JJBS

Volume 12, Number 1,March 2019
ISSN 1995-6673
Pages 99 - 106

Jordan Journal of Biological Sciences

# The Role of the Overexpression of *Suaeda maritima* Choline Monooxygenase and Betaine Aldehyde Dehydrogenase *cDNAs* in the Enhancement of Salinity Tolerance in Different Strains of *E.coli*

Shrikanth Saraswathi Krishnamurthi[1,3*], Sindhu Kuttan[1], Sankararamasubramanian Meenakshisundram[1], Thajuddin Nooruddin[3*] and Ajay Parida[1, 2*]

[1]*Department of Molecular Biology and Biotechnology, M.S. Swaminathan Research Foundation, Taramani Institutional Area, Chennai 600 113;*

[2]*Institute of Life Science, Bhubaneswar 751 023;* [3]*Department of Microbiology, Bharathidasan University, Tiruchirapalli 620 024, India*

*Received May 17, 2018; Revised August 4, 2018; Accepted August 12, 2018*

**Abstract**

Heterologous expression of genes in to *Escherichia coli* helps establish the function of the encoded proteins in complex pathways of higher organisms. This methodology is particularly important in the case of plants where the whole genome sequence information is unavailable. Choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH) are two key enzymes of the glycine betaine biosynthetic pathway in *Suaeda maritima,* a halophyte found growing in the Pichavaram mangroves in Tamil Nadu. The present research is conducted to study the function of *SmCMO* and *SmBADH cDNAs* involved in glycine betaine biosynthetic pathway in *Suaeda maritima* in providing NaCl stress adaptability to *E. coli.* Three different *E. coli* strains namely DH5α, MC4100 and BL21 (DE3) are used for the study. Stability of pET32a*SmCMO+pET32aSmBADH* double recombinants and subsequent analyses for salinity tolerance in each of the strains were performed using *pET32a* protein expression vector. BL21 (DE3) double recombinants showed the maximum level of NaCl tolerance in both Minimal and LB media when compared with that of the other two strains as well as with non-recombinant BL21 (DE3) cells. This study enabled the functional characterization of *S. maritima* glycine betaine pathway genes as well as the additive effect of the *cDNAs* in conferring NaCl tolerance.

**Keywords**: Salinity, CMO, BADH, Glycine betaine, Osmoprotectants, *E. coli.*

## 1. Introduction

Living organisms, whether single-celled bacteria or eukaryotic multi-cellular plants and animals, have evolved various mechanisms to cope with extreme environmental conditions. Mangroves represent those plant communities that have developed morphological as well as physiological mechanisms to withstand harsh environmental conditions. They survive under high salinity, light and temperature, as well as heavy metal polluted soils that are considered unsuitable for the survival of other plants (Cheeseman *et al.* 1997). Previous studies have shown that genes isolated from mangroves and mangrove associated halophytes that were transferred to crop plants have been effective in conferring tolerance to abiotic stresses in crop plants (Rathinasabapathi, 2000; Ashraf and Foolad, 2007; Prashanth *et al.* 2008, Yamanaka *et al.* 2009).

*Suaeda maritima* is herbaceous, succulent, facultative annual halophyte which is native to saline soils of arid and semiarid regions exhibiting a wide range of stress adaptability, and can serve as a potential model for studying the oxidative-stress response in mangroves (Jithesh *et al.* 2006). *S. maritima* is a salt accumulator, and is known to accumulate osmolytes such as proline and glycine betaine (GB) (Moghaeib *et al.* 2004) that helps maintain osmotic equilibrium within the cells and with the external environment. Other species that accumulate GB include halophytic plants such as *Suaeda fruticosa* (Khan *et al.* 2000), *Suaeda aralocaspica, Bienertia sinuspersici* (Park *et al.* 2009) *Salicornia dolichostachya* (Katschnig *et al.* 2013), *Inula crithmoides (*Domenech *et al.* 2016) and halotolerant microbes such as *Ectothiorhodospira halochloris* (Peters and Truper, 1992), *Actinopolyspora halophilia (*Nyyssola *et al.* 2000*), Halomonas elongata* (David *et al.* 2000), *Aphanothece halophytica* (Waditee *et al.* 2003), and *Vibrio sp.* (Yancey *et al.* 1982; Dagmar *et al.* 2005).

Glycine betaine (GB) is also synthesized by most bacteria (Le Rudulier *et al.* 1984), cyanobacteria (Reed *et al.* 1986) and plants (Storey and Jones, 1977; Rhodes and Hanson, 1993). GB acts as a non-toxic cytoplasmic osmolyte, and hence plays a significant role in stress

* Corresponding author. e-mail: ajaydirector18@gmail.com; nthaju2002@yahoo.com; shrimicro3@gmail.com.

adaptation in plants (McNeil *et al.* 1999; Chen and Murata, 2011). Among higher plants, GB is synthesized from choline in a two-step oxidation reaction catalyzed first by a ferredoxin (Fd)-dependent choline monooxygenase (CMO) in to betaine aldehyde (Brouquisse *et al.*1989), followed by a $NAD^+$-dependent betaine aldehyde dehydrogenase (BADH) to give rise to GB (Weigel *et al.* 1986; Sakamoto and Murata, 2002). GB accumulates in the cytoplasm of plants, where it provides osmotic adjustment (Shabir *et al.* 2013).

Under osmotic stress, most bacteria accumulate organic solutes together with $K^+$ in their cytoplasm to build up the internal osmotic strength and thereby prevent osmotic dehydration of the cells (Epstein, 1986). *E. coli* display high versatility in the synthesis and uptake of osmoprotectants depending on the classes of compounds present in the growth medium. Betaines such as GB, proline betaine (stachydrine) and λ-butyrobetaine and other osmolytes such as proline, trehalose and glutamic acid when added in low concentrations stimulate the growth of *E. coli* and other enteric bacteria (Larsen *et al.* 1987; Incharoensakdi *et al.* 2000).

In *E. coli*, four genes, *betA*, *betB*, *betI* and *betT* have been associated with GB synthesis and accumulation, subsequently conferring osmotolerance (ability to grow in the presence of ≥ 0.5 M NaCl) when choline is supplied to the growth medium. *BetA* codes for choline dehydrogenase (CDH), *betB* codes for betaine aldehyde dehydrogenase (BADH), *betI* codes for a putative regulatory protein, and *betT* codes for a high affinity choline transporter (Strom *et al.* 1986).The *E. coli* CDH is a membrane bound, oxygen-dependent flavoprotein independent of soluble cofactors, and contains an N-terminal FAD-binding region (Lamark *et al.* 1991). CDH also catalyzes the oxidation of betaine aldehyde to GB *in vitro*, as it has a lower affinity for betaine aldehyde than betaine aldehyde dehydrogenase (Lamark *et al.* 1992). Due to the $O_2$ requirement of CDH, *E. coli* can utilize choline only under aerobic growth conditions. The *E. coli* BADH is a soluble enzyme with a high affinity for betaine aldehyde, and has a strong preference for $NAD^+$ as electron acceptor (Boyd *et al.* 1991). The *betT* gene is a choline transporter located upstream to the operon and the entire *bet* gene cluster is regulated by the presence of oxygen, choline, and the occurrence of osmotic stress (Andresen *et al.* 1988; Lamark *et al.* 1996).

The present study is conducted to understand the roles if any, of CMO and BADH proteins of *S. maritima* in providing NaCl stress adaptability to *E. coli* in which the *cDNAs* were introduced. As mentioned earlier, *E. coli* itself is a GB accumulator, and can tolerate high salt concentrations. However, there is a difference between plants and bacteria with respect to the synthesis of GB in the first of the two catalytic steps and in the kinetics of the two principal enzymes involved. Therefore, introducing heterologous genes into *E. coli* necessitates a thorough study of its growth responses in the presence of NaCl at different concentrations and also with respect to differences in genotypes of the strains. The present study intends to first screen the NaCl tolerance of three strains DH5α, MC4100 and BL21(DE3) and assay for the stability or successful retention of plasmids in double recombinants (transformation of *pET32a+SmCMO* as well as *pET32a+ SmBADH* in the same cell).

## 2. Materials and Methods

### *2.1. Bacterial Strains*

*E.coli* BL21(DE3), DH5α and MC4100 bacterial strains are used for this study and the genotypes of strains are listed (Supplementary Table 1). The bacterial strains were grown overnight in Luria-Bertani (LB) broth, and were then plated in LB agar. For checking the viability of the strains, they were maintained in both Minimal Medium 63 and LB medium with different concentration of NaCl.

**Table 1.** Growth response of different *E. coli* strains in the presence of NaCl in M63 Medium.

| Strains of *E. coli* | | Control w/ o NaCl | 0.1M NaCl | 0.25M NaCl | 0.5M NaCl | 0.75M NaCl | 1.0M NaCl |
|---|---|---|---|---|---|---|---|
| BL21(DE3) | 1 | ++++ | ++++ | ++++ | ++++ | ---- | ---- |
| | 2 | ++++ | ++++ | ++++ | ++++ | ---- | ---- |
| | 3 | ++++ | ++++ | ++++ | ++++ | ---- | ---- |
| | 4 | ++++ | ++++ | ++++ | ++++ | ---- | ---- |
| DH5α | 1 | ++-- | ++-- | +--- | ---- | ---- | ---- |
| | 2 | ++-- | +--- | +--- | ---- | ---- | ---- |
| | 3 | ++-- | ++-- | +--- | ---- | ---- | ---- |
| | 4 | ++-- | ++-- | +--- | ---- | ---- | ---- |
| MC4100 | 1 | ++++ | ++++ | ++++ | ++-- | ---- | ---- |
| | 2 | ++++ | ++++ | ++++ | +--- | ---- | ---- |
| | 3 | ++++ | ++++ | ++++ | ++-- | ---- | ---- |
| | 4 | ++++ | ++++ | ++++ | +--- | ---- | ---- |

Representation of ++++ indicates very good growth; +++- indicates good growth; ++-- indicates moderate growth; +--- indicates poor growth; ---- indicates no growth.

### *2.2. Growth Kinetics*

Ten mL of each LB was inoculated with a loopful of each of the strains and was incubated at 37°C at 180 rpm for twelve to sixteen hours. 2.5 mL aliquots from each of the overnight grown cultures were then transferred to 250mL flasks containing 100mL of pre-warmed LB. At approximately fifteen-minute intervals starting from the time of inoculation, 1mL each of the samples were aseptically transferred, and their absorbance at 600nm ($A_{600}$) was recorded using a Lambda 3B spectrophotometer. When the growth reached the exponential phase (at ~20 minutes), 2mL each of the samples was taken at thirty-minute intervals. One mL was used for plating, and the remaining 1mL was used for recording the absorbance. Serial dilutions of each of the samples were then prepared and 0.1mL from each of the $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions was plated onto LB-agar plates and incubated at 37°C.

### *2.3. Cloning of SmCMO and SmBADH ORFs into E. coli Protein Expression Vector*

The full-length cDNA sequences of CMO and BADH (NCBI accession nos. JX629239 and JX629240 respectively) previously isolated by RT-PCR and cloned in T/A cloning vector, were analyzed *insilico* using *webcutter2*.0. Primers with restriction sites were designed

based on the web cutter analyses and the MCS of the cloning vector *pET32a* (Amp$^+$) (Novagen Inc., Germany). The ORF of CMO and BADH were amplified using primers with *Bam H1* and *Xho1* overhangs, digested with *BamH1/Xho1* restriction sites (Supplementary Table 2) and cloned onto *pET32a* at the same sites. The clones were named *pET32a+SmCMO* and *pET32a+SmBADH* as 1.3kb and 1.5 kb respectively and 1 Kb Marker was used.

**Table 2.** Growth response of different *E. coli* strains in the presence of NaCl in LB Medium.

| Strains of *E. coli* | | Control (W/O NaCl) | 0.1M NaCl | 0.25M NaCl | 0.5M NaCl | 0.75M NaCl | 1.0M NaCl |
|---|---|---|---|---|---|---|---|
| BL21(DE3) | 1 | ++++ | ++++ | ++++ | ++++ | ++-- | ---- |
| | 2 | ++++ | ++++ | ++++ | +++- | +--- | ---- |
| | 3 | ++++ | ++++ | ++++ | +++- | ++-- | ---- |
| | 4 | ++++ | ++++ | ++++ | +++- | +--- | ---- |
| DH5α | 1 | ++++ | ++++ | ++++ | +--- | +--- | ---- |
| | 2 | ++++ | ++++ | +++- | +--- | ---- | ---- |
| | 3 | ++++ | ++++ | +++- | ++-- | ---- | ---- |
| | 4 | ++++ | ++++ | ++++ | ---- | ---- | ---- |
| MC4100 | 1 | ++++ | ++++ | ++++ | ++-- | +--- | ---- |
| | 2 | ++++ | ++++ | ++++ | ++-- | +--- | ---- |
| | 3 | ++++ | ++++ | ++++ | ++-- | ---- | ---- |
| | 4 | ++++ | ++++ | ++++ | ++-- | ---- | ---- |

Representation of ++++ indicates very good growth; +++- indicates good growth; ++-- indicates moderate growth; +--- indicates poor growth; ---- indicates no growth

*2.4. Competent Cell Preparation*

A single *E. coli* colony of each of the BL21(DE3), DH5α and MC4100 cells was inoculated into 10mL each of LB liquid medium containing ampicillin (0.1 %), and was grown overnight at 37ºC at 180 rpm. One hundred (100) µL each of the overnight grown culture was then inoculated into 100mL LB liquid medium, and was incubated at 37ºC at 180 rpm for two to three hours until the absorbance reached 0.4. It was then kept on ice for fifteen to twenty minutes. Cells were then centrifuged at 5000 rpm for ten minutes at 4ºC. The supernatant was discarded, and the pellet was suspended in 1.5mL of freshly-prepared ice cold TSS. Two hundred (200) µL of competent cells were aliquoted in sterile microfuge tubes. The competent cells were frozen in liquid nitrogen and were stored at -80ºC until use (Chung *et al.* 1989).

*2.5. Transformation*

Two hundred (200) µL of BL21 (DE3), DH5α and MC4100 competent cells, 50ng each plasmid were added and incubated in ice for thirty minutes. Heat shock was given to the cells at 42ºC for ninety seconds and they were immediately kept in ice for five minutes. Eight hundred (800) µL of LB liquid medium was added and kept at 37ºC at 180 rpm for one hour. One hundred (100) µL of the cells was spread plated on to LB agar containing ampicillin (0.1 %), and the plates were incubated at 37ºC overnight (Maniatis *et al.* 1989).

*2.6. Colony PCR*

The colonies from the plates were picked and colony PCR was performed. The PCR reaction was carried out under the following conditions (initial denaturation 94°C - 3 min; denaturation 94°C - 30 sec; annealing 62°C - 45 sec; extension 72°C - 90 sec for 30 cycles and final extension 72°C - 7 min).The products were visualized on a 0.8 % agarose gel, and were observed under UV. The positive colony obtained as a result of colony-PCR was transformed to specific selective competent cells, and were then plated on ampicillin amended plates.

*2.7. Effect of NaCl on the growth of BL21 Transformants*

Overnight grown cultures of BL21 (DE3) transformed with *pET32aSmCMO, pET32aSmBADH* and *pET32aSmCMO+BADH* were plated onto LB agar containing different concentrations of NaCl (Control, 100mM, 200mM, 300mM, 400mM and 500mM). The media was also amended with 100µg/mL ampicillin, 1mM IPTG and 100µM choline chloride. BL21 cells transformed with *pET32a* without an insert served as the control. The plates were incubated at 37ºC overnight and were observed for colony formation.

*2.8. IPTG Induction*

The transformed BL21 (DE3) cells were inoculated into the 10mL LB containing ampicillin (100µg/mL media), and were incubated at 37ºC at 180 rpm overnight. From the overnight grown culture 1mL was added to a flask containing 10mL LB broth with ampicillin (100ug/mL media), and was kept at 37ºC at 180 rpm. When the absorbance reached 0.6, 1mL was taken in an microfuge tube as control. Nine (9) µL of 1M IPTG (1mM final concentration) and 0.5M NaCl concentration was added to the remaining culture, and it was incubated at 37ºC at 180 rpm. One mL culture was withdrawn every three hours. The cells collected at different intervals were then treated with SDS PAGE sample buffer, and were loaded onto 12 % SDS gel. The gel was run at constant 50V. The gel was removed from the glass plate and washed thrice with Milli-Q water. The gel was soaked in staining solution (mix well before use) for twelve hours/overnight in the gel-rocker. The staining solution was removed, and the gel was rinsed twice in Milli-Q water. It was allowed to destain until the background was clear (10-60 min). The bands were then visualized and documented.

## 3. Results and Discussion

*3.1. Growth Response of E. coli Strains*

*E. coli* is known to display innate mechanisms to cope with NaCl stress, including that of GB accumulation. In order to take into account the basal tolerance to NaCl, experiments using three strains of *E.coli* namely DH5α, MC4100 and BL21 (DE3) in nutrient-rich LB medium as well as Minimal medium were carried out in the presence of different concentrations of NaCl.

*3.2. Minimal Medium (M63) with Different NaCl Concentrations*

The evaluation of three different *E. coli* strains for growth in a Minimal medium containing different

concentrations of NaCl (Control, 0.1, 0.25, 0.5, 0.75, 1M) resulted in varying responses among the strains (Table 1). Interestingly, DH5α was not able to tolerate even 0.25M of NaCl in the growth medium. BL21 (DE3) cells were able to tolerate the maximum NaCl concentration (0.5M) and was the best growing strains among the three tested in the present study. MC4100 showed poor growth response at 0.5M NaCl.

### *3.3. Luria Bertanni Medium (LB) with Different NaCl Concentrations*

In the LB medium with different NaCl concentrations (Control, 0.1, 0.25, 0.5, 0.75, 1M), DH5α showed good growth up to 0.5M NaCl unlike in the M63 medium. MC4100 did not grow well in the LB medium as observed in the case of the M63 medium, while BL21 (DE3) could grow well at concentrations of NaCl up to 0.75M (Table 2).

### *3.4. E. coli BL21(DE3) showing maximum level of NaCl Tolerance*

The comparison of the growth response of *E. coli* strains MC4100, DH5α and BL21(DE3) revealed that BL 21(DE3), a widely used strain for protein expression studies grew well in the LB medium and Minimal medium. The same couldn't be said as DH5α and MC4100 showed preference for Minimal medium. Although BL21(DE3) responded well in terms of growth in both the LB as well as the Minimal media, its NaCl tolerance was observed to be better in the LB medium (Figures 1 and 2).

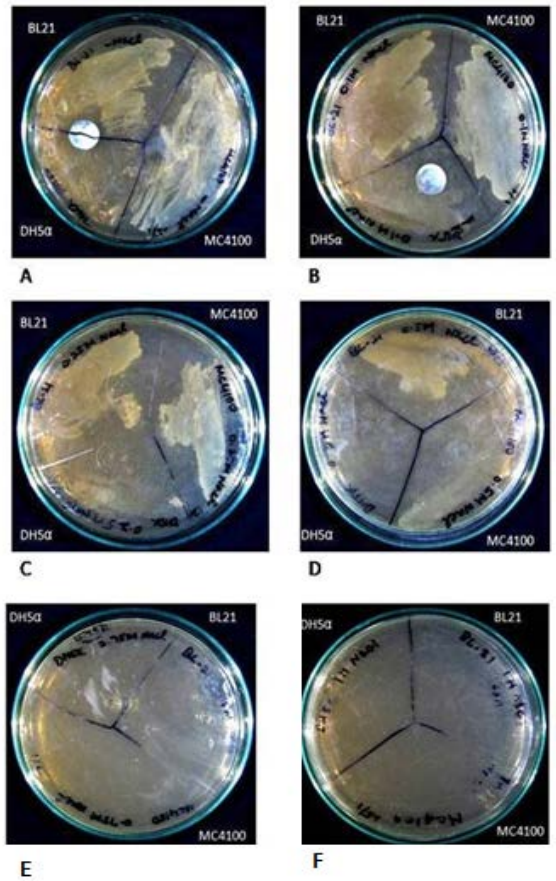



**Figure 1.** Growth response of *E. coli* strains DH5α, MC4100 and BL21(DE3) in M63 medium added with NaCl at different concentrations [A - Control (without NaCl); B in 0.1M; C in 0.25M; D in 0.5M; E in 0.75M; F in 1M].

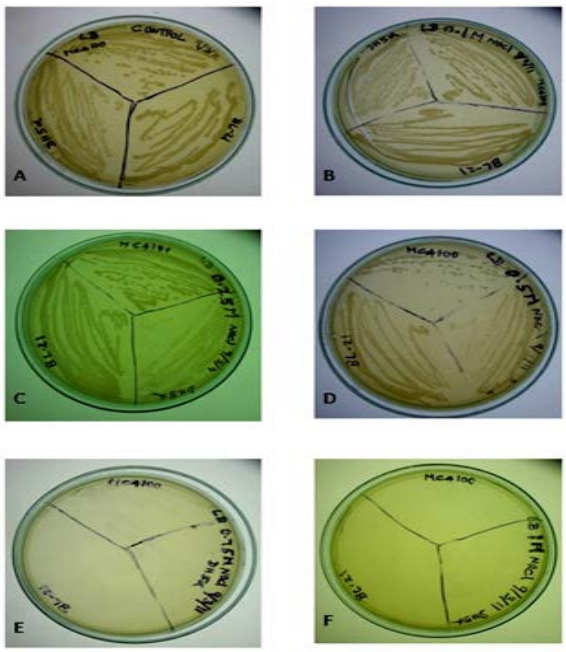



**Figure 2.** Growth response of E.coli strains DH5α, MC4100 and BL21(DE3) in LB medium added with NaCl at different concentrations [A - Control (without NaCl); B in 0.1M; C in 0.25M; D in 0.5M; E in 0.75M; F in 1M].

The difference in growth response could be attributed to genotype differences and the consequent mechanisms that help in achieving tolerance in these strains. It may also be noted that BL21 (DE3) cells are devoid of two proteases (Yadava *et al.* 2005), and such a genotype can possibly aid in protecting proteins from salt-induced misfolding and subsequent degradation (Paliy and Gunasekara, 2007).

Further screening of BL21 (DE3) for NaCl tolerance (Table 3) to narrow down the range of NaCl concentration revealed that the maximum level of tolerance that could be repeated consistently was 0.5M. Moderate to poor growth was observed at higher concentrations. Absorbance (at 600nm) of the culture was maximal at six hours (corresponding to the late log phase of growth- Supplementary Figure 1) at 0.7M NaCl after which the values declined (Figures 3 and 4).

**Table 3**. Growth response of *E. coli* strain BL 21(DE3) between 0.4M to 1M NaCl concentration at different time intervals in LB medium.

| **Strain of *E .coli*** | **NaCl Concentration (Molar)** | **0 hr** | **1 hr** | **3 hrs** | **6 hrs** |
|---|---|---|---|---|---|
| **BL21(DE3)** | Control (W/0 NaCl) | ++++ | ++++ | ++++ | ++++ |
| | 0.4 | +++- | ++++ | ++++ | ++++ |
| | 0.5 | +++- | +++- | +++- | +++- |
| | 0.6 | ++-- | ++-- | ++-- | ++-- |
| | 0.7 | ++-- | +--- | ++-- | ++-- |
| | 0.8 | ---- | ---- | ---- | ---- |
| | 0.9 | ---- | ---- | ---- | ---- |
| | 1.0 | ---- | ---- | ---- | ---- |

Representation of ++++ indicates very good growth; +++- indicates good growth; ++-- indicates moderate growth; +--- indicates poor growth; ---- indicates no growth.

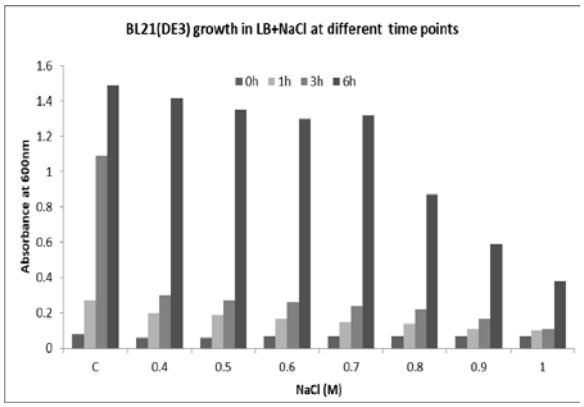



**Figure 3.** Growth response of *E. coli* strain BL21 (DE3) between 0.4M to 1M NaCl concentrations in LB medium.

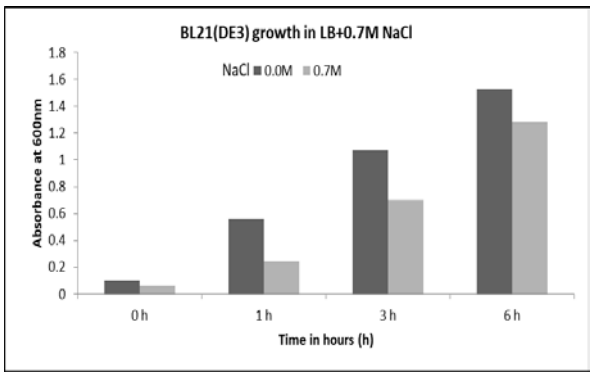
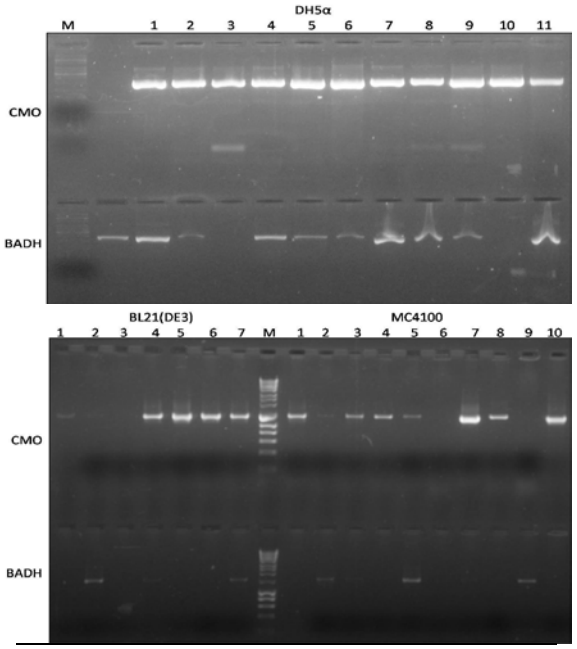



**Figure 4.** Growth of response of *E. coli* strain BL21 (DE3) at different time intervals in LB medium with 0.7M NaCl.

### *3.5. Evaluation of Plasmid Stability in the Three E. coli Strains*

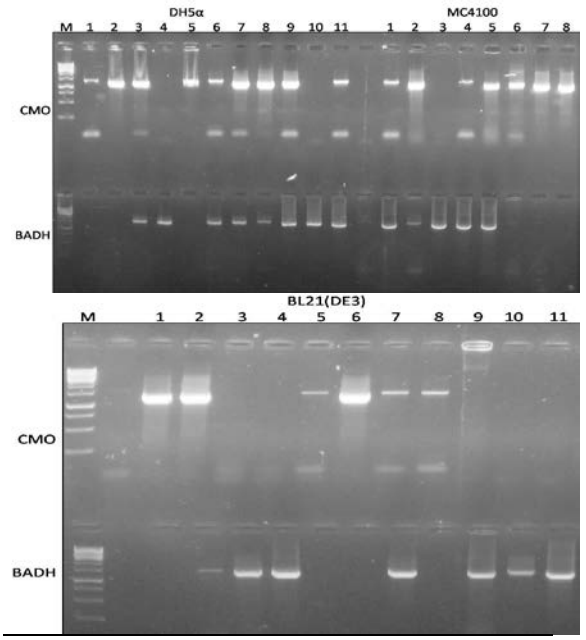
The ability of bacterial cells to take up plasmids could differ significantly based on media composition, pH, and temperature (Hanahan, 1983; Inoue *et al.* 1990). In the present study, two constructs (*pET32a+SmCMO* and *pET32a+SmBADH* referred to, henceforth, as p*SmCMO* and p*SmBADH* for simplicity) were used together at equimolar concentrations to transform the three tested *E. coli* strains and resulted in good transformation efficiency. The possibility of these two constructs co-existing and replicating in the same host was assayed by PCR using corresponding gene-specific primers.

From the results, it is interesting to note that DH5α was able to retain both the plasmids in maximum number of colonies than in BL21 (DE3) or MC4100. It is possible that this particular strain has a genotype which is more suited for the maintenance of plasmids. BL21 (DE3) and MC4100 were examined for the presence of double recombinants at various time points of growth. However, the PCR experiment for the detection of double recombinants at regular intervals during the growth phase did not result in the detection of bands, and hence they were not included in this report (data not shown). Transformation experiments were also repeated to confirm the study's observations. It was found that the initial results were consistently repeated. It is interesting to note that the overnight grown cultures or colonies that were subcultured as patches lost one of the plasmid constructs as compared to the early or mid-log phase fresh cultures, even in the presence of ampicillin selection in the growth medium (Figures 5 and 6). In order to avoid plasmid loss due to frequent sub-culturing experiments to assay for salt tolerance in BL21(DE3), double recombinants were performed only with fresh glycerol stocks of early log phase cultures stored at -80°C (Figure 7a and b).



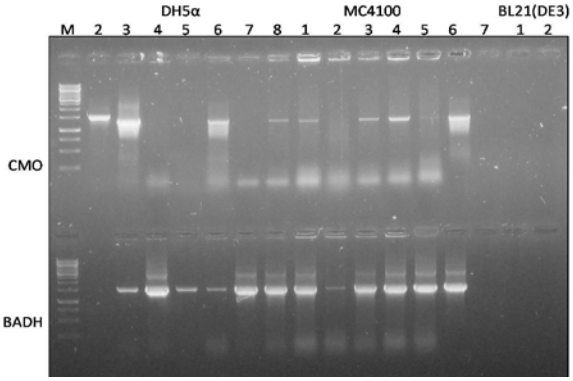

| Strains of *E.coli* | Positives (Lane Nos.) for CMO&BADH |
|---|---|
| BL21(DE3) | 2,4,7 |
| DH5α | 1,4,5,7,8,9 |
| MC4100 | 2,3,5,7, |

**Figure 5.** Identification of positive transformants (for both pET32a+ *SmCMO* and *pET32a+SmBADH*) using gene specific primers.

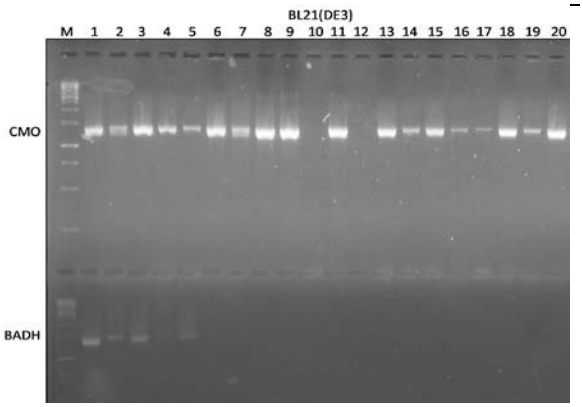



| Strains of *E. coli* | Positives (Lane Nos.) for CMO&BADH |
|---|---|
| BL21(DE3) | 7 |
| DH5α | 3,6,7,8,9 |
| MC4100 | 1,3,4,6,7 |

**Figure 6.** Identification of positive transformants (for both pET32a+*SmCMO* and pET32a+*SmBADH*) using gene specific primers - from overnight cultures from plates.




| Strains of *E. coli* | Positives (Lane Nos.) for CMO&BADH |
|---|---|
| BL21(DE3) | Nil |
| DH5α | 3,8 |
| MC4100 | 1,3,6,7 |




| Strains of *E. coli* | Positives (Lane Nos.) for CMO&BADH |
|---|---|
| BL21(DE3) | 1*,2*,3* |

**Figure 7.** Identification of positive transformants (for both ET32a+*SmCMO* and pET32a+*SmBADH*) using gene specific primers - Top gel performed using batch cultures **(a)**; Bottom panel performed using 3h culture taken from 20 fresh transformants (colonies).

*Positive cultures were maintained as glycerol stocks for further experiments **(b)**.

### *3.6. Sodium Chloride Tolerance of BL21 (DE3) Cells in the Presence of pSmCMO and pSmBADH*

Plasmid constructs for the both the cDNAs involved in GB biosynthetic pathway in *S. maritima* were transferred to competent BL21 (DE3) cells separately (*pSmCMO*, p*SmBADH*) as well as in combination (*pSmCMO+pSmBADH)*. Growth inhibition at higher concentrations of NaCl (0.7M) in IPTG-induced recombinant BL21 (DE3) was observed in the present study in comparison with the uninduced and non-recombinant cells (Figure 8). The results suggest a toxic effect of the recombinant proteins primarily due to high concentrations of NaCl. However at 0.5M NaCl, tolerance was better in the induced recombinants, both in isolation as well as in combination of the individual plasmids (Figure 9).

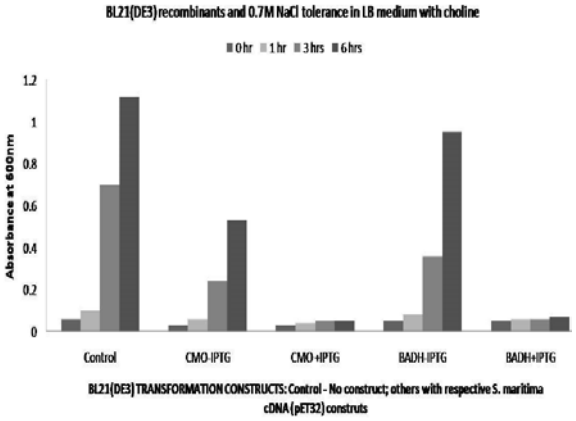



**Figure 8.** Growth response of BL21 (DE3) with different plasmid constructs in 0.7M NaCl containing LB medium supplemented with choline in the presence and absence of IPTG.

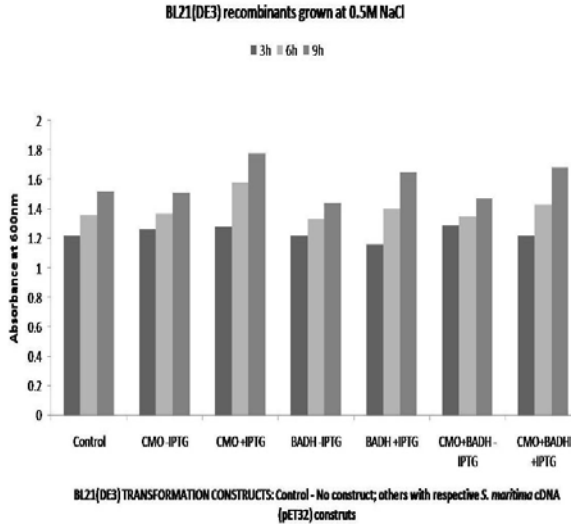



**Figure 9.** Growth response of BL21 (DE3) with different plasmid constructs in 0.5M NaCl containing LB medium in the presence and absence of IPTG**.**

SDS-PAGE of the proteins purified from BL21(DE3) recombinants containing p*SmCMO* and p*SmBADH* in isolation or in combination resulted in higher induction of the CMO polypeptide in case of the *pSmCMO* only recombinant, while the other recombinants were not induced at higher levels. *pSmCMO* expressing BL21(DE3) cells showed a better tolerance to NaCl at all the time points tested. This observation is consistent with earlier reports (Hibino *et al*. 2002) where *E. coli* BL21 cells transformed with spinach CMO accumulated more GB suggesting that the expressed protein was able to better utilize the externally supplied choline. Although in recombinants with both the constructs, BADH at about 80kDa was found to be expressed more, while it was not clear whether CMO was expressed or not, since a band was visible in this molecular weight range in all the protein samples (Figure 10), possibly because of its plasmid being lost or expressed inefficiently due to the less utilization of externally supplied choline. Interestingly, these results show that some of the host proteins were repressed in the induced recombinants.

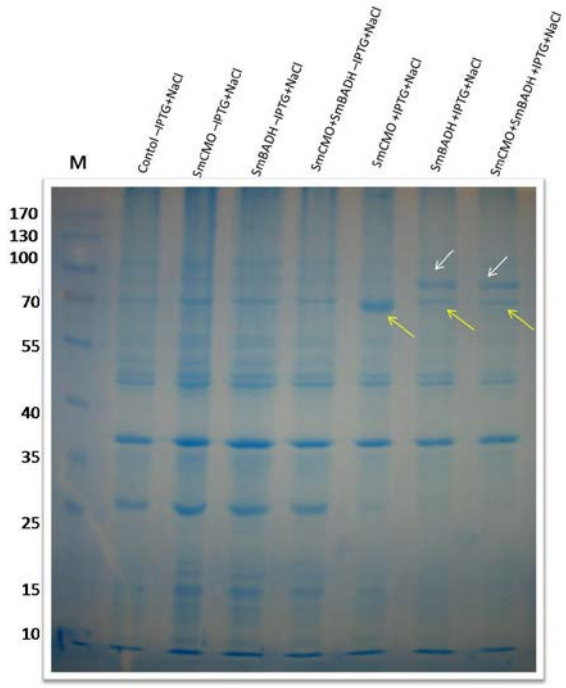



**Figure 10.** SDS PAGE of BL21(DE3) recombinants with different plasmid constructs in 0.5M NaCl containing LB medium in the presence and absence of IPTG. Control – non recombinant. Yellow arrow indicates *SmCMO* polypeptide; White arrow indicates *SmBADH*; M-marker.

## 4. Conclusion

The natural sodium chloride tolerance in *E. coli* is possibly determined by the genotype of the strains, as there is a difference in NaCl-tolerance levels between the DH5α and BL21 (DE3) cells in the LB media, with the difference being more marked in Minimal media. The stability or retention of the two plasmid in the same cell may also depend on the genotype. DH5α, which is generally used for plasmid maintenance, performed better in the present study. Growth of BL21 (DE3) recombinants for *pSmCMO and pSmBADH* proteins, both in isolation and in combination, was inhibited at 0.7M NaCl. This suggests the toxicity of the recombinant proteins when the cells are exposed to high concentrations of NaCl. Sodium chloride tolerance of induced recombinant BL21 (DE3) was better at 0.5M compared to the non-recombinant and uninduced recombinant controls. However, the difference was significant enough to conclusively suggest an additive effect of the cloned proteins from *S. maritima* GB biosynthetic pathway.

## Acknowledgements

The researchers extend their cordial thanks to Professor M.S. Swaminathan for providing facilities and for the constant support to carry out this research. Dr. N. Thajuddin expresses his appreciation for the UGC, New Delhi for BSR one-time grant (F.19-156/2015(BSR). Thanks also go to Ashok Kumar and Lakshmi Priya, MSSRF for giving suggestions and helping in the current research work.

## Conflict of Interest

The authors have no conflict of interest.

## References

Andresen PA, Kaasen I, Styrvold OB, Boulnois G and Strom AR. 1988. Molecular cloning, physical mapping and expression of the bet genes governing the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. *J. Gen. Microbiol.*, **134**:1737-1746.

Ashraf M and Foolad MR. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot.*, **59**:206-216.

Boyd LA, Adam L, Pelcher LE, McHugen A, Hirji R and Selvaraj G. 1991. Characterization of an *Escherichia coli* gene encoding betaine aldehyde dehydrogenase (*BADH*): structural similarity to mammalian *ALDHs* and a plant *BADH*. *Gene*,**103**: 45-52.

Brouquisse R, Weigel P, Rhodes D, Yocum CF and Hanson AD. 1989. Evidence for a ferredoxin-dependant choline monooxygenase from spinach chloroplast stroma. *Plant Physiol.***, 90**: 322-329.

Cheeseman JM, Herendeen LB, Cheeseman AT and Clough BF. 1997. Photosynthesis and photoprotection in mangroves under field conditions. *Plant Cell Environ.*, **20**:579-588.

Chen THH and Murata N. 2011. Glycinebetaine protects plants against abiotic stress: mechanisms and biotechnological applications. *Plant Cell Environ.*, **34**:1-20

Chung CT, Suzzanne LN and Roger HM. 1989. One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U S A*, **86**:2172-2175.

David C, Carmen V, Susanne K, Maria Jesus M, Antonio V, Erhard B and Joaquin JN. 2000. Genes for the synthesis of the osmoprotectant glycine betaine from choline in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. *Microbiology*, **146**:455-463.

Domenech LLP, Tifrea A, Grigore MN, Boscaiu M and Vicente O. 2016. Proline and glycine betaine accumulation in two succulent halophytes under natural and experimental conditions. *Plant Biosystems*, **150(5)**:904-915.

Epstein W. 1986. Osmoregulation by potassium transport in *Escherichia coli*. *FEMS Microbiol. Rev.*, **39**:73-78.

Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, **166**:557-580.

Hibino T, Waditee R, Araki E, Ishikawa H, Aoki K, Tanaka Y and Takabe T. 2002. Functional characterization of choline monooxygenase an enzyme for betaine synthesis in plants. *J. Biol. Chem.*, **277(44)**:41352-41360.

Incharoensakdi A, Nobuyuki M, Takashi H, Yu Ling M, Hiroshi I, Akira H, Tohru F, Tetsuko T and Teruhiro T. 2000. Overproduction of spinach betaine aldehyde dehydrogenase in *Escherichia coli:* structural and functional properties of wild-type, mutants and *E. coli* enzymes. *Eur. J. Biochem.* **267**:7015-7023.

Inoue H, Nojima H and Okayama H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, **96**:23-28.

Jithesh MN, Prashanth SR, Sivaprakash KR and Parida A. 2006. Antioxidative response mechanisms in halophytes: their role in stress defence. *J Genet*, **85(3):**237-254.

Kapfhammer D, Karatan E, Pflughoeft,KJ and Watnick PI. 2005. Role for glycine betaine transport in Vibrio cholerae osmoadaptation and biofilm formation within microbial communities. *Appl. Environ. Microbiol.*, **71(7):**3840-3847

Katschnig D, Broekman R and Rozema J. 2013. Salt tolerance in the halophyte *Salicornia dolichostachya* moss: growth, morphology and physiology. *Environ Exp Bot.,* **92**:32-42.

Khan MA, Ungar IA and Showalter AM. 2000. The effect of the salinity on the growth, water status, and ion content of a leaf succulent perennial halophyte, *Suaeda fruticosa (L)* Forssk. *J Arid Environ.*, **45**:73-84.

Lamark T, Kaasen I, Eshoo MW, Falkenberg P, McDougall J and Strom AR. 1991. DNA sequence and analysis of the bet genes encoding the osmoregulatory choline glycine betaine pathway of *Escherichia coli*. *Mol Microbiol.,* **5**:1049-1064.

Lamark T, Rokenes TP, McDougall J and Strom AR. 1996. The complex *bet* promoters of *Escherichia coli*: regulation by oxygen (*ArcA*), choline (*BetI*), and osmotic stress. *J Bacteriol.,* **178(6):**1655-1662.

Lamark T, Styrvold OB and Strom AR. 1992. Efflux of choline and glycine betaine from osmoregulation cells of *Escherichia coli*. *FEMS Microbiol. Lett.*, **96**:149-154.

Larsen I, Sydnes K, Landfald B, Strom AR. 1987. Osmoregulation in *Escherichia coli* by accumulation of organic osmolytes: betaines, glutamic acid, and trehalose. *Arch Microbiol.,* **147**: 1-7.

Le Rudulier D, Strom AR, Dandekar AM, Smith LT and Valentine RC. 1984. Molecular biology of osmoregulation. *Sci.,* **224**:1064-1068.

Maniatis T, Fritsch EF and Sambrook J. 1989**. Molecular Cloning: a Laboratory Manual**. Cold Spring Harbor Laboratory Press, *Cold Spring Harbor, New York*.

McNeil SD, Nuccio ML and Hanson AD. 1999. Betaines and related osmoprotectants. targets for metabolic engineering of stress resistance. *Plant Physiol.*,**120**:945-949.

Moghaieb REA, Saneoka H and Fujita K. 2004. Effect of salinity on osmotic adjustment, glycinebetaine accumulation and the betaine aldehyde dehydrogenase gene expression in two halophytic plants, *Salicornia europaea*and *Suaeda maritima*. *Plant Sci.,***166**:1345-1349.

Nyyssölä A, Kerovuo J, Kaukinen P, Von Weymarn N and Reinikainen T. 2000. Extreme halophiles synthesize betaine from glycine by methylation. *JBiol Chem.,* **275**:22196-22201

Paliy O and Gunasekara TS. 2007. Growth of *E. coli* BL21 in minimal media with different gluconeogenic carbon sources and salt contents. *Appl Microbiol Biotechnol.,* **73**:1169-1172.

Park J, Okita TW and Edwards GE. 2009. Salt tolerant mechanisms in single-cell C4 species *Bienertia sinuspersici* and *Suaeda aralocaspica* (Chenopodiaceae). *Plant Sci.,* **176**:616-626.

Peters P and Truper HG. 1992. Transport of glycine betaine in the extremely haloalkaliphilic sulphurbacterium *Ectothiorhodospira halochloris*. *J Gen Microbiol.,* **138**:1993-1998.

Prashanth SR, Sadhasivam V and Parida A. 2008. Over expression of cytosolic copper/zinc superoxide dismutase from a mangrove plant *Avicennia marina* in indica rice var *Pusa Basmati-1* confers abiotic stress tolerance. *Transgenic Res.,* **17(2)**:281-291.

Rathinasabapathi B. 2000. Metabolic engineering for stress tolerance: installing osmoprotectant synthesis pathways. *Ann. Bot.,* **86(4)**:709-716.

Reed RH, Borowitzka LJ, Mackay MA, Chudek JA, Foster R, Warr SRC, Moore DJ and Stewart WDP. 1986. Organic solute accumulation in osmotically stressed cyanobacteria. *FEMS Microbiol Rev.*, 2 (1-2):51-56,

Rhodes D and Hanson AD. 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Ann Rev Plant Physiol Plant Mol Biol.,* **44:**357-384.

Sakamoto A and Murata N. 2002. The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. *Plant Cell Environ.,* **25**:163-171.

Shabir HW, Naorem BS, Athokpam H and Javed IM. 2013. Compatible solute engineering in plants for abiotic stress tolerance - role of glycine betaine. *Curr. Genomics*, **14**:157-165.

Storey R and Jones RGW. 1977. Quaternary ammonium compounds in plants in relation to salt resistance. *Phytochem.,* **16**:447-453.

Strom AR, Falkenberg Pand Landfald B. 1986. Genetics of osmoregulation in *Escherichia coli*: uptake and biosynthesis of organic osmolytes. *FEMS Microbiol Rev.,* **39**:79-86.

Waditee R, Tanaka Y, Aoki K, Hibino T, Jikuya H, Takabe T and Takabe T. 2003. Isolation and functional characterization of N-methyltransferase that catalyze betaine synthesis from glycine in a halotolerant photosynthetic organism *Aphanothece halophytica*. *J Biol Chem.,* **278**:4932-4942.

Weigel P, Elizabeth AW and Andrew DH. 1986. Betaine aldehyde oxidation by spinach chloroplast. *Plant Physiol.,* **82(3):**753-759.

Yadava RS, Kumar R and Yadava PK. 2005. Expression of *lexA* targeted ribozyme in *Escherichia coli BL21* (DE3) cells. *Mol Cell Biochem.,* **271**:197-203.

Yamanaka T, Miyama M and Tada Y. 2009. Transcriptome profiling of the mangrove plant *Bruguiera gymnorhiza* and identification of salt tolerance genes by *Agrobacterium* functional screening. *Biosci.Biotechnol.Biochem.,* **73**:304-310.

Yancey PH, Clark ME, Hand SC, Bowlus RD and Somero GN. 1982. Living with water stress: evolution of osmolytes systems. *Sci.,* **217**:1214-1222.