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MINI-REVIEW

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Potential of halotolerant and halophilic microorganisms for biotechnology

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Abstract Halotolerant or halophilic microorganisms, able to live in saline environments, offer a multitude of actual or potential applications in various fields of biotechnology. The technical applications of bacteriorhodopsin comprise holography, spatial light modulators, optical computing, and optical memories. Compatible solutes are useful as stabilizers of biomolecules and whole cells, salt antagonists, or stress-protective agents. Biopolymers, such as biosurfactants and exopolysaccharides, are of interest for microbially enhanced oil recovery. Other useful biosubstances are enzymes, such as new isomerases and hydrolases, that are active and stable at high salt contents. Halotolerant microorganisms play an essential role in food biotechnology for the production of fermented food and food supplements. The degradation or transformation of a range of organic pollutants and the production of alternative energy are other fields of applications of these groups of extremophiles.

Key words Halotolerant/halophilic · Biotechnology · Bacteriorhodopsin · Compatible solutes · Biopolymers · Halofermentation · Waste treatment (biodegradation)

Introduction

As a result of adaptation to their environment, many extremophilic microorganisms have evolved unique properties of considerable biotechnological and, therefore, commercial significance. The objective of this review is to summarize the biotechnological potential of halophilic/halotolerant microorganisms, focusing on recent developments in this area. The taxonomy, physiology, and biochemistry of these organisms, partly including applications, have been reviewed by Galinski and Tindall (1992), Eisenberg et al. (1995), Grant et al. (1998), Ventosa et al. (1998), and Oren (1999). Briefly, there are two strategies to cope with a saline environment. Halophilic Archaea maintain an osmotic balance of their cytoplasm with the hypersaline environment by accumulating high concentrations of salt. This mechanism of osmoregulation requires special adaptations of the intracellular enzymes that have to function in the presence of salt. In contrast, halophilic or halotolerant eubacteria are characterized by a much greater metabolic diversity. Their intracellular salt concentration is low, and they maintain an osmotic balance of their cytoplasm with the external medium by accumulating high concentrations of various organic osmotic solutes; their intracellular enzymes have no special salt tolerance.

Microorganisms able to grow in the presence of salt are found in all three domains of life: Archaea, Bacteria, and Eukarya. Nonhalophilic microorganisms, able to grow in the absence as well as in the presence of salt, are designated halotolerant; those halotolerants that are able to grow above approximately 15% (w/v) NaCl (2.5 M) are considered extremely halotolerant. Microorganisms requiring salt for growth are referred to as halophiles. According to the most widely used definition, that of Kushner (1978), one can distinguish between slight halophiles [many marine organisms; seawater contains about 3% (w/v) NaCl], moderate halophiles [optimal growth at 3%–15% (w/v) salt], extreme halophiles [optimal growth at 25% (w/v) NaCl; halobacteria and halococci], and borderline extreme halophiles [requirement of at least 12% (w/v) salt].

Bacteriorhodopsin

Certain extremely halophilic Archaea contain membrane-bound retinal pigments, bacteriorhodopsin (BR) and halor-

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hodopsin, that enable the organisms to use light energy directly to drive bioenergetic processes by the generation of proton and chloride gradients, respectively (Oren 1994; Lanyi 1995). BR is a 26.5-kDa protein; its transmembrane domain has been revealed as seven helical protein segments. Photons are captured via a retinal chromophore. After the retinal is excited by a photon, a photocycle is initiated in which both the retinal and the protein pass through a series of intermediate conformations. Different states of protonation and absorption maxima result in changed colors of BR. The entire photocycle takes about 10 ms at ambient temperature.

The excellent thermodynamic and photochemical stability of BR has led to many uses in technical applications based on its protonmotive, photoelectric, and photochemical properties. The applications comprise holography, spatial light modulators, artificial retina, neural network optical computing, and volumetric and associative optical memories. A three-dimensional memory is under study. BR is commercially offered in the form of purple membrane patches, isolated from *Halobacterium salinarum* (formerly *H. halobium*) strain S9. It is sold by a Spanish company (COBEL, Barcelona) and a German company (MIB, Munich Innovative Biomaterials) in the lyophilized form with a 75% (by weight) BR content. MIB offers also films and devices made of genetically modified BR for optical information storage and processing. The modified BR (from *H. salinarum* sp. L33) differs from the wild type by a single amino acid exchange and allows adjustment of the membrane lifetime continuously over a range from 10 ms to more than 100 s by changing the extramolecular pH. One of the most interesting applications of BR is its usage as a photosensitive and erasable material for optical information recording and processing, especially in holography. BR films show a high spatial resolution (≥35,000 lines/mm) and an excellent reversibility (>10^6 write/erase cycles); the spectral range is 400–700 nm.

Highly oriented films composed of purple membranes have been obtained by using two kinds of bispecific antibodies with different antigen-binding sites, one binding to a specific side of BR and the other to a phospholipid hapten. These films were used in the construction of a light-sensitive photoelectric device (Koyama et al. 1994). BR-based color sensors that use biotechnologically modified variants of BR were developed, and their ability to recognize colors has been demonstrated (Frydrych et al. 1998). All-optical devices that include BR as photochromic material (e.g., optical switches and modulators, and logic gates, such as optical AND and OR gates), can be used in a wide variety of systems, such as optical signal processes and optical computers (Rao et al. 1998). Other patented applications of BR include its use as a bioelement in a motion sensor (Ackley and Shieh 1998), in an image sensor, or in a biocomputer (Kikura et al. 1998).

A prototype memory subsystem uses BR molecules to store digital bits. The photocycle of BR makes it an ideal AND data-storage gate. Due to the remarkable stability of BR, the data recorded on a BR storage device should be stable for approximately 5 years; the system should operate nearly as fast as semiconductor RAM (Thompson 1996). The optical properties of BR could be exploited to manufacture electronic ink for laptop displays. Electronic ink obtains its color by reflecting ambient light, not from an internal battery-driven light source. This use would be an important contribution to the problem of battery lifetime in portable computing (American Physical Society 1997 online).

Closed shells (vesicles) form spontaneously from the purple membrane of *H. salinarum* in the presence of the detergent octylthioglucoside (OTG) at a protein/OTG ratio of 2:1 by weight (Denkov et al. 1998). The size distribution of the vesicles was almost independent of the incubation conditions (mean radius, 17.9–19 nm). The conditions for vesicle formation and the mechanical properties of the vesicles could be of interest with respect to the application of the BR vesicles as light energy converters.

Another application of BR is the renewal of biochemical energy, i.e., the back conversion from ADP to ATP. Such a solar-driven recycling system could be of interest for biotechnological processes that need large amounts of expensive ATP (Groß 1997). A patented ATP-synthesizing device, useful for bioelements, has been obtained by using BR and ATP synthase (Saito et al. 1992).

Compatible solutes

Osmotically active substances, the so-called compatible solutes, maintain the halotolerant/halophilic cell in positive water balance and are compatible with the cellular metabolism. These low molecular weight substances are highly water-soluble sugars or sugar alcohols, other alcohols, amino acids, or their derivatives (Galinski 1995; da Costa et al. 1998; Ventosa et al. 1998). Compatible solutes have gained increasing interest for biotechnological applications as stabilizers of biomolecules (enzymes, DNA, membranes) and whole cells, salt antagonists, or stress-protective agents.

One of the most abundant osmolytes in nature is ectoine. Ectoines are common in aerobic heterotrophic Eubacteria (Galinski 1995). In contrast to betaine, ectoines and hydroxyectoines can only be obtained by biotechnological procedures. A novel biotechnological process called "bacterial milking" has been established for the production of these two compounds by the extremely halotolerant *Halomonas elongata*. This strain produces, as do other halophiles or halotolerants, the compatible solutes in response to the salinity of the medium. After a high-cell-density fermentation [(to obtain about 48 g/l cell dry weight (dw)], cells were fivefold concentrated using crossflow filtration. When transferred to a low-salinity medium [hypoosmotic downshock from 15% to 3% (w/v) NaCl], bacterial cells rapidly released their solutes to achieve osmotic equilibrium. Subsequent reincubation in a salted medium (hyperosmotic upshock, back to 15% NaCl) resulted in the resynthesis of these compatible solutes. After a regeneration time of 1 day, this procedure could be repeated. After

nine repetitions, 155 mg ectoine/g cell dw per cycle was produced (Sauer and Galinski 1998).

The relative proportion of ectoine and hydroxyectoine in *H. elongata* depends on salinity and temperature. At salinities up to 15% (w/v) NaCl and below 25°C, the strain produced only ectoine. The hydroxyectoine content increased with higher salt concentrations and higher temperatures [50% yield at 20% (w/v) NaCl and 40°C]. In comparison with fed-batch fermentation, bacterial milking is superior as soon as more than two cycles are applied. This process may also be useful for the production of other low molecular weight compounds. The required microorganisms must withstand osmostic shocks and must have a broad salt tolerance. Thus, a biotechnological application of marine cyanobacteria, which produce interesting low molecular weight compounds but are difficult to grow at high cell densities, is unlikely (Sauer and Galinski 1998). A patented process for the recovery of useful metabolites from halophilic/osmophilic and halotolerant/osmotolerant cells that includes bacterial milking is useful for the selective isolation of pure isomers from isomeric mixtures. These substances are accumulated but not metabolized under appropriate conditions (Galinski et al. 1997).

The moderately halophilic *H. elongata* strain KS3, isolated from a salty soil in Thailand and able to grow in the presence of 0.3%–21% (w/v) NaCl and at temperatures of 5°–45°C, also accumulates ectoine and hydroxyectoine under salt-induced hyperosmotic stress. Ectoine production was induced immediately by NaCl addition, whereas hydroxyectoine was detected with a lag in time at NaCl concentrations higher than 1.71 M (Ono et al. 1998). Ectoine, produced by strain KS3, is used to retain and stabilize the activity of enzymes such as amylase, lipase, cellulase, or protease. It is added to the enzymatic solution in an amount of 0.05–50% (w/v), preferably 0.1%–25% (w/v) (Toyoda et al. 1997). Ectoin and ectoin derivatives are further patented as moisturizers in cosmetics for the care of aged, dry, or irritated skin (Motitschke et al. 2000). One of the most promising applications is the use of ectoine as stabilizers in the polymerase chain reaction (PCR) (Sauer and Galinski 1998).

Betaines are the typical compatible solutes of halophilic phototrophic bacteria (Galinski 1995). Nyyssola et al. (2000) studied a novel biosynthetic pathway in two phylogenetically distant extreme halophiles, *Actinopolyspora halophila* and *Ectothiorhodospira halochloris*. They identified a three-step series of methylation reactions from glycine to betaine, catalyzed by methyltransferases. *E. halochloris* methyltransferase genes were expressed in *Escherichia coli*, and betaine accumulation and improved salt tolerance of the heterologous organisms were demonstrated. The efficiency of the methyltransferase pathway for improving the osmotic tolerance of commercially important crops, such as potato, rice, tomato, and tobacco, which do not accumulate betaine, has yet to be tested.

Diglycerol phosphate accumulates under salt stress in the hyperthermophile *Archaeoglobus fulgidus*. This new compatible solute is a potentially useful protein stabilizer, as it exerted a considerable stabilizing effect against heat inactivation of various dehydrogenases and a strong protective effect on rubredoxins (with a fourfold increase in the half-lives) from *Desulfovibrio gigas* and *Clostridium pasteurianum* (Lamosa et al. 2000). Trehalose, an osmolyte in several halotolerant bacteria, could be useful as a cryoprotectant for the freeze-drying of biomolecules, but also for long-term conservation of microorganisms, as the membrane structure is preserved in the presence of this disaccharide (Galinski and Tindall 1992).

Biopolymers

Biosurfactants

Biosurfactants enhance the remediation of oil-contaminated soil and water. By decreasing surface tension, they increase the solubility and thus mobility of hydrophobic hydrocarbons, which may promote degradation. Biosurfactant-producing halophilic/halotolerant microorganisms may thus play a significant role in the accelerated remediation of oil-polluted saline environments. Encouraging results have been obtained in hydrocarbon pollution control in marine biotopes in closed systems, such as oil storage tanks, and several studies indicated potential for pollution treatment in marine environments or coastal areas (Banat et al. 2000). New biosurfactants (trehalose lipids), produced by marine rhodococci during cultivation on *n*-alkanes, could constitute promising surface-active agents for in situ bioremediation of cold marine environments (Yakimov et al. 1999).

The application of biosurfactants for in situ microbially enhanced oil recovery (MEOR) requires organisms that grow and produce these surfactants under the prevailing environmental conditions. Many petroleum reservoirs are anaerobic and have high salinity and temperature. One of the most effective biosurfactants is lychenisin, a cyclic lipopeptide produced by *Bacillus licheniformis* JF-2. The strain grows anaerobically and has been used in core-flood experiments (Thomas et al. 1993). Lychenisin is not affected by the temperatures, pH ranges, and salt concentrations typical of many oil reservoirs (McInerney et al. 1990). A continuous process was developed for production of this lipopeptide, resulting in an increase in the yield from 5.3 to 11.2 mg/l (Lin et al. 1993). Lychenisin is commercialized by Multi-biotech (Geodyne Technology) (Desai and Banat 1997). Another powerful surface-active agent is lychenisin A, produced both aerobically and anaerobically by the thermo- and halotolerant *B. licheniformis* BAS50. Lychenisin A, a mixture of lipopeptides, shows a lowering of surface tension at NaCl concentrations up to 30% (w/v) and is effective at dilute concentrations (Yakimov et al. 1995). Unfortunately, the cost of biosurfactant production is about three to ten times higher than that of the chemical counterparts, which is too expensive for MEOR (Desai and Banat 1997).

Exopolysaccharides

Halophilic exopolysaccharide (EPS) producers are also an interesting source for MEOR where polymers with appropriate properties (high viscosity even at diluted concentrations and high temperatures, pseudoplasticity, resistance to salt and thermal degradation) act as emulsifiers and mobility controllers. Active emulsification of petroleum has been noted for six strains, close to *Halobacterium salinarum, Haloferax volcanii*, and *Halobacterium distributum* (Kulichevskaya et al. 1992). EPS produced by moderately halophilic bacteria of the genus *Halomonas* (up to 2.8 g EPS/l) emulsified hydrocarbons more efficiently than four reference surfactants (Bouchotroch et al. 2000). Maximum EPS production by 19 strains of the species *Halomonas eurihalina* was 1.6 g/l. All EPS studied had an unusually high sulfate content; one strain produced an exopolymer containing significant amounts of uronic acid (Bejar et al. 1998).

Sulfated EPSs are known to interfere with the penetration of viruses into host cells. A stable and effective biological system for the production of this compound was obtained by immobilizing cells of the halophilic cyanobacterium *Aphanocapsa halophytica* on light-diffusing optical fibers. Because of the efficient utilization of light energy, EPS production was tenfold enhanced (Matsunaga et al. 1996). This immobilization method was also successfully applied for enhanced glutamate production by the marine cynobacterium *Synechococcus* sp. (Matsunaga et al. 1991). Other EPS-producing cyanobacteria are the halotolerant *Anabaena* sp. ATCC 33047 (Moreno et al. 1998) and *Cyanothece* sp. ATCC 51142 (Shah et al. 1999). Maximum EPS production by the latter strain was found to occur at 4.5% (w/v) NaCl and pH 7. The gelation of EPS under alkaline conditions was employed to remove dyes from textile effluent.

Liposomes

Liposomes are used in medicines and cosmetics for the transport of compounds to specific target sites in the body. Ether-linked lipids from archaeal halophiles have a high chemical stability and resistance against esterases and thus a higher survival rate than liposomes based on fatty acid derivatives (Galinski and Tindall 1992; Gambacorta et al. 1995). Novel, patented ether lipids were obtained from the extreme halophile *Halobacterium cutirubrum*. Homogeneous liposomes were prepared from emulsions of the total polar ether lipid extracts of the bacteria by pressure extrusion through membranes of various pore sizes. The ether liposomes were stable to attack by phospholipase A2 and B and could be stored for at least 60 days in an atmosphere of air (Choquet et al. 1999).

Poly(γ-D-glutamic acid)

The exopolymer poly(γ-D-glutamic acid) (PGA) can be used as a biodegradable thickener, humectant, sustained-release material, or drug carrier in the food or pharmaceutical industry (Kunioka 1997). Hezayen et al. (2000) reported the first description of a PGA-producing extremely halophilic archaeon related to the genus *Natrialba*. PGA production by this new isolate began at 20% (w/v) NaCl (470 mg PGA/l culture volume was detected after 90 h) and reached its maximum at NaCl saturation.

Lectins

Lectins, selective sugar-binding proteins, are useful tools for cell typing and cell-surface research. They are used as indicators of cell-surface modifications for the detection of malignant cells. Lectins from halophilic Archaea might be useful for archaeal typing and analysis of their cell-surface carbohydrates; they have not yet been exploited because of the high salinity required for the structural integrity of these microorganisms. Gilboa-Garber et al. (1998) defined salt concentration thresholds high enough for archaeal survival and sufficiently low for lectins to bind to them. Concanavalin A was the most reactive lectin because of its glucose and mannose binding.

Bioplastics

Polyhydroxyalkanoates (PHA) are intracellularly accumulated bacterial storage compounds. Properties of some PHAs are comparable to those of polyethylene and polypropylene (Steinbüchel et al. 1997). Such biodegradable plastics could replace oil-derived thermoplastics in some fields. *Haloferax mediterranei* accumulates large amounts (up to 60% of cell dw) of poly (β-hydroxy butyric acid) (PHB). PHB production can be enhanced to about 6 g/l using phosphate limitation and starch, a cheap substrate, as carbon source (Rodriguez-Valera and Lillo 1992). PHB recovery is simplified because the exposure of the halophile to low salt concentrations causes cell lysis (Ventosa and Nieto 1995). Another advantage of the halophile is that it can be cultivated easily in a simple saline open pond without risk of contamination. An extremely halophilic archaeon, isolated from an Egyptian saline soil, was recently described to accumulate PHB as intracellular granules with an amount of about 53% of its cell dw. The most suitable carbon sources were *n*-butyric acid and sodium acetate (Hezayen et al. 2000).

Enzymes

Most halophilic enzymes are inactivated and denatured at concentrations below 1 M NaCl (Adams and Kelly 1995). Their high solubility in highly concentrated NaCl solutions makes the use of standard chromatographic procedures difficult (Eisenberg et al. 1995). Halophilic adaptation of enzymes at a molecular level has been reviewed by Madern et al. (2000).

Hydrolases

Optically active amino acids are widely used as intermediates in the pharmaceutical industry for the synthesis of semisynthetic antibiotics, peptide hormones, and pesticides. A chemoenzymatic route for the synthesis of various D-amino acids involves the conversion of DL-5-substituted hydantoines. They are asymmetrically hydrolyzed to *N*-carbamoyl-D-amino acid by D-specific hydantoinase (dihydropyrimidase). The product is then further chemically converted to the corresponding D-amino acids under acidic conditions. Joshi et al. (2000) patented a novel process for the preparation of D(-)*N*-carbamoylphenylglycine (CPG), using hydantoinase from halophilic *Pseudomonas* sp. ATCC 55940. The strain was isolated from seawater and was described to grow at NaCl concentrations above 7% (w/v) and to produce 5%–6% CPG within 10–15 h. Sudge et al. (1998) screened about 100 bacterial colonies isolated from seawater for hydantoinase activity. Biotransformation by halophilic *Pseudomonas* sp. NCIM 5109 under alkaline conditions allowed the conversion of 80 g D-5-phenylhydantoin/l to 82 g CPG/l within 24 h with a molar yield of 93%. The strain required NaCl for both growth [optimal at 2% (w/v) NaCl] and enzyme production (optimum activity at pH 9–9.5 and 30°C).

β-Galactosidases can be used as a catalyst in the synthesis of galactooligosaccharides, using lactose as substrate and nucleophile (Boon et al. 2000). Such oligosaccharides are used as prebiotics. The extremely halophilic β-galactosidase from *Haloferax alicantei* is optimally active at 4 M NaCl. Purification of the enzyme was facilitated by the ability of sorbitol to stabilize enzyme activity in the absence of salt, which allowed conventional ion-exchange chromatography (Holmes et al. 1997).

Agar is a complex polysaccharide extracted from marine red algae. Few agar-degrading bacteria have been isolated. Commercially available sugar sources from red algae could provide economically profitable feed additives or human food products. A patented agarase system originates from *Alteromonas* sp. (ATCC 43961) that was isolated from a salt marsh and can tolerate a wide temperature, pH, and salinity range. The agarase system was claimed to be of economic importance in the effective production of oligosaccharides, including neoagarobiose, neoagarotetraose, and neoagarohexaose. The enzyme could also be used for the effective control of red algae bloom contaminations or for the treatment of biofouled submerged marine surfaces (Stosz et al. 1995). A new recombinant β-agarase was obtained by introducing and expressing the agarase gene of *Pseudomonas* sp. W7 in *E. coli.* The enzyme was halophilic and had its maximum activity at 0.9 M NaCl, pH 7.8, and 20°–40°C. It catalyzed the hydrolysis of agar and agarose, yielding neoagarotetraose as the main product (Ha et al. 1997). Five gram-negative halophilic and moderately thermophilic bacteria, growing optimally at 2%–3.5% (w/v) NaCl, were the first thermophiles found to degrade agar; they were considered to represent a new genus and species, named *Alterococcus agarolyticus* (Shieh and Jean 1998).

The continuous production of halophilic α-amylase can be performed via whole-cell immobilization of *Halobacterium salinarum* in alginate beads and a polyvinyl alcohol film (Bagai and Madamwar 1997). The cells were osmotically stable and showed continuous enzyme production for 45 days. The stabilized cells could be permeabilized by chloroform treatment without leakage of the intracellular components. Using this procedure, cells can be reused under improved stabilized conditions for biotechnological applications. Mountfort et al. (1998) described a psychrophilic, halophilic, aerotolerant, anaerobic bacterium that was isolated from anaerobic sediments of a high-salinity pond in Antarctica and designated as the new species *Psychromonas antarcticus*. The amylolytic ability of the organism is of biotechnological interest in view of low-temperature bioprocessing of starch. Coronado et al. (2000) isolated, cloned, and characterized the first extracellular amylase-encoding gene, *AmyH*, from the moderate halophile *Halomonas meridiana* DSM 5425. This gene was functional in *Halomonas elongata* and, when cloned, in *E. coli*. *H. meridiana* and *H. elongata* were able to secrete the thermostable α-amylase from *Bacillus licheniformis*. This result demonstrates that members of the genus *Halomonas* are good candidates for use as cell factories to produce heterologous extracellular enzymes.

An extracellular serine protease from the extreme halophile *Halobacterium halobium* (ATCC 43214) was claimed to be an excellent candidate as a catalyst for peptide synthesis, particularly for glycine-containing peptides. The enzyme requires 4 M NaCl for optimal catalytic activity and stability in aqueous solutions (Ryu et al. 1994). Addition of organic solvents, such as dimethylformamide, had a positive effect on enzyme stability in low-salt media. The stabilization of halophilic enzymes by organic cosolvents while lowering the required salt concentration is of practical importance as the corrosive nature of high NaCl concentrations can be avoided. Higher substrate solubilities in the presence of an organic cosolvent can be useful in synthetic reactions catalyzed by enzymes from extreme halophiles (Kim and Dordick 1997).

Recently, 99 extremely halotolerant *Bacillus* strains, most of them able to grow at 20%–25% (w/v) salt, were isolated from hypersaline environments (Garabito et al. 1998). Their discovery could be of great biotechnological potential because many *Bacillus* isolates produce industrially important hydrolases.

Isomerases

A thermostable type I-group B DNA topoisomerase has been isolated and purified from the hyperthermophilic methanogen *Methanopyrus kandleri* (Slesarev 1997). The novel, patented enzyme (designated topisomerase V) is the first type I-group B topoisomerase known from a prokaryote. It relaxes both negatively and positively supercoiled DNA and can unwind closed circular DNA (ccDNA). The enzyme is active over a wide range of temperatures and salt conditions and does not require magnesium or ATP for its

activity, which makes manipulations on DNA more convenient and more efficient. Exploitation of the common features and the differences of type I-group B topoisomerases from eukaryotic and prokaryotic origin will be important for modeling novel drugs. The novel enzyme is recognized by antihuman topoisomerase I antibody; this may be important in further understanding the interaction of human topoisomerase I and cancer chemotherapeutic agents. An aspect of medical utility is the identification of the human ScL-70 antigen as DNA topoisomerase I. Scleroderma (progressive systematic sclerosis) patients may produce high-titer autoimmune antibody directed against human topoisomerase I. It is conceivable that human autoantibody could recognize type I-group B topoisomerases from prokaryotic organisms because type I-group B topoisomerases of higher plants are also recognized, despite the divergence of the kingdoms (Slesarev 1997).

Peptidyl prolyl *cis-trans* isomerase (PPIase) is useful for the regeneration of denatured protein, for the stabilization of proteins, for the production of recombinant protein, and for the development of novel immunosuppressant and physiologically active substances. Iida et al. (1997) patented a novel cyclophilin type PPIase gene by amplifying the genome DNA of *Halobacterium cutirubrum* DSM669.

Food biotechnology

Fermentation products

Halotolerant microorganisms play an essential role in various fermentation processes that occur in the presence of salt. These organisms catalyze the fermentation, thereby producing various compounds that give the characteristic taste, flavor, and aroma to the resulting products.

In the production of pickles (fermented cucumbers), brine strength is increased gradually from 5% to 15.9% (w/v) NaCl. Fermentation of Sauerkraut (pickled cabbage) occurs in the presence of 2.25%–2.5% salt. *Lactobacillus plantarum* is the most essential species in both cases. *Halobacterium salinarum, Halococcus* sp., *Bacillus* sp., pseudomonads, and coryneform bacteria are used in the production of an Asian (Thai) fish sauce (nam pla) in which fish is fermented in concentrated brine (Thongthai and Suntinanalert 1991). The homo-fermentative lactic acid bacterium *Tetragenococcus halophila* is the dominant microorganism during the brining stage in Indonesian soy sauce (kecap) fermentation (Röling and van Verseveld 1996) at a salt concentration of about 18% (w/v). Acetate, produced during bacterial growth in the soy mash, contributes to the taste and is inhibitory to spoiling yeasts. Interestingly, *T. halophila* also produces this compound in the absence of mixed acid fermentation; this might be attributed to not yet identified hydrogen-accepting components in the soy mash (Röling et al. 1999).

It is desirable to control microorganisms during fermentations. Growth of halophilic *Lactobacillus lactis*, which has a deteriorating effect in soy sauce of low salt content, can be prevented by adding to the sauce a product obtained from a culture of the filamentous fungus *Monascus* (Araki et al. 1997). The product has a high storage stability. Halotolerant killer yeasts were isolated from various fermented foods in presence of 6% (w/v) NaCl. One of them, *Pichia farinosa* KK1, produces a stable salt-mediated killer toxin. This toxin showed increasing killer activity with increasing salt concentration [maximum activity at 12% (w/v) NaCl] on certain strains of *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* (Suzuki and Nikkuni 1994).

Food supplements and food colorants

Long-chain polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid, eicosapentaenoic acid, arachidonic acid, and γ-linolenic acid, are attracting considerable attention as dietary supplements to counteract deficiency in essential fatty acids (Nichols and Russell 1999). Currently, many PUFA are obtained from fish oil, which is associated with several problems, such as the undesirable odor and the difficulties of large-scale purification. Also, fish stocks continue to diminish worldwide. A promising alternative source are marine bacteria, which allows conventional purification procedures, rapid production rates, and consistency of product quality and yield. Antarctic marine bacteria, mainly species of *Shewanella* and *Colwellia*, contain significant proportions of PUFA (Nichols et al. 1993; Nichols and Russell 1999).

β-Carotene is used in the food industry as a natural food colorant. As a precursor of vitamin A, it is of importance as an additive in cosmetics, multivitamin preparations, and health food products. New carotenoid-producing bacteria were isolated from saline soil in Egypt. An extremely halophilic bacterium exhibited optimum growth and carotenoid production in presence of 25% (w/v) NaCl. It produced 2.06 mg total carotenoids/g cell dw, including 0.06 mg β-carotene and 0.7 mg canthaxanthin (Asker and Ohta 1999); this was the first report of canthaxanthin production by an extreme halophile. Canthaxanthin is used in cosmetics to decrease the necessary exposure time in sunlight to acquire a tan and to intensify the tan as the compound attaches to the subcutaneous layer of fat.

To reduce the production cost of pigments, a method for the reuse of microbial cells without their breakage has been patented. Halophilic microalgae of the genus *Platymonas* accumulate a red pigment in the stationary phase. This pigment is extracellularly secreted if the algal body is immersed in a solution of a tertiary amine, such as *N,N*-bis(2-hydroxyethyl)glycine and *N*-2-hydroxypiperazine-*N′*-2-ethanesulfonic acid (Matsunaga and Shibazaki 1994).

Halofermentation

The high salt tolerance of extreme halophiles enables their cultivation under nonsterile, and thus cost-reducing, condi-

tions. Because of the highly corrosive nature of salt-rich media, these organisms cannot be cultivated in stainless steel-containing bioreactors. All parts of the bioreactor that are in contact with the medium should be replaced by alternative materials. Hezayen et al. (2000) constructed a novel corrosion-resistant stirred tank bioreactor (6-l culture volume) composed of polyetherether ketone. This reactor was used for the optimization of PHB and PGA production by two extremely halophilic strains (see foregoing) in media containing at least 25% (w/v) NaCl. Cell mass and polymer yield were comparable to flask cultivation.

For the harvesting of cells in fermentation processes, buoyancy of cells is desired. DasSarma et al. (1999) constructed a recombinant vector capable of directing the synthesis of gas vesicles in nonfloating cells. The vector contains genes encoding *Halobacterium halobium* proteins that are required for the synthesis of gas vesicles. Transformed cells float in an aqueous medium and can be separated by skimming or decanting.

Biological waste treatment

Hydrocarbons

The ability of halophiles/halotolerants to oxidize hydrocarbons in the presence of salt is useful for the biological treatment of saline ecosystems contaminated with petroleum products. Successful bioremediation of oil spills has been observed in marine, Arctic, and Antarctic environments (Delille et al. 1998; Margesin and Schinner 1999). In contrast to the assumption of an inverse relationship between oil biodegradation and high salinity, i.e., above 20% (w/v) (Ward and Brock 1978), a new halo- and thermotolerant *Streptomyces albaxialis* was found that was able to degrade crude oil and petroleum products even in the presence of 30% (w/v) NaCl (Kuznetsov et al. 1992). Extremely halophilic Archaea (one of them identified as *Haloferax mediterranei*), able to grow at 10%–25% (w/v) NaCl, utilized oil as the sole carbon source (Zvyagintseva et al. 1995). Kulichevskaya et al. (1992) obtained for the first time an isolate of the *Halobacterium* group from salt-rich stratum fluids of an oil deposit. The strain degraded *n*-alkanes with a C_{10}–C_{30} composition in the presence of 30% (w/v) NaCl.

Salt marshes are occasionally impacted by crude oil spills. Bioremediation may be an effective method in removing oil without damage to the physically sensitive ecosystem. Studies on biodegradation of crude oil in Lousiana salt marshes were performed. Polycyclic aromatic hydrocarbons (PAH) and alkanes degraded simultaneously in microcosm laboratory studies when 0.7 g crude oil/g soil was applied (Jackson and Pardue 1999). Seasonal studies from the same site demonstrated that the mineralization of model alkanes (hexadecane) and PAHs (phenanthrene) was uncoupled (Jackson and Pardue 1997). Low intrinsic degradation rates (0%–3.9%/day) of the alkane component (C_{11}–C_{14}) but high degradation rates (8%–16%/day) of the PAH fraction (naphthalene and phenanthrene) were found (Jackson and Pardue 1999). Nitrogen addition enhanced degradation of total PAHs and many alkanes, especially those larger than C_{20}, while naturally present P was found to be sufficient. In contrast to this result, P significantly increased bioremediation of artificially weathered crude oil, applied at 4,620 kg oil/ha in sediments from a salt marsh near Texas (Wright et al. 1997). Inipol, an oleophilic fertilizer containing N, P, and a dispersant, increased oil degradation significantly, whereas N fertilization (urea and ammonium), with or without P, had no effect.

Woolard and Irvine (1994) demonstrated the applicability of heterotrophic, halophilic bacteria for the treatment of hypersaline wastewaters using a novel periodically operated biofilm reactor (sequencing batch biofilm reactor). A biofilm of halophiles, isolated from the Great Salt Lake, readily developed on the tubing surface and could remove more than 99% of phenol from a synthetic waste brine containing 15% (w/v) NaCl. Hinteregger and Streichsbier (1997) reported the suitability of a moderately halophilic *Halomonas* sp. for the biotreatment of saline phenolic wastewater. This strain degraded 0.1 g phenol/l as the sole carbon and energy source in a model industrial saline wastewater (similar to the wastewaters in the oil industry) containing 1%–14% (w/v) NaCl and exhibited optimum growth on phenol at 5% (w/v) NaCl. Two phenol-degrading microorganisms, *Candida tropicalis* and *Alcaligenes faecalis*, were isolated from Amazonian rain forest soil that had never been contaminated with man-made phenolic compounds (Bastos et al. 2000). The yeast tolerated higher concentrations of phenol and salt than the bacterium and degraded 1.5 g phenol/l in the presence of 15% (w/v) salt within 148 h. The bacterium utilized 1.1 g phenol/l in the presence of 5.6% (w/v) salt within 200 h.

Halogenated organic compounds are of environmental concern because of their persistence and toxicity. The use of methane-metabolizing bacteria has been patented for the inexpensive and efficient bioremediation of seawater contaminated with such hydrocarbons. A halophilic, methane-assimilating *Methylomicrobium* sp. is able to oxidize halogen-containing organic compounds, such as trichloroethylene, in aqueous medium with 2%–6% (w/v) salt (Fuse 1998). A slightly halophilic and alkaliphilic *Nocardioides* sp. (optimum pH and sodium concentration for growth, 9–9.4 and 0.2–0.4 M NaCl, respectively) has a broad spectrum of chlorophenol degradation. The organism could utilize 2,4-dichlorophenol; 2,4,5-trichlorophenol (these compounds are generated from agricultural biocides); and up to 1.6 g 2,4,6-trichlorophenol/l (used as a wood preservative) as the sole energy source (Maltseva and Oriel 1997). Halophilic Archaea, preferably belonging to the genera *Haloarcula*, *Halobacterium* (e.g., strain CB1, DSM11147), and *Haloferax,* are subjected to a patented selection process, in the course of which they are adapted to high concentrations (up to 1 mM) of halogenated hydrocarbons, such as trichlorophenols, or the insecticides lindane and DDT (Oesterhelt et al. 1998). Maltseva et al. (1996) isolated the first moderately halophilic eubacteria able to completely mineralize the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). The

most active strain degraded up to 3 g 2,4-D/l in 3 days; optimal growth on this compound was observed at 3.6%–6% (w/v) NaCl.

2-Hydroxybenzothiazole (OBT), an aromatic hetercyclic compound, is present in wastewaters from the industrial production of the rubber vulcanization accelerator 2-mercaptobenzothiazole. De Wever et al. (1997) reported the first isolation of bacteria able to utilize OBT as sole energy source. Growth on OBT by a *Rhodococcus rhodochrous* strain was not significantly influenced by salt concentrations between 1% and 3% (w/v).

Another source of contaminants are organic solvents. Aerobic transformation of formaldehyde by a moderately halophilic eubacterium in presence of 1%–20% (w/v) salt was described by Oren et al. (1992). Mineralization of *N,N*-dimethylformamide (200 mg/l) by bacterial consortia was observed at various salt (0.2%–7% w/v NaCl) conditions; bacterial growth rate decreased with increasing salinity (Bromley-Challenor et al. 2000). A new halotolerant *Brevibacterium* strain, capable of degrading cyclohexanone (this alicyclic compound is used as a solvent for resins, waxes, fats, etc.) and cyclohexanol in the presence of 10%–15% NaCl, was isolated from an industrial wastewater treatment plant. Cyclohexanone oxidation is inducible; the genes of two enzymes responsible for the oxidation (cyclohexanone monooxygenases) were identified, isolated, and expressed in *E. coli* (Brzostowicz et al. 2000).

Recently, the first halophilic bacteria able to degrade synthetic polymers, such as the novel polyester amide BAK 1095, were found (Wiegand et al. 1999).

Detoxification of chemical warfare agents

Several halophilic isolates produce organophosphorus acid anhydrases (OPAA) with hydrolytic activity against several organophosphorus chemicals and related compounds, such as sarin and soman. These enzymes have considerable potential for the decontamination and demilitarization of chemical warfare agents. G-type nerve agent-degrading enzyme activity was found in halophilic *Alteromonas* JD6.5 (DeFrank et al. 1993). A cloned gene, encoding OPAA from this strain, is used for the production of a recombinant enzyme that is involved in the recently patented enzymatic detoxification of organophosphorus compounds. The composition may be prepared as a dry powder and reconstituted with water when needed, and can be applied to contaminated surfaces or substances (Cheng and DeFrank 2000).

Chemical oxygen demand (COD) removal

Kargi and Uygur (1996) tested the biological treatment of a synthetic saline [0%–5% (w/v) NaCl] wastewater, containing diluted molasses and urea, in an aerated percolator column with immobilized cells on ceramic particles. High COD removal efficiencies at salt concentrations above 4% (w/v) were obtained with halophilic *Halobacter halobium*, whereas salt-tolerant microorganisms from activated sludge were more active at low salt concentrations [1%–2% (w/v)]. Anaerobic treatment of fishery wastewater from the Chilean fishmeal industry, which has a high salt content and high organic load (4–6 kg COD/m^3), was performed using a marine sediment inoculum with methanogenic/sulfate-reducing activity (Aspe et al. 1997).

Alternative energy

Hydrogen is considered to be a likely future energy source because it is easily converted to electricity and easily combustible. The use of photosynthetic bacteria has the advantage that H_2 production can be carried out in light using organic substances that are present in various biological resources. Algal biomass was shown to be appropriate as a material for H_2 production by halotolerant photosynthetic bacteria (Ike et al. 1997). A halophilic bacterial community including photosynthetic bacteria was able to utilize raw starch directly for H_2 production in a single-step culture system in the presence of 3% (w/v) NaCl and light (Ike et al. 1999). Halophilic culture systems would enable cost-saving H_2 production from biomass by means of the use of seawater as a basal culture medium.

Agriculture

Ice-nucleation activity (INA), i.e., the ability to freeze water at subzero temperatures higher than –7° or –5°C, is of advantage in biotechnology for the energy-saving production of artificial snow and ice. In food processing, it is used for ice cream production and efficient freeze concentration without loss of flavor (Lundheim and Zachariassen 1999). The transfer and expression of the responsible gene in nonpathogenic bacteria could be of considerable economic interest. The ice nucleation gene *inaZ* from phytopathogenic *Pseudomonas syringae* has been successfully expressed in various moderately halophilic bacteria (Arvanitis et al. 1995).

Amelioration of soil salinity during crop growth by application of a N-fixing cyanobacterium, *Anabaena torulosa*, was proposed. The cyanobacterium enriched the nitrogen status of moderately saline soils in India, although the fixed N remained confined to the cyanobacterial biomass. The cell-bound sodium remained extracellularly trapped in the mucopolysaccharide sheath of the organism and was thus released after cell death. Removal of the topsoil with the cyanobacterial mats (whereby the fixed N was also removed) decreased the soil salinity significantly (26%–38%) (Apte and Thomas 1997).

One possible strategy to recover saline land for agricultural use is the transfer of halotolerance from halophilic organisms to crops of agronomic value. Transgenic tobacco plants acquired resistance to salt stress after introduction of

the *dnaK1* gene from the halotolerant cyanobacterium *Aphanothece halophytica,* which can grow in saline conditions up to 3 M NaCl. DnaK1 was constitutively expressed. After 3 days of treatment with 0.6 M NaCl, the sodium content in leaves of the transgenic plant remained at levels similar to those in nonstressed plants. Dnak/Hsp70 proteins are known to play a role in the recovery of cells from stress. Herewith, the positive role of heat-shock proteins for enhanced salt tolerance has been demonstrated for the first time (Sugino et al. 1999).

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