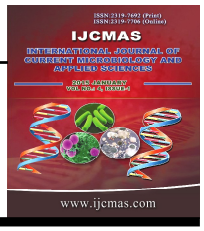
Int.J.Curr.Microbiol.App.Sci (2015) 4(1): 642-658

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 1 (2015) pp. 642-658**
http://www.ijcmas.com



Original Research Article

Salt stress tolerant genes in halophilic and halotolerant bacteria: Paradigm for salt stress adaptation and osmoprotection

Priyanka Das[1,2], Bijay Kumar Behera[1], Dharmendra Kumar Meena[1*], Syed Afrin Azmi[2], Soumendranath Chatterjee[2], Kanti Meena[3] and Anil Prakash Sharma[1]

[1]Central Inland Fisheries Research Institute, Barrackpore, Kolkata, West Bengal, India -700120
[2]Parasitology and Microbiology laboratory, Department of Zoology, University of Burdwan, Burdwan, West Bengal, India
[3]Central Research Institute for Jute and Allied Fibres, Barrackpore, Kolkata, West Bengal, India -700120
**Corresponding author*

Keywords

Bacteria, Compatible solutes, Halotolerant, Halophilic, Osmoregulation, Salt stress tolerant genes

A B S T R A C T

Salinity stress is one of the major factors negatively affecting growth and productivity in living organisms including plants and bacteria resulting in significant losses worldwide. Therefore, it would be fruitful to develop salinity stress tolerant useful species and also to understand the mechanism of stress tolerance that simulate the production of bioactive osmotic compatible solute which are of great significance to cope with hostile salt stress conditions, and to have industrial and pharmaceuticals applications as well. A prerequisite for molecular studies is the identification of genes involved in the accumulation of compatible solutes. In this back drop, this review highlights various studies investigating salt stress tolerant genes from different halophilic / halotolerant bacteria, focusing on recent developments in this area.

Introduction

The damaging effects of salt accumulation in agricultural soils have influenced ancient and modern civilizations. It is estimated that 20% of the irrigated land in the world is presently affected by salinity (Yeo, 1999). Identification of beneficial traits and their use in agriculture and applied sciences based on discovery and use of novel genes has been the key element in meeting the target of food security and sustainability of the food production. With the advent of new biotechnology tools and technique, it has been possible to access genes from diverse biological systems and deploy them in target species. Use of "crystal protein" gene 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 (Jenkins *et al.*, 1991). Therefore, it is imperative to explore the elite and

meticulous type of organism including microbes which are having important genes for enhancing the agricultural production.

Osmoregulation

Osmoregulation is the active regulation of the osmotic pressure of an organism's fluids to maintain the homeostasis of the organism's water content to keep the organism's fluids from becoming too diluted or too concentrated (Solomon *et al.*, 2002). During the course of time osmoconfirmation has evolved a fundamental phenomenon exhibited by bacteria, to uphold the osmotic balance between cellular fluids and external environment (Wood, 2011). With the pace of time microorganisms have continue to develop a complex stress tolerance system to survive with the changes in their external environment. As a result of alteration to their environment, many extremophilic microorganisms have evolved unique properties of considerable biotechnological and commercial significance. Halophilic or halotolerant eubacteria are characterized by a much greater metabolic diversity (Margesin and Schinner, 2001). As their intracellular salt concentration is low, they maintain an osmotic balance of their cytoplasm with the external medium by accumulating high concentrations of various organic osmotic solutes.

Many marine organisms are slight halophiles (with 3% w/v NaCl in sea water). Moderate halophiles optimally grow at 3–15% w/v NaCl; extreme halophiles at 25% w/v NaCl (halobacteria and halococci) and borderline extreme halophiles require at least 12% w/v salt (Kushner and Kamekura, 1988). Behera *et al.* (2012) identified and characterized bacteria *Staphylococcus epidermidis*, (P-30) and tested its survivability in response to varying concentration of NaCl along with *Bacillus cereus*, and it was observed that they could survive up to 20% salt concentration (Behera *et al.*, 2013a, b) (Gene Bank accession #JZ198969-JZ199140), and that was further confirmed with 16 s rRNA and whole genome transcriptome profiling is performed to unwound the real of questionable remarks. Similarly, Behera *et al.* (2014a) screened the bacterial isolates collected from east coast of India and suggested that most of them belong to Firmicutes and Proteobacteria group which might be of great interest from prospecting novel and candidate salt stress tolerant genes (Behera *et al.*, 2014a). Recently, Behera *et al.* (2014b) has reported a bacteria *Halomonas salina* strain CIFRI1 that could survive up to 20% of salt concentration which once again depicted the osmoregulatory survival in face of extreme salt concentration as function of either evolutionary progression or direct nuclear level modification in bacterial genome due to salt stress tolerance genes.

Compatible solutes

Compatible solutes are osmotically active, low molecular weight substances, highly water-soluble sugars or sugar alcohols, other alcohols, amino acids, or their derivatives that make halophilic bacteria versatile in their adaptation to salinity (Ventosa *et al.*, 1998). Compatible solutes keep away the cells from plasmolysis during adverse salt stress arbitrated environmental conditions (Kempf and Bremer, 1998). Compatible solutes exert their effect through changes in solvent structure and/or subtle changes in the dynamic properties of the protein rather than by changing the structure of the protein itself. Compatible solutes have biotechnological applications as stabilizers of biomolecules *i.e.* enzymes, DNA, membranes and whole cells, salt antagonists, stress-protective agents, increase freshness

of foods by stabilizing components and induction of osmolytes in cells can increase protein folding and thereby improve salt tolerance which could be useful in agriculture and xeriscaping (Roberts, 2005; Detkova and Boltyanskaya, 2007).

Ectoine

One of the most abundant osmolytes in nature is ectoine which are common in aerobic heterotrophic Eubacteria (Galinski, 1995). It was first discovered in the extremely halophilic phototrophic sulfobacterium *Ectothiorhodospira halochloris* (Galinski *et al.,* 1985) but later a great variety of halophilic and halotolerant bacteria were found to produce this compound, often together with its 5-hydroxy derivative (Rothschild and Mancinelli, 2001). For instance, *Halomonas elongata*, an extremely halotolerant bacteria had been reported to produce ectoine and hydroxyectoines whose relative proportion depends on salinity and temperature (Margesin and Schinner, 2001). Ectoine synthesis is carried out by the products of three genes: *ectABC* (Ofer *et al.,* 2012) (Fig. 1 and 2). The *ectA* gene codes for diaminobutyric acid acetyltransferase; *ectB* codes for the diaminobutyric acid aminotransferase and *ectC* codes for ectoine synthase (Roberts, 2005).

Ectoines as well as other compatible solutes have been found to improve protein folding and to protect biomolecules such as enzymes, nucleic acids, antibodies and even whole cells against heating, freeze-thawing, drying or chemical treatment (Barth *et al.,* 2000). Industrial and general application of ectoine keeps on increasing day by day: used in dermopharmacy as anti-ageing agents in skin creams, inhibits aggregation of Alzheimer's disease and recently, a clinical trial was initiated to test its efficacy in inhalations against bronchial asthma (Oren, 2010). Ectoines also find applications in the treatment of the mucous membranes of the eye. Ophthalmologic preparations containing these molecules are useful for eye treatment to decrease the dryness syndrome. Moreover, (Detkova and Boltyanskaya, 2007) reported that ectoine is used as components of shampoo, for oral care and as adjuvants for vaccines.

Trehalose

Trehalose, is a non-reducing glucose disaccharide synthesized by *otsA* and *otsB gene*, occurs in a wide variety of organisms, from bacteria and archaea to fungi, plants and invertebrates (Elbein *et al.,* 2003). Trehalose was found to constitute the shells that are secreted by various insects positioned on tree leaves in the Middle East (Richards *et al.,* 2002).

Trehalose plays a crucial role in metabolic homeostasis and abiotic stress tolerance in various organisms (Turan *et al.,* 2012). Trehalose also presents in several common foodstuffs such as bread, wine, beer, vinegar, and honey. Trehalose has several unique properties as a stress metabolite which includes high hydrophilicity, chemical stability, nonhygroscopic glass formation and no internal hydrogen bond formation. In prokaryotes like bacteria trehalose can be used as an external carbon source as it is frequently used as a compatible solute to compete with osmotic stress (Arguelles, 2000). Moreover, trehalose is not only useful as a cryoprotectant for the freeze–drying of biomolecules, but also for long-term conservation of microorganisms, as the membrane structure is preserved in the presence of this disaccharide (Empadinhas and da Costa, 2008) The biosynthetic pathway of trehalose in *Escherichia coli* is represented in Figure 3.

Glycine betaine

Choline oxidase is an important enzyme which accumulates to high levels in the cytoplasm of cells to prohibit dehydration and plasmolysis in adverse hyperosmotic environments (Kempf and Bremer, 1998; Wani *et al.,* 2013). Glycine betaine (N, N, N-trimethyl glycine) is a quaternary ammonium compound found in bacteria, haemophilic archaebacteria, marine invertebrates, plants and mammals synthesized by choline oxidase (Chen and Murata, 2008; Turan *et al.,* 2012). First gene cluster encoding a primary ABC-type transporter for the compatible solute glycine betaine in *Methanosarcina,* a methanogens species has reported (Roessler, 2002). Corollary, Nε-acetyl-β-lysine might be synthesized from lysine by the action of two enzymes, a lysine-2, 3-aminomutase (*ablA)* and a lysine acetyl transferase (*ablB)* (Robertson, 1992) (Fig. 4). Two secondary carriers for the uptake of glycine betaine Na-coupled system which has high-affinity coded by the gene *BetP* and *EctP* which prefers ectoine to glycine betaine have been reported (Peter, 1998; Boscari *et al.,* 2002).

Moreover, different glycine betaine transporter identified in various microorganism, has been reported (Boscari *et al.,* 2002) for instance *BetP* and *EctP* are closely related to glycine betaine transporter OpuD from *B. subtilis*; the choline transporter BetT and the carnitine transporter CaiT from *E. coli*; the glycine betaine transporter BetL from *Listeria monocytogenes* (Sleator and Hill, 2002), and the putative BetP proteins from *Mycobacterium tuberculosis* (Philipp *et al.,* 1996) and from *Haemophilus influenzae* (Fleischmann *et al.,* 1995).

Na^+ and H^+ antiporters

Na^+ and H^+ ions are most commonly involved in cell functioning whose extreme high or low concentrations inhibit the physiological activities of cells (Padan *et al.,* 2001). Different mechanisms are adopted by the cells for maintaining the homeotic balance which includes enhancement of K^+ uptake, elimination of surplus Na^+, re-allocation of Na^+ into other intracellular compartments (such as vacuoles), and biosynthesis of compatible solutes in the cytoplasm to maintain osmotic equilibrium. In prokaryotes, there are five classes of Na^+/H^+ antiporters such as NhaA, NhaB, NhaC, NhaD, and NapA (Krulwich *et al.,* 2009). Among all of them NhaA is the most extensively studied Na^+/H^+ antiporter in both the plasma lemma and tonoplast of *E. coli* which plays a major role in maintaining cell pH and Na^+ homeostasis (Volkmar *et al.,* 1998).

Production of extracellular protease

Proteases constitute one of the most important groups of industrial enzymes with versatile applications including meat tenderization, detergents, cheese-making, de-hairing, baking, waste management and silver recovery (Akcan and Uyar, 2011). Recently, there has been an increased interest in proteases as targets for developing therapeutic agents (Maryanoff, 2004). According to the market research report on world enzymes published in 2007, the world market for enzymes is expected to grow 7.6% per year to $6 billion in 2011 (David *et al.,* 2009). Microbial proteases account for approximately 60% of the total enzyme sales in the world (Banik and Prakash, 2004). One of the several challenges faced by industrial application of microbial proteases is optimal activity and stability in a wide range of salinity. Moreover, halophilic proteases are less suitable for saline fermentation processes, because they need at least 12.5% (w/v) NaCl for expression of high activities (Ventosa *et*

al., 1998). However, halotolerant proteases are active at both low and high concentrations of NaCl. Inherent capability of halotolerant bacteria to grow over an extended range of salt concentrations (3–30% NaCl, w/v) put forward them as candidate for bio-prospecting than their halophilic counterparts, as they need at least 12.5% (w/v) NaCl for expression of high activities (Ventosa *et al.,* 1998). Moreover, marine halotolerant microorganisms show wider distribution, distinct physiological characteristics and nutrient utilization as compared to their terrestrial counterparts and obligate halophiles (Barindra *et al.,* 2006). Most of the Gram-positive or Gram-variable, endospore forming rods with halotolerant properties has been assigned to the genus *Bacillus* (Yoon *et al.,* 2003). *Bacillus* sp. grows in a pH range of 7.0–11.0 and produces extracellular protease and alkaline proteases (Romero, 2007). With this in view, the present investigation treats halotolerant bacteria as a potential source of enzymes (Table 1).

ABC transport cycle through ATP-binding cassette dimer

ABC transporters are transmembrane proteins with representatives in all extant phyla from prokaryotes to humans (Ponte-Sucre, 2009), that utilize the energy of adenosine triphosphate (ATP) hydrolysis to carry out certain biological processes including translocation of various substrates across membranes and non-transport-related processes such as translation of RNA and DNA repair (Davidson *et al.,* 2008). In last decade, researcher have discovered various sub-family of ABC transporters in bacteria, for instance, Boos and Lucht (1996) reported periplasmic maltose binding protein (MBP) which constitutes maltose transport system to mediate nutrient uptake by binding to nutrients with high affinity prior to translocation. Moreover, Chen *et al.* (2001) also ascertained MBP which stimulates the ATPase activity of the membrane-associated porter through transmembrane subunits viz. MalF and MalG and two copies of the ATP binding subunit MalK.

Agricultural significance of salt stress tolerant bacterial genes in salt stress mitigation

Currently, more than 800 million hectares of land throughout the world are affected by levels of salt that could substantially reduce crop productivity (Munns and Tester, 2008). Strategies for alleviation of salt stress involve developing salt-resistant cultivars, leaching excess soluble salts from upper to lower soil depths, flushing soils that contain soil crusts at the surface, reducing salt by harvesting salt-accumulating aerial plant parts in areas with negligible irrigation water or rainfall for leaching, and amelioration of saline soils under cropping and leaching (Bacilio *et al.,* 2004). An alternative is to alleviate salt stress by inoculating crop seeds and seedlings with plant growth promoting bacteria (PGPB). Beneficial effect of PGPB under salinity has been related to hydraulic conductance, osmolyte accumulation, sequestering toxic Na+ ions, maintaining higher stomatal conductance and photosynthetic activities (Dodd and Perez-Alfocea, 2012). Several studies have been accomplished to improve salt tolerance by introducing salt resistant bacterial gene in agriculturally important crop that has been summaries in Table 3 and agriculturally important bacteria have been enumerated in Table 2.

Table.1 Proteases secreted by bacteria

Sr no.	Source species/ strain of bacteria	Nature of source bacteria /proteases secreted	Conducive medium/ salt concentration	Optimum pH and temperature	References
1.	*Bacillus aquimaris* strain VITP4	Halotolerant / Extra cellular	Basal Zobell medium/ 0–4 M	7.5 and 37 °C	Shivanand and Jayaraman, 2009
2.	*Virgibacillus* Dokdonesis Vitp14	Halotolerant/ Extra cellular	5 mM CTAB /1.5 M; NaCl, CaCl2, MgCl2, CuSO4 enhanced the activity	7.0 and 40°C.	Rajeswari *et al.*, 2012
3.	*Bacillus* sp.	Alkalophilic/ Alkaline protease	High alkaline conditions	11 and 60 °C.	Genckal and Tari, 2006
4.	*Bacillus subtilis* AP-MSU 6	Moderately halophilic/alkaline serine protease	Cu^{2+}, Hg^{2+}, Mn^{2+} and Ba^{2} / 0.5 M	9.0 and 40 °C	Maruthiah *et al.*, 2013
5.	*Sinorhizobium* sp. strain BL3	Halophilic / ATPase,		100 mM	
6.	*Pseudoalteromonas ruthenica*	Moderately Halophilic/ Haloprotease CPI	3 to 15% NaCl	Alkaline pH	Sa´nchez-Porro *et al.*, 2009
7.	*Bacillus clausii* I-52	Halotolerant/ Oxidant and SDS-stable alkaline protease	0.4% (w/v) NaCl	11.0 and 45 °C	Joo and Chang, 2005
8.	*Bacillus* sp. HS-4	Halophilic/ Extracellular Alkaline Protease	Ca2+ and Mg2+ ions	8 and 37°C	Shama and Hameed, 2011
9.	*Bacillus halodurans* CAS6	Haloalkaline / Extracellular Alkaline Protease	30% NaCl	9.0 and 50 °C	Annamalai *et al.*, 2013
10.	*Bacillus horikoshii*	Haloalkaline /alkaline protease	2% maltose, 1% sodium citrate, 0.8% NaCl, and 0.6% sodium carbonate to the culturing medium	9 and 37° C	Joo and Choi, 2012
11.	*Salinivibrio* sp. strain AF-2004	Moderately halophilic/ extracellular haloalkaline protease	0–0.5 M NaCl;	8.5 and 65 °C,	Karbalaei-Heidari *et al.*, 2007
12.	*Salinivibrio* sp. strain AF-2004	Moderately halophilic/	7.5 to 10% (w/v) sodium sulfate or 3% (w/v)	9.0 and 32 °C	Amoozegar *et al.*, 2007

		extracellular alkaline metalloprotease	sodium acetate (4.6 U ml^{-1})		
13.	*Halobacillus karajensis* strain MA-2.	Moderately Halophilic/ extracellular protease	55% and 50% NaCl activity and gelatin	8.0-8.5 and 34 °C	Karbalaei-Heidari *et al.*, 2009
14.	*Bacillus subtilis* RSKK96	Extremely halotolerant /extracellular alkaline protease	Salts FeSO4.7H2O and MgSO4.7H2O was found to increase protease production	9.0	Akcan and Uyar, 2011
15.	*Bacillus subtilis* RSKK96	Extremely halotolerant/ Alpha-Amylase	FeSO4, ZnSO4 and CuSO4 inhibited bacterial growth as a result, amylase production	37°C	Ackan and Uyar, 2011
16.	*Bacillus* sp.	Halotolerant / serine alkaline protease	Soybean meal	9.5 and 60°C	Saurabh *et al.*, 2007
17.	*Bacillus licheniformis* Shahed-07	Halotolerant/thermostable α-Amylase	0.5% tryptophan in production medium enhanced the enzyme productivity to two fold	7.5 and 70°C	Rasooli *et al.*, 2008
18	*Bacillus amyloliquefaciens* IIB-14	Haloloterant / alpha amylase	Maltose, glucose, lactose and soluble starch were supplemented as carbon sources.	7 and 70°C	Zar *et al.*, 2013

Fig.1 Biosynthetic pathway of ectoine in *Bacillus halodurans*. The genes ectB, ectA and ectC encode aminotransferase, acetyltransferase and ectoine synthase respectively

L-aspertate-semialdehyde

↓ EctB aminotransferase

L-2, 4- diaminobutyrate

↓ EctA acetyltransferase

N- acetyl -L- 2, 4 - diaminobutyrate

↓ EctC ectoine synthase

Ectoine

(2 –methyl – 1,4,5,6 – tetrahyddropyrimidine – 4 – carboxylic acid)

Table.2 Agriculturally important bacteria and affected crops

Sr no	Name of bacteria	Isolated medium	Mitigated crop	Effects	References
1.	*Hallobacillus* sp. SL3 and *Bacillus halodenitrificans* PU62	Saline habitats	Wheat seedlings	Enhance plant growth under saline stress through direct or indirect mechanisms	Ramadoss *et al.*, 2013
2.	*Brevibacterium epidermidis* RS15, *Micrococcus yunnanensis* RS222, and *Bacillus aryabhattai* RS341	Coastal Soil	Canola plants	Significant increase in root length and dry weight plants	Siddikee *et al.*, 2010
3.	*Escherichia coli.*		Indicia rice cultivar Kasalath	Enhanced stress tolerance in early stages	Prodhan *et al.*, 2008
4.	*Brevibacterium iodinum, Bacillus licheniformis* and *Zhihengliuela alba*	Coastal soil	Red pepper seedlings (Capsicum annuum L.)	Enhancement of growth and salt tolerance	Siddikee *et al.*, 2011
5.	*Rhizobia* and plant-growth-promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate deaminase	rhizosphere soil samples and nodules of mung bean	Mung bean	Efficient for improving seedling growth and nodulation	Ahmad *et al.*, 2011
6.	*Bacillus* and *Bacillus*-derived genera	Salt exposed rhizospheric soil	Wheat	Nitrogen fixation and improved salt stress tolerance	Upadhyay *et al.*, 2009
7.	Mannitol 1-phosphate dehydrogenase (*mtl*D) gene producing bacteria		Transgenic potato plants	Enhanced tolerance to NaCl stress	Rahnama *et al.*, 2011
8.	*Arthrobacter globiformis*		Tomato plant	Higher tolerance to salt stress during seed germination, and subsequent growth of young seedlings and increased water stress resistance	Goel *et al.*, 2011

Table.3 Salt stress tolerance genes from agriculturally imperative bacteria (halotolerant/ halophilic)

Sl. No.	Name of the Gene	Name of the bacteria	References
1.	*KatE*, HPT and NPTII	*Escherichia coli*	Prodhan *et al.*, 2008
2.	*BetS*	*Sinorhizobium meliloti*	Boscari *et al.*, 2002
3.	*katE*	*Escherichia coli* K12	Islam *et al.*, 2003
4.	*ectABC*	*Chromohalobacter salexigens*	Schubert *et al.*, 2007
5.	*ectABC*	*Bacillus halodurans*	Rajan *et al.*, 2008
6.	*codA*	*Arthrobacter globiformis* strain ATCC 8010	Fan *et al.*, 2004
7.	*ectABC*	*Chromohalobacter salexigens*	Calderon *et al.*, 2004
8.	*a-aminoisobutyric acid (AIB)*	*Vibrio costicola*	Kushner *et al.*, 1983
9.	*OpuC and OpuB*	*Listeria monocytogenes*	Fraser *et al.*, 2000
10.	*ablA*	*Methanosarcina mazei* Go¨1	Pfluger *et al.*, 2003
11.	*ectABC*	*Bacillus* species	Kuhlmann and Bremer, 2002
12.	*otsA and otsB*	*Escherichia coli*	Joseph *et al.*, 2010
13.	*PDH45*	*Eschericia coli* BL21cells	Tajrishi *et al.*, 2011
14.	*otsA/otsB, mpgS/mpgP*	*Thermus thermophilus*	Alarico *et al.*, 2005
15.	*BetT*	*Pseudomonas syringae*	Chen and Beattie, 2008
16.	*OtsBA and TreYZ*	*Escherichia coli*	Padilla *et al.*, 2004
17.	*proH, proJ and proA*	*Halobacillus halophilus*	Saum and Muller, 2007
18.	*acd*S	*Hallobacillus* sp. SL3 and *Bacillus halodenitrificans* PU62	Ramadoss *et al.*, 2013
19.	Mpgsmt-sdmt	*Halophilic Methanogen Methanohalophilus portucalensis*	Lai and Lai, 2011
20.	Bacterial mannitol 1-phosphate dehydrogenase (*mtl*D) gene	*mtl*D producing bacteria	Rahnama *et al.*, 2011
21.	codA	*Arthrobacter globiformis*	Goel *et al.*, 2011

Fig.2 The biosynthetic pathway of ectoine and hydroxyectoine

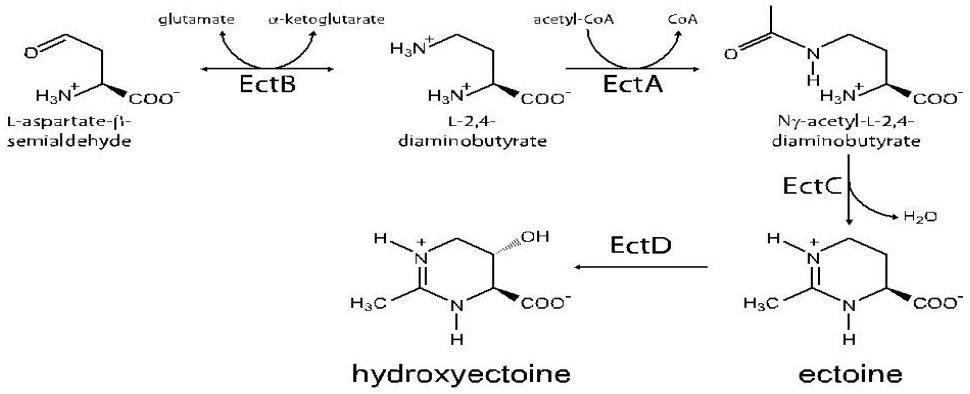


Fig.3 Biosynthetic pathway of trehalose in *Escherichia coli*

UDP-Glucose + Glucose – 6- Phosphate

OtsA

↓ Trehalose -6- phosphate synthase

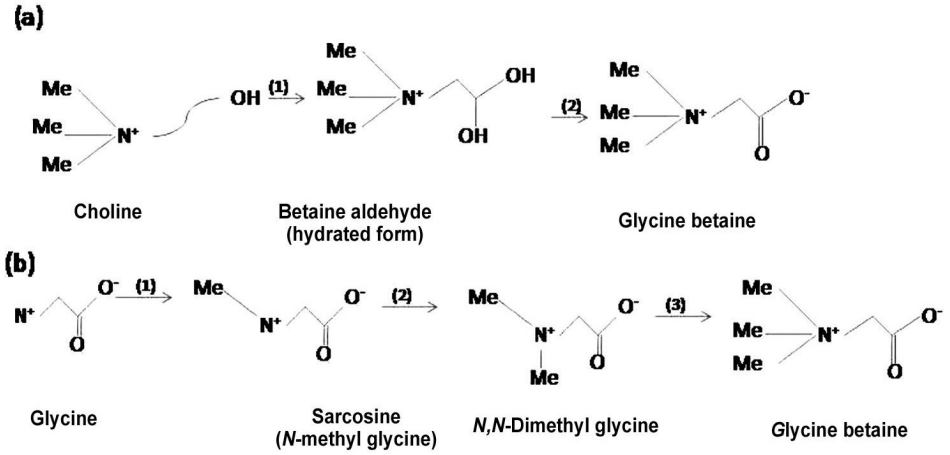
Trehalose -6- Phosphate

OtsB

↓ Trehalose – 6 – phosphate phosphatase

Trehalose

Fig.4 The two main pathways for the synthesis of Glycine Betaine



In changing climatic scenario it is must to pace with various arbitrate biotic variables as general and salinity in particular, for sustainable agricultural production. With this in view, researchers have emphasized upon prokaryotic microorganism, halophilic and halotolerant bacteria to study the signaling pathways and other mechanism. Different biochemical and enzymological studies have revealed that most significant compatible solute, ectoine synthesized from three enzymes (EctABC), is multigenic in nature that studied in selected microbes only. Hence, more comprehensive and advanced technology, whole geneome transcriptome profiling (Fleischmann *et al.*, 1995), studies with wider perspective is warranted to harness the beneficial properties of osmolytes produced from a larger group of novel halophilic/ halotolerant bacteria (Behera *et al.*, 2013a,b). However, in this context, some of recent studies showed that salt resistant bacteria could enhance the salinity tolerance in agricultural crop that later resulted in better acquired of genetic and morphological traits this might be happened due to multigenic nature of salt stress resistant bacterial genes. Although, most of the osmolytes producers are of haloterant group, the study on growth and enzyme production through these microbes has been less explored as

compared to halophilic counterpart. Therefore, it is significant to study the osmoadaptation and osmoprotection mechanism parallel to the molecular phylogeny, designing bioprocesses that improve growth conditions, and then positively influence the productivity of biomass, enzymes or metabolites (Chiara and Mario De, 2002; Behera *et al.,* 2014a, b). From the above review, it is concluded that there is enough scope for prospecting various abiotic stress tolerant genes with special emphasis to salt stress tolerant genes from different species in future which could be used for development of transgenic lines of plants for the agriculture productivity enhancement.

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