MOLECULAR CLONING AND PROTEIN MODELING CONFIRM PRESENCE OF AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) DEAMINASE GENE IN *Bacillus subtilis* EG21 STRAIN

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Bacteria associated with plants employ various tactics and possess several specific genes that enable them to communicate and interact metabolically with plant tissues (Checcucci *et al*., 2017). One of the most widespread genes found in the genomes of bacteria associated with plants is the *acdS* gene, which encodes the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Nascimento *et al*., 2014). This gene is believed to play a role in cleaving ACC, the precursor of the plant stress hormone ethylene. Ethylene is considered key regulator of plants modulating both their developmental processes and responses to environmental stresses. The latter has been reported to be responsible for reducing crop yields by as much as 50% (Lamaoui *et al*., 2018). It has been repeatedly shown in various crops that ethylene biosynthesis is significantly induced during plant exposure to environmental stresses such as salinity and drought.(Fatma *et al*., 2022). Ethylene-mediated stress responses have been described and linked to interactions with numerous stress-related effectors such as reactive oxygen species (ROS), ion transporters, osmolytes, and other cellular compounds regulating various and complex biosynthetic pathways (Tao *et al*., 2015).

Plant growth promoting rhizobacteria (PGPR) are the diverse collection of rhizosphere bacteria that encompass the ability to enhance plant growth and/or yield through wide variety of direct or indirect mechanisms. Their ability to produce ACC deaminase metabolizing ACC (the immediate precursor of ethylene) into alpha-ketobutyrate and ammonia and hence reduce plant-ethylene levels has been well documented (Saleem *et al*., 2007).

ACC deaminase (*acdS*) genes are widely distributed among various bacterial species. While the enzyme was first identified in plant growth-promoting rhizobacteria (PGPR), it has since been found in a diverse range of bacteria, including Pseudomonas, Bacillus, Enterobacter, Azotobacter, and Rhizobium (Anand *et al*., 2021; Gowtham *et al*., 2020; Ma *et al*.,

2003; Sahoo *et al*., 2013 and Saravanakumar and Samiyappan, 2007). It's important to note that *acdS* genes are not exclusive to PGPR. They can be found in various bacterial taxa, highlighting the versatility of this trait in promoting plant growth and stress tolerance (Nascimento *et al*., 2014).

ACC deaminase genes are typically expressed in response to plant-derived signals or environmental stress allowing bacteria to produce this enzyme when it is most needed to promote plant growth and stress tolerance. Bacteria can sense plant-produced molecules, such as ACC or other signaling compounds, which can induce the expression of *acdS* genes. Similarly, stress conditions, such as drought, salinity, or nutrient deficiency, can trigger the up-regulation of *acdS* genes in bacteria (Naing *et al*., 2021). The specific mechanisms regulating ACC deaminase gene expression may vary among different bacterial species. However, it is generally believed that two-component signaling systems, quorum sensing, and transcriptional regulation play important roles in controlling the expression of these genes (Gupta and Pandey, 2019; Jung *et al*., 2020 and Nascimento *et al*., 2014).

The ACC deaminase gene has been a subject of intensive research due to its potential applications in agriculture and plant biotechnology. In recent years, significant advancements have been made in understanding and exploiting this gene. Researchs in this field have focused on developing bacterial strains with higher acdS activity and broader substrate specificity. This has involved introducing/transfer of the *acdS* gene into new strains, and/or enhancing gene expression through genetic engineering techniques, such as overexpression or optimization of the *acdS* gene (Ma *et al*., 2004; Wang *et al*., 2000). Furthermore, multi-trait engineering is another strategy in which ACC deaminase genes have been combined with other beneficial traits, such as nitrogen fixation, phosphate solubilization, and biocontrol abilities, to create multifunctional microbial inoculants (Thomas-Barry *et al*., 2024). Moreover, innovative delivery systems, including immobilized formulations and nanotechnology, have been explored to optimize the application of ACC deaminase-producing bacteria (Chanratana *et al*., 2019 and Conde-Avila *et al*., 2022). Field trials and greenhouse experiments have consistently demonstrated the positive impact of these bacteria on crop yields and stress resilience. The ability of ACC deaminase to reduce ethylene levels, promote nutrient uptake, and enhance plant defense mechanisms has positioned it as a valuable tool for sustainable agriculture and crop improvement.

Previous research on ACC deaminase has encompassed both *in vitro* and *in silico* analyses. The *in-silico* studies have been instrumental in elucidating the genetic and structural characteristics of ACC deaminase genes and proteins (Pramanik and Mandal, 2022; Singh and Kashyap, 2012 and Singh *et al*., 2015). By analyzing the nucleotide and amino acid se-

quences of ACC deaminase genes, researchers have identified conserved domains and motifs essential for enzyme activity. Additionally, protein modeling techniques have been employed to predict the three-dimensional structure of ACC deaminase proteins, providing insights into their potential interactions with substrates and other molecules. These *in silico* analyses have laid the foundation for understanding the molecular mechanisms underlying ACC deaminase function and have guided the development of strategies for enhancing its activity.

To investigate the presence of the *acdS* gene in the bacterial *B. subtilis* EG21 strain, a multi-faceted approach was employed. Initially, microbiological techniques were utilized to isolate and cultivate pure cultures of the strain and qualitatively test for the *acdS* gene activity. Subsequently, genomic DNA was extracted and subjected to PCR amplification using primers targeting the *acdS* gene. The amplified PCR product was then sequenced to obtain the nucleotide sequence. *In silico* analysis of the obtained sequence involved several steps. First, a BLAST search was conducted against public databases to identify homologous sequences and confirm the presence of the *acdS* gene. Second, the deduced amino acid sequence was compared to known acdS proteins to assess sequence similarity and identify conserved domains. Third, protein modeling techniques were employed to predict the three-dimensional structure of the acds protein, allowing for a better understanding of its potential function and interactions with other molecules. By combining these microbiological and computational approaches, the presence of the *acdS* gene in B. subtilis EG21 was confirmed. This comprehensive analysis provides strong evidence for the strain's ability to produce acdS and suggests its potential for promoting plant growth and stress tolerance.

Therefore, in summary this work was undertaken to confirm the presence of ACC deaminase gene (*acdS*) and its activity in the bacterial EG21 strain for further and future studies related to its use to mitigate the deleterious effect of environmental stress on important crops' growth and productivity.

MATERIAL AND METHODS

1. Bacterial strain and culture condition

B. subtilis EG21 strain used in this study was originally isolated from the rhizosphere of cotton plants cultivated in farmland saline soil in Baltim, Kafr El Sheikh Governorate, and northern coast of Egypt. Strain EG21 was identified as *B. subtilis* according to its molecular sequence analysis as well as morphological and biochemical tests (Alfiky *et al*., 2022). This strain was a part of a bigger collection of plant growth promoting and salt stress alleviating rhizobacteria (unpublished data).

2 ACC deaminase activity assay

The ability of rhizobacterial isolates to produce ACC deaminase was qualitatively examined on nitrogen-free minimal medium supplemented with a 3 mM of ACC (Sigma-Aldrich, product No. A3903-250MG) as the sole of nitrogen source. Briefly, bacterial EG21 strain was cultured in the LB medium until overnight, harvested by centrifugation, washed thrice with a 0.1 M of Tris-HCl (pH 8.0) and inoculated on a nitrogen free medium (g/L: 20 g sucrose; 1.0 g K_2HPO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.5 g NaCl; 0.1 g $FeSO_4$ 0.1; 0.005 g Na_2MoO_4; 2.0 g $CaCO_3$ and 15 g agar). Plates with the medium supplemented with (0.2 % w/v) ammonium sulfate as a nitrogen source were served as a positive control. Plates were spot inoculated with a 10 µL of bacterial strain (OD_{600}=1), sealed with parafilm and incubated for 72 h at 28 °C. Bacterial growth was compared between ACC-supplied plates with positive and negative control plates to determine the strain's ability to use ACC when supplied as the sole of nitrogen source in the medium.

3 Polymerase chain reaction (PCR) amplification of ACC deaminase gene (*acdS*)

For identification of the gene encoding ACC deaminase enzyme in the bacterial EG21 strain, DNA was extracted followed by PCR amplification. Bacterial genomic DNA was extracted using GenElute bacterial genomic DNA Extraction Kit (Sigma Aldrich) and confirmed through agarose gel electrophoresis. The forward and reverse primers used for amplification of the *acdS* gene in bacterial EG21 strain were acdS-F GCCAA(G/A)CG(G/T/C)GA(G/A/C)GA CTGCAA) and acdS-R TGCAT(G/C)GA(T/C)TTGCC(T/C)TC (Jha *et al*., 2012).

The PCR reaction was performed in a 25-µl of reaction mixture composed of a 12.5 µl of AccuStart II PCR SuperMix (2x), a 5 µl gDNA, a 1 µl of forward and reverse primers, a 0.5 µl of gel loading dye, and a 5 µl of sterile deionized water. Thermal amplification was performed using initial denaturation step at 94°C for 180 sec., 35 cycles of amplification consisting of i) denaturation at 94°C for 60 sec.; ii) annealing at 54°C for 60 sec.; and iii) primer extension at 72°C for 120 sec, followed by a final extension step at 72°C for 10 min. Obtained PCR product was visualized on 1% agarose gel amended with with peq GREEN DNA dye in 1x Tris-acetae-EDTA buffer. The final outcome was visualized under an UV light using gel documentation system.

The multiple bands observed in the gel electrophoresis image indicated the presence of multiple DNA amplicons and therefore, PCR products could not be directly sequenced. The amplicon in the correct molecular size range (700-800 bp) in reference to the DNA ladder was excised from the gel using scalpel and sharp sterile surgical blade, cleaned with E.Z.N.A.® Gel Extraction Kit (Omega BIO-TEK) following the manufacturer instructions.

4 Cloning of amplified ACC deaminase gene into expression vector

The purified of gel-extracted amplicon for *acdS* gene was then inserted onto the linearized plasmid vector (pCR™4-TOPO®) which features were single, and overhanging 3′ deoxythymidine (T) residues allowing *Taq* polymerase-amplified PCR products to be inserts and ligated efficiently with the vector. The *acdS* gene was cloned using TOPO® TA Cloning® Kits for Sequencing (Invitrogen) following the Kit manufacturer instructions.

5 Transformation of E. coli with the expression vector

The plasmid pCR™4-TOPO® with the ligated *acdS* gene was thereafter used to transform One Shot® TOP10 competent *Escherichia coli* cells following the Kit manufacturer instructions. Briefly, a two µL of cloning reaction containing the plasmid with inserted fragment was added into One Shot® TOP10 competent *E. coli* cells, incubated on ice for 30 min, heat-shocked at 42°C for 30 sec; and 250 µL of S.O.C. medium was added for the recovery of *E. coli* competent cell to maximize the transformation efficiency.

6 Screening and confirmation of successful transformation

Using sterile glass beads, the competent cells were spread on freshly prepared plates containing LB medium amended with a 50 µg/mL of kanamycin for selection of successful transformants. Upon successful transformation, three colonies were independently picked and cultured overnight in LB medium amended with a 50 µg/mL of kanamycin.

The plasmids were isolated from the overnight cultured cells using Zymo-PURE™ Plasmid Miniprep Kit (Zymo Research) following the manufacturer instructions. Restriction digestion with EcoR I was performed on isolated plasmids to confirm successful transformation and the presence of inserted *acdS* gene fragment. Briefly, the analytical restriction digestion was performed in a 20 µL reaction containing a 16 µL of nuclease-free water, a 2 µL of 10x Buffer EcoR I, a 1 µL of plasmid DNA (1µg/µL), and a 1 µL of EcoR I (Thermo Fisher Scientific). The beforementioned ingredients were gently mixed, spun down for a few seconds in a microcentrifuge, and incubated at 37°C for one hour using Eppendorf Thermomixer C (Eppendorf, Germany). For enzyme inactivation, the reaction mixtures were incubated at 65°C for 20 min. Following the digestion, a 3-4 µL of loading dye was added before proceeding to gel electrophoresis analysis.

7 DNA sequencing and sequence analysis

Plasmids were recovered from three independent and verified insert-containing colonies and sequenced with the help of sequencing primers provided in the cloning Kit using BigDye Terminator Cycle Sequencing Ready Reaction Kit version (PE Applied Biosystems, USA). The reactions were performed in an auto-

mated sequencer (ABI Prism 310 Genetic Analyzer, PE Applied Biosystems). Obtained sequences were analyzed for homology with translated amino acid sequences using the basic sequence alignment BLAST program to in the publicly available National Centre for Biotechnology Information database [website http://www .ncbi.nlm.nih.gov/BLAST]. Translation of the obtained gene sequence and prediction of deduced protein was performed with EXPASY tools (http://www.au.expasy.org/).

Alignment and phylogenetic reconstructions were performed using the function "build" of the Environment for Tree Exploration (ETE) computational framework (Huerta-Cepas *et al*., 2016) as implemented on the GenomeNet (https://www. genome.jp/tools/ete/). ML tree was inferred using PhyML v20160115 (Guindon *et al*., 2010) and Branch supports are computed out of 100 bootstrapped trees. Similarity percentage was calculated using the basic sequence alignment BLAST program against the NCBI database [website http://www.ncbi.nlm.nih. gov/BLAST].

8 Molecular modeling and model evaluation

The deduced protein sequence from EXPASY was used as the building block (target) for the modelling of three-dimensional structure of ACC deaminase protein based on sequence homology to previously described structures using SWISS-MODEL web server (https://swissmodel.expasy.org/). The process for building the 3D model of our cloned gene started with using the target amino acid sequence as query to search for available template proteins structures against the SWISS-MODEL template library SMTL (Biasini *et al*., 2014). Possible templates were ranked according to the anticipated score qualities of resulting models, specifically GMQE and QSQE that identified as Global Model Quality Estimate and Quaternary Structure Quality Estimate, respectively (Biasini *et al*., 2014 and Waterhouse *et al*., 2018). The target sequence was also examined using BLAST Protein tool at the NCBI to confirm the selection of the best possible template protein featuring highest sequence identity and coverage. The selected template was also examined in RCSB Protein Data Bank (https://www.rcsb.org/). Modeling of the protein was carried out using SWISS-MODEL ProMod3 modelling engine on the web server based on the alignment between our target sequence and the best matching protein template.

The quality and accuracy of the 3D structure of the obtained protein model was evaluated by Global Model Quality Estimate (GMQE) score (Waterhouse *et al*., 2018). The PyMOL Molecular Graphics System (Schrödinger, LLC) was used to visualize and analyze the generated three-dimensional model structure. The Root Mean Square deviation (RMSd) score was measured using PyMOL software and used as a parameter to measure the fitting of the carbon backbone of both

target and template models. Ramachandran plot statistics showed the allowed and disallowed regions of torsion angle values that were used for the assessment of the quality of modeled protein 3D structures (Vaseeharan *et al*., 2012).

RESULTS AND DISCUSSION

Qualitative examination confirmed the ability of EG21 strain to produce ACC deaminase as shown in Fig. (1), which prompted the need for further examination using molecular and bioinformatic tools to confirm this ability.

1.Amplification of *acdS* gene from *B. subtilis* EG21 strain

The PCR reaction used for the amplification of the targeted 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene (*acdS*) in the bacterial *B. subtilis* EG21 strain was performed. The obtained results from the PCR reactions exhibited several amplicons of different sizes including one at the expected size range (700-800 bp) in reference to the DNA ladder (Fig. 2, A). Several attempts were carried out to optimize PCR reaction conditions by changing the annealing temperature and/or the extension time to eliminate the problem of non-targeted amplifications. However, when this problem was not resolved through optimization of the PCR reaction conditions, we resorted to cut the band corresponding to the expected size from the gel followed by molecular cloning and sequencing to confirm the presence of this gene.

2. Description of successful cloning of ACC deaminase gene

The gel-band corresponding to the expected molecular size was cut from the gel and cleaned before proceeding with gene cloning. The cloning vector (Fig. 2, D) used to clone the *acdS* gene in this study was contained two antibiotic resistance genes (kanamycin and ampicillin). Successful and efficient ligation of the amplified fragment into the cloning vector was achieved due to the presence of overhanging 3´ deoxythymidine (T) residues in the plasmid cloning sites and the presence of a single unpaired deoxyadenosine (A) to the 3´ ends of the PCR products. These latter (A) residues are the result of the non-template-dependent terminal transferase activity of the *Taq* polymerase used in the PCR reactions (Horn, 2005). Following successful insertion of amplified gene into plasmid vectors, these latter were introduced into *E. coli* competent cells.

Transformed *E. coli* competent cells were plated on the LB agar plates amended with the antibiotic kanamycin. Only bacterial cells that have successfully up-taken the recombinant plasmids were able to grow and thrive in the presence of kanamycin in the growth medium (Fig 2, B). On other hand, non-transformant cells were not able to survive due to the absence of the kanamycin resistance gene in their genetic makeup. Several independent colonies were picked and independently propagated for plasmid recovery.

In order to verify successful insertion of the *acdS* gene fragment into the plasmid, restriction digestion was performed with the restriction enzyme EcoR I and the result (Fig. 2, C) confirmed the presence of DNA band at the expected size. The analytical restriction digestion resulted in two bands on the gel with molecular weights of ~750 bp and 4.0 kb corresponding to the inserted amplicon and the plasmid, respectively. The recombinant plasmids were recovered and subjected to DNA sequencing.

3.ACC deaminase gene sequencing and alignment analysis

Band of ACC deaminase gene in expected size (700-800 bp) was amplified from *B. subtilis* EG21 strain using primer designed on the basis of the consensus regions of known ACC deaminase genes. Sequence analysis of amplified band showed that detected sequence is 729 bp length and encodes for an open reading frame (ORF) of a 600 nucleotide and precursor protein of a 200 amino acids on the first frame of the positive strand from nucleotide position 130 to 729 (Fig. 3). The conserved domain of pyridoxal phosphate dependent enzyme was detected in the characterized sequence with e-value 1.54e-133 using online conserved domain detector on the (NCBI) site [https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi]. Activity of this catalytic residue catalyzes the conversion of 1-aminocyclopropane-L-carboxylate (ACC), a precursor of the plant hormone ethylene, to alpha-ketobutyrate and ammonia-pyridoxal. Identified sequence was deposited in NCBI database (Ac. OQ409922.1).

Sequence analysis of the amplified band with a 729 bp in length using the basic sequence alignment BLAST program against the NCBI database [website http:// www.ncbi.nlm.nih.gov/BLAST] revealed marked sequence similarities with closely related reference of *acdS* genes sequences from different bacterial species (Fig. 4).

Alignment analysis revealed that the highest similarity (99.59%) was detected with *asdS* gene sequence of *Bacillus subtilis* strain MBPSB207 (Ac. JQ995371.1) followed by 96.01% with *asdS* gene from *both of Klebsiella pneumoniae* (Ac. JN625725.1) and *Pseudomonas putida* strain AM15 (Ac. EF011160.1) and by 95.46 % with *Bacillus cereus* (Ac. JN625726.1). Phylogenetic analyses of the *acdS* isolated sequence and the closely related reference sequences were placed and the compared sequences in two main clusters with a 100% bootstrap support value. The acdS sequence of *B. subtilis* EG21 strain was placed as a sister group to the sequence of *Bacillus subtilis* strain MBPSB207, with a 100% bootstrap support value (Fig. 5). This relationship was supported with the high sequence similarity (99.59%).

a. Protein modelling between ACC deaminases from EG21 strain and *Cyberlindnera saturnus*

Aligning the target protein sequence with the selected template (1J0E)

from the PDB was conducted to determine how closely and globally aligned the carbon atoms in the two models (Fig. 6). The obtained results revealed that the executive root mean square deviation (RMSd) between the positioning of the carbon atoms in the target protein model and 1J0E was 0.078. The lower the RMSd value when closer to zero, indicate that these two structures were more closely related , which suggested that the target was well aligned with 1J0E.

The generated 3D model showed series of α-helices of both protein models (EG21 in green while 1J0E in Turquoise) featuring high similarity value between the two models with the exception that 1J0e was crystallized as crystallographic dimer, while EG21 was as a monomer. Furthermore, since we have shown that the positioning of the carbon atoms backbone between the two models were very similar globally, further alignment at key residues level between the two models was equally important. The key active site motifs in 1J0E protein model were pinpointed from the primary citation reporting its crystal structure (Ose *et al*., 2003). Comparing and visualizing the sequences at the pinpointed key active sites of the two models in PyMOL molecular visualization system revealed identical motifs. Such feature is a high importance since this active site is highly conserved in this class of enzymes (Nascimento *et al*., 2014).

b. Model validation

Homology modeling of the ACC deaminase deduced through cloning from EG21 strain was carried out using SWISS-MODEL web server powered with ProMod3 modeling engine. The stereochemical validation of the generated model using Ramachandran conformation map was investigated to evaluate its structural rationality. Structure assessment of the generated model through Ramachandran plot (Fig. 7, A) showed that 93.16% of the amino acids were positioned in the favored region. Furthermore, additional plot statistics revealed that Mol Probity score=1.62, Clash Score=3.92, Ramachandran Outliers=0.85%, Rotamer Outliers=0.79%, C-Beta Deviations=1, Bad Bonds=1/3640, Bad Angles=35/4916, and Cis Non-Proline=3/450 reflected good fitting features of the generated model. QMEAN Z-score was reported to be -1.14 for the generated model indicated that the proposed homology model was in the reliable and accepted range. QMEAN Z-score has been described as a useful parameter providing an estimation for the "degree of nativeness" of the structural features obtained in the model (Benkert *et al*., 2011). It has been reported that QMEAN Z-scores are reported in the range from zero (denoting excellent quality) to -4 (or below, denoting models with low quality) (Santhoshkumar and Yusuf, 2020).

Also, when pointed that having over 90% of the amino acid residues of the generated model in the favored region is a characteristic of a fitting model of

good quality (Vaseeharan *et al*., 2012). Therefore, we can conclude that our generated model based on the target protein is of a good quality.

The "Local Quality" provide a score value for each residue of the model (*x*-axis) and the expected similarity to the selected template model (*y*-axis). It has been reported that residues with a score above 0.6 to be of acceptable quality (Santhoshkumar and Yusuf, 2020). As illustrated in Fig. (7, B), most of the residues featured local quality estimates above 0.7 indicative of reliable modeling. Finally, the GMQE value of 0.85 and QMEAND (Co Global score) of 0.82 ± 0.05 (Fig. 7, C) confirmed the reliability of the generated model and affirm its good quality. Overall, the 3D structure of the modeled protein had met and satisfied the requested threshold for model quality and reliability.

SUMMARY

The aim of this work was to confirm the presence of ACC deaminase gene in the plant growth promoting and salt-stress ameliorating bacterial EG21 strain isolated from cotton plants in salt affected soil. TheEG21 Strain was qualitatively evaluated for its ACC deaminase activity. The ACC deaminase gene (*acdS*) was amplified from extracted genomic makeup and cloned into pCR™4-TOPO® plasmid cloning vector. The recombinant plasmids were used to transform *E. coli* competent cells which were screened for successful transformants. Recombinant plasmids were recovered and used for sequence analysis. The obtained sequence of the *acdS* gene as well as the deduced amino acid sequence of the corresponding protein were analyzed using the Basic Local Alignment Search Tool (BLAST) that available in the GenBank databases for nucleotide and protein sequences. Bioinformatic tools were used to model the *acdS* gene. Finally, the 3D structure of the modeled protein had met and satisfied the requested threshold for model quality and reliability.

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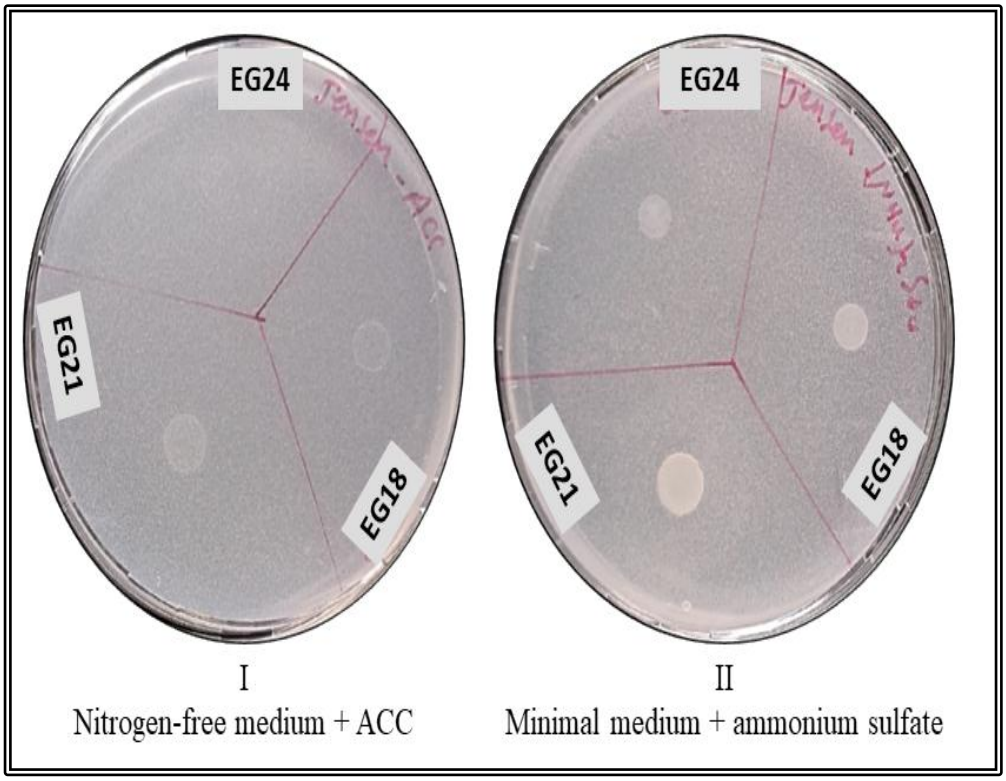

Fig. (1). Qualitative assessment of ACC deaminase activity of bacterial strains. (I), growth of different bacterial strains including strain *B. subtilis* EG21 on minimal medium (MM) supplemented with ACC as the sole nitrogen source in the medium confirms its ability of to produce ACC deaminase (I) in comparison to bacterial strains growing on MM supplemented with ammonium sulfate as nitrogen source.

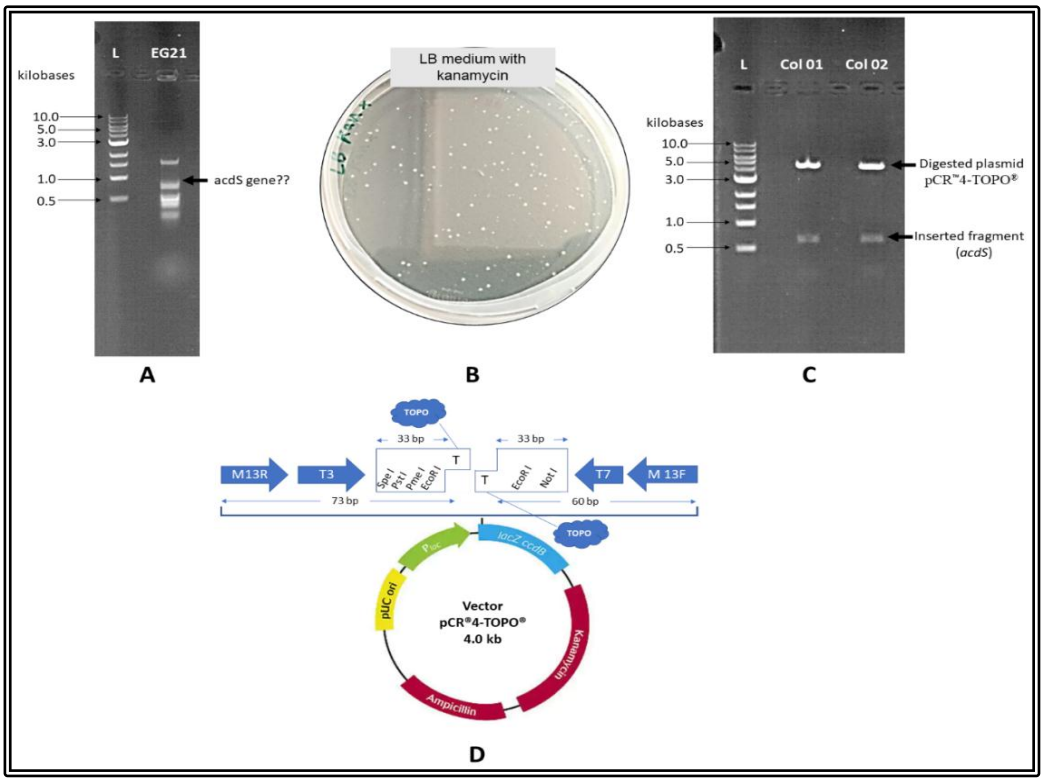

Fig. (2). Key steps in cloning of ACC deaminase gene from strain *B. subtilis* EG21. (A), PCR amplification of ACC deaminase gene; (B), successful *E. coli* transformants grown on selective medium (LB with 50 µg/mL kanamycin); (C), restriction digestion of recovered plasmid with enzyme EcoR I confirmed successful insertion of cloned fragment; and (D), map of the cloning plasmid used in this study..

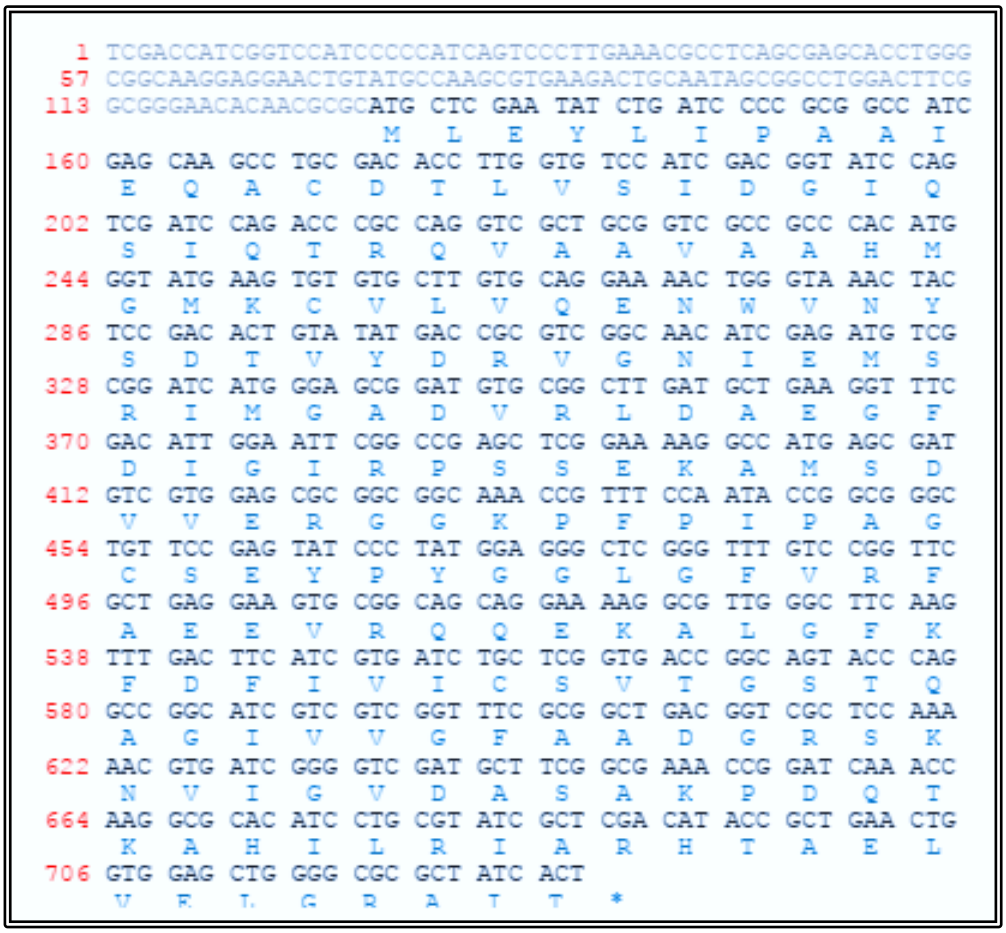

Fig.(3) . Nucleotide sequence and deduced amino acid sequence of characterized *asdS1* gene isolated from *B. subtilis* EG21 strain using primer designed based on the consensus regions of known ACC deaminase genes. Numbers on the left indicate nucleotide positions. The deduced amino acid sequence is shown in single-letter code below the nucleotide sequence. The asterisk denotes the translation stop signal.

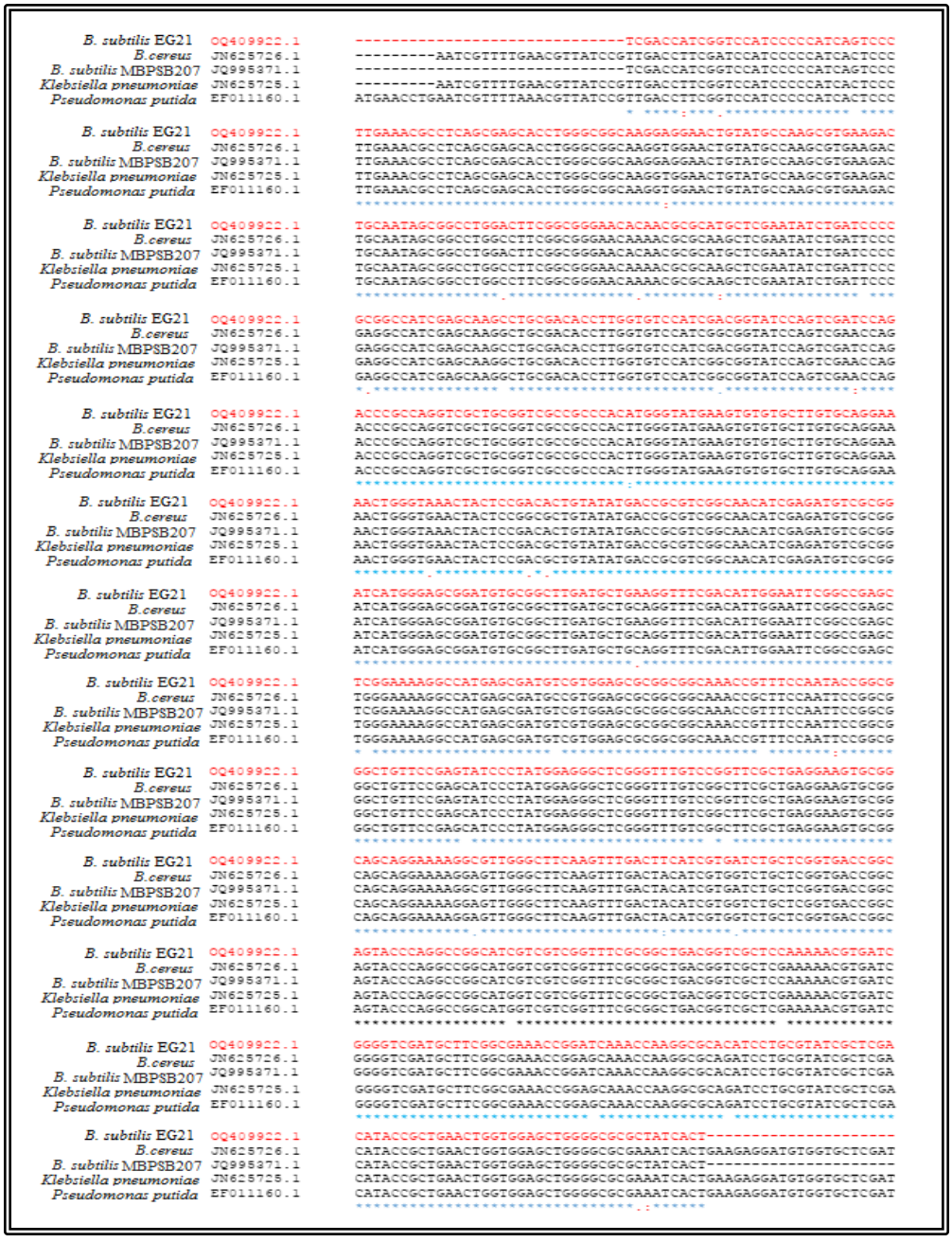

Fig. (4). Optimized alignment of ACC deaminase gene (*acdS1*) isolated from *B. subtilis* EG21 strain with respect to most closely related *acdS* genes from *Bacillus subtilis* strain MBPSB207 (Ac. JQ995371.1), *Klebsiella pneumoniae* (Ac. No JN625725.1), *Pseudomonas putida* strain AM15 (Ac. EF011160.1), *Bacillus cereus* (Ac. JN625726.1). high similarity within the conserved regions is marked with blue asters, Gaps are marked with dashes.

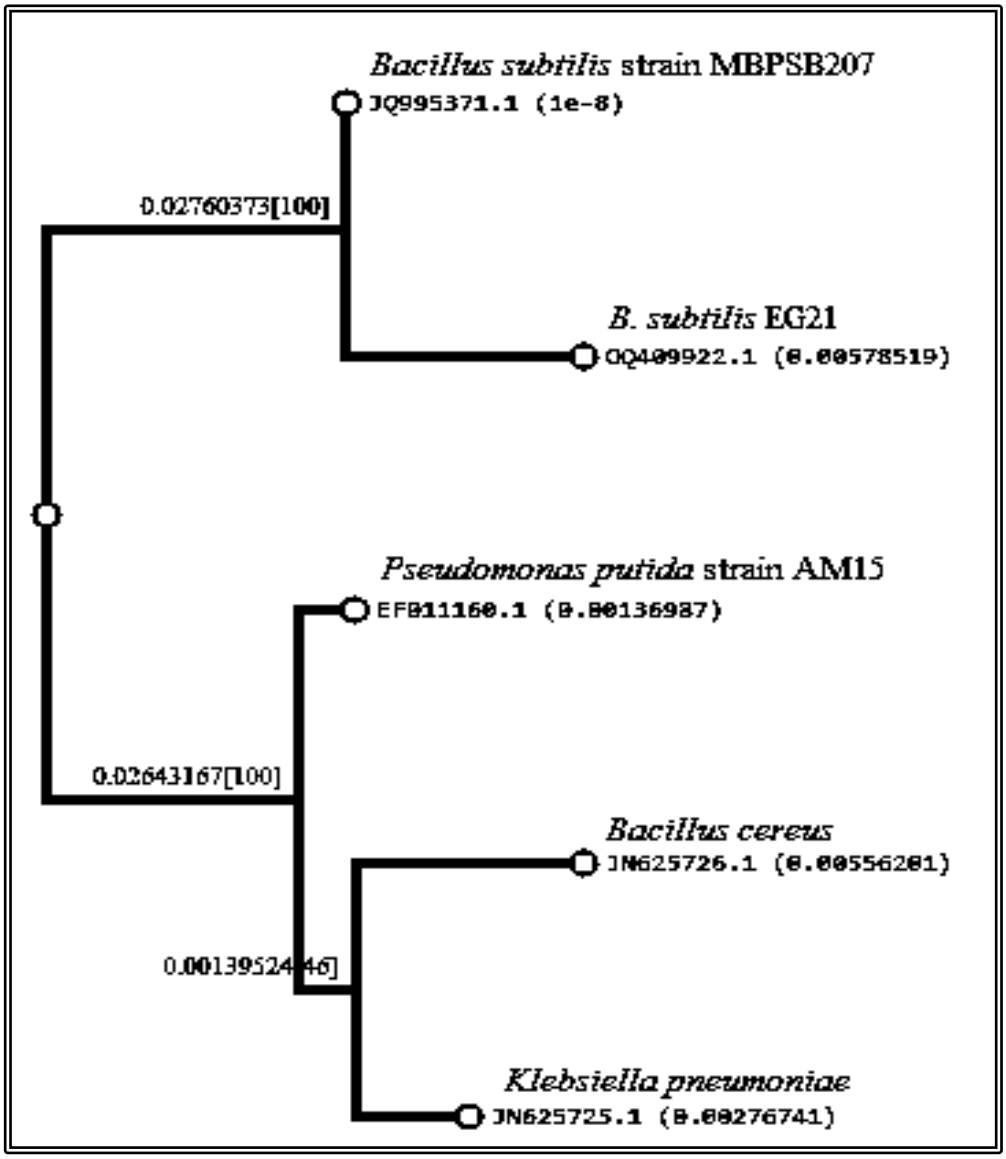

Fig. (5). Phylogenetic tree showing the relationships of the ACC deaminase gene (acdS1) isolated from *B. subtilis* EG21 strain with respect to the acdS genes of other bacteria. The tree was inferred using using PhyML v20160115. The numbers at branching points refer to bootstrap values based on 100 replicates.

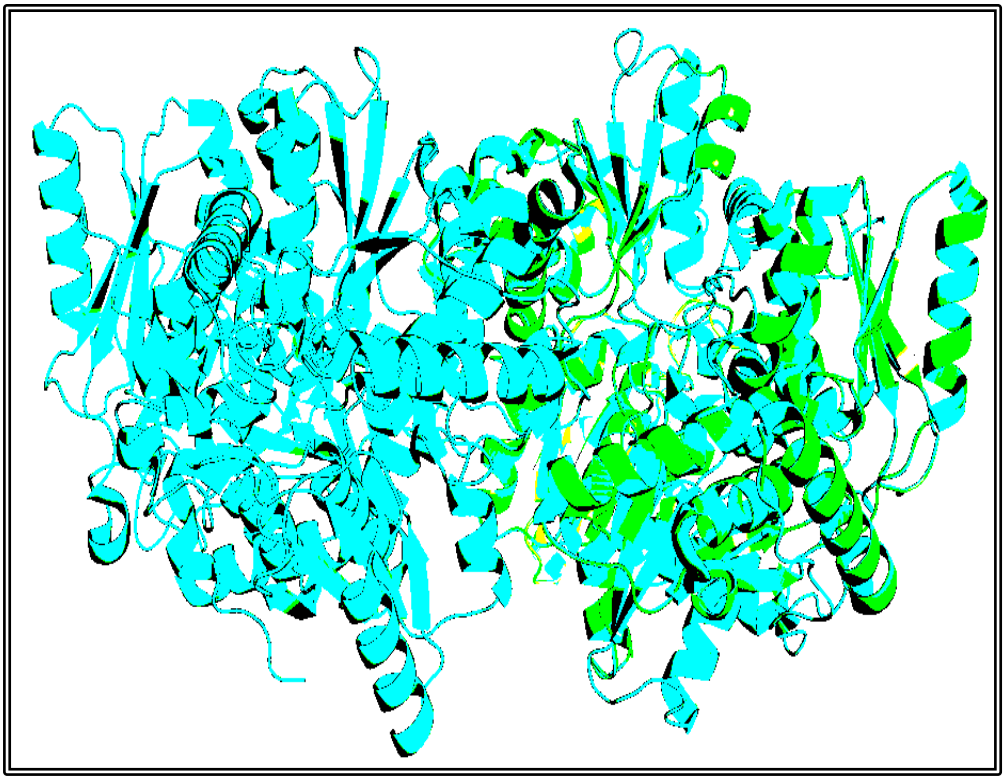

Fig. (6). Protein modelling of cloned ACC deaminase gene from *B. subtilis* EG21 aligned to the crystal structure of the ACC deaminase (ID=1J0e) from the yeast *Cyberlindnera saturnus* retrieved from Protein Data Bank (PDB).

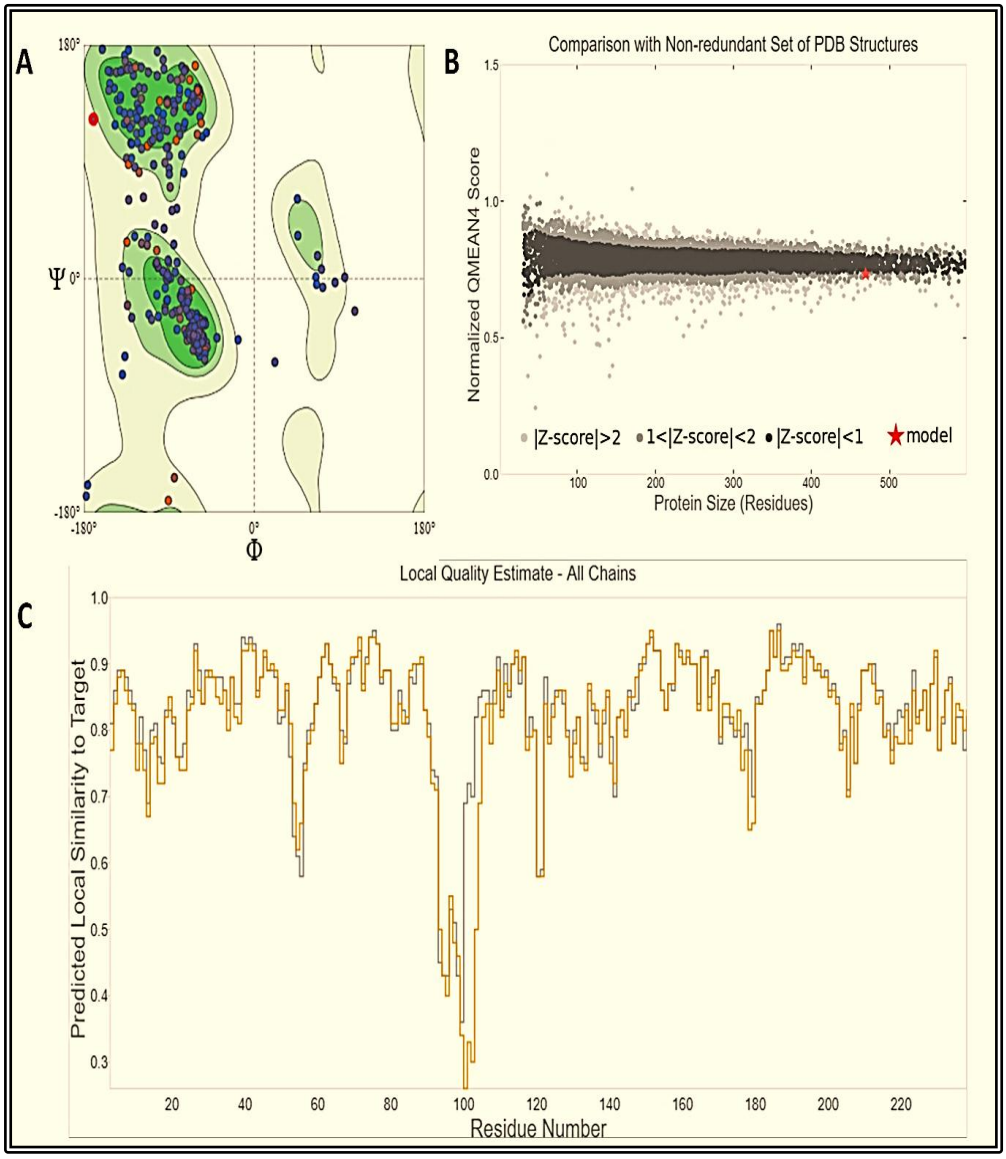

Fig. (7). Structure and quality assessment of the generated model. (A), Ramachandran plot showing that 93.16% of the amino acids are positioned in the favored region. (B), Comparison with a non-redundant set of PDB structure, protein size residue. (C), Local quality estimation showing the score of selected template predicted local similarity to target (*y*-axis) plotted against the target protein residue number (*x*-axis) for both protein chains A (blue) and B (red).