# CHAPTER II

# LITERATURE REVIEW

## 2.1 Overview of fermented fish (pla-ra)

Pla-ra is the traditional fermented fish product that made from fresh water fish such as *Channa striata* (Striped snake head fish, Chon), *Trichogaster trichopterus* (Gourami, Kra-dee), *T. leeri* (Kradee- nang), *Cyclocheilichthys repasson* (Silver Carp, Soi), *Puntius gonionotus* (Barb, Ta-pien). (National Research Journal of Thailand, 1981-1982). Marine fish namely *Johnius argentatus* (Silver johnfish), *Rastrelliger neglectus* (Shortbodied mackerel), *Rachycentron canadus* (Cobia), and *Caranx leptolepis* (Slender trevally) were also used for making pla-ra and were famous among people who live in the north-eastern part of Thailand (Sangjindavong, 2005).

Pla-ra was classified into 2 types according to the ingredients. The product which was processed by adding roasted rice was called pla-ra Khao-kuo and the one which bran added was called pla-ra ram (Pooscreepab, 1996). The production of pla-ra in Thailand was 20,000 - 40,000 tons/year and the value of pla-ra was about 800 million baht/year but the value of pla-ra for exporting was more than 20 million baht/ year (Anonymous, 2000). Pla-ra was popular among the people who live in every region of Thailand especially in the northern and northeastern parts of Thailand. Pla-ra was popular among ASEAN countries such as Myanma, Laos, Vietnam and Cambodia. Pla-ra is an ingredient in Thai foods such as: Namprik Pla-ra, Som Tam Pla-ra, Kang Lao, Namya, Pla-ra Sub, and Pla-ra Song Kraung (Poosereepab, 1996; Yamprayoon and Sukkho, 1999).

### 2.1.1 Fermentation of pla-ra

According to Amano (1962), the fermentation of pla-ra involes the combined effect of fish and microbial enzymes supplied in the form of starter cultures on fish flesh and entrails with added salt.

The preparation methods vary from simple to complicated processes by using salt and raw material in appropriate ratios. Generally, freshwater fish are de-scaled, de-headed, eviscerated, and washed with trap water and then rapidly dried. The prepared fish is mixed with salt in a fish to salt ratio of 3-5:1 by weight and then left at ambient temperature for 12-24 hours before packing in an earthenware jar and letting it ferment for 1 month. Then, salted fish is added with roasted rice or rice bran in a salted fish to roasted rice or rice bran ratio of 4-5:1 by weight. It is put in earthenware jars and held at least 6-12 months.

The fermentation process depends on the enzyme from the fish gut and microorganisms. Both roasted rice and rice bran or roasted rice bran are use as carbon and nitrogen source for lactic acid bacteria (LAB). In addition, various enzymes, including the proteolytic enzyme amylase and lipase, produced from microorganism cause chemical changes in the product.

Various kinds of processed pla-ra product are developed for market. Pla-ra cube and pla-ra powder are two main products in the market. The processing steps are as follows. Pla-ra is processed by high heat treatment to soften the fish scales and then dried and ground as pla-ra powder or dried and formed as cubes for pla-ra cubes. Pla-ra powder is packed in glass bottles or plastic bags, whereas pla-ra cubes are wrapped individually and pack in laminated bags. Processed pla-ra can be used as one of the ingredients for many recipes. In addition, there is product development to add more value to pla-ra powder and pla-ra cubes by supplementing them with herbal ingredients such as ginger or roasted rice. Iodated pla-ra is one being studied to enhance the nutritional value of the product. The production process flowsheet for pla-ra is shown in Figure 2.1.

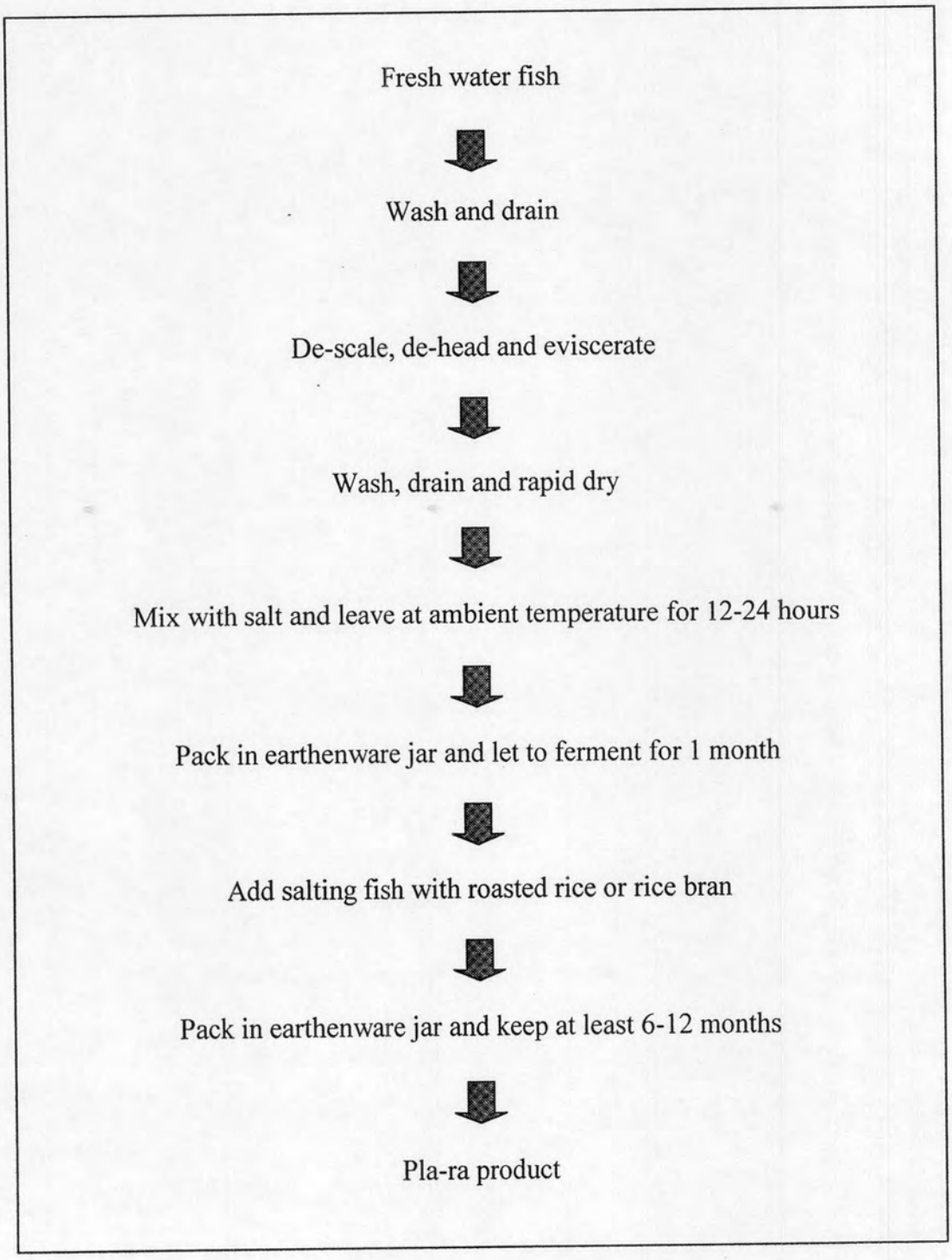


**Figure 2.1** Process of pla-ra fermentation (Krusong, 2004).

### 2.1.2 Nutrient compositions of pla-ra

This product is rich in various nutrient, particularly amino acids and peptides, and contains a high concentration of NaCl. The nutrient compositions of pla-ra product as pla-ra powder and pla-ra submerge are shown in Tables 2.1 and 2.2, respectively.

**Table 2.1** Nutrient compositions of pla-ra powder

| Nutrients | The composition per 100 grams edible portion |
|---|---|
| Energy (Kcal) | 147 |
| Water (g) | 52.5 |
| Protein (g) | 15.3 |
| Fat (g) | 8.0 |
| Carbohydrate (g) | 3.4 |
| Dietary fiber (g) | 0.5 |
| Ash (g) | 20.3 |
| Calcium (mg) | 22 |
| Phosphorous (mg) | 20 |
| Iron (mg) | 3.4 |
| Thaimin (mg) | 0.02 |
| Riboflavin (mg) | 0.16 |
| Niacin (mg) | 0.8 |

(Source: Nutritive Value of Thai foods Bangkok: Ministry of Public Health, 1992)

**Table 2.2** Nutrient compositions of pla-ra submerge (100g)

| Nutrient | Pla-ra | |
|---|---|---|
| | **Meat** | **Juice** |
| Carbohydrate (g) | 1.75 | - |
| Lipid (g) | 6 | 0.6 |
| Protein (g) | 14.15 | 3.2 |
| Energy (kcal) | 117.5 | 18 |
| Vitamin A (IU) | 195 | 2 |
| Vitamin B1(mg) | 0.02 | - |
| Vitamin B2(mg) | 0.16 | - |
| Niacin (mg) | 0.6 | - |
| Ca (mg) | 939.55 | - |
| P (mg) | 648.2 | 76.5 |
| Fe (mg) | 4.25 | 42.9 |

(Source: Faculty of Medicine, Khonkaen University, Thailand)

### 2.1.3 Microbiology of pla-ra

Pla-ra is rich in various nutrients, particularly amino acids and peptides, therefore, many kinds of microorganisms were isolated and acted as the protein degradation and provided a typical flavor to pla-ra. The strains of *Bacillus subtilis*, *B. licheniformis*, *Micrococcus* sp., *Pediococcus* sp., *P. halophilus*, *Staphlococcus* sp., *S. epidermidis*, *Lactobacillus acidipiscis*, *Weissella thailandensis* were found (Krusong, 2004; Tanasupawat et al., 2000). Otherwise, the product contained high concentration of NaCl, which allowed various halophilic bacteria to thrive (Lopetcharat et al., 2001). *Piscibacillus salipiscarius* and *Halobacterium piscisalsi* (Tanasupawat et al., 2007; Yacha., 2008). Prachasitthisak et al. (2005) has been investigated for the microbiological quality of 151 pla-ra were samples from sold in the market in 29 Thai provinces were halophilic bacteria ranged from $10^2$ to $10^7$ cfu/g and the total viable bacterial counts ranged 10-$10^7$ cfu/g as shown in Table 2.3.

**Table 2.3** Microbiological quality of pla-ra from 29 Thai provinces

| Microorganisms | Number of sample | Number of organisms per gram |
|---|---|---|
| Total bacterial count | 151 | $1.0 \times 10 - 7.3 \times 10^7$ |
| Halophilic bacteria | 151 | $3.0 \times 10^2 - 8.5 \times 10^7$ |
| Yeast and mold | 151(140) | $<10\text{-}8.5 \times 10^7$ |
| Coliforms | 151(151) | <3 |
| *E. coli* | 151(151) | <3 |
| *Salmonella* spp. | 151(151) | Absent* |
| *S. aureus* | 151(151) | <3 |
| *B. cereus* | 151(9) | <3-1,100 |
| *C. perfringens* | 151(145) | <3-3.6 |

(Source: Prachasitthisak et al., 2005)

## 2.2 Overview of halophilic Bacteria

Halophiles are "salt-loving" organisms that flourish in saline environments which distributed all over the world, in natural brines in arid, coastal and deep-sea locations, underground salt mines and artificial salterns. Although salts are essential for all life forms, halophiles are distinguished by their requirement of saline to hypersaline conditions for growth. To describe microorganisms according to their behavior toward salt, different classification schemes have been devised. Larsen (1962) proposed four groups of micro-organisms inhabiting saline environments: 1)non-halophiles, those which grow best in the medium containing <2% salt(NaCl), and 2)slight, 3)moderate, and 4)extreme halophiles as those which grow best in the medium containing 2-5%, 5-20%, and 20-30% salt(NaCl), respectively. Kushner (1993) expanded Larsen's (1962) definition, and proposed the classification of microorganisms response to salt(NaCl) in which they grow best. Five groups were defined: 1) non-halophilic microorganisms, <0.2 M (1% ) salt; 2) slight halophiles, 0.2-0.5 M (1-3%) salt; 3) moderate halophiles, 0.5-2.5 M (3-15%) salt; 4) borderline extreme halophiles, 1.5-4.0 M (9-23%) salt; and 5) extreme halophiles, 2.5- 5.2 M (15-32%) salt (Figure 2.2). In contrast, the halotolerant organisms grow best in media containing <0.2M (1%) salt and also can tolerate high salt concentrations. By the

way, extremely halotolerant organisms will be those whose optimum salt concentration for growth can reach 2.5M NaCl or more, and halotolerant those whose optimum growth rate is established below 1M NaCl. In fact, the unusual group capable of growing over a range of salt concentrations from zero to saturation, with optimum growth rate in the presence of salt, have been given the name of haloversatile (Horikoshi and Grant, 1998)(Table 2.4), or euryhaline (Vreeland, 1987) by other authors. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations with requirement or tolerance for salts sometimes dependent on nutritional factors and environmental.

**Table 2.4** Classification of microorganisms according to salt resistance

| Category | Salt concentration(M) | |
|---|---|---|
| | Range | Optimum |
| Nonhalophile | 0-0.1 | <0.2 |
| Slight halophile | 0.2-2-0 | 0.2-0.5 |
| Moderate halophile | 0.4-3.5 | 0.5-2.0 |
| Borderline halophile | 1.4-4 | 2.0-3.0 |
| Extreme halophile | 2.0-5-2 | >3.0 |
| Halotolerant | 0->1.0 | <0.2 |
| Haloversatile | 0->3.0 | 0.3-0.5 |

(Source: Kushner, 1993)

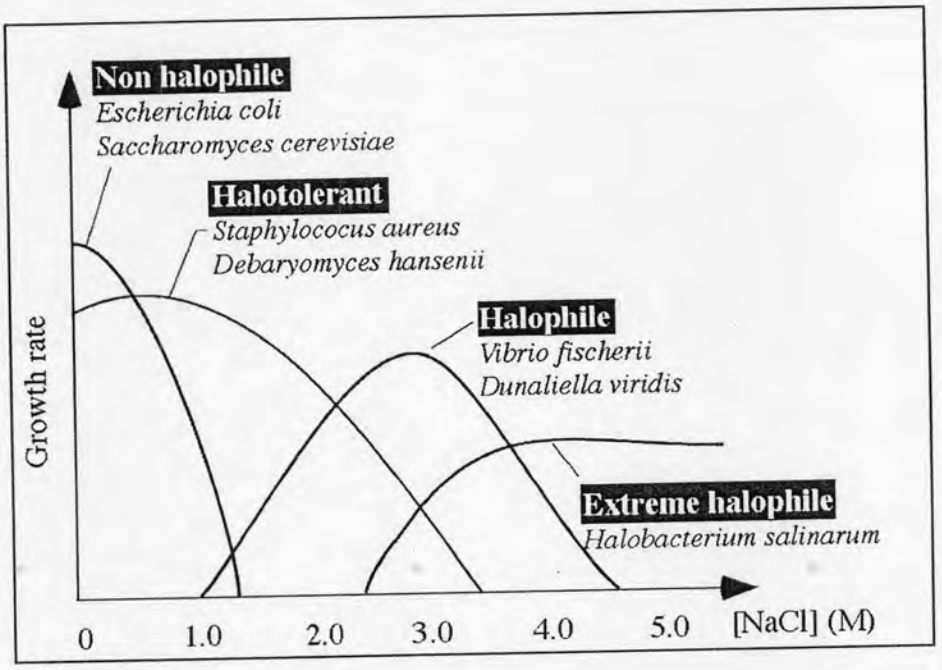


**Figure 2.2** Scheme representing growth rate patterns of salt resistance categories (Horikoshi and Grant, 1998).

### 2.2.1 Physiology of halophilic bacteria

Halophilic bacteria used amino acids as energy source and required a number of growth factors mainly vitamins. Electron transport chains containing cytochromes a, b and c were presented in halophilic bacteria and energy was conserved during aerobic growth via a proton motive force (Ventosa, Nieto and Oren 1998). Some strains of halophilic bacteria could grow in anaerobic condition.

Halophilic bacteria could withstand the osmotic forces that accompanied to their life in a high solute environment by accumulating organic compounds intracellulary that referred as compatible solutes (Margesin and Schinner, 2001). These compounds counteracted the tendency of the cell to become dehydrated under conditions of high osmotic strength by placing the cell in positive water balance with its surroundings and can be accumulated compatible solutes at high concentrations without interfering with cellular metabolism (Moral, Severin, Romos-Cormenzana, Truper and Galinski, 1994). Halophilic bacteria thrive in high osmolarity environment and excrete compatible solutes from their cytoplasm or uptake from the medium to

achieve osmotic balance (Robert, 2000). In the case of *Halobacterium*, cells pumped large amounts of $K^+$ from the environment into the cell. The concentration of $K^+$ inside the cell was considerably greater than that the concentration of $Na^+$ outside the cell. Therefore, *Halobacterium* used an inorganic ion as a compatible solute and remained in positive water balance (Moral et al., 1994).

Cell wall of halophilic bacteria was stabilized by sodium ions. In low sodium environment, the cell wall of extremely halophilic bacteria was broken down. Extremely halophilic bacteria was similar to gram negative bacteria but cell wall was quite different. Sodium ion ($Na^+$) bound to outer surface of cell wall that was absolutely essential for maintaining cellular integrity. When insufficient $Na^+$ was presented, the cell wall was broken apart and the cell was lysed. Although extremely halophilic bacteria did not contain peptidogycan in the cell wall, the cell wall was composed of glycoprotein. The negative charges contributed by the carboxyl groups of amino acids in the cell wall. Glycoprotein was shielded by $Na^+$. When $Na^+$ was diluted, the negatively charged regions of the proteins actively repel each other and cell lysed (Margesin and Schinner, 2001). Cytoplasmic protein of halophilic bacteria contained very low levels of hydrophobic amino acids which probably represented an evolutionary adaptation to highly ionic cytoplasm of halophilic. The ribosomes of halophilic bacteria also required high $K^+$ levels for stability while ribosomes of nonhalophiles required no $K^+$. It appeared that the halophilic bacteria was highly adapted, both internally and externally, to life in highly ionic environment (Ventosa et al., 1998). Cellular component of *Halobacterium salinarium* exposing to the external environment required high $Na^+$ for stability while internal components required $K^+$ (Matheson, Sprott, McDonald and Tessier, 1976). Lipid membrane of halophilic bacteria had a low $H^+$ and $Na^+$ permeability at high salt concentration. Vossenberg et al. (1999) show that pH and the salt concentration influenced on the proton and sodium ion permeability in lipid membrane of the halophiles. The proton permeability in lipid membrane of halophlies was independent of the salt concentration and was essentially constant between pH 7.0-9.0 Thus, the membranes of halophiles were stable over a wide range of salt concentrations, slightly alkaline pH and were well adapted to halophilic conditions.

### 2.2.3 Habitat of halophilic bacteria

Halophilic bacteria can thrive in hypersaline environments. Life is represented in both athalassohaline and thalassohaline conditions. Thalassohaline environments contain sodium and chloride ions as the predominant ions. While athalassohaline environments are potassium, magnesium and sodium. One characteristic shared by athalassohaline and thalassohaline environments is pH which usually near neutral to slightly alkaline (Litchfield and Gillevet, 2002).

Dead Sea is the athalassohaline ecosystem. There are reports on the characterization of members of archaea and bacteria (Oren, Gurevich, Gemmell and Teske, 1995). Several halophilic bacteria are found as follows: *Halobacterum* spp., *Halococcus* spp., *Halobaculum gomorrense*, *Haloarcula marismortui*, *Haloarcula vallismortis*, *Haloferax volcanii*, *Flavobacterium*, *Pseudomonas*, *Chromobacterium*, *Halomonas* and *Bacillus*, particularly *B. marismortui*. (Chookietwattana, 2003). A novel species of the genus *Halorubrum* was isolated in the Atacama Saltern. Many microorganisms were found on thalassohaline systems. A new genus belonging to the archaea domain, *Halogeometricum*, was isolated and characterized by Montalvo-rodriguez et al. (1998). Several new species belonging to the genera *Haloterrigena*, *Haloferax* and *Haloarcula* have been also reported (Asker and Ohta, 2002). In addition, anovel genus belonging to bacteria domain can be found in this environment. The genus *Thermohalobacter* was isolated from a saltern (Cayol et al., 2000). A new genus of halophilic bacteria, *Salinibacter*, was isolated and characterized (Anton et al., 2002) from a similar environment.

Several halophiles are often found on salted food, such as salted fish, meat and other food products. Villar, Ruiz-Holgado and Sanchez (1985) found that *Pediococcus halophilus* was a dominant bacterium at the end of the curing process of anchovies. While *Halomonas salina* was isolated from fully cured wet and dry bachalao (dried salted codfish) that contains about 19% salt (Vihelmsson, Hafsteinwsson and Kristjansson 1996). *Pseudomonas beijerinckii* and *Halomonas halodenitrificans* were isolated from salted beans preserved in brine and meat curing brines, respectively. Moreover, *Halobacterium* sp., *Halococcus* sp., *Halobacillus* sp. SR5-3, *Filobacillus* sp. RF2-5, *Lentibacillus salicampi*, *Lentibacillus halophilus* sp.nov., and *L. juripiscarius* sp. nov were isolated from Thai fish sauce (Thongthai

and Suntinanalert, 1991; Hiraga et al., 2005; Namwong et al., 2005, 2006;Tanasupawat et al., 2006).

**2.2.4 Systematics of halophilic bacteria**

There are several problems associated with the accuracy of conventional method for halophilic bacterium systematics (Kushner, 1993). First, the diverse physiology of halophilic bacteria is by no means constant because it is affected by salt concentration. The salt requirement and tolerance properties of the bacteria are highly variable and may vary according to the growth temperature and the nature of the nutrients available (Ventosa et al., 1998b). Halophilic bacteria produce a variety of colonial characteristics from pigmented to non-pigmented according to the salt concentration of the media. Second, halophilic bacteria do not grow fast especially the group of extremely halophilic bacteria. Many of them need natural brines and a variety of nutrients such as pressed fish juices and milk for their growth, as well as yeast extract to support their growth. Third, some lots of peptone of the Difco brand, a common constituent in many biochemical test media, inhibited the growth of halophilic archaeobacteria while the Oxoid brand did not (Vreeland, 1993b). This was shown to be due to the high concentration of bile salts in the Difco Bacto-peptone, compounds to which halophilic archaeobacteria were especially sensitive.

Therefore, a polythetic view involving the combination of conventional and modern bacterial systematics is needed for halophilic bacterial systematics. Whenever the chemical data and/or the molecular data disagreed with taxonomic clusters produced by phenotypic means, preference should be provisionally given to the latter clusters until further research resolves the discrepancy (Vreeland, 1993b).

During the last two decades, there have been many attempts to provide a firm systematics base for halophilic bacteria. All of this work used different phenotypic feature tests and analytical systems to cluster the organisms. A successful taxonomy for halophiles has not been developed. Basically, the optimum growth conditions for all of the halophilic bacterial strains under study needed to be established prior to characterization steps. Unfortunately, this taxonomic methodology for the study of halophilic bacterial diversity is very time consuming and costly. Vreeland (1993b) recommended the test methodology for halophilic bacteria in that all tests and test media must be modified by the addition of salts and/or increasing

incubation times to account for the requirements of halophiles (this reference book also provides more details on test methodology for halophiles). Presently, the determination of phenotypic and chemotaxonmic characterization along with 16S rRNA gene sequences are commonly used for halophilic bacterial systematics (Stan-lotter et al., 2002; and Yoon et al., 2003).

Historically, the taxonomy of halophilic bacteria was based upon a few phenotypic characteristics with little attention given to either biochemistry or phylogenic of the organisms (Vreeland, 1993b). Once nucleic acid techniques based on 16S rRNA were developed, it was revealed that the extremely halophilic archaeobacteria and the halophilic eubacteria have a different phylogenetic branch. Most extreme halophiles are archaeobacteria while the moderate and slight halophiles are members of archaeobacteria and eubacteria.

Moderately and extremely halophilic bacteria are the most important group in hypersaline habitats, and receive much attention from microbiologists. There are few studies on slightly halophilic bacteria since the earlier studies have concentrated on particular habitats such as the Great Salt Lake, Dead Sea, Wadi Natrun, Lake Magadi, and solar salterns. These habitats have one or more harsh environmental conditions such as high salinity, high temperature, low oxygen availability, high nutrient availability, high light intensity, and extremely alkalinity. Only moderately and extremely halophilic bacteria survive and play a major ecological role. The slightly halophilic bacteria constitute a low proportion of the total microbial population (Rodriguez-Valera, 1988) in such habitats. In addition, slightly halophilic bacteria are similar to bacteria present in common environments making them uninteresting for scientific studies when compared to moderately and extremely halophilic bacteria. Due to the scarcity of information concerning the taxonomy of slightly halophilic bacteria, only the revision on the taxonomy of extremely and moderately halophilic bacteria is available.

#### 2.2.4.1 Extremely halophilic bacteria

The extremely halophilic bacteria (halobacteria) are members of the class *Halobacteria* (order *Halobacteriales* and family *Halobacteriaceae*) (Grant et al., 2001). They are rods, coccus or a multitude of involution forms from disks to triangle. They require at least 1.5 M (~9%) NaCl for growth and lack muramic acid-

containing peptidoglycan in the cell envelope. Their colonies are various shades of red due to the presence of $C_{50}$ carotenoids (bacterioruberins). Their intracellular enzymes have a requirement for high levels of KCl, over 3M and up to 5M. Their cytoplasmic membrane is composed of phytanyl ether lipids. They are insensitive towards many antibiotics and occur in hypersaline habitats such as salt lakes, soda lakes, and salterns. The family *Halobacteriaceae* consists of 14 genera: *Haloarcula*, *Halobacterium*, *Halobaculum*, *Halococcus*, *Haloferax*, *Halogeometricum*, *Halorubrum*, *Haloterrigena*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronomonas*, and *Natronorubrum*.

**2.2.4.2 Moderately halophilic bacteria**

Moderately halophilic bacteria are bacteria that require at least 0.5 M (~3%) NaCl for growth. They constitute very heterogeneous groups. In general, most halophiles within the bacteria are moderate rather than extreme halophiles (Oren, 2002). Moderately halophilic eubacteria are both heterotrophs and phototrophs. The heterotrophs include gram-negative and gram-positive moderate halophiles. Gram-negative species of moderately halophilic bacteria are *Deleya halophila*, *Desulfohalobium retbaense*, *Desulfovibrio halophilus*, *Flavobacterium halmephilum*, *Haloanaerobacter chitinovorans*, *Haloanaerobacter saccharolytica*, *Haloanaerobium praevalens*, *Halobacteroides halobius*, *Halomonas halodenitrificans*, *Halomonas halodurans*, *Halomonas elongata*, *Halomonas eurihalina*, *Halomonas subglaciescola*, *Paracoccus halodenitrificans*, *Pseudomonas beijerinckii*, *Pseudomonas halophila*, *Sporohalobacter lortetii*, *Sporohalobacter marismortui*, *Spirochaeta halophila,* and *Salinivibrio costicola*. Species of gram-positive moderate halophiles are *Micrococcus halobius*, *Sporosarcina halophila*, *Marinococcus halobius,* and *Marinococcus albus*. Phototrophs include *Ectothiorhodospira vacuolata*, *Rhodospirillum salexigens*, and *Rhodospirillum salinarum* (Ventosa, 1989).

All the members of moderately halophilic eubacteria and halotolerant, contain chemically peptidoglycans comprising peptides (short amino acids chains) and glycans, N-acetyl(glycolyl)muramic acid (NAM or M) and N-acetylglucosamine (NAG or G). Some of the amino acids are only found in cell walls, but not in other cellular proteins i.e., D- amino acids, e.g D-alanine and diaminopimelic acid, DAP. The family *Bacillaceae* contain *meso*-diaminopimelic acid

as the diagnostic cell wall peptidoglycan. The family *Halomonaceae* is the presence of the hydrophobic amino acid leucine, which is expected to add to the overall hydrophobicity of the cell wall.

Polar lipids of halophilic bacteria are glycerol bound to fatty acids with ester links. The lipid side chains are fatty acids i.e., Saturated fatty acid ($C_{13:0}$, $C_{14:0}$, $C_{15:0}$ and $C_{16:0}$) and Unsaturated fatty acids (iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{16:0}$). The major polar lipids present in most species are phosphoglycerol (PG) and diphosphoglycerol (DPG). Additional types of lipids may occur such as are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), diphosphatidylglycerol (cardiolipin, CL) and glycolipids. Generally the content of negatively charged phospholipids (PC, CL) increases at the expense of neutral phospholipids (PE) as salinity increases (Vreeland et al., 1992).

**Gram-positive moderate halophiles**

**Genus *Virgibacillus*.** The genus *Virgibacillus* was firstly proposed by Heyndrickx et al. (1998). It was originally assigned to the genus *Bacillus* named *Bacillus pantothenticus* based on nutritional analysis (Proom and Knight, 1950). From the polyphasic study by Heyndrickx et al. (1998) including amplified rDNA restriction analysis (ARDRA), fatty acid profile, SDS-PAGE whole-cell protein and phenotypic characteristic show that the *B. pantothenticus* is distinct from other *Bacillus* species to warrant the status of a separate genus for which they had proposed the name *Virgibacillus*. Otherwise, this genus could be distinguished from members of *Bacillus* rRNA group aerobic endospore-forming bacteria, such as *Halobacillus*, *Paenibacillus*, *Brevibacillus* and *Aneurinbacillus*.

Members of the genus *Virgibacillus* were gram positive rods (0.3-0.7 x 2.0-6.0 μm). Cells arrangement occurred singly, pair, short or long chain. Colony was small, circular, low convex and slightly transparent to opaque. This genus was catalase-positive, motile and produced endospores. Spores were usually spherical to ellipsoidal and located at terminal or subterminal position. It usually hydrolyzed gelatin, aesculin and casein. Moreover, it could produce acid from a variety of carbohydrate, depending on species. Growth of genus *Virgibacillus* was noticed between 15°C and 50°C with the optimum temperature at about 28°C or 37°C. Growth of *Virgibacillus* was stimulated by 4-10% NaCl. The G + C in DNA of

*Virgibacillus* was 36-43 mol%, which was less than *Bacillus* (32-69% mol) (Heyrman et al., 2003). Cell wall peptidoglycan of *Virgibacillus* contained *meso*-diaminopimelic acid type.

At the present, members of the genus *Virgibacillus* comprised 14 species including as *V. pantothenticus* (Heyndrickx et al., 1998), *V. proomii* (Heyndrickx et al., 1999), *V. salexigens*, *V. carmonensis*, *V. necropolis*, *V. marismortui* (Heyrman et al., 2003), *V. halodenitrificans* (Yoon et al., 2004), *V. dokdonensis* (Yoon, Kang, Lee, Lee, and Oh, 2005), *V. koreensis* (Lee, Lim, Lee, Park and Kim, 2006), *V. olivae* (Quesada et al, 2007), *V. halophilus* (An et al., 2007), *V. chiguensis* (Wang, et al., 2008), *V. kekensis* (Chen et al., 2008c) and *V. salarius* (Hua et al., 2008). The differential characteristic of *Virgibacillus* species show in Table 2.5.

**Genus *Gracilibacillus*.** The genus *Gracilibacillus* was established by Waino et al. (1999). Member of this genus are Gram-positive, motile, endospore-forming rods or filaments, with menaquinone 7 (MK-7) as the predominant respiratory quinine. Growth of genus *Gracilibacillus* was noticed between 4°C and 50°C with the optimum temperature at about 30°C or 37°C. From the salt requirement, the member of these genus were described as halophilic and halotolerant. The G + C in DNA of *Gracilibacillus* were 37- 40% mol. Cell wall peptidoglycan contained *meso*-diaminopimelic acid type. At the time of writing, there are eight recognized species, *Gracilibacillus dipsosauri* (Lawson et al., 1996; Wainø et al., 1999) *Gracilibacillus halotolerans* (Wainø et al., 1999.) *Gracilibacillus orientalis* (Carrasco et al., 2006.) *Gracilibacillus boraciitolerans* (Ahmed et al., 2007) *Gracilibacillus lacisalsi* (Jeon et al. 2008.) *Gracilibacillus halophilus* (Chen et al., 2008a.) *Gracilibacillus quinghaiensis* (Chen et al., 2008b.) *Gracilibacillus saliphilus* (Tang et al., 2008.) The differential characteristic of *Gracilibacillus* species show in Table 2.6.

**Table 2.5** Characteristics of species belonging to the genus *Virgibacillus*

Strain 1, *V. carmonensis;; 2, V. chiguensis; 3, V. dokdonensis; 4, V. halodenitrificans; 5, V. halophilus; 6, V. kekensis; 7, V. koreensis; 8, V. marismortui; 9, V. necropolis; 10, V. olivae; 11, V. pantothenticus; 12, V. proomii; 13, V. salaries; 14, V. salexigens* (Lee, Lim, Lee, Park and Kim, 2006; Hua et al., 2008).

| Strain | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spore shape | E,S | E,S | E,S | E | E | E,S | E | E | E | E | E,S | E,S | E,S | E |
| Spore position | ST | T,ST | T,ST | T,ST | ST | T | T | T,ST | C,T,ST | T,ST | T,ST | T,ST | ST | C,T,ST |
| Anaerobic growth | - | + | + | + | - | - | + | - | - | - | + | + | - | + |
| Temp. range | 10-40 | 15-55 | 15-50 | 10-45 | 5-45 | 10-50 | 10-45 | 15-50 | 10-40 | 20-45 | 15-50 | 15-50 | 10-50 | 15-50 |
| Growth at/in: | | | | | | | | | | | | | | |
| 0 % NaCl | - | + | + | - | + | + | - | - | W | + | - | - | - | - |
| 20% NaCl | - | + | + | + | - | + | + | + | - | + | + | - | + | + |
| pH 10 | ND | - | - | - | + | + | - | - | ND | - | - | - | + | + |
| Hydrolysis of: | | | | | | | | | | | | | | |
| Starch | ND | - | + | - | ND | + | ND | - | ND | + | ND | | - | - |
| Casein | + | + | + | + | ND | - | ND | + | + | + | + | + | + | + |
| Gelatin | - | + | + | + | + | - | - | + | W | + | + | V | W | + |
| Aesculin | W | + | + | - | ND | - | + | + | - | + | + | + | + | + |
| Nitrate reduction | + | + | - | + | + | + | - | + | + | + | V | - | - | + |
| Acid from: | | | | | | | | | | | | | | |
| D-Glucose | - | + | + | + | + | + | W | + | W | - | - | + | + | W |
| myo-Inositol | - | ND | + | - | - | - | - | - | - | - | - | | ND | - |
| D-Fructose | - | + | + | + | + | - | + | + | W | + | - | + | + | W |
| D-Galactose | - | + | + | + | W | - | - | - | - | - | - | + | - | W |
| D-Mannose | - | + | + | + | + | - | - | + | W | - | - | + | + | - |
| L-Rhamnose | - | ND | - | - | - | - | - | - | - | - | + | V | - | - |
| Major polar lipid | DPG, PG | DPG, PG, PE,PLs | DPG, PG, PE,PLs | DPG, PG, PLs | ND | DPG, PG, PLs | DPG, PG, PLs | DPG, PG, PE,PLs | DPG, PG, PLs | ND | DPG, PG, PE,PLs | DPG, PG, PE,PL | DPG, PG, PE | DPG, PG, PLs |
| DNA G+C content (mol%) | 38.9 | 37.3 | 36.7 | 38 | 42.6 | 41.8 | 41 | 39-42.8 | 37 | 33.4 | 36.9-38.3 | 36.8-37 | 37.3 | 36-40 |

+, positive; - negative; ND, no data; NG, no growth

**Table 2.6.** Characteristics of species belonging to the genus *Gracilibacillus*
Strains: 1, *G. boraciitolerans*(Ahmed et al., 2007); 2, *G. dipsosauri* (Lawson et al., 1996; Wainø et al., 1999); 3, *G. halophilus*(Chen et al. , 2008a); 4, *G. halotolerans*; 5, *G. lacisalsi*(Jeon et al., 2008); 6, *G. orientalis*(Carrasco et al., 2006); 7, *G. quinghaiensis* (Chen et al. 2008b); 8, *G. saliphilus*(Tang et al., 2008).

| Characteristic | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| Colony color | | Pink | White | Creamy white | Creamy white | Creamy | Creamy | Creamy white to pink | Creamy white |
| Spore shape | | S,E | S | E | E | S | S | S | S |
| Anaerobic growth | | - | + | - | - | - | - | - | FA |
| NaCl range (%, w/v) | | 0-11 | 0-15 | 7-30 | 0-20 | 0.5-18 | 3-20 | 0.5-8 | 1-22 |
| Optimum growth in NaCl (%) | | 0.5-3 | 3 | 15 | 0 | 5-7 | 10 | 1-3 | 10-15 |
| Temperature range (°C) | | 16-37 | 28-50 | 28-60 | 6-50 | 15-50 | 4-45 | 4-45 | 4-45 |
| Optimum temperature (°C) | | 25-28 | 45 | 45-50 | 47 | 40 | 37 | 37 | 28-37 |
| pH range | | 6-10 | | 6-9 | 5-10 | 5.5-10 | 5-9 | 6-8.5 | 6-8 |
| Optimum pH | | 7.5-8.5 | 7.5 | 7 | 7.5 | 7.5-8 | 7.5 | 7-7.5 | 7 |
| Nitrate reduction | | - | + | + | + | + | - | + | + |
| $H_2S$ production | | - | - | ND | + | ND | - | - | - |
| Voges-Proskauer reaction | | + | - | - | - | ND | ND | - | + |
| Hydrolysis of: Aesculin | Aesculin | + | + | + | + | | + | + | + |
| Casein | | - | - | - | - | + | - | - | - |
| Gelatin | | - | + | + | + | ND | + | - | ND |
| Starch | | - | + | + | + | + | + | - | + |
| Urea | | - | - | - | + | + | - | + | + |
| Major polar lipid | | DPG,PG | DPG,PG | DPG,PG | DPG,PG | DPG,PG | DPG,PG,PE,PL,APL | DPG,PG | DPG,PG,PE,PL,APL,GL |
| DNA G+C content (mol%) | | 35.8 | 39.4 | 42.3 | 38 | 38.8-39 | 37.1 | 40.9 | 40.1 |

+, positive; - negative; ND, no data; NG, no growth

**Genus *Halobacillus*.** The genus *Halobacillus* was created by Spring et al. (1996) through the reclassification of *Sporosarcina halophila* as *Halobacillus halophilus* and the description of two novel species, *Halobacillus litoralis* and *Halobacillus trueperi*. They are Gram-positive, spore-forming, rod-shaped or spherical to oval cells. Motile by means of flagella. Endospores resist heating at 75°C for at least 10 min. Moderately halophilic, strictly aerobic, and chemoorganotrophic. Catalase and oxidase are produced; DNase positive and urease negative. Nitrate is not reduced to nitrite. The Voges-Proskauer reaction is negative. The G+C content of the DNA ranges from 40 to 43 mol% (as determined by the thermal denaturation method).

The genus *Halobacillus* can be differentiated clearly from other related genera based on the cell-wall peptidoglycan type based on L-Orn–D-Asp (Spring et al., 1996; Shida et al., 1997; Yoon et al., 2001), with the exception of that for *H. campisalis* and *H. seohaensis* , which is based on *meso*-diaminopimelic acid.

At the time of writing, the genus comprises 16 species with validly published names, with the addition of *Halobacillus salinus* (Yoon et al., 2003), *H. karajensis* (Amoozegar et al., 2003), *H. locisalis* (Yoon et al., 2004), *H. yeomjeoni* (Yoon et al., 2005), *H. dabanensis* and *H. aidingensis* (Liu et al., 2005), *H. profundi* and *H.kuroshimensis* (Hua et al., 2007), *H. campisalis* (Yoon et al.,2007) , *H. faecis* (An et al., 2007), *H. mangrovi* (Soto-Ramirez et al., 2008), *H. seohaensis* (Yoon et al., 2008) and *H. alkaliphilus* (Cao et al., 2008).

**Table 2.7** Characteristics of species belonging to the genus *Halobacillus*
Strain 1, *H. aidingensis*(Liu et al., 2005); 2, *H. alkaliphilus*(Cao et al., 2008); 3, *H. campisalis*(Yoon et al.,2007); 4, *H. dabanensis*(Hua et al., 2007); 5, *H. faecis*(An et al., 2007); 6, *H. halophilus*; 7, *H. karajensis*(Amoozegar et al., 2003); 8, *H. kuroshimensis*(Hua et al., 2007).

| **Characteristic** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
|---|---|---|---|---|---|---|---|---|
| Cell morphology | Rods | Cocci | Cocci or oval-shaped | Rods | Rods | Cocci or oval-shaped | Rods | Rods |
| Flagellation | Peritrichous | ND | Peritrichous | Peritrichous | Absent | Single or peritrichous | Absent | Absent |
| Spore shape/position | E/C,ST | S | S/C | E/C,ST | E,S/C | S/C | E,S/C,ST | E,S/C, |
| Colony colour | Orange | Pale Orange | Light yellow | Cream to orange | Orange | Orange | Cream or white | Yellow–orange |
| Maximum temp for growth (°C) | 40 | 45 | 41 | 50 | 45 | 40 | 49 | 48 |
| Growth at: | | | | | | | | |
| 4 °C | - | - | + | - | - | - | - | - |
| pH 5.0 | - | - | - | + | - | - | - | - |
| pH 5.5 | - | - | + | + | + | - | - | + |
| 0.5% NaCl | + | + | + | + | + | - | - | + |
| 25% NaCl | - | - | - | + | - | - | - | + |
| Hydrolysis of: | | | | | | | | |
| Aesculin | - | ND | + | - | ND | - | + | + |
| Casein | + | ND | + | + | ND | + | + | + |
| Gelatin | + | ND | - | - | + | + | + | + |
| Starch | - | ND | + | + | ND | + | + | + |
| Tween 80 | + | ND | - | - | ND | - | - | + |
| Acid production from | | | | | | | | |
| D-Fructose | + | + | + | + | + | - | + | + |
| D-Galactose | - | - | + | - | + | - | - | - |
| Maltose | + | + | - | + | + | - | + | + |
| Sucrose | + | + | + | + | + | - | - | + |
| D-Xylose | - | + | - | + | - | - | - | - |
| Cell-wall type | L-Orn-D-Asp | L-Orn-D-Asp | DAP | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp |
| DNA G+C | 42.2 | 43.5 | 42.1 | 41.4 | 46.5 | 40.1-40.9 | 41.3 | 42.1 |

+, positive; - negative; ND, no data; NG, no growth.

**Table 2.7** (Cont.)
Strain 9, *H. litoralis*; 10, *H. locisalis*(Yoon et al., 2004); 11, *H. mangrovi*(Soto-Ramirez et al., 2008); 12, *H. profundi*(Hua et al., 2007); 13, *H. salinus*(Yoon et al., 2003); 14, *H. seohaensis* (Yoon et al., 2008); 15, *H. trueperi*; 16, *H. yeomjeoni*(Yoon et al., 2005).

| **Characteristic** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** |
|---|---|---|---|---|---|---|---|---|
| Cell morphology | Rods | Rods | Rods | Rods | Rods | Rods | Rods | Long rods filamentous |
| Flagellation | Peritrichous | Single | ND | Absent | Peritrichous | Single | Peritrichous | Single |
| Spore shape/position | E,S/C,ST | E | ND | E,S/C, | E | E/C,ST | E,S/C,ST | E/C,ST |
| Colony colour | Orange | Lightorange–yellow | Cream | Pale yellow | Pale orange–yellow | Yellowishwhite | Orange | Light yellow |
| Maximum temp for growth (°C) | 43 | 42 | 50 | 47 | 45 | 38 | 44 | 48 |
| Growth at: | | | | | | | | |
| 4 °C | - | - | - | - | - | + | - | - |
| pH 5.0 | - | + | - | - | + | - | - | - |
| pH 5.5 | - | + | - | + | + | - | - | - |
| 0.5% NaCl | + | - | - | + | + | - | + | + |
| 25% NaCl | + | - | - | + | - | - | + | - |
| Hydrolysis of: | | | | | | | | |
| Aesculin | - | + | - | + | + | - | - | - |
| Casein | - | - | + | + | + | + | - | + |
| Gelatin | + | - | + | - | + | - | + | + |
| Starch | - | + | + | + | - | + | - | - |
| Tween 80 | - | - | + | + | + | - | - | + |
| Acid production from | | | | | | | | |
| D-Fructose | + | + | - | + | + | - | + | - |
| D-Galactose | + | - | - | - | W | - | + | - |
| Maltose | + | - | - | + | + | - | + | + |
| Sucrose | + | + | - | + | + | + | + | + |
| D-Xylose | - | - | - | + | - | + | - | - |
| Cell-wall type | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp | L-Orn-D-Asp | DAP | L-Orn-D-Asp | L-Orn-D-Asp |
| DNA G+C | 42 | 44 | 45.7 | 43.3 | 45 | 39.3 | 43 | 42.9 |

+, positive; - negative; ND, no data; NG, no growth.

**Gram-negative moderate halophiles**

**Genus *Salinivibrio.*** The genus *Salinivibrio* is included in the family *Vibrionaceae*, belonging to the γ-subclass of the class *Proteobacteri*a. This genus was created from *Vibrio costicola* based on the significant phenotypic and genotypic differences between this species and other *Vibrio* species. Kita-Tsukamoto et al. (1993) compared partial 16S rRNA sequences (approximately 600 nucleotides) of a large number of *Vibrio* species, and the resulting data also demonstrated that *V. costicola* is not related to other *Vibrio* species or related organisms. Mellado et al (1996), confirmed the phylogenetic positions of six *Vbrio costicola* strains were determined by direct sequencing and analysis of their PCR-amplified 16S ribosomal DNAs. A comparative analysis of the sequence data revealed that the moderate halophile *V. costicola* forms a monophyletic branch that is distinct from other *Vbrio* species and from other moderately halophilic species belonging to the gamma subclass of the *Proteobacteria.*(Figure 2.3). In addition, the primary sequence of the 16s rRNA genes of *V.* cos*ticola* has insertions at helices between positions 183 and 193 *(Escherichia coli* 16S rRNA gene sequence numbering) and positions 207 and 214. These differences in the primary sequence, which produce significant secondary structure changes, are found in the sequences of all strains of *V. costicola,* but are not present in the sequences of any *Vibrio* or *Photobacterium* species. Because of the significant phenotypic and genotypic differences between *V costicola* and other *Vibrio* species, as well as other bacteria belonging to the gamma subclass of the *Proteobacteria,* Mellado et al (1996), transferred that *V. costicola* to a new genus, the genus *Salinivibrio.*

At present, the genus comprises two species, *S. costicola* subsp. *costicola* (Smith, 1938; Garcia et al., 1987), *S. costicola* subsp. *vallismortis* (Huang et al., 2000), *S. costicola* subsp. *alcaliphilus.* (Romano et al., 2005) and *S. proteolyticus* (Amoozegar et al., 2008). They are moderately halophilic bacteria which are distributed in salted meats, brines and hypersaline environments and grow in the presence of 0-20% NaCl.

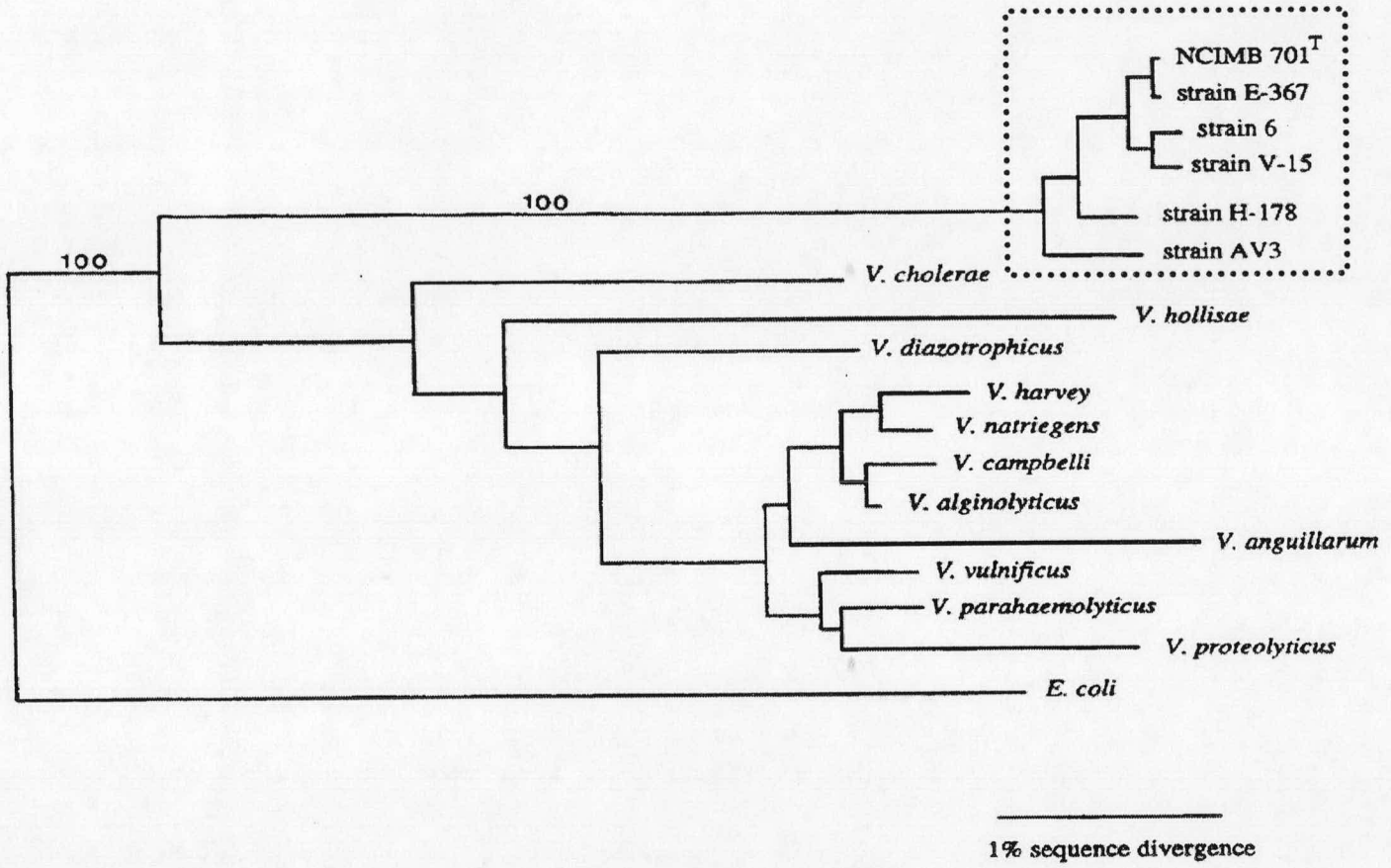


**Figure 2.3** Phylogenetic tree of six *V. costicola* strains and other species of the genus *Vibrio* based on 16S rDNA sequences (Mellado et al.,1996).

**Table 2.8** Characteristics of species belonging to the genus *Salinivibrio*

Strains: 1, *S. costicola* subsp. *costicola*; 2, *S. costicola* subsp. *alcaliphilus*(Romano et al., 2005); 3, *S. costicola* subsp. *vallismortis*(Huang et al., 2000); 4, *S. proteolyticus*(Amoozegar et al., 2008).

| Characteristic | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Colony color | Cream | Cream pink | Cream white | Cream white |
| Oxygen requirement | FA | A | FA | FA |
| Flagellation | One polar flagellum | ND | One or two polar flagella† | One polar flagellum |
| NaCl range (%, w/v) | 2-18 | 2-22 | 0-12.5 | 1-17 |
| Optimum growth in NaCl (%) | 10 | 9 | 2.5 | 5 |
| Temperature range (°C) | 15-45 | 10-45 | 20-50 | 10-45 |
| Optimum temperature (°C) | 34 | 37 | 37 | 32-35 |
| pH range | 6-9 | 6-10 | 5.5-8.2 | 5-9.5 |
| Optimum pH | 8 | 8 | 7.3 | 8-8.5 |
| Voges-Proskauer | + | - | + | + |
| Nitrate reduction | - | + | - | - |
| Esterase (C4) | + | - | + | w |
| Naphthanol-AS-BI phosphohydrolase | + | - | w | - |
| N-acetyl-β-glucosaminidase | + | - | + | w |
| Hydrolysis of : Aesculin | - | + | - | - |
| Starch | - | - | + | + |
| L-Tyrosine | + | w | - | - |
| Acid from: D-Cellobiose | - | + | + | - |
| D-Xylose | - | - | - | + |
| Utilization of: D-Cellobiose | + | - | + | - |
| Crotonic acid | - | + | - | ND |
| D-Fructose | - | - | + | + |
| Glycine | - | + | - | + |
| Malonic acid | - | + | - | + |
| Succinate | - | + | - | + |
| G+C content (mol%) | 49.9 or 50.0* | 49.3† | 50.0† | 49.5 |
| Source of isolation | Cured meat and salterns* | Saltish spring† | Death valley† | Hypersaline lake |

+, positive; - negative; ND, no data; NG, no growth.

**Genus *Chromohalobacter*** Among the bacterial families that form part of the Gamma-proteobacteria, the family *Halomonadaceae* is characterized as being represented by several halophilic, halotolerant and non-halophilic species that belong to different genera. The family *Halomonadaceae* includes the genera *Halomonas*, *Carnimonas*, *Chromohalobacter*, *Cobetia* and *Zymobacter*. Members of the genus *Chromohalobacter*, which currently has nine species, form a monophyletic group included in the family *Halomonadaceae*. Ventosa et al. (1989) reclassified '*Chromobacterium marismortui*' as *Chromohalobacter marismortui* and other species were subsequently placed in the genus *Chromohalobacter* as follows: *Chromohalobacter canadensis* and *Chromohalobacter israelensis* (Arahal et al., 2001a), *Chromohalobacter salexigens* (Arahal et al., 2001b), *Chromohalobacter sarecensis* (Quillaguama et al., 2004), *Chromohalobacter nigrandesensis* (Prado et al., 2006), *Chromohalobacter beijerinckii* (Pec¸onek et al., 2006), *Chromohalobacter salarius* (Aguilera et al., 2007) and *Chromohalobacter japonicus* (Sánchez-Porro et al., 2007). They were motile and rod-shaped moderately halophilic bacterium. The rod shape was measured about 0.6-1.2x1.5-4.2 μm and occur singly, in pairs and in short chains. It was aerobic, non-spore forming and Gram-negative. Cells were straight or sometimes slightly curved. They were tolerate at salt concentrations up to 30%(w/v). The broader ranges of temperature and pH observed for growth were 5-45 °C and pH 5.0-10. Strains reduced nitrate, but $H_2S$ was not produced. Urease and phenylalanine deaminase were negative, but not nitrate reduction, catalase and oxidase. Casein, DNA, aesculin, gelatin, starch and Tween 80 were not hydrolyzed. The cell wall peptidoglycan contain diaminopimelic acid. The predominant menaquinone is MK-7. The differentiated characteristics of *Chromohalobacter* species show in Table 2.9.

**Table 2.9.** Characteristics of species belonging to the genus *Chromohalobacter*

Strains: 1, *C. japonicus*(Sánchez-Porro et al., 2007); 2, *C. marismortui*; 3, *C. canadensis* (Arahal et al., 2001a), 4, *C. beijerinckii*(Pec¸onek et al., 2006); 5, *C. israelensis*(Arahal et al., 2001a); 6,*C. salexigens*(Arahal et al., 2001b); 7, *C. sarecensis*(Quillaguama et al., 2004); 8, *C. nigrandesensis*(Prado et al., 2006); 9, *C. salaries*(Aguilera et al., 2007).

| Characteristics | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Pigmentation | Cream | Brown Yellow | White | Light yellow | Cream | White Cream | Brown | Black | Yellow |
| Oxidase | - | + | - | + | - | - | + | - | - |
| Nitrate reduction | + | - | + | + | + | + | - | - | + |
| Simmons citrate | + | - | | + | ND | + | - | + | ND |
| Hydrolysis of: | | | | | | | | | |
| Gelatin | + | - | - | - | v | - | +/- | ND | - |
| Aesculin | - | - | + | - | + | v | +/- | - | - |
| NaCl range (%) | 5-25 | 1-30 | 3-25 | 0-25 | 3.5-20 | 0.9-25 | 0-25 | 0.5-25 | 3-25 |
| Acid production from: | | | | | | | | | |
| D-galactose | + | + | ND | + | ND | + | + | + | + |
| Maltose | + | + | - | +- | + | + | - | + | - |
| Sucrose | - | + | - | - | + | + | - | + | - |
| D-glucose | + | + | ND | + | ND | + | + | + | + |
| D-trehalose | - | + | - | - | - | - | + | + | - |
| D-Xylose | + | + | ND | + | ND | + | - | + | - |
| Lysine decarboxylase | - | - | + | - | + | - | - | ND | + |
| G+C contents (mol%) | 62.9 | 62.1-64.9 | 62 | 60.7 | 65 | 64.2-66 | 56.1 | 59.8 | 63.6 |

+, positive; - negative; ND, no data; NG, no growth.

### 2.2.4.3 Halotolerant bacteria

They were tolerant high salt concentration but do not require them for growth. The slight halophile grow optimally in the media containing <2% salt but may grow in a NaCl concentration of 10% of more. In contrast, nonhalophiles grow optimally at less than 0.2 M NaCl. Halotolerant organisms can grow both in high salinity and in the absence of a high concentration of salts. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations with requirement or tolerance for salts sometimes depending on environmental and nutritional factors. Such as *Jeotgalicoccus*, *Pseudomonas, Moraxella, Flavobacterium, Acinetobacter*, some *Bacillus* sp. and *Vibrio* they isolated from sea fish and shell. This recently, many research found *Bacillus* sp. grew in 15% NaCl, and optimally in 1% NaCl; they were therefore regarded as halotolerant bacteria.

The ubiquity of *Bacillus* species and closely related bacteria in fermented fish products implied their importance in the preparation of these food materials. The results of several studies have supported this conjecture. For example, Itoh et al. (1993) verified the participation of bacteria in fish sauce fermentations. *Bacillus* strains have been isolated from fermented fish in Vietnam and Japan (Crisan and Sands,1975). Researchers have isolated proteolytic *Halobacillus* (Choorit and Prasertsan, 1992) and *Bacillus* from fermented fish in Thailand (Chaiyanan et al., 1999). Isolates of *Bacillus* species and moderately halophilic bacteria were recovered from Korean fermented seafoods (Yoon et al., 2001). Bacillus species have been isolated from nam-pla, a Thai fermented fish sauce (Saisithi et al., 1966; Crisan and Sands, 1975). Noguchi et al., (2004) isolated four *Bacillus* strains from nuoc mam, a Vietnamese fish sauce

**Genus *Bacillus* sp.**

***Bacillus vietnamensis*** This species was isolated from nuoc mam, a Vietnamese fish sauce. Cells are rod-shaped, measuring 0.5–1.0 by 2.0–3.0 mm, Gram-positive and aerobic. Ellipsoidal spores developed centrally in the cells and sporangia were not swollen. Cells are motile with peritrichous flagella. The strains tested produced catalase and oxidase. They grew in 15% NaCl, and optimally in 1% NaCl. In addition, this species grew at pH 10.0 as alkaliphilic bacteria. The major cellular fatty acids of was anteiso-$C_{15:0}$ (48.3±8.6 %), iso-$C_{15:0}$ (16.2±3.7 %),

anteiso-$C_{17:0}$ (13.6±4.9%) and iso-$C_{16:0}$ (11.2±1.8 %). Fatty acids occurring in minor amounts were iso-$C_{14:0}$ (3.7±1.8 %), $C_{14:0}$ (1.3±0.8 %), $C_{15:0}$ (1.0±0.5 %), $C_{16:0}$ (2.8±0.6%) and iso-$C_{17:0}$ (1.3±1.0 %). The isolates contained menaquinone 7(MK-7), cell-wall peptidoglycan of *meso*-Diaminopimelic acid. (Noguchi et al., 2004).

***Bacillus aquimaris*** Cells are aerobic rods, 0.5–0.7x1.2–3.5 mm. Gram-variable, motile by means of peritrichous flagella. Central ellipsoidal endospores are observed in large, swollen sporangia. Colonies are circular to slightly irregular, slightly raised, pale orange-yellow in colour and 2–4 mm in diameter after 3 days at 30 °C on MA. Optimal growth occurs in the presence of 2–5% (w/v) NaCl. Growth is poor in the absence of NaCl, but occurs in the presence of up to 18% (w/v) NaCl. Growth does not occur under anaerobic conditions on MA. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. The G+Ccontent is 38 mol%.

***Bacillus marisflavi*** Cells are aerobic rods, 0.6–0.8 x1.5–3.5 mm. Gram-positive, but Gram-variable in older cultures. Motile by means of a single polar flagellum. Central or subterminal ellipsoidal endospores are observed in swollen sporangia. Colonies are smooth, circular to slightly irregular, slightly raised, pale yellow in colour and 2–4 mm in diameter after 3 days at 30 °C on MA. Optimal growth occurs in the presence of 2–5% (w/v) NaCl. Growth occurs in the presence of 0–16% (w/v) NaCl. Growth does not occur under anaerobic conditions on MA. Catalase-positive. Oxidase-negative. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The major fatty acids are anteiso-$C_{15:0}$ and iso-$C_{15:0}$. The G+C content is 49 mol%.

**Table 2.10** Characteristics of *B. vietnamensis* JCM 11124$^T$. (Noguchi et al., 2004), *B. marisflavi* TF-11$^T$ and *B. aquimaris* TF-12$^T$(Yoon et al., 2003b).

| **Characteristics** | **JCM11124$^T$** | **TF-11$^T$** | **TF-12$^T$** |
|---|---|---|---|
| Colony color | Cream orange yellow | Pale yellow | Pale orange-yellow |
| Flagellation | Peritrichous, Single polar | Single polar | Peritrichous |
| Oxygen requirement | aerobic | aerobic | aerobic |
| NaCl range (%, w/v) | 0-15 | 0-16 | 0-18 |
| Optimum growth in NaCl (%) | 1 | 2-5 | 2-5 |
| Temperature range (°C) | 10-40 | 10-47 | 10-44 |
| Optimum temperature (°C) | 30-40 | 30-37 | 30-37 |
| pH range | 6.5-10 | 4 5-7 | 4.5-9 |
| Optimum pH | 6-8 | 6-8 | 6-7 |
| Oxidase | + | - | - |
| Hydrolysis of : Aesculin | + | + | - |
| Starch | + | - | + |
| L-Tyrosine | + | - | - |
| Acid from: D-Cellobiose | - | + | - |
| D-Mannitol | + | + | - |
| D-Mannose | - | + | - |
| Melibiose | - | + | - |
| D-Xylose | - | + | - |
| Raffinose | - | + | + |
| D-Xylose | - | + | - |
| G+C content (mol%) | 43 | 49 | 38 |

+, positive; - negative; ND, no data; NG, no growth

### 2.2.5 Application of halophilic microorganisms

Moderately halophilic bacteria have the potential for exciting and promising applications. Not only do many of them produce compounds of industrial interest (enzymes, polymers, and osmoprotectans), but also they possess useful physiological properties which can facilitate their exploitation for commercial purposes. First, most of them can grow at high salt concentrations, minimizing the risk of contamination. Second, they are easy to grow, and their nutritional requirements are simple: the majority can use a large range of compounds as their sole carbon and energy source. In spite of all this, the moderately halophilic bacteria have not yet been used extensively for biotechnological purposes. The current industrial applications and the possibilities of their biotechnological applications are summarized as follows.

#### 2.2.5.1 Compatible solutes

Moderate halophiles accumulate high cytoplasmatic concentrations of low-molecular-weight organic compounds to cope with the osmotic stress and to maintain positive turgor pressure. The ability to produce and accumulate high concentrations of these compounds makes moderate halophiles useful for the biotechnological production of these osmolyte. Some compatible solutes, especially glycine, betaines, and ectoines, may be used as stress protectants (against high salinity, thermal denaturation, desiccation, and freezing) and stabilizers of enzymes, nucleic acids, membranes and whole cells (Galinski, 1993). The industrial applications of these compounds in enzyme technology are most promising (Ventosa and Nieto, 1995). The other compatible solutes such as trehalose, glycerol, proline, ectoines, sugars, and hydroxyectoine from halophilic bacteria showed the highest efficiency of protection of lactate dehydrogenase against freeze-thaw treatment and heat stress. Ectoine was also the most effective freeze-stabilizing agent for phosphofructokinase (Wohlfarth et al., 1989).

Ectoine and its derivatives have found interesting applications as moisturizers in cosmetics for the care of aged, dried, or irritated skin (Motitschke et al., 2000). The Merck Co., Darmstadt, Germany has recently introduced and reported multiple cosmetic benefits for the skin with respect to immune system of the Langerhans cells, formation of heat shock proteins, and protection of membrane

integrity (Beyer et al., 2000). Ectoine also reduces the formation of "sunburn cells" in the skin following UV radiation (Bunger et al., 2000). Ectoine and hydroxyectoine are now commercially produced by Bitop (Witten, Germany) (see http://www.bitop.de). Ectoine is industrially produced from *Halomonas elongata,* and hydroxyectoine from *Marinococcus* M52. Chemical synthesis of ectoine can easily be achieved, but is not economically competitive with biotechnological production due to the price of the precursors.

**2.2.5.2 Halophilic bacteria in food products**

A characteristic flora of halophilic bacteria is associated with cured salted fish. Examination of the microbial community of cured salted anchovies showed dominance of *Pediococcus halophilus,* with optimal growth between 60 and 100 g/l salt and it tolerated up to 150 g/l (Villar et al., 1985). Moderately halophilic bacteria were also abundant on cured salted cod (bachalao). Viable counts up to $10^7$ CFU/g were reported. These consisted of two types of cream to pinkish colonies that grew between 5 and 250 g/l NaCl. These colonies have not been characterized further (Vilhelmsson et al., 1996).

Halophilic Bacteria are also involved in the production of traditionally fermented salted foods in the Far East. "Nukazuke", a paste of fermented fish in bran that contains between 10 and 15% salt, contains many halophilic cocci that are involved in the fermentation process. Lactic acid is the main product of their metabolism. Viable counts between $10^2$and $10^7$per gram have been obtained (Kuda et al., 2001).

The microbial community involved in the production of fermented salted puffer fish ovaries in rice bran ("fugunoko nukazuke") in Japan has been investigated in more detail. The fermentation process here lasts up to 1-2 years, and salt concentrations from about 13% up to 30% are being used. Among the microorganisms abundantly found in the fermented material are *Tetragenococcus halophilus* and *Tetragenococcus muraticus, Pseudomonas*-like halophiles and also *Archaea* (Kobayashi et al., 1995, 2000). *Halomonas alimentaria* is an important component of the microbial community that develops during the preparation of "jeotgal", a traditional Korean fermented seafood (Yoon et al., 2002).

Also related to the food industry is the commercial production of the flavoring agents 5'-guanylic acid (5'-GMP) and 5'-inosinic acid from RNA, using the halophilic nuclease H of "*Micrococcus varians* subsp. *halophilus*". This enzyme degrades RNA at 60°C and 120 g/l NaCl. At these conditions an excellent yield is achieved, as there is little activity of contaminating 5'-nucleotidases (Kamekura et al., 1982).

In the preparation of Thai fish sauce (nam pla, a food condiment widely used in Southeast Asia), moderate halophiles and halotolerant bacteria are used (*Bacillus* spp., coryneform bacteria, and, more rarely, pseudomonads, most of these tolerating up to 20 to 30% salt). Extremely halophilic red archaea are also found during the process (Thongthai, and Suntinanalert. 1991).

**2.2.5.3 The biodegradative potential**

Industrial wastewaters often contain both high concentrations of toxic organic compounds and high salt concentrations, sometimes accompanied by high levels of toxic inorganic ions. In Japan, a biological treatment system used to clean up wastewater (salt concentration about 150 g/l) generated during the production of pickled plums. The system was reported to reduce the chemical oxygen demand of the wastewater by 70-90%. Two salt tolerant bacteria were isolated from the system that grew optimally at salt concentrations between 0-100 g/l but could tolerate up to 200g/l. These isolates were tentatively identified as *Staphylococcus* sp. and *Bacillus cereus* (a species not otherwise known as highly halotolerant) (Kubo et al., 2001). It has been suggested that halophilic bacteria might be utilized to remove phosphate from saline environments, as a cheaper alternative to chemical approaches (Ramos-Cormenzana, 1989). Among the toxic compounds shown to be broken down at high salt concentrations by halophilic bacteria are formaldehyde (Azachi et al., 1995; Oren et al., 1992), phenol and other aromatic compounds, organophosphorus compounds, and others. Biodegradation of hypersaline wastewaters containing phenol was achieved by a *Halomonas* strain has been isolated with effectively degrades phenol, chloroaromatic compounds such as the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) as carbon and energy sources.(Hinteregger and Streichsbier, 1997; Maltseva et al., 1996; Oriel et al., 1997).

Several moderate halophiles that degrade hydrocarbons, including hexadecane and phenanthrene, have been isolated from Organic Lake, Antarctica (McMeekin et al., 1993). Some halophilic bacteria show a surprisingly high tolerance to heavy metal ions (Nieto, 1991). Halophilic bacteria have been identified that can perform biotransformations of selenium and uranium and aid in their bioremediation. From a selenium-contaminated hypersaline evaporation ponds in the San Joaquin Valley, California, built to reduce the volume of agricultural drainage water high in selenate, a number of *Halomonas* like bacteria were isolated that tolerate up to 2 M selenate . These organisms accumulated selenate and volatilized it to the relatively nontoxic dimethylselenide (D'Souza et al., 2001).

**2.2.5.4 Polymers**

Bacterial polysaccharides are of great value as enhancers of oil recovery because of their surfactant activity and bioemulsifying properties. Since the conditions in oil deposits are often saline, the use of salt-resistant surfactants may be advantageous. *Haloferax mediterranei* produces exopolysaccharides (up to 3 g/L) with pseudoplastic behavior that are resistant to pH, heat, and shear. They show higher viscosity at dilute concentrations and elevated temperatures than commercial polymers such as xanthan gum (Galinski and Tindall, 1992; and Ventosa et al., 1998b). The polymer displays a remarkable immunomodulating activity *in vitro.* It enhances the proliferativc effect of human lymphocytes as a response to the presence in blood of the anti-CD3 monoclonal antibody (Perez-Fernandez et al., 2000). New *Halomonas* isolates from Morocco also produce interesting exopolysaccharides, and may find applications as emulsifiers with potential in the oil industry (Bouchotroch et al., 2000).

**2.2.5.5 Enzymes**

A number of extra - and intracellular enzymes from moderately halophilic bacteria have been isolated and characterized. A considerable amount of effort has been dedicated to the study of extracellular salt-tolerant enzymes of the moderately halophilic bacteria, especially toward the use of such enzymes in biotechnological processes. These include hydrolases (amylases, nucleases, phosphatases, and proteases), which are currently of commercial interest.

Halophiles from the archaeal domain provide the main source of extremely halophilic enzymes. The potentials of halophiles and haloenzymes have been reviewed previously. The production of halophilic enzymes, such as xylanases, amylases, proteases and lipases, has been reported for some halophiles belonging to the genera *Acinetobacter*, *Haloferax*, *Halobacterium*, *Halorhabdus*, *Marinococcus*, *Micrococ-cus*, *Natronococcus*, *Bacillus*, *Halobacillus* and *Halothermothrix* (Oren, 2002; Madon et al., 2000).

However, many of these enzymes have not been investigated in detail or application. Although the halophilic enzymes can perform enzymatic functions identical to those of their non-halophilic counterparts, these enzymes have been shown to exhibit substantially different properties, especially, the requirement for high salt concentrations (1-4M NaCl) for activity and stability and a high excess of acidic over basic amino residues (Mevarech et al., 2000). It is argued that the high negative surface charge of halophilic proteins makes them more soluble and renders them more flexible at high salt concentrations, conditions under which non-halophilic proteins tend to aggregate and become rigid. This high surface charge is neutralized mainly by tightly bound water dipoles (Madern et al., 2000; Mevarech et al., 2000; Da Costa et al., 1998; Danson and Hough, 1997).

## 2.3 Overview of protease

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Protease catalyzes the cleavage of peptide bonds in other proteins. Proteases are degradative enzymes which catalyze the total hydrolysis of proteins (Figure 2.4).

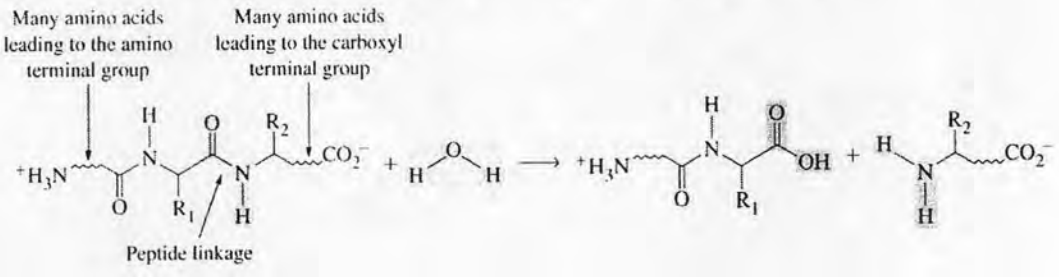


**Figure 2.4** Catalytic reaction of protease

Proteolytic enzymes are involved in a great variety of physiological processes and their action can be divided into two different categories:

1) Limited proteolysis, in which a protease cleaves only one or a limited number of peptide bonds of a target protein leading to the activation or maturation of the formely inactive protein *e.g* conversion of prohormones to hormones.

2) Unlimited proteolysis, in which proteins are degraded into their amino acid constituents. The proteins to be degraded are usually first conjugated to multiple molecule of the polypeptide ubiquitin. This modification marks them for rapid hydrolysis by the proteasome in the presence of ATP. Another pathway consists in the compartmentation of proteases *e.g* in lysosomes. Proteins transferred into this compartment undergo a rapid degradation.

**2.3.1 Classification of protease**

According to the Nomenclature Committee of the Internatioal Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria (Barett, 1994).

**2.3.1.1 Type of reaction catalyzed.**

Proteases are grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on the location of the enzymatic action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate.

**2.3.1.2 Chemical nature of the catalytic site.**

Based on the functional group present at the active site, proteases are further classified into four prominent groups, including serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Guzzo et al., 1990)

Serine proteases: Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. This class comprises two distinct families. The

chymotrypsin family which includes the mammalian enzymes such as chymotrypsin, trypsin or elastase or kallikrein and the substilisin family which include the bacterial enzymes such as subtilisin.

Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Some of the serine proteases are inhibited by thiol reagents such as *p*-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities including esterolytic and amidase activity.

Aspartic proteases: Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin, retropepsin, and enzymes from pararetroviruses.

The aspartic proteases are inhibited by pepstatin (Fitzgerald et al., 1990). They are also sensitive to diazoketone compounds such as diazoacetyl-DL-norleucine methylester (DAN) and 1, 2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin. Microbial aspartic proteases can be broadly divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* spp.

Cysteine/thiol proteases: Cysteine proteases occur in both prokaryotes and eukaryotes. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin- like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Papain is the best-known cysteine protease. Cysteine

proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents.

Metalloproteases: Metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by the requirement for a divalent metal ion for their activity. The metallo proteases may be one of the older classes of proteases and are found in bacteria, fungi as well as in higher organisms. They differ widely in their sequences and their structures but the great majority of enzymes contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity. Because of they require a metal ion for their activity, so inhibited by metal chelating agents such as EDTA but not by sulfhydryl agents or DFP. For example carboxypeptidase, thermolysin, collagenase.

### 2.3.2 Protease of halophilic bacteria

Halophiles from the archaeal domain provide the main source of extremely halophilic enzymes. The potentials of halophiles and haloenzymes have been reviewed previously (Oren, 2002; Eichler, 2001; Madern et al., 2000; Hough and Danson, 1999; Sellek and Chaudhuri, 1999; Da Costa et al., 1998; Danson and Hough, 1997). The production of halophilic enzymes, such as xylanases, amylases, proteases and lipases, has been reported for some halophiles belonging to the genera *Acinetobacter*, *Haloferax*, *Halobacterium*, *Halorhabdus*, *Marinococcus*, *Micrococcus*, *Natronococcus*, *Bacillus*, *Halobacillus* and *Halothermothrix* (Oren, 2002; Eichler, 2001; Madern et al., 2000; Sellek and Chaudhuri, 1999; Da Costa et al., 1998; Danson and Hough, 1997; Adams et al., 1995).

However, many of these enzymes have not been investigated in detail or application (Sellek and Chaudhuri, 1999). Although the halophilic enzymes can perform enzymatic functions identical to those of their non-halophilic counterparts, these enzymes have been shown to exhibit substantially different properties, especially, the requirement for high salt concentrations (1-4 M NaCl) for activity and stability and a high excess of acidic over basic amino residues (Mevarech et al., 2000). It is argued that the high negative surface charge of halophilic proteins makes them more soluble and renders them more flexible at high salt concentrations,

conditions under which non-halophilic proteins tend to aggregate and become rigid. This high surface charge is neutralized mainly by tightly bound water dipoles (Madern et al., 2000; Mevarech et al., 2000; Da Costa et al., 1998; Danson and Hough, 1997).

Although halophilic microorganisms have attracted much attention in recent years, most studies have been performed in halobacteria. However, moderately halophilic bacteria represent an excellent model of adaptation to frequent changes in extracellular osmolality and constiute an interesting group of microorganisms from a biotechnological point of view. Thus, many of them accumulate intracellular organuc osmolytes named "compatible solutes" which can be used as stabilizers of enzymes and whole cells (Da Costa et al., 1997: Ventosa et al., 1998; Nieto et al., 2000) and they produce halophilic exoenzymes that could be of commercial interest and could be used in biodegradation processes. They have the advantage that most species are able to grow in a wide range of salinities, in contrast to the more strict requirements of salt presented by halobacterium. For example *Gracilibacillus* and *Tetragenococcus* strains can growth in absence NaCl and cell reduce size in low concentration of salts(Thongsanit et al., 2002)

**2.3.2.1 Protease-producing extremely halophilic bacteria.**

A few protease from extreme halophiles, member of the archael phylogenetic branch, have been characterized by Norberg and Hofsteen (1969); Stepnov et al. (1992); Studdert et al. (1997); Ryu et al. (1994); Gimenez (2000).

**2.3.2.2 Protease-producing moderately halophilic bacteria.**

Protease from moderately halophilic bacteria have not been extensively studied. Some of their works on protease from moderately halophilic bacteria are as follows : Duong Van Qua et al. (1981), Sanchez-Porro et al. (2003), and Namwong et al. (2005).

In previous study, only extracellular protease produced by unidentified moderately halophilic bacterium, designated *Psseudomonas* sp. strain A-14, was purified. The molecular weight of this enzyme was estimated to be 12,000 Da, The optimum pH for activity was 8.0, and the enzyme presented maximal at 18% NaCl concentration (Qua et al., 1981).

In 2003, the protease CP1 produced by the moderately nalophilic bacterium, *Pseudoalteromonas* sp. CP76 has been purified and characterized in detail by Sanchez-Porro et al., (2003). The enzyme is a homodimer with a subunit size 38 kDa. The enzyme is moderately thermophilic , presenting optimum activity at 55 °C, at pH 8.5. An interesting feature of this protease is salt tolerance over a wide range of NaCl concentration (0-20% NaCl). These characteristic make the protease CP1 interesting for its application in biotechnological process. The protease activity was inhibited by EDTA, PMSF and Pefabloc. No significant inhibition was detected with E-64, bestatin, chymostatin or leupeptin. According to this result and the sequencing of the amino terminal region of the purified enzyme, the protease CP1 has been classified as a serine metalloprotease.

In order to improve the production of the protease CP1 for industrial application, the growth condition of *Pseudoalteromonas* sp.CP76 for optimum protease activitty were studied. The production was optimal in saline medium containing 7.5% NaCl, supplemented with sucrose, fructose and glycerol. This study constitutes the first report on the purification and in-depth characterization of proteolytic enzyme from a moderately halophilic microorganism.

Hiraga, et al. (2005) and Namwong et al. (2006) studied the protease-producing bacteria were screened from fish sauce in Thailand. An isolated moderately halophilic bacterium, strain RF2-5 was identified and named as *Fillobacillus*. The molecular weight of the purified enzyme was estimated to be 49 kDa. The enzyme showed the highest activity at 60 °C and pH 10-11 under 10%NaCl and was highly stable in the presence of about 25% NaCl. The activity was strongly inhibited by PMSF, Chymostatin, and α-microbial alkaline proteinase inhibitor (MAPI). The N-terminal 15 amino acid sequence of the purified enzyme showed about 67% identity of the serine proteinase from *Bacillus subtilis* 168 and *Bacillus subtilis* (Natto). Strain SR5-3 was identified and named as *Halobacillus*. The enzymatic properties of the SR5-3 protease was estimated to be 36.9 kDa, was identified to be a serine protease, to be a chymotrypsin-type or a subtilisin-type, but not a trypsin-type protease, optimal pH and temperature of the SR5-3 protease were pH 10.0 and 50 °C using MCA-substrate in the presence of 20% NaCl and without

$Ca^{2+}$ ion. The SR5-3 protease almost lost its activity after 2 h at 70°C under the presence of 2 mM $Ca^{2+}$

In 2005-2007, protease-producing halophilic bacteria, *Salinivibrio* sp., were purified and characterization. Lama et al. (2005) have purified and characterized a metalloprotease from *Salinivibrio* sp. $18AG^T$ was identified and named as *Salinivibrio costicola* subsp. *alcaliphilus.* (Ramano et al., 2005) The protease was to be 38 kDa, strongly inhibited by PMSF and its show optimal activity at 2% NaCl, 60°C in presence 2Mm $CaCl_2$ while in the absence $CaCl_2$ optimum activity was 50°C. The enzyme had an optimum pH 8.0. The synthesis of the enzyme in culture medium was influenced by the presence of gelatin. In 2007, Salinivibrio sp AF2004 was isolared from hypersaline lake and it produced two kind of protease. First report is zince metalloptrotease was monomeric protease with a relative molecular mass of 38 – 43 kDa by SDS-PAGE and gel filtration chromatography. The apparent optimum temperature for the enzymatic activity was found to be 65°C, pH 8.5 in presence of 0-0.5M NaCl. The N-terminal amino acid sequence showed high similarity to the zinc-metalloprotease from *Vibrio* species (Karbalaei-Heidari et al., 2007). The secondly reported, is serine metalloprotease, molecular mass was 31 kDa by SDS-PAGE and 29 kDa by gel filtration. The optimum activity was art 55°C, pH 8.5 and 0-0.5 M NaC. This enzyme stable in organic solvent than in absence organic solvents.