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Research Paper

Functional characterization of a major compatible solute in Deep Sea halophilic eubacteria of active volcanic Barren Island, Andaman and Nicobar Islands, India

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ABSTRACT

Ectoine, a cyclic tetrahydropyrimidine (2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) is a compatible solute, serves as a protective compound in many halophilic eubacterial cells under stress. In this study, the ectoine biosynthesis genes (*ect*A, B and C) from the genomic DNA of a deep sea eubacteria, *Bacillus clausii* NIOT-DSB04 was PCR amplified, cloned into the expression vector pQE30 with a 6 × histidine tag and expressed in M15 cells. The lysates of induced cells with diaminobutyric acid aminotransferase and ectoine synthase disclosed two clear expressed bands with molecular masses of 46 kDa and 15 kDa as estimated by SDS-PAGE. The recombinant ectoine synthase activity of the expressed cells was at higher level than that of uninduced cells. In silico sequence and phylogenetic analysis of nucleotides and amino acids revealed that the *ect*A, B and C sequences of *Bacillus clausii* NIOT-DSB04 were conserved in many eubacteria.

1. Introduction

Most halophilic and halotolerant bacteria adapt to various environmental stress by accumulating low-molecular weight organic compounds called compatible solutes. These compatible solutes equilibrate the external osmotic pressure and support intracellular turgor which is higher than that of surrounding environment. Among halophilic microorganism, variety of heterotrophic and methanogenic archaea, photosynthetic, lithotrophic and heterotrophic bacteria have been reported to biosynthesis compatible solutes (Oren et al., 1997). Compatible solutes also play a major role in protection of cells and its components from freezing, desiccation and temperature stress (Muller et al., 2005). Among the compatible solutes, ectoine is the most extensively studied and has been found to be distributed in many chemoheterotrophic halophilic eubacteria (Vargas et al., 2006). The cyclic amino acid ectoine was originally discovered as an osmoprotectant in oxygenic phototrophs of the *Ectothiorhodospira* group (Galinski et al., 1985) and subsequently found in many other Gram-negative and Gram-positive bacteria (Kempf and Bremer, 1998).

Ectoine protects the halophilic bacterial cells bio membranes, proteins, enzymes and nucleic acids against high or low temperature, salt concentration and low water activity. The organic osmolyte ectoine are amphoteric, water-binding organic molecules compatible with the cellular metabolism without adversely affecting the physiological processes of the bacterial cells (Galinski and Truper, 1994). Ectoine have been used in cosmetics as stabilizers, since it protects the skin cells against different damaging factors such as heating, freezing, desiccation and UV radiation (Graf et al., 2008). Various investigations underline the outstanding antiaging properties and immunoprotective potential of ectoine on skin (Pfluecker et al., 2005).

The biosynthetic pathways of ectoine have been elucidated in many halophilic eubacteria and usually occur in four enzymatic steps (Peters et al., 1990). First being the conversion of aspartate semialdehyde into diaminobutyric acid by diaminobutyrate transaminase (*ect*B), which is subsequently acetylated to *N*-γ-acetyldiaminobutyrate by diaminobutyrate acetyl transferase (*ect*A). The cyclic condensation of this compound by ectoine synthase (*ect*C) leads to the formation of ectoine (Prabhu et al., 2004). Since ectoine have gained more importance in biotechnology research, the necessity in elucidation of the genes responsible for ectoine biosynthesis in halophilic and halotolerant

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eubacteria isolated from extreme environment is of great demand for the development of new technologies for ectoine production. To the best of our knowledge, no report on functional characterization of ectoine biosynthesis genes from deep sea eubacteria, *Bacillus clausii*. In this study, ectoine biosynthesis gene cluster (*ect*ABC) from *Bacillus clausii* NIOT-DSB04 was characterized and determined the diversity and phylogenetic relationship of *ect*A, B and C genes with other eubacteria.

2. Material and methods

2.1. Growth conditions and molecular identification

The deep sea halophilic eubacterial strain NIOT-DSB04 was isolated from the deep sea core sediment and grown aerobically in alkali bacillus medium containing (1% (w/v) peptone, 0.5% (w/v) yeast extract, 1% (w/v) glucose, 0.1% (w/v) K_2HPO_4, 1% (w/v) Na_2CO_3 and incubated at room temperature for 24 h). The genomic DNA was extracted by following the method described by Ausubel et al. (1994). Approximately 1 ml of NIOT-DSB04 culture grown overnight was centrifuged at 10,000 ×*g* for 1 min in a 3 K30 centrifuge (Sigma, Germany). The pellet was resuspended in 100 µl of MilliQ water and subjected to cell lysis in a boiling water bath for 5 min. After centrifugation for 5 min at 5000 ×*g*, 1 µl of supernatant was used for PCR amplification. The 16S rDNA was PCR amplified using the universal eubacterial primers, 16S f (5′-ACTCAAAGGAATTGACGG-3′) and 16S r (5′-TACGG CTACCTTGTTACGACTT-3′). The amplicon was cloned in a T/A cloning vector according to the manufacturer's instructions in the InsTAclone PCR Cloning kit (MBI Fermentas, USA). The sequencing was performed on an ABI PRISM 377 genetic analyzer (Applied Biosystems, USA) using the dye termination method. The acquired 16S rDNA sequences were used in a homology search with the available sequences in GenBank using BLAST provided by NCBI (http://www.ncbi.nlm.nih.gov) for pairwise identities. Multiple sequence alignment of the sequences was performed using the CLUSTAL-X version 1.81 program, and the phylogenetic tree was constructed with the MEGA version 5.0 program using neighbor-joining analysis.

2.2. PCR amplification of ectoine biosynthesis genes

*Ect*ABC gene cluster of *B. clausii* NIOT-DSB04 was amplified by PCR using gene specific primers. 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, USA), 1.25 U of *Pfu* DNA polymerase (MBI Fermentas), 1 × *Pfu* buffer; 2.5 mM of $MgSO_4$ and remaining autoclaved millipore water. Amplification was performed in a Master cycler (Eppendorf, Germany) 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 1.5% agarose gel along with DNA molecular weight marker (MBI Fermentas) and documented in gel documentation system (UVP BioSpectrum Imaging system, USA).

2.3. Molecular cloning and sequencing

The *ect*ABC PCR amplicons were purified by MinElute Gel purification Kit (Qiagen, Germany) and cloned into pDrive (Qiagen), according to the manufacture's instructions. The pDrive-*ect*ABC construct was transformed into *E. coli* JM109 (*recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (rK-mk+), e14–(*mcrA*–), *supE*44, *relA*1, Δ(*lac-proAB*)/F′ [*traD*36, *proAB* +, *lac* Iq, *lacZ*ΔM15]). White colonies were selected for PCR amplification with vector primers M13f-M13r (MBI Fermentas) and the clones with the correct insert as judged by size were sequenced on an ABI PRISM 377 genetic analyzer (Applied Biosystems Inc., USA).

2.4. Heterologous expression of ectABC genes

The recombinant plasmid pDrive-*ect*ABC construct were double digested with *Sac*I and *Bam*HI (MBI Fermentas) and purified by MinElute Gel purification Kit. The purified *ect*ABC gene was recloned into pQE30 expression vector (Qiagen), which had previously been digested and purified. The resulting recombinant expression vector pQE30-*ect*ABC 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_{600} 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 1 × SDS-PAGE sample buffer and lysed in boiling water bath for 3 min. The cells were centrifuged at 4000 *g* for 20 min and the supernatant was checked for expression of soluble proteins. The expression of the target proteins were analyzed by SDS-PAGE as described by Laemmli (1970). The molecular mass was estimated by SDS-PAGE with protein ladder (Sigma-Aldrich, USA).

2.5. Preparation of enzyme extract

Enzymes extraction was performed as described previously (Nakayama et al., 2000). 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 25 µg of lysozyme at 37 °C for 10 min. The mixture was then incubated with 0.5 mg of DNase I and 2.0 mg of RNase A at 37 °C for 15 min. The cell lysate was then treated with 1 mM phenylmethylsulfonyl fluoride [PMSF] (Sigma-Aldrich, USA), 0.5 mM EDTA, 0.1 M NaCl and centrifuged at 12,000 ×*g* for 25 min. The protein concentration was determined according to the method of Bradford (1976) with bovine serum albumin as the standard.

2.6. Enzyme assays

2.6.1. Diaminobutyric acid aminotransferase activity

The enzyme activity of 2,4-diaminobutyric acid (DABA) aminotransferase (*ect*B) was assayed as described previously (Nakayama et al., 2000). DABA aminotransferase activity was determined by the amount of glutamate produced in the reverse reaction. Briefly, 200 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 20 °C for 30 min, and the reaction was stopped by boiling the mixture for 10 min. The concentration of the released glutamate was determined by L-glutamic acid kit (Megazyme, USA).

2.6.2. Diaminobutyric acid acetyltransferase activity

DABA acetyltransferase (*ect*A) activity 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. A coupled spectrometric test was employed. The acylation activity of *ect*A was analyzed using a coupled spectrometric test with the detection at 410 nm.

2.6.3. Ectoine synthase activity

Ectoine synthase (*ect*C) activity was performed as described previously (Nakayama et al., 2000). Briefly, the reaction was carried out in a 150 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

20 °C for 15 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_2 column (LiChroCART 250-4 NH_2; 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.

2.7. 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 (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/) 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 sequences generated in this study have been deposited in the GenBank database under the accession numbers MH020162, MH020163 and MH020164.

3. Results and discussion

3.1. Molecular identification of the potent strain

The 16S rDNA sequences (1535 bp) generated in this study were deposited in GenBank under accession number MH114929. Upon analysis with the BLASTN program and phylogenetic analysis, it was established that the deduced nucleotide sequences of NIOT-DSB04 were highly homologous (99.87%) with the reported 16S rDNA sequences of *Bacillus clausii* (GenBank accession no. CP012475). Based on the morphological, biochemical characteristics and phylogenetic analysis, the deep sea halophilic eubacteria was identified as *B. clausii* NIOT-DSB04.

3.2. PCR amplification and molecular cloning of ectoine biosynthesis genes

The *ect*A, B and C genes encode the diaminobutyric acid acetyltransferase, diaminobutyric acid aminotransferase and ectoine synthase respectively. Together these proteins constitute the ectoine biosynthetic pathway. The ectoine biosynthesis genes *ect*A, B and C from deep sea bacteria, *Bacillus clausii* were PCR amplified and encoded by polynucleotides of 537 bp, 1281 bp and 405 bp. The *ect*A, B and C genes encodes polypeptides of 178, 426 and 134 amino acids with calculated molecular masses of 19,816, 46,713, 15,321 Da. The amplicons were purified from the agarose gel and cloned into pDrive cloning vector. The positive clones were selected and screened for the presence of insert by PCR amplification using specific primers, which also exhibited the specific products. The recombinant transformants of *ect*A, B and C genes were also confirmed by double digestion with *Sac*I and *Sma*I restriction enzyme, which released full gene along with flanking region of the vector.

3.3. Molecular characterization of ectoine biosynthesis genes

The recombinant expression vector pQE30-*ect*ABC 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 *ect*A protein was determined by acylation assay. The acylation activity in the expressed cells was 3.5 mU/mg, which is three times more than that of control cells. Aminotransferase assay was employed to determine the activity of *ect*B protein. The aminotransferase activity of the expressed cells was

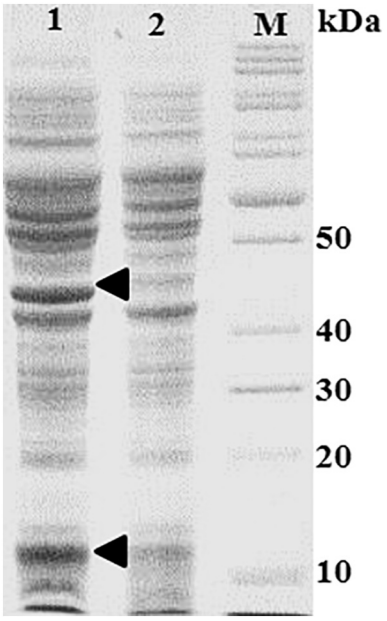
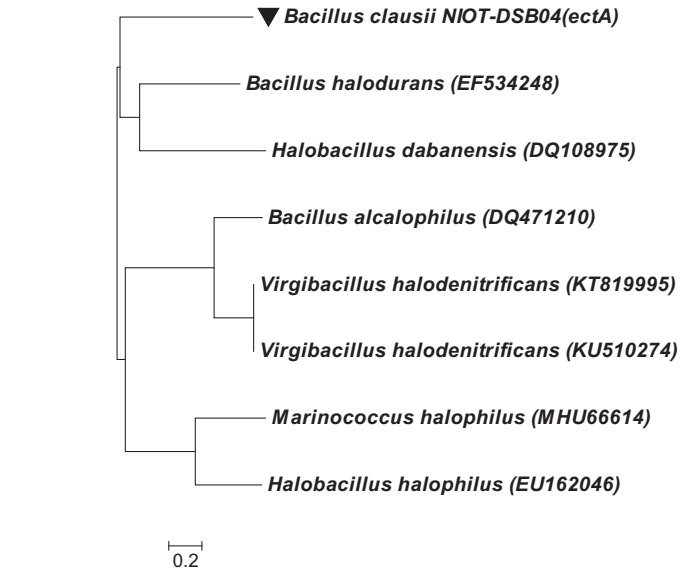


Fig. 1. Coomassie brilliant blue stained SDS-PAGE analyses of the expressed *ect*B and *ect*C of *Bacillus clausii*. Lane 1, Total protein of the induced *ect*B and C gene cassette; Lane 2, Total protein of the uninduced *ect*B and C gene cassette; Lane M, Protein molecular mass marker. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

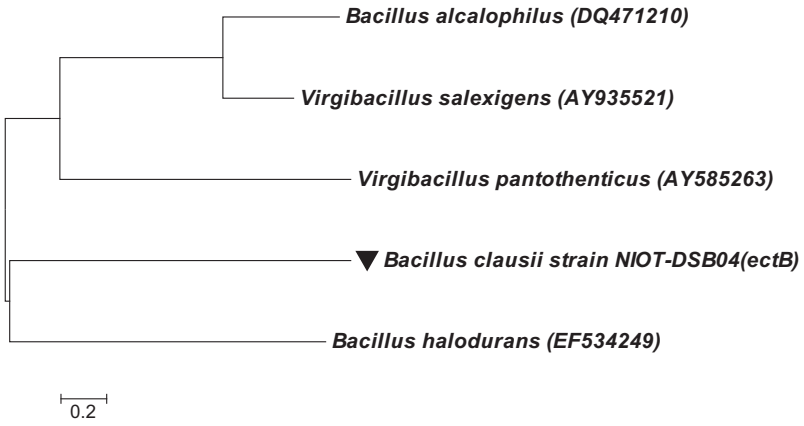
5 mU/mg, which is considerably higher than that of uninduced cells. The ectoine synthase activity of the expressed cells was also in the higher side 6.5 mU/mg, which is higher than that of uninduced cells. The ectoine biosynthesis genes *ect*A, B and C have been functionally characterized in *Marinococcus halophilus*, *Bacillus pasteurii*, *Methylomicrobium alcaliphilum* and *Chromohalobacter salexigens* (Louis and Galinski, 1997; Kuhlmann and Bremer, 2002; Reshetnikov et al., 2006; Schubert et al., 2007). Expression of the ectoine biosynthesis genes was analyzed by SDS-PAGE. The lysates of induced cells showed two clear expressed bands with molecular masses of 46 kDa and 15 kDa that correspond to *ect*B and *ect*C, which was not present in non-induced cells (Fig. 1). The expressed protein band of *ect*A gene was not detectable in SDS-PAGE due to its instability and it has already been reported (Schubert et al., 2007).

3.4. Sequence analysis of ectA, B and C genes

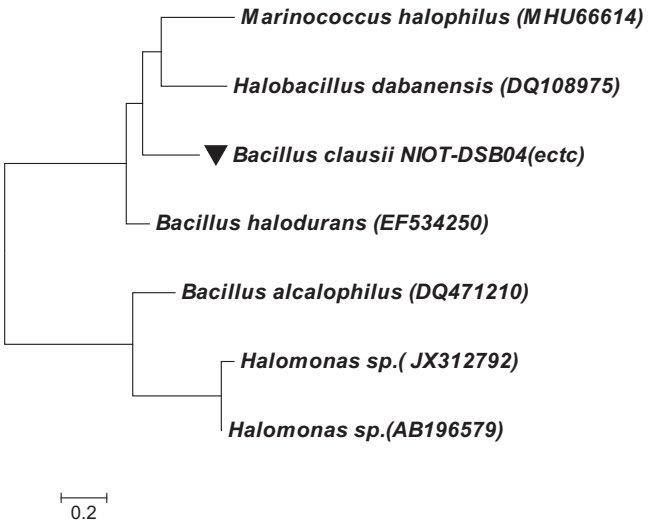
The *ect*A, B and C sequences from *Bacillus clausii* NIOT-DSB04 were analyzed with reported amino acid sequences of other eubacteria viz. *Bacillus halodurans* (GenBank Accession No EF534248, EF534249 and EF534250), *Marinococcus halophilus* (MHU66614), *Bacillus alcalophilus* (DQ471210), *Virgibacillus salexigens* (AY935521), *Virgibacillus pantothenticus* (AY585263), *Virgibacillus halodenitrificans* (KT819995), *Virgibacillus halodenitrificans* (KU510274), *Halobacillus dabanensis* (DQ108975), *H. halophilus* (EU162046), *Halomonas* sp. (JX312792) and *Halomonas* sp. (AB196579) 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 Gcn5-related *N*-acetyltransferase family. The *ect*A gene encodes proteins of 178 amino acids with the pI value of 5.25. The polynucleotide sequences of *ect*A disclosed a high similarity with other bacteria: *B. halodurans* with 68.74% identity, *B. alcalophilus*, 69.06% identity; *H. dabanensis*, 80% identity; *M. halophilus*, 73.82% identity and *V. halodenitrificans* with 76.27% identity. The amino acid analysis of *ect*A revealed that, the amino acid sequence of *B. halodurans* and *M. halophilus* has the maximum identity of 71% and



(a)



(b)



(c)

Fig. 2. The phylogenetic tree analysis of ectoine biosynthesis genes. (a) Amino acid sequences of *ect*A gene. (b) Amino acid sequences of *ect*B gene. (c) Amino acid sequences of *ect*C gene.

59% respectively with that of *B. clausii* NIOT-DSB04. All the other sequences had less than 45% similarity with the amino acid sequence of *B. clausii* NIOT-DSB04.

The amino acid analysis of *ect*B gene suggests that the encoded protein belongs to the Aminotransferases class-III pyridoxal-phosphate family. The *ect*B gene encodes proteins of 426 amino acids with the pI value of 5.32. Among the ectoine biosynthesis genes, the nucleotide sequences of *ect*B gene showed highest similarity with other bacteria compared to *ect*A gene. *B. halodurans* with 72.19% identity, *B. alcalophilus* sequence has 70.06% identity, *V. salexigens*, 68.04% identity; *V. pantothenticus*, 67.94% identity and *V. halodenitrificans* with 68.04% identity. The amino acid sequence analysis revealed that, only *B. halodurans* and *B. alcalophilus* has the maximum identity of 78% and 72% with the amino acid sequence of *B. clausii* NIOT-DSB04.

The amino acid analysis of *ect*C gene suggests that the encoded protein belongs to the ectoine synthase. The *ect*C gene encodes proteins of 134 amino acids with the pI value of 5.05. The amino acid sequences of *ect*C showed a considerable homology with ectoine synthase from other bacteria. *B. halodurans* with 70.88% identity, *B. alcalophilus*, 73.40% identity; *V. dokdonensis*, 67.67% identity; *Virgibacillus* sp., 67.67% identity; *Halobacillus halophilus*, 68.34% identity; *B. lehensis,* 75.70% identity and *Bacillus* sp. has 72.60% identity. The amino acid sequence of *B. halodurans* and *B. alcalophilus* has maximum similarity of 76% and 78% with the amino acid sequence of *B. clausii* NIOT-DSB04 than other eubacteria.

3.5. Phylogenetic tree construction and analysis of ectoine biosynthesis genes

Phylogenetic tree analysis of nucleotide and amino acid sequences of *ect*A revealed a single cluster pattern for *B. clausii*, *B. halodurans* (EF534248) and *H. dabanensis* (DQ108975). The phylogenetic tree of nucleotide and amino acid sequences of *ect*B gene also revealed the grouping of *B. clausii* and *B. halodurans* (EF534249) in a single cluster as that of *ect*A. Nucleotide analysis of *ect*C gene revealed that *B. clausii* was clustered together with *B. halodurans* (EF534250) and *M. halophilus* (MHU66614) and formed as a single cluster. On phylogenetic analysis, *ect*C gene was found to have highest similarity between bacterial species compared to the *ect*A and *ect*B genes. Based on phylogenetic analysis, *B. clausii* and *B. halodurans* were found to be clustered together for all the genes (Fig. 2a, b & c). The bacterial species switched to different clusters at nucleotide indicates the divergence among the organisms and the degree of divergence in the sequences. Even though 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). In this paper, we report the heterologous expression of ectoine biosynthetic genes from the deep sea halophilic bacterium, *B. clausii* NIOT-DSB04 in *E. coli* M15. The engineered *E. coli* strain has potential industrial application since it produces ectoine at high rates and can avoid the complex down streaming process associated with the conventional bioprocess.

Authors' contribution

The research concept and the experiments were executed by LA and BM. NVV, RK and GD analyzed the data and reviewed the manuscript. All authors approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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