

**IDENTIFICATION AND CHARACTERIZATION OF  
BACTERIAL GENES IMPARTING  
OSMOTOLERANCE IN *BACILLUS HALODURANS*  
AND *ESCHERICHIA COLI* ISOLATED FROM  
SALTED FISH**

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## **DECLARATION**

I hereby declare that the thesis entitled “Identification and characterization of bacterial genes imparting osmotolerance in *Bacillus halodurans* and *Escherichia coli* isolated from salted fish” is a record of bonafide research work done by me under the supervision and guidance of Dr. Nirmala Thampuran, and it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles of this University or any other University or Society.

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# **INTRODUCTION**

## 1. INTRODUCTION

Soil salinity is a major constraint to food production because it limits crop yield and restricts the use of land previously uncultivated. The United Nations Environment Program estimates that approximately 20% of agricultural land and 50% of cropland in the world is salt-stressed (Flowers and Yeo, 1995). Most of the crops are salt sensitive in contrast to halophytes, which are native flora of saline environments. Genetic engineering has been used as a relatively fast and precise way to improve salt tolerance in crop plants. Much effort has been made to identify and isolate genes from a variety of sources, including viruses, bacteria, fungi, animals and humans, useful for transfer to plants. Various mechanisms have been elucidated for increased stress tolerance in eukaryotic as well as prokaryotic systems. Out of these, osmolyte biosynthesis is the well-studied and efficient system for development of transgenic plants.

Increase in agricultural productivity has to out-pace the rate of growth in population. This has to happen despite all the adverse abiotic and biotic stress factors under the climate change regime. India, with more than one billion population to be provided with adequate food and nutrition cannot relax. Besides, sustainability of the agricultural production systems, profitability and greater competitiveness in the world market make the task of meeting the targeted growth in agriculture a formidable challenge. Genetic enhancement has been successful in meeting the demands of the everincreasing population largely because of discovery and use of novel genes. The dwarfing genes in wheat and rice, and rust resistance genes in wheat are some of the examples, which stand testimony to the power of genetic technology that ushered in green revolution and subsequently helped sustaining the productivity gains. With the

advent of new biotechnology tools and techniques, it has been possible to access genes from diverse biological systems and deploy in target species. Use of crystal protein genes from the soil bacterium *Bacillus thuringiensis* in genetic engineering of crops like cotton clearly depicts how genes from evolutionarily distant organisms can bring new revolution in agricultural production. Besides Bt genes, several other genes have also been prospected, validated and are being deployed to gain commercial advantage. These efforts encourage prospecting of novel genes for future deployment to enhance and sustain agricultural productivity.

Therefore, the great challenge for the coming decades will be increasing the food production by developing crop varieties with enhanced abiotic stress tolerance and higher water-use efficiency. This needs identification, cloning and functional characterization of genes that confer resistance towards salt and stress. Genes for salt resistance can be isolated from plants as well as microorganisms. Efforts are being made globally to isolated genes from microbes and plants and employ them for genetically engineering salt tolerance of crops. The marine environment, being the reservoir of such salt tolerant species, is a promising source for such genes. In this project, identification and functional characterization of two major osmolytes; ectoine and glycine betaine from bacterial isolates in salted fishes were studied in detail.

Halophiles are salt loving organisms that inhabit hypersaline environments. They include mainly of prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Among halophilic microorganism, variety of heterotrophic and methanogenic archaea, photosynthetic, lithotrophic, and heterotrophic bacteria, photosynthetic and heterotrophic eukaryotes have been reported (Oren, 2002 a). Examples of well

adapted and widely distributed extremely halophilic microorganisms include archaeal *Halobacterium* spp., cyanobacteria such as *Aphanothece halophytica*, and the green algae *Dunaliella salina*. Among multicellular eukaryotes, species of brine shrimp and brine flies are commonly found in hypersaline environments.

Microorganisms have developed a complex stress management mechanism to cope with the changes in their external environment (Sleator and Hill, 2002). One such environmental parameter is the osmolality of the external growth medium. The ability to adapt to changes in the osmolality of the external environment is therefore a challenge for growth and survival of the prokaryotic cells (Csonka and Hanson, 1991).

To avoid plasmolysis, most halophilic eubacteria cope with these conditions by accumulating small, highly water-soluble organic compounds, the so-called compatible solutes (Brown, 1976; Kempf and Bremer, 1998). Ectoine, a compatible solute was first discovered in the extremely halophilic phototrophic sulfobacterium *Ectothiorhodospira halochloris* (Galinski *et al.*, 1985). Ectoine have recently gained importance in biotechnology as it has been used in dermatopharmacy as anti ageing agents in skin creams, as components of shampoo, for oral care and as adjuvants for vaccines (Vargas *et al.*, 2006; Anburajan *et al.*, 2008 a). These osmolytes enable organisms to adapt to a wide range of salt concentrations by adjusting the cytoplasmic solute pool to the osmolarity of the surrounding environment. 1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (ectoine) represent the predominant class of osmolytes in aerobic chemoheterotrophic eubacteria (Severin *et al.*, 1992; Frings *et al.*, 1993; Galinski, 1995) and it is an excellent osmoprotectant (Ono *et al.*, 1999). So far, to our knowledge no group has reported the functional characterization of ectoine

biosynthesis genes from *B. halodurans*. In this study ectoine biosynthesis gene cluster (*ectABC*) from *B. halodurans* was functionally characterized and determined the diversity and phylogenetic relationship of *ectA*, *B* and *C* genes in *B. halodurans* with other eubacteria.

Different classes of chemical compounds, including polyols, sugars, methylamines, and linear and cyclic amino acids and betaines, have been found to act as compatible solutes. Besides functioning as osmotic counterweights, compatible solutes were shown to protect biomolecules and whole cells against denaturation caused by heating, freezing, desiccation, or chemical agents (Schubert *et al.*, 2007). These properties of the compatible solutes have gained more commercial attention.

Among the compatible solutes, Glycine betaine is an effective osmoprotectant. Glycine betaine (*N,N,N*-trimethylglycine) is a quaternary ammonium compound that occurs naturally in a wide variety of plants, animals and microorganisms (Lamark *et al.*, 1991). Numerous *in vitro* experiments have indicated that betaine acts as an osmoprotectant by stabilizing both the quaternary structure of proteins and membrane structures against the adverse effects of high salinity and extreme temperatures. Up to date three enzymatic systems have been described for the formation of glycine betaine from choline (Andresen *et al.*, 1988). Biosynthesis of betaine is catalyzed in a single step reaction by choline oxidase in soil bacterium, *Arthrobacter globiformis* (Fan *et al.*, 2004). Recently, bacterial glycine betaine synthesizing enzymes have become a major target in developing stress tolerant crop plants of economic interest (Hayashi *et al.*, 1997).

Salting and drying has been used for a long time as a method of fish preservation (Huss and Valdimarsson, 1990). Production of canned salted fishes is a

traditional activity in the fishing industry in the countries around the Mediterranean Sea. The annual world production of the salted fishes is estimated at around 2,50,000 tones (Arason and Arnason, 1992). In fishing vessels, fresh fish is stored in wooden boxes covered with ice. Sometimes these boxes are not properly cleaned and cause fish contamination. When salting is used for the preservation of these fishes, spoilage due to tissue autolysis and microbial action is competitive with salt penetration (Filsinger, 1987). Microbial ecology of salted fish products is markedly influenced by the water activity of the product (Chandler, 1988). In salted fishes the NaCl concentration in the fish flesh has reached saturation (19%), makes the fish with a tough consistency and yellowish colour. Earlier studies shows that pink discolouration in the salted fish is due to the growth of extreme halophiles (Bjarnason, 1986). Microbial spoilage of the salted fishes is due to the presence of extremely halophilic archaea and is also responsible for the reddening of salted fishes (Beatty and Fougere, 1957). Moderate halophilic bacteria from salted fish are also found to cause reddening and associated spoilage (Vilhelmsson *et al.*, 1996). A broad range of bacteria inhabit in salted fishes. Among these moderate halophilic bacteria are the dominating floras. Moderate halophilic bacteria were also recovered from seafoods (Yoon *et al.*, 2001). Moderately halophilic bacteria are a heterogeneous group of microorganisms characterized by growth over a wide range of salt concentrations. The optimum growth of these bacteria is at the concentration between 0.5 and 2.5 M NaCl (Kushner, 1978). Several studies have been made of the microbiology and microbial ecology of hyper saline waters (Volcani, 1940; Brisou *et al.*, 1973; Nissenbaum, 1975; Post, 1977).

The commercial uses of halophiles are quite significant; fermentation of soy and fish sauces,  $\beta$ -carotene production (Ben Amotz and Avron, 1989), and aquaculture. The novel and unique properties of many of these organisms suggest that they have even greater potential for biotechnology (Rodriguez Valera and Garcia Lillo, 1992). Halophiles can survive and flourish in environments that limit the growth of most other organisms. Hypersaline environments are ubiquitous and they are spreading as a result of irrigation and other uses of fresh water. Many natural geological formations, such as petroleum reserves are associated with hypersaline brines. Many industrial processes also use salts and frequently release brine effluent into the environment. Halophiles are likely to be useful for bioremediation of contaminated hypersaline brine (Cohen, 2002). Halophiles produce a large variety of stable and unique biomolecules that may be useful for practical applications. Halophilic microorganisms produce hydrolytic enzymes such as DNAases, lipases, amylases, gelatinases and proteases capable of functioning under conditions that lead to precipitation or denaturation of most proteins (Porro *et al.*, 2002). Halophilic proteins compete effectively with salts for hydration, a property that may result in resistance to other low water activity environments, such as in the presence of organic solvents. Novel halophilic biomolecules may also be used for specialized applications, e.g. bacteriorhodopsin for biocomputing (Spudich, 1993), gas vesicles for bioengineering floating particles, pigments for food colouring, and compatible solutes as stress protectants.

**The objectives of this study were**

- Isolation and identification of *Bacillus halodurans* and *Escherichia coli* from salted fishes.
- Identification of ectoine (*ectABC*) and choline dehydrogenase (*betA*) genes from the above organisms.
- Functional characterization and enzymatic assays of ectoine and choline dehydrogenase.
- *In silico* sequence analysis of ectoine and glycine betaine biosynthesis genes using bioinformatics tools.

**REVIEW  
OF  
LITERATURE**

## 2. REVIEW OF LITERATURE

Surviving in an extreme environment to thrive is detrimental to the majority of life. The majority of known extremophiles are varieties of archaea and bacteria. They are classified, according to the conditions in which they exist as; thermophiles, hyperthermophiles, psychrophiles, halophiles, acidophiles, alkaliphiles, barophiles and endoliths.

The discovery of extremophiles points out the extraordinary adaptability of primitive life forms and further raises the prospect of finding at least microbial life elsewhere in the Solar System and beyond. There is also growing support for the idea that extremophiles were among the earliest living things on Earth.

**Table 2.1 Classification and examples of extremophiles**

<b>Environmental parameter</b>	<b>Type</b>	<b>Definition</b>	<b>Examples</b>
Temperature	Hyperthermophile	growth >80°C	<i>Pyrolobus fumarii</i> , 113°C
	Thermophile	growth at 60-80°C	<i>Synechococcus lividis</i>
	Mesophile	growth at 15-60°C	<i>Homo sapiens</i>
	Psychrophile	growth <15°C	<i>Psychrobacter</i>
Radiation			<i>Deinococcus radiodurans</i>
Pressure	Barophile	Weight loving	Unknown
	Piezophile	Pressure loving	For microbe, 130 MPa
Gravity	Hypergravity	>1g	None known

	Hypogravity	<1g	None known
Vacuum		Tolerates vacuum (space devoid of matter)	tardigrades, insects, microbes, seeds
Desiccation	Xerophiles	Anhydrobiotic	<i>Artemia salina</i> ; nematodes, microbes, fungi, lichens
Salinity	Halophiles	Salt loving (2-5 M NaCl)	<i>Halobacteriaceae</i> , <i>Dunaliella salina</i>
pH	Alkaliphile  Acidophile	pH >9  Low pH loving	<i>Natronobacterium</i> , <i>Bacillus firmus</i> OF4, <i>Spirulina</i> spp. (all pH 10.5) <i>Cyanidium caldarium</i> , <i>Ferroplasma</i> spp. (pH: 0)
Oxygen tension	Anaerobe  Microaerophil  Aerobe	Cannot tolerate O <sub>2</sub>  Tolerates a few O <sub>2</sub>  requires O <sub>2</sub>	<i>Methanococcus jannaschii</i>  <i>Clostridium</i>  <i>Homo sapiens</i>
Chemical  Extremes	Gases  Metals	Can tolerate high concentrations of gases (metalotolerant)	<i>Cyanidium caldarium</i>  <i>Ferroplasma acidarmanus</i> (Cu, As, Cd, Zn); <i>Ralstonia</i> spp. CH34 (Zn, Co, Cd, Hg, Pb)

## 2.1 HALOPHILES

Halophilic microorganisms or "salt loving" microorganisms live in environments with high salt concentration that would kill most other microbes. Halotolerant and halophilic microorganisms can grow in hypersaline environments, but only halophiles specifically require at least 0.2 M of salt for their growth. Halotolerant microorganisms can only tolerate media containing <0.2 M of salt. Distinctions between different kinds of halophilic microorganisms are made on the basis of their level of salt requirement and salt tolerance. Larsen (1962) proposed 4 groups of microorganisms inhabiting saline environments: nonhalophiles, those which grow best in the medium containing <2% salt (NaCl), and slight, moderate, and extreme halophiles as those which grow best in the medium containing 2-5%, 5-20%, and 20-30% salt (NaCl), respectively. Kushner (1993) expanded Larsen's (1962) definition, and proposed the classification of microorganism's response to salt (NaCl) in which they grow best. Five groups were defined: 1) non halophilic microorganisms, <0.2 M (~1%) salt; 2) slight halophiles, 0.2-0.5 M (~1-3%) salt; 3) moderate halophiles, 0.5-2.5 M (~3-15%) salt; 4) borderline extreme halophiles, 1.5-4.0 M (~9-23%) salt; and 5) extreme halophiles, 2.5-5.2 M (~15-32%) salt. The halotolerant grow best in media containing <0.2 M (~1%) salt and also can tolerate high salt concentrations. This description is widely referred to in many reports (Arahal and Ventosa, 2002; Ventosa *et al.*, 1998; and Yoon *et al.*, 2003).

The saline content in halophilic environments is usually 10 times the saline content of normal ocean water. Normal ocean water has a salinity level of 30 percent. Some environments that halophiles live in are the Great Salt Lake in Utah, Owens Lake in California and the Dead Sea. These microorganisms use osmotic pressure and

chemical substances like sugars, alcohols, amino acids to cope salt inside the cell. Osmotic pressure is relationship of fluids inside and outside of a cell. Healthy cells keep the pressure the same in inside and outside of the cells. Halophiles are not like other extremophiles since; the proteins inside the microorganisms play the most important role of making it possible to survive in extreme saline environments.

Halophiles are coated with a special protein covering, which is used to allow only certain levels of saline/salt into the cell. This covering helps to seal in water with the right level of saline. Using the diffusion process, the halophiles maintain the salinity at the right level all of the time.

## **2.2 EUKARYOTIC HALOPHILES**

Multicellular eukaryotes like, *Tilapia* spp. can tolerate salinity to the range of 1 molL<sup>-1</sup> NaCl. A variety of obligate and facultative halophytic plants, e.g. *Atriplex halimus* and *Mesembryanthemum crystallinum*, can survive in moderately high saline soils (Watson *et al.*, 1994). There are also a surprising number of invertebrates that can survive in hypersaline environments. Some examples are rotifers such as *Brachionus angularis* and *Keratella quadrata*, Tubellarian worms such as *Macrostomum* spp., copepods such as *Nitocra lacustris* and *Robertsonia salsa*, ostracods such as *Cypridis torosa*, *Paracyprideinae* spp., *Diacypris compacta*, and *Reticypris herbsti*. Some insects from hypersaline environments include brine flies *Ephydra hians* and *E. gracillis* and brine shrimp *Artemia franciscana* and related species (Jason and Brian, 1995). Some hypersaline environments help to support many birds, one of the most spectacular of which is the pink flamingo.

### **2.2.1 ALGAE**

The growth of green algae will be supported in moderately high salinity level (1–3.5 molL<sup>-1</sup> NaCl) (Mohapatra *et al.*, 1998). These are obligately aerobic, photosynthetic, unicellulareukaryotic microorganisms, some species of which produce large quantities of orange coloured  $\beta$ -carotene at high salinities. Green algae of the genus *Dunaliella*, e.g. *Dunaliella salina*, *D. parva* and *D. viridis*, are ubiquitous and are the main source of food for brine shrimps and the larvae of brine flies. Most species of green algae are moderate halophiles, with only a few extremely halophilic species, e.g. *Dunaliella salina* and *Asteromonas gracilis*, which can grow even in saturated NaCl (Elloumi *et al.*, 2006). Algae predominantly use polyols as compatible solutes. In *Dunaliella salina*, glycerol is synthesized in response to osmotic stress. The cytoplasmic concentration of glycerol can reach 7 molL<sup>-1</sup> when grown in medium containing 5 molL<sup>-1</sup> NaCl and can constitute over 50% of the dry weight of the cells (Marin *et al.*, 1998). The intracellular sodium concentration has been reported to be less than 100 mmolL<sup>-1</sup> over a wide range of external salt concentrations. During moderate stress, glycerol does not leak out of cells but is metabolized and transformed into osmotically inactive reserve material.

### **2.2.2 PROTOZOA**

A large variety of protozoa are cell wall less chemoheterotrophic protists that ingest algae and bacteria, have been described from hypersaline environments. Identified species include the moderate halophile *Fabrea salina* from a west Australian lake (Post *et al.*, 1983), and the extreme halophile *Porodon utahensis* from the Great Salt Lake (Satyanarayana *et al.*, 2005). Although protozoa are known to regulate osmotic pressure in freshwater with contractile vacuoles that expel water,

their mechanism of osmoregulation in hypersaline brine has not been investigated in detail.

### **2.2.3 FUNGI**

Fungi and yeasts are chemoheterotrophic cell walled eukaryotes, some of which are well adapted to tolerate hypersaline environments (Adler and Gustaffson, 1980). They grow best under aerobic conditions on carbohydrates at moderate temperatures and acidic to neutral pH. *Debaromyces hansenii* is halotolerant yeast isolated from sea water, which can grow aerobically up to salinities of 4.5 mol L<sup>-1</sup> NaCl (Adler *et al.*, 1985). It produces glycerol as a compatible solute during the logarithmic phase and arabitol in the stationary phase (Larsson and Gustafsson, 1987). A saprophytic hyphomycete, *Cladosporium glycolicum* was found growing on submerged wood panels at a salinity exceeding 4.5 molL<sup>-1</sup> NaCl in the Great Salt Lake (Satyanarayana *et al.*, 2005). Halophilic fungi, like *Polypaecilum pisce* and *Basipetospora halophila*, have also been isolated from salted fish (Kathryn and Ailsa, 1993).

## **2.3 PROKARYOTIC HALOPHILES**

### **2.3.1 CYANOBACTERIA**

Cyanobacteria are bacterial prokaryotes that are characterized by the presence of chlorophyll and phycobilin pigments and carry out oxygenic photosynthesis. They dominate the planktonic biomass and form microbial mats in many hypersaline lakes (Hamada *et al.*, 2001). The top brown layer of microbial mats contains a common unicellular cyanobacterial species, *Aphanothece halophytica*. It can grow over a wide range of salt concentrations, from 2–5 molL<sup>-1</sup> NaCl, is an extreme halophile with an optimum salt concentration of 3.5 molL<sup>-1</sup> (Wutipraditkul *et al.*, 2005). It uses

glycine betaine as the major compatible solute, which it can take up from the medium or synthesize from choline (Waditee *et al.*, 2003). *A. halophytica* and similar unicellular *cyanobacteria* have been described from the Great Salt Lake, Dead Sea, Solar Lake and artificial solar ponds. The diversity of cyanobacteria occurring in hypersaline environments have not been studied extensively (Caumette *et al.*, 1994).

### **2.3.2 SULFUR OXIDIZING BACTERIA**

Below the cyanobacteria and the phototrophic bacteria in microbial mats are the halophilic, filamentous, carbon dioxide fixing bacteria that can oxidize hydrogen sulfide to sulfate (Ollivier *et al.*, 1991). Examples include the filamentous *Achromatium volutans* from Solar Lake, *Beggiatoa alba* from Guerrero Negro, and *B. leptiformis* from Solar Lake (Widdel, 1988). A unicellular halophilic, chemoautotrophic sulfur oxidizing bacterium, *Thiobacillus halophilus* from a hypersaline western Australian Lake, has also been reported (Post *et al.*, 1983).

### **2.3.3 HALOBACTERIA**

Common species of halobacteria are rod, cocci, disc shaped, triangular and even square shaped species exist (Hamamoto *et al.*, 1998). Many are pleiomorphic, especially when the ionic conditions of the media are altered, and most lyse below the NaCl level of 1–1.5 molL<sup>-1</sup>. Halobacteria are classified as archaea, and are also called halophilic archaea or haloarchaea belongs to the family Halobacteriaceae. The physiology of halophilic and moderately halophilic bacteria is affected by salt concentration (Kushner, 1993). The salt requirement and tolerance properties of the bacteria are highly variable according to the growth temperature and the nature of the nutrients available (Ventosa *et al.*, 1998). Halophilic bacteria produce a variety of colonial characteristics from pigmented to non pigmented according to the salt

concentration of the media. Second, halophilic bacteria do not grow fast especially the group of extremely halophilic bacteria. Many of them need natural brines and a variety of nutrients such as pressed fish juices for their growth, as well as yeast extract to support their growth. The determination of phenotypic characterization along with 16S rRNA gene sequences is commonly used for halophilic and moderately halophilic bacterial identification (Ihara *et al.*, 1997; Stan lotter *et al.*, 2002; and Yoon *et al.*, 2003). Most extreme halophiles are archaeobacteria while the moderate halophiles are members of archaeobacteria and eubacteria. Moderately and extremely halophilic bacteria are the most important group in hypersaline habitats. There are few studies on moderately halophilic bacteria, since the early studies have concentrated on particular habitats such as the Great Salt Lake, Dead Sea, Wadi Natrun, Lake Magadi, and solar salterns. These habitats have harsh environmental conditions such as high salinity, high temperature, low oxygen availability, high nutrient availability, high light intensity, and extremely alkalinity. Only moderately halophilic bacteria survive and play a major ecological role. The moderately halophilic bacteria constitute a low proportion of the total microbial population (Rodriguez Valera, 1988) in such habitats.

### **2.3.3.1 EXTREMELY HALOPHILIC BACTERIA**

The extremely halophilic bacteria are members of the class *Halobacteria* (order *Halobacteriales* and family *Halobacteriaceae*) (Grant *et al.*, 2001). They are rods, coccus or a multitude of involution forms from disks to triangle. They require at least 1.5 M (~9%) NaCl for growth and lack muramic acid containing peptidoglycan in the cell envelope. Their colonies are various shades of red due to the presence of C50 carotenoids (bacterioruberins) (Kushwaha *et al.*, 1975). Their intracellular

enzymes have a requirement for high levels of KCl, over 3 M and up to 5 M. Their cytoplasmic membrane is composed of phytanyl ether lipids. They are insensitive towards many antibiotics and occur in hypersaline habitats such as salt lakes, soda lakes, and salterns. The family *Halobacteriaceae* consists of 14 genera: *Haloarcula*, *Halobacterium*, *Halobaculum*, *Halococcus*, *Haloferax*, *Halogeometricum*, *Halorubrum*, *Haloterrigena*, *Natrialba*, *Natrinema*, *Natronobacterium*, *Natronococcus*, *Natronomonas*, and *Natronorubrum*.

### **2.3.3.2 MODERATELY HALOPHILIC EUBACTERIA**

Moderately halophilic bacteria are bacteria that require at least 0.5 M (~3%) NaCl for growth. They constitute very heterogeneous groups. The taxonomy of moderately halophilic bacteria presented by Ventosa (1989) is divided into two groups: moderately halophilic eubacteria and moderately halophilic archaeobacteria. In general, most halophiles within this group are moderate rather than extreme halophiles (Oren, 2002 a). Moderately halophilic eubacteria are both heterotrophs and phototrophs. The heterotrophs include Gram negative and Gram positive moderate halophiles. Gram negative species of moderately halophilic bacteria includes; *Deleya halophila*, *Desulfohalobium retbaense*, *Desulfovibrio halophilus*, *Flavobacterium halmephilum*, *Haloanaerobacter chitinovorans*, *Haloanaerobacter saccharolytica*, *Haloanaerobium praevalens*, *Halobacteroides halobius*, *Halomonas halodenitrificans*, *Halomonas elongata*, *Halomonas eurihalina*, *Halomonas subglaciescola*, *Paracoccus halodenitrificans*, *Pseudomonas beijerinckii*, *Pseudomonas halophila*, *Sporohalobacter lortetii*, *Sporohalobacter marismortui*, *Spirochaeta halophila*, and *Vibrio costicola*. Species of Gram positive moderate halophiles are *Bacillus halodurans*, *Micrococcus halobius*, *Sporosarcina halophila*,

*Marinococcus halobius*, and *Marinococcus albus*. Phototrophs include *Ectothiorhodospira vacuolata*, *Rhodospirillum salexigens*, and *Rhodospirillum salinarum* (DasSarma, 2001; Ventosa, 1989).

### **2.3.3.3 MODERATELY HALOPHILIC ARCHAEOBACTERIA**

The halophilic *Archaea* are the extreme halophiles. The exclusion is the methanogenic group of bacteria (Oren, 2002 a). Cytoplasmic membranes of methanogenic archaeobacteria contain phytanyl ether lipids. (Zinder, 1993). Their intracellular salt concentration of these bacteria is higher than that of most bacteria, about 0.6 mol/L KCl, but is significantly lower than for the extremely halophilic archaeobacteria. Species of moderately halophilic archaeobacteria are *Methanohalophilus mahii*, *Methanohalophilus zhilinae*, *Methanohalophilus halophilus* and *Methanohalophilus portucalensis* (Oren, 2002 b).

### **2.3.3.4 HABITATS OF HALOPHILIC AND MODERATELY HALOPHILIC BACTERIA**

Hypersaline environments originated from two sources, seawater and non seawater. The seawater environment is called thalassohaline (Oren, 2002 b), while the non seawater environment is referred to as athalassohaline (Demergasso *et al.*, 2004). Thalassohaline environments contain sodium chloride as the predominant salt. The pH of thalassohaline environments is usually near neutral to slightly alkaline. In contrast to thalassohaline environments, dominant ions in athalassohaline environments are potassium, magnesium, or sodium (Litchfield and Gillevet, 2002). The saline habitats such as; saline waters, saline soils, and salted foods have been studied intensively and are summarized as follows:

### **2.3.3.5 SALINE WATER**

The best known environment for halophilic bacteria is saline water. Water with salinity more than 3% is considered to be saline (De Dekker, 1983). Examples of saline water are the ocean, the sea, salt lake, and saltern. The diversity of halophilic bacteria in saline water has been studied in detail (Ventosa, 1989).

### **2.3.3.6 SALINE SOIL**

Saline soil is a common hypersaline habitat of both halophilic and moderately halophilic bacteria. The soil habitat is inherently heterogeneous and therefore a wide range of the salinity might be present in saline soil at different soil depths (Grant, 1991). The salinity of saline soil is much more variable than saline waters (Quesada *et al.*, 1982). Microbial diversity in saline soil environments have not been studied intensively (Ramos Cormenzana, 1989). Therefore very little information is available. Many plants such as salt tolerant species and halophytes are adapted to grow in saline soil. They play various ecological roles involving nutrient cycle and plant microbial interactions that are different for each saline soil (Rodriguez Valera, 1993). Rodriguez Valera (1988) also stated that there was an abundance of halophilic and moderately halophilic bacteria in saline soil and that the dominant types belong to genera of *Alcaligenes*, *Bacillus*, *Micrococcus*, and *Pseudomonas*.

### **2.3.3.7 SALTED FOOD**

Food in nature is naturally a poor source of salt (sodium chloride). Food becomes salty when salt is added for the preservation and to make them more appealing. Salt has been used to preserve food for thousands of years. Another use of salt has been that of a flavor enhancer. Food preservation often uses salting in combination with smoking and curing. Examples of salted food are salted fish, salted

hides, bacon, ham, sausage, lunchmeats, hot dogs, crackers, cheese, chips, soy sauce, paste, and sauerkraut. *Halomonas salina* was isolated from fully cured wet and dry bachalao that contains about 19% salt (Vilhelmsson *et al.*, 1996). *Pediococcus halophilus* was the dominant bacterium at the end of the curing process of anchovies (Villar *et al.*, 1985). According to DasSarmas, (2001), several halophilic and moderately halophilic bacteria are isolated from salted food. *Pseudomonas beijerinckii* has been isolated from salted beans preserved in brine. *Halomonas halodenitrificans* has been isolated from meat curing brines. *Vibrio costicola* has been isolated from Australian bacon. *Halobacterium* spp. and *Halococcus* spp. were isolated from Thai fish sauce (Thongthai and Suntainalert, 1991).

#### **2.3.4 BACILLUS HALODURANS**

This is one of a group of rod shaped, Gram positive, aerobic bacteria widely found in saline soil (Zhang *et al.*, 2008). *Bacillus halodurans* is an alkaliphilic bacterium (Takami and Horikoshi, 1999) that can grow well at pH 7–10.5 in saline environments. It is the most systematically characterized strain in physiologically, biochemically and genetically, among those in the collection of alkaliphilic bacillus isolates. *Bacillus halodurans* genome contains 112 transposase genes, indicating that transposase have played an important evolutionary role in horizontal gene transfer and also in internal genetic rearrangement in the genome. To cope with the external hyperosmotic shifts, *B. halodurans* will synthesis an osmolyte, ectoine. The biosynthetic pathway for ectoine consists of three steps and has been well characterized in many halophiles (Peters *et al.*, 1990; Galinski and Truper, 1994; Ono *et al.*, 1999; Kuhlmann and Bremer, 2002; Schubert *et al.*, 2007), the first being the conversion of aspartate semialdehyde, an intermediate in amino acid metabolism, to

L-2, 4-diaminobutyric acid. This reaction is followed by acetylation to *N*- $\gamma$ -acetyldiaminobutyric acid. The last step is the cyclic condensation reaction leading to formation of tetrahydropyrimidine, L-ectoine.

Functional characterization of ectoine biosynthesis genes from *B. halodurans* is not well characterized physiologically, biochemically and genetically (Horikoshi, 1999; Takami and Horikoshi, 1999).

### **2.3.5 *ESCHERICHIA COLI***

*E. coli* has independent proline transport systems, *PutP*, *ProP* and *ProU* which are all involving in osmoregulation (Culham *et al.*, 1993). Among these three, *PutP* system is required for the transport of proline when this metabolite is used as a carbon or nitrogen source; this system is not involved in osmoregulation. The other two systems, *ProP* and *ProU* are responsible for the accumulation of proline and glycine betaine to high levels and hence allow the cells to cope with conditions of hyperosmotic stress. *ProP* encodes a constitutive, low affinity transport system whose expression is stimulated severalfold during osmotic shock. *ProU* encodes a binding protein dependent high affinity transport system whose transcription is induced by elevated osmolarity *in vivo* and by potassium glutamate *in vitro*. *ProU* is an operon composed of three genes, *proV*, *proW* and *proX*. In *E. coli*, the biosynthetic pathway of glycine betaine from choline has been well characterized at the genetic level (Landfald and Strom, 1986), it has been shown that four genes encoding choline dehydrogenase (*betA*), betaine-aldehyde dehydrogenase (*betB*), a putative regulator (*betI*), and a choline transporter (*betT*) are clustered in the *bet* operon (Andresen *et al.*, 1988). The enzymes involved in the biosynthesis of betaine have been cloned and

characterized from bacteria and plants (Weretilnyk and Hanson, 1990; Rathinasabapathi *et al.*, 1997; Gadda and Wilkins, 2003).

Previous studies reports the resistance towards salinity and low temperature in transgenic tobacco expressing the two *E. coli* genes *betA* and *betB* (Lilius *et al.*, 1996; Holmstrom *et al.*, 2000), signifying the practical applications of choline dehydrogenase. Choline dehydrogenase (*betA*) of *E. coli* catalyses the first step in the synthesis of betaine, the oxidation of choline. However, this enzyme also catalyses the second step, the dehydrogenation of betaine aldehyde to betaine (Landfald and Strom, 1986). Choline dehydrogenase catalyzes the four electron oxidation of choline to glycine betaine via a betaine aldehyde intermediate (Tsuge *et al.*, 1980). Only nominal studies on characterization of choline dehydrogenase from *E. coli* have been reported to date.

### **2.3.6 OTHER HALOPHILIC ORGANISMS**

Among them, ectoine was accumulated by the phototrophic bacterium *Ectothiorhodospira halochloris* (Galinski *et al.*, 1985) and the moderately halophilic eubacteria like *Vibrio costicola* (Ronit *et al.*, 1990). Some of the symbiotic soil bacterium *Sinorhizobium meliloti* has the capacity to synthesize the osmoprotectant glycine betaine from choline-*O*-sulfate and choline. (Mandon *et al.*, 2003).

## **2.4 ADAPTATION OF HALOPHILIC AND MODERATELY HALOPHILIC BACTERIA IN HIGH OSMOTIC STRESS**

Availability of water is the most important prerequisite for life of any living cell. The ability of an organism to adapt to changes in external osmotic pressure and the development of mechanisms of osmoregulation are fundamental to its survival (Csonka, 1989). In general, exposure of microorganisms to hypersaline environments

triggers rapid fluxes of cell water along the osmotic gradient out of the cell. This causes a reduction in turgor, dehydration of the cytoplasm and is consequently lethal. Halophilic and moderately halophilic bacteria have adapted genotypically and phenotypically to grow in hypersaline environments. Therefore they are not affected by these conditions (Imhoff, 1993). The degree of salt dependency and salt tolerance of these microorganisms is distinguished by their levels of salt requirement and salt tolerance that reflects the differences in osmoadaptation to hypersaline environments (Russell, 1989). There are three mechanisms available for adaptation of halotolerant and halophilic microorganisms to high-osmolarity environments: (1) the recognition of osmotic imbalance by an osmosensor; (2) the accumulation of osmolytes or compatible solutes in response to the imposed pressure difference; and (3) the stabilization of macromolecules under the new intracellular conditions (Russell, 1989).

#### **2.4.1 OSMOREGULATION BY HALOPILIC AND MODERATELY HALOPHILIC BACTERIA**

The responses of halophilic bacteria to osmolarity shifts are on the genetic level and the enzymatic level (RoeBler and Muller, 2001). This conserved mechanism is known to have two component regulatory systems. These consist of a sensor protein, which detects the signal and a regulator protein that binds DNA and controls gene expression. The two component regulatory systems that respond to the osmotic changes are the EnvZ/OmpR and the KdpD/KdpE. The EnvZ/OmpR has been found in the *Eubacteria* while the KdpD/KdpE has been found in the *Archaea* (RoeBler and Muller, 2001). The EnvZ is a transmembrane histidine kinase that monitors osmolarity changes on both sides of the cytoplasmic membrane through the OmpR

(Bartlett and Roberts, 2000). It functions as a dimer with a part in the cytoplasm and a part extending outside the membrane. It has three separate enzymatic activities. The KdpD, a sensor kinase, is autophosphorylated under a decrease in turgor pressure condition. Subsequently, the phosphoryl group is transferred to the response regulator, KdpE, which then acts as a transcriptional activator for the *KdpABC* operon encoding a primary ABC type K<sup>+</sup> transporter. This allows the cell to counteract the stress by increasing the internal osmolarity through the accumulation of K<sup>+</sup> via the KdpABC ATPase (Poolman *et al.*, 2002).

#### **2.4.2 ACCUMULATION OF COMPATIBLE SOLUTES BY HALOPHILIC AND MODERATELY HALOPHILIC BACTERIA**

Microorganisms in hypersaline environments have to balance their cytoplasm with the osmotic stress exerted by the external medium. The osmotic balance in these microorganisms can be achieved by the accumulation of compatible solutes. The solutes can be accumulated at high concentrations without interfering with cellular processes (Brown, 1976). However, when the turgor becomes too high, microorganisms need to excrete compatible solutes from their cytoplasm. Halophilic eubacteria accumulate compatible solutes by either *de novo* synthesis or uptake from the medium. Compatible solutes can be classified into two major groups: inorganic ions (K<sup>+</sup>, Mg<sup>2+</sup>, and Na<sup>+</sup>) and organic solutes (betaine, ectoine and trehalose). The accumulation of inorganic ions is found in the *Archaea* of the family *Halobacteriaceae* and the *bacteria* of the order *Haloanaerobiales*. The accumulation of specific organic solutes, organic solutes strategy, is found in all other species of halophilic bacteria (Da Costa *et al.*, 1998). However, it may be a combined function of inorganic ions and organic solutes, since the inorganic ions are insufficient to

provide osmotic balance with the external medium (Ventosa *et al.*, 1998). The salt in cytoplasm strategy is strictly confined to those environments in which salinity is high and relatively stable (Yancey *et al.*, 1982). The major type of intracellular ion is K<sup>+</sup>, which is less harmful for the enzymes in the cytoplasm than Na<sup>+</sup> intracellularly at high concentration. The intracellular ion concentrations can be varied among different species of halophilic bacteria and even within the same species by means of transport, synthesis or catabolism

## 2.5 OSMOLYTES

Since microorganisms lack systems for active water transport, environmental changes in osmolarity are compensated by transport or synthesis of compatible solutes, a group of inert organic solutes that can be accumulated in the intracellular milieu to very high levels without affecting metabolic function of the cell (Giovanni *et al.*, 1999). These compatible solutes are known as osmolytes, hence they involve in the osmoregulation.

Osmolytes tend to occur at high intracellular concentrations; they do not have unique chromophores and were not considered in much detail and in most cases even identified until high resolution NMR spectroscopy became a routine analytical method. From the 1970s onward, a variety of NMR approaches have been used to identify the organic solutes accumulated by halotolerant and halophilic organisms. Early natural abundance <sup>13</sup>C NMR studies of cell extracts identified novel solutes, such as ectoine (Galinski *et al.*, 1985), several β-amino acids (Robertson *et al.*, 1989; Lai *et al.*, 1991) and di-*myo*-inositol-1,1'-phosphate (DIP), the last associated with hyperthermophiles (Scholz *et al.*, 1992; Ciulla *et al.*, 1994). More recent methods using <sup>1</sup>H NMR and two-dimensional experiments have significantly increased the

sensitivity of solute detection (Fan, 1997).  $^1\text{H}$  NMR methodology can also be used to detect and quantify osmolytes in cell cultures without extraction (Motta *et al.*, 2004). Other analytical methods such as HPLC have been used, often to quantify specific solutes as long as an appropriate detection method is available. Refractive index detection is the most general (Kets *et al.*, 1996), but specific classes of molecules can be derivatized for rapid and sensitive detection solutes containing free amino groups (Kunte *et al.*, 1993). More recent advances have improved on the sensitivity of these other assays. For example, the combination of anion exchange chromatography and pulse amperometric detection is a very sensitive method that can detect osmolytes such as ectoine after hydrolytic cleavage of the pyrimidine ring (Riis *et al.*, 2003). The methodology is sufficiently sensitive that it can be used to screen colonies on agar for solutes.

Organic osmolytes fall into three general chemical categories: (i) noncharged solutes, (ii) anionic solutes and (iii) zwitterionic solutes

### **2.5.1 NONCHARGED SOLUTES**

Few molecules that are polar but lack any formal charges have been identified as osmolytes in halophilic bacteria, although they are well represented in eukaryotes. For example, glycerol is prevalent osmolyte in marine and halophilic *Dunaliella* (Borowitzka and Brown, 1974; Petrovic *et al.*, 2002). Glycerol accumulation is also a characteristic of halotolerant yeast *Debaryomyces hansenii* as well as the black yeast *Hortea werneckii*, and adaptation of this eukaryotic organism to high NaCl requires glycerol accumulation (Petrovic *et al.*, 2002). *Myo*-inositol, another polyol is used as an osmolyte in several eukaryotes (Scholz *et al.*, 1992). Neither of these polar noncharged solutes has been identified as an osmolyte in bacteria or archaea.

However, negatively charged derivatives of both glycerol and inositol are accumulated by archaea.

### **2.5.1.1 CARBOHYDRATES**

Few carbohydrates are used for osmotic balance, perhaps because those with a reducing end are chemically reactive, and these noncharged solutes would be likely to react with surface amino groups. To avoid this, the reactive end of the sugar forms a glycosidic bond with a small neutral molecule, either glycerol or glyceramide. The neutral derivatized sugars glucosyl glycerol and  $\alpha$ -mannosylglyceramide (Silva *et al.*, 1999) have been detected in few bacteria.

#### **2.5.1.1.1 TREHALOSE**

Trehalose is a nonreducing glucose disaccharide that occurs in a wide variety of organisms, from bacteria, archaea, fungi, plants, and invertebrates (Empadinhas and da Costa, 2008). It protects numerous biological structures against various kinds of stress, including desiccation, oxidation, heat, cold, dehydration, and hyperosmotic conditions. In addition, trehalose is a source of carbon and energy and a signaling molecule in specific metabolic pathways (Elbein *et al.*, 2003). To date, five different enzymatic systems have been described for trehalose synthesis: TPS/TPP, TreS, TreY, TreZ, TreP, and TreT enzymes (Avonce *et al.*, 2006).

#### **2.5.1.1.2 SUCROSE**

Sucrose is a non-reducing disaccharide of glucose and fructose that is widely distributed in plants (Lunn, 2002). In prokaryotes, however only freshwater and marine cyanobacteria as well as some proteobacteria are known to accumulate sucrose. In these bacteria, sucrose behaves as a compatible solute in osmotic stress. The pathways for the synthesis of sucrose were first characterized in higher plants and

later in green algae and in cyanobacteria (Lunn, 2002). One pathway involves two steps catalyzed by sucrose-6-phosphate synthase (SPS) and sucrose-6-phosphate phosphatase (SPP) through a phosphorylated intermediate (Lunn and MacRae, 2003), much like the TPS/TPP pathway for trehalose. An alternative pathway, which was found in higher plants and in some filamentous cyanobacteria of the genus *Anabaena*, uses a sucrose synthase (SuS) that catalyzes the condensation of ADP-glucose and fructose into sucrose (Curatti *et al.*, 2000).

#### **2.5.1.1.3 AMINO ACIDS AND PEPTIDES**

Two solutes in this class have been identified as osmolytes: (i) carboxamine and (ii) acetylated neutral glutamine dipeptide. In both solutes, modifications mask the charged  $\alpha$ -amino and  $\alpha$ -carboxyl groups. N- $\alpha$ -Carbamoyl-L-glutamine 1-amide, an unusual amino acid derivative is accumulated by halophilic phototrophic bacterium *Ectothiorhodospira marismortui* (also known as *Ectothiorhodospira mobilis*) (Galinski and Oren, 1991). The dipeptide N-acetyl glutaminyl glutamine amide is synthesized by several halophilic purple sulfur bacteria (Smith and Smith, 1989 and D'Souza Ault *et al.*, 1993).

#### **2.6 ORGANIC ANIONS**

Cells have a negative potential inside and often quite high intracellular K<sup>+</sup>. Negatively charged solutes could serve to balance high intracellular K<sup>+</sup> as well as counteract osmotic pressure. Indeed, at lower external NaCl, many bacteria synthesize ectoine and archaea synthesize L- $\alpha$ -glutamate as an osmolyte. In methanogens, high NaCl often causes the cells to switch from anionic glutamate isomers to the zwitterionic solute N $\epsilon$ -acetyl- $\beta$ -lysine for osmotic balance (Sowers and Gunsalus, 1995). Anionic solutes used by bacteria and archaea for osmotic balance can have a

carboxylate supply the negative charge or contain phosphate or sulfate groups (Roberts, 2005).

### **2.6.1 BETA GLUTAMATE**

Methanogens tend to accumulate beta glutamate as well as alpha glutamate for osmotic balance. In *Methanothermococcus thermolithotrophicus*, both the glutamate levels increase with increasing external NaCl (Robertson *et al.*, 1990). However, there appears to be a threshold for the glutamates in this organism. The negatively charged glutamates are accumulated when the external NaCl is less than 1 M. In that regime, the total intracellular glutamates occur at concentrations comparable to the intracellular K<sup>+</sup>. Above 1 M NaCl, zwitterionic N-acetyl-lysine becomes the major solute (Robertson *et al.*, 1992; Ciulla and Roberts, 1999). The accumulation of the zwitterions at high NaCl could indicate that it is now energetically too rich to increase K<sup>+</sup> and hence the anionic glutamates that aid in neutralizing much of the K<sup>+</sup> are not needed. While most studies identifying alpha glutamate have concentrated on methanogens, this solute has been detected in a few bacteria. It has been detected in the Gram positive organism *Nocardiopsis halophila*, which also accumulates the zwitterionic hydroxyectoine (DasSarma and Arora, 2002).

### **2.6.2 POLYOLS**

The polyols; glycerol, arabitol, sorbitol, mannitol and inositol are archetypal compatible solutes of halotolerant fungi and few algae and plants (Grant, 2004). Bacteria rarely use polyols as compatible solutes, with the few exceptions including *Zymomonas mobilis* and *Pseudomonas putida* (Kets *et al.*, 1996). Unlike most heterotrophic bacteria, *Z. mobilis* can convert sucrose into glucose and sorbitol and *P. putida* accumulates mannitol when exposed to osmotic stress. The accumulation of a

polyol by *Z. mobilis* seems to reflect convergent evolution for osmoadaptation. Many organisms have developed a strategy to modify a readily available and abundant sugar into a compatible solute (Kets *et al.*, 1996). Cyanobacteria can synthesize glucosyl glycerol and use it as a compatible solute in salt stress. This rare compatible solute is a polyol derivative structurally related to galactosyl glycerol, found in some red algae (Karsten *et al.*, 2003). The halotolerant proteobacteria *Pseudomonas mendocina* and *Stenotrophomonas rhizophila* also produce glucosyl glycerol (Mikkat *et al.*, 2000 and Roder *et al.*, 2005).

### **2.6.3 ZWITTER SOLUTES**

Free polar amino acids in cells might be expected to play a role in osmotic balance. However, neutral amino acids are not accumulated to high concentrations, presumably because they are intermediates in protein biosynthesis. High and varying concentrations of these compounds could affect diverse cell pathways. Instead, many bacterial and archaeal cells synthesize and accumulate a few zwitterionic molecules derived from amino acids as compatible solutes (Roder *et al.*, 2005).

#### **2.6.3.1 BETAINE**

Betaine is a neutral chemical compound with a positively charged ammonium ion or phosphonium ion and a negatively charged carboxylate group. Betaines serve as organic osmolytes in biological systems, for the protection against osmotic stress, drought, high salinity and high temperature. Intracellular accumulation of betaines is enzyme function, protein structure and membrane integrity, permits water retention in cells and thus protecting the cells from dehydration.

Glycine with the primary amine methylated to form a quaternary amine, is found in halophilic bacteria of diverse phylogenetic affiliation (Imhoff and Rodriguez

Valera, 1984). Betaine concentrations vary with external NaCl. Imhoff and Rodriguez Valera (1984) showed that for the eight halophiles examined, the average betaine concentrations in 3, 10 and 20% NaCl were  $0.21 \pm 0.2$ ,  $0.65 \pm 0.06$ , and  $0.97 \pm 0.09$  M. A number of methanogens have also been observed to accumulate betaine when grown in rich medium (Robertson *et al.*, 1990). In contrast to the large number of bacteria that transport betaine into the cell as an osmolyte, there are only a few bacteria; *Actinopolyspora halophila* and *Halomonas elongate*, one methanogen *Methanohalophilus portucalensis* FDF1 are able to synthesize betaine either by oxidation of choline or methylation of glycine (Nyysola *et al.*, 2000; Roberts *et al.*, 1992).

#### **2.6.3.1.1 BIOSYNTHESIS OF BETAINE**

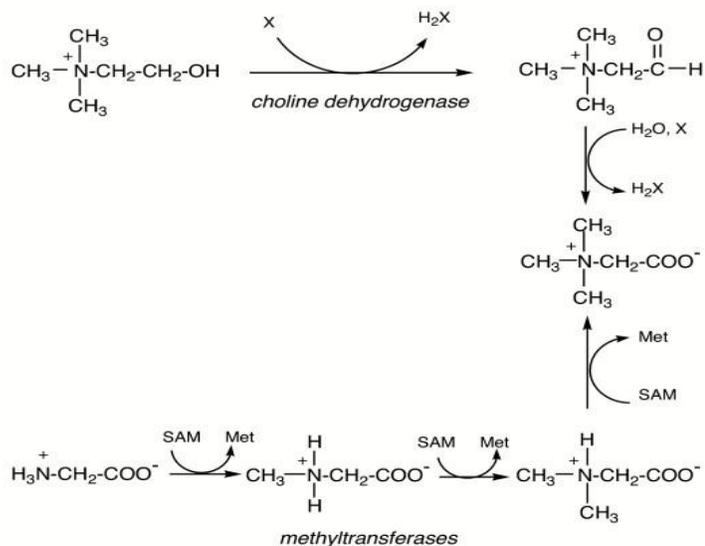
Microorganisms have two different metabolic pathways for synthesizing betaine. The oxidative pathway can occur with a single soluble enzyme (choline oxidase in Gram positive soil bacteria) (Rozwadowski *et al.*, 1991) and require two distinct soluble enzymes (choline monooxygenase and betaine aldehyde dehydrogenase in higher plants) (Rathinasabapathi *et al.*, 1997), or it can occur with a membrane associated system coded by four genes in the *bet* operon (in marine invertebrates and bacteria including *Escherichia coli*). The *bet* operon system has been studied genetically (Andresen *et al.*, 1988), with genes choline dehydrogenase (*betA*), betaine aldehyde dehydrogenase (*betB*), a choline transporter (*betT*) and a putative regulator (*betI*). Choline dehydrogenase is the enzyme, catalyzes the chemical reaction. The substrate of this enzyme is choline, whereas its products are betaine aldehyde and reduced acceptor. This enzyme belongs to the family of oxidoreductases.

Choline dehydrogenase catalyses the conversion of exogenously supplied choline into the intermediate glycine betaine aldehyde, as part of a two step oxidative reaction leading to the formation of osmoprotectant betaine. This enzymatic system can be found in both Gram positive and Gram negative bacteria. As in *Escherichia coli* (Andresen *et al.*, 1988), *Staphylococcus xylosus* (Rosenstein *et al.*, 1999) and *Rhizobium meliloti* (*Sinorhizobium meliloti*) (Pocard *et al.*, 1997) this enzyme is found associated in a transcriptionally co induced gene cluster with betaine aldehyde dehydrogenase, the second catalytic enzyme in this reaction. Other Gram positive organisms have been shown to employ a different enzymatic system, utilising a soluble choline oxidase or type III alcohol dehydrogenase instead of choline dehydrogenase (Boch *et al.*, 1996).

The genes from the extreme halophile *Ectothiorhodospira halochloris* encoding the biosynthesis of glycine betaine from glycine were cloned into *Escherichia coli* (Weymarn *et al.*, 2000). The accumulation of glycine betaine and its effect on osmotolerance of the cells were studied in detail. In mineral medium with NaCl concentrations from 0.15 to 0.5 M, the accumulation of glycine betaine by both endogenously and exogenously stimulated the growth of *E. coli*. The intracellular levels of glycine betaine and the cellular yields were clearly higher for cells receiving glycine betaine exogenously than for cells synthesizing it (Weymarn *et al.*, 2000). The lower level of glycine betaine accumulation in cells synthesizing it is most likely a consequence of the limited availability of precursors (eg. *S*-adenosylmethionine) rather than the result of a low expression level of the genes.

Choline dehydrogenase catalyzes the four electron oxidation of choline to glycine betaine *via* a betaine aldehyde as the intermediate, which is then oxidized to

betaine by the *betB* gene product. In *Pseudomonas*, an electron acceptor other than O<sub>2</sub> is used for choline oxidation with suggestions that PQQ is the acceptor (Russell and Scopes, 1994). In *Actinopolyspora halophila*, choline is oxidized to betaine aldehyde and then to betaine (Nyyssola and Leisola, 2001). Glycine betaine synthesizing enzymes such as choline dehydrogenase have been shown to have enhanced tolerance towards various environmental stresses such as hypersalinity, freezing and high temperatures. Choline dehydrogenase has been inadequately characterized in its biochemical and kinetic properties, mainly because its purification has been hampered by its instability in the *in vitro* condition. Functional characterization of *betA* gene, which codes for choline dehydrogenase from the moderate halophile *Halomonas elongata* has been functionally characterized (Gadda and Wilkins, 2003).



**Fig. 2.1 Biosynthetic pathway of betaine in bacteria and archaea**

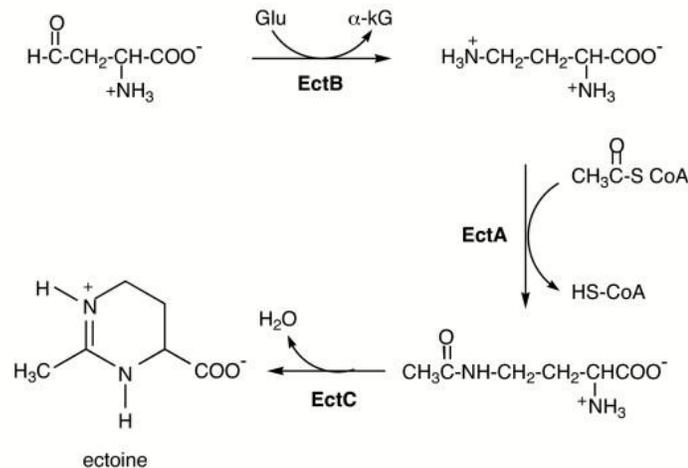
### **2.6.3.1.2 ECTOINE AND HYDROXYECTOINE**

Ectoine, a cyclic tetrahydropyrimidine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) can almost be considered a marker for halophilic and moderate halophilic bacteria (Galinski, 1995). As shown in Fig. 2.2, it is synthesized by a wide range of halotolerant and halophilic bacteria. This solute was first detected in the halophilic, phototrophic *Halorhodospira halochloris* (Galinski *et al.*, 1985). The intracellular ectoine concentration was shown to increase with increased extracellular NaCl. It is also the major osmolyte in bacterial strains isolated from alkaline, hypersaline Mono Lake (Ciulla *et al.*, 1997). More recently it has also been observed in the moderately halophilic methylotrophic bacteria *Methylophilum marina*, *M. terricola*, and *Methylophilum* spp. (Doronina *et al.*, 2000, 2003). A variant of this solute, hydroxyectoine, has been detected in halotolerant *Sporosarcina pasteurii* grown in high osmolarity medium (Kuhlmann and Bremer, 2002).

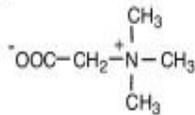
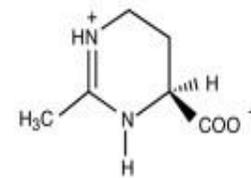
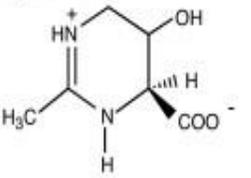
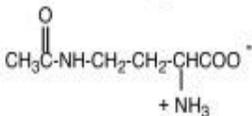
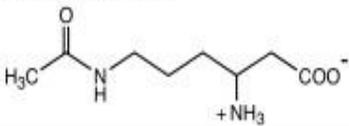
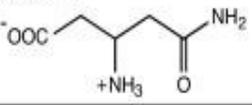
### **2.6.3.1.3 BIOSYNTHESIS OF ECTOINE**

The biosynthesis and regulation of ectoine in cells have been studied in several different eubacteria (Peters *et al.*, 1990; Galinski and Truper 1994; Ono *et al.*, 1999; Kuhlmann and Bremer, 2002; Schubert *et al.*, 2007). Biosynthesis of ectoine in *H. elongata* has been studied in the greatest detail (Ono *et al.*, 1999). The precursor molecule for ectoine biosynthesis is aspartate semialdehyde, which is an intermediate in amino acid metabolism (Peters *et al.*, 1990). As shown in Fig. 2.2, the aldehyde is converted to L-2,4-diaminobutyric acid, which is then acetylated to form N $\gamma$ -acetyldiaminobutyric acid. The final step is the cyclization of this solute to form ectoine. The genes for biosynthesis of this solute were identified after the isolation of salt sensitive mutants led to cloning of genes (Canovas *et al.*, 1997). Ectoine synthesis

is carried out by the enzymatic action of three genes: *ectABC*. The *ectA* gene codes for diaminobutyric acid acetyltransferase; *ectB* codes for the diaminobutyric acid aminotransferase; *ectC* codes for ectoine synthase (Canovas *et al.*, 1998). The ectoine biosynthetic genes were functionally characterized in *H. elongata*. The first enzyme (260 kDa complex of 44 kDa subunits) generates the diaminobutyrate by transaminating the aspartate semialdehyde with glutamate. Both pyridoxal 5'-phosphate and  $K^+$  are necessary for the diaminobutyrate aminotransferase activity (Ono *et al.*, 1999). The activity of aminotransferase is activated by 0.5 M NaCl (and similarly by KCl). The last enzyme, ectoine synthase is also activated by NaCl (Roberts, 2005). This suggests that ectoine accumulation is partially regulated by intracellular cations.



**Fig. 2.2 Biosynthetic pathway of ectoine**

Zwitterionic solutes:	Occurrence:
betaine 	<u>Halotolerant:</u> <i>Thioalkalivibrio versutus</i> ; <i>Actinopolyspora</i> sp. <u>Halophilic:</u> <i>Actinopolyspora halophila</i> ; <i>Halorhodospira halochloris</i> <i>Methanohalophilus portucalensis</i> FDF1; <i>Methanosarcina</i> <i>thermophila</i> ; <i>Synechococcus</i> sp. DUN 52
ectoine 	<u>Halotolerant:</u> <i>Sporosarcina pasteurii</i> .; <i>Brevibacterium epidermidis</i> ; <i>Thioalkalimicrobium aerophilum</i> ; <i>Vibrio cholerae</i> and <i>V. costociola</i> <u>Halophilic:</u> <i>Chromohalobacter israelensis</i> ; <i>Chromohalobacter</i> <i>saalexigens</i> ; <i>Halorhodospira halochloris</i> ; <i>Halomonas elongata</i> , <i>H.</i> <i>variabilis</i> and phylogenetically related organisms; <i>Methylarcula</i> <i>marina</i> and <i>M. terricola</i> ; <i>Methylophaga alcalica</i> and <i>Methylophaga</i> <i>natronic</i> ; aerobic, halophilic isolates from Mono Lake
hydroxyectoine 	<u>Halophilic:</u> <i>Halomonas elongata</i> ; <i>Nocardiopsis halophila</i>
Nγ-acetyldiaminobutyrate 	<u>Halotolerant:</u> <i>Halomonas elongata</i> CHR63
Nε-acetyl-β-lysine 	<u>Halotolerant:</u> <i>Methanosarcina thermophila</i> ; <i>Methanothermococcus</i> <i>thermolithotrophicus</i> ; <i>Methanosarcina mazei</i> Gö1 <u>Halophilic:</u> <i>Methanohalophilus portucalensis</i> FDF1; <i>Methanohalophilus</i> Z7302
β-glutamine 	<u>Halophilic:</u> <i>Methanohalophilus portucalensis</i> FDF1

### Zwitterionic organic osmolytes in eubacteria and archaea

## 2.7 BIOTECHNOLOGICAL APPLICATION OF OSMOLYTES

Halophilic bacteria provide a high potential for biotechnological applications for two reasons: The majority can use a large range of compounds as their sole carbon and energy source and most of the bacteria can grow at high salt concentrations, minimizing the risk of contamination (Rodriguez Valera, 1993). Moreover, several genetic tools developed for the nonhalophilic bacteria can be applied to the halophiles, and hence their genetic manipulation seems feasible (Ventosa *et al.*, 1998). The current industrial applications and the possibilities of their biotechnological applications are summarized as follows: Halophilic bacteria have the ability to produce compatible solutes, which are useful for the biotechnological applications. Few important compatible solutes like glycine betaine and ectoine can be used as stress protectants against high salinity, thermal denaturation, desiccation, freezing and also as stabilizers of enzymes, nucleic acids, membranes and whole cells (Galinski, 1993). The industrial applications of these compounds in enzyme technology are most promising (Ventosa and Nieto, 1995). Ectoine, a viable osmolyte have recently gained importance in biotechnology as it has been used in dermatopharmacy as anti ageing agents in skin creams, as components of shampoo, for oral care and as adjuvants for vaccines (Vargas *et al.*, 2006). The other compatible solutes such as trehalose, glycerol, proline, ectoine and hydroxyectoine from halophilic bacteria showed the highest efficiency of protection of lactate dehydrogenase against freeze thaw treatment and heat stress. Among the compatible solutes, ectoine was the most effective freeze stabilizing agent for phosphofructokinase (Wohlfarth *et al.*, 1989). Secondly, halophilic bacteria produce a number of extra and intra cellular enzymes and antimicrobial compounds that are

currently of commercial interest (Kamekura and Seno, 1990). Halophilic bacteria can produce enzymes that have optimal activity at high salinity, which is advantageous for insensitive industrial processes. Several extracellular enzymes have been reported to be produced from halophilic bacteria such as amylases from *Micrococcus halobius* (Onishi, 1970) and *Halobacterium halobium* (Good and Hartman, 1970), nucleases from *Micrococcus varians* (Kamekura and Onishi, 1974 a) and *Bacillus* spp. (Onishi, *et al.*, 1983) as well as proteases from *Halobacterium salinarum* (Norberg and Hofsten, 1969), *Bacillus* spp. (Kamekura and Onishi, 1974 b) and *Pseudomonas* spp. (Makino *et al.*, 1981). A serine protease from the family of *Halobacteriaceae* showed high activity at 75-80°C in the presence of 25% (w/v) salt (Galinski and Tindall, 1992). Halophilic bacteria produce a variety of hydroxyalkanoates, which are useful for thermally processed plastics (Vreeland, 1993). *Haloferax mediterranei* produces exopolysaccharides (up to 3 g/L) with pseudoplastic behavior that are resistant to pH, heat, and shear. They show higher viscosity at dilute concentrations and elevated temperatures than commercial polymers such as xanthan gum (Galinski and Tindall, 1992; Ventosa *et al.*, 1998). Mainly, the application of halophilic and moderate halophilic bacteria in environmental biotechnology is possible for the recovery of saline soil, the decontamination of saline or alkaline industrial wastewater and the degradation of toxic compounds in hypersaline environments. Woolard and Irvine (1992) reported the utilization of biofilm from a moderately halophilic bacterium isolated from the saltern at the Great Salt Lake, Utah, for the treatment of hypersaline wastewater containing phenol. Benzoate and other aromatic compounds could be degraded by *Pseudomonas halodurans* by cleavage of aromatic rings (Rosenberg, 1983). Hayes *et al.*, (2000) stated that *Chromohalobacter marismortui* or

*Pseudomonas beijingensis*, moderately halophilic bacteria, isolated from a hypersaline spring in Utah could have utilized organophosphates as phosphorus sources for growth.

### **2.7.1 THERMAL DNA MELTING AND DNA STABILITY**

A first approach to compatible solute and nucleic acid interactions should aim at the effect of compatible solutes on the melting of double stranded DNA (dsDNA). Rees and co-workers (Rees *et al.*, 1993) showed that glycine betaine in high concentrations eliminates the GC dependency of dsDNA melting, a phenomenon counteracting and being counteracted by the influence of inorganic ions. Similar effects were reported from several other groups; DNA helix destabilization by proline and its possible role in osmoadaptation (Rajendrakumar *et al.*, 1997), effects of phenoxazine derivatives (Chandramouli *et al.*, 2004) and lowering of dsDNA  $T_m$  by trehalose (Spiess *et al.*, 2004).

### **2.7.2 PCR IMPROVEMENT**

In a polymerase chain reaction we have to take care of the proteins and their interactions with DNA in addition to thermal denaturation of the DNA template and formation of new DNA duplexes. To complicate matters the enzymatic reaction itself might be influenced by the addition of solutes or co solvents (Kurz, 2008). Additives which improve PCR reactions are highly sought after since even a simple standard application to amplify a certain region of DNA might not work properly with a particular sequence. Possible reasons might range from the GC content of the template to the complexity of compounds in a diagnostic PCR on clinical samples (Abu Al Soud and Radstrom, 1998). Cations, mainly  $K^+$  and  $Na^+$  are among the main inhibitors of a PCR and  $Mg^{2+}$  – which is needed by the DNA polymerase, has a

reliant impact on PCR specificity (Ignatov *et al.*, 2003). Glycine betaine is the first solute used to counteract the effect of NaCl (Weissensteiner and Lanchbury, 1996). They introduced the term *cosolute* for such additives. Ever since betaine has been used as a PCR facilitator: as a single compound (Hengen, 1997; Shamma *et al.*, 2001; Shamma *et al.*, 2001) in combination with DMSO (Baskaran *et al.*, 1996; Kang *et al.*, 2005; Shi and Jarvis, 2006; Sahdev *et al.*, 2007) or together with BSA. It has since been used on templates with varying GC content, to enhance formation of long PCR products (Diakou and Dovas, 2001; Chen *et al.*, 2004), in low temperature PCR with heat labile polymerases (Lakobashvili and Lapidot, 1999) and in diagnostic PCR. Sugars like trehalose or sucrose have also been used with similar success as have been low molecular weight sulfones, amides and sulfoxides (Chakrabarti and Schutt, 2002). In addition, recent data demonstrates that synthetic derivatives of ectoines can also act as powerful PCR enhancer (Kurz, 2008).

The properties of osmolytes make them suitable for a variety of uses in biotechnology as long as one can generate reasonable quantities either *in vivo* or *in vitro*. Insoluble or misfolded overexpressed proteins can often be partially denatured and refolded in the presence of osmolytes. A specific example is the use of osmolytes to enhance the yield of folded, functional cytotoxic proteins directed to the periplasm of *E. coli* (Barth *et al.*, 2000). Cells grown in 4% NaCl with 0.5 M sorbitol and supplemented with 10 mM betaine can accumulate large amounts of the target protein in the periplasm. Several osmolytes (notably betaine, ectoine) have been shown to be useful in PCR amplification of GC rich (72.6% GC) DNA templates with a high  $T_m$ . In particular, ectoine was shown to outperform regular PCR enhancers by reducing the  $T_m$  of DNA (Schnoor *et al.*, 2004). Organic osmolytes have also been used as

cryoprotectants. In a recent study, the ability of betaine to act as a cryoprotectant during freezing of diverse bacteria was examined (Roberts, 2005). Betaine is often much better than two common cryoprotectant mixtures such as serum albumin and trehalose/ dextran, particularly under conditions simulating longterm storage (Cleland *et al.*, 2004). Betaine is as effective as glycerol for liquid nitrogen freezing of halophilic archaea, and neutrophilic Fe-oxidizing bacteria (Roberts, 2005). Choline dehydrogenase (EC 1.1.99.1) catalyzes choline to glycine betaine via a betaine aldehyde intermediate. Such a reaction is of considerable interest for biotechnological applications in that transgenic plants engineered with bacterial glycine betaine synthesizing enzymes have been shown to have enhanced tolerance towards various environmental stresses, such as hypersalinity, freezing, and high temperatures.

**MATERIALS  
AND  
METHODS**

### **3. MATERIALS AND METHODS**

#### **3.1. MATERIALS**

##### **3.1.1 SAMPLES**

Representative samples of dried salted fishes were obtained from retail fish shops in Cochin, Kerala, India. The samples were collected in the month of December 2005. Salted fish samples selected for the study were

SF1 *Scoliodon* spp.

SF2 *Thrissina thryssa*

##### **3.1.2 SAMPLING**

Salted fish samples were collected in sterile polythene bags and carried to the laboratory. Analysis for various bacteriological and physico chemical parameters were initiated within 2 h of sample collection as per the procedure outlined in 3.2.2

##### **3.1.3 INSTRUMENTS USED**

- I. Serological water baths (Beston, India)
- II. Incubators, Hot air oven and Deep freezers (Labline, India)
- III. Shaker incubator (Innova 4230, USA)
- IV. Autoclave (Osprey, UK)
- V. Spectrometer (Varion Cary 100 Bio, Australia)
- VI. Stomacher (Lab blender 400, Seward Medical, UK)
- VII. Quebec colony counter
- VIII. Microscope (Zeiss, USA)
- IX. Centrifuge (Eppendorf 584 R, USA)
- X. Microfuge (Genei, India)
- XI. Thermal cycler (MJ Research PTC-150, USA)

- XII. MilliQ water unit (Millipore, USA)
- XIII. Cooling centrifuge (Remi C24)
- XIV. Gel documentation system (Alpha Imager 1440, USA)
- XV. UV transilluminator (Genei, India)
- XVI. pH meter (Cyberscan 510, USA)
- XVII. Weighing balance (Sartorius, Germany)

### **3.1.4 BACTERIAL GROWTH MEDIA**

#### **3.1.4.1 DEHYDRATED MEDIA**

The growth media used in the study were from Oxoid (Hampshire, UK), Difco (Becton, Dickinson and Co., USA), Merck (Darmstalt), Sigma (USA) and HiMedia Laboratories Ltd. (Bombay). The list of the media/broth, staining solutions, test reagents and kits used are given below.

- a. Nutrient agar (Oxoid)
- b. Salt agar (Oxoid)
- c. Eosin Methylene Blue Agar (Oxoid)
- d. Lactose broth (Merck)
- e. Simmon's citrate agar (Oxoid)
- f. Triple Sugar Iron agar (Oxoid)
- g. Lead acetate agar (Oxoid)

#### **3.1.4.2 COMPOUNDED MEDIA**

The following list of media was prepared as per the procedure outlined in USFDA (2001).

- a. Aminoacid decarboxylase broth

- b. Hugh and Leifson's glucose oxidative/fermentative medium (H&L glucose medium)
- c. Methyl Red and Voges Proskauer (MRVP medium)
- d. Modified MacConky's broth (for MPN)
- e. Normal saline (NS)
- f. Sugar fermentation broth (glucose, galactose, maltose, sucrose, dulcitol, trehalose, raffinose, salicin, lactose, arabinose, cellobiose, xylose, sorbitol, inositol)
- g. Tryptone broth (Indole medium)

Other compounded media used in this study are

i) Luria-Bertani (LB) broth

Tryptone	: 1 gm
Yeast extract	: 0.5gm
NaCl	: 0.5gm
D/W	: 100 ml

ii) Luria-Bertani (LB) agar

Tryptone	: 1 gm
Yeast extract	: 0.5gm
NaCl	: 0.5gm
Agar	: 1.5gm
D/W	: 100 ml

iii) Salt agar

NaCl	: 5.8 g
Peptone	: 0.5 g

Casein	: 0.5 g
Agar	: 1.5 g
D/W	: 100 ml

iv) High salt casein agar

NaCl	: 25 g
MgCl <sub>2</sub> ·6H <sub>2</sub> O	: 2 g
Casein	: 0.5 g
Yeast extract	: 0.5 g
KCl	: 0.2 g
CaCl <sub>2</sub> ·H <sub>2</sub> O	: 0.02 g

v) Alkaline bacillus medium

Peptone	: 1 g
Glucose	: 1 g
Yeast extract	: 0.5 g
K <sub>2</sub> HPO <sub>4</sub>	: 0.1 g
Na <sub>2</sub> CO <sub>3</sub> solution	: 10 ml
Agar	: 1.5 g
D/W	: 100 ml

### 3.1.5 DYES, CHEMICALS AND OTHER REAGENTS

#### I) DYES AND REAGENTS

The following list of dyes and reagents was prepared as per the procedure outlined in USFDA (2001).

- Andrade's indicator (for sugar fermentation tests)
- Crystal violet (for Gram's staining)

- Methyl Red indicator (for MR test)
- Saffranine (for Gram's staining)
- Gram's iodine (for Gram's staining)
- Kovac's cytochrome oxidase reagent
- Kovac's indole reagent
- TTC (Triphenyl tetrazolium chloride)
- Voges Proskauer reagent
- Coomassie brilliant blue (for SDS-PAGE)

## **II) ANTIBIOTICS (HIMEDIA, INDIA)**

Ampicillin

Kanamycin

## **III) MOLECULAR BIOLOGY REAGENTS AND CHEMICALS**

### a) PCR primers (Sigma, India)

Primers hydrated to a stock concentration of 100  $\mu\text{M}$  using sterile MilliQ water and then an aliquot of the primer stock solution was diluted to a working concentration of 10  $\mu\text{M}$  using sterile MilliQ water. The stock and working primer solutions were stored at  $-20^{\circ}\text{C}$  until used.

### b) dNTP's (MBI Fermentas, Germany)

### c) PCR buffer (MBI Fermentas)

### d) *Taq* DNA polymerase (MBI Fermentas)

### e) *Pfu* DNA polymerase (MBI Fermentas)

### f) $\text{MgCl}_2$ (MBI Fermentas)

### g) Agarose (Sigma, USA)

- h) TAE (Tris Acetate EDTA) buffer
- i) Acrylamide/Bis-Acrylamide (Sigma)
- j) Ammonium per sulphate (Sigma)
- k) Sodium Dodecyl Sulfate (Sigma)

Stock solution (50 X)

Tris base	: 242 gm
Glacial acetic acid	: 57.1 ml
0.5 M EDTA	: 100 ml
Distilled water	: 1 L

Working solution (1 X)

20 ml of the stock TAE buffer was mixed with 980 ml of distilled water to prepare the working solution of 1 X TAE buffer.

Sample buffer (SDS-PAGE)

Distilled water	: 3.8 ml
Tris-HCl 0.5 M, pH 6.8:	1ml
Glycerol	: 0.8 ml
10% SDS	: 1.6 ml
2- mercaptoethanol	: 0.4 ml
1% Bromophenol blue:	0.4 ml

Stock electrode buffer

Tris base	: 9 g
Glycine	:43.2 gm
SDS	:3 gm

#### 7.5% separating gel

Distilled water	:4.85 ml
Tris-HCl (1.5 M)	:2.5 ml
10% SDS	: 100 $\mu$ l
Acrylamide	: 2.5 ml
APS 10%	: 50 $\mu$ l
TEMED	: 5 $\mu$ l

#### 4% Stacking Gel

Distilled water	:6.1 ml
Tris-HCl (1.5 M)	:2.5 ml
10% SDS	: 100 $\mu$ l
Acrylamide	: 1.33 ml
APS 10%	: 50 $\mu$ l
TEMED	: 10 $\mu$ l

- l) 6 X gel loading buffer
- m) DNA ladder (MBI Fermentas)
- n) Ethidium Bromide (0.5 mg/ml)
- o) Acetic acid (SRL, India)
- p) Restriction enzymes (MBI Fermentas)

#### 3.1.6 KITS USED

- I. MinElute Gel purification Kit (Qiagen, Germany)
- II. PCR purification Kit (Qiagen, Germany)
- III. Expression Kit (Qiagen, Germany)

### 3.1.7 BACTERIAL CULTURES

The list of bacterial cultures used in this study is given in the Table 3.1, which included the bacterial host used for cloning and expression studies.

## 3.2 METHODS

### 3.2.1 PHYSIOCHEMICAL ANALYSIS OF SALTED FISH SAMPLES

Salted fish samples were analysed for salinity using a refractometer (Atago, Japan). Briefly the salted fish samples were mashed thoroughly with sterile distilled water in a Mortar and Pestle. Later, two drops of the sample fluid was placed on the measuring surface of the refractometer's prism. The salinity was measured by the shadow line across the refractometer's internal scale.

**Table 3.1 Bacterial cultures used in this study**

<b>Strains</b>	<b>Experiment</b>
<i>E.coli</i> JM109	Transformation (Cloning)
<i>E. coli</i> M15 (pREP4)	Transformation (Expression)

### **3.2.2 BACTERIOLOGICAL ANALYSIS**

Dry fish samples were quantitatively studied for total plate count (TPC) and the identification of *Escherichia coli* and *Bacillus halodurans*.

#### **3.2.2.1 QUANTITATIVE ANALYSIS**

The total plate count of salt tolerant bacterial population was estimated in three agar media with different salt levels as per the details given in 3.2.2.3

#### **3.2.2.2 PREPARATION OF SAMPLES**

10 g of salted fish samples were transferred into the sterile stomacher bag (Seward Medical, London, UK) containing 90 ml 0.85% normal saline (NS). This was blended in a stomacher (Lab blender 400, Seaward Medical, London, UK) for 60 seconds at normal speed to obtain  $10^{-1}$  dilution. Using a sterile pipette, 1 ml of the supernatant was aseptically transferred into the 9 ml NS tube and mixed well to obtain  $10^{-2}$  dilution. Similarly other required dilutions to  $10^{-4}$  were prepared for inoculation.

#### **3.2.2.3 ENUMERATION OF SALT TOLERANT MICROORGANISMS**

One ml of the appropriate dilutions were pipetted to marked sterile Petri plates containing about 15-18 ml of salt agar, nutrient agar and high salt casein agar with the salt concentration of 5, 3, 25% of NaCl respectively (Atlas, 1946). The plates were later incubated at 37°C for 3-7 days in sealed polythene bags. After the incubation period, the bacterial colonies were counted using Quebec colony counter. The TPC per gram of the sample was calculated as,

$$\text{TPC/g sample} = \text{Average count} \times \text{Dilution factor}$$

#### **3.2.2.4 ISOLATION OF BACTERIA**

Representative colonies developed in quarter of the Petri plates were isolated randomly and purified in the same media, in which they were isolated. The purified isolates were maintained and stored in room temperature till further analysis.

### **3.3 MORPHOLOGICAL OBSERVATIONS**

All the samples were observed for morphological features, Gram reaction and motility.

#### **3.3.1 GRAM'S STAINING**

Gram's staining was performed according to H.C.Gram (1884). Staining can reveal the size of the organism and at the same time differential staining can differentiate the Gram positive and Gram negative bacteria. Hence Gram staining is preferred. Gram staining procedure involves the use of two dyes namely crystal violet (primary stain), safranin (the secondary stain) a mordant Gram's iodine and a decolorizer ethyl alcohol (95%). Gram positive bacteria retain the violet color of the primary stain and appear violet while the gram negative form appears pink in color. The mordant seems to form an insoluble complex and fix the color to the bacterial cell. The lipid content present in the cell wall of bacteria is readily dissolved by the action of ethyl alcohol (Aneja, 1996).

A thin smear of the culture was prepared with sterile saline on a clean glass slide. The smear was dried and heat fixed. Slides were added with crystal violet and left for few minutes. Then the slide was washed with water. The smear was covered in iodine solution for 1 minute and then treated with fresh ethyl alcohol. After washing, it was followed with addition of counter stain safranin and it was left for 30

seconds. Then the slide was washed, dried and examined under the microscope at 100 X magnifications.

### **3.3.2 KOPELEFF AND BEERMAN'S MODIFIED GRAM'S STAINING**

(Gimenez, 1965)

A thin smear of the bacterial growth from agar plate was made on the slide and heat fixed. The smear was covered with methyl violet and allowed to react for one minute. The slide was rinsed with tap water. A few drops of Gram's iodine was added to cover the smear and allowed to react for 30 seconds to one minute. The slide was rinsed with tap water. The slide was then subjected for decolourisation with 70% ethanol. Then the slides were flooded with safranin for one minute. Washed with distilled water and observed under oil immersion objective.

### **3.3.3 MOTILITY (Armitage *et al.*, 1977)**

The hanging drop method was followed to observe the motility of the isolates. Vaseline was applied on four corners of the cover slip with the help of the applicator stick. One loopful of culture was placed on the center of the cover slip and the cavity slide was placed over it. The slide was examined under the microscope to observe the motility of the organisms.

## **3.4 IDENTIFICATION BY BIOCHEMICAL TESTS**

To identify and characterize the bacterial colonies isolated from the plate culture the following biochemical tests were carried out.

### **3.4.1 INDOLE TEST**

Indole is a nitrogen containing compound formed from the degradation of the amino acid tryptophan by definite bacteria. Tryptophan present in the culture media

was converted into indole pyruvic acid and ammonia by the enzyme tryptophanase. Indole reacts with aldehydes to produce a red color product.

The inoculum size of 0.1 ml was inoculated to the tryptone broth and incubated at 37°C for 24 hrs. After completion of test, 1.0 ml of Kovac's reagent was added. The positive result was indicated by a bright fuchsin red color at the interface of the reagent and the broth within seconds after adding the reagent.

#### **3.4.2 METHYL RED TEST (MR)**

Methyl red test was used to identify the change in the pH of the medium. Specific microbes such as enteric bacteria can ferment glucose to pyruvate and produce mixed acid and other end products due to excess acid production. So that the pH of the broth is reduced to acidic this can be detected by pH indicator.

The inoculum size of 0.1 ml was inoculated to the MRVP medium and incubated at 37°C for 24 hrs. After completion of test, 5 drops of the methyl red solution was added directly to the broth. The development of a stable red color in the medium indicated the positive result.

#### **3.4.3 VOGES PROSKAUER TEST (VP)**

Some organisms ferment glucose, pyruvate and produce butylenes, glycol and acetone which were more neutral in nature.

The inoculum size of 0.1 ml was inoculated to the MRVP medium and incubated at 37°C for 24 hrs. After completion of test, 1 ml of the culture broth was transferred to a clean test tube. 0.6 ml of solution A was added to the tube, followed by 0.2 ml of solution B. The tube was shaken gently to expose the medium to atmospheric oxygen and allowed to remain undisturbed for 10 to 15 minutes. A

positive result was indicated by the development of a red colour within 15 minutes or more but not over than 1 hr. after adding the reagents.

#### **3.4.4 CITRATE UTILISATION TEST**

Some of the bacteria were capable of utilizing citrate as the sole carbon source and ammonium phosphate as the sole source of nitrogen. Consequently, the pH of the medium will change and was indicated by a color change of the medium.

Simmon's citrate medium slants were prepared and inoculated the tube with a loop full of inoculum and incubated at 37°C for 24 – 48 hrs. Change in the color from green to blue indicated the positive reaction.

#### **3.4.5 TRIPLE SUGAR ION AGAR TEST**

This test is mainly performed to analyse the bacteria's ability to ferment lactose, sucrose, and glucose, H<sub>2</sub>S and CO<sub>2</sub> production. Acid production in the media is indicated by the change in colour of medium to yellow. An alkaline reaction of the medium was indicated by development of purple color. Production of H<sub>2</sub>S was indicated by the formation of black color as H<sub>2</sub>S combines with ferrous ammonium sulphate.

TSI medium slants were prepared and a loopful of culture was surface streaked on the slant and incubated at 37°C for 48 hrs.

#### **3.4.6 CATALASE TEST**

Most aerobic organisms were capable of splitting H<sub>2</sub>O<sub>2</sub> to release free O<sub>2</sub>. The release of the O<sub>2</sub> gas can be visualized as white bubbles when a few drops of H<sub>2</sub>O<sub>2</sub> were added to the culture.

The bacterial cells were transferred to the surface of a glass slide. 1–2 drops of 3% hydrogen peroxide were added over the cells. Rapid appearance of gas bubbles indicated the positive result.

#### **3.4.7 NITRATE TEST**

The inoculum size of 0.1 ml was inoculated to the nitrate reduction broth and incubated at 37°C for 48 hrs. 0.1ml of sulphalinic acid was added to each culture tube, followed by few drops of  $\alpha$  - naphthalamine and mixed thoroughly. The development of a red color indicated a positive result while no red color developed indicates the negative result. The result of a negative test was confirmed by adding small amounts of zinc powder. The true negative test was indicated by the development of the red color.

#### **3.4.8 OXIDASE TEST**

The filter paper (Whatman no. 40) was placed into a petri plate and wet with 0.5 ml of 1% dimethyl-p-phenylenediamine dihydrochloride. The bacterial cells were streaked onto the reagent zone of the filter paper. The development of a deep blue color at the inoculation site within 10 seconds indicated the positive result.

#### **3.4.9 HYDROGEN SULFIDE PRODUCTION**

The bacterial isolates were stabbed deep into the lead acetate agar slant and also streaked on the surface of the slant. The slants were incubated at 37°C for 24 hrs. Development of brownish color on the surface and along the line of the stab indicated the positive result.

#### **3.4.10 GELATIN HYDROLYSIS**

Each bacterium to be tested was stabbed deep into the gelatin medium with 10% (w/v) marine salts containing: NaCl, 8.1g; MgCl<sub>2</sub>, 0.7g; MgSO<sub>4</sub>, 0.96g; CaCl<sub>2</sub>,

0.036g; KCl, 0.2g; NaHCO<sub>3</sub>, 0.006g and NaBr, 0.0026g (Rodriguez Valera *et al.*, 1980). The inoculated gelatin medium was incubated at 37°C for 24 hrs. Gelatin hydrolysis was indicated by the liquefaction of the medium after the tube was kept at 4°C for 20-30 minutes.

#### **3.4.11 CASEIN HYDROLYSIS**

Casein hydrolysis was tested on plates composed of 10% marine salts with 5% skim milk powder (Cowan and Steel, 1974). Hydrolysis of casein by caseinase positive organism will be indicated by a clear zone around the line of growth, by degrading the milk protein in the agar medium.

Casein agar plates were prepared and plated with the bacterial isolates. The plates were incubated at 37°C for 24 hrs. After incubation, the plates were observed for a clear zone around the line of growth.

#### **3.4.12 STARCH HYDROLYSIS**

Starch hydrolysis was performed with 10% marine salts containing 1% starch. When iodine is added to starch agar medium, the iodine starch complex gives a characteristic dark brown or deep purple color reaction. This indicated the positive result.

Starch agar plates were prepared and plated with the bacterial isolates. The plates were incubated at 37°C for 24 hrs. After incubation, the starch agar plate was flooded with Gram's iodine. The plates were later observed for a clear zone around the line of growth.

#### **3.4.13 SUGAR FERMENTATION TESTS**

Sugar fermentation from glucose, fructose, lactose, mannitol, trehalose, maltose, sucrose, salicin, mannose, cellobiose, rhamnose, dulcitol, xylose, raffinose,

sorbitol, adonitol, inulin, galactose, inositol and arabinose were detected in the basal medium: 10% (w/v) marine salts (Rodriguez Valera *et al.*, 1980), 1% (w/v) Peptone, 0.5% (w/v) yeast extract and 0.001 % (w/v) phenol red. Carbohydrates were sterilized by filtration and added to the previously autoclaved basal medium at a final concentration of 1% (w/v). Inverted Durham's tubes were used to visualize gas production. The test samples were inoculated and incubated at 37°C for 24 hrs. After incubation the tubes were observed for acid and gas production.

### **3.5 PHYSIOLOGICAL CHARACTERIZATION**

#### **3.5.1 GROWTH AT DIFFERENT SALT CONCENTRATION**

Growth at different salt concentration was determined on nutrient agar medium with 0.5, 2, 5, 10, 20% NaCl. The growth on plate was rated as stringent, moderate, good, profused and negative based on visual observation. The plates were incubated at 37°C for 48 hrs. The result was considered positive if there was visible growth after 2 days of incubation.

#### **3.5.2 GROWTH AT DIFFERENT pH LEVELS**

The pH range for growth was determined in the nutrient broth media, in which the pH was adjusted to 8.0, 9.0 and 10.0 with HCl or KOH. If necessary, the pH was readjusted after sterilization. The nutrient broth tubes were incubated at 37°C for 48 hrs. The result was considered positive if there was visible growth after 2 days of incubation.

#### **3.5.3 GROWTH AT DIFFERENT TEMPERATURE LEVELS**

Growth range at various temperatures was determined in the nutrient broth media at 15, 25, 30, 35 and 45°C. The tubes were incubated at 37°C for 48 hrs. The result was considered positive if there was visible growth after 2 days of incubation.

### **3.6 IDENTIFICATION OF *BACILLUS HALODURANS***

Identification and biochemical characterization of *B. halodurans* using conventional method was carried out as per (Nielsen *et al.*, 1995) and used as a source for the identification of operon encoding for ectoine biosynthesis (*ectABC*). Briefly the method of identification consisted the following protocol.

#### **3.6.1 PREPARATION OF SAMPLE**

10 g of salted fish samples were transferred into the sterile stomacher bag (Seward Medical, London, UK) containing 90 ml 0.85% normal saline (NS). This was blended in a stomacher (Lab blender 400, Seaward Medical, London, UK) for 60 seconds at normal speed to obtain  $10^{-1}$  dilution. Using a sterile pipette, 1 ml of the supernatant was aseptically transferred into the 9 ml NS tube and mixed well to obtain  $10^{-2}$  dilution. Similarly other required dilutions were prepared for inoculation.

#### **3.6.2 GROWTH MEDIUM AND CONDITIONS OF *B. HALODURANS***

0.5 ml of  $10^{-3}$  to  $10^{-5}$  dilutions was used to spread in growth medium. Enumeration and isolation of the bacteria was done on Alkali bacillus agar medium. The plates were incubated at room temperature for 48 hrs in sealed polythene bags.

#### **3.6.3. IDENTIFICATION OF *B. HALODURANS***

Identification of the bacterial isolate was performed with the procedures outlined in 3.4.1 – 3.4.13

##### **3.6.3.1 ISOLATION AND CONFIRMATION OF *B. HALODURANS***

Characteristic well separated colonies from alkali bacillus agar plates were subjected to culture morphology, hydrolysis of Tween 40 and 60, casein, gelatin and starch. The positive isolates were selected and proceeded for nitrate reduction, optimal growth analysis with pH range of 9-10 and salt tolerance analysis with 9-12%

NaCl. *B. halodurans* isolate was further confirmed by the carbohydrate utilization tests with L-arabinose, galactose, xylitol, inositol and mannose (Nielsen *et al.*, 1995).

#### **3.6.4 16S rDNA ANALYSIS**

16S rDNA analysis is a sensitive molecular method, in which the isolate can be identified using ribosomal approach. This method is a confirmatory method for identification of bacterial species. In this study, this identification procedure for *B. halodurans* was performed using the following procedures.

##### **3.6.4.1 PREPARATION OF GENOMIC DNA**

Genomic DNA extraction from *B. halodurans* was performed as described by Moore, (1995). 1.5 ml of the late exponential phase bacterial cells were transferred into a microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was discarded. The clean cells were suspended in 200 µL of lysis buffer and incubated at 37°C for 30 minutes. Then, 30 µL of 10% SDS and 10 µL of proteinase K (20 mg/mL) were added. The tube was gently mixed and incubated at 37°C for 1 hour. An equal volume of phenol: chloroform was added and the tube was centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube. Twice volume of cold absolute ethanol was added to the supernatant and gently mixed to precipitate the DNA. The tube was then centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was discarded. The precipitated DNA was washed with 500 µL of 70% ethanol, dried it at 37°C for 30 minutes. The DNA pellet was resuspended with 50 µL of TE buffer and kept overnight at 4°C to dissolve the precipitated DNA.

#### **3.6.4.2 PCR AMPLIFICATION OF 16S rDNA OF BACTERIAL ISOLATE**

The 16S rDNA of halophilic bacterium isolate was amplified by PCR using primers 27f and 1525r (Table 3.2). The PCR was performed using a PTC-150 Mini cycler (MJ Research, Waltham, MA, U.S.A.) with a primary heating step for 2 minutes at 95°C, followed by 30 cycles of denaturation for 20 seconds at 95°C, annealing for 60 seconds at 55°C, and extension for 2 minutes at 72°C, then followed by a final extension step for 7 minutes at 72°C. Each 25 µL reaction mixture contained 2 µL of genomic DNA, 14.25 µL of MilliQ water, 2.5 µL of 10× buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 1.5 µL of MgCl<sub>2</sub> (25 mM), 2.5 µL of dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration), 1.0 µL of each primer (20.0 pmoles/µL), and 0.25 µL of *Taq* DNA polymerase (MBI Fermentas, U.S.A.). The PCR amplified product was analyzed on 1% agarose gel containing ethidium bromide (0.5 µg/mL), along with 1 kb DNA molecular weight marker (MBI Fermentas) and documented using a gel documentation system (Alpha Imager 1220, Alpha Innotech Corporation, San Leandro, CA, U.S.A.).

**Table 3.2 Oligonucleotide primers used for PCR amplification of 16S rDNA**

<b>Primer designation</b>	<b>Sequence (5'–3')</b>	<b>Reference</b>
27f	AGAGTTTGATCMTGGCTCAG	Lawson <i>et al.</i> , 1996
1525r	AAGGAGGTGWTCCARCC	Lawson <i>et al.</i> , 1996

#### **3.6.4.3 SEQUENCING OF PCR AMPLICONS**

The PCR amplicon was purified by MinElute Gel purification Kit (Qiagen, Hilden, Germany) and was sequenced on an ABI PRISM 377 genetic analyzer (Applied Biosystems Inc., Foster City, CA, USA).

### **3.7 PCR AMPLIFICATION, CLONING, EXPRESSION AND ENZYME ACTIVITY OF *ectABC* GENE CLUSTER OF *B. HALODURANS***

The functional characterization and *in silico* sequence analysis of choline dehydrogenase from *E. coli* isolate were determined as per the procedure reported (Anbu Rajan *et al.*, 2008 a, b). Molecular characterization studies were performed using the following procedures.

#### **3.7.1 DNA EXTRACTION**

Genomic DNA extraction from *E. coli* was performed, following the method of Ausubel *et al.*, 1994. Briefly, 1.5 ml of over night culture was transferred into microfuge tube and centrifuged at 10,000 rpm for 1 to 2 minutes. The supernatant was discarded and the pellet was dried on filter paper. 100 µl of Milli Q water was added

to the pellet, and the pellet was gently tapped. The pellet was subjected for heating in boiling water bath for 5 minutes. The lysate was centrifuged at 5,500 rpm for 5 minutes. Pellet was discarded and the supernatant was used for PCR amplification.

### 3.7.2 PCR AMPLIFICATION OF *ectABC* GENE CLUSTER

PCR amplification of *ectABC* gene cluster of *B. halodurans* was performed by using gene specific primers. A pair of specific primers was designed (Table 3.3). PCR was performed in 50 µl of reaction mixture which contained 50 ng of genomic DNA, 0.5 µM of each primer, 200 µM each of dNTP ((MBI Fermentas),

**Table 3.3 Oligonucleotide primers used for PCR amplification of *ectABC* gene cluster**

<b>Primer designation</b>	<b>Sequence (5'–3')</b>	<b>Reference</b>
EO1	CGTATGCAGGTTCAATGCAATGAAAAAGCC	Anbu Rajan <i>et al.</i> , 2008
EO2	GCATTATTCGTCGTCGACTACCG	Anbu Rajan <i>et al.</i> , 2008

1.25 U of *Pfu* DNA polymerase (MBI Fermentas), 1X *Pfu* buffer; 2.5 mM of MgSO<sub>4</sub> and remaining autoclaved millipore water. Amplification was performed in a PTC-150 Mini cycler (MJ Research) with the following conditions; initial denaturation at 94°C for 3 min, followed by 30 repeated cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 2 min and final extension at 72°C for 5 min. The PCR amplified product was analyzed on 1% agarose gel along with DNA molecular weight marker (MBI Fermentas) and documented using a gel documentation system (Alpha Imager).

### **3.7.3 CLONING OF *ectABC* GENE CLUSTER**

The *ectABC* PCR product was purified by MinElute Gel purification Kit (Qiagen) and cloned into pDrive (Qiagen). Briefly, the ethidium bromide stained DNA fragment from TAE agarose gel was excised without disrupting the fragment. The gel slice was weighed in an already weighed empty microfuge tube. (Maximum weight of gel slice being 400 mg). For every 1 volume of the gel slice, 3 volume of binding buffer was added. (1 mg of weight equals to 1 µl of volume). The gel slice was incubated at 50°C for 5 to 10 minutes in a heat block. 1 volume of isopropanol equal to the original gel slice volume was added after complete dissolution. A spin column was placed in a clean, fresh 2 ml collection tube. 800 µl of sample was added to the spin column and centrifuged at 10,000 rpm for 1 minute. The filtrate was discarded and the spin column was replaced in the same collection tube. 750 µl of diluted wash buffer was added to the spin column. The spin column-collection tube assembly was centrifuged at 10,000 rpm for 1 minute. The filtrate was discarded and the spin column with the same collection tube was subjected to centrifugation for an additional minute at 10,000 rpm to remove any residual diluted wash buffer. The spin column was again placed in a new 2 ml collection tube and 30 µl of elution buffer was

added to the centre of the spin column and centrifuged for at 10,000 rpm for 1 minute. The spin column was discarded, and the collection tube was capped. The purified DNA was confirmed for its presence by gel electrophoresis on 1.5% agarose gel. The purified *ectABC* amplicons were ligated to the cloning vector pDrive with the reaction profile as mentioned in Table 3.4.

### **3.7.4 TRANSFORMATION OF *ectABC* GENE CLUSTER**

The pDrive-*ectABC* construct was transformed into *E. coli* JM109 (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (rK-mk+), *e14*-(*mcrA*-), *supE44*, *relA1*,  $\Delta$ (*lac-proAB*)/F' (*traD36*, *proAB*+, *lac Iq*, *lacZ* $\Delta$ M15) by following the procedure in (Sambrook and Russell 2001).

#### **3.7.4.1 PREPARATION OF COMPETANT CELLS**

A single bacterial colony was selected from plates that have been incubated for 16-20 hrs at 37°C. 1 ml of overnight culture was transferred into 100 ml of LB broth in a one liter flask and incubated at 37°C with vigorous agitation till the O.D. attains 0.4. Transfer the bacterial cells were transferred into sterile disposable ice cold 50 ml polypropylene tubes. Later the cultures were cooled to 0°C by storing the tubes on ice for 10 minutes. The cells were recovered by centrifugation at 2,700g for 10 minutes at 4°C. The medium was decanted from the cell pellet, and the tubes were kept in an inverted position on a pad of paper towel for one minute to allow the last traces of medium to drain away. The pellet was dissolved in 30 ml of ice cold magnesium chloride or calcium

**Table 3.4. Cloning reaction profile for *ectABC* gene cluster**

<b>Reagents</b>	<b>Volume (µl)</b>
PCR product	10
10X Buffer	2.5
T4 DNA Ligase	1
Plasmid	1.5
Nuclease free water	5
<b>Total</b>	<b>20</b>

chloride solution (80mMgCl<sub>2</sub>, 20mM CaCl<sub>2</sub>) with swirling or gentle vortexing. The cells were recovered by centrifugation at 2,700g for 10 minutes at 4°C. The medium was decanted from the cell pellet, and the tubes were kept in an inverted position on a pad of paper towel for one minute to allow the last traces of medium to drain away. The pellet was suspended by gentle vortexing in 2 ml of ice cold 0.1M CaCl<sub>2</sub> for each 50 ml of original culture. The cells were used directly for transformation studies.

#### **3.7.4.2 TRANSFORMATION PROCEDURE**

200 ml of CaCl<sub>2</sub> treated suspension of competent cells were directly transferred into chilled, sterilized 17×100mm polypropylene tubes using a chilled micropipette tip. To each tube containing competent cell suspension ligated DNA mixture was added. The contents of tubes were mixed by swirling gently. And the

tubes were kept on ice for 30 minutes. The tubes are then transferred to a rack placed in preheated 42°C circulating water bath. The tubes are placed in the rack for exactly 90 seconds without shaking. Rapidly the tubes were transferred to an ice bath allowing the cells to chill for 1- 2 minutes. 800 µl of SOB medium was transferred to each tube. The culture was incubated for 45 minutes in a water bath set at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. The appropriate volume of transformed competent cells was transferred on to SOB medium containing 20mM MgSO<sub>4</sub> and the appropriate antibiotic. The plates were stored at room temperature until the liquid has been absorbed. The plates were further incubated at 37°C for overnight and the transformants were selected by blue white selection.

### **3.7.5 CHARACTERIZATION OF THE RECOMBINANT PLASMID**

The white recombinant colonies were picked up and inoculated in 5 ml LB broth containing ampicillin and incubated at 37°C. The plasmids were isolated from the overnight culture by alkaline lysis (Sambrook and Russell, 2001).

#### **3.7.5.1 PREPARATION OF BACTERIAL CELLS**

2 ml of rich medium containing the appropriate antibiotic was inoculated with a single colony of transformed bacteria and incubated overnight at 37°C. 1.5 ml of the overnight culture was transferred into a microfuge tube and centrifuged at maximum speed for 30 seconds at 4°C. After centrifugation, the supernatant was discarded and the bacterial pellet was dried as feasible.

#### **3.7.5.2 LYSIS OF BACTERIAL CELLS**

The bacterial pellet was resuspended in 100 µl of ice cold alkaline lysis solution I by vigorous vortexing. 200 µl of freshly prepared alkaline lysis solution II

was added to each of the bacterial suspension and the contents were mixed by inverting the tube rapidly for 5 minutes. 150 µl of ice cold alkaline lysis solution III was added and dispersed through the viscous bacterial lysate by inverting the tube several times, the tubes were kept on ice for 3–5 minutes. The bacterial lysate was centrifuged at maximum speed for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. An equal volume of phenol-chloroform was added; the organic and aqueous phases were then mixed by vortexing and centrifuged the emulsion at maximum speed for 2 minutes at 4°C. The aqueous upper layer was later transferred to a fresh tube.

#### **3.7.5.3 RECOVERY OF PLASMID DNA**

The nucleic acids were precipitated from supernatant by the addition of two volumes of ethanol at room temperature. The solution was mixed by vortexing and then allowed to stand for two minutes at room temperature. The precipitated nucleic acid was collected by centrifugation at maximum speed for 5 minutes at 4°C. The supernatant was discarded and the tube was kept in an inverted position on a paper towel to allow all of the fluid to drain away. 1 ml of 70% ethanol was added to the pellet and inverted in the closed tube several times; the DNA was recovered by centrifugation at maximum speed for 2 minutes at 4°C. The supernatant was discarded and the tubes were kept open for few minutes at room temperature. The nucleic acid was dissolved in 50µl of TE (pH 8) containing 20µg/ml DNase free RNaseA and used for restriction analysis.

#### **3.7.5.4 RESTRICTION ANALYSIS OF *ectABC* GENE INSERT**

The recombinant plasmid with *ectABC* gene cluster was double digested with *SacI* and *BamHI* (MBI Fermentas) restriction enzymes. The detailed reaction profile

is given in Table 3.5. The reaction mixture was incubated overnight at 37°C in a water bath. The digested product was analyzed on 1.5% agarose gel electrophoresis.

### 3.7.6 SEQUENCING OF *ectABC* GENES

The sequencing was performed initially with vector primers M13 forward and M13 reverse (MBI Fermentas) and later by primer walking. The sequencing was performed with automatic ABI PRISM 377 genetic analyzer (Applied Biosystems).

**Table 3.5. Restriction analysis profile of *ectABC* gene cluster**

<b>Reagents</b>	<b>Volume (µl)</b>
Recombinant plasmid	2
Enzyme buffer (10X)	2
<i>Bam</i> HI (10U/µl)	0.5
<i>Xba</i> I (10U/µl)	0.5
Nuclease free water	15
<b>Total</b>	<b>20</b>

### **3.7.7 PROTEIN EXPRESSION OF *ectABC* GENE CLUSTER**

The recombinant plasmid pDrive-*ectABC* construct were double digested with *SacI* and *BamHI* (MBI Fermentas) and purified by MinElute Gel purification Kit. The purified *ectABC* gene was recloned into pQE30 expression vector (Qiagen), which had previously been digested and purified. The resulting recombinant expression vector pQE30-*ectABC* cassette was transformed into *E. coli* M15(pREP4). A single colony of the recombinant culture was inoculated into 5 ml of LB broth containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin, and incubated overnight at 37°C. About 2-5 ml of the culture was transferred into 50 ml of LB containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin and incubated at 37°C, until OD<sub>600</sub> value reached 0.6. Isopropyl-β-D-thiogalactoside [IPTG] (MBI Fermentas) was then added into the culture at the final concentration of 1 mM and was continuously incubated at 37°C for 4 h. The induced bacterial cells were harvested by centrifugation and resuspended in 1X SDS-PAGE sample buffer and lysed in boiling water bath for 3 min. The cells were centrifuged at 4,000 g for 20 min and the supernatant was checked for expression of soluble proteins.

### **3.7.8 ELECTROPHORETIC SEPARATION OF EXPRESSED PROTEIN**

The expression of the target proteins were analysed by SDS-PAGE as described by Laemmli, (1970). It is based on the principle that, in the presence of 10% SDS and 2-mercaptoethanol, proteins dissociated into their subunits and bind large quantities of the detergent which mask the charge of the proteins and giving a constant charge to mass ratio. Thus, the proteins will move according to their molecular weight in an electric field. In this discontinuous buffer system, the separating (resolving gels) and stacking gels are made up in the electrode buffer, Tris-glycine. During

electrophoresis, the leading ion is chloride while the trailing ion is glycine. In this experiment, 7.5% gel concentration was used for the effective separation of proteins.

The separating gel was prepared and immediately transferred the solution to the gel casting apparatus. Little quantity of water was added on the top of the gel to level it and kept for 45 min for solidification. The stacking gel was prepared in the same way. Immediately after pouring the stacking gel, the comb was placed over the apparatus, and kept for 30 minutes for solidification. Later, the comb was removed and the apparatus was transferred to the sandwich clamp assembly into the inner cooling core. Rinsed the apparatus and wells with electrode buffer and filled the inner chamber of the apparatus completely and the outer chamber to the optimum level. 10  $\mu$ l of the sample was added into the wells using a sterile syringe. The electrode lid was placed at proper position and connections were established. The power of 200 V was supplied. Electrophoresis was carried out for 45 min approximately until the dye reaches the bottom. Subsequently, the gel was removed and is placed in a big petridish containing the stain, Coomassie blue. Kept for 30 min, and transferred the gel into 7% acetic acid for destaining. 7% acetic acid was changed intermittently till the gel got completely destained.

The molecular mass of the protein was estimated with protein ladder (MBI Fermentas).

### **3.7.9 PREPARATION OF ENZYME EXTRACT**

Enzyme extraction was performed as described by Ono *et al.*, 1999. Briefly, the cells obtained from a 100 ml culture were resuspended with 1 ml of 50 mM Tris HCl buffer (pH 8.0) and treated with 20  $\mu$ g of lysozyme at 37°C for 5 min. The mixture was then incubated with 0.5 mg of DNase I and 2.0 mg of RNase A at 37°C

for 10 min. The cell lysate was then treated with 1 mM phenylmethylsulfonyl fluoride [PMSF] (Sigma, St. Louis, MO, USA), 0.5 mM EDTA, 0.1 M NaCl and centrifuged at 10,000 g for 20 min. To remove endogenous low molecular weight metabolites, the supernatant was passed through a column (12 by 40 mm) of Sephadex G-25 (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) equilibrated with 50 mM Tris-HCl buffer, pH 9.0, and crude proteins were eluted using the same buffer. The protein concentration was determined according to the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

The cell pellets were resuspended in 50 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA, treated with lysozyme (50 mg/ml)<sup>21</sup> at 30°C for 1 min, and disrupted with a French pressure cell press (SLM Aminco Instruments, Inc., Urbana, Ill.) at 15,000 lb/in<sup>2</sup>, once. After the addition of PMSF to a final concentration of 1 mM, the lysate was centrifuged at 10,000 g for 20 min. The supernatant was diluted with the same buffer to 500 ml, treated with 0.25% protamine sulfate to remove nucleic acids, and centrifuged at 10,000 g for 20 min. The ratio of the absorbance at 260 nm to that at 280 nm of this supernatant was in the range of 1.2 to 1.4. This supernatant was used as enzyme extract for the further study.

### **3.7.10 ENZYME ASSAYS**

The enzyme activity of *ectABC* proteins was performed with the following procedures as described previously by Ono *et al.*, (1999).

#### **3.7.10.1 DETERMINATION OF DABA AMINOTRANSFERASE ACTIVITY**

DABA aminotransferase activity was determined by the amount of glutamate produced in the reverse reaction. Briefly, 100 µl reaction mixture consisting of 5 mM 2-oxoglutarate, 10 mM DABA, 10 µM pyridoxal phosphate (PLP), 50 mM Tris HCl

buffer (pH 8.5), and 25 mM KCl was incubated at 25°C for 30 min, and the reaction was stopped by boiling the mixture for 5 min. The concentration of the released glutamate was determined by L-glutamic acid kit of Roche (R-Biopharm GmbH, Darmstadt, Germany).

#### **3.7.10.2 DETERMINATION OF DABA ACETYLTRANSFERASE ACTIVITY**

Activity of DABA acetyltransferase (*ectA*) was assayed by acylation assay as described previously (Schubert *et al.*, 2007). Briefly, the reaction mixture contained 0.3 mM Ellman's reagent [5,5'-Dithiobis(2-nitrobenzoic acid)] in 60 mM Tris HCl buffer (pH 8.5), 0.4 mM NaCl, 2 mM acetyl-coenzyme A and 30 mM diaminobutyrate. The acylation activity of *ectA* was analysed using a coupled spectrometric test with the detection at 412 nm.

#### **3.7.10.3 DETERMINATION OF ECTOINE SYNTHASE ACTIVITY**

Ectoine synthase (*ectC*) activity was performed as described previously (Ono *et al.*, 1999). Briefly, the reaction was carried out in a 100 µl mixture consisting of 10 mM ADABA, 0.6 M NaCl, 1 mM DABA, 50 mM Tris-HCl buffer (pH 9.5). The reaction mixture was incubated at 15°C for 10 min and stopped by 0.3% of trifluoroacetic acid (TFA). The amount of ectoine produced by the *ectABC* gene cluster was determined by HPLC (Nagata and Wang, 2001). Ectoine was detected in a HPLC system (L-7400; Hitachi, Tokyo) using an NH<sub>2</sub> column (LiChroCART 250-4 NH<sub>2</sub>; Merck, NJ, USA) at 30°C with an acetonitrile-water (85%, v/v) as the mobile phase and a flow rate of 1.0 ml/min was applied. UV detection at 210 nm was used. The identification and quantification of ectoine were carried out using ectoine (Sigma) as the standard.

#### **3.7.10.4 HPLC ANALYSIS OF ECTOINE**

The transformants with *ectABC* gene cluster were extracted with 80% ethanol and subjected to isocratic HPLC analysis for quantification of ectoine (Nagata and Wang, 2001). The HPLC analysis was performed using NH<sub>2</sub> column (LiChroCART 250-4 NH<sub>2</sub>; Merck, NJ) at 30°C with acetonitrile-water (85%, vol/vol) as the mobile phase at a flow rate of 1.0 ml/min. UV detection at 210 nm was used. The identification and quantification of ectoine were carried out using ectoine standard (Sigma).

#### **3.7.10.5 *IN SILICO* SEQUENCE ANALYSIS**

The nucleotide sequences obtained were compared against database sequences using BLAST (Altschul *et al.*, 1990) provided by NCBI (<http://www.ncbi.nlm.nih.gov>) and were aligned and clustered using CLUSTAL-X version 1.81 program (Thompson *et al.*, 1997). The output alignments were imported into the GeneDoc program (<http://www.psc.edu/biomed/genedoc/>) and BioEdit version 7.05 program ([www.mbio.ncsu.edu/BioEdit/](http://www.mbio.ncsu.edu/BioEdit/)) to calculate the percent identities among the nucleotide and amino acid sequences. The molecular masses and the theoretical pI values of the polypeptides were predicted using the ProtParam tool (<http://www.expasy.org/tools/protparam.html>).

#### **3.8 IDENTIFICATION OF *ESCHERICHIA COLI***

Microbial identification and biochemical characterization of *E. coli* in this study was carried out as per the standard protocol of [U.S. Food and Drug Administration (USFDA, 2001)] and used as a source of the gene encoding the choline dehydrogenase (*betA*). Briefly the method of identification consisted the following protocol.

### **3.8.1 PREPARATION OF SAMPLE**

10 g of salted fish samples were transferred into the sterile stomacher bag (Seward Medical, London, UK) containing 90 ml 0.85% normal saline (NS). This was blended in a stomacher (Lab blender 400, Seaward Medical, London, UK) for 60 seconds at normal speed to obtain  $10^{-1}$  dilution. Using a sterile pipette, 1 ml of the supernatant was aseptically transferred into the 9 ml NS tube and mixed well to obtain  $10^{-2}$  dilution. Similarly other required dilutions were prepared for inoculation.

### **3.8.2 MOST PROBABLE NUMBER (MPN) DETECTION OF *E. COLI***

Identification of *E. coli* was established by the three tube most probable number (MPN) procedure. Briefly, the aliquots of serially diluted samples were inoculated into MacConkey's broth (Oxoid, UK) and incubated at 37°C for 24 hrs. Positive tubes were subjected to 2<sup>nd</sup> step MPN procedure in brilliant green lactose bile broth (Oxoid, UK) broth at 44.5°C.

### **3.8.3 ISOLATION AND CONFIRMATION OF *E. COLI***

The *E. coli* isolate was further confirmed by streaking a loopful of culture from positive MPN tubes on eosin methylene blue (EMB) agar and incubated overnight at 37°C. After incubation, the plates were observed for green metallic sheen colour formation. The isolates exhibiting positive reaction were further subjected to (IMViC) test for confirmation of *E. coli* isolate (3.4). The typical *E. coli* colonies were purified and maintained for further studies.

### **3.9 FUNCTIONAL CHARACTERIZATION AND SEQUENCE ANALYSIS OF CHOLINE DEHYDROGENASE (*betA*) FROM *E. COLI***

The functional characterization and *in silico* sequence analysis of choline dehydrogenase from *E. coli* isolate were determined as per the procedure reported

(Anbu Rajan *et al.*, 2010). Molecular characterization studies were performed using the following procedures.

### **3.9.1 DNA EXTRACTION**

Genomic DNA extraction from *E. coli* was performed, following the method of Ausubel *et al.*, 1994. Briefly, 1.5 ml of over night culture was transferred into microfuge tube and centrifuged at 10,000 rpm for 1 to 2 minutes. The supernatant was discarded and the pellet was dried on filter paper. 100 µl of Milli Q water was added to the pellet, and the pellet was gently tapped. The pellet was subjected for heating in boiling water bath for 5 minutes. The lysate was centrifuged at 5,500 rpm for 5 minutes. Pellet was discarded and the supernatant was used for PCR amplification.

### **3.9.2 PCR AMPLIFICATION OF *betA* GENE**

PCR amplification of *betA* gene cluster of *B. halodurans* was amplified by PCR using gene specific primers. A pair of primers were designed and used to amplify the *betA* gene (Table 3.6). PCR was performed in 50 µl of reaction mixture which contained 50 ng of genomic DNA, 0.5 µM of each primer, 200 µM each of dNTP (MBI Fermentas, Hanover, Maryland, USA), 1 U of *Pfu* DNA polymerase (MBI Fermentas), 1X *Pfu*

**Table 3.6 Oligonucleotide primers used for PCR amplification of *betA* gene**

<b>Primer designation</b>	<b>Sequence (5'–3')</b>	<b>Reference</b>
bAF	CGTATGCAATTTGACTACATCATT	Anbu Rajan <i>et al.</i> , 2010
bAR	GCATCATTTTTTCGCTCTCACCG	Anbu Rajan <i>et al.</i> , 2010

buffer; 2.5 mM of MgSO<sub>4</sub> and remaining autoclaved millipore water. Amplification was performed in a PTC-150 Mini cycler (MJ Research, Waltham, MA, U.S.A.) with the following conditions; initial denaturation at 94°C for 3 min, followed by 30 repeated cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 2 min and final extension at 72°C for 5 min. The PCR amplified product was analyzed on 1.5% agarose gel along with DNA molecular weight marker (MBI Fermentas) and documented using a gel documentation system (Alpha Imager).

### **3.9.3 CLONING OF *betA* GENE**

The *betA* PCR product was purified by MinElute Gel purification Kit (Qiagen) and cloned into pDrive (Qiagen). Briefly, the ethidium bromide stained DNA fragment from TAE agarose gel was excised without disrupting the fragment. The gel slice was weighed in an already weighed empty microfuge tube. (Maximum weight of gel slice being 400 mg). For every 1 volume of the gel slice, 3 volume of binding buffer was added. (1 mg of weight equals to 1 µl of volume). The gel slice was incubated at 50°C for 5 to 10 minutes in a heat block. 1 volume of isopropanol equal to the original gel slice volume was added after complete dissolution. A spin column

was placed in a clean, fresh 2 ml collection tube. 800 µl of sample was added to the spin column and centrifuged at 10,000 rpm for 1 minute. The filtrate was discarded and the spin column was replaced in the same collection tube. 750 µl of diluted wash buffer was added to the spin column. The spin column-collection tube assembly was centrifuged at 10,000 rpm for 1 minute. The filtrate was discarded and the spin column with the same collection tube was subjected to centrifugation for an additional minute at 10,000 rpm to remove any residual diluted wash buffer. The spin column was again placed in a new 2 ml collection tube and 30 µl of elution buffer was added to the centre of the spin column and centrifuged for at 10,000 rpm for 1 minute. The spin column was discarded, and the collection tube was capped. The purified DNA was confirmed for its presence by gel electrophoresis on 1.5% agarose gel. The purified *betA* amplicon was ligated to the cloning vector with the reaction profile as mentioned in Table 3.7.

#### **3.9.4 TRANSFORMATION OF *betA* GENE**

The pDrive- *betA* construct was transformed into *E. coli* JM109 (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (rK-mk+), *e14*-(*mcrA*-), *supE44*, *relA1*,  $\Delta$ (*lac-proAB*)/F' (*traD36*, *proAB*+, *lac Iq*, *lacZ* $\Delta$ M15) by following the procedure in (Sambrook and Russell, 2001).

##### **3.9.4.1 PREPERATION OF COMPETANT CELLS**

A single bacterial colony was selected from plates that have been incubated for 16-20 hrs at 37°C. 1 ml of overnight culture was transferred into 100 ml of LB broth in a one liter flask and incubated at 37°C with vigorous agitation till the O.D. attains 0.4. Transfer the bacterial cells were transferred into sterile disposable ice cold 50 ml polypropylene tubes. Later the cultures were cooled to 0°C by storing the tubes

on ice for 10 minutes. The cells were recovered by centrifugation at 2,700g for 10 minutes at 4°C. The medium was decanted from the cell pellet, and the tubes were kept in an inverted position on a pad of paper towel for one minute to allow the last traces of medium to drain away. The pellet was dissolved in 30 ml of ice cold magnesium chloride or calcium chloride solution (80mMgCl<sub>2</sub>, 20mM CaCl<sub>2</sub>) with swirling or gentle vortexing. The cells were recovered by centrifugation at 2,700g for 10 minutes at 4°C. The medium was decanted from the cell pellet, and the tubes were kept in an inverted position on a pad of

**Table 3.7. Cloning reaction profile for *betA* gene**

<b>Reagents</b>	<b>Volume (µl)</b>
PCR product	10
10X Buffer	2.5
T4 DNA Ligase	1
Plasmid	1.5
Nuclease free water	5
<b>Total</b>	<b>20</b>

paper towel for one minute to allow the last traces of medium to drain away. The pellet was suspended by gentle vortexing in 2 ml of ice cold 0.1M CaCl<sub>2</sub> for each 50 ml of original culture. The cells were used directly for transformation studies.

#### **3.9.4.2 TRANSFORMATION PROCEDURE**

200 ml of CaCl<sub>2</sub> treated suspension of competent cells were directly transferred into chilled, sterilized 17×100mm polypropylene tubes using a chilled micropipette tip. To each tube containing competent cell suspension ligated DNA mixture was added. The contents of tubes were mixed by swirling gently. And the tubes were kept on ice for 30 minutes. The tubes are then transferred to a rack placed in preheated 42°C circulating water bath. The tubes are placed in the rack for exactly 90 seconds without shaking. Rapidly the tubes were transferred to an ice bath allowing the cells to chill for 1- 2 minutes. 800 µl of SOB medium was transferred to each tube. The culture was incubated for 45 minutes in a water bath set at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. The appropriate volume of transformed competent cells was transferred on to SOB medium containing 20mM MgSO<sub>4</sub> and the appropriate antibiotic. The plates were stored at room temperature until the liquid has been absorbed. The plates were later incubated at 37°C for overnight and the transformants were selected by blue white selection.

#### **3.9.5 CHARACTERIZATION OF THE RECOMBINANT PLASMID**

The white recombinant colonies were picked up and inoculated in 5 ml LB broth containing ampicillin and incubated at 37°C. The plasmids were isolated from the overnight culture by alkaline lysis (Sambrook and Russell, 2001).

### **3.9.5.1 PREPERATION OF BACTERIAL CELLS**

2 ml of rich medium containing the appropriate antibiotic was inoculated with a single colony of transformed bacteria and incubated overnight at 37°C. 1.5 ml of the overnight culture was transferred into a microfuge tube and centrifuged at maximum speed for 30 seconds at 4°C. After centrifugation, the supernatant was discarded and the bacterial pellet was dried as feasible.

### **3.9.5.2 LYSIS OF BACTERIAL CELLS**

The bacterial pellet was resuspended in 100 µl of ice cold alkaline lysis solution I by vigorous vortexing. 200 µl of freshly prepared alkaline lysis solution II was added to each of the bacterial suspension and the contents were mixed by inverting the tube rapidly for 5 minutes. 150 µl of ice cold alkaline lysis solution III was added and dispersed through the viscous bacterial lysate by inverting the tube several times, the tubes were kept on ice for 3–5 minutes. The bacterial lysate was centrifuged at maximum speed for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. An equal volume of phenol-chloroform was added; the organic and aqueous phases were then mixed by vortexing and centrifuged the emulsion at maximum speed for 2 minutes at 4°C. The aqueous upper layer was later transferred to a fresh tube.

### **3.9.5.3 RECOVERY OF PLASMID DNA**

The nucleic acids were precipitated from supernatant by the addition of two volumes of ethanol at room temperature. The solution was mixed by vortexing and then allowed to stand for two minutes at room temperature. The precipitated nucleic acid was collected by centrifugation at maximum speed for 5 minutes at 4°C. The supernatant was discarded and the tube was kept in an inverted position on a paper

towel to allow all of the fluid to drain away. 1 ml of 70% ethanol was added to the pellet and inverted in the closed tube several times; the DNA was recovered by centrifugation at maximum speed for 2 minutes at 4°C. The supernatant was discarded and the tubes were kept open for few minutes at room temperature. The nucleic acid was dissolved in 50µl of TE (pH 8) containing 20µg/ml DNase free RNaseA and used for restriction analysis.

#### **3.9.5.4 RESTRICTION ANALYSIS OF *betA* GENE INSERT**

The recombinant plasmid with *ectABC* gene cluster was double digested with *HindIII* and *BamHI* (MBI Fermentas) restriction enzymes. The detailed reaction profile is given in Table 3.8. The reaction mixture was incubated overnight at 37°C in a water bath. The digested product was analyzed on 1.5% agarose gel electrophoresis.

#### **3.9.6 SEQUENCING OF *betA* GENE**

The sequencing was performed initially with vector primers M13 forward and M13 reverse (MBI Fermentas) and later by primer walking. The sequencing was performed with automatic ABI PRISM 377 genetic analyzer (Applied Biosystems).

#### **3.9.7 EXPRESSION OF RECOMBINANT CHOLINE DEHYDROGENASE**

The recombinant plasmid pDrive-*betA* constructs was double digested with *HindIII* and *BamHI* (MBI Fermentas) and purified by MinElute Gel purification Kit. The purified *betA* gene was sub-cloned into pQE30 expression vector, which had previously been digested and purified. The resulting recombinant expression vector pQE30-*betA* cassette was transformed into *E. coli* M15 (pREP4). A single colony of the recombinant culture was inoculated into 5 ml of LB broth containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin, and incubated overnight at 37°C. About 2.5 ml of the culture was

**Table 3.8. Restriction analysis profile of *betA* gene**

<b>Reagents</b>	<b>Volume (<math>\mu</math>l)</b>
Recombinant plasmid	2
Enzyme buffer (10X)	2
<i>Hind</i> III (10U/ $\mu$ l)	0.5
<i>Bam</i> HI (10U/ $\mu$ l)	0.5
Nuclease free water	15
<b>Total</b>	<b>20</b>

transferred into 50 ml of LB containing 100  $\mu$ g/ml of ampicillin and 25  $\mu$ g/ml of kanamycin and incubated at 37°C, until OD<sub>600</sub> value reached 0.6. Isopropyl- $\beta$ -D-thiogalactoside [IPTG] (MBI Fermentas) was then added into the culture at the final concentration of 1 mM and was continuously incubated at 37°C for 4 h. The induced bacterial cells were harvested by centrifugation and resuspended in 1X SDS-PAGE sample buffer and lysed in boiling water bath for 3 min. The cells were centrifuged at 14,000 g for 5 min and the supernatant was checked for expression of soluble proteins. The expression of the target proteins were analysed by (SDS-PAGE).

### **3.9.8 SDS-PAGE ANALYSIS OF EXPRESSED PROTEIN**

The expression of the target proteins were analysed by SDS-PAGE as described previously by Laemmli, (1970). In this experiment, 10% gel concentration was used for the effective separation of choline dehydrogenase.

The separating gel was prepared and immediately transferred the solution to the gel casting apparatus. Little quantity of water was added on the top of the gel to level it and kept for 45 min for solidification. The stacking gel was prepared in the same way. Immediately after pouring the staking gel, the comb was placed over the apparatus, and kept for 30 minutes for solidification. Later, the comb was removed and the apparatus was transferred to the sandwich clamp assembly into the inner cooling core. Rinsed the apparatus and wells with electrode buffer and filled the inner chamber of the apparatus completely and the outer chamber to the optimum level. 10  $\mu$ l of the sample was added into the wells using a sterile syringe. The electrode lid was placed at proper position and connections were established. The power of 200 V was supplied. Electrophoresis was carried out for 45 min approximately until the dye reaches the bottom. Subsequently, the gel was removed and is placed in a big petridish containing the stain, Coomassie blue. Kept for 30 min, and transferred the gel into 7% acetic acid for destaining. 7% acetic acid was changed intermittently till the gel got completely destained.

The molecular mass of the protein was estimated with protein ladder (MBI Fermentas).

### **3.9.9 CELL EXTRACTION**

Luria Bertani broth (50 ml) supplemented with 100  $\mu$ g/ml of ampicillin and 25  $\mu$ g/ml of kanamycin was inoculated with 2.5 ml of overnight cultures of *E. coli*

M15 (pREP4) with pQE30-*betA* cassette. The cells were incubated at 37°C until OD<sub>600</sub> value reached 0.6. IPTG was added to 1 mM final concentration, and the culture was grown for a further 5 h. The cells were harvested by centrifugation at 10,000 g for 10 min and were resuspended with 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF] (Sigma) and 2 mM dithiothreitol [DTT] (Sigma). The cells were subjected to sonication for 3 min in 1 min pulses with discontinuous cooling in ice, using SonicMan multiwell sonicator (Matrical, Spokane, WA, USA). The cell debris was removed by centrifugation at 12,000 g at 4°C for 15 min.

#### **3.9.10 ENZYME ASSAY**

The concentration of choline dehydrogenase was determined by the method of Bradford by using bovine serum albumin as the standard. The oxidase activity of the enzyme was measured with 10 mM choline (Sigma) as the substrate in 50 mM potassium phosphate (pH 7.0), by monitoring the rate of oxygen consumption. The dehydrogenase activity of the enzyme was measured with 1 mM phenazine methosulfate (Sigma) as the primary electron acceptor using a coupled assay in which the enzymatically reduced phenazine methosulfate is spontaneously reoxidized by molecular oxygen. One unit of enzymatic activity corresponds to the conversion of a micromole of oxygen per minute.

#### **3.9.11 *IN SILICO* SEQUENCE ANALYSIS**

The nucleotide sequences obtained were compared against database sequences using BLAST provided by NCBI (<http://www.ncbi.nlm.nih.gov>) and were aligned and clustered using CLUSTAL-X version 1.81 program. The output alignments were imported into the GeneDoc program (<http://www.psc.edu/biomed/genedoc/>) and

BioEdit version 7.05 program ([www.mbio.ncsu.edu/BioEdit/](http://www.mbio.ncsu.edu/BioEdit/)) to calculate the percent identities among the nucleotide and amino acid sequences. The molecular masses and the theoretical pI values of the polypeptides were predicted using the ProtParam tool (<http://www.expasy.org/tools/protparam.html>). The secondary structure prediction was performed using the PROTEAN program (DNASTAR).

**RESULTS  
AND  
DISCUSSION**

## 4. RESULTS AND DISCUSSION

### 4.1 MICROBIAL DIVERSITY OF SALTED FISHES

The major objective of this part of the study was to isolate the halophilic bacteria, which are moderately halophilic in nature and which could be used for further genetic studies. For this purpose, randomly selected samples of salted fishes like *Scoliodon* spp. and *Thrissina thryssa* were collected from different retail markets in Cochin, Kerala, India and were used for the study. About 75% of the isolates obtained from these samples were grown optimally in 3-10% NaCl media and were thus considered as moderate halophiles as per Kushner, 1985.

*Scoliodon* spp. and *Thrissina thryssa* were analyzed for the halophilic composition of bacteria by inoculating the sample on to salted agar media (5% NaCl), nutrient agar (3% NaCl), high salt casein (25% NaCl), selective media like EMB agar (3% salt) were also used. The count observed for the sample in three different media are given in the (Table 4.1). Nutrient agar with 3% NaCl showed elevated count in both the samples analysed and *Scoliodon* spp. had slightly higher count than *Thrissina thryssa*. Compared to high salt casein agar, agar with 3-5% salt promoted better growth. Among these, nutrient agar with 3% NaCl showed slightly higher count than 5% salt agar. A total of 93 cultures were isolated and the source of the isolates are given in the (Table 4.2)

All the 93 isolates were observed for their morphology, size and pigmentation pattern. Biochemical properties like Gram's staining, motility, catalase, oxidase, utilization of sugars (arabinose, glucose, fructose, lactose, trehalose, maltose, sucrose, salicin, mannose, cellobiose, rhamnose, dulcitol, xylose, raffinose, sorbitol, adonitol, inulin, galactose, inositol and mannitol), gelatin, starch, casein hydrolysis, hydrogen

sulfide, nitrate, citrate utilization, MR-VP, triple sugar ion and indole tests were performed. These isolates will be further studied for their salt tolerance, growth at different pH range and temperature.

**Table 4.1 Growth of microorganisms isolated from *Scoliodon* spp. and *Thrissina thryssa***

Sample	No. of samples	Average count range CFU/gm		
		Salted agar (5% NaCl)	Nutrient agar (3% NaCl)	High Salt Casein (25% NaCl)
<b>Shark</b> ( <i>Scoliodon</i> spp.)	<b>6</b>	3.3x10 <sup>4</sup> -5.5x10 <sup>4</sup>	4.2x10 <sup>4</sup> - 9.7x10 <sup>4</sup>	1.1x 10 <sup>4</sup> - 1.2x10 <sup>4</sup>
<b>Anchovies</b> ( <i>Thrissina thryssa</i> )	<b>6</b>	1.1x10 <sup>4</sup> - 1.7x10 <sup>4</sup>	1.9x10 <sup>4</sup> - 2.7x10 <sup>4</sup>	-

**Table 4.2 Number of cultures isolated from *Scoliodon* spp. and *Thrissina thryssa***

Samples	No. of isolates			
	Total	Salted agar (5% NaCl)	Nutrient agar (3% NaCl)	High Salt Casein (25% NaCl)
<b>Shark</b> ( <i>Scoliodon</i> spp.)	38	17	21	-
<b>Anchovies</b> ( <i>Thrissina</i> <i>thryssa</i> )	55	15	30	10

## **4.2 MORPHOLOGICAL OBSERVATIONS**

Out of the 48 isolates from dried shark (*Scoliodon* spp.), 42 (87.5%) were Gram positive cocci and remaining 6 (12.5%) were Gram positive rods. In the case of isolates from dried anchovies (*Thrissina thryssa*), out of 45 isolates 43 (95.5%) isolates were Gram positive rods and 2 (4.4%) were Gram positive cocci (Table 4.3).

### **4.2.1 PIGMENTATION PATTERN**

All isolates were observed for their colony colour. Out of 93 isolates, 21.5% isolates were whitish; 27.9% isolates were yellowish; 34.4% isolates were creamy; 13.9% isolates were pinkish and 1% of the isolates were orange in colour (Table 4.4).

### **4.2.2 CHARACTERIZATION OF ISOLATES**

All the isolates were subjected to biochemical tests for characterization of isolates to further level.

#### **4.2.2.1 CARBOHYDRATE UTILIZATION**

Of 93 total isolates, 5.3% isolates utilized arabinose. 13.9% utilized glucose. 9.6% utilized fructose. 2.1% utilized lactose. 9.6% utilized trehalose. 13.9% utilized mannitol. 13% utilized maltose. 16% utilized sucrose. 9.6% utilized salicin. 10.7% mannose. 8.6% utilized cellobiose. 5.3% utilized rhamnose. 10.7% utilized dulcitol. 15% utilized xylose. 11.8% utilized raffinose. 14% utilized sorbitol. 7.5% utilized adonitol. 11.8% utilized inulin. 6.4% utilized galactose and 8.6% utilized inositol (Table 4.5).

#### **4.2.2.2 PHENOTYPIC CHARACTERS**

Of total isolates, 21.5% of isolates were found to be motile. 19.3% isolates revealed positive result for H<sub>2</sub>S. About 29% of the isolates were found to reduce nitrate. 11.8% of the isolates were found to utilize citrate. 18.2% isolates revealed positive result for Methy Red test. 22.5% isolates revealed positive result for Voges Praskeur. 22.5% isolates exhibited positive result for triple sugar iron agar test.

**Table 4.3 Morphological characterization of isolates**

<b>Morphology</b>	<b>No. of isolates</b>
Gram positive rods	45
Gram positive cocci	48

**Table 4.4 Pigmentation pattern of isolates**

<b>Colony pigment</b>	<b>No. of isolates</b>
White	20
Yellow	26
Cream	32
Pink	13
Orange	1

**Table 4.5 Carbohydrate utilization**

<b>Utilization of carbohydrates</b>	<b>No. of isolates</b>
Glucose	13
Fructose	9
Arabinose	5
Mannitol	13
Trehalose	9
Lactose	2
Maltose	12
Sucrose	15
Salicin	9
Mannose	10
Cellobiose	8
Rhamnose	5
Dulcitol	10
Xylose	14
Raffinose	11
Sorbitol	13
Adonitol	7
Inulin	11
Galactose	6
Inositol	8

Out of 48 isolates from shark (*Scoliodon* spp.) samples, all isolates were found catalase positive; 56.25% isolates were oxidase positive and remaining 43.75% isolates were oxidase negative. Of 45 isolates from anchovy's (*Thrissina thrissa*) samples, all isolates were catalase positive and in case of cytochrome oxidase test, 51.1% were positive and 48.8% were found negative. No isolates produce indole from tryptophan (Table 4.6).

#### **4.2.2.3 EXTRACELLULAR HYDROLYTIC ACTIVITIES**

Of the total isolates, 29% of the isolates were found to hydrolyze starch by producing amylase. About 18.2% isolates were found to hydrolyze casein and 12.9% isolates were found hydrolyze gelatin (Figure 4.7).

### **4.3 PHYSIOLOGICAL CHARACTERIZATION**

#### **4.3.1 GROWTH AT DIFFERENT SALT CONCENTRATION**

Growth at different salt concentration was determined on nutrient agar medium with 0.5, 2, 5, 10, 20% NaCl. Out of 93 isolates 80.6% isolates were grown in 10% NaCl; 77.4% isolates were grown in 2% NaCl; 75.2% isolates were grown in 0.5% NaCl; 83.8% isolates were grown in 5% NaCl and 10.75% isolates grown in 20% NaCl. Optimum NaCl range for growth was 2-10% NaCl among the 93 isolates (Figure 4.8).

#### **4.3.2 GROWTH AT DIFFERENT pH LEVELS**

The pH range for growth was determined in the nutrient broth media, with the pH of 8.0, 9.0 and 10.0. Out of 93 isolates 95.6% isolates were found to have good growth at pH 8, 91.3% isolates had good growth at pH 9 and 60.21% isolates grown at pH 10. The optimum range of pH was found between 7-9 (Figure 4.9).

**Table 4.6 Phenotypic characterization of isolates**

<b>Tests</b>	<b>No. of isolates</b>
Cytochrome oxidase	50
Catalase	93
Indole production	Nil
Motility	20
Hydrogen Sulfide production	18
Nitrate reduction	27
Citrate utilization	11
Methyl Red	17
Voges Proskeur	21
Triple Sugar Iron agar test	21

**Table 4.7 Extracellular hydrolytic activity of isolates**

<b>Tests</b>	<b>No. of isolates</b>
Gelatin hydrolysis	12
Casein hydrolysis	17
Starch hydrolysis	27

**Table 4.8 Growth at different salt concentrations**

<b>NaCl (%)</b>	<b>No. of isolates</b>
0.5%	70
2.0%	72
5.0%	78
10.0%	75
20.0%	10

**Table 4.9 Growth at different pH levels**

<b>pH levels</b>	<b>No. of isolates</b>
pH 8	89
pH 9	85
pH 10	56

**Table 4.10 Growth at different temperatures**

<b>Temperature</b>	<b>No. of isolates</b>
15°C	39
25°C	63
30°C	89
35°C	93
40°C	79

#### **4.3.3 GROWTH AT DIFFERENT TEMPERATURES**

Growth range at various temperatures was determined in the nutrient broth media at 15, 25, 30, 35 and 45°C. The optimal growth temperature of 93 isolates was determined at 30°C (Figure 4.10).

#### **4.4 ISOLATION AND IDENTIFICATION OF *B. HALODURANS***

All the isolated organisms were subjected for the DNA isolation and checked for the *ectABC* gene cluster and choline dehydrogenase (*betA*) gene using their gene specific primers. Among all the isolates, only one was found to have the positive amplicon for *ectABC* gene cluster and it was identified as *Bacillus halodurans* TCJAR01 using 16S rDNA approach. Moreover, only one isolate revealed positive result for choline dehydrogenase gene product, and it was identified and confirmed as *Escherichia coli* TCJAR023 using conventional methods.

Morphological, physiological and biochemical characterization of *Bacillus halodurans* TCJAR01 were determined using classical methods (Nielsen *et al.*, 1995). It is a Gram positive, spore forming rod, motile, oxidase positive, catalase negative, utilized glucose, fructose, galactose, sucrose, lactose and arabinose. Hydrolysis of Tween 40, 60, casein, gelatin and starch is obtained. The hydrolysis of Tween 20 and reduction of nitrate were not observed. The isolate also exhibited good growth on nutrient agar plates with 15% NaCl and at pH 10.0. Moreover, good growth was observed in the temperature range of 15-55°C. These properties bout with the previous report for the identification of *B. halodurans* (Nielsen *et al.*, 1995). The sequence of the 16S rDNA gene, 1502 bp (Fig. 4.1), of our isolate GenBank accession number (GU367604) showed 100% identity to that of the previously reported *B. halodurans* C-125 (BA000004), (Takami and Horikoshi, 1999) and *B. halodurans* MS-2-5 (AB359904), (Murakami *et al.*, 2008). Thus the bacterial strain was identified and confirmed as *B. halodurans*, on the basis of these results.

#### **4.5 CHARACTERIZATION OF *ectABC* GENE CLUSTER OF *B. HALODURANS* TCJAR01**

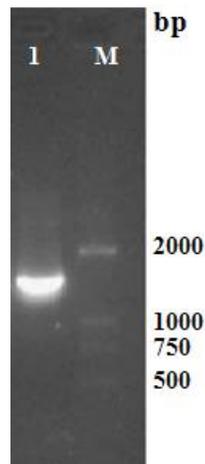
##### **4.5.1 PCR AMPLIFICATION, CLONING AND SEQUENCING**

The *ectA*, *B* and *C* genes encode the diaminobutyric acid acetyltransferase, diaminobutyric acid aminotransferase and ectoine synthase respectively and together these proteins constituted the ectoine biosynthetic pathway (Fig. 2.2). The ectoine biosynthesis genes of *B. halodurans* TCJAR01 were cloned in pDrive and sequenced. The ectoine operon was of 2389 bp length (Fig. 4.2) comprising of *ectA*, *B* and *C* genes, which are 570 bp, 1284 bp and 370 bp respectively; encoding putative proteins of 189, 427 and 129 amino acids (Fig. 4.3, 4.4, 4.5). Molecular masses of *ectA*, *B* and

*C* proteins are 21048, 47120 and 14797 Da respectively based on the *in silico* estimates with isoelectric points of 4.81, 4.84 and 4.93 respectively. The nucleotide sequences generated in this study has been deposited in the GenBank with the accession nos. EF534248, EF534249, EF534250

#### 4.5.2 EXPRESSION OF *ectABC* GENE CLUSTER IN PROKARYOTE

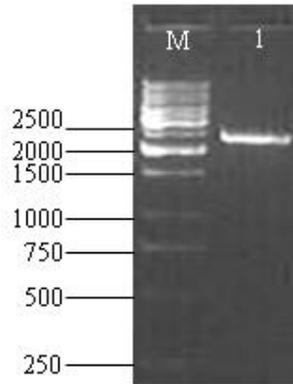
The *E. coli* transformants harboring recombinant pQE30-*ectABC* gene cassette was induced with IPTG. Expression of the *ectABC* operon was analysed by SDS-PAGE electrophoresis of the lysates of induced and noninduced cells. The lysates of induced cells showed two clear expressed bands with molecular masses of 41 kDa and 15 kDa that correspond to *ectB* and *C* (Fig. 4.6). The expressed protein band of *ectA* gene was not detectable in SDS-PAGE due to its instability (Schubert *et al.*, 2007).



**Fig 4.1 16S rDNA amplicon of *Bacillus halodurans* TCJAR01**

Lane M -1 kb DNA Ladder

Lane 1 -Amplicon of 16S rDNA (1502 bp)



**Fig 4.2 PCR amplicon of *ectABC* operon in *Bacillus halodurans* TCJAR01 with primers EO1 and EO2**  
Lane M - 1 kb DNA Ladder

```

ttgcaggttc aatgcaatg aaaaagcctt caaaggaggattc attattaac agtcaaatt
L Q V Q C N E K A F K G G F I I N S Q I
gcaaccgccc tcccaaaacg ttggataccaca attacgataggaaa accaacagtggaa
A T A P P K T L D T T I T I G K P T V E
gatggcgcgg cc atgtggga actggttaaca agtcaacgctc gacacgaatt caccttac
D G A A M W E L V N K S T L D T N S P Y
aagtacattat gatgtg cgaataactttg cgggaaacg tgtgtgtgc gaaaggaaaac gaa
K Y I M M C E Y F A E T C V V A K E N E
agattggtcgg gatttgtaacggc tttattccac ctgaacacca agatggttat atttgtt
R L V G F V T A F I P P E H Q D V I F V
tggcaaat tgggtgttg actcatcaca acgcgggaa aggtctcgc atccaaattg cttcaa
W Q I G V D S S Q R G K G L A S K L L Q
gaattaattt cacgtgata tctgtagca atgtaaattat gttgaggca actgttaccct
E L I S R D I C S N V N Y V E A T V T P
tcaaataaag cttcacag gctttgtttc aaaagctag ctcgggagt ataactca atgt
S N K A S Q A L F Q K L A R E Y N T Q C
gaagtgagtg aatgtttct ctgaagattt attcctc tggcgat gatcatg aagctgaatta
E V S E C F S E D L F P G D D H E A E L
actttccgta tcgggtccc cctcacc cgtaa
T F R I G P L H P -

```

**Fig 4.3 *ectA* gene with nucleotide and its corresponding amino acid sequences of *Bacillus halodurans* TCJAR01**

```

atgagccaaacagatatgaacggttttgaacaattggaatcgggaagtccgaagttattgc
M S Q T D M N V F E Q L E S E V R S Y C
cgtagctttccaaccggtttcaccaaagcaaaaggttataaaatgtgggacgaagccggg
R S F P T V F T K A K G Y K M W D E A G
aaagagtacattgatttttctctggggcagggccttaaac tacggacataatgatgag
K E Y I D F F S G A G A L N Y G H N D E
aaaatgaaaaaagcgcttgggactacattatggatgacggaattacccattctctcgat
K M K K A L V D Y I M D D G I T H S L D
atggccacaacgccaaggaaagtttttacagaaatttcatgatgctattttaaaaccg
M A T T P K G K F L Q K F H D V I L K P
agaaaccttgattataaagtgatgttccctggacc tactgggacaaataccgtagaaagt
R N L D Y K V M F P G P T G T N T V E S
gogttaaagc ttggcgaaagtgactggccgtacggatattat tagctttactaacgga
A L K L A R K V T G R T D I I S F T N G
ttcattggcatgacgatcggttcccttctgtgactgggaacctattcaagcgaagggg
F H G M T I G S L S V T G N S F K R K G
gocggaatccctctcagcaacgtagtgactatgoccttatgacaaatttgctcagtgaaagc
A G I P L T N V V T M P Y D N F V S E S
cttgatacactcgattacttggagcgtcttttagaggatggcgggttcaggtgttgaaatt
L D T L D Y L E R F L E D G G S G V E I
cctgctgcgatgatccttgaacgggttcaaggagaagggcgaat taatgocgctcgata
P A A M I L E T V Q G E G G I N A A R T
gaatgggttgacgagtagagaagatttgcaaacgctggggaattctcctcattattgat
E W L Q R V E K I C K R W G I L L I I D
gacgttcaagctggagtgggacgcactggcacgttcttttagc tttgaagacgcagggatt
D V Q A G V G R T G T F F S F E D A G I
acacctgacatcgtatgtttgtcaaaatcaatcgggtggc tttgggc ttcggttagcgatt
T P D I V C L S K S I G G F G L P L A I
accctcttccgacctgagctagacatttgggcaccagggtgaacacaacggaacgttccgt
T L F R P E L D I W A P G E H N G T F R
gaaataaaccacggtttgtcacagcgaccgaagccttgtcttattgggaagatgatcc
G N N H A F V T A T E A L S Y W E D D S
ttcgaaaaagacattcaagaaaaatcagcaacgatctctgatttcc ttgtaagctcgtg
F E K D I Q E K S A T I S D F L V K L V
acggaataccctgaaataaaaggggaagtaaaaggaaggggtttaggtc ggaatcgcg
T E Y P E I K G E V K G K G F M V G I A
cttgatgtagaaggctttgcttcaaaagtgacggaagaagcattttcccgcggtctcata
S D V E G F A S K V T E E A F S R G L I
atggagacttcaggtccgaacgatgaagtgtttaagctattcccgccactcaccatcgat
M E T S G P N D E V F K L F P P L T I D
gatgaaggggttagaaaaaggggttagctatcattgaagaaagc atcaaagctcttgtcgaa
D E G L E K G L A I I E E S I K A L V E
accaaagagcttgctcatgcaataa
T K E L V M O -

```

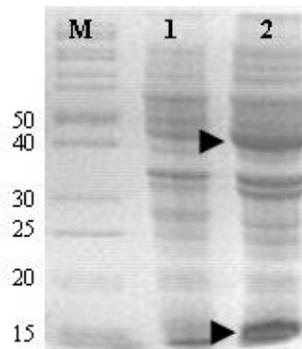
**Fig 4.4** *ectB* gene with nucleotide and its corresponding amino acid sequences of *Bacillus halodurans* TCJAR01

```

atgaaagtcggttaaattagaagatatcattggaacagaacaagaagttaaaggggagaac
M K V V K L E D I I G T E Q E V K G E N
tggacgagtcgcagactgctcttaaaaaaagatggaatgggctattccgtccatgacact
W T S R R L L L K K D G M G Y S V H D T
atcattaaagcagggacagaaacgcacatttgggtatcagaaccacctcgaagccgtgtac
I I K A G T E T H I W Y Q N H L E A V Y
tgcatcgaagcgaaggtgaagttgagacagtc aaagacggc aaagtgtggccgattaag
C I E G E G E V E T V K D G K V W P I K
gcgaatgaaatttacgcacttgatgagcagcagacgaacatttgcacgcgcaaaaaccgat
A N E I Y A L D E H D E H L L R A K T D
atgcgatgggttggcgtctttaaccaccacaattacagggaaagagaccatgatgaaat
M R M V C V F N P P I T G K E T H D E N
gggtgtgtatccgctagtcgacgacgaataa
G V Y P L V D D E -

```

**Fig 4.5** *ectC* gene with nucleotide and its corresponding amino acid sequences of *Bacillus halodurans* TCJAR01



**Fig. 4.6** SDS-PAGE analyses of the expressed *ectB* and *ectC* of *Bacillus halodurans* TCJAR01

Lane M - Protein molecular mass marker

Lane 1 - Total protein of the uninduced *ectABC* gene cassette

Lane 2 - Total protein of the induced *ectABC* gene cassette.

The arrow (►) indicates the position of the expressed *ectB* (41 kDa) and *ectC* (15 kDa)

### **4.5.3 FUNCTIONAL CHARACTERIZATION OF ECTOINE BIOSYNTHESIS GENES**

The recombinant expression vector pQE30-*ectABC* cassette was transformed into *E. coli* M15 (pREP4). The expression of the ectoine biosynthetic genes was confirmed by determining the activity of the individual enzymes. The functional activity of *ectA* protein was determined by acylation assay. The acylation activity in the expressed cells was 5 mU/mg, which is three times more than that of control cells (Table 4.11).

Aminotransferase assay was employed to determine the activity of *ectB* protein. The aminotransferase activity of the expressed cells was 6 mU/mg, which is considerably higher than that of uninduced cells.

The ectoine synthase activity of the expressed cells was also in the higher side 8 mU/mg, which is higher than that of uninduced cells.

The ectoine biosynthesis genes *ectA*, *B* and *C* have been functionally characterized in *Marinococcus halophilus*, *Bacillus pasteurii*, *Methylomicrobium alcaliphilum* and *Chromohalobacter salexigens* (Louis and Galinski, 1997; Kuhlmann and Bremer, 2002; Reshetnikov *et al.*, 2006; Schubert *et al.*, 2007). The expressed protein band of *ectA* gene was not detectable in SDS-PAGE. The instability of the *ectA* has already been reported (Ono *et al.*, 1999; Schubert *et al.*, 2007) and elucidates the absence of the *ectA* protein band in the SDS PAGE.

### **4.5.4 SEQUENCE AND PHYLOGENETIC ANALYSIS OF *ectA*, *B* and *C* GENES**

The *ectA*, *B* and *C* sequences from *B. halodurans* TCJAR01 were analyzed with reported amino acid sequences of other eubacteria viz. *Marinococcus halophilus*

(GenBank accession no. U66614), *Bacillus alcalophilus* (DQ471210), *Bacillus pasteurii* (AF316874), *Virgibacillus salexigens* (AY935521), *Virgibacillus pantothenicus* (AY585263), *Halobacillus dabanensis* (DQ108975), *Halomonas elongata* (AF031489) using Clustal X program. Homology search of the nucleotide and deduced amino acid sequence was performed using BLAST program.

The amino acid analysis revealed that the *ectA* gene encoded protein belongs to the GNAT family. The *ectA* gene encodes proteins of 189 amino acids with the pI value of 4.81. The amino acid sequences of *ectA* showed a high similarity with other bacteria, as given below

For *B. alcalophilus*, 60% identity with 165 amino acid overlap was observed. Similarly for *H. dabanensis*, 46% identity with 150 amino acid overlap; *V. salexigens*, 43% identity with 151 amino acid overlap; *V. pantothenicus*, 43% identity with 150 amino acid overlap; *B. pasteurii*, 41% identity with 174 amino acid overlap; *H. elongata*, 36% identity with 166 amino acid overlap and *M. halophilus*, 73% identity with 156 amino acid overlap (Fig. 4.7). The amino acid analysis of *ectA* revealed that, the amino acid sequence of *B. alcalophilus* and *M. halophilus* has the maximum identity of 60% and 73% respectively with that of *B. halodurans* TCJAR01. All the other sequences had less than 50% similarity with the amino acid sequence of *B. halodurans* TCJAR01. The phylogenetic tree at nucleotide and amino acid level of *ectA* (Fig. 4.8 & 4.9) revealed the phylogenetic similarity of *ectA* genes from *B. halodurans* TCJAR01 with other organisms. The bacterial species switched to different clusters for *ectA* gene at nucleotide and amino acid level indicating divergence among the organisms and the degree of divergence in the sequences.

However *B. halodurans* TCJAR01, *M. halophilus* and *B. alcalophilus* were grouped in the same cluster in both the phylogenetic trees.

<i>Stage of cells</i>	<i>Specific activity of enzyme (mU/mg)</i>		
	<b>Diaminobutyric acid aminotransferase</b>	<b>Diaminobutyric acid acetyltransferase</b>	<b>Ectoine synthase</b>
Before induction	1.6	2.1	2.5
After induction			
1 hr	2.0	3.0	3.4
3 hr	2.5	5.2	5
4hr	5	6	8

**Table 4.11 Activity of enzymes involved in ectoine biosynthesis in *Bacillus halodurans* TCJAR01 at different time intervals**

```

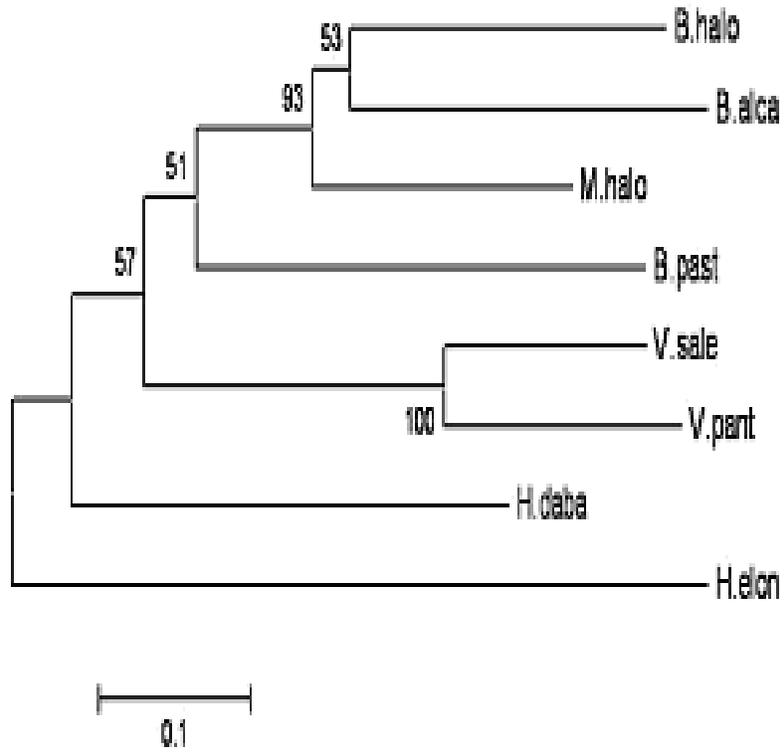
      *      20      *      40      *      60      *      80
E. halo : ---MQVQCNEKAFKGGFIIINSQI&T&APPKTLDTTITIGKQVVDG&A&V&ML&MK&S-T&T&T&S&P&K&V&M&C&E&S&A&E&T&C&Q&A&K&E&M&E-
M. halo : -----METNMTG---TNGSVD-----S&V&F&D&K&Q&V&E&D&D&V&D&L&K&M&S-T&T&D&T&S&P&K&V&M&C&E&S&A&E&T&C&Q&A&K&E&M&D-
E. alca : -----MTL&P---TKQTE-----S&L&L&T&Q&P&K&Q&D&D&V&D&L&K&M&S-T&T&D&T&S&P&K&V&M&C&E&S&A&E&T&C&Q&A&K&E&G&H-
B. past : -----MF&W&I&SK&Q&S&T&A&W&A&E&Q&E&E-----T&V&F&R&V&P&E&D&D&K&K&A&H&Q&M&L&H&M&E&P&G&W&L&D&L&S&S&P&S&P&K&V&M&C&E&S&A&E&T&C&Q&A&K&E&M&E-
V. sale : -----MT&K&T&S&V&I&E&Q-----D&K&F&R&P&E&K&D&D&K&A&H&Q&M&L&H&M&E&P&G&W&L&D&L&S&S&P&S&P&K&V&M&C&E&S&A&E&T&C&Q&A&K&E&S&E&G-
V. pant : -----D&K&F&R&P&E&K&D&D&K&A&H&Q&M&L&H&M&E&P&G&W&L&D&L&S&S&P&S&P&K&V&M&C&E&S&A&E&T&C&Q&A&K&E&S&E&G-
H. daba : M&S&M&L&S&E&W&Q&M&F&Q&K&I&R&E&V&I&P&I&P&T&T&E&D&G&K-----H&Z&Q&F&S&V&E&K&S&Q&E&S&P&L&L&H&E&N&I&P&I&D&L&M&S&S&P&S&P&K&V&M&C&E&S&A&E&T&C&Q&A&K&E&G&E-
H. elon : -----M&N&A&T&T&E&P&F&T&P&S&A&D&L&A&K&P&S&V&A&D&V&W&G&H&E&A&S&P&F&I&R&K&E&P&D&T&G&G&K&Y&L&A&K&S&C&P&P&I&D&R&S&A&S&P&L&A&T&Q&R&E&S&C&A&P&A&T&N&E&E&G

      *      100      *      120      *      140      *      160      *
E. halo : R&V&G&F&V&I&A&F&I&P&E&H-Q&V&E&V&Q&Q&I&G&D&S&S&Q&R&K&G&L&A&S&M&L&Q&E&L&S&E&D&I&C&S&M&Q&V&E&T&P&S&K&A&S&Q&A&M&Q&K&L&R&E&Y&N&Q&C&E&S&E&C
M. halo : E&V&G&F&V&I&A&F&I&P&E&K-Q&V&E&V&Q&Q&I&G&D&T&S&Q&R&K&G&L&A&S&M&L&Q&E&L&S&E&D&V&C&E&M&Q&V&E&T&I&P&S&K&A&S&Q&A&M&Q&K&L&A&Q&K&R&E&V&T&S&E&C
E. alca : E&V&G&F&V&I&A&R&P&M&R-Q&V&E&V&Q&Q&I&G&K&P&S&E&Q&M&G&I&A&Q&L&Q&E&L&H&R&S&D&H&M&P&A&D&Q&E&T&I&P&S&K&A&S&Q&A&M&Q&K&L&A&R&D&L&A&E&V&S&E&R&F
B. past : Q&V&G&F&V&I&G&L&H&T&T&E&Y&G&P&M&R&Y&L&A&S&C&S&D&E&T&Q&R&K&G&L&A&S&E&P&I&Q&E&L&H&R&S&D&H&M&P&A&D&Q&E&T&I&P&S&K&A&S&Q&A&M&Q&K&L&A&R&D&L&A&E&V&S&E&R&F
V. sale : E&V&G&F&V&I&G&F&I&H&M&K-P&M&R&Y&L&A&S&C&S&D&E&T&Q&R&K&G&L&A&S&E&P&I&Q&E&L&H&R&S&D&H&M&P&A&D&Q&E&T&I&P&S&K&A&S&Q&A&M&Q&K&L&A&R&D&L&A&E&V&S&E&R&F
V. pant : E&V&G&F&V&I&G&F&I&H&M&K-P&M&R&Y&L&A&S&C&S&D&E&T&Q&R&K&G&L&A&S&E&P&I&Q&E&L&H&R&S&D&H&M&P&A&D&Q&E&T&I&P&S&K&A&S&Q&A&M&Q&K&L&A&R&D&L&A&E&V&S&E&R&F
H. daba : E&V&G&F&V&I&G&F&I&H&S&S-P&M&R&Y&L&A&S&C&S&D&E&T&Q&R&K&G&L&A&S&E&P&I&Q&E&L&H&R&S&D&H&M&P&A&D&Q&E&T&I&P&S&K&A&S&Q&A&M&Q&K&L&A&R&D&L&A&E&V&S&E&R&F
H. elon : E&V&G&F&V&I&G&F&I&H&S&S-P&M&R&Y&L&A&S&C&S&D&E&T&Q&R&K&G&L&A&S&E&P&I&Q&E&L&H&R&S&D&H&M&P&A&D&Q&E&T&I&P&S&K&A&S&Q&A&M&Q&K&L&A&R&D&L&A&E&V&S&E&R&F

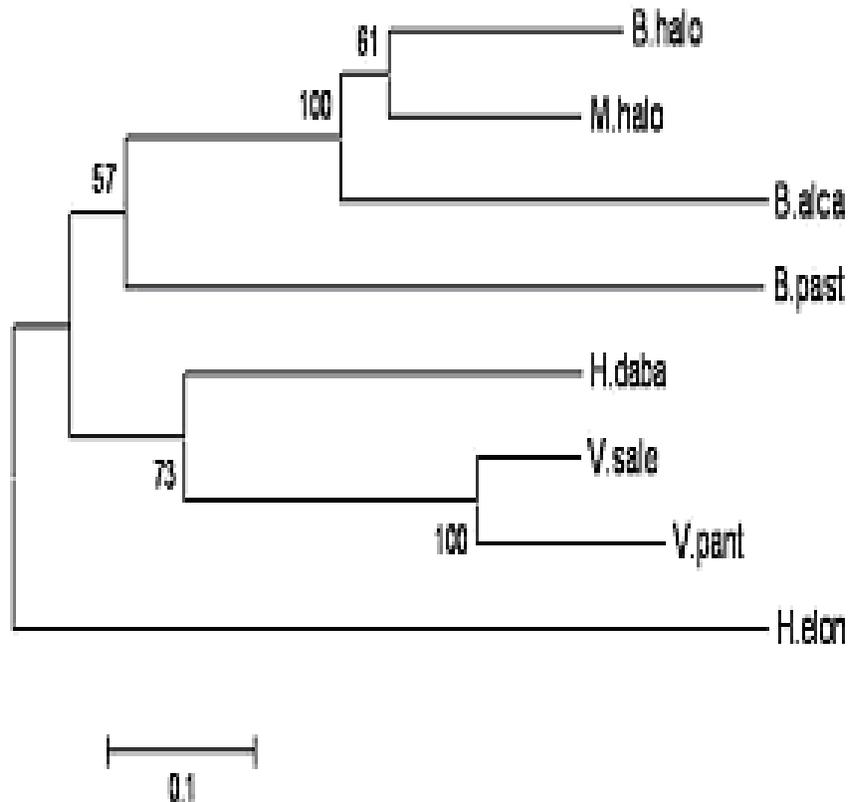
      180      *      200
E. halo : F&E&E&L&P&E&G&D&D&H&E&D&L&T&R&I&G&P&L&K&P-----
M. halo : F&E&E&L&P&E&G&D&D&H&E&D&L&T&R&I&G&P&L&K&P-----
E. alca : F&E&E&L&P&E&G&D&D&H&E&D&L&T&R&I&G&P&L&S&S-----
B. past : F&E&E&L&P&E&G&D&D&H&E&D&L&T&R&I&G&P&P&Q&V-----
V. sale : F&L&S&I&D&P&E&R&T&G&E&D&D&Q&L&K&I&G&P&F&Q&K&M&N&E
V. pant : F&L&S&V&D&P&E&R&T&G&E&D&D&Q&L&K&I&G&P&I&R&R&A&M&N&K
H. daba : F&E&E&D&P&E&G&E&H&E&Q&D&N&T&R&I&G&P&L&K&N-----
H. elon : F&E&I&D&Q&L&G&G-E&R&D&P&E&M&L&R&I&G&P&P&Q&T&D&Q&I-

```

**Fig. 4.7 Multiple alignments of *ectA* amino acid sequences from different organisms using GeneDoc programme.** The alignment was performed using amino acid sequences from *B. halo* (*Bacillus halodurans* TCJAR01), this work; *M. halo*, from *Marinococcus halophilus*; *B. alca*, from *B. alcalophilus*; *B. past*, from *B. pasteurii*; *V. sale*, from *Virgibacillus salexigens*; *V. pant*, from *Virgibacillus pantothenicus*; *H. daba*, from *Halobacillus dabanensis*; *H. elon*, from *Halomonas elongata*. Shaded boxes show region of sequence similarity.



**Fig. 4.8** The phylogenetic tree analysis of *ectA* nucleotide sequences from *Bacillus halodurans* TCJAR01 and other organisms using MEGA program

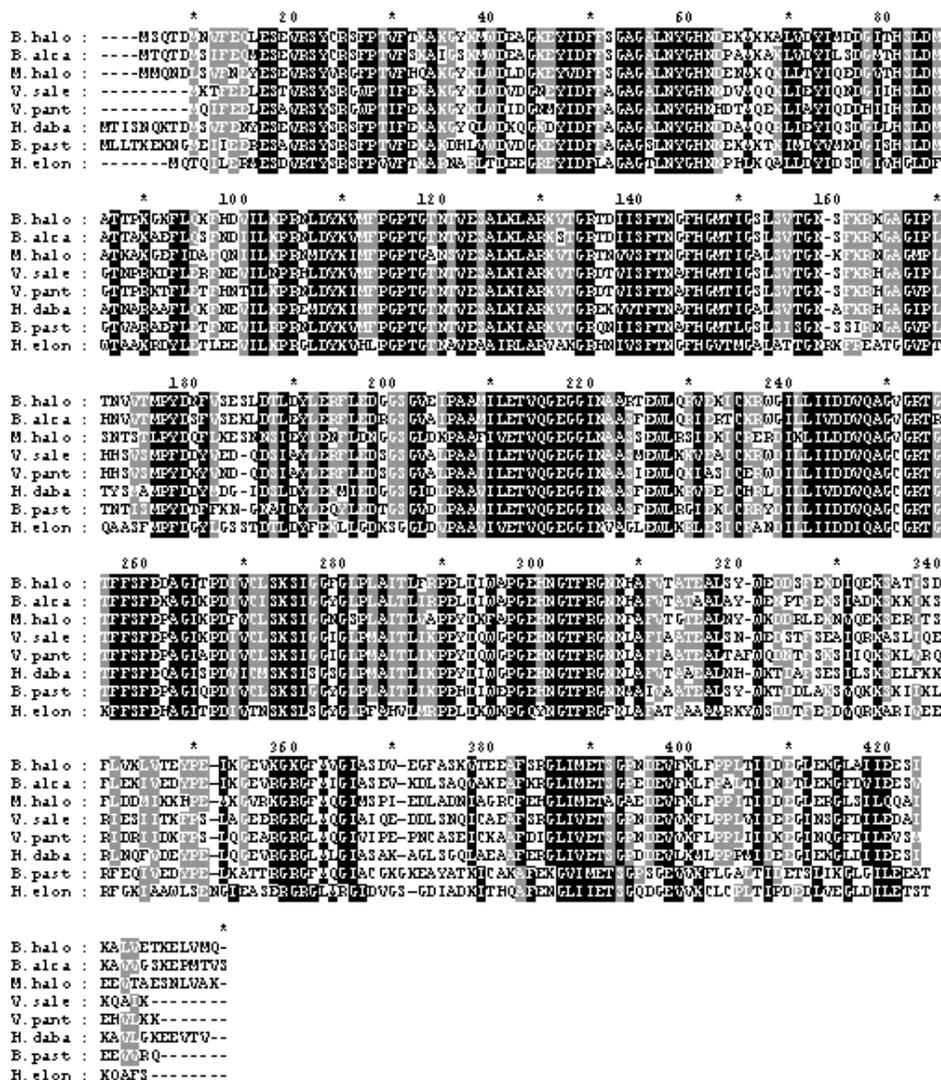


**Fig. 4.9** The phylogenetic tree analysis of *ectA* amino acid sequences from *Bacillus halodurans* TCJAR01 and other organisms using MEGA program

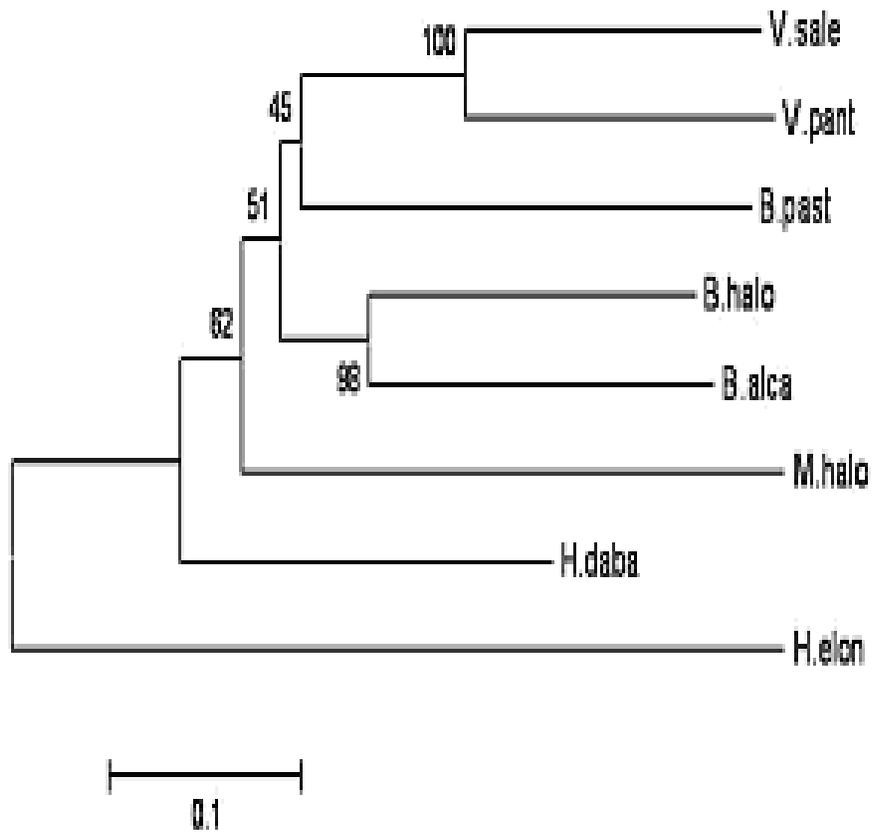
The amino acid analysis of *ectB* gene suggests that the encoded protein belongs to the GabT family. The *ectB* gene encodes proteins of 427 amino acids with the pI value of 4.84. Among the ectoine biosynthesis genes, the amino acid sequences of *ectB* gene showed highest similarity with other bacteria compared to *ectA* and *B* genes. *B. alcalophilus* sequence has 81% identity with 423 amino acid overlap; *H. dabanensis*, 66% identity with 423 amino acid overlap; *V. salaxigens*, 67% identity with 414 amino acid overlap; *V. pantothenicus*, 65% identity with 414 amino acid overlap; *B. pasteurii*, 65% identity with 413 amino acid overlap; *H. elongata*, 55% identity with 408 amino acid overlap and *M. halophilus* has 66% identity with 427

amino acid overlap (Fig. 4.10). The amino acid sequence analysis revealed that, only *B. alcalophilus* has the maximum identity of 81% with the amino acid sequence of *B. halodurans* TCJAR01. The phylogenetic tree of nucleotide and amino acid sequences of *ectB* gene also revealed the grouping of *B. halodurans* TCJAR01 and *B. alcalophilus* in a single cluster as that of *ectA* (Fig. 4.11 & 4.12).

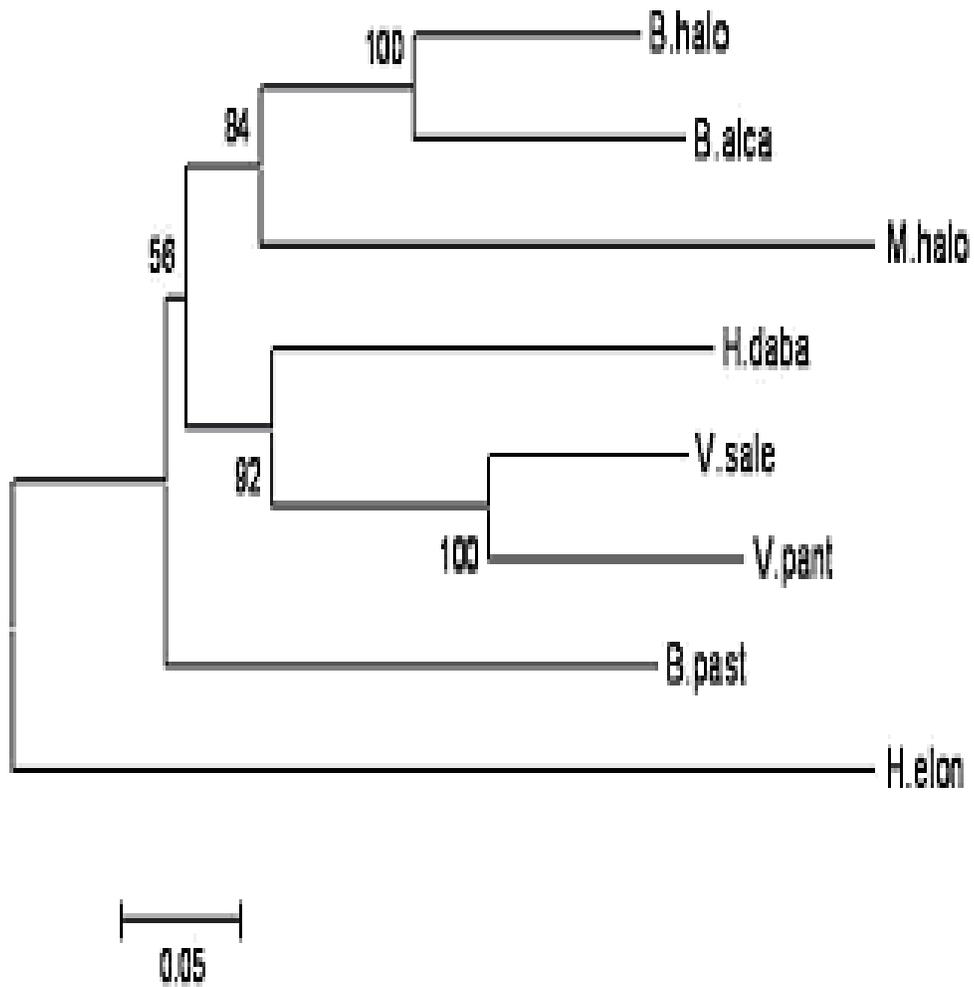
The amino acid analysis of *ectC* gene suggested that the encoded protein belongs to the ectoine synthase. The *ectC* gene encodes proteins of 129 amino acids with the pI value of 4.93. The amino acid sequences of *ectC* showed a considerable homology with ectoine synthase from other bacteria. *B. alcalophilus* sequence has 80% identity with 128 aa overlap; *H. dabanensis*, 62% identity with 128 aa overlap; *V. salexigens*, 60% identity with 128 aa overlap; *V. pantothenicus*, 59% identity with 128 aa overlap; *H. elongata*, 55% identity with 130 aa overlap; *B. pasteurii*, 66% identity with 129 aa overlap and *M. halophilus* has 68% identity with 124 aa overlap (Fig. 4.13). The amino acid sequence of *B. alcalophilus* and *M. halophilus* has the maximum similarity of 80% and 68% with the amino acid sequence of *B. halodurans* TCJAR01 than other eubacteria. In phylogenetic tree



**Fig. 4.10** Multiple alignments of *ectB* amino acid sequences from different organisms using GeneDoc programme. The alignment was performed using amino acid sequences from *B. halo* (*Bacillus halodurans* TCJAR01), this work; *M. halo*, from *Marinococcus halophilus*; *B. alca*, from *B. alcalophilus*; *B. past*, from *B. pasteurii*; *V. sale*, from *Virgibacillus salexigens*; *V. pant*, from *Virgibacillus pantothenicus*; *H. daba*, from *Halobacillus dabanensis*; *H. elon*, from *Halomonas elongata*. Shaded boxes show region of sequence similarity.



**Fig. 4.11** The phylogenetic tree analysis of *ectB* nucleotide sequences from *Bacillus halodurans* TCJAR01 and other organisms using MEGA program



**Fig. 4.12** The phylogenetic tree analysis of *ectB* amino acid sequences from *Bacillus halodurans* TCJAR01 and other organisms using MEGA program

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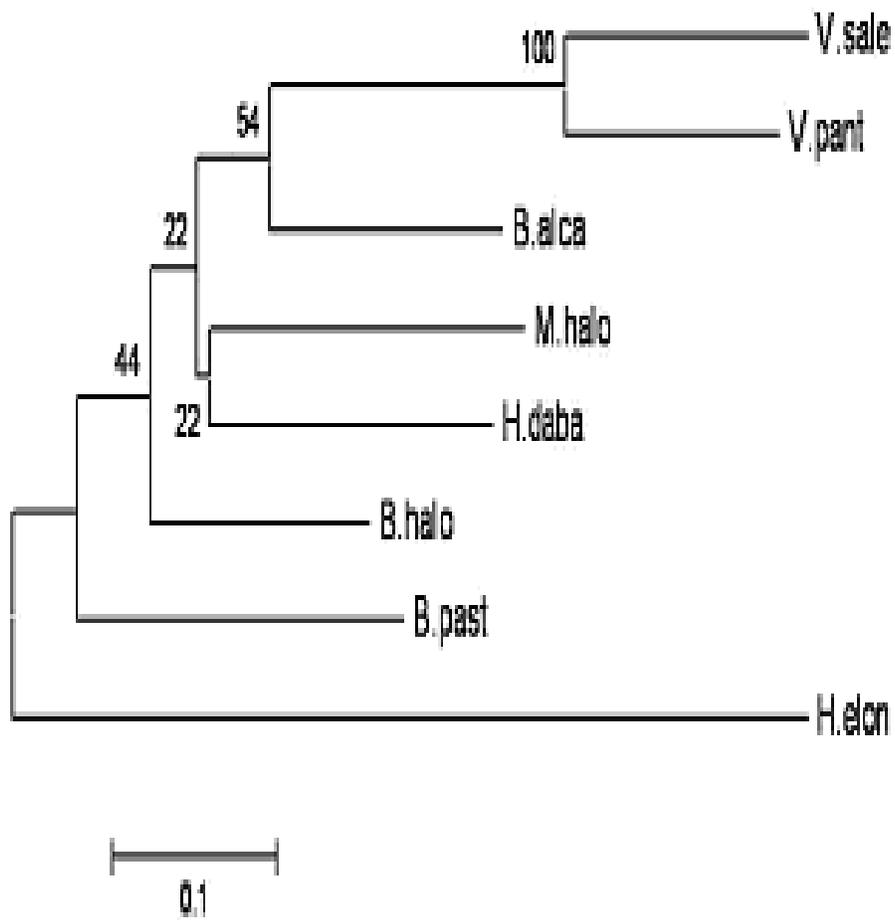
      *           20           *           40           *           60
Bhalo : KKQKIDDDIIGTDEQKKE--ATSRLLLEKQGGSGSMDHTIYKSGTETHIQKQKHEAQA
Bhalo : KKQKIDDDIIGTDEQKKE--ATSRLLLEKQGGSGSMDHTIYKSGTETHIQKQKHEAQA
Mhalo : KKQKIDDDIIGTDEQKKE--ATSRLLLEKQGGSGSMDHTIYKSGTETHIQKQKHEAQA
Vsale : KIKKIDDDIIGTDEQKKE--ATSRLLLEKQGGSGSMDHTIYKSGTETHIQKQKHEAQA
Upant : KIKKIDDDIIGTDEQKKE--ATSRLLLEKQGGSGSMDHTIYKSGTETHIQKQKHEAQA
Hdaba : KIKKIDDDIIGTDEQKKE--ATSRLLLEKQGGSGSMDHTIYKSGTETHIQKQKHEAQA
Bpast : KIKKIDDDIIGTDEQKKE--ATSRLLLEKQGGSGSMDHTIYKSGTETHIQKQKHEAQA
Helon : KIKKIDDDIIGTDEQKKE--ATSRLLLEKQGGSGSMDHTIYKSGTETHIQKQKHEAQA

      *           80           *           100          *           120
Bhalo : CIEGEGELETQDDKQKQKQKANEVVALDEMDDELE-ANTDQRMQCWFNPPITCKTIDDM
Bhalo : CIEGEGELETQDDKQKQKQKANEVVALDEMDDELE-ANTDQRMQCWFNPPITCKTIDDM
Mhalo : CIEGEGELETQDDKQKQKQKANEVVALDEMDDELE-ANTDQRMQCWFNPPITCKTIDDM
Vsale : CIEGEGELETQDDKQKQKQKANEVVALDEMDDELE-ANTDQRMQCWFNPPITCKTIDDM
Upant : CIEGEGELETQDDKQKQKQKANEVVALDEMDDELE-ANTDQRMQCWFNPPITCKTIDDM
Hdaba : CIEGEGELETQDDKQKQKQKANEVVALDEMDDELE-ANTDQRMQCWFNPPITCKTIDDM
Bpast : CIEGEGELETQDDKQKQKQKANEVVALDEMDDELE-ANTDQRMQCWFNPPITCKTIDDM
Helon : CIEGEGELETQDDKQKQKQKANEVVALDEMDDELE-ANTDQRMQCWFNPPITCKTIDDM

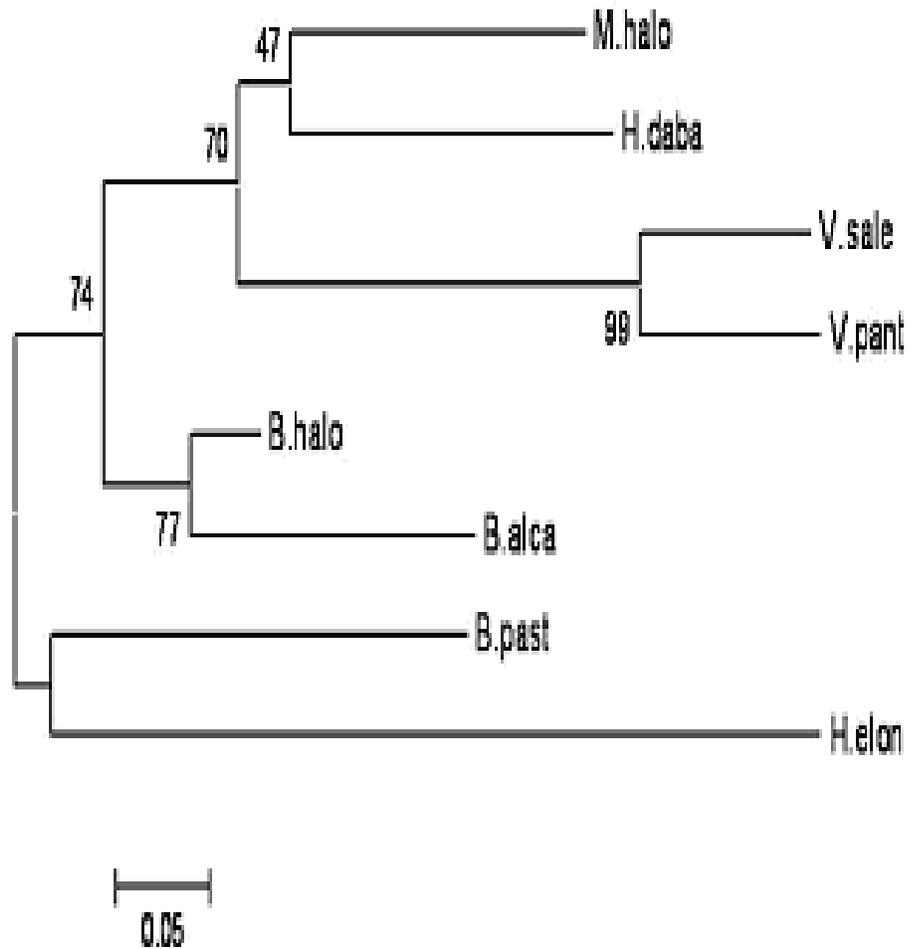
      *
Bhalo : VPELVDE---
Bhalo : VPELVDE---
Mhalo : VPELVDE---
Vsale : VPELVDE---
Upant : VPELVDE---
Hdaba : VPELVDE---
Bpast : VPELVDE---
Helon : VPELVDE---

```

**Fig. 4.13 Multiple alignments of *ectC* amino acid sequences from different organisms using GeneDoc programme.** The alignment was performed using amino acid sequences from *B. halo* (*Bacillus halodurans* TCJAR01), this work; *M. halo*, from *Marinococcus halophilus*; *B. alca*, from *B. alcalophilus*; *B. past*, from *B. pasteurii*; *V. sale*, from *Virgibacillus salexigens*; *V. pant*, from *Virgibacillus pantothenicus*; *H. daba*, from *Halobacillus dabanensis*; *H. elon*, from *Halomonas elongata*. Shaded boxes show region of sequence similarity.



**Fig. 4.14** The phylogenetic tree analysis of *ectC* nucleotide sequences from *Bacillus halodurans* TCJAR01 and other organisms using MEGA program



**Fig. 4.15** The phylogenetic tree analysis of *ectC* amino acid sequences from *Bacillus halodurans* TCJAR01 and other organisms using MEGA program

analysis, a diverged mode of clustering was observed (Fig. 4.14 & 4.15). Nucleotide analysis of *ectC* gene revealed that *B. halodurans* TCJAR01 was clustered separately from other eubacteria, however in the amino acid analysis *B. halodurans* TCJAR01 and *B. alcalophilus* were grouped in the single cluster. Eventhough the ectoine biosynthesis pathway is evolutionary well conserved with respect to the genes and

enzymes involved, some differences in their organization and regulation could occur in various halophilic bacteria (Reshetnikov *et al.*, 2006).

#### **4.5.5 HPLC ANALYSIS OF ECTOINE**

In order to determine whether the recombinant *ectABC* gene cluster was able to produce ectoine, HPLC analysis of ethanolic extract culture supernatant was done. There was an additional peak in the culture supernatant drawn 16 h after induction, which was not present in uninduced control. The retention times of the peak correlated well with those of authentic ectoine standard. The transgenic *E. coli* synthesized 4.6 mg/l ectoine (Fig. 4.16). The ectoine synthesised in this study is relatively higher quantity than the previous report of Schubert *et al.*, 2007.

#### **4.6 ISOLATION AND IDENTIFICATION OF *ESCHERICHIA COLI* TCJAR023**

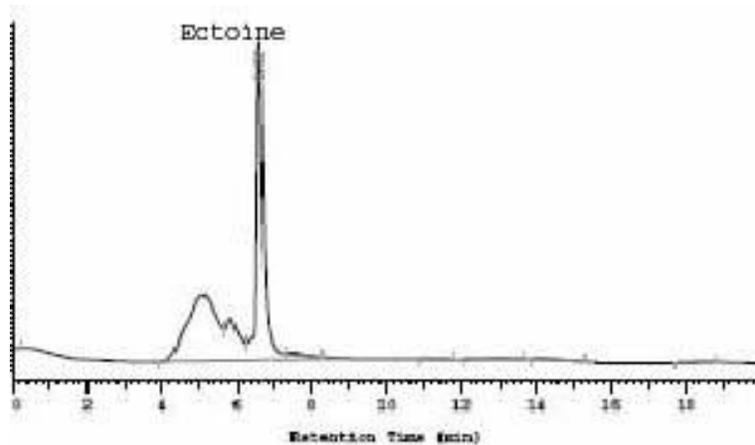
The identification and biochemical characterization of *E. coli* TCJAR023 was performed as per [U.S. Food and Drug Administration (USFDA)]. The detailed characteristic feature of the *E. coli* isolate in this study is presented in (Table 4.12). Further, the growth with metallic sheen formation was also observed in eosin methylene blue agar. Thus the bacterial strain was identified and confirmed as *E. coli* TCJAR023, on the basis of these results.

#### **4.7 FUNCTIONAL CHARACTERIZATION OF CHOLINE DEHYDROGENASE FROM *E. COLI* TCJAR023**

##### **4.7.1 PCR AMPLIFICATION, CLONING AND SEQUENCING**

The choline dehydrogenase gene, *betA* encodes polynucleotide of 1671 bp long (Fig. 4.17). The polypeptides of choline dehydrogenase comprised of 556 amino acids (Fig. 4.18) with the calculated molecular masses of 61848 Da., based on the *in*

*in silico* estimates. The protein of *betA* gene has the isoelectric point of 5.45. The *betA* gene from *E. coli* TCJAR023 was PCR amplified, cloned in pDrive and transformed in *E. coli* JM109. Plasmid pDrive with *betA* gene insert was confirmed by nucleotide sequencing. The *in silico* sequence analysis of *betA* gene revealed a total of thirty one base substitutions at the nucleotide level with that of the sequences deposited in the GenBank. (accession nos. X52905, M77738) (Fig. 4.19). However only two of these changes translated into change of amino acid. The differences were observed at positions 133 and 452, which resulted in the amino acid substitution of valine with methionine and of threonine with valine residue, respectively (Fig. 4.20). No internal stop codons were observed due to the base substitution in the gene.

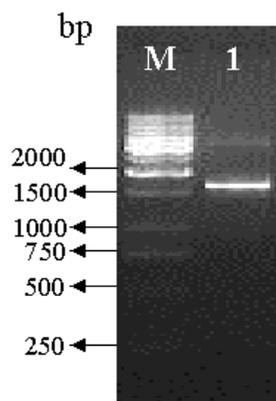


**Fig. 4.16 HPLC chromatogram of ectoine from**

*Bacillus halodurans* TCJAR01

**Table 4.12 Characteristic features of *Escherichia coli* TCJAR023**

Test	Standard observation	<i>Escherichia coli</i> TCJAR023
H <sub>2</sub> S production	-	-
Indole	+	+
Methyl Red	+	+
Voges Proskauer	-	-
Citrate	-	-
Urea	-	-
Motility	+	+
Glucose	+	+
Lactose	+	+
Sucrose	+	+
Oxidase	-	-



**Fig. 4.17 PCR amplicon of *betA* from *Escherichia coli* TCJAR023 with primers bAF and bAR**

Lane M -1 kb DNA Ladder

Lane 1 -Amplicon of *betA* gene (1671 bp)

atgcaatttgactacatcattattgggtgccggctcagccggcaacggtctcgcctaccggt  
M Q F D Y I I I G A G S A G N V L A T R  
ctgactgaagatccgaatacctccgtgctgctgcttgaagcgggcgcccgactatcgc  
L T E D P N T S V L L L E A G C P D Y R  
tttgacttccgcacccagatgcccgagcgtggcggtcccactacagggcaaacgctac  
F D F R T Q M P A A L A F P L Q G K R Y  
aactgggctatgaaacagagcctgaaccgtttatgaataaccgcccgcagtgagtgcgga  
N W A Y E T E P E P F M N N R R M E C G  
cgcggtaaaggcctgggtggatcgtcgtgatcaacggcatgtgctacatccgtggcaat  
R G K G L G G S S L I N G M C Y I R G N  
gcgctggatctcgataaactgggcgcaagaacccggtctggagaactggagttatctcgac  
A L D L D N W A Q E P G L E N W S Y L D  
tgctgccctactaccgcaaggccgagactcgcgatgtgggcgagaacgactaccacggt  
C L P Y Y R K A E T R D V G E N D Y H G  
ggcgatggcccggtgagcgtcaccacctccaaacccggcgctcaatccgctgtttgagcg  
G D G P V S V T T S K P G V N P L F E A  
atgattgaagcggcgctgcaggcaggctaccgcgcacggacgatctcaacggctatcag  
M I E A G V Q A G Y P R T D D L N G C Y Q  
caggaaggttttggcccgatggatcgcaccgctcacgccgcagggccgtcgcgccagtacc  
Q E G F G P M D R T V T P Q G R R A S T  
gcgcgggtatctcgatcaggccaaatcgcgccctaacctgaccattcgtactcagcc  
A R C Y L D Q A K S R P N L T I R T H A  
atgaccgatcacatcatttttgacggcaaacgcgcggtggcgctcgaatggctggaaggc  
M T D H I I F D C K R A V G V E W L E C  
gacagcaccatcccaaccgcgcaacggccaacaagaagtgtgttatgtgcaggcgcg  
D S T I P T R A T A N K E V L L C A G A  
attgcctcaccgcagatcctgcaacgctccggcgctcggcaacgctgaactgctggcggag  
I A S P Q I L Q R S C V G N A E L L A E  
tttgatattccgctggtgcatgaattaccgcgctcggcgaataatcttcaggatcatctg  
F D I P L V H E L P C V G E N L Q D H L  
gagatgatctgcaatagagtgcaagaaccgggtttccctctaccctgccctgcagtg  
E M Y L Q Y E C K E P V S L Y P A L Q W  
tggaaaccagccgaaatcgggtgcggagtggtgtttggcggcactggcggttggtgccagc  
W N Q P K I G A E W L F G G T G V G A S  
aaccactttgaagcaggtggatttattcgcagccgtgaggaatttgcgctggccgaatatt  
N H F E A G C F I R S R E E F A W P N I  
cagtagcatttccctgccagtagcgattaaactataacggctcgaatgcagtgaaagagcac  
Q Y H F L P V A I N Y N G S N A V K E H  
ggttccagtgccagtcggctcaatgcgctcgccaagccgtggcgatgtgcggattaaa  
G F Q C H V G S M R S P S R G H V R I K  
tcccgcgaccgcaccagcatccggcgattctgtttaactacatgtcgcacgagcaggac  
S R D P H Q H P A I L F N Y M S H E Q D  
tggcaggagttccgcgacgcaattcgcctcaccgcgagatcatgcatcaaccgcgctg  
W Q E F R D A I R I T R E I M H Q P A L  
gatcagtatcgtggccgcgaaatcagccccggcagcggaaatgtcagacggatgaacagctc  
D Q Y R G R E I S P C T E C Q T D E Q L  
gatgagttcgtgcgtaaccacgcccgaacccgcttccatccgtgcggtacctgcaaaatg  
D E F V R N H A E T A F H P C G T C K M  
ggttacgacgagatgtccgtggttgacggcgaaggccgcgctacacggggttagaaggcctg  
G Y D E M S V V D G E G R V H G L E G L  
cgtgtggtggatgcgtcgattatgccgcagattatcaccgggaatttgaacgccacgaca  
R V V D A S I M P Q I I T G N L N A T T  
attatgattggcgagaaaatagcggatattgattcgtggacaggaagcgtgccgaggagc  
I M I G E K I A D M I R G Q E A L P R S  
accggcgggatattttgtggcaaatgggatgccggtgagagcgaaaaaatga  
T A G Y F V A N G M P V R A K K -

Fig 4.18 *betA* gene with nucleotide and its corresponding amino acid sequences

from *Escherichia coli* TCJAR023

Upon BLAST analysis it was found that the deduced amino acid sequence of *betA* was highly homologous to choline dehydrogenase of reported strains; 99% identity with *E. coli* (accession no. X52905) and 98% identity with *E. coli* (accession no. M77738).

To authenticate the nucleotide variation in the sequence of *betA*, the gene was again PCR amplified with the genomic DNA from *E. coli* TCJAR023 with *Taq* DNA polymerase (Dynazyme II, Espoo, Finland). The underlying principle for this strategy was that feasible misincorporations of nucleotides in gene amplification might occur at different positions by using different DNA polymerases and PCR protocols. The PCR product was cloned in pTZ57R/T (MBI Fermentas) and sequenced. The nucleotide sequence of the *betA* gene cloned in pTZ57R/T was the same as dogged using pDrive-*betA* cassette. This result confirms that the base divergence was conserved in the genome and not due to the external parameters.

```

      10      20      30      40      50      60      70      80      90     100
EU032535ATGCAATTGACTACATCATATTGTTGGCCGGCTCGAGCCGGCCAGCTCTCCGCTACCCGCTGACTGAGGATCCGATACCTCCGCTGCTGCTTGAAG
X52905  T.....
H77738  T.....
      110     120     130     140     150     160     170     180     190     200
EU032535CGGGCGGGCCGACTATCGCTTTGACTTCCGACCCAGTGGCCGCAAGCGCTGGCGTTCCCACTACAGGGCCAAACGCTACAACTGGGCTTATGAAACGAA
X52905  .....T.C.A.G.....T.....G.....
H77738  .....T.C.A.G.....T.....G.....
      210     220     230     240     250     260     270     280     290     300
EU032535GCCTGACCGTTTATGATACCCGCCGATGGAGTGGCGGACGGGTTAAGGCCCTGGGTGGATCGTCCGCTGATCAACGGCATGTGCTACATCCGCTGGCAT
X52905  A.....T.....
H77738  A.....T.....
      310     320     330     340     350     360     370     380     390     400
EU032535GGCCTGATCTCGATACCTGGGCGCAAGAACCCGGTCTGAGGACTGGAGTTATCTCGACTGCCTGCCCTACTACCGCAAGGCCGAGACTCGCATGTGG
X52905  .....C.C.....A.....
H77738  .....C.C.....A.....
      410     420     430     440     450     460     470     480     490     500
EU032535GGCGAAGCACTACACCGGTGGCCGCTGGCCGGTGGAGCTCACCCCTCCAAACCCGGCTCATCCGCTGTTTGAAGCGATGTTGAGCGGGCGTGGCA
X52905  .T.A.....T.....C.T.....
H77738  .T.A.....T.....C.T.....
      510     520     530     540     550     560     570     580     590     600
EU032535GGCGAGCTACCCGGCCAGCGAGTCTCAACGGCTATCAGCAGGAGGTTTTGGCCCGATGATCGCACCGTCCGCGCGAGGGCCGTCGCGCCAGTACC
X52905  G.....T.....C.....
H77738  G.....T.....C.....
      610     620     630     640     650     660     670     680     690     700
EU032535GGCCGCGGTTATCTCGATCAGGCCAATCGGCGCCCTAACCTGACCACTCGTACTCAGCCATGACCATCAATATTTTGGCCGCAACGGCGGTGG
X52905  .T.C.....T.....T.....
H77738  .T.C.....T.....T.....
      710     720     730     740     750     760     770     780     790     800
EU032535GCCTGATGAGTGGTGGAGGCGACAGCACATCCGACCCCGCCAGCGCCACCAAGGATGCTGTTATGTGCAAGCCGCGATTGCTCCAGCGCAATCCT
X52905  .....
H77738  .....
      810     820     830     840     850     860     870     880     890     900
EU032535GCACCGCTCCGGCGTGGGCAACGCTGATCTGCTGGCGGAGTTTGAATATCCGCTGGTGGATTAACCCGGCGTCCGGCAATCTTCAGGATCATCTG
X52905  .....
H77738  .....
      910     920     930     940     950     960     970     980     990     1000
EU032535GAGTGTATCTCGATATGAGTGGCAAGAACCCGTTTCCCTACACCTGCCCTGCACTGGTGGAAACAGCCGAAATCGGTGGCGAGTGGCTGTTGGCG
X52905  .....
H77738  .....
      1010    1020    1030    1040    1050    1060    1070    1080    1090    1100
EU032535GCACTGGCGTTGGTGGCCGACACCTTTGAGGCGGTTGATTTTATTCGACCGCTGGGATTTGCTGGCCGATATTCACTACCTTTCTGCGCGT
X52905  .....
H77738  .....
      1110    1120    1130    1140    1150    1160    1170    1180    1190    1200
EU032535AGGGATTACTATACCGGCTCGATGCGGTGAAAGGCGAGTTTTCACTGCGCACGTCGGCTCAATGGCTCGCCAGCGCGTGGGCTTGGCGGATTA
X52905  .....
H77738  .....
      1210    1220    1230    1240    1250    1260    1270    1280    1290    1300
EU032535TCCCGGACCCGCAACGACATCCGGCGATTCTGTTTAACTACATGTCGACAGGCAAGGCTGGCGAGGATTCCGCGACGCAATTCGATCACCCGCGAA
X52905  .....
H77738  .....
      1310    1320    1330    1340    1350    1360    1370    1380    1390    1400
EU032535TCACTGATCAACCCGCGCTGATCGATATCGTGGCCGCAATCAAGCCCGGCAAGGATGTCAAGCGGATGAAACGCTCGATGAGTTCTGCGGTACCA
X52905  .....TGTC.....C.....
H77738  .....TGTC.....C.....
      1410    1420    1430    1440    1450    1460    1470    1480    1490    1500
EU032535CGCCGAAACCGGCTTCCATCCGTCGGTACCTGCAATATGGGTTTACAGCAGATGTCCGTGTTGACGGCGAAAGGCCGCTACACGGGTTAAGAGGCGCTG
X52905  .....
H77738  .....
      1510    1520    1530    1540    1550    1560    1570    1580    1590    1600
EU032535CGTGTGGTGGATGCGTCAATATGCCGCAATTTATCCCGGATTTGAAAGCCAGCAGCAATTTGATTTGGCGAAGGATAGCGGATGATTTCTGGAC
X52905  .....
H77738  .....
      1610    1620    1630    1640    1650    1660    1670
EU032535AAGGAGCGCTGCCAGGAGGACCGGGGATTTTTGTGGCAATGGATGCCGCTGAGAGCGAATTAATCA
X52905  .....
H77738  .....

```

**Fig. 4.19** BioEdit analysis of nucleotide substitutions of choline dehydrogenase (*betA*) in *E. coli* TCJAR023 [EU032535], with (*betA*) genes from *E. coli* strains [X52905 & M77738].

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          10      20      30      40      50      60      70      80      90      10
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
EU032535HQEDYIIIQAGSAGNVLRLRLTEPMTSVLLLEAGGPDYKFDERTQHPAALAEPLQGKRYMAYETEPEPFHNRREHCGRGKLGGSLLINGHCYIRGN
X52905.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
M77738.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

          110     120     130     140     150     160     170     180     190     20
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
EU032535ALILDWAQEPGLENSYLDCLPFYKKAETEDVGENDYHGGDGPVSVTTSKPGVMPLEEAHLEAGVQAGYFKTDDLNGYQQEGSGPHDRTVTPQGERAST
X52905.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
M77738.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

          210     220     230     240     250     260     270     280     290     30
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
EU032535ARGYLDQAKSRPMLTIKTHAHTDHIIFDGKRAVGVFWLRGDSSTIPTRRTANKEVLLCAGAIRSPQILQRSGVGNRAELLAEFDIPLVHELPGVGENLQDHL
X52905.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
M77738.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

          310     320     330     340     350     360     370     380     390     40
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
EU032535EHYLQYECREPVSLYPALQVWVQPKIQAEWLFGGTGVGASMHFEAGGFIRSRERFAWPMIQYHFLPVRLNYNGSMAVKEHGFQCHVGSRRSPSRGHVKIK
X52905.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
M77738.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

          410     420     430     440     450     460     470     480     490     50
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
EU032535SRDPHQHPALIFMYHSHEQDWQEEFRDAIRITREIRHQPALDQYRGEIISPGTSCQTDEQLDEFVRMHAEATAEHPCGTCKHGYDEHSVVDGEGRVHGLEGL
X52905.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
M77738.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

          510     520     530     540     550
.....|.....|.....|.....|.....|.....|
EU032535RVVDAIHPQIITGNLNATTIHIKELADHIRGQEALPRTAGTFVANGHPVRAKK
X52905.....|.....|.....|.....|.....|.....|
M77738.....|.....|.....|.....|.....|.....|

```

**Fig. 4.20 BioEdit analysis of amino acid substitutions of choline dehydrogenase (*betA*) in *E. coli* TCJAR023 [EU032535], with (*betA*) genes from *E. coli* strains [X52905 & M77738].**

#### **4.7.2 PROTEIN EXPRESSION**

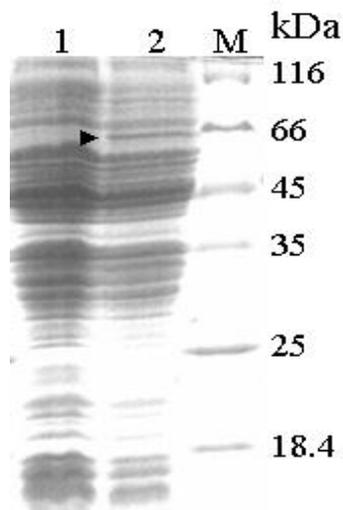
The expression vector pQE30 carrying the *betA* gene cassette, downstream of the inducible promoter was transformed in *E. coli* M15 (pREP4). To validate the expression, the *betA* gene cassette was induced with IPTG. The proteins of the induced and noninduced *E. coli* M15 (pREP4) cells were visualized by SDS-PAGE. The lysate of induced cells showed one clear expressed band with molecular masses of 63 kDa, which corresponds to choline dehydrogenase (Fig. 4.21).

#### **4.7.3 PURIFICATION OF CHOLINE DEHYDROGENASE**

The expressed choline dehydrogenase enzyme was then subjected to 50% ammonium sulfate saturation and the purification was performed using Hi-Prep 16/10 DEAE Fast Flow column (Table 4.13). The follow on purified enzyme was stabilized with 2 M sorbitol and 20% glycerol, at pH 7.0. The purified protein was visualized by SDS-PAGE (Fig. 4.22).

#### **4.7.4 FUNCTIONAL ANALYSIS OF CHOLINE DEHYDROGENASE**

Choline dehydrogenase is capable of using either choline or betaine aldehyde as a substrate (Landfald and Strom, 1986; Tsuge *et al.*, 1980), The ability of the enzyme to catalyze both oxidative steps in the conversion of choline to glycine betaine has previously been observed in choline dehydrogenase from *Halomonas elongata* (Gadda and Wilkins, 2003) and rat liver mitochondria (Tsuge *et al.*, 1980) and in choline oxidase from *Arthrobacter globiformis* (Ikuta *et al.*, 1977). In this study, the choline dehydrogenase activity was dogged by monitoring the rate of oxygen consumption in the presence of 1 mM phenazine methosulfate as the primary electron acceptor (Gadda and Wilkins, 2003) with different concentrations of choline as the substrate for the

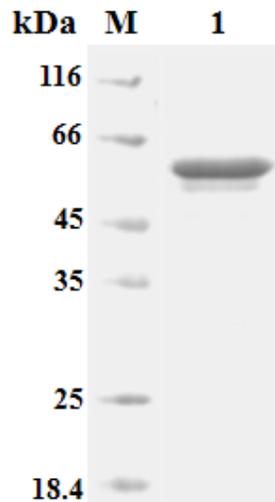


**Fig. 4.21 SDS-PAGE analyses of the expressed *betA* of *E. coli* TCJAR023**

Lane M - Protein molecular mass marker  
 Lane 1- Total protein of the uninduced *betA* gene  
 Lane 2- Total protein of the induced *betA* gene  
 The arrow (►) indicates the position of the expressed *betA* (63 kDa)

**Table 4.13. Purification of recombinant choline dehydrogenase from  
*E. coli* TCJAR023**

Stages	Total protein (mg)
50% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> saturation	18
Hi-Prep 16/10 DEAE	7



**Fig. 4.22 SDS-PAGE analyses of purified choline dehydrogenase from *E. coli* TCJAR023**

Lane 1 - Purified choline dehydrogenase

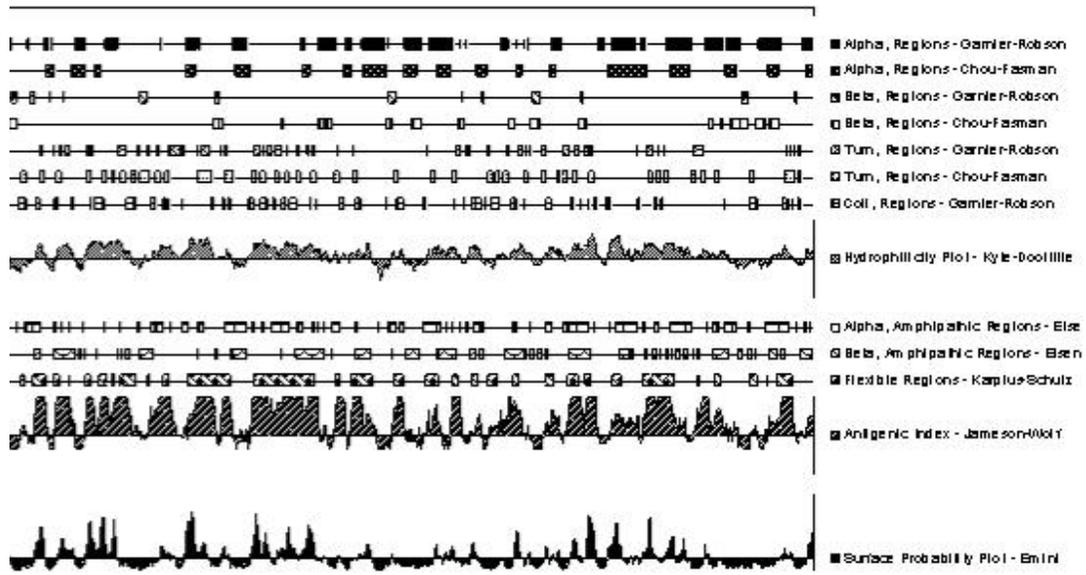
Lane M - Protein molecular mass marker

recombinant enzyme. The activity of choline dehydrogenase with 10 mM choline was 8.8  $\mu\text{mol}$  of  $\text{O}_2/\text{min}/\text{mg}$  of cultured cells. The declined activity of the enzyme was observed with the decrease in the concentration of substrate. In the presence of 2.5 mM choline, the specific activity was lowered to 3  $\mu\text{mol}$  of  $\text{O}_2/\text{min}/\text{mg}$  of cultured cells. These results are consistent with previously reported data showing that the product of the enzymatic oxidation of choline, glycine betaine, can be formed *in vivo* when *E. coli* cells containing a choline transporter and *betA* from *H. elongata* are incubated with [*methyl-14C*]choline (Canovas *et al.*, 2000).

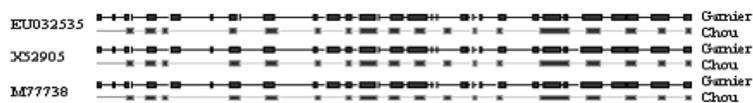
#### **4.7.5 SECONDARY STRUCTURE PREDICTION ANALYSIS**

The secondary structure of choline dehydrogenase protein was predicted to have the alpha-helical structure with maximum hydrophilic molecules. The prediction analysis also revealed the presence of acidic amino acids, regions with high

antigenicity and very high backbone chain flexibility (Fig. 4.23). Upon analysis of *betA* protein, the predicted charge at pH 7.0 was 15.45 with the isoelectric point of 5.52. Common amino acids include 58% glycine, 45% alanine, 41% glutamic acid, 40% leucine, 37% arginine, 33% proline and 30% each of isoleucine and aspartic acid. Upon structural analysis, both Chou-Fasman and Garnier-Robson prediction of alpha helix structures showed a maximum similarity, suggesting that the active domains of the enzyme from *E. coli* TCJAR023 have the considerable identity with the database reports (Fig. 4.24).



**Fig. 4.23 Secondary structure prediction of choline dehydrogenase using PROTEAN** (Garnier-Robson and Chou-Fasman methods). The analysis was performed using choline dehydrogenase from *E. coli* TCJAR023 [EU032535], with *E. coli* strains [X52905 and M77738].



**Fig. 4.24 Secondary structure prediction analysis of choline dehydrogenase using PROTEAN.** The analysis was performed using choline dehydrogenase from *E. coli* TCJAR023 [EU032535], with *E. coli* strains [X52905 & M77738].

**SUMMARY  
AND  
CONCLUSION**

## 5.0. SUMMARY AND CONCLUSION

In recent times, agriculture based research is mainly focused in functional characterization of halotolerant genes from marine origin for their potential benefits. In marine origin sources, salted fishes have long been recognized to inhabit salt tolerant bacterial species. To cope with the osmolytic environmental stress, bacteria inhabit in marine sources will accumulate osmotically active solutes like, potassium, proline, glutamic acid, glutamine,  $\alpha$ -aminobutyric acid, ectoine, and glycine betaine. The focus of the current study was to functionally characterize the major halotolerant genes, ectoine and glycine betaine from the bacterial isolates from salted fishes.

Salted fish samples like *Scoliodon* spp. and *Thrissina thryssa* were selected in this study. From the samples a total of 142 aerobic halophiles were isolated, in which 38 from shark (*Scoliodon* spp.) and 55 from anchovies (*Thrissina thryssa*) were randomly selected and characterized in detail for the isolation of *Bacillus halodurans* and *Escherichia coli*.

The *ectABC* gene cluster involved in the ectoine biosynthesis has been isolated and characterized from *Bacillus halodurans* TCJAR01. *Bacillus halodurans* is an alkaliphilic bacterium that can grow well at pH 7–10.5 in saline environments. So far, no group has reported the functional characterization of *ectoine* biosynthesis genes from *B. halodurans* isolated from salted fishes. *B. halodurans* is well characterized physiologically, biochemically, and genetically. *ectABC* gene cluster from *Bacillus halodurans* was heterologously expressed in *E. coli* and the production of ectoine was confirmed by HPLC analysis. The activity of the enzymes coded by the *ectA*, *B* and *C*

genes were found to be higher in induced transgenic cells compared to the uninduced cells. Phylogenetic analysis revealed sequence identities ranging from 36-73% for *ectA* gene, 55-81% for *ectB* gene and 55-80% for *ectC* gene indicating that the enzymes are evolutionarily well conserved.

Glycine betaine, is a quaternary ammonium compound that occurs naturally in a wide variety of plants, animals and microorganisms. The biosynthetic pathway for the production of glycine betaine from choline has been well characterized at the genetic level in *E. coli*. Choline dehydrogenase catalyzes the oxidation of choline to glycine betaine via betaine aldehyde in glycine betaine biosynthesis. Glycine betaine is a compatible solute, able to restore and maintain osmotic balance of living cells under stress. Choline dehydrogenase (*betA*) gene encoding for *glycine betaine* biosynthesis from *Escherichia coli* TCJAR023 was cloned and homologously expressed in *E. coli* M15 (pREP4). The recombinant *E. coli* strain showed a fourfold increase in the activity of choline dehydrogenase enzyme with choline as substrate and phenazine methosulfate as electron acceptor, compared to the control strain. The sequence *betA* gene reported in this study contains several base substitutions with that of reported sequences in GenBank, resulting in the altered amino acid sequences of the translated proteins.

In conclusion, the salted fishes will remain an excellent source for the isolation of moderately halophilic bacteria than other marine sources. Further, the novel halotolerant genes isolated from the bacterial inhabitants of salted fishes can be used in agricultural research for the development of transgenic plant species of economically important.

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**LIST  
OF  
PUBLICATIONS**

## 7.0 LIST OF PUBLICATIONS BASED ON THIS RESEARCH

- Anbu Rajan, L.**, Toms C. Joseph, Nirmala Thampuran, Roswin James, Ashok Kumar, K., Viswanathan, C. and Kailash C. Bansal 2008, a. Cloning and heterologous expression of ectoine biosynthesis genes from *Bacillus halodurans* in *Escherichia coli*. **Biotechnology Letters**, 30: 1403-1407
- Anbu Rajan, L.**, Toms C. Joseph, Nirmala Thampuran, Roswin James, Viswanathan, C. and Kailash C. Bansal 2008, b. Characterization and phylogenetic analysis of ectoine biosynthesis genes from *Bacillus halodurans*. **Archives of Microbiology**, 190: 481-487
- Anbu Rajan, L.**, Toms C. Joseph, Nirmala Thampuran and Roswin James. 2010, Functional characterization, secondary structure prediction and analysis of ectoine biosynthesis genes from *Bacillus halodurans*: an osmolyte involved in stress tolerance. **World Journal of Microbiology and Biotechnology**, DOI: 10.1007/s11274-010-0379-6
- Anbu Rajan, L.**, Toms C. Joseph, Nirmala Thampuran and Roswin James. 2010, Studies on microbial diversity of the salted fishes under aerobic conditions. **Microbiology Research**, DOI:10.4081/mr.2010.e4.
- Anbu Rajan, L.**, Toms C. Joseph, Nirmala Thampuran and Roswin James. 2010, Functional characterization and sequence analysis of choline dehydrogenase from *Escherichia coli*. **Genetic Engineering and Biotechnology Journal** (Accepted for publication)

Toms C. Joseph, **Anbu Rajan, L.**, Nirmala Thampuran and Roswin James 2010,  
Functional characterization of trehalose biosynthesis genes from *Escherichia coli*. **Molecular Biotechnology**, 46: 20-25