Chapter 14
Isolation and Screening of Halophilic Bacteria for Production of Hydrolytic Enzymes

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Abstract There is a great interest on study of extreme microorganisms because of their special characteristics in biotechnology. Particularly, halophilic microorganisms which live in saline environments throughout of their different adaptation mechanisms produce metabolites with great potential. Some of its biomolecules has been studied and applied in industrial processes, such as exopolyssaccharides, carotenoid pigments, bacteriorhodopsin etc. beside certain enzymes especially hydrolases (pectinases, amylases, proteases, lipases, etc.) are important. Recent researches on halophilic microorganisms and their biomolecules have increased around the world. Saline environments such as saline lakes or saline soils are excellent sources for isolation of halophilic microorganisms. However, few saline environments have been studied in depth in order to evaluate the special characteristics of halophilic biomolecules. In this review, the importance of halophilic microorganisms for biotechnological industries, methods for their isolation; techniques for physiological, taxonomical and molecular characterization have been highlighted so as to establish them as important source for enzyme production.

Keywords Halophilic bacteria • Hydrolytic enzymes • Biomolecules • Biotechnology • Molecular identification

14.1 Introduction

Because of its salt tolerance, halophilic microorganisms are considered extreme organisms (Meseguer 2004), According to salt requirements, halophilic microorganisms are classified as (i) extreme halophiles which require 3.5–5 M NaCl; (ii)

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moderately halophiles which grow at 0.5–3.5 M NaCl; (iii) weakly halophilies: need 0.3–0.5 M of NaCl and (iv) halo-tolerant which not need salt for growth but their growth is supported at high levels of salt in its medium. In addition to its salt requirements, halophilic microorganisms can tolerate alkaline pH and survive in hot and dry places (Madigan et al. 2004; Ramírez et al. 2004). Its capacity to grow under high salt concentration is due to adaptation mechanisms such as accumulations of compatible solutes in the cytoplasm. These substances act as biological structures stabilizing and allowing adaption in front of cold, heat, desiccation, etc. Principally, compatible solutes form a water layer generating a cell hydration in presence of high salt in the medium (Kunte et al. 2002; Ramírez et al. 2004). These special characteristics developed scientifically interest not only for studies of isolation and search for new species, but to explore the possibility of searching for biomolecules which may have potential in biotechnological processes (Table 14.1).

14.1.1 Enzymes

The most important biotechnological application is halophilic enzymes. The major characteristics that they exhibit are because of large proportion of acidic amino acids, principally, aspartic and glutamic acid, allowing a hydration of protein surface because of presence of carboxylic groups of these amino acids (Lanyi 1974; Pundak and Eisenberg 1981). It has been observed that sodium chloride has a role to stimulate important biological processes such as transcription, translation and transport activity of halophilic enzymes (Averhoff and Müeller 2010). The principal class of enzyme produced by halophilic microorganism is hydrolases. It has been reported the commercial use of halophilic hydrolases e.g., amylases for starch degradation and detergents formulation while proteases are applied during fish oil hydrolysis. More studies on halophilic enzymes are still in progress (Karan et al. 2012).

Table 14.1 Biotechnological applications of biomolecules from halophilic microorganisms

Biomolecules	Application	Reference
Enzymes	Starch hydrolysis, microalgae saccharification (amylases), chitin conversion (chitinases), fish oil hydrolysis (lipases)	Karan et al. (2012)
Bacteriorhodopsin	Holography, spatial light modulators, artificial retina, neural network optical computing, and volumetric and associative optical memories	Margesin and Schinner (2001)
Biopolymers	Exo-polysaccharides: emulsifiers and mobility controllers, remove dyes from textile effluent used in alkaline conditions, pharmaceutical, food, and petroleum industries	Ventosa and Nieto (1995)
	Liposomes: compound in medicines and cosmetics, drug carrier in food or pharmaceutical industry	
Halocins	Natural antibiotics, inhibition of Na+/H+ antiporter generating a protection against the myocardium ischemia and reperfusion injury	Schiraldi et al. (2002)
Carotenoid pigments	Food colorant, additive in cosmetics, multivitamin preparations, etc.	Margesin and Schinner (2001)

14.1.2 Bacteriorhodopsin

Because of its photochromic property, bacteriorhodpsin has potential to be used as material for optic memories elaboration, holographic storage material, modelers spatial light, computer memories, etc. The studies of bacteriorhodopsin are focus on halophilic Archea particularly *H. salinarum* and *H. halobium* (Oesterhelt et al. 1991; Meseguer 2001).

14.1.3 Biopolymers

This is another attractive application because they can be used as emulsifiers, thickeners etc. The halophilic microorganisms can produce liposomes which are used as transporters of compound in medicine, cosmetology and polyhydroxyalkanoates to generate biodegradable polymers: Specially, halophilic polysaccharides such as sulfated polysaccharides produced from *Halomonas* sp., others with substantial quantity of fucose are produced from *Salipiger mucescens* and have a high potential and value (Ventosa and Nieto 1995; Margesin and Schinner 2001).

14.1.4 Halocins

These compounds have potential to be used as myocardial protector. These have role to play as preserving agents in food and leather industries and in control of infectious bacteria (Torreblanca et al. 1994 Schiraldi et al. 2002; Karthikeyan et al. 2013).

14.1.5 Carotenoid Pigments

Some halophilic microorganisms are source of carotenoids such as β-carotene vitamin A precursor, which have application as food additive, food coloring, cosmetics product and as drug component (Schiraldi et al. 2002). Now-a-days some of these applications are commercialized but others are in the process of development at pilot scale (Meseguer 2004). However, the tremendous diversity of halophilic microorganisms found in nature is still far from being fully exploited (Oren 2002).

14.2 Saline Environments

Alkaline hyper-saline environments are rich source of diverse microorganisms, especially halophile, alkalophile and haloalkalophile microorganisms which are useful for the development of new bioprocess and microbial products of

Table 14.2 Ionic composition of some saline environments (Madigan et al. 2004)

Concentration (g/L)				
Ion	Sea water	Great Salad Lake	Dead Sea	Zugm Lake (Egypt)
Na^+	10.5	105	40.1	142
K^+	0.38	6.7	7.7	2.3
Mg^+	1.27	11	44	<0.1
Ca^{2+}	0.4	0.3	17.2	<0.1
Cl^-	19	18.1	225	155
Br^-	0.065	0.2	5.3	–
SO^{4-}	2.65	27	0.5	23
HCO^{3-}	0.14	0.7	0.2	67
pH	8	7.7	6.1	11

commercial interest. Around the world, there are many saline environments, majority are located in dry and hot zones (Delgado-García 2011), being most of them originated by evaporation of seawater (called thalassohaline environments) where salt is composed mainly by sodium and chloride. When evaporation proceeds, some changes occur in the ionic composition because of precipitation of $CaSO_4$ and other minerals; these habitats are called athalassohaline (Oren 2002). Predominance of different ions depends on topography, geology and climatic conditions. The principal cation is sodium, while the anion is chloride (Table 14.2) (Madigan et al. 2004). Microbial life has been adapted to environments that combine high salt concentration with extremely high pH values. Alkaline soda lakes in Africa, India, China with pH values of 11 and higher salt concentrations about 300 g/L proved a source of potential halophilic microorganisms (Oren 2002).

Around of the world there are many saline habitats like Dead Sea (Israel), Great Salt Lake (USA) and others environments in Korea, and Spain. However, such studies cover only a limited part of the tremendous diversity among the halophiles. Nowadays, there are more investigations of saline environments around the world; particularly in Mexico exist many saline environments and saline saltern. Specially, in the north of Mexico Cuatro Ciénegas basin is important for its aquatic habitats, which has high salt concentrations; and it has been described as an important biodiversity reservoir within the Chihuahuan desert. This zone has saline soils with high levels specially of sodium chloride and others salts such as magnesium, calcium, potassium, sulphate and carbonates (Castro et al. 2011). However, Cuatro Ciénegas has negligible phosphorus levels (Elser et al. 2005; Castro 2007), and has been described as a location with high levels of species endemism (Escalante et al. 2008).

On the other hand, solar salterns found around the world, are extremely hyper saline habitats containing microbial systems (Sabet et al. 2009). Mexico has some solar salterns for example, Las Coloradas in Yucatan State, and Guerrero Negro in Baja California Sur State. Especially the last has been studied for almost 30 years, and it is described as a place with great diversity of microbial systems, especially halophilic microorganisms (Ley et al. 2006; Escalante et al. 2009). About soda lakes, in Mexico exist the former soda Texcoco Lake which has been suffered many

disturbances because of human activities. Disturbances include drainage of the lake since seventeenth century to halt flooding of the city and irrigation of the generated soils with sewage sludge since the 1970s (Ortega-Guerrero et al. 1997). However, some studies revealed presence of bacteria, actinomycetes, denitrifiers and others (Luna-Guido et al. 2000). Around the world, there are many examples of saline environments where halophilic microorganisms have been isolated. But, still there is lack of information about new halophilic species, and biotechnological applications of these microorganisms. In the next paragraphs, we will discuss different techniques for isolation, physiological, taxonomical and molecular characterization of halophilic microorganisms. In addition, screening techniques for enzyme production will also be discussed.

14.3 Culture Media for Isolation of Halophilic Microorganisms

For isolation of halophilic microorganisms and enzyme production, salts-added culture medium is used. There are reported different culture media depending of the halophilic microorganism of interest. In the case of extreme halophiles and halotolerant, eubacteria may interfere in the isolation of the desirable moderate halophilic microorganisms. Specific microorganisms need specific nutrients per example diverse vitamins. For this reason, it is an important criterion for selection of the best culture medium according to the microorganism of interest. One of the best media for isolation of halophilic bacteria is named halophilic medium (HM) specific for moderate halophiles because of its low Mg^{2+} content, which does not support good growth of extreme halophiles (Ventosa et al. 1982). However, there is other culture medium composition that is more specific for moderate halophiles such as *Halobacillus* genera, where in all cases, pH should be adjusted at 7.0 (Amoozegar et al. 2003). Composition of different culture media reported for halophilic microorganisms isolation is shown in the next paragraphs.

(a) **Halophilic medium** (**HM**) (**g/L**): NaCl (178), KCl (Amoozegar et al. 2003), $MgSO_4 \cdot 7H_2O$ (1), $CaCl_2 \cdot 2H_2O$ (0.36), NaBr (0.23) $NaHCO_3$ (0.06), $FeCl_3$ (trace), yeast extract (10), glucose (1), agar (24) and distilled water (1,000 mL) (Ventosa et al. 1982).

(b) **HALO medium** (**g/L**): sodium citrate (10), sodium thiosulfate (10), sodium collate (3), saccharose (20), NaCl (25), Iron citrate (1), potassium dihydrogen phosphate (2), magnesium sulfate (5) and distilled water (1,000 mL) (Castro 2011).

(c) **Marine agar** (**Difco**) (**g/L**): peptone (5), yeast extract (1), ferric citrate (0.1), NaCl (8.8), $MgSO_4 \cdot 7H_2O$ (8.8), $CaCl_2 \cdot 2H_2O$ (1.8), sodium sulfate (3.24), KCl (0.55), Na_2HCO_3 (0.16), KBr (0.08), strontium chloride (34), boric acid (22), sodium silicate (4), sodium fluoride (2.4), ammonium nitrate (1.6), disodium phosphate (8), agar (15) and distilled water (1,000 mL). This medium is used for different halophilic microorganisms such as *Halobacillus* sp. specified by DSMZ institute (Leibniz-Institute DSMZ, German Collection of Microorganisms and Cell Cultures).

(d) **Nutritive agar modified adding NaCl as salt source**. The quantity of NaCl depending on the halophilic microorganism to isolate: (1) extreme halophilic: need about 3.5–5 M of salt, (2): moderately halophilic: growth at 0.5–3.5 M of salt, (3) weakly halophilic: need 0.3–0.5 M of salt (Delgado-García et al. 2013; Meseguer 2004).

(e) **Specific culture medium for *H. trueperi* and *H. karajensis* (g/L)**: NaCl (100), $MgSO_4{\cdot}7H_2O$ (5), casein peptone (5), yeast extract (3) (Amoozegar et al. 2003).

There are other culture media, specifically for moderate halophiles, however in the case of the extreme halophiles, it is possible to use the culture medium for *Halobacterium salinarun* NRC-1 due to is the major extreme halophilic bacteria studied. It is important considered that extreme halophiles will tolerate a fairly generous salinity range and ionic composition (Rainey and Oren 2006).

(f) **Culture medium for extreme halophilic microorganisms**, **specifically for *Halobacterium salinarum* NRC-1**, **basal medium** (**g/L**): NaCl (250), $MgSO_4{\cdot}7H_2O$ (20), trisodium citrate (3), KCl (2), tryptone (5), yeast extract (3), distilled water (1), trace metals (0.1 mL): $ZnSO_4$ $7H_2O$ (1.32), MnSO4 H_2O (0.34), Fe (NH_4) $2SO_4$ $6H_2O$ (0.82), $CuSO_4$ $5H_2O$ (0.14), distilled water (200 mL) (Rainey and Oren 2006).

Specifically, for isolation of halophilic Archea, it is important to perform a concentrated seawater solution. According to Rodriguez-Valera et al. (1980) and Torreblanca et al. (1986) this solution should be contain salts in approximately the same proportions as found in sea water. Seawater composition is described below.

(g) **Seawater solution** (**stock solution**) (**g/L**): NaCl (240), $MgCl_2$ $6H_2O$ (30), $MgSO_4$ $7H_2O$ (35), KCl (7), NaBr (0.8), $NaHCO_3$ (0.2), is important to add double-distilled water to near the final required volume and dissolve the salts completely. Finally, add 5 mL $CaCl_2$ $2H_2O$ (slowly) from a 1 M sterile stock solution and adjust pH up to 7.5 (Rainey and Oren 2006).

14.4 Culturing of Soil Samples on Solid Media

Agar is the most commonly used gelling agent for halophiles. For this reason, there are different methods to optimize culturing and isolate different halophilic microorganisms.

14.4.1 Modified Pour Plate

First 10 g of soil was added into 90 mL of sterile distilled water. The Stirred should be vigorously to dissolve major part of soil sample. This was the first dilution of sample (10^{-1}), then, in other flask with 90 mL of sterile distilled water 10 mL of the first dilution was placed. It is important to maintain in agitation the first dilution. This step was repeated to obtain a dilution approximately of 10^{-8}. Finally, agar

medium was poured into Petri plate and when this almost solidifies 1 mL of the diluted soil suspension was stirred to homogenized agar medium. This step was repeated for each dilution. This technique was used principally to avoid loss microbial diversity (Fig. 14.1) (Madigan et al. 2004; Delgado-García et al. 2013).

14.4.2 Soil Granules

First of all, little soil granules should be placed with a sterile spatula into the agar plate. After that, soil granules should be having a distance about 1 cm among themselves (Fig. 14.2). This technique of soil granules allow to obtain more colonies of bacteria, besides this technique is easy and cheap (Aquilanti et al. 2004; Delgado-García 2011).

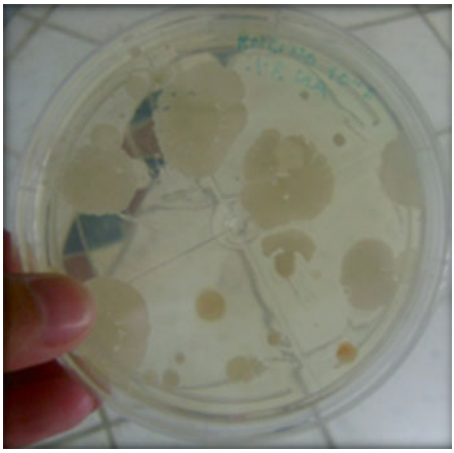

Fig. 14.1 Pour plate technique using a 10^{-5} soil dilution (Delgado-García 2011)

Fig. 14.2 Soil granules technique (Delgado-García 2011)

These types of culturing methods can be used to isolate all kinds of halophilic microorganisms but, in the case of extreme halophilic microorganism, plates should be placed in sealed plastic containers to prevent moisture loss during the incubation period. Incubation time depends on the halophilic microorganism type, per example if objective is to isolate moderate halophilic microorganisms about 2 or 3 days of incubation is sufficient but, in the case of extreme halophilic microorganisms, it could take about 2 or 4 weeks to obtain colonies. The optimum temperature for halophilic bacteria is 37 °C (Rainey and Oren 2006).

14.5 Evaluation of Halophilic Capacity

The salt tolerance evaluation allows selecting the best halophilic strains and determination of the group where each strain belongs.

14.5.1 Growth Kinetics

In order to known adaption of a specific cell to a culture medium with low or high salt concentration, growth kinetics was studied. For this study, it was important to select a range of salt concentrations to establish optimum salt concentration for each strain. First, from a liquid culture with a specific strain is recommended to perform a calibration curve using spectrophotometric methods (λ 600 nm). Then, each strain is grown at different salt concentration, covering the range mentioned for halophilic microorganisms (1) extreme halophilic: need about 3.5–5 M of salt, (2): moderately halophilic: growth at 0.5–3.5 M of salt (3) weakly halophilic: 0.3–0.5 M of salt. After that, it was important to grow the selected strain (s) to specific conditions and to determine bacterial growth in the medium with different salt concentrations at specific time (hours, days, weeks), in this case, it was took 1 mL of the culture and was determinate the absorbance using spectrophotometric methods (λ 600 nm). Later, data was converted and a graphic (cell/mL vs. time) was performed. With this method, it was possible to identify which strain assimilated more rapidly salt from culture medium and optimum salt concentration for microorganism growth (Delgado-García et al. 2013).

14.5.2 Halo Formation in Saline Medium

Formation of a halo by a microorganism in a medium supplemented with salt (NaCl) is an indicative of its halophilic capacity. One of the widely used medium is salt-mannitol agar (g/L): meat extract (10), pluripepetone (10), d-mannitol (10), NaCl (different concentrations), phenol red (0.25), agar (15) (Delgado-García 2013).

After the medium is prepared, place a bacterial colony on the salt-mannitol agar and incubate to specific grow conditions, then, determine halo formation and measure its diameter and quantify days for halo formation. Finally a graphic of halo diameter vs. time is made.

14.5.3 In Vitro Sodium Capture

This technique allows determining halophilic potential of the tested strains, which may use sodium utilization by different metabolic pathways. First, all lab material used in this technique should be washed several times with a nitric acid 10 % (w/v) solution to eliminate sodium and potassium traces, after growing the tested microorganism at a specific salt concentration and grow conditions, sodium capture could be determinate by atomic absorption spectroscopy. It is important to use a sodium standard and control (culture medium with salt concentration without microbial inoculum) (Table 14.3). In addition, it is very important to perform dilutions of the culture medium, approximately 1: 1,000, which will be used for the analysis by atomic absorption spectroscopy. Finally, statistical analysis should be performed to compare the results and deduce which strain is the best sodium scavenger.

The halophilic capacity determination of each microorganisms is a key factor, because it has been mentioned that sodium chloride increase the metabolic activity in halophilic microorganisms (Mudryk and Donderski 1991). Based on these studies, it is possible to have an idea about sodium chloride quantity requirements for halophilic microorganism's growth, because different studies have indicated that halophilic microorganisms require high chloride concentrations to stimulate DNA transcription, translation and transport activity (Lanyi 1974; Pundak and Eisenberg 1981).

14.6 Identification of Halophilic Microorganisms

Phylogeny is the study of evolution and development of species. The comparison of genomic sequences of some macromolecules is the best and reliable technique for inference on the phylogenetic relationships (Herrera 2011). Specially, the study of 16S rDNA gene is the most used to establish phylogenetic and taxonomic

Table 14.3 Specifications for the atomic absorption spectroscopy technique used for study of sodium in vitro capture to determinate the halophilic capacity of the strain (Sánchez-Leal and Arguello 2006; Delgado-García et al. 2013)

Component	Characteristic
Flame	Air-acetylene
Air flux	13.58 L/min
Acetylene flux	2.25 L/min
Lamp current	5 mA
Wavelength	589 nm
Slot width	0.5 nm

relationships among bacteria. Sequence of this gene has been used because is a highly conserved allowing determinate difference among bacteria and construct a dendrogram or phylogenetic tree for their representation (Woese et al. 1990; Delgado-García 2011). Recent advances on mass sequencing will speed a better phylogeny and systematic of organisms. However, still studies on 16S rDNA sequences allow new perspectives on diversity, given to the generation of a new knowledge and classification of bacteria groups and its evolution (Herrera 2011).

14.6.1 DNA Isolation

Although different DNA isolation techniques have been reported in literature, in the next paragraph we will describe one methodology which has offered high DNA quality. First, obtain a cellular pellet by centrifugation (200 g × 10 min) from a liquid culture medium, using per example 200 µL of biomass and 800 µL of sterile distilled water. Then, re-suspend the cellular pellet in a lysozyme solution (5 mg/mL) and incubate at 50 °C for 1 h. After that, apply a thermal shock (1 min in ice and 1 min at 90 °C) for 3 times. Later, add 200 µL of buffer TE 1X and 200 µL of SDS (sodium dodecylsulfate) 10 % and incubate for 1 h in dry bath, then add 100 µL of NaCl (5 M) and 80 µL of CTAB (hexadecyltrimethylammonium bromide) buffer and incubate at 65 °C for 30 min. After that, add an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuge at 1,174 g × 5 min separate the aqueous phase and add 600 µL of isopropyl alcohol, then incubate for 24 h at −20 °C. Later, add 1 mL of alcohol (70 %) and centrifuge at 1,174 g × 5 min. Finally, remove supernatant and add 100 µL of Buffer TE 1X (Maloy 1989; Zavala-Castro 2005; Delgado-García 2011).

14.6.2 Microorganisms Identification by Specific Genes Amplification

One of the most important techniques and most used around the world is the polymerase chain reaction (PCR) (Mullis and Faloona 1987). This technique involves in vitro enzymatic amplification of a specific DNA region located between two DNA regions whose sequence is known. Using a PCR reaction is possible obtain until millions of copies of a gene or DNA region. The PCR technique is indispensable because allow establish new challenges to study and better understand the role of some genes (Mullis and Faloona 1987; Saiki et al. 1988).

There are different PCR variants, for example, RT-PCR used when the sample is a complementary DNA from mRNA. This conversion is due to reverse transcription by an enzyme named reverse transcriptase which converts mRNA in cDNA (Tamay de Dios et al. 2013). Other PCR variant is nested-PCR based in two consecutive

rounds of amplification, the first PCR is performed using a pair of external primers. The resulting amplification product is transferred to another tube containing a second primer pair, which is nested. In other words they are internal to the first the initial pair. There are other variants of PCR such multiplex PCR, multiplex with touchdown PCR, etc. however, the basis is the same, amplify a gene to obtain millions of copies (Bartlett and Stirling 2003; Olmos et al. 2003).

14.6.2.1 16S rDNA

It has been reported predominance of bacteria than other microorganisms (fungi, yeast, etc.) in halophilic group. By this reason, there are more studies of isolation, and characterization of bacteria (Vreeland and Hochstein 1992). Also the major representatives of halophilic Archea are prokaryotic (Prescott et al. 2004). Different primers have been used for amplification of the 16S rDNA gene such as those described in Table 14.4 (Guillén-Cruz et al. 2005), which were efficient to identify bacterial strains to specie level (Delgado-García et al. 2013). For PCR reaction is important to add 14.5 µL of sterile distilled water 2.5 µL buffer TE 10X 1 µL of $MgCl_2$ (50 mM), 0.5 µL of dNTP's (20 mM) 2 µL of each primer (10 pM), 0.5 µL of *Taq* polymerase (5 U/mL) and 2 µL of sample DNA. Temperatures used for PCR reactions are: 94 °C (5 min) of initial denaturation followed by 35 cycles (94 °C/1 min, 65 °C/1 min and 72 °C/1 min) and final extension of 72 °C for 8 min (Flores-Gallegos 2009).

There are other useful primers (Table 14.4) for bacteria identification (Muyzer et al. 1995). Specifically GM3 and GM4 universal primers amplify nearly the complete sequence of 16 rDNA of the Bacteria domain, while other primers amplify the V6 and V8 region of 16S rDNA (Table 14.5). Specifically, these primers amplify a short sequence (approximately 58 bp) from the hyper-variable region V6, which is variable enough that allow to distinguish among most closely related bacterial species and also it is useful for differentiation among genera in ecological studies (Lane 1991; Grosskopf et al. 1998).

Table 14.4 Primers used for 16S rDNA amplification (Muyzer et al. 1995; Guillén-Cruz et al. 2005)

Primer	Sequence
16S rDNA forward	5′-AGGAGGTGATCCAACCGCA-3′
16S rDNA reverse	5′-AACTGGAGGAAGGTGGGAT-3′
GM3 forward	5′-AGAGTTTGATCMTGGC-3′
GM4 reverse	5′-TACCTTGTTACGACTT-3

Table 14.5 Primers for amplification of V6 and V8 regions from 16S rDNA (Grosskopf et al. 1998)

Primer	Sequence
A-109	5′-AC(G/T)GCTCAGTAACAGTAACACGT-3′
GC-515	5′-AC(G/T)GCTCAGTAACAGTAACACGT-3′

Table 14.6 Primers used for gene amplification from bacterial strains, during taxonomical studies

Gene	Primers
RNA polymerase subunit β	*rpoβ*F
	5′-GCGAAGTGTTAGAATTACC-3′
	*rpoβ*R
	5′- TCGTATTCTAACCATGCGCC-3′
	Band size = 450 bp
DNA gyrase subunit β topoisomerase II gene	UP-2r
	5′-AGCAGGGTACGGATGTGCGAG-3′
	UP-2Sr
	5′-AGCAGGGTACGGATGTGCGAGCC-3′
	Band size = 1,260 bp

14.6.2.2 Other Genes for Taxonomic Studies

Other gene sequences different to ribosomal ones have been used to differentiate bacterial strains to specie or subspecies level. One of the most used is *rpoβ* gene (RNA polymerase subunit β) sequence because predict similarities among genomes especially in bacteria (Table 14.6). The *rpoβ* gene sequence is more informative than that of 16S rDNA because of its size (4,200 bp). This gene has conserved and alternating variable regions and is used for taxonomic and phylogenetic studies, especially when it is suspected that the tested strain may be new specie (Meintanis et al. 2006). PCR reaction temperatures are: 95 °C/10 min (initial denaturing), 35 cycles (95 °C/1 min, 53 °C/1 min, 72 °C/1 min) and 72 °C/1 min for final extension (Delgado-García 2013).

The *gyrβ* (DNA gyrase subunit β topoisomerase II gene) (Table 14.6) is other gene used for taxonomic studies. This gene allows discriminate highly related species and is possible identify to subspecie level, being an excellent taxonomic marker; the sequence of this gene is about 12,000 bp. PCR reaction temperatures are: 94 °C/5 min (initial denaturing), 35 cycles (94 °C/1 min, 60 °C/1 min, 72 °C/2 min) and 72 °C/5 min for final extension (Yamamoto and Harayama 1995; Li- Tang et al. 2007).

14.6.3 Metagenomics Studies

Metagenomics is defined as the direct genetic analysis of genomes contained with an environmental sample. The analysis start with the cloning of environmental DNA, followed by functional expression screening and then is complemented by direct random shotgun sequencing of the environmental DNA (Tyson et al. 2004; Venter et al. 2004). Direct metagenomic sequencing is an appealing route for investigate microbial community composition, because, it provides simultaneous insight into phylogenetic composition and metabolic capabilities of uncultivated populations (Allen and Banfield 2005; Wilmes et al. 2009).

Metagenomic protocol starts with a soil or water sample, followed by genomic DNA extraction. Then, ligation of heterologous genomic DNA and restriction digested vector is performed; later, the vector is inserting into *E. coli* cells. This procedure allows construct a genomic library and study the DNA sequences in two ways: heterologous gene expression (Function-driven analysis) or by DNA detection by hybridization with specific or by vector-primed insert sequencing (Sequence-driven analysis) (Schloss and Handelsman 2003).

14.6.4 Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE)

Genetic fingerprints technique provides a pattern of profile of the genetic diversity in a microbial community. One of the fingerprint techniques that have been used in microbial ecology is the electrophoretic separation in high resolution polyacrylamide gels. Separation of DNA fragments in DGGE and TGGE is based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in poly-acrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) or a linear temperature gradient, which is created by two water baths attached to a cooling plate under gel (Muyzer and Smalla 1998).

By using DGGE or TGGE, 50 % of the sequence variants can be detected in DNA fragments up to 500 bp (Myers et al. 1985). This percentage can be increased to nearly 100 % by the attachment of a GC-rich sequence, to one side of the DNA fragment. A sequence of guanines and cytosines is added to the 5′-end of one the PCR primers, co-amplified and thus introduced into the amplified DNA fragments (Sheffield et al. 1992). DNA bands in DGGE and TGGE profiles can be visualized using ethidium bromide or SYBR green I (Myers et al. 1985).

14.7 Biochemical and Hybridization Techniques for Taxonomic Studies

Generally, taxonomic studies are used when it is suspected that the tested microorganisms may be new species. Biochemical studies allow knowing cell wall markers for establish relationship and differences among different species of one genus. Polyphasic is one important development for bacterial taxonomy; it has been used to integrate the different kinds of data and information (phenotype, genotypic and phylogenetic) on microorganisms and essentially indicates a consensus type of taxonomy. The principal aim of taxonomy is to give classification to bacteria. Phenotypic methods comprise all those that are not directed toward DNA or RNA; therefore, they also include the chemotaxonomic techniques. The chemotaxonomy

is considered one of the essential milestones in development of modern bacterial classification. These types of studies refer to application of analytical methods to collect information on various chemical constituents of cell to classify bacteria (Vandame et al. 1996).

14.7.1 Isoprenic Quinones

Respiratory quinones are lipidic terpenoids constituting the bacterial cytoplasmatic membrane, which play important roles on electron transport, oxidative phosphorylation and possibly, active transport (Ventosa et al. 2004). Two major structural groups, naphtoquinones, which occur less commonly in bacteria and the menaquinones have been reported (Vandame et al. 1996). The study of the respiratory quinones, size and chain saturation allows to determine phylogenetic relations in a bacteria group (Da Costa et al. 2011). In addition, quinones are biomarks of bacterial populations and their study allow understand the respiratory function and electron transport (Irvan 2006).

To determine isoprenic quinone type, the following protocol is used. First, 4 g of lyophilized biomass are added to 50 mL of hexane. Sample is maintained in agitation for 24 h. After that, hexane is evaporated and sample is dried by nitrogen flux. Then, the solvent system of hexane and ethyl acetate (96:4) is prepared and sample is dissolved with chloroform. Later, sample is placed into a TLC (10×10 cm) and putting into the chromatographic camera for elution. Finally, TLC is dried and observed under UV light, quinone mark are reveal with cerium-sulfate 0.1 %. Quinones purification is performed from the sample charged in a TLC (20×20 cm). The protocol is the same as described above. After the band is obtained, it is cut off from the TLC and purified using methanol-chloroform (2:1) and filtered. Finally, sample is dissolve in chloroform-d_3 and analyzed by H^1 NMR and LC/MS techniques for isoprene units and molecular weight determination (Nicolaus et al. 2001; Romano et al. 2001).

14.7.2 Polar Lipids Composition

The polar lipids are also biomarkers for bacteria identification. Lipid distribution is not identical for all species, for this reason is useful to show evolutionary changes (Barton 2005). A variety of lipids are present in bacteria cells. Polar lipids are the major constituents of the lipid bi-layer of bacterial membranes and have been studied frequently for classification and identification purposes. Phospholipids of cell wall are the most important for their proportion (allow cell or organelles integrity by forming a semi-permeable barrier), also form an active biological material essential for biosynthesis and transport processes (Setlmann and Holst 2002; Barton 2005).

Other types of lipids, such as sphingo-phospholipids, occur in only a restricted number of taxa and are important within these groups of microorganisms (Jones and Krieg 1984; Vandame et al. 1996).

A protocol for determination of polar lipids is as follows: First, 4 g of lyophilized biomass are added to 50 mL of chloroform-methanol-water (65:25:4) and maintained in agitation for 24 h, hexane is evaporated and dried by nitrogen flux. Then, a solvent system chloroform-methanol-water (65:25:4) is prepared and sample is dissolve with chloroform. After that, sample is placed into a TLC (20×20 cm), and then put into the chromatographic camera for elution. Finally, TLC is dried and revealed to know the polar lipid composition with different solutions such as α-naftol (glycolipids), Dittmer (phospholipids), cerium-sulfate 0.1 % (total lipids) and ninhydrin (amino lipids) (Nicolaus et al. 2001). Identification of specific phospholipids is performed by bi-dimensional TLC. The system solvents are chloroform-methanol-water (65:25:4) (one dimension) and chloroform-methanol-acetic acid-water (80:12:15:4) (two dimension) (Fig. 14.3). First, TLC is placed in one system solvent, after that, it is dried and placed in the other system solvent. Finally, TLC is dried and reveal with Dittmer. In this case, for phospholipids identification is important to use standards such as: phosphatidyl serine (PS), phosphatidic acid (AP), inositol phosphatidil (PI), phosphatidil glycerol (PG), phosphoryl ethanol amine (PEA), phosphatidil coline (PC1 and PC3), cardiolipin (CLBH) (Nicolaus et al. 2001).

Phospholipids comprise about 10 % of the bacterial cell dry weight, and each mole of lipids requires about 32 mol of ATP for its synthesis. Thus, phospholipids synthesis requires significant energy investment by the cell, and advantages of maintaining fine control over the pathway are obvious. Bacteria have a pathway where phospholipids are catalyzed by a series of discrete proteins: enzymes are mainly integral inner membrane proteins. The key activated intermediate component during bacterial phospholipids synthesis is CDP-diacylglycerol, which comprises only 0.05 % of the total phospholipids pool.

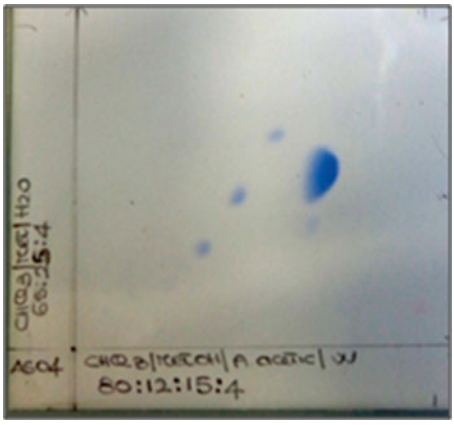

Fig. 14.3 Identification of phospholipids by bidimensional TLC

14.7.3 Fatty Acids Methyl Esters (FAME) Composition

Fatty acids are the major constituents of lipids and lipopolysaccharides and have been used extensively for taxonomic purposes. More than 300 different chemical structures of fatty acids have been identified. The variability in chain length, double-bond position, and substituent groups has proven to be very useful for characterization of bacterial taxa. Mostly, total cellular fatty acid fraction is extracted, but particular fractions such as polar lipids have been analyzed. Cellular fatty acid methyl ester content is a stable parameter, proving that highly standardized culture conditions are used. This method is cheap and rapid and has reached a high degree of automation (Vandame et al. 1996).

Fatty acids between 9 and 20 carbons in length have been used to characterize bacterial genera and species. Simple mutations or plasmid loss or gain do not alter fatty acid composition of an organism. There is an automated system, the MIDI Sherlock Microbial Identification System, which identifies microorganisms based on unique FAME patterns of known strains (Whittaker et al. 2003). The protocol is as follows: lipids are extracted; their hydrolysis is performing by TLC using hexane/Et2O (96:4) and is detected with I_2 vapor. FAME is analyzed by TLC on silica gel using α-naftol as developer. Finally, products are analyzed by GC/MS (Heat et al. 2002; Romano et al. 2007).

14.7.4 DNA-DNA Hybridization

The bacterial specie definition mentioned above is found upon whole genomic DNA-DNA hybridization values (Wayne et al. 1987). Results of this analysis are given in percent of DNA-DNA hybridization and decrease in thermal stability of the hybrid, it is used to delineate species. The percent of DNA binding or the DNA-DNA hybridization value or relative binding ratio is an indirect parameter of sequence similarity between two entire genomes (De Ley et al. 2006). DNA-DNA hybridization is often performed under standard conditions that are not necessarily optimal or stringent for all bacterial DNAs. Generally, optimal conditions for hybridization are preferred, because the optimal temperature curve for hybridization is rather broad (about 5 °C). The hybridization under optimal conditions requires a temperature between 22 and 26 °C, below the melting temperature, measured or calculated at equal salt concentration (Johnson 1991).

First, DNA sample should have a concentration of 1 ng/mL for an efficient DNA-DNA hybridization. One protocol for DNA-DNA hybridization is as follows: DNA is denatured by 10 min at 100 °C followed by quick immersion in water-ice bath. An amount of 50–80 ng/dot of DNA from tested strain is blotted on a positively charged nylon membrane (Roche, Germany). Dots are washed twice by 0.1 × SSC. Then, DNA is cross-linked to nylon by 3 min UV exposure and by 1 h under-vacuum at 120 °C. 1 μg of DNA shared by ultrasonic treatment is dioxigenin-dUTP labeled

over night in 20 μL reaction mixture using a nucleotide random priming procedure (Dig DNA Labeling kit, Roche). After that, membranes are pre-hybridized for 3 h at 41 °C in Dig-Easy-Hyb solution and hybridized over night at 41 °C, using a roller tube hybridization incubator, in Dig Easy-Hyb solution containing 20 pg/mL of DIG labeled probe, heat denatured as above described or by 10 min at 68 °C in DIG-Easy-Hyb solution. Then, membrane washes are performed: twice for 5 min at room temperature in 2×SSC solution containing 0.1×SDS, twice for 15 min at 68 °C in 0.1×SSC solution containing 0.1×SDS. Later, immune detection is performing using the anti-dioxigenin AP antibody. Finally, chemi-luminicence is quantified in condition of time-exposure linearity by using a Versa-DOC 400 (BioRad). The DNA-DNA homology percentage is calculated according to Jahnke (1994) (Romano et al. 2007).

14.8 Biotechnological Potential of Halophilic Microorganisms and Enzymes

Enzyme production of halophilic microorganisms is poorly exploited to commercial level. One advantage of these enzymes is its capacity to catalyze reactions under extreme conditions, principally under high salt concentrations, but also are able to be stable at alkaline pH and some of them are thermostable (Enache and Kamekura 2010). These properties allow use them in industrial processes that use hard physicochemical conditions, or use them under low quantity of substrate or in highly concentrated substrates, but the most interesting characteristic of these enzyme is their capacity to catalyze reaction under organic solvents (Setati 2009; Oren 2010).

Hydrolases such as amylases, pectinases, pullulanase, DNases, xylanases, lipases, cellulases, inulinases, etc. is the major enzyme group produced by halophilic microorganisms (Table 14.7) (Cojoc et al. 2009; Rohban et al. 2009; Enache and Kamekura 2010). Halophilic enzymes have other applications per example as flavoring agent, or for pulp and paper industry, detergents formulation, oil fish hydrolysis, etc. (Kamekura and Onishi 1974; Karan et al. 2012).

14.8.1 Detection of Enzyme Production

For enzyme production by halophilic microorganisms is important first to know the enzyme type produced, so it is essential to perform an enzymatic screening. For the screening is crucial to select the substrate to produce the enzyme of interest. There is a simple, rapid and cheap method, which use only a microplate to perform the enzymatic screening. The protocol is as follows: First, culture medium with the specific substrate is prepared 250 μL of each agar is placed in a microplate well (Fig. 14.4). After that, wells are inoculated with the tested strain using the puncture

Table 14.7 Hydrolases produced by diverse halophilic bacteria and their industrial applications (Delgado-García 2012; Karan et al. 2012;)

Enzyme	Microorganisms (examples)	Applications
Amylases	*Haloferax mediterranei*, *Halobacterium salinarum*, *Nesterenkinia halobia*, *Halomonas meridiana*, *Halobacillus* sp.	Saccharification of marine microalgae, starch hydrolysis and textile, food, brewing and distilling industries
Proteases	*Bacillus* sp., *Halobacillus* sp., *Virgibacillus* sp. *Natrialba magadii*	Peptide synthesis, fish sauce preparation, detergents formulations
Lipases	*Salinivibrio* sp. *Natrococcus* sp.	Detergent additives, in the food and paper industries, enantioselective biocatalysis
Xylanases	*Halobacillus* sp., AS-04, *Bacillus pumilus*	Pulp and paper industry, baking industry for increasing loaf volume
DNase	*Micrococcus varians*, *Bacillus* sp., N23-2	Acid 5′-guanilic and acid 5′-inosinic as flavor agents
Cellulases and pullulanases	*Bacillus* sp.	Biocatalysis in organic solvents and super critic fluids
Chitinases	*Halobacterium salinarum* NRC-1, *Planococcus riftoensis*	Oligosaccharide synthesis, bioconversion of chitin from fish, crab or shrimp, treatment of chitinous waste

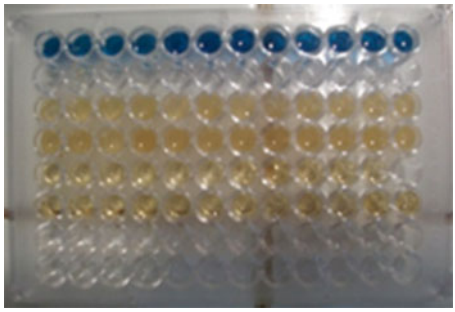

Fig. 14.4 Microplate with agar in each well for enzymatic screening of halophilic microorganism using hydrolysis halos formation. Each well has a substrate specific where the strain will be inoculate (Delgado-García 2013)

technique. Then, microplate is covered and incubated at specific temperature. Finally, each well is reveal and observed with stereoscope, using halo formation as an indicative of the hydrolysis mechanism (Delgado-García et al. 2013).

14.8.2 Genetic Aspects of Halophilic Enzymes

Generally, the halophilic enzymes reported are typically secreted into the extracellular environment throughout the growth cycle of halophilic bacteria in the presence of suitable substrates which would act as direct inducers of respective gene according to the enzyme produced (Setati 2010). It is reported that halophilic proteins are

acidic in nature and the increase in acidic amino acids is compensated by decrease in basic amino acids. The frequency of proline and glycine is higher in case of extreme halophiles. Significant increase in the frequency of proline among extreme halophiles is observed in the genes reported as regulators of ion transporters (*aca4*, *esi47*, *hal3*, *hal*), transporters (*ema1*, *gbuA*), osmotic tolerance proteins (*gpd1*, *relA*) and salt toxicity target (*hal2*). On the other hand, high frequency of glycine among extreme halophile is observed in the genes reported to regulators of ion transporters (esi47, hal3, hal5), transporters (*ena1*, *ena2*, *gbuA*, *hal11*), osmotic tolerance protein (*cysK*, *gdp1*, *relaA*) and chaperones (*dnaK*, *groEL*) (Anwar and Chauhan 2012).

There are different studies for gene identification of specific halophilic enzyme per example the amylase produced by *Halomonas meridiana* and codified by *amyH* belongs to the already proposed family of α-amylases composed of the enzymes from *Alteromonas haloplanktis*, *Thermonospora curvata*, *Streptomyces* sp., insects and mammals due to the amino acid homology. The *AmyH* contains the four highly conserved regions in amylase enzymes. The invariant amino acid residues are also conserved in the *AmyH* sequence (Coronado et al. 2000).

A solvent stable protease produced by *Geomicrobium* sp. EMB2 a moderately halophilic bacterium has been characterized. The gene had a coding capacity of 375 amino acids. Also, the amino terminus of the mature extracellular purified protease matched with 65th amino acid onwards of the predicted polypeptide sequence. According to gene bade date, the protease clustered with serine protease marine gamma proteobacterium HTCC2207, *Shewanella loihica* PV-4, *Renibacterium salmoninarum* and other related *Bacillus* sp. and show a 40 % of amino acid hydrophobic residues (Karan et al. 2011).

14.9 Conclusion

In this chapter are discussed diverse methods and techniques for isolation and screening of halophilic bacteria with capacity to produce hydrolytic enzymes. Use of these techniques may help to perform a deep study for characterization of this kind of microorganisms, and generate valuable information for future studies on halophilic microorganisms and enzyme and search for more biotechnological applications. In the near future, halophilic bacteria will be used for their enzymes principally in biocatalysis processes, where the use of organic synthesis is indispensable. Halophilic enzymes may perform a reaction at low aqueous activity and tolerate organic solvents as culture medium which allow use them in pharmaceutical, food and chemical industries. In addition, enantioselectivity and stereoselectivity are very important characteristics of hydrolytic enzymes and are very important in bio-catalysis process. However, studies on halophilic enzymes until now are non significant because there are only few reports about genetic and proteomic studies. For this reason, it will be interesting to know more about genetic of halophilic enzymes and in a future performed studies of cloning, recombinant DNA or over-expression of halophilic enzymes, contributing to generation of new enzymes appropriate for modern biotechnological industries.

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