Functional Characterization and Sequence Analysis of Choline Dehydrogenase from *Escherichia coli*

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Abstract

Choline dehydrogenase catalyzes the oxidation of choline to glycine betaine via betaine aldehyde as intermediate. Biotechnological applications of glycine betaine in transgenic plant improvement have been shown to have enhanced tolerance towards hypersalinity and freezing. It can also restore and maintain osmotic balance of living cells under stress. In this study, choline dehydrogenase (*betA*) gene from *E. coli* was cloned and homologously expressed in *E. coli* M15 (pREP4). The recombinant enzyme was purified by column chromatography using DEAE Sepharose. The purified enzyme 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 *betA* gene sequence 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.

Keywords: Osmolyte; betA; glycine betaine; choline dehydrogenase.

1. Introduction

Microorganisms are often exposed to changes in the solute concentrations in their environment [1]. Increase in the osmolarity results in the changes in intracellular solute concentration and cell volume. To cope with the environment stress, bacteria accumulate osmotically active solutes, including potassium, proline, glutamic acid, glutamine, α-aminobutyric acid, ectoine and betaine [2]. In addition they can accumulate α-glutamate and the β-amino acids; *N*-acetyl-β-lysine, β-glutamine, and β-glutamate as compatible solutes in response to high external NaCl levels [3]. Among the compatible solutes, Glycine-betaine is an effective osmoprotectant [4]. Glycinebetaine (*N*,*N*,*N*-trimethylglycine) is a quaternary ammonium compound that occurs naturally in a wide variety of plants, animals and microorganisms [5]. 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 [6]. To date three enzymatic systems have been described for the formation of glycine betaine from choline. Biosynthesis of betaine is catalyzed in a single step reaction by choline oxidase in soil bacterium, *Arthrobacter globiformis* [7]. In higher plants, such as spinach [8], sugar beet and amaranth [9] betaine biosynthesis is catalyzed by choline monooxygenase in combination with betaine aldehyde dehydrogenase. In *E. coli*, the biosynthetic pathway for the production of glycine betaine from choline has been well characterized at the genetic level [10]; 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 [11]. The enzymes involved in the biosynthesis of betaine have been cloned and characterized from bacteria and plants [12-14].

Recently, bacterial glycine betaine synthesizing enzymes have become a major target in developing stress tolerant crop plants of economic interest. Previous studies reports the resistance towards salinity and low temperature in transgenic tobacco expressing the two *E. coli* genes *betA* and *betB* [15], 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 [10]. Choline dehydrogenase catalyzes the four-electron oxidation of choline to glycine betaine via a betaine aldehyde intermediate [16]. Only nominal studies on characterization of choline dehydrogenase from *E. coli* have been reported to date. In this study, we report the characterization and structural analysis of *betA* gene coding for the choline dehydrogenase in *E. coli*. Moreover, the sequence analysis of choline dehydrogenase from our isolate shows several base substitutions with that of reported sequences in GenBank, resulting in the altered amino acid sequences of the translated proteins.

2. Methods

2.1 Bacterial strains and plasmids

E. coli TCJAR023 was isolated from a finfish; Rohu (*Labeo rohita*) procured from local fish markets in Cochin, Kerala, India. Microbial identification and biochemical characterization of *E. coli* was carried out as per U.S. Food and Drug Administration (USFDA) and used as a source of the gene encoding the choline dehydrogenase (*betA*). *E. coli* JM109 was used for cloning and *E. coli* M15 (pREP4) was used as expression host. The plasmids used for cloning and expression was pDrive and pQE30 (Qiagen, Hilden, Germany) respectively.

2.2 DNA extraction and gene amplification

Genomic DNA extraction from *E. coli* TCJAR023 was performed following the method of Ausubel et al. [17]. Plasmid DNA was purified from the cells by alkali lysis method. Plasmids and PCR products were recovered from agarose gel using MinElute Gel purification Kit (Qiagen) following the manufacturer's instructions. A pair of primers bAF (5′-CGTATGCAATTTGACTACATCATT-3′) and bAR (5′-GCATCATTTTTTCGCTCTCACCG-3′) were designed and used to amplify the *betA* gene. PCR amplification was done with 30 cycles of 1 min at 94°C, 1.5 min at 50°C and 2 min at 72°C. Additional extension was carried out for 5 min at 72°C using high fidelity PCR enzyme mix (MBI Fermentas, Maryland, USA).

2.3 Cloning and sequencing

The PCR product of *betA* gene was purified using Qiagen PCR purification Kit and cloned into pDrive according to the manufacturer's instructions. The pDrive-*betA* construct was transformed into *E. coli* JM109 (*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsdR*17 (*rK-mk*+), e14– (*mcrA*–), *supE*44, *relA*1, Δ(*lac-proAB*)/F' [*traD*36, *proAB*+, *lac I*q, *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, Perkin Elmer Co., Foster City, CA, USA).

2.4 Expression of recombinant choline dehydrogenase

The recombinant plasmid pDrive-*betA* construct was double digested with *Hind* III and *Bam* HI (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 Luria–Bertani (LB) broth containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin, and incubated overnight at 37°C. 2·5 ml of the overnight culture was transferred into 50 ml of LB containing the corresponding antibiotics and incubated at 37°C, until OD_{600} value reached 0.6. 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 14 000 *g* for 5 min and the supernatant was checked for expression of soluble proteins. The expression of the target proteins were analyzed by SDS-PAGE. The SDS-PAGE was performed as described previously [18]. The molecular mass was estimated by SDS-PAGE with protein ladder (MBI Fermentas).

2.5 Cell extraction and enzyme purification

The IPTG induced *E. coli* M15 (pREP4) cells with pQE30-*betA* cassette 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-Aldrich Co., St. Louis, MO, USA) 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.

The purification of choline dehydrogenase was performed as described previously [14] with slight modifications. Briefly, the supernatant was subjected to 50% ammonium sulfate saturation and incubated on ice for 20 min, and centrifuged at 10 000 *g* for 10 min. The resulting pellet was suspended in 2.5 ml of 0.5 mM EDTA, 15 mM potassium phosphate (pH 7.0) and centrifuged at 10 000 *g* for 10 min. Later, the supernatant was transferred into a HiPrep 16/10 DEAE Fast Flow column (Amersham Pharmacia Biotech Inc,

NJ, USA) equilibrated with 0.5 mM EDTA and 15 mM potassium phosphate (pH 7.0). Equilibrated buffer solution was also used as mobile phase at a flow rate of 1.0 ml/min. The elution was suspended in the mixture of 2 M sorbitol, 20% glycerol, 15 mM potassium phosphate (pH 7.0) and stored at -20°C.

2.6 Enzyme assay

The concentration of choline dehydrogenase was determined by the method of Bradford [19] 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 [14]. 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.

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 [20]. 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 secondary structure prediction was performed using the PROTEAN program (DNASTAR, Inc., Madison, USA).

The nucleotide sequences generated in this study have been deposited in NCBI GenBank under accession number EU032535.

3. Results

3.1 Cloning and sequence analysis

The choline dehydrogenase gene, *betA* encodes the polypeptides comprised of 556 amino acids with the calculated molecular masses of 61 848 Da., based on the *in silico* estimates. The pI value of choline dehydrogenase is 5.45. Choline dehydrogenase of *E. coli* TCJAR023 is 1 671 bp long (Fig. 1). The *betA* gene from *E. coli* 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 (Fig. 2) with that of the sequences deposited in the GenBank. (accession nos. X52905, M77738). 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. 3). No internal stop codons were observed due to the base substitution in the gene. 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 yet again PCR amplified with the genomic DNA from *E. coli* TCJAR023 with *Taq* DNA polymerase (Dynazyme II, 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.

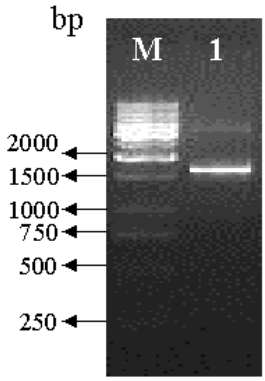

Fig. 1: Result from PCR with the primers bAF and bAR. Lane M: DNA molecular weight marker; Lane 1: Amplicon of the *betA*.

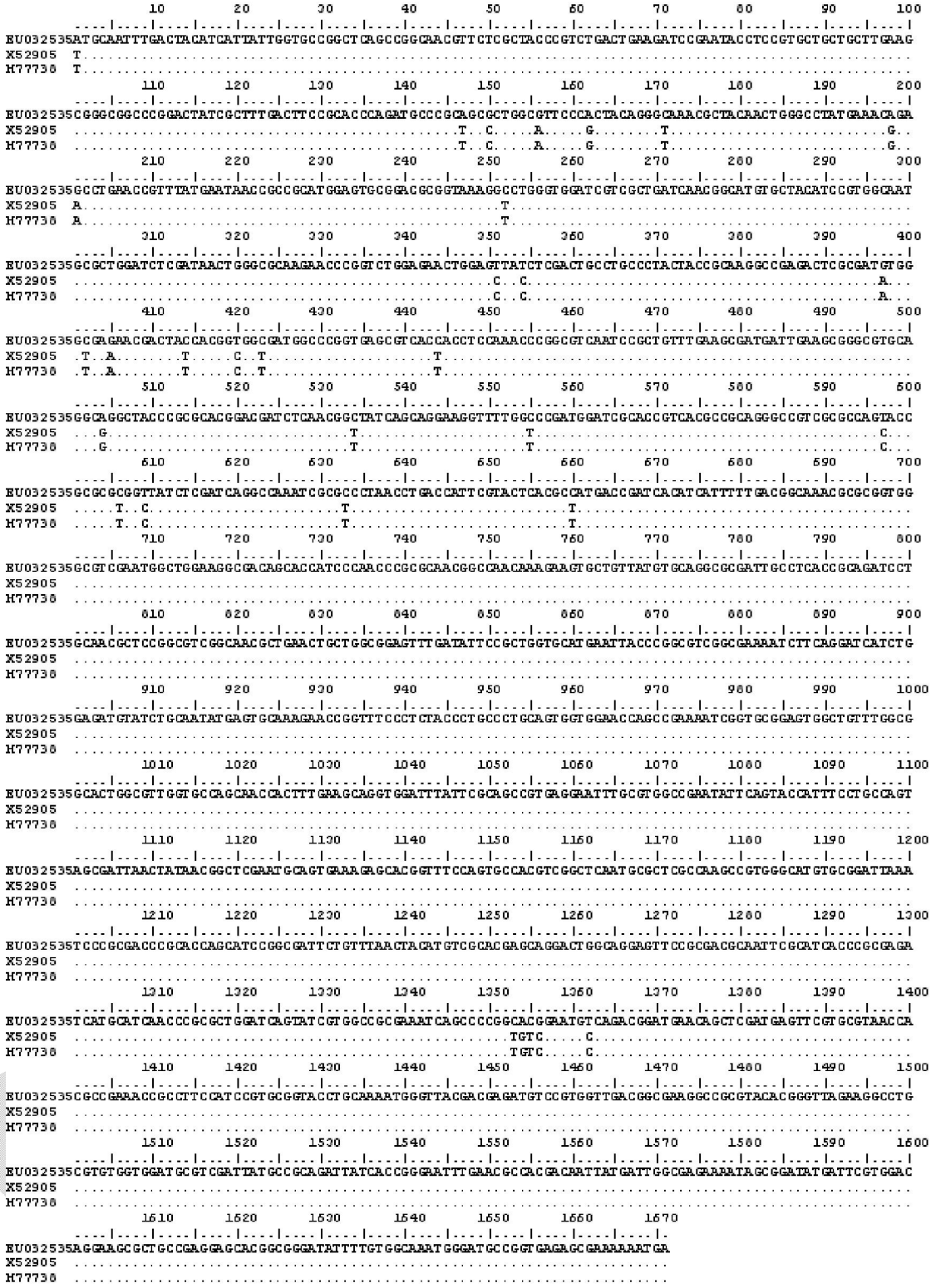

Fig. 2: BioEdit analysis of nucleotide substitutions of choline dehydrogenase (*betA*) in *E. coli* TCJAR023 [EU032535], with *E. coli* strains [X52905 & M77738].

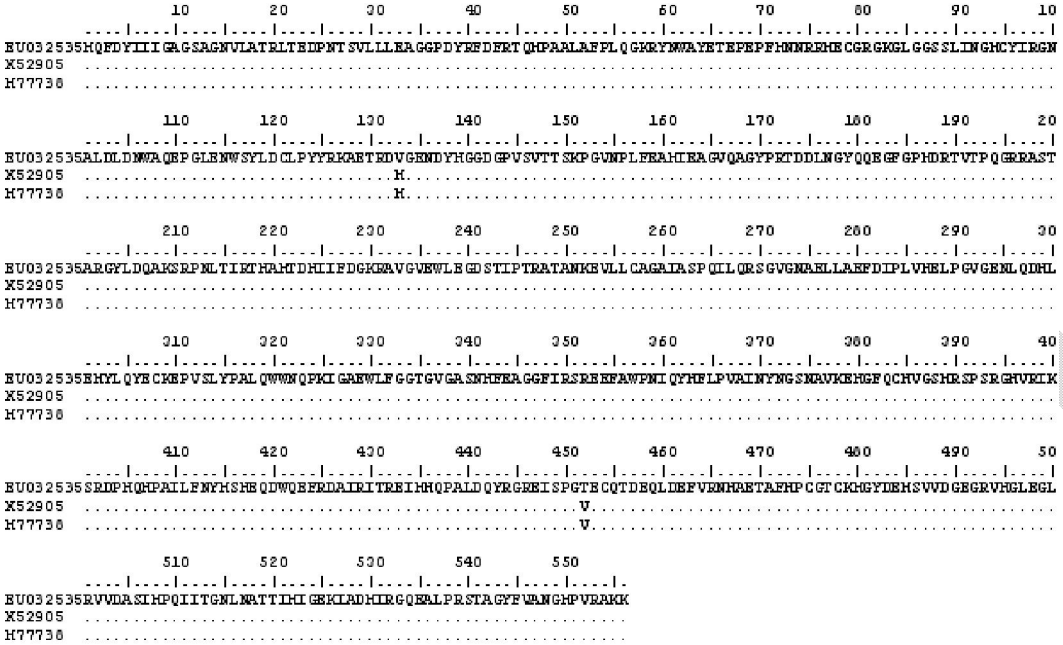

Fig. 3: BioEdit analysis of amino acid substitutions of choline dehydrogenase (*betA*) in *E. coli* TCJAR023 [EU032535], with *E. coli* strains [X52905 & M77738].

3.2 Protein expression and enzyme purification

The expression vector pQE30-*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 1.0 mM IPTG. The proteins of the induced and non-induced *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 correspond to choline dehydrogenase (Fig. 4). 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 1). The resultant purified enzyme was stabilized with 2 M sorbitol and 20% glycerol, at pH 7.0 [14]. The purified protein was visualized by SDS-PAGE (Fig. 4).

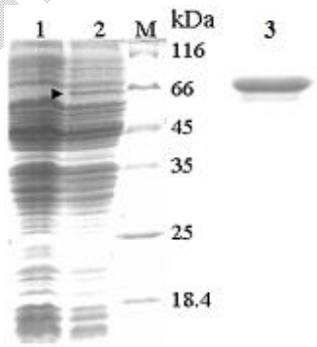

Fig. 4: SDS-PAGE analyses of the expressed and purified choline dehydrogenase from *E. coli*. The arrow (►) indicates the position of the expressed *betA*. Lane 1: Total protein of the uninduced pQE30-*betA* gene cassette; Lane 2: Total protein of the induced pQE30-*betA* gene cassette; Lane M: Protein molecular mass markers; Lane 3: Purified choline dehydrogenase

Table 1. Purification of recombinant choline dehydrogenase.

Stage of cells	Total units (µmol of O_2/min)	Total protein (mg)	Specific activity (µmol of O_2/min)
Cell free extract	17	31	0.2
50% $(NH_4)_2SO_4$ saturation	12	18	0.4
Hi-Prep 16/10 DEAE	16	7	1.1

3.3 Functional analysis of choline dehydrogenase

Choline dehydrogenase activity was determined by measuring the rate of reaction with choline as the substrate and 1 mM phenazine methosulfate as the primary electron acceptor in the assay reaction mixture. The activity of choline dehydrogenase with 10 mM choline was 8.8 µmol of O_2/min/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 µmol of O_2/min/mg of cultured cells.

3.4 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 much acidic amino acids, regions with high antigenicity and very high backbone chain flexibility (Fig. 5). 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% praline and 30% each of isoleucine and aspartic acid.

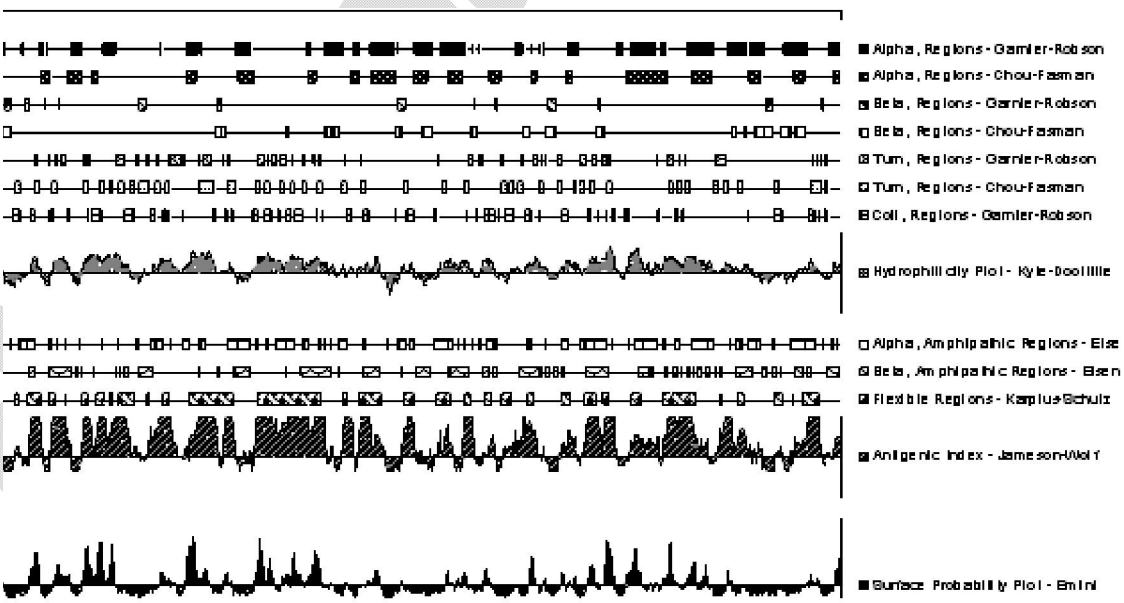

Fig. 5: Secondary structure prediction of choline dehydrogenase using PROTEAN.

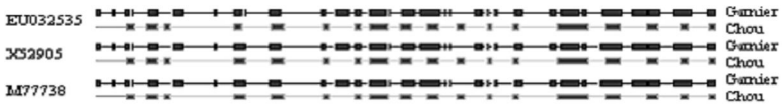

Fig. 6: Secondary structure analysis using PROTEAN. The analysis was performed using choline dehydrogenase from *E. coli* TCJAR023 [EU032535], with *E. coli* strains [X52905 & M77738].

4. Discussion

Based on the sequence analysis, it was previously reported that the *betA* gene of *E. coli* codes for choline dehydrogenase [13]. So far, only minimum reports on the characterization of choline dehydrogenase have been reported [14, 16]. In this study, we cloned and characterized the *betA* gene from *E. coli* cells under the control of inducible promoter. Evaluation of the deduced amino acid sequence of *betA* gene with reported choline dehydrogenase sequences in the database revealed a maximum similarity. However, the sequence analysis of *betA* of our isolate showed several base substitutions with that of reported sequences, resulting in the altered amino acid sequences of the translated protein. The activity of the enzyme using choline as the substrate and phenazine methosulfate as the electron acceptor revealed the expressed enzyme from *betA* is choline dehydrogenase. The ability of the choline dehydrogenase to catalyze both oxidative reactions in the conversion of choline to glycine betaine has been previously reported in choline dehydrogenase from *Halomonas elongata* [14], rat liver mitochondria [16] and in choline oxidase from *Arthrobacter globiformis* [7]. Choline dehydrogenase is capable of using either choline or betaine aldehyde as a substrate [16]. In this study, we used choline as the substrate and the enzyme activity rate was measured by the rate of oxygen consumption in the presence of 1 mM phenazine methosulfate as the primary electron acceptor. The enzyme activity of choline dehydrogenase from recombinant *E. coli* with pQE30-*betA* cassette showed a fourfold-enhanced activity relative to the control strain. Moreover, the catalytic rate of the enzyme was in the declined manner with that of the concentration of substrate by 10 mM to 2.5 mM. These observations signify that the enzyme activity is maximum, only in the presence of appropriate concentration of the substrate. These results are consistent with previously reported data [14] that, the specific activity of the enzyme was 11.8 µmol of O_2/min/mg with 10 mM choline and 8 µmol of O_2/min/mg with 5 mM choline. The secondary structure prediction results also revealed considerable similarity with the reported choline dehydrogenases from *E. coli* strains. 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. 6).

In conclusion, based on the expression and enzymatic analysis, the enzyme encoded by *betA* in *E. coli* TCJAR023 is a choline dehydrogenase. To our knowledge, this study represents the first instance in which a choline dehydrogenase from *E. coli* has been cloned and characterized in detail. Moreover, the determination of protein structure modification due to the nucleotide substitutions will certainly provide the basis for performing site-directed mutagenesis to improve the production and configuration of the osmolytes of biotechnological interest. This, in turn, has great potential for biotechnological applications aimed at genetically engineering stress tolerance in crop plants of economic interest.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

The research concept was developed by LAR and TCJ; research experiments were performed by LAR and RJ; NT organized for and provided the necessary facilities.

Acknowledgement

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