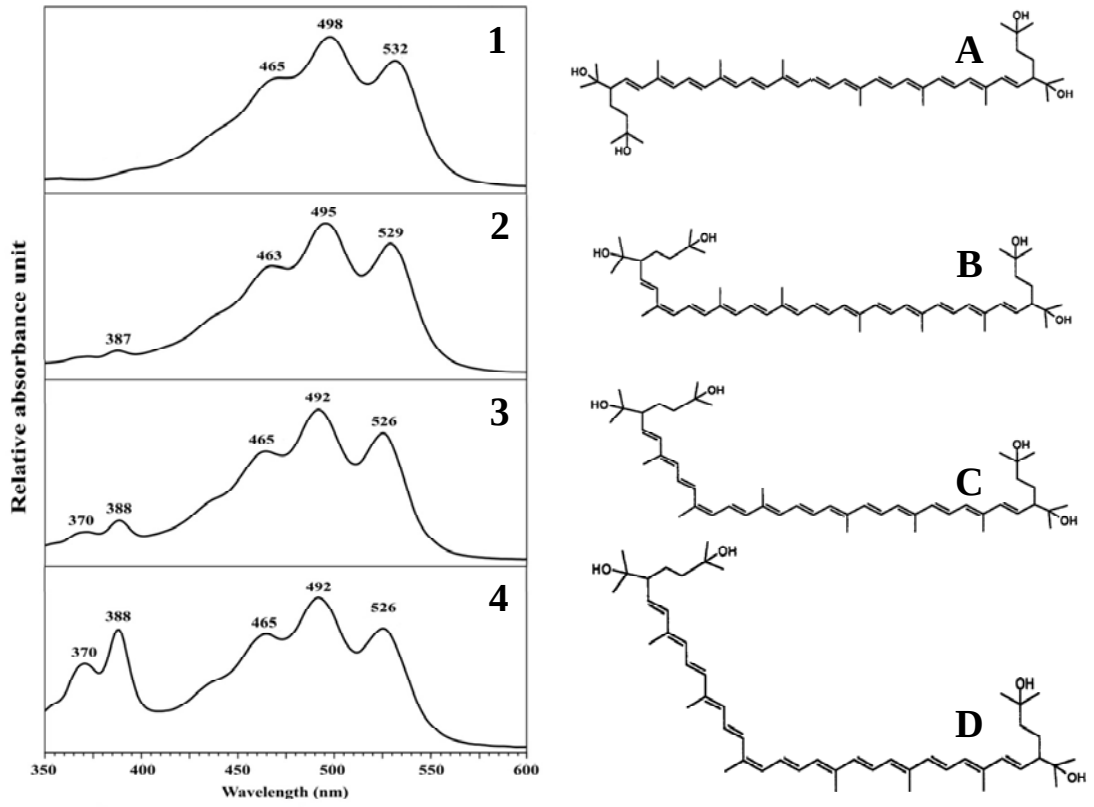

Figure 15. Absorption spectra of carotenoid fractions at retention of 15.17 min (1), 15.84 min (2), 16.40 min (3), and 16.88 min (4) separated by HPLC using an Inertsil ODS-3 C_{18} reversed-phase column (4.6 mm × 250 mm) and their tentatively identified structures. trans-bacterioruberin (A), 5Z-bacterioruberin (B), 9Z-bacterioruberin (C), and 13Z-bacterioruberin (D).

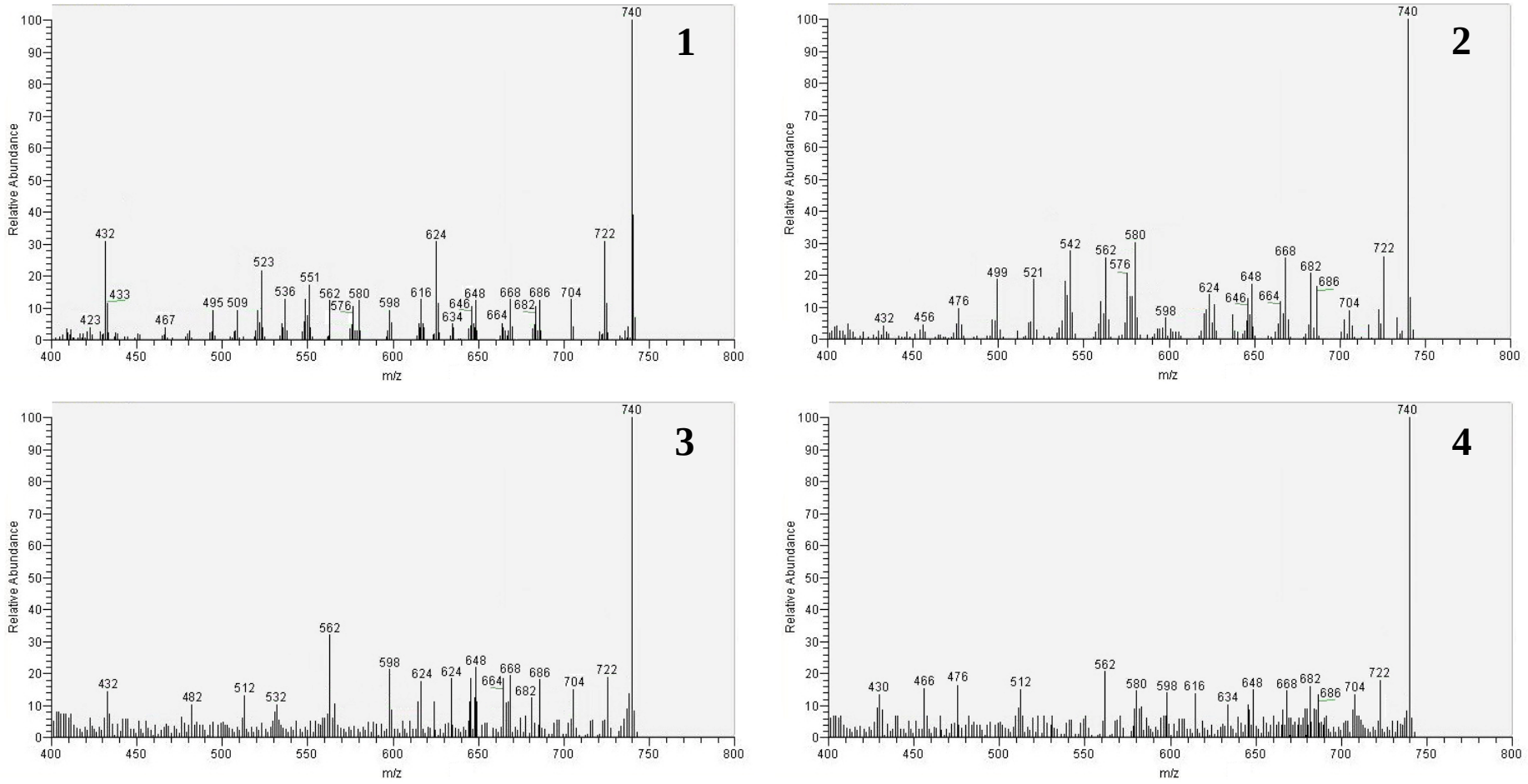

Figure 16. Mass spectra (EI mode) of major carotenoids fractions at retention of 15.17 min (1), 15.84 min (2), 16.40 min (3), and 16.88 min (4) separated by an Inertsil ODS-3 C_{18} reversed-phase column (4.6 mm × 250 mm).

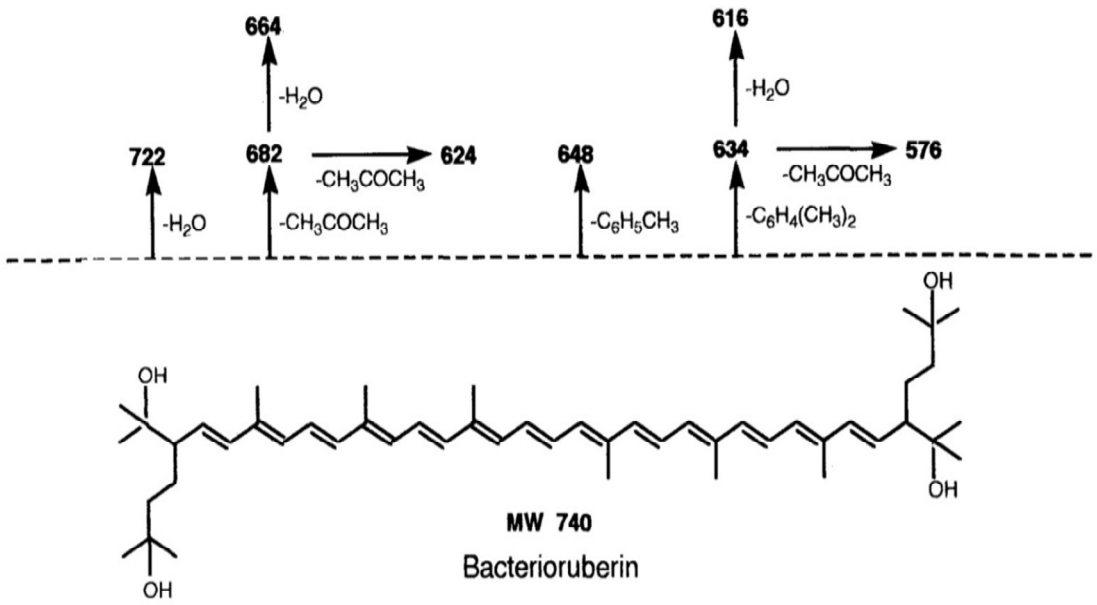

Figure 17. Chemical structure of bacterioruberin and fragmentation schemes from mass spectra.

Optimization of growth and carotenoid production

The effects of NaCl concentration, initial pH, temperature, and incubation time on carotenoid production of strain HM3 are shown in Figure 18. Growth and carotenoid production by strain HM3 were studied in the range of NaCl concentration of 15-30% (w/v) based on the concentration of NaCl that supports its growth. The maximum growth and production of carotenoid were observed at 25% NaCl (Figure 18.1). The medium salinity was the most important on the pigment content the *Halobacteriaceae*. *Haloferax* species are brightly pigmented when grown in the lower salinity range enabling growth (150 g/L or below), while at higher salt concentrations (250 g/L) they may appear almost colorless (Kushwaha *et al*., 1982; Rodriguez-Valera *et al*., 1980). *Haloferax mediterranei*, a species that is only weakly pigmented at high salinity, produces massive amounts of bacterioruberin pigments when incubated at 50 g/L salt, a concentration too low to support growth (D'Souza *et al*., 1997). The growth and carotenoid production increased in the pH ranges of 4.5-7.0 with maximum at pH 7.0 (Figure 18.2). The growth and carotenoid production by

strain HM3 did not occur below 20 °C and above 50 °C. The maximum growth and production of carotenoid occurred at 37 °C. (Figure 18.3). The growth curve of strain HM3 was characterized by a long lag phase (about 2 days). Pigment production by the strain was observed after 4 days of incubation, as the culture became slightly red in color. Growth and carotenoid production increased to a maximum after 7 days of incubation, followed by a decrease in both growth and carotenoid production. (Figure 18.4).

Plackett-Burman design was used for initial screening of medium components. The growth and carotenoid production under Plackett-Burman experimental design are shown in Table 10. Table 11 represents the contribution of effect (%), F-value, and *P*-value of effective components from the results. The components were screened at confidence level of 95% based on their effects. The significant values implied that the effects of glucose and meat extract were significant. Rest of the components showing the significant values over 0.05 were considered insignificant.

Table 12 represents the growth and carotenoid production. The variables used for analysis were meat extract and glucose. The centre point in the design was repeated five times for estimation of error. The experimental results of CCD were fitted into a quadratic polynomial equation as follows. Final equation in terms of coded factors for growth and carotenoid production:

$$Y_1 = 3.35 + 0.60\ A + 0.14\ B + 0.25\ AB + 0.052\ A^2 + 0.10\ B^2$$
$$Y_2 = 6.73 + 1.21\ A + 0.27\ B + 0.49\ AB + 0.11\ A^2 + 0.21\ B^2$$

where Y_1 and Y_2 is the response variable (growth and carotenoid production) and A and B are the concentrations of meat extract and glucose, respectively.

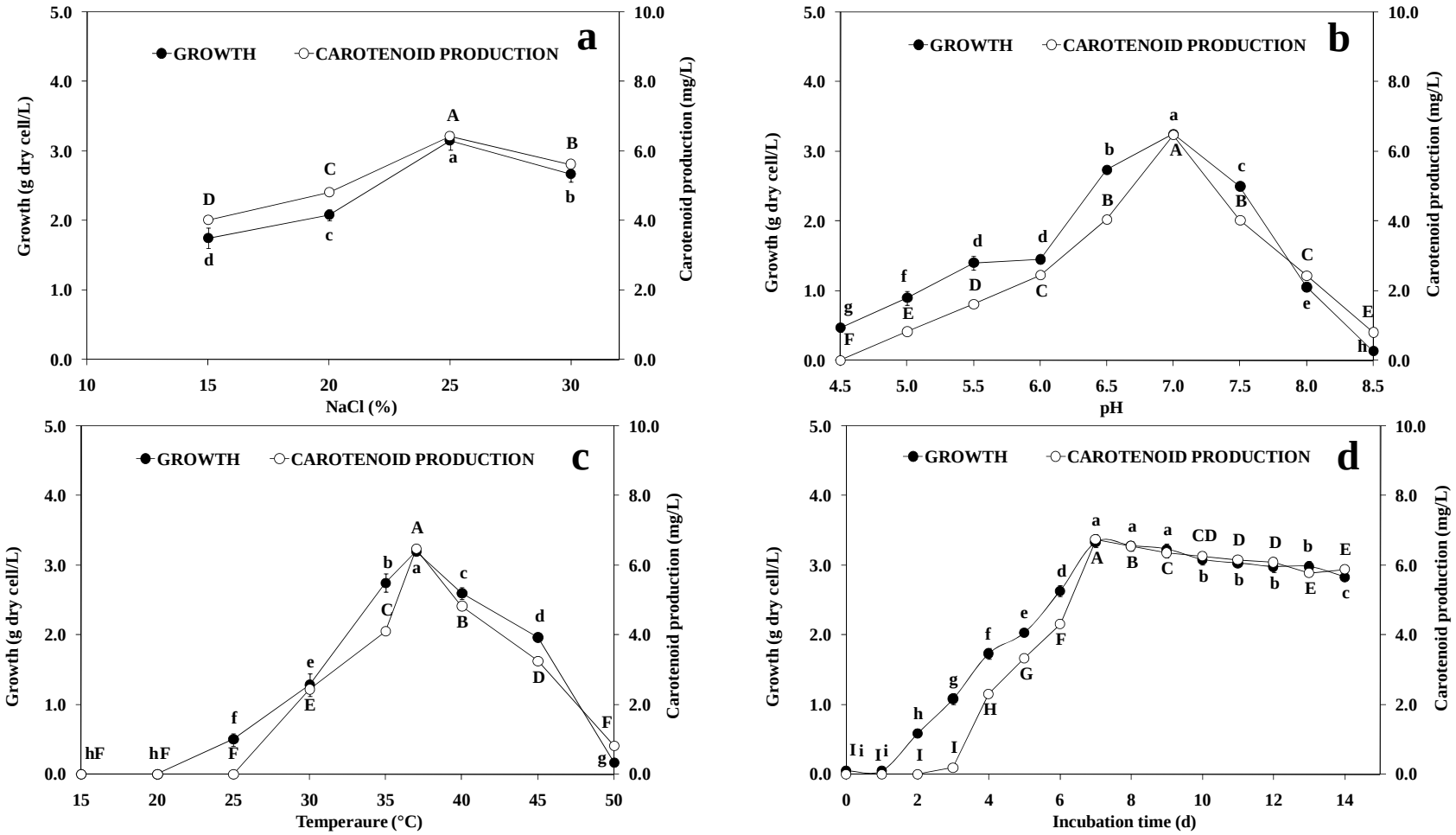

Figure 18. Optimization of growth and carotenoid production of *Hbt. salinarum* HM3 under different NaCl concentrations (a), initial pHs (b), temperatures (c), and incubation times (d).

Table 10. Plackett-Burman experimental design matrix along with the observed response (growth and carotenoid production)

Run	Variables											Growth	Carotenoid production
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	g dry cell/L	mg/L
1	-	-	-	-	-	-	-	-	-	-	-	0.71 ± 0.01^{l}	1.22 ± 0.01^{i}
2	-	-	-	+	-	+	+	-	+	+	+	2.15 ± 0.06^{j}	4.05 ± 0.04^{g}
3	+	-	+	+	-	+	+	+	-	-	-	4.37 ± 0.08^{b}	8.26 ± 0.21^{a}
4	-	+	+	+	-	-	-	+	-	+	+	3.47 ± 0.05^{d}	6.84 ± 0.13^{c}
5	+	-	+	+	+	-	-	-	+	-	+	3.25 ± 0.04^{e}	6.61 ± 0.08^{d}
6	+	-	-	-	+	-	+	+	-	+	+	4.23 ± 0.01^{c}	8.25 ± 0.00^{a}
7	-	+	-	+	+	-	+	+	+	-	-	2.41 ± 0.01^{i}	5.52 ± 0.10^{f}
8	+	+	-	-	-	+	-	+	+	-	+	4.69 ± 0.07^{a}	8.36 ± 0.07^{a}
9	-	+	+	-	+	+	+	-	-	-	+	1.91 ± 0.01^{k}	3.90 ± 0.12^{h}
10	+	+	+	-	-	-	+	-	+	+	-	2.64 ± 0.05^{g}	5.75 ± 0.14^{e}
11	-	-	+	-	+	+	-	+	+	+	-	2.75 ± 0.04^{f}	5.51 ± 0.14^{f}
12	+	+	-	+	+	+	-	-	-	+	-	2.54 ± 0.04^{h}	5.56 ± 0.04^{f}

X_1–X_{11} represent different assigned variables; the sign '+' is for high concentration of variables and '-' is for low concentration of variables related to Table 6.

Table 11. Statistical analysis of medium components for growth and carotenoid production by Plackett-Burman design

Medium components	Effect (%)	F - Value	*P* - Value
Growth			
Glucose	44.53	94.12	< 0.0001
Meat extract	42.33	89.47	< 0.0001
Yeast extract	6.26	13.24	0.051
Casitone	2.25	4.76	0.072
Xylose	1.78	3.76	0.100
Beef extract	0.94	0.15	0.767
Casamino acid	0.64	0.09	0.817
Peptone	0.45	0.05	0.862
Tryptone	0.36	0.02	0.903
Gelysate peptone	0.25	0.01	0.935
Glycerol	0.20	0.01	0.955
Carotenoid production			
Glucose	46.27	78.11	< 0.0001
Meat extract	41.73	70.45	< 0.0001
Yeast extract	4.39	7.42	0.055
Beef extract	2.51	4.23	0.086
Xylose	1.54	2.60	0.158
Tryptone	1.25	0.39	0.645
Glycerol	0.81	0.11	0.792
Peptone	0.55	0.08	0.822
Gelysate peptone	0.46	0.07	0.836
Casitone	0.35	0.06	0.853
Casamino acid	0.13	0.02	0.911

Table 12. Experimental and predicted values of growth and carotenoid production using central composite design (CCD)

Run	Variables / levels		Growth (g dry cell/L)		Carotenoid production (mg/L)	
	Meat extract (g/L)	Glucose (g/L)	Exp.	Predicted	Exp.	Predicted
1	10	5	3.75	3.73	7.54	7.5
2	7.5	7.5	3.34	3.35	6.7	6.73
3	7.5	7.5	3.45	3.35	6.92	6.73
4	7.5	11.04	3.63	3.76	7.29	7.54
5	7.5	7.5	3.33	3.35	6.69	6.73
6	7.5	7.5	3.32	3.35	6.66	6.73
7	5	10	2.91	2.8	5.84	5.62
8	7.5	7.5	3.33	3.35	6.68	6.73
9	10	10	4.6	4.49	9.24	9.02
10	11.04	7.5	4.24	4.31	8.52	8.65
11	5	5	3.04	3.02	6.11	6.07
12	3.96	7.5	2.55	2.61	5.11	5.24
13	7.5	3.96	3.37	3.37	6.77	6.78

$\propto$ is the axial spacing and the signs '+' and '-' represent the positive and negative directions, respectively. Value of $\propto$ is 1.4.

The analysis of variance (ANOVA) for the model of growth and carotenoid production were performed and are summarized in Table 13 and 14. The *P*-values of both parameters were found to be <0.0001 indicating that the quadratic model is significant. The efficacy of the model for both growth and carotenoid production were indicated by the high values of R-squared and adjusted R-squared. The good precision and reliability of the experiment were also seen by a relatively lower value of the coefficient of variation (CV = 2.68% for both growth and carotenoid production).

Table 13. Analysis of variance (ANOVA) for the quadratic model growth[a]

Source	Sum of Squares	df	F Value	*P*-value Prob > F
Model	3.37	5	78.32	< 0.0001
A - Meat extract	2.87	1	335.95	< 0.0001
B - Glucose	0.15	1	17.32	0.004
AB	0.24	1	28.12	0.001
A^2	0.02	1	2.24	0.179
B^2	0.08	1	8.96	0.020
Residual	0.06	7		
Lack of Fit	0.05	3	5.47	0.067
Error	0.01	4		
Corrected total	3.40	12		

[a] $R^2 = 0.982$; adj. $R^2 = 0.970$; CV = 2.68%; adequate precision ratio = 29.934.

Table 14. Analysis of variance (ANOVA) for the quadratic model for carotenoid production[a]

Source	Sum of Squares	df	F Value	*P*-value Prob > F
Model	13.57	5	78.65	< 0.0001
A - Meat extract	11.65	1	337.55	< 0.0001
B - Glucose	0.59	1	16.99	0.005
AB	0.97	1	28.12	0.001
A^2	0.08	1	2.33	0.171
B^2	0.32	1	9.32	0.019
Residual	0.24	7		
Lack of Fit	0.20	3	5.67	0.064
Error	0.05	4		
Corrected total	13.81	12		

[a] $R^2 = 0.982$; adj. $R^2 = 0.970$; CV = 2.68%; adequate precision ratio = 29.983.

Figure 19 shows the behavior of growth and carotenoid production. Increase in meat extract concentration showed increased growth and carotenoid production. Similarly, increase in glucose concentration also showed an increased protease production but not at the level observed with meat extract. However, the interaction effect of both components was found to be highly significant (*P*-value of AB = 0.001), implying that both the components were essential for growth and carotenoid production. From the study of response surface plot, the optimal concentrations of meat extract and glucose were 10 g/L. The growth and carotenoid production were found at 4.60 g dry cell/L and 9.24 mg/L, respectively. It is noted that the carotenoid production was increased 1.5 folds compared to the un-optimized medium (6.07 mg/L).

Carotenoid production under non-aseptic condition

The growth and carotenoid production of strain HM3 cultured in the optimized medium under aseptic and non-aseptic media are shown in Figure 20. The highest growth and carotenoid production were found at 7 days of incubation at 4.60 g dry cell/L and 9.24 mg/L, respectively. The results suggested that the model was valid to the growth and carotenoid production of strain HM3. In addition, there was slight increase ($P>0.05$) in growth and carotenoid production when cultured in the non-aseptic medium. The results were related to the study of Asker and Ohta (2002) which indicated that the growth and carotenoid production of *Hfx. alexandrinus* were lower when cultured in a heat-aseptic medium compared to a non-aseptic medium and a filter-aseptic medium. They suggested that heat treatment may cause the loss of vitamins that help stimulate the growth of *Hfx. alexandrinus* cells.

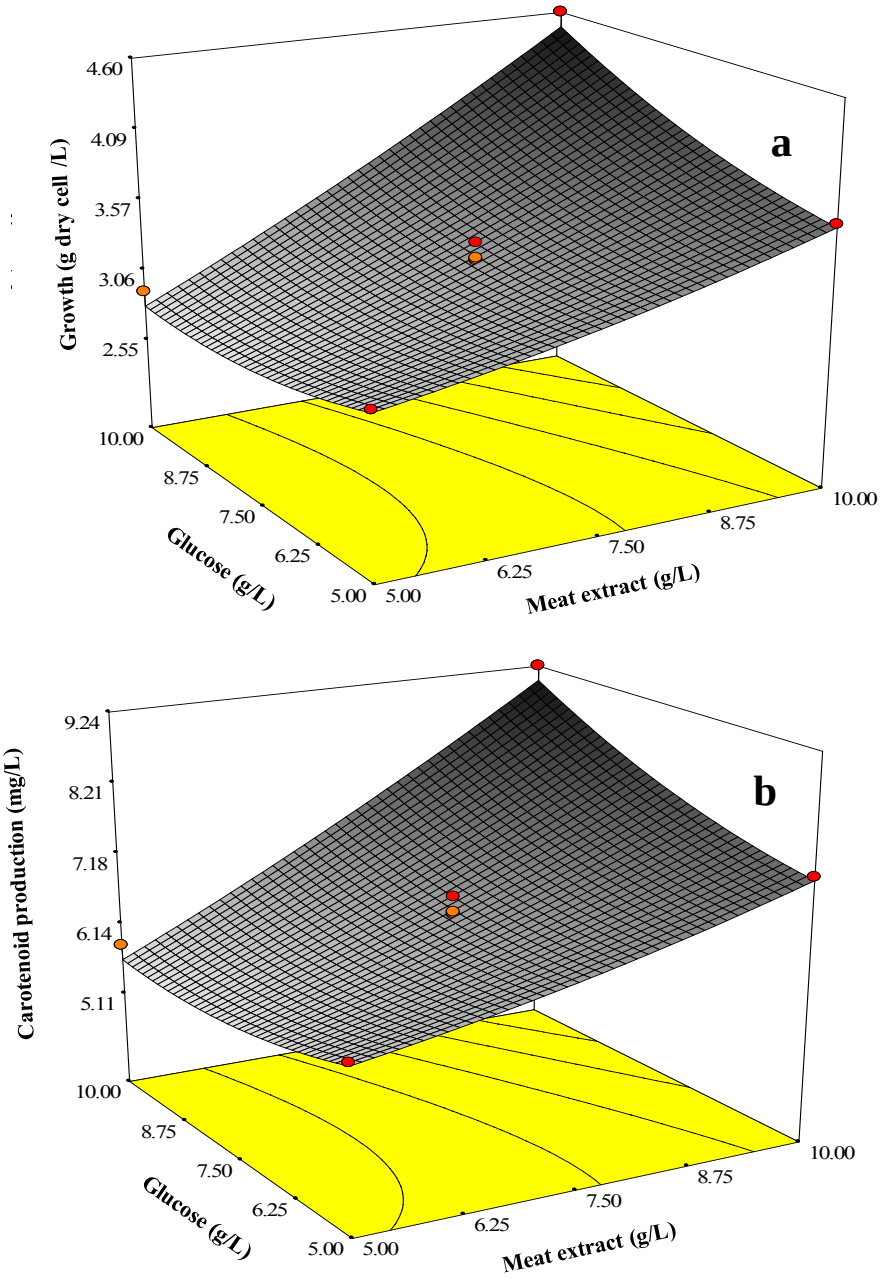

Figure 19. Three-dimensional response plot showing the effect of meat extract and glucose on growth (a) and carotenoid production (b).

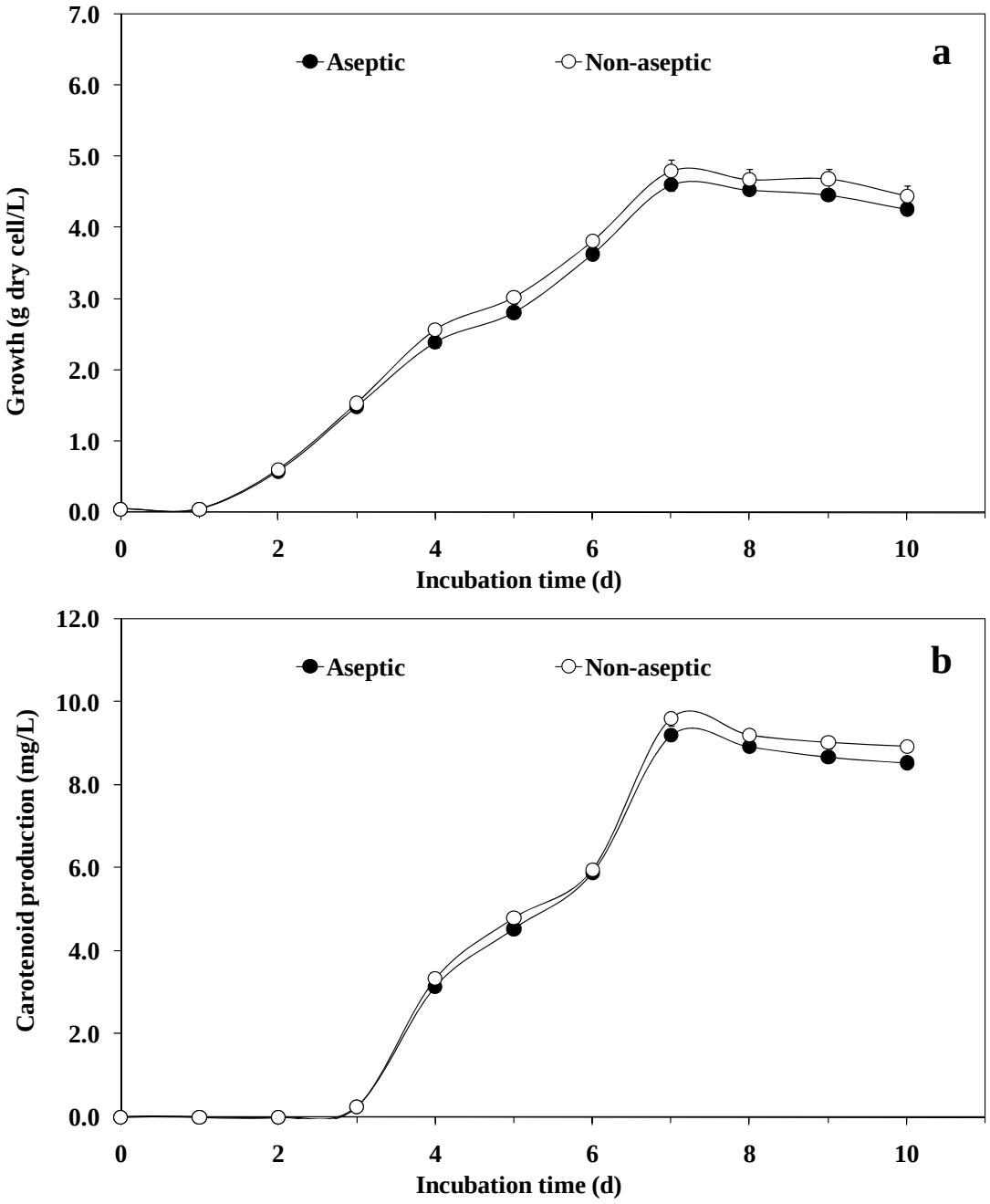

Figure 20. Growth (a) and carotenoid production (b) in the optimized medium under aseptic and non-aseptic conditions in flask cultivation. Mean ± SD (standard deviation) from three determinations

2.5 Conclusion

Out of 124 red extremely halophilic archaea isolated from salt fermented foods, *Hbt. salinarum* HM3 exhibited the highest carotenoid production. On the basis of chromatography and mass spectroscopy, the carotenoids produced by strain HM3 were identified as bacterioruberin, the main peak being (all-trans) and the others *cis* isomers (probably 5Z, 9Z, and 13Z, respectively). The optimized medium was composed of (L^{-1}) 250 g NaCl, 10 g meat extract, 10 g glucose, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4.7H_2O$, 0.036 g $FeCl_4.4H_2O$, and 0.00036 g $MnCl_2.4H_2O$ (pH 7.0). The carotenoid production was 9.24 mg/L which increased about 1.5-fold via statistical optimization.

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CHAPTER 3

HALOBACTERIUM PISCISALSI SP. NOV., FROM FERMENTED FISH (*PLA-RA*) IN THAILAND

3.1 Abstract

A Gram-negative, motile, rod-shaped, extremely halophilic archaeon, designated strain HPC1-2^T, was isolated from *pla-ra*, a salt-fermented fish product of Thailand. Strain HPC1-2^T was able to grow at 20-60 °C (optimum at 37-40 °C), at 2.6-5.1 M NaCl (optimum at 3.4-4.3 M NaCl) and at pH 5.0-8.0 (optimum at pH 7.0-7.5). Hypotonic treatment with less than 1.7 M NaCl caused cell lysis. The major polar lipids of the isolate were $C_{20}C_{20}$ derivatives of phosphatidylglycerol, methylated phosphatidylglycerol phosphate, phosphatidylglycerol sulfate, triglycosyl diether, sulfated triglycosyl diether and sulfated tetraglycosyl diether. The G+C content of the DNA was 65.5 mol%. 16S rRNA gene sequence analysis indicated that the isolate represented a member of the genus *Halobacterium* in the family *Halobacteriaceae*. Based on 16S rRNA gene sequence similarity, strain HPC1-2^T was related most closely to *Halobacterium salinarum* DSM 3754^T (99.2%) and *Halobacterium jilantaiens*e JCM 13558^T (97.8%). However, low levels of DNA-DNA relatedness suggested that strain HPC1-2^T was genotypically different from these closely related type strains. Strain HPC1-2^T could also be differentiated based on physiological and biochemical characteristics. Therefore, strain HPC1-2^T is considered to represent a novel species of the genus *Halobacterium*, for which the name *Halobacterium piscisalsi* sp. nov. is proposed. The type strain is HPC1-2^T (=BCC 24372^T =JCM 14661^T =PCU 302^T).

3.2 Introduction

Pla-ra is a fermented fish product from Thailand made by mixing freshwater fish with salt and roasted rice and leaving this mixture to ferment at room

temperature for 6-12 months (Phithakpol *et al*., 1995). The product is rich in various nutrients, particularly amino acids and peptides and contains a high concentration of salt (about 1.2-3.1 M NaCl), conditions that are suitable for the growth of halophilic bacteria (Saisithi *et al*., 1966; Phithakpol *et al*., 1995; Tanasupawat and Komagata, 2001; Tanasupawat *et al*., 2007; Chamroensaksri *et al*., 2008). Nevertheless, no extremely halophilic bacteria have previously been isolated from *pla-ra* and in particular none of the genus *Halobacterium*, family *Halobacteriaceae*, which are reported to inhabit highly saline environments (Elazari-Volcani, 1957; Thongthai *et al*., 1992; Yang *et al*., 2006). At the time of writing, the genus *Halobacterium* comprises fifteen species with validly published names. Of these, all but three species, namely *Halobacterium salinarum* (Ventosa and Oren, 1996), *Halobacterium noricense* (Gruber *et al*., 2004) and *Halobacterium jilantaiense* (Yang *et al*., 2006), have subsequently been transferred to other genera or classed as synonyms of already described species. Here, the taxonomic properties of a novel, extremely halophilic archaeon, strain HPC1-2^T, isolated from *pla-ra* are described.

3.3 Materials and Methods

Isolation procedure, strains and growth conditions

Strain HPC1-2^T was isolated from *pla-ra* samples collected from local markets in Thailand. Samples were plated on agar plates of halophilic medium comprising of (L^{-1}) 250 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4$. $7H_2O$, 0.036 g $FeCl_4$. $4H_2O$, 0.00036 g $MnCl_2$. $4H_2O$, 20 g agar (pH 7.2) and incubated at 37 °C for 1-2 weeks. A pure culture was obtained by repeated transfers of separate colonies on agar plates of the same medium. Unless otherwise stated, strains were grown in liquid (with shaking at 200 rpm) or on agar plates of the halophilic medium and cultivated at 37 °C for 1-2 weeks.

Morphological, cultural and physiological characteristics

Phenotypic tests were performed in accordance with the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al*., 1997). Growth at various temperatures (20-60 °C) was examined. NaCl requirement was determined in the above medium containing various NaCl concentrations (0-5.1 M). Similarly, the requirement of the strains for Mg^{2+} was tested in halophilic medium supplemented with 0-1.0 M $MgSO_4$. $7H_2O$. Growth was determined by measuring culture turbidity at 600 nm. Cell motility and morphology were examined by phase contrast and transmission electron microscopy of liquid cultures grown for 7 days. Gram staining was carried out as described by Dussault (1955). Colony morphology was observed by growth on agar plates of the halophilic medium after incubation at 37 °C for 7 days.

Biochemical characteristics

Anaerobic growth was tested on agar plates in the presence of nitrate (1 g/L), L-arginine (1 g/L), or DMSO (10 g/L). Catalase and oxidase activities and the hydrolysis of casein, gelatin, starch and Tween 80 were tested according to the methods of Barrow and Feltham (1993). Casamino acids were omitted from the test medium for determination of hydrolysis of casein and gelatin. Additional enzyme activities were determined by using API test kits (API ZYM and API 20E) at 37 °C as recommended by the manufacturer (bioMérieux). Utilization of sugars, alcohols, amino acids and organic acids and acid production from various substrates were determined in modified Leifson medium supplemented with 0.01% (w/v) yeast extract and 4.3 M NaCl, but lacking casitone and Tris-HCl (Leifson, 1963). Nitrate reduction, H_2S formation and indole production were tested as described by Oren *et al*. (1997). Tests for DNase, urease, methyl red, the Voges-Proskauer reaction, lysine and ornithine decarboxylases were performed as described by Gerhardt *et al*. (1981). Determination of antibiotic susceptibility was tested according to the methods of Stan-Lotter *et al*. (2002). The susceptibility of antibiotics was tested by spreading cell suspensions onto halophilic medium agar plates and then the antibiotic paper discs (6

mm in diameter) were applied on the medium. Zones of inhibition were measured following 14 days of incubation at 37°C. Sensitivity was considered as strong when the zone of inhibition extended more than 3 mm beyond the antibiotic disc with the following antibiotics: gentamicin (10 μg), neomycin (30 μg), rifampicin (30 μg), nalidixic acid (30 μg), choramphenicol (30 μg), bacitracin (10 μg), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg), ampicilin (10 μg) and novobiocin (5 and 30 μg).

DNA G+C content

The G+C content was determined by the method of Tamaoka and Komagata (1984) by using a reversed-phase HPLC.

Quinones and polar lipids analyses

Menaquinones were analyzed as described by Komagata and Suzuki (1987). Polar lipids were determined according to the method of Minnikin *et al*. (1984).

Sequencing of 16S rRNA

DNA was isolated and purified according to the method of Saito and Miura (1963). The 16S rRNA gene sequence of strain HPC1-2^{T}, comprising 1,375 bp, was PCR-amplified with primers D30F (5′-ATTCCGGTTGAT CCTGC-3′; positions 6-12 according to the *Escherichia coli* numbering system) and D56R (5′-CTTGTTACGACTT-3′; positions 1492-1509). The amplified DNA fragment was separated by agarose gel electrophoresis and was recovered by using a GenElute Minus EtBr Spin Column (Sigma). The sequence was determined by using the BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.0 (Applied Biosystems) in an ABI PRISM 310 genetic analyser (Applied Biosystems) with the following primers: D30F, D33R (5′-TCGCGCCTGCGCCCCGT-3′; positions 344-360), D34R (5′-GGTCTCGCTCGTTGCCTG-3′; positions 1096-1113) and D56R.

The sequence was compared with reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center for Biotechnology Information database by using BLAST searches. The alignment was subjected to phylogenetic analysis with the neighbour-joining method (Saitou and Nei, 1987) by using programs in the CLUSTAL_X (Thompson *et al*., 1997). Confidence in the branching pattern was assessed by analysis of 1,000 bootstrap replicates.

DNA-DNA hybridization

DNA-DNA hybridization was performed as reported by Ezaki *et al*. (1989) and levels of relatedness were determined by using the colorimetric method given by Tanasupawat *et al*. (2000).

3.4 Results and Discussion

Phenotypic characteristics

Cells of strain HPC1-2^T were motile rods (0.5-1.0 × 1.0-2.0 μm) that possessed peritrichous flagella (Figure 21). On halophilic agar medium, colonies of strain HPC1-2^T were circular, smooth, translucent and red. Cell lysis occurred in 1.7 M NaCl solution. Cells stained Gram-negative. Strain HPC1-2^T was capable of growing over a wide concentration range of NaCl from 2.6-5.1 M. It grew optimally in the presence of 3.4-4.3 M NaCl, similar to most extremely halophilic Archaea (Grant, 2001). It also grew over a wide range of $MgCl_2$ concentrations from 0-1 M, with optimum growth at about 0.5-0.6 M. Strain HPC1-2^T grew in the temperature range 20-60 °C (optimum 37-40 °C) and pH range 5.0-8.0 (optimum pH 7.0-7.5). Catalase and oxidase activity tests were positive. No indole formation was observed in the presence of tryptophan. Cells hydrolysed gelatin, casein and Tween 80, but not starch. Strain HPC1-2^T showed anaerobic growth in the presence of DMSO, nitrate and L-arginine. Nitrate was not reduced and no gas formation was observed.

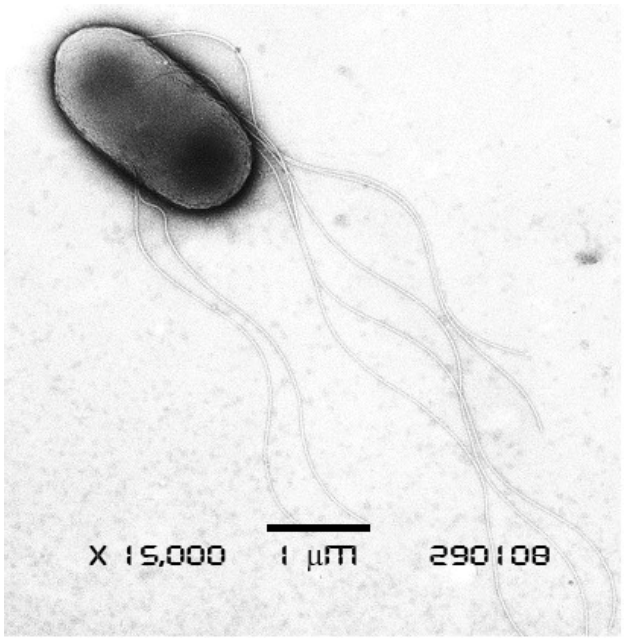

Figure 21. Transmission electron micrograph of cells of strain HPC1-2^T grown in halophilic medium at 37 °C. Bars, 1µm.

The strains were sensitive to bacitracin (10 µg), neomycin (30 µg) and novobiocin (5 µg), but not to ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), rifampicin (30 µg), streptomycin (10 µg) or tetracycline (30 µg).

Polar lipids and quinones

Two-dimensional TLC revealed that strain HPC1-2^T possessed $C_{20}C_{20}$ derivatives of phosphatidylglycerol (PG), methylated phosphatidylglycerol phosphate (PGP-Me), phosphatidylglycerol sulfate (PGS), triglycosyl diether (TGD), sulfated triglycosyl diether (S-TGD) and sulfated tetraglycosyl diether (S-TeGD) and pigments (Figure 22). The overall polar lipid pattern was similar to those of *Hbt. salinarum* DSM 3754^T (Ventosa and Oren, 1996) and *Hbt. jilantaiense* JCM 13558^T (Yang *et al*., 2006). The presence of S-TGD can be used to distinguish the above three taxa from *Hbt. noricense* (Gruber *et al*., 2004). Strain HPC1-2^T had menaquinones MK-8 (98%) and MK-8 (H_2) (2%).

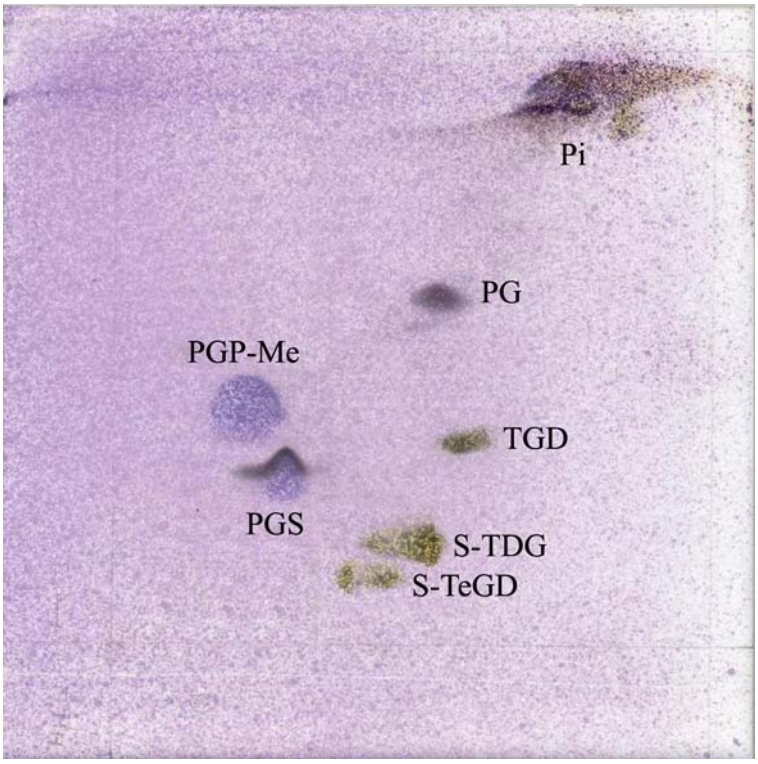

Figure 22. Two-dimensional TLC of polar lipids extracted from strain HPC1-2^{T}. Separation of components was achieved by developing the plate with chloroform/methanol/water (65:25:4 by vol., horizontal direction) and then with chloroform/methanol/acetic acid/water (85:12:15:4 by vol., vertical direction). Spots were visualized by staining with anisaldehyde reagent and then heated to 150 °C for 10 min. The glycolipids (yellow-green) and phospholipids (blue) were further identifed based on the relative mobility (Yang *et al*., 2006). Pi, Pigment; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; TGD, triglycosyl diether; S-TGD, sulfated triglycosyl diether; S-TeGD, sulfated tetraglycosyl diether.

DNA G+C content

The DNA G+C content of strain HPC1-2^T was 65.5 mol%, which was similar to that of *Hbt. salinarum* DSM 3754^T (Ventosa and Oren, 1996) and *Hbt. jilantaiense* JCM 13558^T (Yang *et al*., 2006) but not to that of *Hbt. noricense* A1^T (Gruber *et al*., 2004). The range of G+C contents of recognized species of the genus *Halobacterium* are 57-60 mol% for the minor DNA component and 67.1-71.2 mol% for the major DNA component (Grant, 2001). Characteristics that distinguish strain HPC1-2^T from recognized members of the genus *Halobacterium* are summarized in Table 15.

From these results, newly isolated strain HPC1-2^T was classified as extremely halophilic aerobic archaea because (i) TLC of whole-organism methanolysates revealed that they contain ether-linked isoprenoid lipids, (ii) they could grow in the presence of antibiotics effective against eubacteria (ampicillin, kanamycin and chloramphenicol) but not in the presence of antibiotics effective against eukaryotes (pravastatin and anisomycin), (iii) they could grow aerobically only in the presence of high concentration of NaCl and lysed at low levels of salinity and (iv) they possessed bacterial rhodopsins (Sugiyama *et al*., 1994; Tateno *et al*., 1994) which have been found only in members of the order *Halobacteriales* (Grant and Larsen, 1989).

16S rRNA gene sequences and phylogenetic tree

Figure 23 shows the phylogenetic tree constructed by the neighbour-joining method in which strainHPC1-2^T clustered with the type strains of species of the genus *Halobacterium*. Levels of 16S rRNA gene sequence similarity between strain HPC1-2^T and *Hbt. salinarum* DSM 3754^T, *Hbt. jilantaiense* JCM 13558^T and *Hbt. noricense* A1^T were 99.2, 97.8 and 96.6%, respectively.

Table 15. Differential characteristics between strain HPC1-2^T and recognized *Halobacterium* species. Taxa: 1, strain HPC1-2^T; 2, *Hbt. salinarum* DSM 3754^T; 3, *Hbt. noricense* A1^T; 4, *Hbt. jilantaiense* JCM 13558^T.

Characteristic	1	2	3	4
Cell morphology	Rods	Small rods	Irregular rods	Rods
Pigmentation	Red	Red	Light red	Red
pH range for growth	4.5-8.5	5.5-8.5	5.2-7.0	5.5-8.5
Temperature range for growth (°C)	20-60	20-55	28-50	22-55
Optimum NaCl concentration for growth (M)	3.4-4.3	3.5-4.5	2.5-3.0	3.1-3.5
Optimum Mg^{2+} concentration for growth (M)	0.5-0.6	0.5-0.6	0.6-0.9	0.5-0.6
Oxidase	+	+	-	+
Hydrolysis of:				
Casein	+	+	-	+
Gelatin	+	+	-	+
Tween 80	+	-	-	-
Anaerobic growth with DMSO	+	+	-	+
Acid production form glycerol	+	+	+	-
Utilization of carbon sources:				
Maltose	-	+	-	-
D-Xylose	-	+	-	-
Enzyme assay (API ZYM)				
Acid phosphatase	+	+	-	+
Alkaline phosphatase	+	+	-	+
Cystine arylamidase	-	-	+	-
Esterase (C_4)	+	-	+	+
Valine arylamidase	-	-	+	-
DNA G+C content (mol%)	65.5	67.1-71.2	54.5	64.2

Data for *Hbt. salinarum*, *Hbt. noricense* and *Hbt. jilantaiense* were obtained from Grant (2001), Gruber *et al*. (2004) and Yang *et al*. (2006), respectively. +, Positive; -, negative

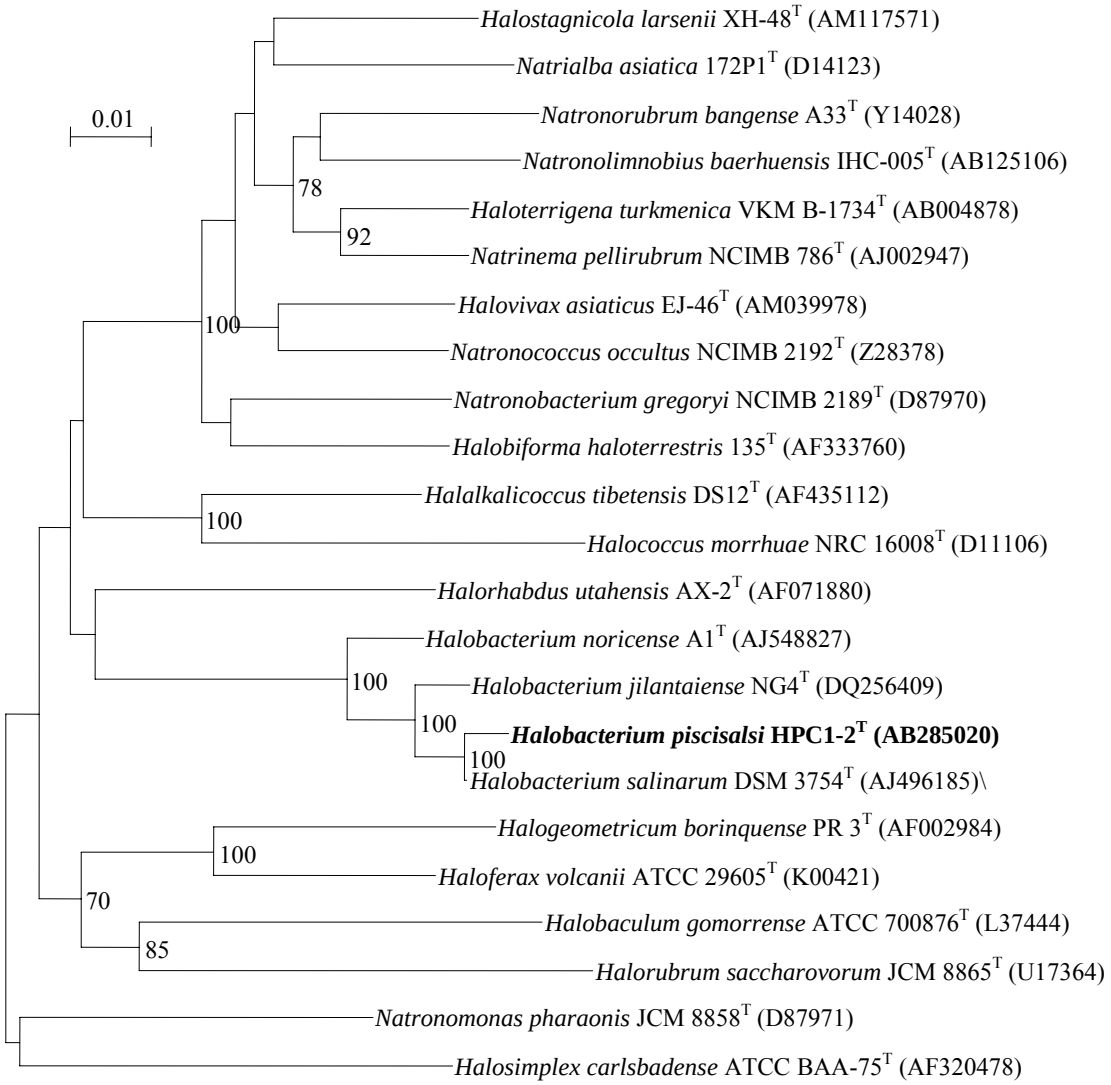

Figure 23. Phylogenetic tree showing the relationships between strain HPC1-2^T and related archaeal species based on 16S rRNA gene sequences. The branching pattern was generated according to the neighbour-joining method. Bootstrap percentages above 70%, based on 1,000 replications, are shown at the nodes. Bar, 0.01 substitutions per 100 nucleotide positions.

DNA-DNA hybridization

Strain HPC1-2^T showed levels of DNA-DNA relatedness of only 36.5±2.5% with *Hbt. salinarum* DSM 3754^T and 19.3±2.1% with *Hbt. jilantaiense* JCM 13558^T. In view of the unclear results of the methods described above, DNA-DNA hybridizations were carried out to clarify the taxonomic position of the strains. DNA-DNA homology values higher than 70% are generally accepted for a definition of species (Wayne *et al*., 1987). These values are the mean ± SD obtained from three independent determinations. The results presented herein indicate that strain HPC1-2^T represents a novel species of the genus *Halobacterium*, for which the name *Halobacterium piscisalsi* sp. nov. is proposed.

Description of *Halobacterium piscisalsi* sp. nov.

The descriptions of *Halobacterium piscisalsi* (pis.ci.sal´si. L. n. *piscis* fish; L. adj. *salsus* salted, salt; N.L. gen. n. *piscisalsi* of salted fish) were summarized as follows: cells are rod-shaped (0.5-1.0 × 1.0-2.0 mm) and motile by means of peritrichous flagella. Cells stain Gram-negative and are chemo-organotrophic. Colonies on plates of halophilic agar medium are red, smooth, circular and elevated. Requires at least 2.6 M NaCl for growth; optimal growth occurs at 3.4-4.3 M NaCl. Cell lysis occurs upon hypotonic treatment with less than 1.7 M NaCl. Growth occurs at 0-1 M $MgCl_2$, optimally at around 0.5-0.6 M $MgCl_2$. The temperature and pH ranges for growth are 20-60 °C (optimum at 37-40 °C) and 5.0-8.0 (optimum at pH 7.0-7.5). Grows anaerobically in the presence of nitrate, L-arginine and DMSO. Catalase- and oxidase- positive. Nitrate and nitrite are not reduced. Methyl red, Voges-Proskauer reaction and indole production are also negative. Casein, gelatin and Tween 80 are hydrolysed, but starch is not. The following substrates are utilized for growth: lactose, melezitose, glycerol and citrate. Acid is produced from glycerol. Does not utilize L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, myo-inositol, inulin, maltose, D-mannitol, D-mannose, melibiose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose, D-xylose, acetate, L-glycine, L-alanine, L-arginine, Laspartate, L-glutamate or L-lysine. Produces alkaline phosphatase, esterase

(C_4), esterase lipase (C_8), lipase (C_{14}), acid phosphatase and naphthol-AS-BI-phosphohydrolase, but not leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, Nacetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, lysine decarboxylase, ornithine decarboxylase, urease or DNase. Sensitive to bacitracin (10 μg), neomycin (30 μg) and novobiocin (5 μg), but not to ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), rifampicin (30 μg), streptomycin (10 μg) or tetracycline (30 μg). The major polar lipids are $C_{20}C_{20}$ derivatives of PG, PGP-Me, PGS, TGD, STGD and S-TeGD. The predominant menaquinone is MK-8. The G+C content of the DNA is 65.5 mol%.

The type strain, HPC1-2^T (=BCC 24372^T =JCM 14661^T =PCU 302^T), was isolated from *pla-ra* (salt-fermented fish product) in Thailand.

3.5 Conclusion

On the basis of growth requirements, poor utilization of carbohydrates, antibiotic susceptibility, menaquinone content, overall phospholipid composition, DNA G+C contents and 16S rRNA gene sequence analysis, strains HPC1-2^T is considered to represent a single species of the genus *Halobacterium.* However, it was differentiated from recognized *Halobacterium* species based on levels of DNA-DNA relatedness, phenotypic and chemotxonomic characteristics. These results suggested that the strains HPC1-2^T represent a novel species of the genus, for which the name *Halobacterium piscisalsi* sp. nov. is proposed.

3.6 Reference

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CHAPTER 4

ANTIOXIDANT ACTIVITY OF BACTERIORUBERIN FROM *HALOBACTERIUM SALINARUM* HM3

4.1 Abstract

The antioxidant activities of bacterioruberin from *Halobacterium salinarum* HM3 were assessed *in vitro* with respect to radical scavenging, singlet oxygen quenching abilities and plasmid relaxation assay compared with other carotenoids. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-Azinobis-3-ethylbenzo thizoline-6-sulphonate (ABTS) radical scavenging activity of bacterioruberin were highest among tested carotenoids with the effective concentration for 50% scavenging (EC_{50}) of 3.88 and 15.80 mM, respectively. The reducing power (OD_{700}) of bacterioruberin was 3.27. Singlet oxygen quenching ability of bacterioruberin was higher than other tested carotenoids, with the quenching rate constants (k_q) of 2.48×10^{10}, 1.18×10^{10}, 1.49×10^{10}, 2.15×10^{10} and 1.24×10^{10} M^{-1} s^{-1} for bacterioruberin, astaxanthin, β-carotene, lycopene, lutein, respectively. Bacterioruberin also exhibited protective effect on DNA damage by the attack of hydroxyl radical ($OH^{\bullet}$) generated from the Fenton reaction. The higher antioxidant effect of bacterioruberin may be attributed to its distinct chemical structure with 13 conjugated double bonds and the additional hydroxyl group substitutions on both sides of the molecule, compared with other tested carotenoids.

4.2 Introduction

One of the important characteristics of carotenoids is the ability to act as antioxidants, thus protecting cells and tissues from damaging effects of free radicals and singlet oxygen. The free radicals and singlet oxygen produced in the body by the normal aerobic metabolism are highly reactive (Darley-Usmar and Halliwell, 1996). These oxidants can react with various components of living cells,

such as proteins, DNA, or lipids, and cause structural changes leading to many diseases; carotenoids have been found to be important in protecting against diseases and age related phenomena caused by oxidants (Halliwell, 1996; Elliot, 2005). Antioxidative properties of many of the carotenoids, particularly that of β-carotene and lycopene, have been established (Kiokias and Gordon, 2004). The antioxidant mechanism of carotenoids is attributed to their ability to quench singlet oxygen and scavenge free radicals (Palozza and Krinsky, 1992; Hirayama *et al*., 1994)

Halobacterium salinarum, an extremely halophilic archaeon, produced large amount of red membrane consisting of C_{50}-carotenoids called bacterioruberin under aerobic cultivation. It has been shown that bacterioruberin of *Hbt. salinarum* is highly resistant to the lethal effects of DNA-damaging agents (Shahmohammadi *et al*., 1998). Additionally, protective mechanisms such as membrane pigments, including C_{50} carotenoid, bacterioruberin and intracellular KCl were found to be important in UV and gamma-ray protection in *Hbt. salinarum* through the scavenging of hydroxyl radicals (Shahmohammadi *et al*., 1998). Saito *et al*. (1994) found that the hydroxyl radical-scavenging ability of bacterioruberin is greater than that of β-carotene. However, there was no study on the mechanism of antioxidant activity of bacterioruberin. Therefore, the antioxidant activity of bacterioruberin was evaluated using the radical scavenging activity, the singlet oxygen quenching capacity, and plasmid relaxation assay compared with astaxanthin, β-carotene, lycopene, and lutein.

4.3 Materials and Methods

Chemicals and reagents

Astaxanthin, β-carotene, and lutein were kindly provided by DSM (Basel, Switzerland). Lycopene, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-3-ethylbenzothizoline-6-sulphonate (ABTS) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All carotenoids were stored at -20°C in nitrogen-packed vials, and solutions were made immediately before use. All other solvents and chemicals were of analytical grade.

Preparation of bacterioruberin

Hbt. salinarum HM3 was isolated from the salt-fermented crab collected from local markets in Thailand. The strain was grown in 200 mL of a halophilic medium composed of (L^{-1}): 250 g NaCl, 5 g casamino acid, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4.7H_20$, 0.036 g $FeCl_4.4H_20$, 0.00036 g $MnCl_2.4H_20$, and cultivated at 200 rpm, 37 °C for 1 week. The wet cells were obtained by centrifugation at 10,000 × g at 4 °C for 20 min and extracted with ethanol (Asker and Otha 1999). The cell debris was removed by centrifugation. The colorless polar impurities (salts, polar lipids) were precipitated by overnight cooling in a deep freezer and removed by centrifugation. The ethanol was evaporated by using rotary evaporator (R-114, Buchi, Switzerland). The dry bacterioruberin was kept in nitrogen-packed vials, and solutions were made immediately before use.

ABTS radical scavenging activity

ABTS radical scavenging activity was determined by ABTS assay as per the method of Arnao *et al*. (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing with methanol to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV-1601 spectrophotometer (Helios-alpha, Unicam, England). Fresh ABTS solution was prepared for each assay. An aliquot of each carotenoid (50 µL, 0.5-5 mM) in a mixture of acetone and MeOH in the ratio 1:1 (v/v) was mixed with 950 µL of ABTS solution and the mixture was left at room temperature for 30 min in dark. The absorbance was then measured at 734 nm. The scavenging activity was calculated based on the percentage of ABTS radical scavenged. EC_{50} value (mg/mL) is the effective concentration at which 50% of the ABTS radicals were scavenged and was obtained by interpolation from linear regression analysis from the plot of scavenging activity against the concentration of sample.

DPPH radical scavenging activity

The DPPH assay was done according to the method of Jiménez-Escrig *et al*. (2000) with some modifications. An aliquot of each carotenoid (50 µL, 1-20 mg/mL) in a mixture of acetone and MeOH in the ratio 1:1 (v/v) was mixed with 950 µL of 100 µM DPPH (prepared with methanol). The mixture was shaken vigorously and then left to stand at room temperature for 30 min in the dark. The absorbance was measured spectrophotometrically at 580 nm against an acetone/MeOH (1/1, v/v) blank. The lower absorbance indicated the stronger scavenging activity. EC_{50} value (mg/mL) is the effective concentration at which 50% of the DPPH radicals were scavenged and was obtained by interpolation from linear regression analysis from the plot of scavenging activity against the concentration of sample.

Reducing power

The reducing power was measured according to that reported by Yen and Chung (1999). An aliquot of each sample (0.5 mL, 0.5-10 mM) in acetone/MeOH (1/1, v/v) was mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of 1% $K_3Fe(CN)_6$ followed by incubating at 50 °C for 20 min. After adding 0.5 mL of 10% trichloroacetic acid, the mixture was centrifuged at 3750 × *g* for 10 min (Centrifuge). The supernatant (0.5 mL) was then taken out and mixed with 0.5 mL of methanol and 100 µL of 0.1% ferric chloride immediately. After 10 min reaction, the absorbance was determined at 700 nm. The reducing power was expressed as an increase in A_{700} after blank substraction.

Singlet oxygen quenching

Singlet oxygen luminescence measurements were carried out using excitation from a Lumonics HY200 Nd:YAG laser (FWHM 8 ns) at 355 nm and a liquid-nitrogen cooled germanium photodiode/amplifier (North Coast model EO-817P) as a detector (Tinkler, *et al*., 1996). Luminescence was detected from the front face of the sample, with a 355 nm holographic notch filter (Kaiser Optical) and a 1270

nm interference filter (Newport) interposed between the sample and the detector to markedly reduce laser scatter and sensitizer emission reaching the detector. The signal from the amplifier was digitized using a digital oscilloscope (Tektronix model 2432A) and transferred to a computer for analysis. The singlet oxygen luminescence decay traces were all fitted to a bi-exponential kinetic analysis, and the first-order rate constant for singlet oxygen decay was extracted.

Plasmid relaxation assay

Plasmid relaxation assay was determined by modified method of Ishikawa *et al*. (2002). DNA strand damages were measured by converting circular double-stranded super-coiled plasmid DNA into nicked circular and linear forms. Reactions were performed in 50 µL of solution containing 10 µL of super-coiled pUC18 plasmid DNA (750 ng), 10 µL of 10 mM phosphate buffer (pH 7.8), 5 µL of 3.5% hydrogen peroxide, 5 µL of 100 µM ferric chloride and 20 µL of the tested samples at various concentrations. The mixtures were incubated at 37 ºC for 30 min, and the reactions were stopped by adding 1 µL of 5 mM EDTA. The 20µL of reaction mixtures was mixed with 5 µL of loading dye, and 15 µL of the mixture was separated on 1 % agarose gel electrophoresis. DNA bands (supercoiled, linear, and open circular) were stained with ethidium bromide and quantified by scanning the intensity of bands with Quantity One programme (version 4.2.3, BioRad Co., USA). Evaluation of the protective effects on DNA was based on the increase or decrease in the percentage of supercoiled monomers compared to that of the control.

Statistical analysis

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

4.4 Results and Discussion

DPPH radical scavenging activity

The DPPH radical scavenging activity of bacterioruberin and other carotenoids tested is presented in Figure 24A. The DPPH radical scavenging activity increased with the increasing concentration of all studied carotenoids. Scavenging of DPPH, a stable free radical, is one of the major methods commonly used to evaluate the antioxidative activity. The antioxidants react with the stable free radical i.e. 1,1-diphenyl-2-picrylhydrazyl (deep violet color) and convert it to 1,1-diphenyl-2-picrylhydrazine with discoloration. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant through the hydrogen donating ability (Bondet *et al*., 1997). The effective concentration for 50% scavenging (EC_{50}) determined by using a linear regression equation increased in the order bacterioruberin < lycopene < β-carotene < astaxanthin < lutein (Figure 24B). Comparison of the structures of the carotenoids tested revealed that two structural features modulated the scavenging ability: length of conjugated double bonds (hydrocarbon carotenoids and xanthophylls) and addition of chemical groups on the β-ionone ring (xanthophylls) (Figure 25). Within the hydrocarbon carotenoid group, in terms of the length of the conjugation system, lycopene and β-carotene which both have 11 conjugated double bonds, should have the same antioxidant capacity. However, in the case of β-carotene the effective length of the conjugated system is reduced, because two double bonds are in cyclohexene rings that are not planar with the rest of the molecular backbone. Consequently, the antioxidant activity of lycopene is much more effective than that of β-carotene. However, we have found that lycopene is much more reactive than the other carotenoids tested, except bacterioruberin.

Compared with the tested xanthophylls, bacterioruberin was found to be the most reactive on scavenging $DPPH^{\bullet}$ free radicals, followed by astaxanthin and lutein. This order of reactivity might also be justified by the length of the conjugated double bonds of each carotenoids: bacterioruberin (13), astaxanthin (11), and lutein (10), were expected to generate the more stable carotenoid radical intermediates,

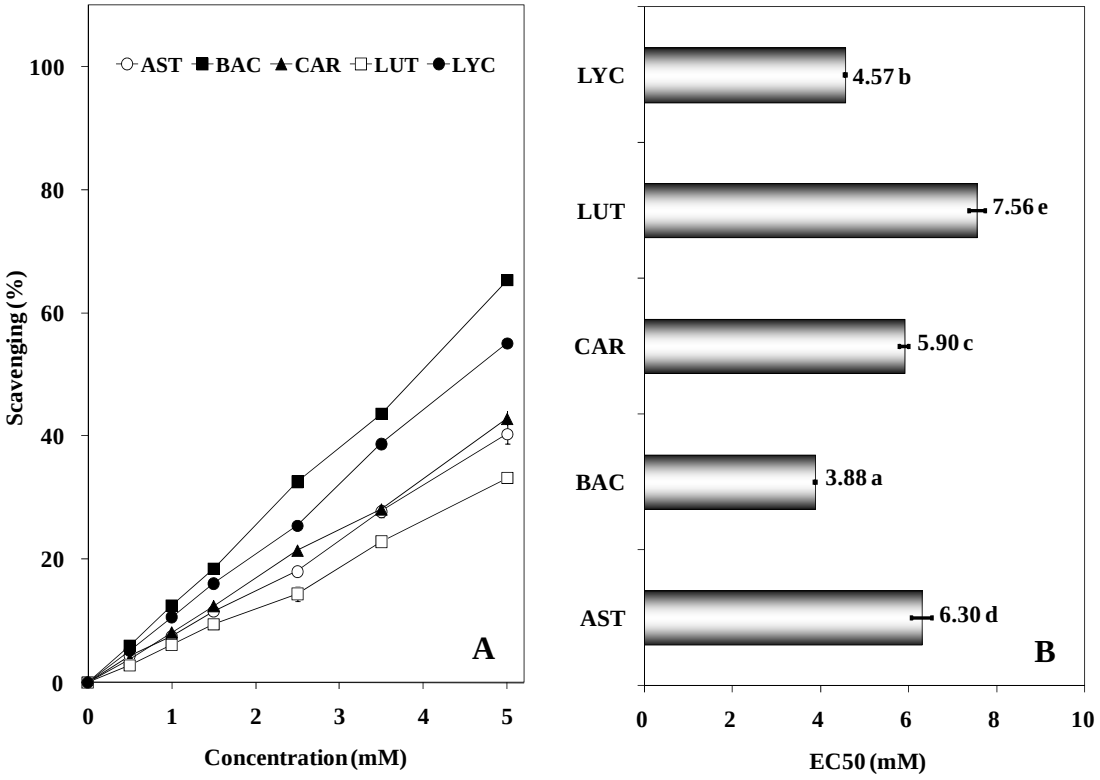

Figure 24. DPPH radical scavenging activity of carotenoids. Astaxanthin (AST); bacterioruberin (BAC); β-carotene (CAR); lutein (LUT); lycopene (LYC). Bars represent the standard deviation (n = 3). The different letters in the column denote the significant differences (P<0.05).

whereas lutein, having only 10 conjugated double bonds, was expected to have the least antioxidant efficiency for DPPH$^{\bullet}$. On the other hand, the presence of functional groups on the terminal rings of these xanthophyll compounds apparently modulated the radical scavenging. The presence of one hydroxyl group on each of the terminal rings, as in astaxanthin and lutein, decreased the antioxidant activity. However, the presence of four hydroxyl groups of bacterioruberin results in the increase in antioxidant activity.

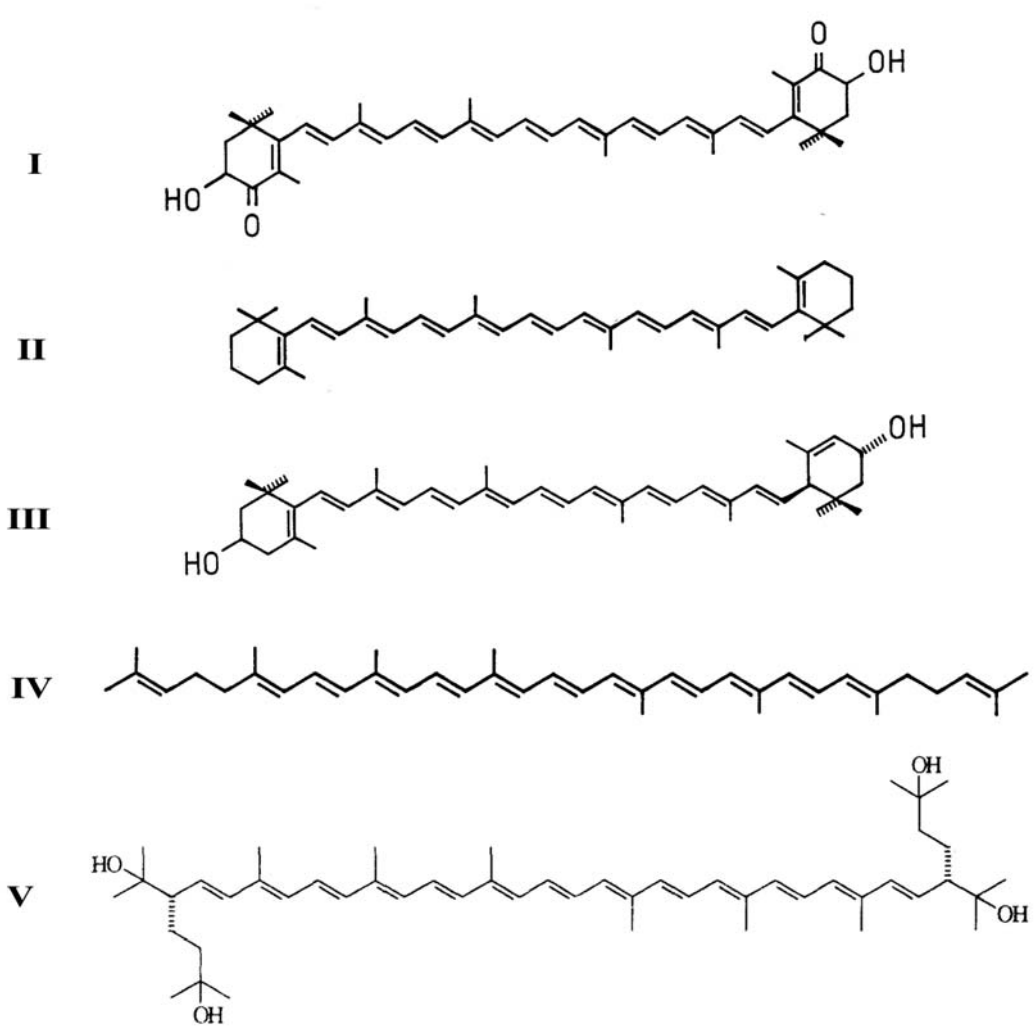

Figure 25. Structures of carotenoids. Astaxanthin (I), β-carotene (II), lutein (III), lycopene (IV), bacterioruberin (V).

ABTS radical scavenging activity

The ABTS radical scavenging activity of bacterioruberin was found to be higher than that of other studied carotenoids (Figure 26A). The ABTS radical scavenging assay is one of the popular indirect methods of determining the antioxidative capacity of compounds. In the absence of antioxidants, the ABTS radical is rather stable, but it reacts energetically with an H atom donor and is converted into a noncolored form of ABTS. The EC_{50} values calculated from the

regression equations show that the order of potency of the samples tested for ABTS radical scavenging was bacterioruberin > lycopene > β-carotene > astaxanthin > lutein (Figure 26B). The results showed that the relative abilities of the carotenoids to scavenge the ABTS radical were influenced by the number of conjugated double bonds as well as the position of functional groups (carbonyl and hydroxyl groups). The electron rich conjugated double bond structure is primarily responsible for the excellent ability of carotenoids to physically quench singlet oxygen without degradation and for the chemical reactivity with free radicals, and for its instability toward oxidation (Britton, 1995; Krinsky, 1994). The ABTS radical scavenging activity of carotenoids increases depending on the extension of the conjugated double bond molecular orbitals (Miller *et al*., 1996). Terao (1989) suggested that the electron-withdrawing character of the carbonyl oxygen atoms in echinenone, canthaxanthin and astaxanthin reduced the unpaired electron density in the 11-double-bonded carbon skeleton, resulting in a decrease in the reactivity of the hydrogen atoms/electrons on the carbon atoms in carotenoid skeleton with the ABTS radical cation. Miller *et al*. (1996) reported that the presence of hydroxyl groups in the terminal rings reduced the ABTS radical scavenging activity of carotenoids. However, Tian *et al*. (2007) suggested the presence of hydroxyl groups on the side of conjugated double bonds of deinoxanthin at C-1′ enhanced the scavenging activity. A hydroxycarotenoid with a chemical structure similar to deinoxanthin has been reported to have greater antioxidant properties than lycopene and its derivatives because of the presence of a hydroxyl group at position C-1′ (Albrecht *et al*, 2000). The highest radical scavenging activity of bacterioruberin among studied carotenoids might be due to the longest conjugated double bond and presence of four hydroxyl groups at the terminal of structure.

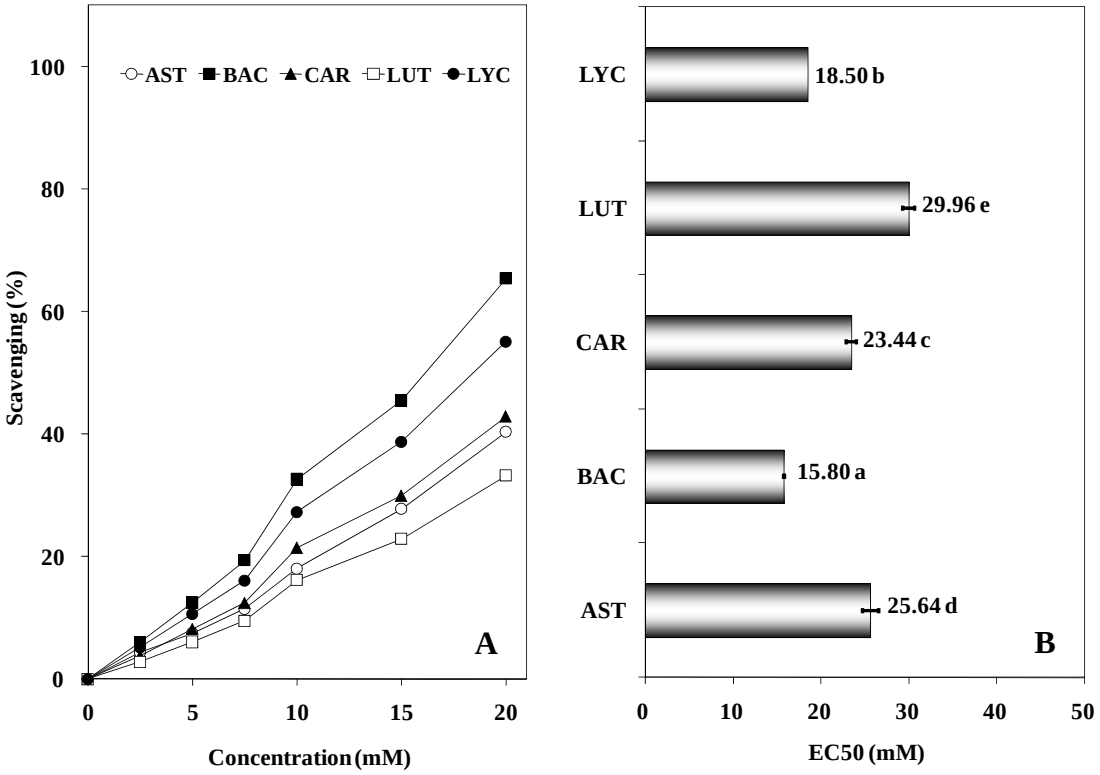

Figure 26. ABTS radical scavenging activity of carotenoids. Astaxanthin (AST); bacterioruberin (BAC); β-carotene (CAR); lutein (LUT); lycopene (LYC). Bars represent the standard deviation (n = 3). The different letters in the column denote the significant differences (P<0.05).

Reducing power

Bacterioruberin was the strongest radical reducer when compared with other carotenoids (Figure 27). In this method, ferric-ferricyanide complex is reduced to the ferrous form depending on the presence of antioxidants (Amarowicz *et al*., 2004). Reducing power was increased with increasing concentration of each carotenoid. Reducing power of a compound is also a supporting feature for its antioxidant activity. Martínez and Barbosa (2008) suggested that the reducing properties of carotenoids are generally associated with the ability to donate a hydrogen atom which could be assessed by carotenoid-hydrogen (CAR-H) bond

dissociation energy (ΔE) and the number of reactive positions. The homolytic dissociation of the CAR-H bond (HAT mechanism) of the reaction is governed by the ΔE of the H atom). The lower the ΔE value, the easier the H abstraction and the more important the role played by carotenoid as an antiradical.

Singlet oxygen ($^1O_2$) quenching activity

The kinetics curves of $^1O_2$ quenching with or without the presence of carotenoids are shown in Figure 28. Quenching of singlet oxygen by carotenoids is known to occur primarily through an energy transfer mechanism ($^1O_2 + CAR \rightarrow {}^3O_2 + {}^3CAR$). The rate constants obtained for the carotenoids studied are summarized in Table 16. The results demonstrated that bacterioruberin is a stronger radical scavenger than two carotenes (lycopene and β-carotene), and two xanthophylls (astaxanthin and lutein). The remarkable antioxidant activity of bacterioruberin may be ascribed to its chemical structure. There are four hydroxyl group substitutions on both sides of the conjugated double bond system and an additional double bond at C-3', 4' position of bacterioruberin.

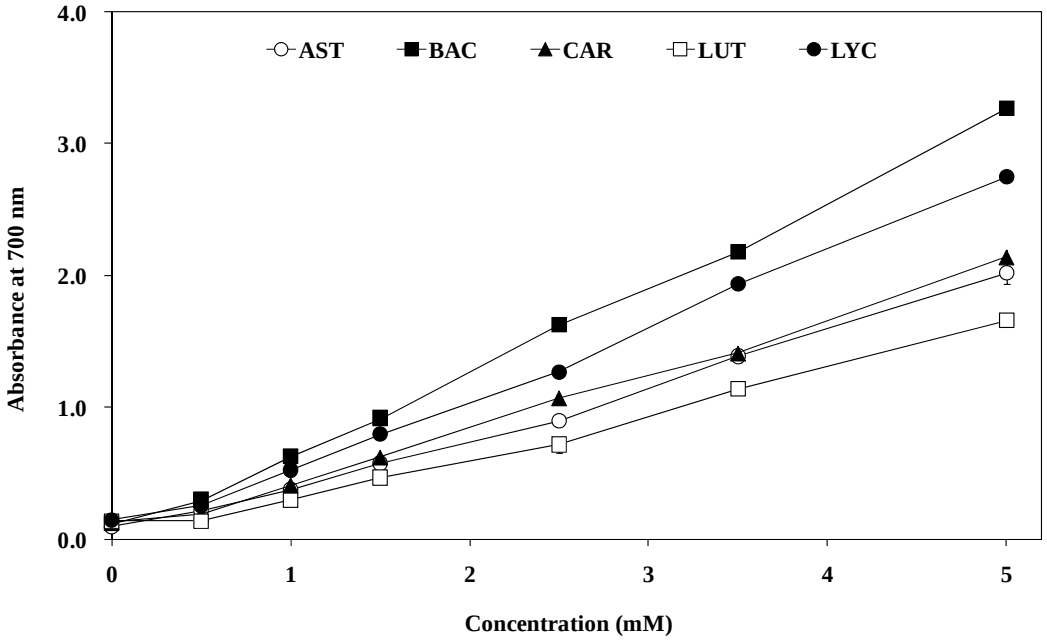

Figure 27. Reducing power of carotenoids. Astaxanthin (AST); bacterioruberin (BAC); β-carotene (CAR); lutein (LUT); lycopene (LYC). Bars represent the standard deviation (n = 3). Mean ± SD (standard deviation) from three determinations.

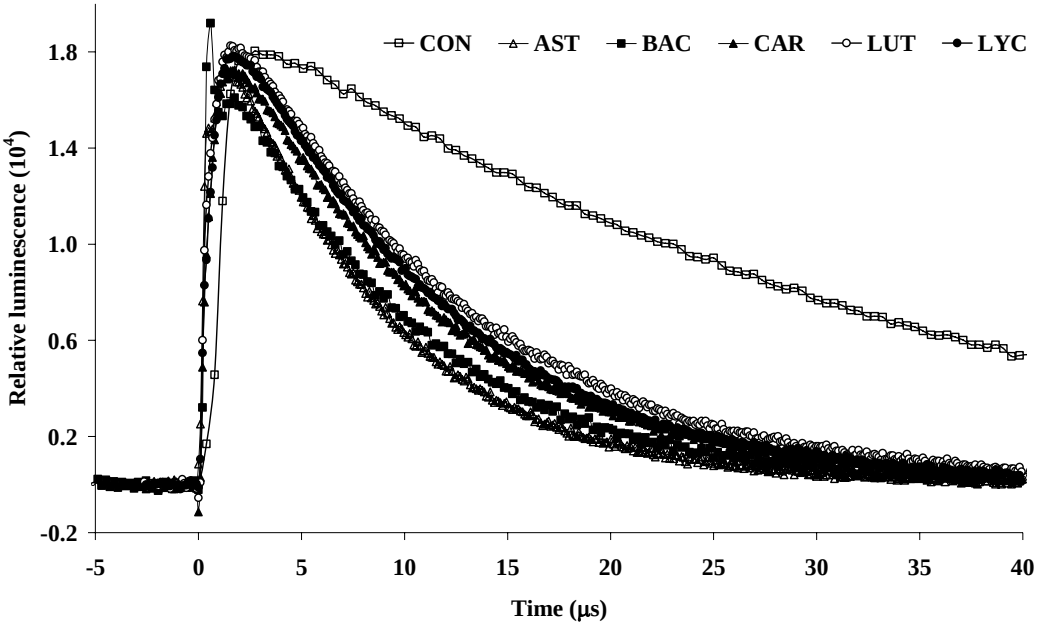

Figure 28. Singlet oxygen quenching of carotenoids. Astaxanthin (AST); bacterioruberin (BAC); β-carotene (CAR); lutein (LUT); lycopene (LYC).

Table 16. Singlet oxygen quenching rate constant for various carotenoids

Carotenoid	Conjugated double bonds	Functional groups	Singlet oxygen quenching k_q / 10^{10} $M^{-1}s^{-1}$
Bacterioruberin	13	4 OH	2.48
Astaxanthin	11 (+2, C = O)	2 OH	1.18
β-Carotene	11	-	1.49
Lycopene	11	-	2.15
Lutein	10	2 OH	1.24

Plasmid relaxation assay

Effects of bacterioruberin on DNA were investigated using a hydroxyl radical-induced DNA breaks system *in vitro*. With the attack of hydroxyl radical ($OH^{\bullet}$) generated from the Fenton reaction, supercoiled plasmid DNA was broken into the relaxed form. The extent of DNA damage was represented by the percentage of the supercoiled (SC) form in DNA bands, and the antioxidant effects of tested samples can be demonstrated by comparing the percentage values of the SC form in the tested samples and in the control (DNA treated by $OH^{\bullet}$). Figure 29a shows the effect of bacterioruberin on $OH^{\bullet}$-induced DNA damage. The SC form (96.84%, Lane 1) in the DNA control was totally broken into the relaxed form by the $OH^{\bullet}$ generated from the Fenton reaction ($H_2O_2 + Fe^{2+}$). H_2O_2 or Fe^{2+} treatment alone resulted in no significant damage on DNA. The protective effect of bacterioruberin on DNA can be inferred from the higher recovery (55.4%) of the SC form compared to the extent of DNA damage. Astaxanthin, β-carotene, lutein and lycopene also exhibited some protective effect on DNA (32.4%, 30.5%, 23.2%, and 35.7% recovery of the SC form respectively). The protective effect of bacterioruberin was significantly higher than that of studied carotenoids ($P<0.05$) as shown in Figure 29b.

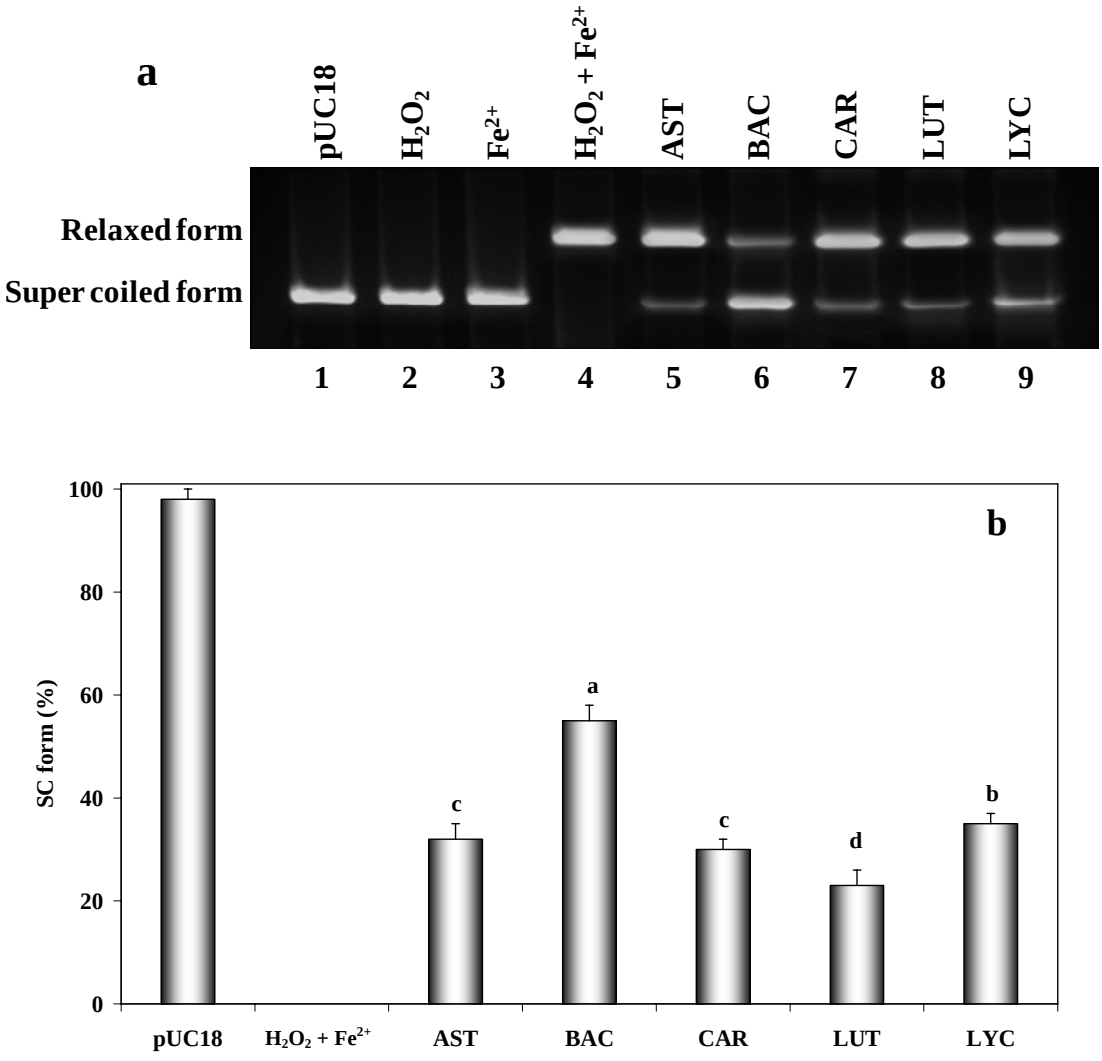

Figure 29. Agarose gel electrophoresis plasmid DNA breaks by •OH generated from Fenton reaction. (a) Lane 1-3, pUC18 plasmid DNA; Lane 4, plasmid DNA treated with $FeSO_4$ and H_2O_2; Lanes 5-9, DNA treated with $FeSO_4$ and H_2O_2 in the presence of astaxanthin (AST), bacterioruberin (BAC), β-carotene (CAR), lutein (LUT), and lycopene at concentration of 1 mM, respectively. (b) Relative intensity of supercoiled DNA from the tested samples in comparison with that of control. Experiments were performed three times and the values are represented as mean ± SD.

4.5 Conclusion

Bacterioruberin from *Hbt. salinarum* HM3 exhibited higher antioxidant activities compared to that of astaxanthin, β-carotene, lycopene, and lutein. It is presumed that the presence of more conjugated double bond and the additional hydroxyl group substitutions on both sides of the molecule might be responsible for the higher antioxidant activities.

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CHAPTER 5

STABILITY OF BACTERIORUBERIN FROM *HALOBACTERIUM SALINARUM* HM3 AND ITS APPLICATION IN SURIMI GEL

5.1 Abstract

Stabilities of bacterioruberin from *Halobacterium salinarum* HM3 upon heating and light exposure were investigated both in soybean oil and when it was incorporated into surimi as a coloring additive. In soybean oil, approximately 20% of the content and 40% of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of bacterioruberin decreased after heating at 90 °C for up to 24 h. Upon exposure to light at the intensity of 5,000 lux for 24 h, reductions in both content and DPPH-radical scavenging activity of bacterioruberin were approximately 10 and 20%, respectively. Incorporation of bacterioruberin into surimi at the concentrations of 100 and 200 mg/kg resulted in orange pink color with the L*, a*, and b* values in the ranges of 51.2-67.6, 15.7-31.7, and 15.7-23.4, respectively. At the concentrations of bacterioruberin added, no differences in the breaking force and deformation of surimi gels were observed (P>0.05). During storage at the refrigeration temperatures with the light exposure at 2,500 lux for 7 days, the color differences (ΔE^*_{ab}) and thiobarbituric acid reactive substances (TBARS) values of surimi gels packed in polypropylene bags were higher than those packed in aluminum bags. In polypropylene bag, however, surimi gels added with bacterioruberin had lower ΔE^*_{ab} and TBARS values than control with no bacterioruberin added. From the results, bacterioruberin exhibited relatively good stability upon heating at elevated treatments and light exposure. In addition to its color, bacterioruberin also exerted the antioxidative activity resulting in lower oxidation of lipids in surimi gels during storage.

5.2 Introduction

The C-50 carotenoids bacterioruberin have been found in some halophilic aerobic archaea that gives them a striking red color and seems to protect them from strong sunlight in their natural environments. Bacterioruberin composed of 13 conjugated dienes while other dietary carotenoids such as lycopene, β-carotene, astaxanthin, and canthaxanthin had only 11 conjugated double bonds. The high numbers of conjugated double bond acquire more red appearance. Beside their color properties, carotenoids pigments have the ability to act as chain-breaking antioxidants. The ability of carotenoids to quench singlet molecular oxygen ($^1O_2$*) is well known (Jung and Min, 1991) and reactions with radicals species have also been studied (Jorgensen and Skibsted, 1993), including the prevention of lipid peroxidation (Packer, 1993). Conn *et al*. (1991) indicated that the ability to quench $^1O_2$* increases with increasing number of conjugated double bond. In addition, Saito *et al*. (1997) found that the hydroxy radial-scavenging ability of bacterioruberin is greater than that of β-carotene.

Currently, surimi seafood was colored to mimic shellfish (crab, lobster, shrimp, and scallop) and salmon. Various kinds of both synthetic and natural colorants were used. Due to increasing consumer demand, natural colorants are more favored. The commonly used natural colorants include carmine, paprika oleoresin, annatto, caramel, canthaxanthin, and β-carotene (Lauro *et al*., 2005). Furthermore, a new range of colors for surimi that features both existing and new shades is required. In most colored surimi-based products, Lauro *et al*. (2005) suggested that the colorants should have various properties including the correct hue, not contribute objectionable flavor, compatible with surimi, not affect gel formation or the texture of the final product, survive under heat cooking, stable to light during storage, and not bleed after thermal processing. Based on the color and antioxidative properties, bacterioruberin should be an alternative source of colorant in surimi seafood. Therefore, the stabilities of bacterioruberin from *Halobacterium salinarum* HM3 upon heating and light exposure were investigated both in soybean oil and when it was incorporated into surimi from bigeye snapper (*Priacanthus tayenus*) as a coloring additive.

5.3 Materials and Methods

Chemicals and reagents

6-Hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Refined soybean oil was purchased from the local market. All chemicals used in this study were of analytical grade or HPLC grade. The polypropylene and aluminum foil bags were purchased from MMP Packing Group Co., Ltd.

Preparation of bacterioruberin

Halobacterium salinarum HM3 was isolated from the salt-fermented crab collected from local markets in Thailand. The strain was grown in 200 mL of a halophilic medium composed of (per liter): 250 g NaCl, 5 g casamino acid, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g $MgSO_4.7H_20$, 0.036 g $FeCl_4.4H_20$, 0.00036 g $MnCl_2.4H_20$, and cultivated at 200 rpm, 37 °C for 1 week. The wet cells were obtained by centrifugation at 10,000 ×*g* at 4 °C for 20 min and extracted twice with ethanol (Asker and Otha 1999). The cell debris was removed by centrifugation. The colorless polar impurities (salts, polar lipids) were precipitated by overnight cooling in a deep freezer and removed by centrifugation. The ethanol was evaporated by using a rotary evaporator (R-114, Buchi, Switzerland). A colorant was prepared by dissolving the dry bacterioruberin in soybean oil with gently stirring under dim light in a refrigerator to make a bacterioruberin stock solution of 50 g/L. The stock was diluted with the soybean oil at the concentration of 4 mg/L and its absorption spectra were determined between 350 and 600 nm using a spectrophotometer (Helios-alpha, Unicam, England). The extinction coefficients for bacterioruberin in soybean oil and ethanol were calculated by measuring the absorbances at the respective maximum wavelength (λ_{max}).

Thermal stability of bacterioruberin

Bacterioruberin was dissolved in soybean oil at the concentration of 4 mg/L (the reading absorbance at 505 nm ~ 1.0) and thermostated in a waterbath (Memmert, WNB22, Germany) at the desired temperatures. The changes in bacterioruberin content and radical scavenging activity were followed at 70, 80 and 90 °C for 0, 1, 3, 6, 12, 18, and 24 h. At time designated, the samples were cooled to room temperature (25 °C) in ice bath. The absorption spectrum was recorded at 350-600 nm by a spectrophotometer (Helios-alpha, Unicam, England) setting up a blank with the control soybean oil which was heated under the same condition as the samples. The bacterioruberin content was estimated by using the absorbance and extinction coefficients respective to the wavelength dependencies. The DPPH radical scavenging activity of colorant was determined as described by Espín *et al*. (2000) and Jiménez-Escrig *et al*. (2000) with slight modification. The stock solution was prepared by dissolving 24 mg DPPH with 100 mL ethyl acetate and then stored at -20 °C. The working solution was obtained by mixing stock solution with ethyl acetate to obtain an absorbance of 0.6 units at 580 nm. The colorants (150 µL) were allowed to react with 2,850 µL of the DPPH solution for 30 min in the dark, and then the absorbance was taken at 580 nm. A standard curve was prepared using Trolox in the range of 0 to 1,000 µM. The activity was expressed as µmol Trolox equivalent (TE)/ mL colorant. The peroxide value (PV) was determined by the modified method of Hornero-Méndez *et al*. (2001).

Effect of light exposure

Bacterioruberin in soybean oil (4 mg/L) was exposed to fluorescent light at 5,000 lux in a Versatile Environment Test Chamber (MLR-350T, Sanya, Japan) for 0, 1, 3, 6, 12, 18, 24 h at 20 °C. The sample was agitated with a magnetic stirrer (IKAMAG, RO 10, Malaysia). The bacterioruberin content, DPPH radical scavenging activity and PV were determined as described previously.

Kinetic studies

The degradation of bacterioruberin and loss in DPPH radical scavenging activity were calculated by using the standard equation for a first-order reaction given below:

$$\ln C = \ln C_0 - kt$$

where C, the concentration at time t; C_0, the concentration at time zero; k, the first-order rate constant (h^{-1}); t, the incubation time (h).

Temperature dependence of bacterioruberin degradation and loss in DPPH radical scavenging activity were determined by Arrhenius equation:

$$k = k_0 \cdot e^{-Ea/RT}$$

where E_a: the activation energy (kcal/mol); k: the rate constant; k_0: the pre-exponential factor; R: the universal gas constant (1.987 kcal/mol); and T: the absolute temperature (°K).

Acute oral toxicity determination (LD_{50})

Acute oral toxicity of bacterioruberin of *Halobacterium salinarum* HM3 was studied following the OECD Guideline No. 423 (OECD, 2001) and conducted in compliance with Good Laboratory Practice Standards. Fifty Wistar rats were allocated to five groups (5 of each sex) and acclimatized to the laboratory environment for one week prior to study. The rats were fasted for 16 h prior to dosing while drinking water was available ad libitum. Bacterioruberin in soybean oil at the concentrations of 125, 250, 500, and 1,000 mg/mL were orally fed to the rats at the dosages of 125, 250, 500, and 1,000 mg/kg body weight. The rats of the control group were dosed with soybean oil. All animals were examined for signs of toxicity daily. The individual body weights were determined on day 0 (start of administration

period) and thereafter at weekly intervals to calculate cumulative body weight gains during the study.

Surimi gel preparation

Frozen surimi from bigeye snapper grade SA (breaking force of 600-800 g; deformation of 12-14 mm) obtained from Man A Frozen Food Co, Ltd. (Songkhla, Thailand) was used. Frozen surimi was partially thawed at 4 °C for 2-3 h, cut into small pieces and placed in a Moulinex Masterchef 350 mixer (Paris, France). The moisture was adjusted to 80% and 2.5% salt was added. Bacterioruberin was dissolved in soybean oil at the concentrations of 10 and 20 g/L and added into surimi paste at 1% (v/w) which corresponded to 100 and 200 mg/kg, respectively. Soybean oil was added as a control. The mixture was chopped for 5 min at 4 °C. The paste was stuffed into a polyvinylidine chloride casing with a diameter of 2.5 cm and both ends were sealed tightly with approximately 10 cm in the length. The surimi gels were incubated at 40 °C for 30 min, followed by heating at 90 °C for 20 min in a water bath (Memmert, Schwabach, Germany). The surimi gels were immediately cooled in iced water for 30 min and stored at 4 °C overnight prior to analysis.

Refrigerated storage test

The surimi gels were cut into a cylinder shape of 2.5 cm in length. Ten pieces of gels were packed in either polypropylene or aluminum foil bags, sealed, and stored at 4 °C under a fluorescent light (2,500 lux). The changes in color, texture, lipid oxidation, and microstructures of surimi gels were monitored at 0, 1, 3, 5, and 7 days of storage.

Bleeding test

The surimi gels added with bacterioruberin at the concentration of 200 mg/kg (colored gel) and the surimi gels without bacterioruberin (white gel) were packed by surface touching in polypropylene bag. Five replicates were prepared,

cooked by steaming for 50 min, and then cooled to room temperature (25 °C) in ice bath. The treated white gels were separated and measured the L*, a*, and b* values. The degree of color transfer (bleeding) was calculated as the color difference (ΔE^*_{ab}) between the treated white gels and a reference white gels. The reference white gels were prepared by steaming.

Color measurement

The color of surimi gels were measured using a colorimeter (JP7100F, Juki Corporation, Tokyo, Japan). Measurements of L*, a*, and b* were made on five replicate samples of each treatment. The total color difference (ΔE^*_{ab}) was calculated as $\Delta E^*_{ab} = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$ (Sharma, 2003).

Determination of bacterioruberin content

A sample (5 g) was homogenized with 25 ml of ethanol. The mixture was centrifuged at 5,500 ×*g* for 25 min. The absorbance of the supernatant was scanned at 300-600 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). The bacterioruberin content was calculated using the extinction coefficient (2500) and expressed as mg/kg sample.

Texture analysis

The breaking force and deformation of surimi gel were measured by a texture analyser (Model TA-XT2, Stable Micro Systems, Godalming, Surrey, England) equipped with a cylindrical plunger (5 mm diameter; 60 mm/min penetration speed) according to the method of Benjakul *et al*. (2001). Gels were equilibrated and tested at room temperature (25 °C). Five replicate samples of each treatment were analyzed.

Determination of thiobarbituric acid reactive substances (TBARS)

TBARS was determined as described by Benjakul and Bauer (2001). A sample (5 g) was homogenized with 25 ml of TBARS solution (0.375% TBA, 15% TCA, and 0.25 N HCl). The mixture was heated for 10 min in boiling water to develop a pink colour. Then the mixture was cooled with a running water and centrifuged at 5,500 ×*g* for 25 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). The TBARS value was calculated from the standard curve of malonaldehyde and expressed as mg malonaldehyde/kg sample.

Scanning electron microscopy

The surimi gels collected at 0 and 7 days were fixed with 2.0% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), and fixed with osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2) at room temperature (28-30 °C). The fixed specimens were successively dehydrated in ethanol solutions of increasing strength and then dried to a critical point using a Polaron CPD 7501 critical point drier (Uckfield, East Sussex, England). The specimens were coated with a gold layer and observed with a JEOL scanning electron microscope (Model JSM 5200, Tokyo, Japan).

Statistical analysis

One-way ANOVA was used and mean comparison was performed by Duncan's multiple range test (Steel and Torrie, 1980). Statistical analysis was carried out using SPSS statistic program (Version 10.0) for Window (SPSS Inc. Chicago, IL).

5.4 Results and Discussion

Absorption spectra and extinction coefficients

The UV-Visible spectra of bacterioruberin extracts in ethanol and soybean oil at the concentrations up to 500 μM were found to be independent of the two solvents used (Figure 30). The spectra of bacterioruberin in ethanol and soybean oil are almost identical except for a large bathochromic shift of absorption maxima (Figure 30). The results indicated that all peaks were red shifted about 12 nm with increasing solvent polarity, suggesting a strong dependence of absorption and emission spectra of bacterioruberin with the solvent polarity. Since polarities of the ground and excited state of a chromophore are different, a change in the solvent polarity will lead to differential stabilization of the ground and excited states, and thus, a change in the energy gap between these electronic states. Sachindra and Mahendrakar (2005) showed that the λ_{max} of astaxanthin in soybean oil (487 nm) was higher than that in ethanol (478 nm). An extinction coefficient of 2500 in both ethanol and soybean oil for bacterioruberin at 493 and 505 nm was observed. The λ_{max} and extinction coefficients determined were used for further experiments to determine bacterioruberin content.

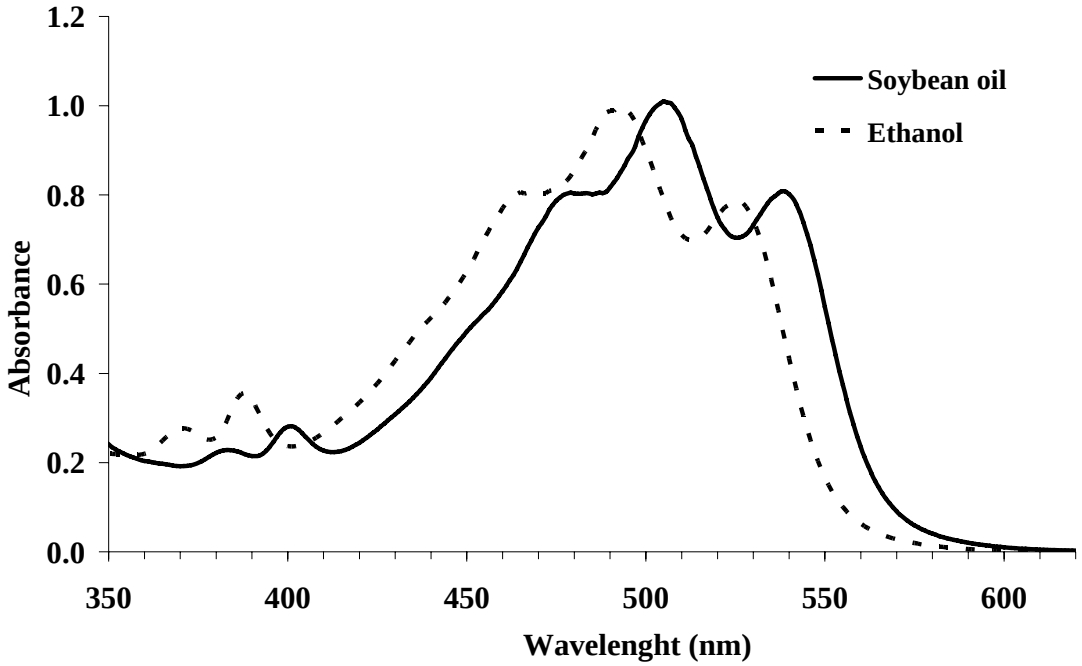

Figure 30. Spectra of bacterioruberin (500 μM) in ethanol (dashed line) and soybean oil (solid line).

Thermal stability

The bacterioruberin content slightly decreased during incubation and retained at 87, 81, and 78 % of the initial content when incubated for 24 h at 70, 80, and 90 °C, respectively (Figure 31a). The degradation kinetics for the bacterioruberin followed a first-order kinetic model. The rate constants (*k*) for degradation were determined by linear regression of the ratio of ln (C/C_0) versus time, and the results are shown in Table 17. The rate constants increased with the increasing temperature, and the activation energy was calculated to be 26.5 kJ mol^{-1}. The first-order kinetic models were found in the degradation of carotenoids under heating including lycopene in hexane (Lee and Chen, 2002), lycopene, β-carotene, and lutein in safflower seed oil (Henry *et al*., 1998).

The radical scavenging activities of bacterioruberin gradually decreased after incubation at 70, 80, and 90 °C for 24 h (Figure 31b). Incubation of bacterioruberin at 70 and 80 °C resulted in decreasing of scavenging activities in the

same extent. The noticeable difference was found when the incubation temperature was increased to 90 °C. Frankel (1989) suggested that the unsaturated oil were prone to autoxidation initiated by free radicals, especially at elevated temperature. Burton (1989) reported that β-carotene could inhibit a free radical in the absence of light. The decrease of bacterioruberin content was probably caused by the degradation of pigment due to the antioxidant activity against lipid peroxidation. Anguelova and Warthesen (2000) showed that lycopene, β-carotene and α-carotene were degraded when using to inhibit the lipid peroxidation. They also suggested that degradation of β-carotene and α-carotene was accompanied by the formation of oxygenated derivatives eluting in the HPLC analysis as single peaks before the corresponding carotenes (450 nm).

The PV of bacterioruberin in soybean oil compared with soybean oil after incubated at 70, 80, and 90 °C are shown in Figure31 c. The PV was very low at the beginning and gradually increased during incubation time. After incubated at 70, 80, and 90 °C for 24 h, the PV of soybean oil were 13.5, 17.5, and 21.7 meq/kg, respectively while that of soybean oil with bacterioruberin were 9.6, 12.5, and 15.5 meq/kg, respectively. The decreases in PV were related to the study of Steenson and Min (2000) which both 50 ppm β-carotene and 50 ppm lycopene decreased the PV of soybean oil heated at 60 °C.

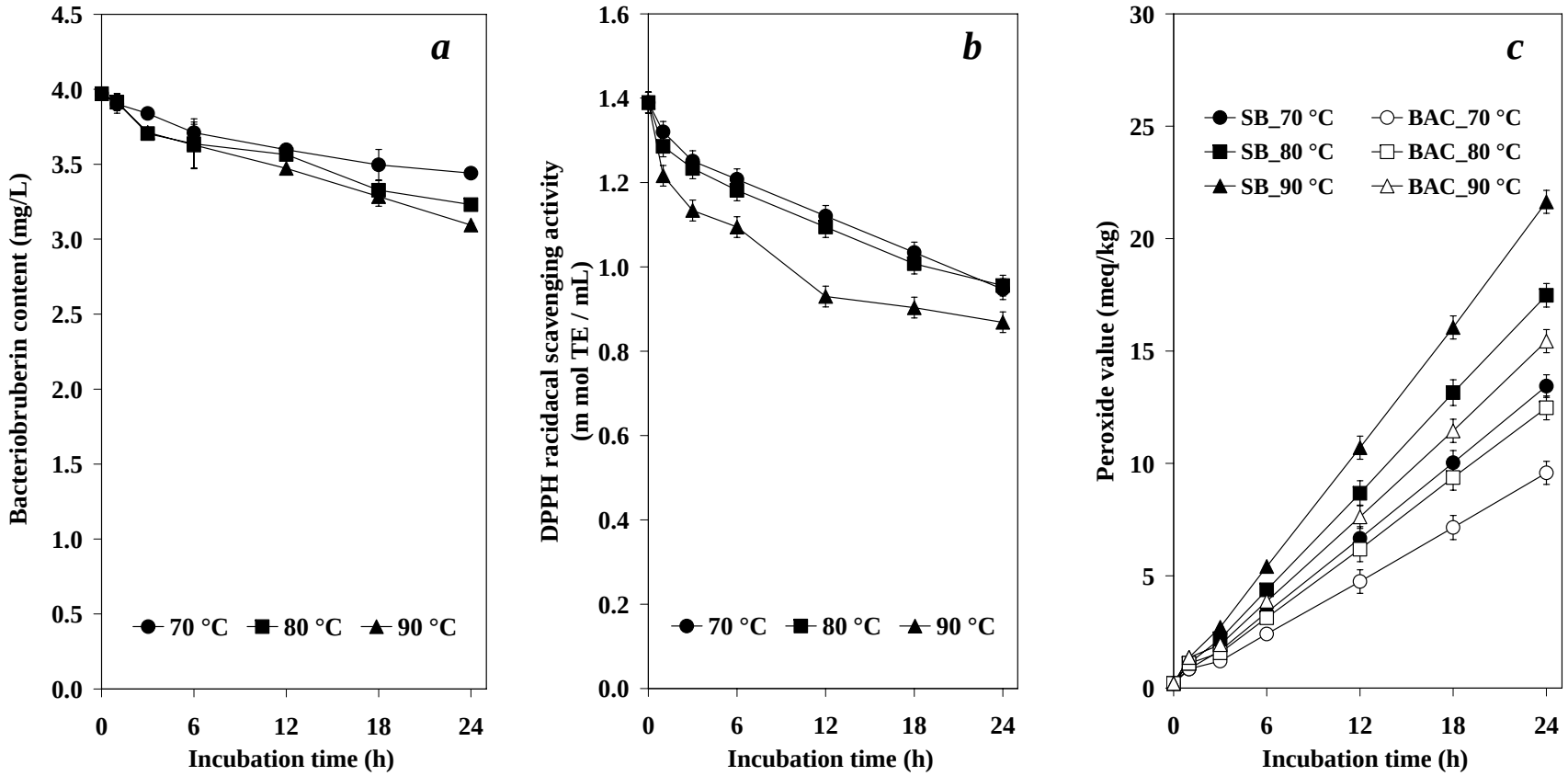

Figure 31. Changes in content (*a*), radical scavenging activity (*b*), and peroxide values (*c*) of bacterioruberin in soybean oil during heating at different temperatures.

Table 17. Thermal degradation of bacterioruberin in soybean oil at various temperatures

Temperature (°C)	k (1/h)[a]	R^2
70	0.006	0.950
80	0.008	0.948
90	0.010	0.978

[a] The degradation kinetics for the bacterioruberin at each temperature followed a first-order kinetic model and calculated using the following formula: $k = -\ln (C/C_0)/t$ where C: the total amount of lycopene after heating; C_0: the initial amount of bacterioruberin; *t*:heating time.

Effect of light exposure

The effect of light exposure at 5,000 lux on the stability of bacterioruberin after incubation for 24 h at 20 °C is shown in Figure 32a. Bacterioruberin was relatively stable during the light exposure for 24 h in which only about 10% of bacterioruberin was degraded. The residual radical scavenging activity was remained up to 80% during condition tested (Figure 32b). The degradation of bacterioruberin during light exposure was found to fit a first-order kinetic model with a rate constant of 0.0029 h^{-1}. Steenson and Min (2000) suggested that the oxidation of the soybean oil stored under light conditions was caused by singlet oxygen oxidation. Furthermore, many studies suggested that dietary carotenoids e.g. β-carotene and lycopene were an effective quencher of singlet oxygen (Foote and Denny, 1968; Warner and Frankel, 1987; Jung and Min, 1991; Edge *et al*., 1997). The PV of bacterioruberin in soybean oil compared with soybean oil after exposure under light at 5,000 lux is shown in Figure 32c. After incubated under light exposure for 24 h, the PV of soybean oil with and without bacterioruberin were 13.5 and 9.7, respectively. The decreases in PV were related to the study of Steenson and Min (2000) which both 50 ppm β-carotene and 50 ppm lycopene decreased the PV of soybean oil under light storage at 1,650 lux.

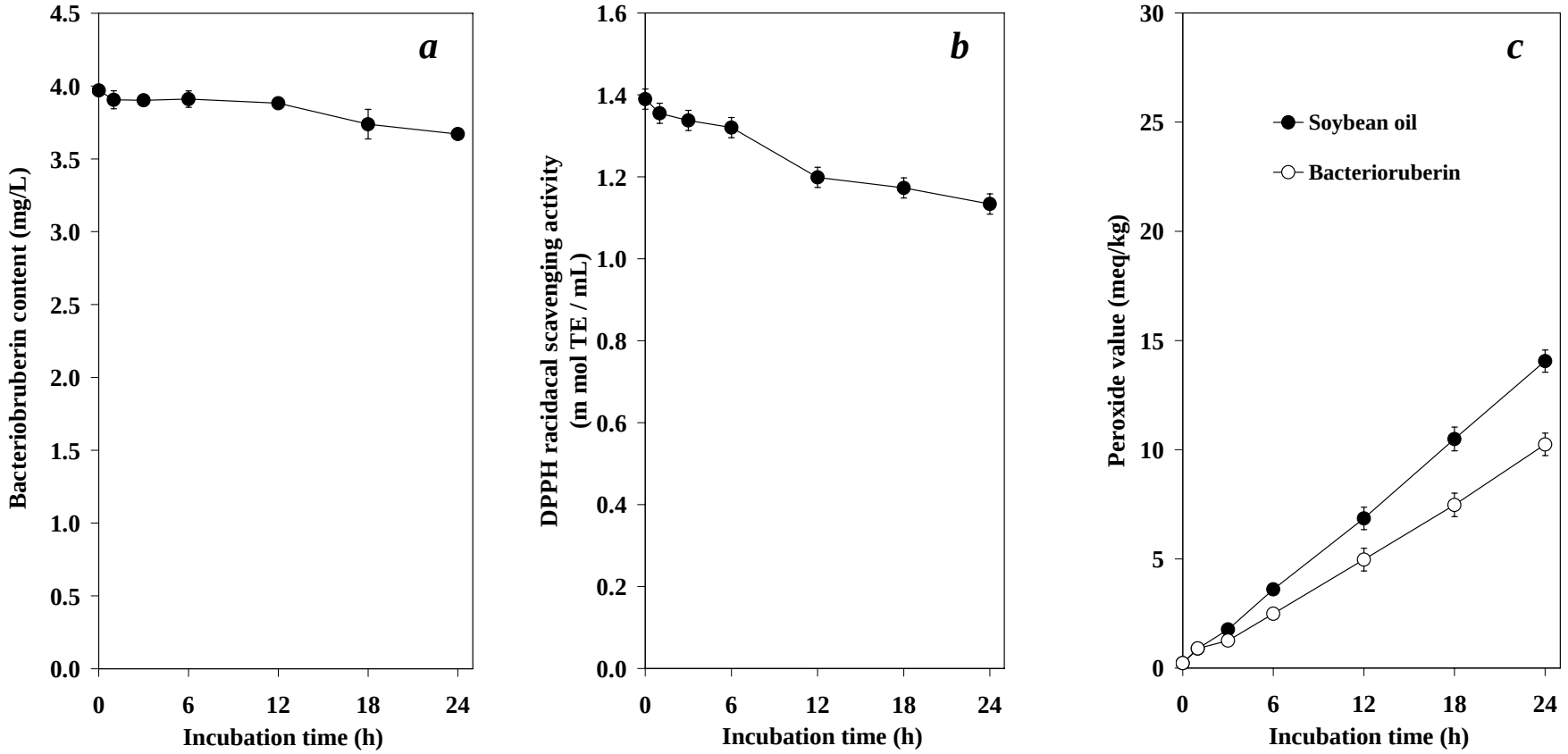

Figure 32. Changes in content (*a*), radical scavenging activity (*b*), and peroxide values (*c*) of bacterioruberin in soybean oil during light exposue illumination at 5,000 lux.

Acute oral toxicity (LD_{50})

The body weight gain of male and female Wistar rats fed bacterioruberin at 0, 125, 250, 500, and 1,000 mg/kg/day are shown in Table 18. During the first week, a slightly decrease in the body weight gain were found in both male and female rats fed with bacterioruberin ($P<0.05$). However, in the second week, no significant differences ($P>0.05$) were found in the body weight gain of male rats. The decreases in body weight gain of female rats were found in female receiving bacterioruberin at 1,000 mg/kg/day. No deaths were observed in any of the groups during the 14 days, and all survival rats did not show any significant gross pathological lesion. The no-observed-adverse-effect level (NOAEL) of various dietary carotenoids were reported as their highest dosages evaluated. Stewart *et al*. (2008) showed that the NOAEL of astaxanthin from *Haematococcus pluvialis* for male and female rats were determined as 465 and 557 mg/kg/day, respectively. Mellert *et al*. (2002) reported the NOAEL for lycopene CWD and Lyco Vit products was 3000 mg/kg/day (equivalent to 324 mg synthetic lycopene/kg/day). Nabae *et al.* (2005) judged the NOAEL of β-carotene derived from *Blakeslea trispora* was at least 5.0% (3,127 mg/kg/day for males, 3362 mg/kg/day for females).

Color

The color parameters of surimi gels with and without bacterioruberin addition were shown in Table 19. The surimi gels without bacterioruberin (white surimi gel) exhibited the L*, a*, and b* at the values of 80.07, -2.29, and 5.69, respectively. Incorporation of bacterioruberin into surimi at the concentrations of 100 and 200 mg/kg resulted in orange pink color with the L*, a*, and b* values in the ranges of 51.2-67.6, 15.7-31.7, and 15.7-23.5, respectively. The addition of bacterioruberin at 200 mg/kg exhibited the same L*, a*, b* values of surimi gels as canthaxanthin at 250 mg/kg (50.2, 35.7, and 24.9, respectively) and paprika oleoresin (51.6, 30.3, and 26.2, respectively) (Lauro *et al*., 2005).

Table 18. Body weight gain (g) of male and female Wistar rats orally dosed bacterioruberin at 0, 125, 250, 500 and 1,000 mg/kg body weight

Bacterioruberin (mg/kg body weight)	Bodyweight gain (g)			
	7 d		14 d	
	Male	Female	Male	Female
0	23.0 ± 1.0^{a}	30.7 ± 3.2^{a}	80.7 ± 4.2^{a}	44.3 ± 5.5^{b}
125	21.3 ± 1.5^{ab}	26.3 ± 3.1^{ab}	71.3 ± 3.8^{a}	54.3 ± 3.8^{a}
250	21.3 ± 0.6^{ab}	23.0 ± 4.4^{b}	73.3 ± 10.5^{a}	40.7 ± 1.5^{bc}
500	19.7 ± 1.5^{b}	22.3 ± 2.3^{b}	74.7 ± 7.6^{a}	38.0 ± 4.4^{bc}
1000	20.7 ± 1.5^{ab}	22.3 ± 1.5^{b}	74.7 ± 6.5^{a}	35.3 ± 2.1^{c}

Mean ± SD (standard deviation) from five rats.

^{a,b,c} Different letters in the same row denote significant differences ($P < 0.05$).

No color bleeding of surimi gel containing bacterioruberin was observed in white surimi under steaming (Table 20). In most colored surimi-based products it is important that the color must not bleed or migrate into the white part of the surimi or to a neighboring surimi in the same package, resulting in a less appealing look of the product.

During refrigerated storage under lighting, the color differences of white surimi gels stored in aluminum foil bag were not significantly different ($P>0.05$) during storage for 7 days. The increases in color difference of surimi gels without bacterioruberin were observed after stored in polypropylene bag for more than 5 days ($P<0.05$). In Pacific whiting surimi gel, the decrease in L* value was also found at 7 days of refrigerated storage (Pipatsattayanuwong *et al*., 1995). This was a result of protein decomposition and denaturation that led to the loss of translucency of protein (Ledward, 1992). For the surimi gels added with bacterioruberin at both levels, the slight increase in the color difference was found at about 2.6. The results were related to the study of Lauro *et al*. (2005) which showed that the color differences of surimi gels adding with carmine, paprika color, canthaxanthin, β-carotene, and annatto were in the ranges of 1.4-3.2 after 7 days storage.

Table 19. Color determinations of surimi gels added with bacterioruberin at 0, 100, and 200 mg/kg packed in either aluminium foil (AF) or polypropylene (PP) bags during storage under lighting (2,500 lux) at 4 °C

Storage time (d)	Package	L*	a*	b*	ΔE^*_{ab}
no bacterioruberin					
0		80.07 ± 0.17[a]	-2.29 ± 0.19[a]	5.69 ± 0.21[a]	
3	AF	79.69 ± 0.27[a]	-2.32 ± 0.13[a]	5.75 ± 0.04[a]	0.39 ± 0.26[c]
	PP	79.71 ± 0.23[a]	-2.31 ± 0.15[a]	5.75 ± 0.04[a]	0.37 ± 0.23[c]
5	AF	79.49 ± 0.13[a]	-2.28 ± 0.14[a]	5.65 ± 0.01[a]	0.59 ± 0.13[c]
	PP	77.42 ± 0.61[b]	-2.19 ± 0.14[a]	5.47 ± 0.04[b]	2.66 ± 0.61[b]
7	AF	79.52 ± 0.15[a]	-2.28 ± 0.11[a]	5.64 ± 0.01[a]	0.55 ± 0.14[c]
	PP	73.92 ± 1.04[b]	-2.12 ± 0.13[a]	5.27 ± 0.07[b]	6.17 ± 1.03[a]
Bacterioruberin (100 mg/kg)					
0		67.19 ± 0.62[b]	15.79 ± 0.32[a]	15.76 ± 0.32[a]	
3	AF	67.57 ± 0.20[b]	15.68 ± 0.14[a]	15.68 ± 0.15[a]	0.41 ± 0.19[d]
	PP	67.47 ± 0.19[b]	15.69 ± 0.15[a]	15.67 ± 0.14[a]	0.32 ± 0.17[b]
5	AF	66.80 ± 0.03[b]	15.69 ± 0.11[a]	15.67 ± 0.11[a]	0.41 ± 0.02[d]
	PP	69.71 ± 0.13[a]	15.24 ± 0.13[b]	15.22 ± 0.13[b]	2.60 ± 0.13[b]
7	AF	67.71 ± 0.04[b]	15.66 ± 0.11[a]	15.64 ± 0.11[a]	0.55 ± 0.04[c]
	PP	69.69 ± 0.18[a]	15.20 ± 0.14[b]	15.19 ± 0.14[b]	2.62 ± 0.17[a]
Bacterioruberin (200 mg/kg)					
0		51.95 ± 0.65[b]	31.73 ± 0.52[a]	23.55 ± 0.64[a]	
3	AF	52.15 ± 0.14[b]	31.59 ± 0.07[a]	23.41 ± 0.06[a]	0.31 ± 0.12[b]
	PP	52.10 ± 0.09[b]	31.54 ± 0.11[a]	23.40 ± 0.04[a]	0.32 ± 0.08[b]
5	AF	51.67 ± 0.02[b]	31.55 ± 0.01[a]	23.42 ± 0.01[a]	0.35 ± 0.01[b]
	PP	54.87 ± 0.13[a]	29.93 ± 0.10[b]	21.27 ± 0.09[b]	2.65 ± 0.14[a]
7	AF	51.66 ± 0.04[b]	31.54 ± 0.03[a]	23.41 ± 0.01[a]	0.37 ± 0.04[b]
	PP	54.90 ± 0.21[a]	27.00 ± 0.10[b]	20.28 ± 0.07[b]	2.68 ± 0.14[a]

Mean ± SD (standard deviation) from five determinations.

[a,b] Different letters in the same column at the same level of bacterioruberin denote significant differences ($P < 0.05$).

Table 20. Color bleeding of surimi gels added with bacterioruberin 200 mg/kg

Sample	L*	a*	b*	ΔE^*_{ab}
Surimi	79.70 ± 0.19	-2.30 ± 0.06	5.77 ± 0.04	0
Surimi+bacterioruberin 200 mg/kg	79.76 ± 0.34	-2.32 ± 0.03	5.78 ± 0.04	0.26

Mean ± SD (standard deviation) from five determinations.

Color difference (ΔE^*_{ab}) was compared with reference surimi gel.

Changes in lipid oxidation

The TBARS values of surimi gels added with and without bacterioruberin stored under lighting are shown in Table 21. At all levels of bacterioruberin added, no significant changes in TBARS values of surimi gels packed in aluminium foil bags were observed during 7 days of storages (P>0.05). Nevertheless, an increase in TBARS values was observed in surimi gels stored in polypropylene bag. The differences in TBARS values between surimi gels stored in aluminium foil and polypropylene bags indicated that the lipid oxidation was induced by light exposure. The lipid oxidation was rapidly increased in surimi gels without bacterioruberin followed by addition of bacterioruberin at 100 and 200 mg/kg, respectively. After storage for 7 days, the TBARS values of surimi gels added with bacterioruberin at 100 and 200 mg/kg decreased about 28.8 and 33.7% of that without bacterioruberin, respectively. The results suggest that the higher amount of bacterioruberin added the slower rate of lipid oxidation occurred. Generally, marine fish muscle typically has a high content of polyunsaturated fatty acids and is consequently prone to oxidative reaction (Stamman *et al*., 1990; Harris and Tall, 1994). Lipid oxidation in Som-fug produced from bigeye snapper was also reported by Riebroy *et al*. (2006). During storage under lighting, the light should induce the lipid oxidation of soybean oil added. Furthermore, the soybean oil added in surimi gel also caused the lipid oxidation under lighting storage. Soybean oil contains chlorophyll which is an excellent singlet oxygen sensitizer. Photosensitizers such as chlorophyll pheophytins, porphyrins, riboflavin, myoglobin, and synthetic colorants

Table 21. TBARS values of surimi gels added with bacterioruberin at 0, 100, and 200 mg/kg packed in aluminium foil (AF) or polypropylene (PP) bag during storage under lighting (2,500 lux) at 4 °C for 7 days (mg malondialdehyde/kg)

Bacterioruberin (mg/kg)	Package	Storage time (d)				
		0	1	3	5	7
0	AF	1.45 ± 0.05^{aA}	1.48 ± 0.07^{aB}	1.51 ± 0.08^{aC}	1.51 ± 0.09^{aC}	1.50 ± 0.08^{aC}
	PP	1.45 ± 0.05^{eA}	1.68 ± 0.08^{dA}	1.96 ± 0.06^{cA}	2.30 ± 0.03^{bA}	2.88 ± 0.09^{aA}
100	AF	1.40 ± 0.06^{aA}	1.43 ± 0.06^{aB}	1.49 ± 0.06^{aBC}	1.50 ± 0.08^{aC}	1.53 ± 0.05^{aC}
	PP	1.40 ± 0.05^{dA}	1.65 ± 0.05^{cA}	1.74 ± 0.05^{bcAB}	1.83 ± 0.06^{bB}	2.05 ± 0.08^{aAB}
200	AF	1.41 ± 0.07^{bA}	1.46 ± 0.07^{aB}	1.50 ± 0.06^{aC}	1.52 ± 0.05^{aC}	1.53 ± 0.05^{aC}
	PP	1.41 ± 0.09^{dA}	1.60 ± 0.05^{cA}	1.70 ± 0.04^{bB}	1.80 ± 0.06^{aB}	1.91 ± 0.06^{aB}

Mean ± SD (standard deviation) from three determinations.

^{a,b,c,d} Different letters in the same row denote significant differences as the effect as the effect of storage time ($P < 0.05$).

^{A,B,C} Different letters in the same column denote significant differences as the effect of packing and amount of bacterioruberin added ($P < 0.05$).

in foods can absorb energy from light and transfer it to triplet oxygen to form singlet oxygen (Foote and Denny, 1968; Lledias and Hansberg, 2000).

Retention of bacterioruberin

The residual contents of bacterioruberin in surimi gels during storage test are shown in Table 22. The bacterioruberin contents in surimi gels were about 95% of the initial levels added. The decrease might be due to the effect of heating during gel preparation. During storage in aluminium foil bag under lighting for 7 days, the bacterioruberin contents of surimi gels remained constant ($P>0.05$). In polypropylene bag, however, a slight decrease ($P<0.05$) in bacterioruberin content was found in surimi gels added with 100 and 200 mg/kg bacterioruberin after 3 and 5 days of storage, respectively. The decrease in bacterioruberin contents should be related to the retardation of lipid oxidation. As previously discussed, the lipid oxidation in the surimi gel was singlet oxygen oxidation. The quenching ability of singlet oxygen by carotenoids was well known.

Textural and microstructural properties

The breaking force and deformation of surimi gels were not significantly different during storage time in all treatments groups (Table 23). The average breaking force and deformation of surimi gels were 708.2 ± 2.1 and 12.3 ± 0.3, respectively. These values were related to the study of Benjakul *et al*. (2004) which soybean oil was added into surimi gels at 1%. The microstructure of surimi gels with bacterioruberin at 200 mg/kg storage at 4 °C for 7 days in both aluminium foil and polypropylene bags are shown in Figure 33. All surimi gels had a fine fibrous matrix with a well-organized three-dimensional network. The results confirmed that addition of bacterioruberin at both levels had no effect on gel-forming ability of surimi gels ($P>0.05$).

Table 22. Bacterioruberin content (mg/kg) of surimi gels added with bacterioruberin at 100 and 200 mg/kg packed in aluminium foil (AF) or polypropylene (PP) bag during storage under lighting (2,500 lux) at 4 °C for 7 days

Bacterioruberin (mg/kg)	Package	Storage time (d)				
		0	1	3	5	7
100	AF	95.33 ± 1.53^{aA}	93.33 ± 3.33^{aA}	92.17 ± 4.83^{aA}	91.05 ± 3.58^{aA}	92.67 ± 3.88^{aA}
	PP	95.33 ± 1.53^{aA}	92.67 ± 2.50^{aA}	87.83 ± 3.92^{bA}	85.50 ± 2.26^{bB}	86.50 ± 2.17^{bB}
200	AF	191.00 ± 3.46^{aA}	190.00 ± 6.45^{aA}	188.67 ± 5.47^{aA}	187.17 ± 4.36^{aA}	185.17 ± 6.18^{aA}
	PP	191.00 ± 3.46^{aA}	188.33 ± 4.13^{aA}	187.33 ± 8.55^{aA}	162.17 ± 6.59^{bB}	163.17 ± 9.70^{bB}

Mean ± SD (standard deviation) from three determinations.

^{a,b,c} Different letters in the same row denote significant differences ($P < 0.05$).

^{A,B} Different letters in the same column at the same concentration of bacterioruberin denote significant differences ($P < 0.05$).

Table 23. Breaking force and deformation of surimi gels added with bacterioruberin at 0, 100, and 200 mg/kg packed in aluminium foil (AF) or polypropylene (PP) bag during storage under lighting (2,500 lux) at 4 °C for 7 days

Bacterioruberin (mg/kg)	Package	Storage time (d)				
		0	1	3	5	7
Breaking force (g)						
0	AF	707.8 ± 3.8	708.2 ± 2.7	708.3 ± 3.5	708.2 ± 3.3	707.2 ± 3.4
	PP	707.8 ± 3.8	706.9 ± 2.7	707.9 ± 3.3	708.3 ± 2.9	708.3 ± 2.6
100	AF	709.4 ± 2.0	708.0 ± 2.0	708.0 ± 1.5	707.5 ± 1.2	708.1 ± 1.2
	PP	709.4 ± 2.0	708.5 ± 1.7	707.5 ± 1.5	708.7 ± 1.5	708.7 ± 1.5
200	AF	707.9 ± 1.7	708.9 ± 1.4	708.6 ± 1.3	708.6 ± 1.5	708.8 ± 1.6
	PP	707.9 ± 1.7	708.6 ± 1.7	709.4 ± 1.3	709.3 ± 1.8	708.7 ± 1.8
Deformation (mm)						
0	AF	12.13 ± 0.13	12.63 ± 0.15	12.24 ± 0.07	12.15 ± 0.19	12.03 ± 0.20
	PP	12.13 ± 0.13	12.63 ± 0.15	12.16 ± 0.12	12.09 ± 0.17	12.16 ± 0.26
100	AF	12.16 ± 0.08	12.64 ± 0.33	12.06 ± 0.33	12.12 ± 0.27	12.16 ± 0.33
	PP	12.16 ± 0.08	12.59 ± 0.32	12.27 ± 0.39	12.39 ± 0.21	12.31 ± 0.31
200	AF	12.26 ± 0.10	12.59 ± 0.38	12.35 ± 0.31	12.24 ± 0.39	12.30 ± 0.37
	PP	12.26 ± 0.10	12.89 ± 0.42	12.20 ± 0.24	12.13 ± 0.35	12.00 ± 0.28

Mean ± SD (standard deviation) from five determinations.

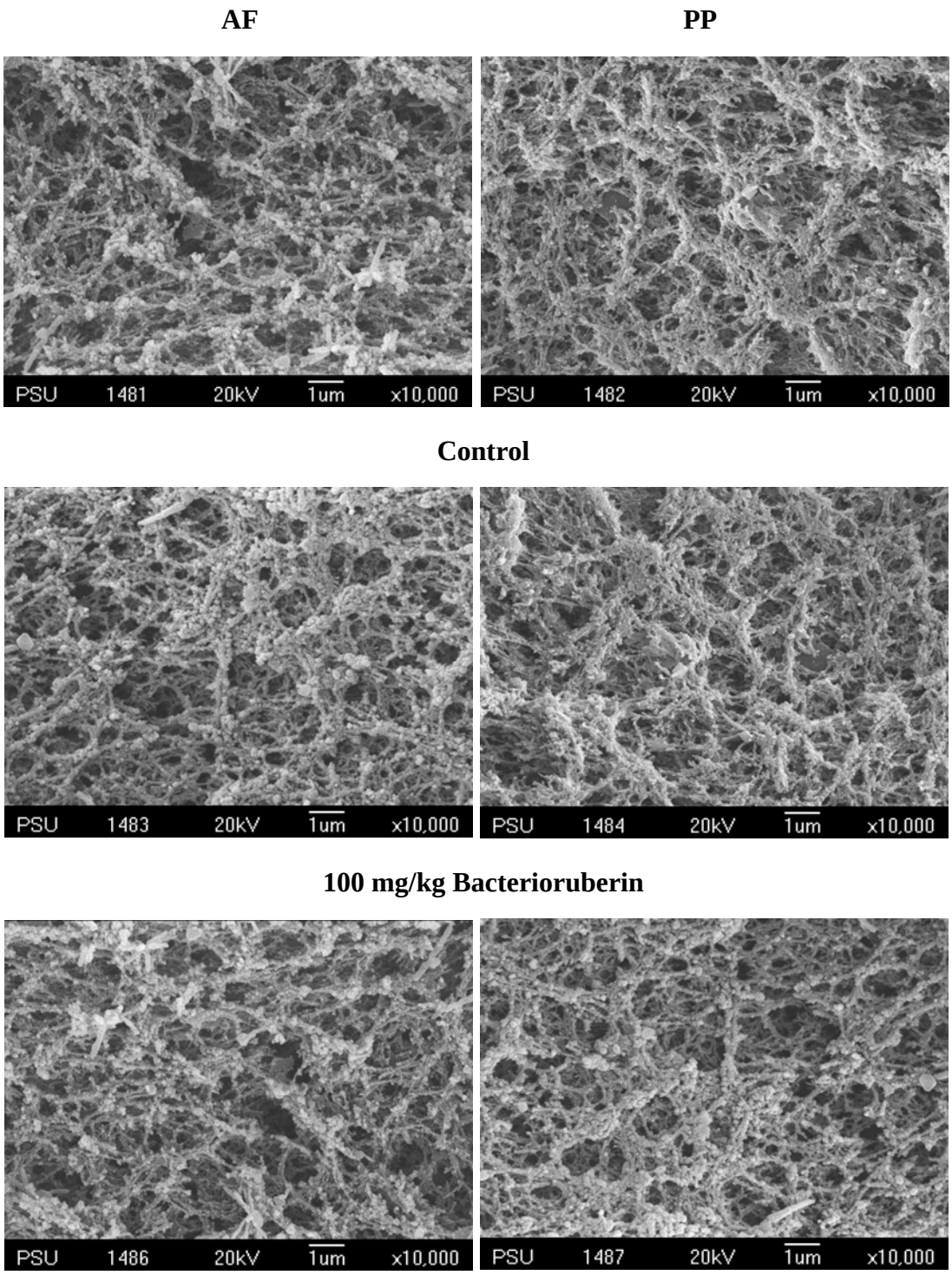

Figure 33. Microstructure of surimi gels added with bacterioruberin at 0, 100, and 200 mg/kg packed in aluminium foil (AF) or polypropylene (PP) bag during storage under lighting (2,500 lux) at 4 °C for 7 days.

5.5 Conclusion

Bacterioruberin exhibits good stability under heating and light exposure both in soybean oil and in surimi. During refrigerated storage, color of surimi gels added with bacterioruberin slightly changed (ΔE^*_{ab} ~2.7). In addition to color, bacterioruberin can prevent the lipid oxidation in surimi gels during storage. Bacterioruberin can be used as a colorant in surimi gel without effect on the gel-forming ability.

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CHAPTER 6

SUMMARY AND FUTURE WORKS

6.1 Summary

Out of 124 red extremely halophilic archaea isolated from salt fermented foods in Thailand, strain HM3 exhibited the highest carotenoid-producing ability followed by AS133, HM322, and HPC1-2, respectively. On the basis of their polar lipid compositions and growth characteristics, all isolates were assigned to the genus *Halobacterium*. Based on the 16S rDNA sequence similarity value and DNA-DNA relatedness value, AS133, HM3, and HM322 were a member of *Halobacterium salinarum*. Bacterioruberin was found to be the main carotenoid in all of the selected strains based on the absorption spectra and HPLC chromatogram. Under optimization using statistical approach (Plackett-Burman and Central composite design), meat extract and glucose were identified as important factors controlling the growth and carotenoid production. The carotenoid production was 9.24 mg/L which increase 1.5-fold.

On the basis of growth requirements, poor utilization of carbohydrates, antibiotic susceptibility, menaquinone content, overall phospholipid composition, DNA G+C contents and 16S rRNA gene sequence analysis, strains HPC1-2^T is considered to represent a single species of the genus *Halobacterium.* However, it was differentiated from recognized *Halobacterium* species based on levels of DNA-DNA relatedness and differences in whole-cell protein patterns. These results suggested that the strains HPC1-2^T represent a novel species of the genus, for which the name *Halobacterium piscisalsi* sp. nov. is proposed.

Bacterioruberin exhibited the highest DPPH and ABTS radical scavenging activity when compared to astaxanthin, β-carotene, lycopene, and lutein. The effective concentration for 50% scavenging (EC_{50}) of DPPH and ABTS were found at 3.88 and 15.80 mM, respectively. The reducing power (OD_{700}) of bacterioruberin was 3.27. Singlet oxygen quenching ability of bacterioruberin was

higher than other tested carotenoids, with the quenching rate constants (k_q) of 2.48×10^{10}, 1.18×10^{10}, 1.49×10^{10}, 2.15×10^{10} and 1.24×10^{10} M^{-1} s^{-1} for bacterioruberin, astaxanthin, β-carotene, lycopene, lutein, respectively. Bacterioruberin also exhibited protective effect on the plasmid DNA.

Bacterioruberin exhibits good stability under heating and light exposure both in soybean oil and in surimi. During refrigerated storage, color of surimi gels added with bacterioruberin slightly changed (ΔE*ab ~2.7). In addition to color, bacterioruberin can prevent the lipid oxidation in surimi gels during storage. Bacterioruberin can be used as a colorant in surimi gel without effect on the gel-forming ability.

6.2 Future works

1. Estimation of bacterioruberin accessibility from *Halobacterium salinarum* HM3 determined by an *in vitro* digestion method.
2. Cost evaluation for bacterioruberin production of *Halobacterium salinarum* HM

APPENDIX

CULTURE MEDIA AND IDENTIFICATION METHODS

1. Halophilic medium

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

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

2. Gram staining

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

3. Anaerobic growth in the presence of L-arginine

Anaerobic growth in the presence of L-arginine was determined by inoculating the archaea into on agar plates in the presence of L-arginine and incubated for 1-2 weeks. Cultures were incubated under an anaerobic condition at 37°C. Growth was determined by measuring culture turbidity at 600 nm. A positive reaction for hydrolysis L-arginine was also shown by a colour change of the indicator to red.

L-arginine agar medium

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

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

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

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

Nitrate broth

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

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

Nitrate reduction test reagent

Sulphanilic acid solution

Sulphanilic acid	0.8	g
5 N Acetic acid	100	mL

Dissolve by gentle heating in a fume hood.

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

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

Dissolve by gentle heating in a fume hood.

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

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

DMSO medium

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

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

6. Indole test

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

Tyrptone broth

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

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

Kovacs' reagent

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

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

7. Acid production test

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

Modified Leifson medium

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

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

8. Oxidase test

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

9. Catalase test

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

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

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

Casein agar medium

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

Skim milk 1% (w/v)

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

Gelatin agar medium

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

Gelatin 10% (w/v)

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

Starch agar medium

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

Starch	10%	(w/v)

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

Tween 80 agar medium

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

Tween 80	2	mL

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

Lugol's iodine

KI	40	g
Distilled water	100	mL

10% Trichloroacetic acid

Trichloroacetic acid (TCA)	5	g
Distilled water	100	mL

Add conc. TCA into the distilled water.

11. Antibiotics test

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

Antibiotic paper disc (6 mm Ø)		
Gentamicin	10	µg
Neomycin	30	µg
Rifampicin	30	µg
Nalidixic acid	30	µg
Choramphenicol	30	µg
Bacitracin	10	µg
Kanamycin	30	µg
Streptomycin	10	µg
Tetracycline	30	µg
Ampicilin	10	µg
Novobiocin	5 and 30	µg

12. Dried cells preparation

Dried cells of the selected strain was prepared by inoculating 250 µL of stock culture into 5 mL of halophilic medium (Appendix 1) and incubated at 37 °C in a shaker incubator at 200 rpm for 7 days. After that, inoculum was added at 5% (v/v) into halophilic liquid medium and incubated at 37 °C in a shaker incubator at 200 rpm for 7 days. Wet cell paste was obtained by centrifugation of cultured broth at 10,000 ×*g* at 4 °C for 10 min. The cells were washed twice with 25% (w/v) NaCl and centrifuged at 10,000 ×*g* (4°C) for 10 min. The obtained wet cell paste was freeze dried and kept at 4°C until used.

13. Quinones (Komagata and Suzuki, 1987)

Dried cells (100-500 mg) (Appendix 12) were extracted with chloroform: MeOH (2:1) overnight. The suspension was then filtered and dried under rotary evaporator. The dried sample was Dissolve with a small amount of acetone and applied onto a silica gel thin layer chromatography (TLC) (Merck no. 1.05744). The applied TLC was then developed by 100% benzene and the band of menaquinone was detected by using a UV lamp (254 nm). The menaquinone band was scraped and

Dissolve with HPLC acetone. The suspension was filtered and dried up with N_2 gas. The menaquinone sample was analyzed by HPLC.

14. Polar lipids

Dried cells (150-300 mg) (Appendix 12) were added with 3 mL of MeOH: 0.3% NaCl aq. (100:10) and 3 mL of petroleum ether. The solution was mixed for 15 min. The lower layer was added with 1 mL of petroleum ether and then the solution was mixed for 2-5 min. The lower layer was added with chloroform: MeOH: water (90:100:30) and mixed for 1 h. The upper layer was transferred into another tube. The lower layer was extracted again with chloroform: MeOH: water (50:100:40) and the supernatant was transferred to the upper layer tube. The upper layer tube was added with 1.3 mL of chloroform and water. The final lower layer was dried with N_2 gas (<37°C). The polar lipid fraction was Dissolve with 60 μL of chloroform: MeOH (2:1) and applied to 3 plates of two-dimensional silica high performance thin layer chromatography (HPTLC) no. 1.05633 and was developed with the following solvent systems.

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

Ninhydrin solution

Ninhydrin	0.5	g
1-Butanol saturated in water	100	mL

Dittmer and Lester reagent

Solution A

MoO_3	4.011	g
25 N H_2SO_4	100	mL

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

Solution B

Molybdenum powder	0.178	g
Solution A	50	mL

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

Note: Before spraying, mixed solution A (50 mL) plus solution B (50 mL) plus water (100 mL). The final solution was greenish yellow in color. If too little water was used it will be blue; if too much, yellow. The spray was stable for months.

Anisaldehyde reagent

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

Dragendorff's reagent

Solution A

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

Solution B

KI	40	g
Distilled water	100	mL

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

15. DNA base composition

Chromosomal DNA was isolated from cells grown in halophilic agar plate for 7 days according to the method of Tamaoka (1994) and Saito and Miura (1963). Cells were harvested and suspended in 10 mL of saline-EDTA buffer pH 8.0. The cell suspension was with 1.0 mL of 10% (w/v) SDS and incubated at 50°C for 10 min. The phenol extraction was then carried out by adding an equal volume of phenol: chloroform (1:1) to the sample to remove protein and other debris. The upper layer of the mixture was collected after centrifugation at 12,000 ×*g* for 20 min. Chromosomal DNA was precipitated with two volumes of ice cold absolute ethanol. DNA was Dissolve with 0.1× SSC and treated with RNase A, RNase T_1 and protease K solution at 37°C for 1 h to remove RNA and protein, respectively. Chromosomal DNA was stored in 0.1× SSC at 4°C.

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

Saline-EDTA buffer pH 8.0

NaCl	8.76	g
EDTA	37.22	g

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

10% (w/v) SDS

Sodium dodecyl sulphate	10	g
Distilled water	90	mL

Dissolve and make up to 100 mL with distilled water.

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

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

20× SSC

NaCl	17.5	g
Sodium citrate	8.8	g
Distilled water	950	mL

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

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

0.15 M NaCl

NaCl	0.84	g

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

RNase A solution

RNase A	20	mg
0.15 M NaCl	10	mL

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

0.1 M Tris-HCl (pH 7.5)

Tris(hydroxymethyl)aminomethane	1.21	g
Distilled water	90	mL

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

RNase T_1 solution

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

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

50 mM Tris-HCl (pH 7.5)

Tris(hydroxymethyl)aminomethane	605	mg
Distilled water	90	mL

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

Proteinase K

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

Use freshly prepared solution.

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

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

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

Nuclease P_1 solution

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

Dissolve and store at 4°C.

0.1 M Tris-HCl (pH 8.1)

Tris(hydroxymethyl)aminomethane	1.21	g
Distilled water	90	mL

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

Alkaline phosphatase solution

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

16. 16S rRNA analysis

16.1. 16S rRNA amplification by PCR

The PCR was performed in a total volume of 50 μL containing 1 μL of DNA sample, 0.25 μL of *Taq* DNA polymerase, 5 μL of 10× polymerase buffer, 4 μL of dNTP mixture, 2.5 μL of 10 μM forward and reverse primers of D30F (5'-ATTCCGGTTGATCCTGC-'3, positions 6 to 12 according to the *Escherichia coli* numbering system) and D56R (5'-CTTGTTACGACTT-'3, position 1492-1509), and 34.75 μL of deionized water. A DNA Thermal Cycler (Gene Amp® PCR System

2400; Perkin Elmer) was used with a temperature profile of 3 min at 95°C (denaturing of DNA), 15 s at 55°C (primer annealing) and 1 min at 72°C (polymerization) and a final extension for 5 min at 72°C. The PCR amplified products were analyzed by running 5 µL of the reaction mixture on a 1% agarose gel in Tris-acetate EDTA buffer. Agarose gel was stained in an ethidium bromide solution and examined under UV-transilluminator (UVP Inc.) to visualize the amplified 16S rRNA band.

16.2. 16S rRNA sequencing

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

16.3. 16S rRNA sequence analysis and phylogenetic tree construction

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

5× Tris-acetate EDTA (TAE) buffer

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

Dissolve and dilute 5× before used.

Agarose gel

Agarose	1.6	g
1× TAE buffer	100	mL

Dissolve and melt in microwave for 2-3 min.

17. DNA-DNA hybridization

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

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

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

0.1 M Tris-HCl (pH 9.0)

Tris(hydroxymethyl)aminomethane	1.21	g
Distilled water	90	mL

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

100× Denhardt solution

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

Dissolve and adjust to 100 mL with distilled water.

100 mM Tris-HCl (pH 7.6)

Tris(hydroxymethyl)aminomethanein	12.1	g
Distilled water	950	mL

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

10 mM Na_2-EDTA (pH 7.6)

Na_2-EDTA	0.47	g
Distilled water	950	mL

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

TE buffer

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

Make up to 1 L with distilled water.

10 mg/mL Salmon sperm DNA

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

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

Prehybridization solution

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

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

Hybridization solution

Prehybridization solution	100	mL
Dextran sulfate	5	g

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

Phosphate-buffer saline (PBS)

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

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

Solution I

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

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

Solution II

Streptavidin-POD	1	µL
Solution I	4	mL

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

Solution III

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

Solution III was freshly prepared.

2 M H_2SO_4

H_2SO_4	22	mL
Distilled water	178	mL

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

Ethidium bromide solution (10 mg/mL)

Ethidium bromide	1	g
Distilled water	100	mL

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

Gel loading buffer

Bromophenol blue	0.025	g
Distilled water	17	mL
Glycerol	3	mL

Dissolve and store in light-tight container in refrigerator.