Egyptian Journal Of

GENETICS AND CYTOLOGY

INTERNATIONAL JOURNAL DEVOTED TO GENETICAL

AND CYTOLOGICAL SCIENCES

Published by

THE EGYPTIAN SOCIETY OF GENETICS

Volume 40 **July 2011** **No. 2**

CLONING AND EXPRESSION ANALYSIS OF BETAINE ALDEHYDE DEHYDROGENASE FROM *Pseudomonas fluorescens*

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Pseudomonas fluorescens are gram-negative rod shaped bacteria that improve plant health and nutrition (Rodriguez and Pfender, 1997; de Bruijn *et al.*, 2007). Most studies describe *P. fluorescens* as a psychrotrophic bacterium unable to grow at temperatures greater than 32°C and therefore as an avirulent bacterium to humans (Naseby *et al.*, 2001). It has many positive effects on the plant health as it enhances the production of plant growth hormones, it boosts the suppression of pathogens (especially fungi and oomycetes), and it directs the elicitation of plant defense responses (Haas and Defago, 2005).

Many micro-organisms use glycine betaine (GB) as a sole carbon, nitrogen, and energy source. GB is an efficient

osmoprotectant, which is accumulated to counteract drought and high salinity environments, and to maintain a positive cell turgor, needed for cell extension growth. Betaine aldehyde dehydrogenase (BADH) catalyzes the final step of glycine betaine from choline (Chen and Murata, 2002; Hanson *et al.*, 1985; He *et al.*, 2004). Several reports have demonstrated the purification of BADHs from several species (Arakawa *et al.*, 1987; Hibino *et al.*, 2001; Ishitani *et al.*, 1993). Overexpression of BADH may protect intracellular enzymes and organelles against the elevation of intracellular ionic strength or temperature, which results in increase of tolerance of salt and osmotic stresses in many organisms (Kempf and Bremer, 1998; Welsh, 2000; Li *et al.*, 2006).

Beside its function in the production of glycine betaine, BADH is a key enzyme for the growth of many pathogens (Velasco-Garcı´a *et al.*, 2000). The growth arrest of the pathogen can be accomplished by blocking choline degradation, abolishing synthesis of the osmoprotectant glycine betaine, and accumulating the BADH substrate, betaine aldehyde, which is highly toxic to the pathogen (Boch *et al.*, 1996; Sage *et al.*, 1997).

In this investigation, the full length of *bet*B gene, *PfBADH*, coding for BADH enzyme was isolated from *Pseudomonas fluorescens*, cloned in *Escherichia coli* and identified by studying the gene sequence and its expression analysis. Cloning the full length gene of PfBADH is a starting point for the strategic improvement of commercial crops grown under biotic and abiotic stress conditions using gene transfer techniques.

MATERIALS AND METHODS

Physiological studies

The *Pseudomonas fluorescens* strain was obtained from the Bio-fertilizer Production Unit; Soil, Water and Environmental Research Institute; Agricultural research center; Giza; Egypt. To confirm its characteristic features, *Pseudomonas fluorescens* was examined according to Bergey's manual of systematic bacteriology (Staley *et al.*, 2005). The propagated bacteria appeared fluorescent, with yellow green diffusible pigments. The bacteria were tested for trehalose utilization, production of Pyocyanin and the Geraniol utilization according to Staley *et al.* (2005). The Lecithinase activity test was performed according to Watson *et al.* (1993).

betB gene amplification and cloning

Genomic DNA was isolated from *Pseudomonas fluorescens* using Wizard® Genomic DNA Purification Kit (Promega). Amplification of *bet*B was performed using its specific oligonucleotide primers (5`ggaattccatatggcccgtttcgaactgcaaaaactc3`, and 5`aagcttttagaacaccgaggcgtagtcgcccag3`). The PCR fragment of *bet*B (1.5 kb) was purified from the agarose gel using QIAquick PCR Purification Kit (Qiagen). Cloning of the PCR fragment was per-

formed using the pGEM T-Easy cloning kit (Promega). The construct was used to transform the *E. coli* strain DH5α (Stratagene). After selecting the white clones, the construct was isolated using Wizard® Plus SV® Minipreps DNA Purification System (Promega). The positive transformants were sequenced at JenaGen labs Corporation, Germany. The *bet*B clone was digested with *Nde*I and *Hind*III, and ligated into pCAL-n vector (Stratagene). The resulting construct designated as pCAL-*bet*B was used to transform the *E. coli* strain XL1-blue (Stratagene), the positive transformants were selected and DNA sequencing was performed at JenaGen labs Corporation, Germany. Total protein was extracted from control and transformed *E. coli* after IPTG induction. The induced level of the BADH was assessed by sodium docecyl sulfate polyacrylamide gel electrophoresis (SDS -PAGE). One μg of total soluble protein from the control and transformed *E. coli* was separated on 8% SDS-PAGE gel (Sambrook *et al.*, 2005).

Sequence analysis

The PfBADH clone was sequenced using a Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 DNA sequencer (Applied Biosystems). A homology search was performed using BLASTX against the NCBI protein database (http://www.ncbi.nlm.nih.gov). Sequences of plant and micro organisms BADH proteins that showed similarity to the PfBADH protein were obtained from the NCBI nonredundant and dbEST data sets using BLASTX or BLASTP (ver. 2.0.10) (Altschul *et al.*, 1997). The full amino acid sequences of the proteins were aligned using CLUSTAL W ver. 1.8 (Thompson *et al.*, 1994) and subjected to phylogenetic analysis. Phylogenic trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) with parsimony and heuristic search criteria and 1000 bootstrap replications to assess branching confidence.

RESULTS AND DISCUSSION

The species of *Pseudomonas fluorescens*, non-pathogenic bacteria of kingdom Monera, is found in abundance in a wide range of terrestrial and aquatic habitats. They are recognized for their ability to partially or completely degrade pollutants such as polycyclic aromatic hydrocarbons (Gilcrease and Murphy, 1995; Caldini, *et al.*, 1995).

We have confirmed the strain used is *Pseudomonas fluorescens* using micro biological experments. The identified strain has the ability to grow well at 36°C, can grow at 4°C, but can't grow at 41°C. It has no ability to grow on medium contains trehalose or geraniol. Moreover, it is positive to Lecithinase activity.

Molecular cloning of P. fluorescens's HABD gene

Using a general definition, betaine aldehyde dehydrogenase is an oxidizing enzyme that catalyses the oxidation of betaine aldehyde with NAD^+ and water to

betaine and NADH. It catalyzes the final and irreversible step in the synthesis of glycine betaine from choline (Lin *et al.*, 1996).

Betaine aldehyde dehydrogenase genes have been cloned and characterized from many prokaryotic and eukaryotic species such as *Sinorhizobium meliloti*, carrot, *Pseudomonas aeruginosa*, *Hordeum vulgare* and *Avena sativa*. (Arakawa *et al.*, 1990; Roland *et al.*, 1997; Roberto *et al.*, 1999; Livingstone *et al.*, 2003; Kumar *et al.*, 2004).

We successfully isolated BADH clone with 1473-bp open reading frame encoding 490 amino acid residues (Fig. 1). Full length gene was submitted to GenBank under accession number AB687490. The main ORFs of the isolated gene encodes protein with predicted molecular masses of 53.2 KDa (pI 4.97), which corresponds to a single subunit of the tetrameric BADH enzyme (Valenzuela-Soto *et al.*, 2003).

Pseudomonas fluorescens has a variety of strains, two of them are most important namely, *Pseudomonas fluorescens* PFO-1 and *Pseudomonas fluorescens* Pf-5, which are capable of suppressing many soil born diseases. Amino acid sequence alignments between PfBADH isolated in this study and BADH isolated from *Pseudomonas fluorescens* Pf-5 strain, *Pseudomonas fluorescens* PFO1 strain, and *Pseudomonas aeruginosa* was performed. Alignment showed an extensive conservation of 99% identity with BADH of *Pseudomonas fluorescens* Pf-5 strain, 92% identity with BADH of *Pseudomonas fluorescens* PFO1 strain, and 83% identity with BADH sequence of *Pseudomonas aeruginosa* (Fig. 2).

Amino acids from 18 to 479 showed high similarities to Aldehyde dehydrogenase family; pfam00171. This family of dehydrogenases acts on aldehyde substrates. Members use NADP as a cofactor. The family includes the following members: The prototypical members are the aldehyde dehydrogenases EC:1.2.1.3, Succinate-semialdehyde dehydrogenase EC:1.2.1.16, Lactaldehyde dehydrogenase EC:1.2.1.22 and Benzaldehyde dehydrogenase EC:1.2.1.28 (Steinmetz *et al.*, 1997; Marchler-Bauer *et al.*, 2011).

Amino acids from 26 to 484 shows high similarity to $NAD(P)^+$-dependent aldehyde dehydrogenase superfamily; cl11961. The aldehyde dehydrogenase superfamily (ALDH-SF) of $NAD(P)^+$-dependent enzymes, in general, oxidize a wide range of endogenous and exogenous aliphatic and aromatic aldehydes to their corresponding carboxylic acids and play an important role in detoxification. Besides aldehyde detoxification, many ALDH isozymes possess multiple additional catalytic and non-catalytic functions such as participating in metabolic pathways, or as binding proteins, or osmoregulants, to mention a few. The enzyme has three domains, a $NAD(P)^+$ cofactor-binding domain, a catalytic domain, and a bridging domain; and the ac-

tive enzyme is generally either homodimeric or homotetrameric. The catalytic mechanism is proposed to involve cofactor binding, resulting in a conformational change and activation of an invariant catalytic cysteine nucleophile. The cysteine and aldehyde substrate form an oxyanion thiohemiacetal intermediate resulting in hydride transfer to the cofactor and formation of a thioacylenzyme intermediate. Hydrolysis of the thioacylenzyme and release of the carboxylic acid product occurs, and in most cases, the reduced cofactor dissociates from the enzyme (Marchler-Bauer *et al.*, 2011).

Multiple alignments of the predicted amino acid sequence of PfBADH with BADH proteins isolated from *Pseudomonas fluorescens* Pf-5 strain, *Pseudomonas fluorescens* PFO1 strain, and *Pseudomonas aeruginosa* was performed using clustallX software. We have identified the conserved regions for NAD binding site, that contain conserved double β-α- β-α-β-motif, which are common structural feature of many enzymes that bind NAD. It also contains conserved amino acids for polypeptide binding site needed for BADH activity *in vivo* (Fig. 2).

Neighborjoining method (NJ), with 1000 bootstrap replications to assess branching, was used to determine the evolutionary relatedness of PfBADH to other BADH proteins isolated from other species (Fig. 3). It showed that BADH proteins isolated from microorganisms consistute a distinct clade. Moreover, PfBADH was on a seperate clade than that of BADH isolated from *Pseudomonas aeruginosa*. PfBADH showed a high degree of similarity with the BADH from *Pseudomonas fluorescens* Pf-5 (99%), *Pseudomonas brassicacearum* (97%), *Pseudomonas syringae* (91%).

The induction of pfBADH enzyme was confirmed in the transformed *E. coli.* Total protein was extracted from transformed *E. coli* after IPTG induction. Total protein was analyzed by denaturating sodium dodicylsulphat-polyacrelamide gel electrophoresis (SDS -PAGE). Figure (4) shows different protein patterns of the treated cultures of *Pseudomonas fluorescens* and IPTG induced *E. coli* indicating the presence of ~55 KDa band.

In this work, the full length of *bet*B gene, PfBADH, coding for BADH enzyme was isolated from *Pseudomonas fluorescens*, cloned in *Escherichia coli* and identified by studying the gene sequence and expression. Cloning a full length gene responsible for drought stress is a starting point for the strategic improvement of commercial crops grown under abiotic stress conditions using gene transfer techniques. Some plants produce the compatible solute, glycine betaine (Gorham, 1995) which is produced from choline- in response to osmotic stress. Using this fact, bacterial choline oxidases may be introduced into the plant that is unable to synthesize glycine betaine to improve its osmotic tolerance (McCue and Hanson, 1990).

SUMMARY

The plant-growth promoting bacteria *Pseudomonas fluorescens*, can utilize glycine betaine (GB) as a sole carbon, nitrogen, and energy source. GB, an important osmoprotectant for many bacteria, can be derived from choline or carnitine. *P. fluorescens* uses the betaine aldehyde dehydrogenase (BADH) for assimilating carbon and nitrogen from choline or choline precursors. Moreover, BADH is also, used to catalyzes the final step in the synthesis of the GB from choline, which is a solute that is able to restore and maintain the osmotic balance of living cells protecting them against the high-osmolarity stress in their surrounding medium. In this study, the full length of *bet*B gene, PfBADH, coding for BADH enzyme was isolated from *Pseudomonas fluorescens*, cloned in *Escherichia coli* and identified by study the gene sequence and expression using SDS-PAGE. The identified gene encoded a peptide of 490 amino acids. The main ORFs of PfBADH encoded protein with predicted molecular mass of 53.2 KDa (pI 4.97). Comparison of the deduced polypeptide of PfBADH with other BADH proteins revealed high similarity. The expression of the *PfBADH* gene in *E. coli* was demonstrated and detected using SDS-PAGE.

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(A)

ATGGCCCGGTTCGAACTGCAAAAACTCTACATCGACGGCGGCTACACCGATGCCGGCAGC
GACGCCACTTTCGATGCCATCAACCCGGCCAACGGTGAAGTTCTCGCACAAGTGCAGCGT
GCGACCAAGGAAGACGTTGAACGCGCCGTGGTCAGCGCCGAGAAAGGCCAGAAGATCTGG
GCCGCCATGACCGCCATGGAGCGTTCGCGCTTCCTGCGTCGCGCCGTGGAAATCCTCCGC
GAGCGCAACGACGAACTGGCCGCCCTGGAAACCCTGGACACCGGCAAGGCCTTCTCCGAA
ACCAAGTACGTGGACATCGTAACCGGCGCCGACGTGCTGGAATACTACGCAGGCCTGGTA
CCGGCCATCGAAGGCGAGCAGATCCCGCTGCGCACCACTTCCTTCGTCTACACCCGTCGC
GAGCCCCTGGGCGTAGTGGCCGGTATCGGCGCGTGGAACTACCCGATCCAGATCGCCCTG
TGGAAATCCGCTCCAGCCCTGGCGGCCGGTAACGCGATGATCTTCAAACCGAGCGAAGTG
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GTGTTCAACGTCCTGACCGGCAGCGGCCGCGAAGTCGGCACCTGGCTGACCGAGCACCCG
CGCATCGAGAAGGTCTCCTTCACCGGCGGCACCGACACTGGCAAGAAAGTCATGGCCAGC
GCTTCCAGCTCCTCGCTGAAAGACGTGACCATGGAACTGGGCGGCAAGTCGCCACTGATC
ATCTTCGACGACGCCGACCTGGATCGCGCCGCCGACACCGCCATGATGGCCAACTTCTAC
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GCTTTCGAAGCCAAGATCGCCGAGCGCGTTGCGCGCATCCGCATCGGCAACCCGGAAGAC
GAAAACACCAACTTCGGCCCGCTGGTCAGTTTCCCGCACATGGAAAGCGTGCTGGGCTAC
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ATGACCATCGTCCGTGAAGAAATCTTCGGCCCGGTCATGGCGATCCTGACCTACGAAACC
GAAGAAGAAGTGATCCGCCGCGCCAACGACACCGACTTCGGCCTGGCTGCCGGCCTGGTC
ACCAAGGATCTGAACCGGGCTCACCGCGTTATTCATCAGCTCGAAGCCGGTATCTGCTGG
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GTGGGCCGTGAGAACGGCATCAGCTCGCTGAACAACTTCACCCGCATCAAGTCGGTGCAG
GTCGAGCTGGGCGACTACGCCTTGGTGTTCTAA

(B)

MARFELQKLYIDGGYTDAGSDATFDAINPANGEVLAQVQRATKEDVERAVVSAEKGQKIW
AAMTAMERSRFLRRAVEILRERNDELAALETLDTGKAFSETKYVDIVTGADVLEYYAGLV
PAIEGEQIPLRTTSFVYTRREPLGVVAGIGAWNYPIQIALWKSAPALAAGNAMIFKPSEV
TSLTTLKLTEIYTEAGLPDGVFNVLTGSGREVGTWLTEHPRIEKVSFTGGTDTGKKVMAS
ASSSSLKDVTMELGGKSPLIIFDDADLDRAADTAMMANFYSSGQVCTNGTRVFVPSHLKA
AFEAKIAERVARIRIGNPEDENTNFGPLVSFPHMESVLGYIAKCKEEGARVLCGGERLTD
GEFAKGAFVAPTVFTDCTDDMTIVREEIFGPVMAILTYETEEEVIRRANDTDFGLAAGLV
TKDLNRAHRVIHQLEAGICWINAWGESDAKMPVGGYKQSGVGRENGISSLNNFTRIKSVQ
VELGDYALVF

Fig. (1): (A) Nucleotide sequence of the isolated PfBADH from *Pseudomonas fluorescens*, representing 1473 bp as obtained from the ABI PRISM 310 DNA sequencer, (B) The deduced 490 amino acids residues from the ORF.

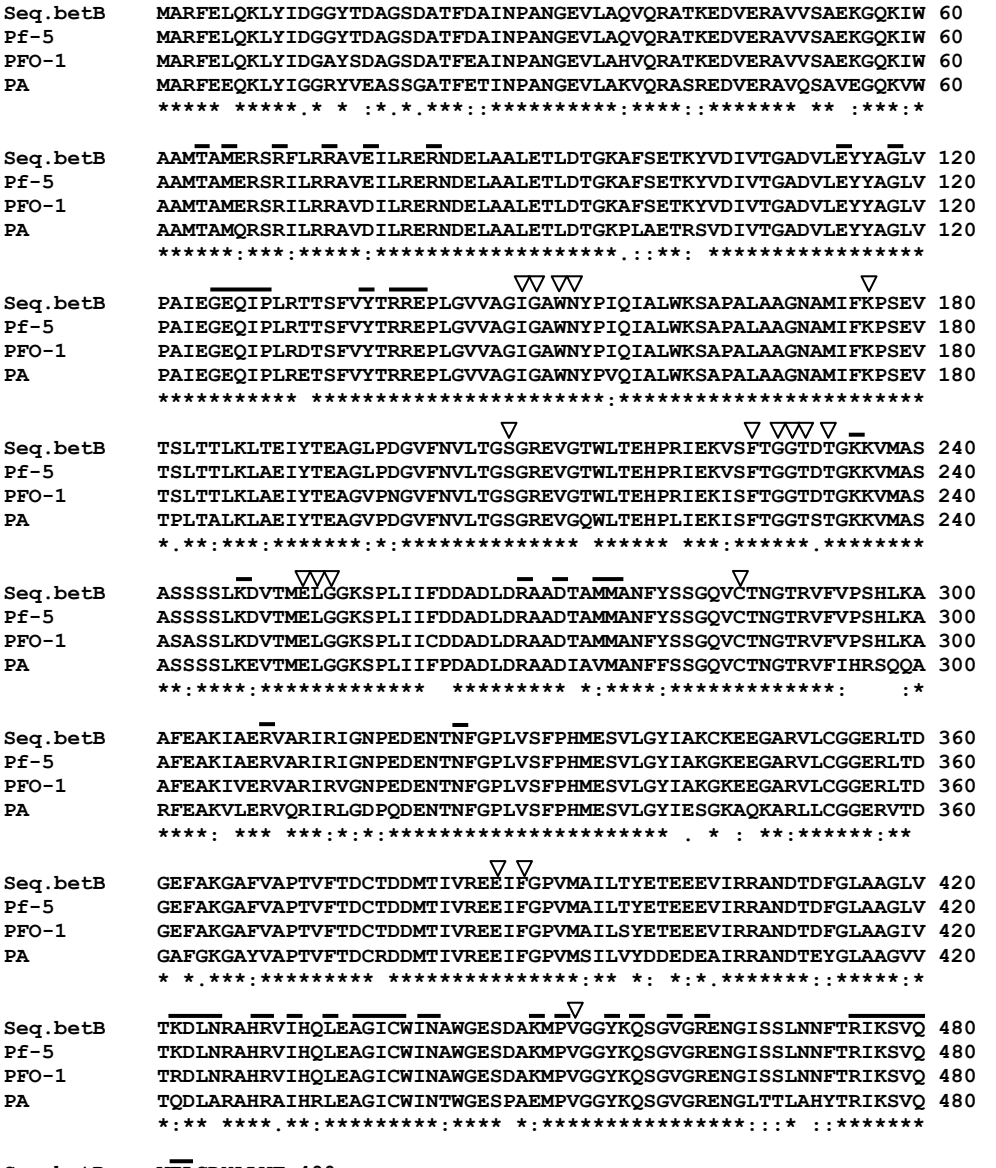

Fig. (2): The recombinant BADH sequence with conservation of 99% identity with the annotated BADH sequence of *Pseudomonas fluorescens* Pf-5 (PF-5), with conservation of 95% identity with *Pseudomonas fluorescens* PFO-1(PFO-1), and with conservation of 82% identity with *Pseudomonas aeruginosa* (PA) from the database. Triangles show the conserved amino acids for NAD binding site. Solid lines show conserved amino acids for polypeptide binding site.

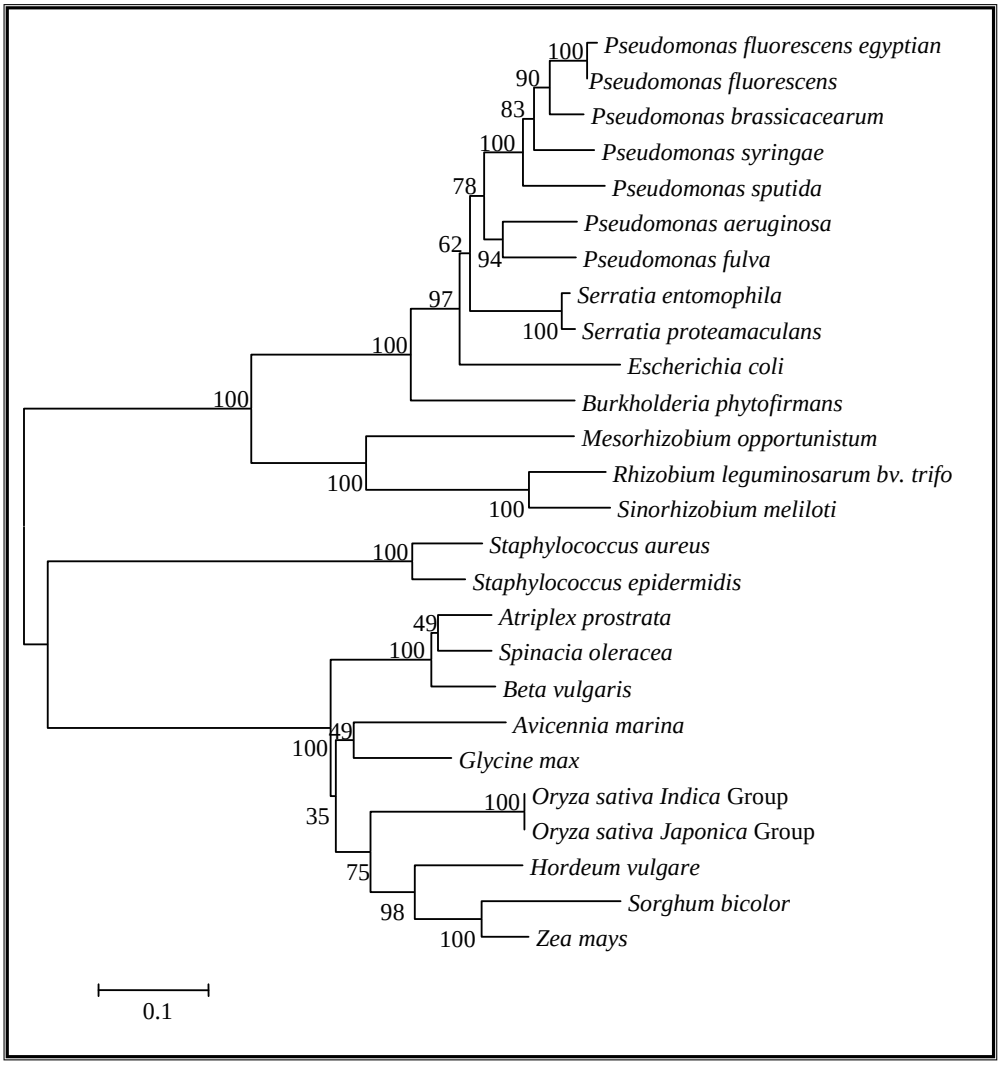

Fig. (3): Phylogenetic analysis of Betaine aldehyde dehydrogenase enzyme. A tree generated from the alignment of the amino acid sequence of BADH with those of microorganisms and plant-encoded isozymes of Betaine aldehyde dehydrogenase protein family was subjected to phylogenetic analysis.

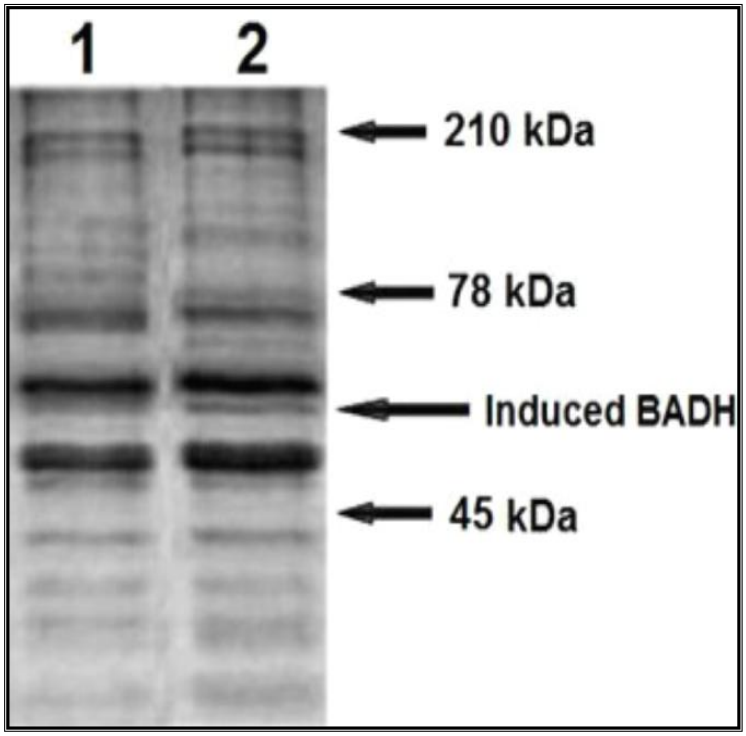

Fig. (4): Electrophoretic mobility of total protein extracted from control *E. coli* and transformed *E. coli* bacteria. The total protein was separated using SDS-PAGE electrophoresis and stained with commassie brilliant blue stain.
Lane (1): Control transformed *E. coli* total protein pattern;
Lane (2): IPTG induced transformed *E. coli* total protein pattern (~ 55kDa BADH).