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Research Article

Cloning, Prokaryotic Expression and Functional Characterization of *NifH* Gene from the Associative Nitrogen-Fixing Bacteria *Klebsiella Variicola* DX120E

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Background: Biological nitrogen fixation (BNF) is a unique mechanism in which microorganisms utilize the nitrogenase enzyme to catalyze the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). Fe protein, encoded by the *nifH* gene, is an essential component of the nitrogenase in *Klebsiella variicola* DX120E. However, the function of this gene in regulating nitrogen fixing activity is still unclear.

Objectives: The objective of this study was to reveal the function of *nifH* gene in associative nitrogen-fixing bacteria *Klebsiella variicola* DX120E and micro-sugarcane system by immunoassay and gene editing.

Materials and Methods: In the current investigation, the *nifH* gene was cloned in a pET-30a (+) vector and expressed in *Escherichia coli*. The NifH protein was purified and used to immunize rabbit, and then the serum was collected and purified to obtain rabbit anti-NifH polyclonal antibodies. The CRISPR-Cas9 system was applied to produce *nifH* mutant strains, and the nitrogen-fixing enzyme activity, gene, and protein expression were analyzed.

Results: Both *in vitro* and *in vivo* NifH proteins were detected by Western blotting, which were 43 and 32 kDa respectively. The expression of *nifD* and *nifK* genes was decreased, and nitrogenase activity was reduced in the *nifH* mutant strain.

Conclusion: The *nifH* gene mutant weakened the nitrogenase activity by regulating the expression of Fe protein, which suggests a potential strategy to study the nitrogen fixation-related genes and the interactions between endophytic nitrogen-fixing bacteria and sugarcane.

Keywords: Antibody, Fe protein; *Klebsiella variicola* DX120E, Knockout, Nitrogenase, Prokaryotic expression

1. Background

Biological nitrogen fixation (BNF) refers to the process in which atmospheric N (N_2) is reduced to ammonia (NH_3) molecule (1). BNF has been classified into rhizobium-legume symbiotic nitrogen (N) fixation, associative N fixation (ANF), and autogenous N fixation system. To reduce the application of chemical fertilizers and environmental pollution, BNF has received high attention (2) recently. Various of endophytes associative N fixation bacteria have been reported to promote

sugarcane growth, which are identified to be a valuable source of nitrogen for sustainable crop production as well as to maintain soil fertility (3).

N-fixing microorganisms have shown diversity in different environments. The diazotroph observations are often characterized in terms of measured in-situ abundance of the *nifH* gene, which is an essential gene in nitrogen fixation, encoding a structural component of the nitrogenase enzyme (4-5). The reduction of dinitrogen by Mo-dependent nitrogenase passes through the transient interaction of two-component proteins known as Fe protein and MoFe protein under the two-component regulatory system. Fe-protein has two identical subunits connected via the 4Fe-4S cluster and a γ_2-dimer composed of identical subunits encoded by *nifH* (6). Nitrogen fixation genes expression in foreign hosts such as *Escherichia coli* and yeast was achieved in 1985 (7). *Klebsiella* spp. is one of the N-fixing species isolated from sugarcane, and some studies have reported the NifH protein exists in *Klebsiella pneumoniae* (8). However, the *nifH* gene in *Klebsiella variicola*, a novel species associated with plant (9-10), is less reported.

Klebsiella variicola DX120E is an endophytic BNF bacteria isolated from the roots of sugarcane, which has a wide range of characteristics, including N fixation, solubilization of mineral phosphates, production of siderophore, secretion of growth hormone, with positive effects on sugarcane growth. ^{15}N isotope dilution assay has proven that DX120E strain successfully fixed N_2 in the sugarcane variety ROC22 (11), but the mechanism of the interactions between sugarcane and this microorganism remains unknown.

2. Objectives

The objective of the current study was to characterize the fuction of the DX120E *nifH* gene in *Klebsiella variicola* through gene editing (CRISPR-Cas system) and assess the nitrogen fixation capacity regulated by Fe protein antibodies in the microbe-sugarcane system.

3. Materials and Methods

3.1. Molecular Cloning of NifH and Prokaryotic Expression

Klebsiella variicola DX120E, preserved in glycerol at -80 °C, was inoculated on LB solid media after gradient dilution and cultured overnight at 28 °C. The *nifH* gene was cloned into vector pMD18-T (Takara, Japan) and transformed into *E. coli* DH5α cells. Positive plasmids were sequenced using sanger sequencing technology by Qingke Biotechnology Co., Ltd. Guangzhou, China. The sequence data were compared with the NCBI database's open reading frame (ORF) of the *nifH* gene (GenBank: KF732646.1). The pMD18-T-*nifH* and pET-30a vector were digested with EcoRI and Not I restriction enzymes (Takara, Japan) by incubating at 37 °C overnight, and the target DNA fragments were purified. The T4 DNA ligase (Takara, Japan) was used to ligate the target fragments to express in the vector. The plasmid pET-30a-*nifH* was re-extracted and transformed into *E. coli* BL21 (DE3) cells for protein expression. All the samples were electrophoretically separated (50 V, 30 min; 120 V, 2.5 h) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% separation gel and 5% stacking gel (12).

3.2. Antibody Production

According to the 1:5 (v/v) ratio of the original bacterial solution, precooled bacterial lysis buffers (50 mM Tris-HCl (pH 8.5 to 9.0), 2 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 1 $mg.mL^{-1}$ lysozyme) were added and the resuspended bacteria were broken by sonication in an ice bath. Subsequently, the pellet was washed with 2 M and 4 M urea containing 1% Triton X-100 and dissolved in 8 M urea. The recombinant protein was identified by SDS-PAGE (12) .

Two Japanese white rabbits were used in the immunization experiment. On the first day, these rabbits were immunized with 0.3 mg of recombinant protein immunogenic and sterile Freund's complete adjuvant (Freund's incomplete adjuvant and *Mycobacterium tuberculosis*) by subcutaneous multi-point injection. On the 12th, 26th, and 40th days, the rabbits were immunized with 0.15 mg recombinant protein and Freund's incomplete adjuvant (lanolin: mineral oil = 1: 1.5). The inoculated rabbits were kept in good conditions during the experiment, and blood samples were collected on the 52th day. The antisera were obtained by centrifugation, and indirect ELISA was used to test the titer of the antisera at indicated time points. The antigen was adhered to the solid phase carrier by coupling sulfolink coupling gel with prepared immunogen sulfhydryl, and the antibody was obtained through affinity purification. The final concentration of the antibodies was measured after repacking in an aseptic EP tube.

3.3. Construction of NifH Mutants and Replenishment Strains

PCR reaction was conducted using the strain DX120E genomic DNA as a template along with *nifH* up-F/*nifH* up-R, *nifH*-down-F/*nifH* down-R, *nifH*-MCS-F/*nifH*-MCS-R primers, respectively, to amplify up/down homologous fragments of *nifH* gene, and the plasmid pBBR1MCS2 was amplified in PCR by using the primers MCS-*nifH*-F/MCS-*nifH*-R. The PCR amplified products of *nifH* up and down fragments were purified and subjected to fusion PCR in a total volume of 50 μL comprising of 25 μL 2×Superpfu PCR mixture, 2 μL *nifH*-UP-F (10 μM), 2 μL *nifH* down-R (10 μM), 4 μL up recovery fragment, 4 μL down recovery fragment, and 13 μL ddH_2O. The PCR parameters were set as 94 °C 5 min, 32 cycles (94 °C 30 s, 50 °C 30 s, 72 °C 30 s), and 10 °C hold on. The fused UD fragments with A were ligated to the T vector and sequenced. The prepared knockouted plasmid (pKLCas9Tc-UP-down) and repair homology arm fragments (pBBR1MCS2-Tac-*nifH*) were electro-transformed into strain DX120E to obtain positive clones through screening. The correctly sequenced positive plasmid was transformed into strain DX120E, and finally, the mutant strain Δ*nifH* and C (Δ*nifH*) were obtained.

3.4. Analysis of Biological Characteristics of NifH mutants

3.4.1. Determination of Growth Curve and Nitrogenase Activity

The strain DX120E, Δ*nifH*, and C (Δ*nifH*) were inoculated into LB medium and put for shaking culture at 28 °*C*. Samples were taken every 4 h to measure the OD_{600}. An acetylene reduction assay was used to determine the nitrogenase activity (13).

3.4.2. Bacterial RNA Extraction and RT-QPCR

Trizol (Solarbio, China) (14) was used to extract the bacterial RNA. Snap frozen tissues were ground in liquid nitrogen using a mortar and pestle. One milliliter of Trizol per to 50-100 mg of tissue was added. The samples were solubilized for 5 min at room temperature, 0.2 mL of chloroform per mL of Trizolwas added, mixed vigorously by vortex for 30 sec and the mixture was allowed to stand for 2-3 min at room temperature. The mixture was centrifuged at maximum speed of 12,000 ×g for 10 min at 4 °C, and the clear upper phase was transferred to a fresh tube (0.5-0.6 mL). Then 0.5 mL of isopropanol was added per milliliter of the clear phase, and mixed vigorously by vortexing for 10 min. After centrifuging at 12,000 ×g for 10 min at 4 °C, the supernatant was decanted and precipitated， the obtained RNA was dried with a pulled Pasteur pipette, then the RNA precipitate was washed with the mixture of ethanol and Trizol (1:1). After re-centrifuged at 12,000 ×g for 2 min at 4 °C, the liquid was discarded. The pellet was allowed to dry upside down at room temperature for 5 to 10 min. An appropriate amount of DEPC-treated water was added to dissolve the RNA precipitate. The RNA concentration at OD_{260} was measured by IMPLEN (NanoPhotometer® P-ClassP 300). RT-qPCR was conducted using HiScript® III 1st Strand cDNA Synthesis Kit (+gDNA wiper) and ChamQ SYBR Color qPCR Master Mix (High ROX Premixed) (Vazyme Biotech Co., Ltd, China). The reference 16S RNA and target gene primers were shown in **Table 1**. The $2^{-\Delta\Delta Ct}$ method (15) was used for expression analysis.

3.4.3. Extraction of Protein and Western Blotting Analysis

Sugarcane plantlets (ROC22) were cultured and kept in the Guangxi Key Laboratory of Sugarcane Biology at Guangxi University. 200 single sugarcane plantlets were planted in 50 bottles, and each bottle had 4 plantlets and 25 mL (1/10 MS) liquid medium. In the treatment group, 25 bottles of culture medium were inoculated with 100 μL of bacterial suspension, and in the control group, each bottle was inoculated with an equal volume of sterile water. Samples were collected on the 0, 3rd, and 6th days of culturing and maintained in a sterile environment during the experiment. The phenol extraction method was used to extract the total protein from the samples (16).

A 50 mL volume of bacterial suspension was centrifuged at 8000 × g (4 °C) for 5 min, and the pellet was washed and dissolved in 1 mL of lysis buffer. The total plant protein, induced recombinant protein and bacterial protein were denatured at 100 °C for 5 min and separated by SDS-PAGE (12). Western blotting was conducted according to Taylor and Posch (17).

Table 1. Primers sequences used for PCR amplification

Name	Sequence (5´→ 3´)	Annealing temperature
nifH-UP-F	TAGCGGCCCA TTCA TCGACA	55°C
nifH-UP-R	TGGTGAGGATCCTGTGCGGTGACTTCTCCTGTTGTTGA	
nifH-down-F	AACAGGAGAAGTCACCGCACAGGATCCTCACCATGACCAA	55°C
nifH-down-R	CCGGTTTA TCCAGCGCCTTG	
nifH-ter-F	ATGACCATGCGTCAATGCGCTA	58°C
nifH-ter-R	TCAGGCCGCGTTTTCTTCAG	
nifH sgRNA	A TGCGTCAA TGCGCTA TCTA	
nifH-MCS-F	ATATACCATGGAAGCTTACATGACCATGCGTCAATGCGCTA	55°C
nifH-MCS-R	CGGTATCGATCTCGAGTCAGGCCGCGTTTTCTTCAG	
MCS-*nifH*-F	AAGAAAACGCGGCCTGACTCGAGATCGATACCGTCGA	55°C
MCS-*nifH*-R	TGACGCATGGTCATGTAAGCTTCCATGGTATATCTCCTTCTT	
MCS2-JD-F	ACACTGCTTCCGGTAGTCAA	55°C
M13R	AGCGGATAACAATTTCACACAGG	
16SF	CTGGAACTGAGACACGGTCC	55°C
16SR	GGAGTTAGCCGGTGCTTCTT	
*nifD*F	CCGACCAAAATCGCCGAATC	55°C
*nifD*R	CGCCATCTGCCCCTCATATT	
*nifK*F	AGCTTTATCGGCAGCCATGT	55°C
*nifK*R	CCGGTCACCAGATTGAGCTT	
*nifH*F	GACGAACTGATCATCGCCCT	55°C
*nifH*R	GGTCGTACTCGATCACCGTC	

4. Results

4.1. Molecular Cloning and Bacterial Expression

The 882 bp *nifH* gene was obtained in the PCR reaction using strain DX120E genomic DNA as the template. The bacterial colonies were easily screened by white (recombinant) and blue (non-recombinant) colors. The plasmid of the bacterial strain with the pET-30a-*nifH* sequence was extracted and sequenced through sanger sequencing. The results were consistent with the known ORF of the *nifH* gene (GenBank: KF732646.1) (18). Double digestion of the plasmid was done with the restriction enzymes EcoRI and NotI to divide the pET-30a *nifH* vector into two parts with sizes of 5392 bp and 889 bp, respectively. These results showed that the full-length *nifH* gene was cloned successfully into the pET-30a (+) vector, and the prokaryotic expression vector pET-30a-*nifH* was effectively constructed **(Fig. 1A, 1B)**. The pET-30a-*nifH* was transferred into *E. coli* BL21 (DE3) and positive colonies were used to construct a prokaryotic expression system (*nifH*-ORF). The recombinant pET-30a-NifH protein was successfully expressed in a prokaryotic expression system **(Fig. 2A)**. The positive strains were cultured at 37 °C in IPTG with a final concentration of 0.5 mM for different lengths of time. The NifH protein band of 43 kDa could be seen starting at 2 h post-induction. The full expression content was induced at 6 h **(Fig. 2B)**. Finally, 0.5 mM IPTG and 6 h culture time were selected for induction culture according to the experiment's protocol. The induced protein was precipitated mainly by ultrasonic treatment **(Fig. 2C)**, indicating that the recombinant protein of pET30a-*nifH* was mainly in the form of an inclusion body in this expression system. The amounts of induced proteins identified through Western blotting were 5 ng and 10 ng in response to the mouse anti-His tag antibody. A distinct band of 43 kDa in size was discovered in Western blotting (**Fig. 2D**).

4.2. Antibody Production

SDS-PAGE showed that the inclusion body protein bands were clear. Immunogen concentration was 6 $\mu g \cdot mL^{-1}$, suitable for immune conditions (**Fig. 3A**). The antisera were tested by indirect ELISA, and

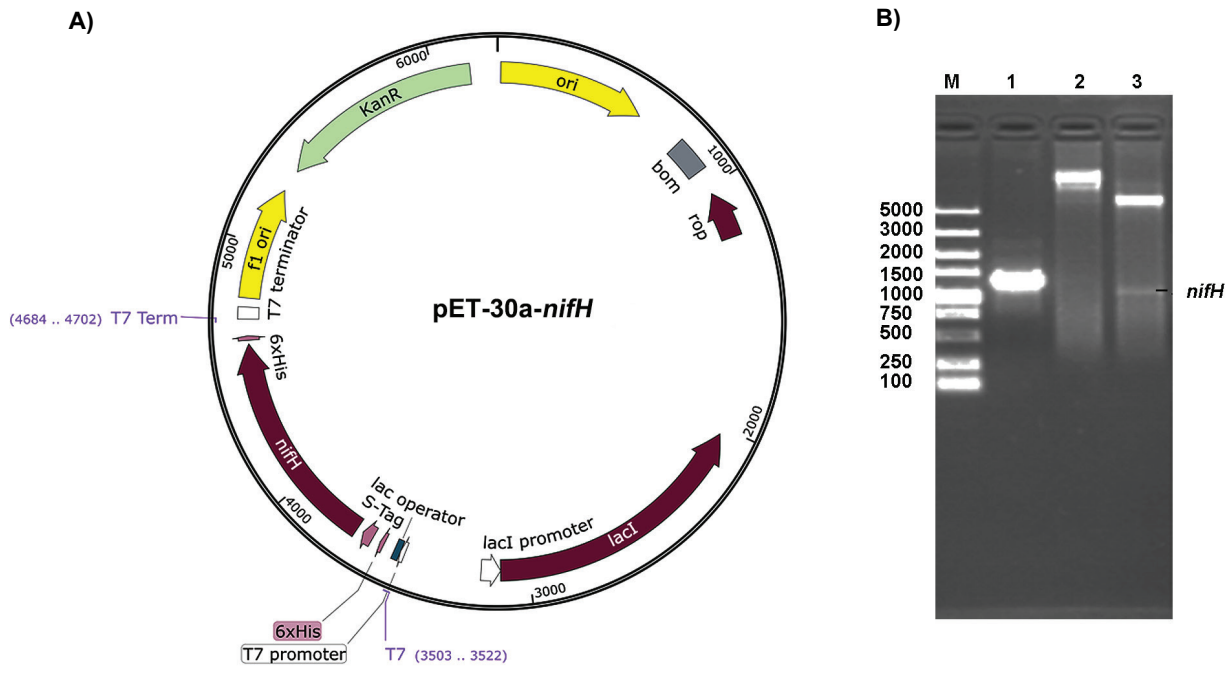


Figure 1. Construction and verification of pET-30a-*nifH*. A) pET-30a-*nifH* clone model, **B)** PCR and double enzyme digestive verification of pET-30a-*nifH*. M: DL 5000bp DNA Marker; 1: T7 universal primer PCR positive control; 2: pET-30a *nifH* plasmid; 3: Double digested product.

the result showed that the titer of antisera reached 1:512000 (**Fig. 3B**). To reduce the influence of background noise in Western blotting, the antisera affinity was purified using the specific antigen-antibody, and the final concentration of the antibody was 2.51 µg. mL^{-1}.

4.3. Western Blotting Analysis of Immunogen and Endogenous NifH Proteins

It is considered that the polyclonal antibodies of the NifH protein could confidently recognize the recombinant protein. The amounts of identified induced protein through Western blotting were 5 ng and 10 ng, respectively, in response to the anti-NifH antibody in rabbits. A distinct band of 43 kDa in size was discovered in Western blotting. At the same time, the total protein from *Klebsiella variicola* DX120E was detected by Western blotting with our anti-NifH antibody, and the protein band was visible **(Fig. 3C)**. These results verified that the rabbit polyclonal antibody could recognize both the exogenous and endogenous NifH proteins.

4.4. NifH Protein Expression Analysis of Tissue Cultured Sugarcane Seedlings

Tissue cultured seedlings of sugarcane variety ROC22 and *Klebsiella variicola* DX120E were co-cultured for 0, 3, and 6 days, and total protein was extracted by the phenol extraction method. The expression of ROC22 NifH protein in the seedlings was found to be higher at 6th day (0.31) than that at 0 day (0.24) determined by Western blotting **(Fig. 3D)**. It confirmed that the antibody could detect protein expression. Moreover, N-fixing strains could enter sugarcane seedlings from the inoculation site and migrate into the aboveground part.

4.5. Effects of NifH Gene Deletion on N-Fixing Bacteria

4.5.1. Screening of Mutant Strains

The *nifH*-UP-F/*nifH*-UP-R and *nifH*-down-F/*nifH*-down-R were used to amplify the upstream and downstream homology arms of *nifH*, respectively **(Fig. 4A)**, and the 1096 bp fusion fragments of the upstream and downstream arms were obtained by fusion PCR **(Fig. 4B)**. The knockouted plasmid was electroporated,

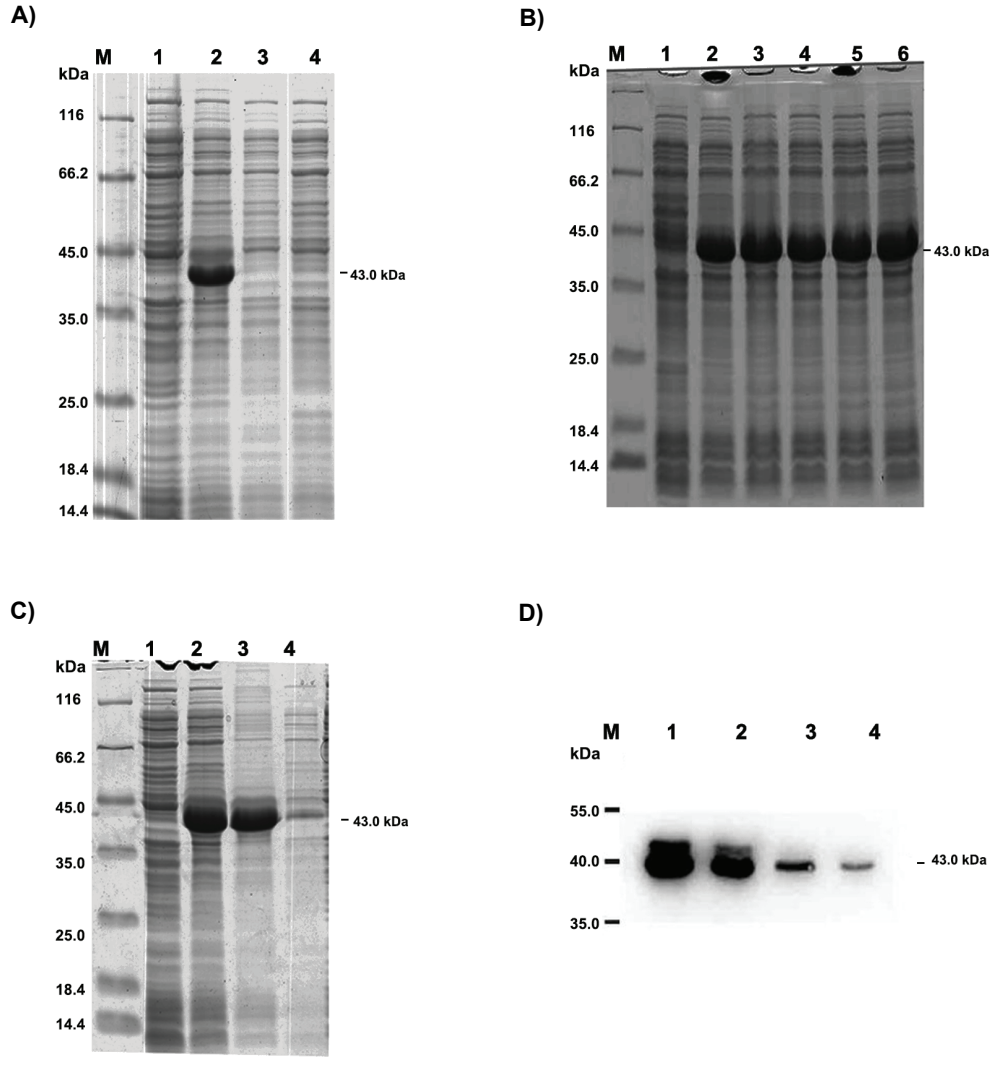


Figure 2. Prokaryotic expressions. A) Induced expression identification of pET-30a-*nifH*. M: Protein marker, 1: pET-30a-*nifH* no induction; 2: pET-30a-*nifH* induction; 3: pET-30a(+) with no induction; 4: pET-30a(+) induced, **B)** pET-30a-*nifH* induced at different times. M: Protein marker, 1: no induction; 2: 2 h; 3: 3 h; 4: 4 h; 5: 5 h 6: 6 h, **C)** Soluble identification of pET-30a-*nifH*. M: Protein marker, 1: no induction; 2: induction; 3: induced pellet; 4: induced supernatant, **D)** His-Tag label protein identification. M: Protein marker; 1: 100 ng; 2: 50 ng; 3:10 ng; 4: 5 ng

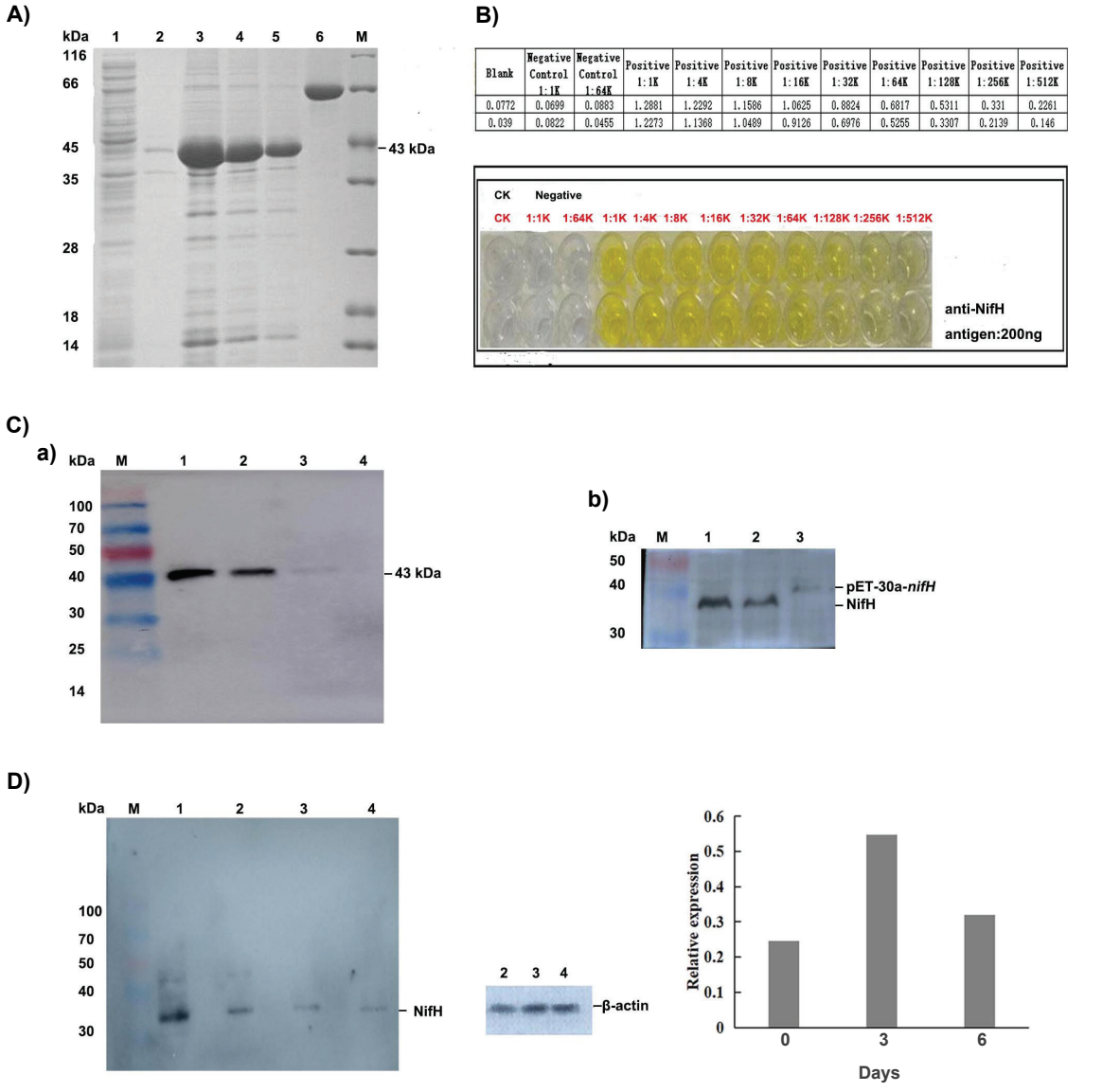
and the homology arm fragment was repaired, so the knockouted strain Δ*nifH* was identified **(Fig. 4C, 4D)**. At the same time, the MCS-*nifH* replenishment plasmid was constructed to transform the knockouted strain to obtain the knockout replenishment strain C (Δ*nifH*) **(Fig. 4E,4F**). The above strains were used in the following physiological and biochemical experiments.

4.5.2. Effects on Growth

Growth curve analysis showed no significant variation among wild-type strains, mutant strains, and complementary strains of strain DX120E in LB medium at 37 °C, and all the strains showed maximum growth rate at 16 h and later started gradual decline **(Fig. 5A)**.

4.5.3. Effect of NifH Mutation on Nitrogenase Activity

The acetylene reduction method was applied to detect nitrogenase activity in wild-type strain DX120E, *nifH* mutant strains, and replenishment strains during the N fixation process. It was found that mutation of the *nifH*



Blank	Negative Control 1:1K	Negative Control 1:64K	Positive 1:1K	Positive 1:4K	Positive 1:8K	Positive 1:16K	Positive 1:32K	Positive 1:64K	Positive 1:128K	Positive 1:256K	Positive 1:512K
0.0772	0.0699	0.0883	1.2881	1.2292	1.1586	1.0625	0.8824	0.6817	0.5311	0.331	0.2261
0.039	0.0822	0.0455	1.2273	1.1368	1.0489	0.9126	0.6976	0.5255	0.3307	0.2139	0.146

Figure 3. Antibody preparation and expression analysis of NifH protein. A) Detection of urea dissolution of recombinant protein. M: Protein marker; 1: supernatant 1; 2: supernatant 2; 3: 2-fold dilution; 4: 5-fold dilution; 5: 10-fold dilution; 6: 0.4 mg/mL BSA. **B)** Indirect ELISA of titer. **C): a)** Detection of pET-30a-NifH protein. M: Protein marker; 1: 10 ng pET-30a-NifH; 2: 5 ng pET-30a-NifH; 2: 1 ng pET-30a-NifH; 2: 500 pg pET-30a-NifH, **b)** Detection of strain DX120E endogenous NifH protein. M: Protein marker;1-2: NifH; 3: pET-30a-NifH. **D)** Western Blotting analysis of NifH protein at different time after inoculation. M: Protein marker; 1: total protein in strain DX120E; 2: NifH protein in seedling of sugarcane variety ROC22 inoculated with strain DX120E for 3 days; 3: NifH protein in seedling of sugarcane variety ROC22 inoculated with strain DX120E for 6 days; 4: NifH protein in seedling of sugarcane variety ROC22 at 0 day of inoculation with strain DX120E .

gene decreased the nitrogenase activity of the strain. However, the replenishment strain restored the N-fixing ability which was significantly higher than that in the wild-type strain, which suggested that mutation of the *nifH* gene had lowered the strain's N-fixing capability **(Fig. 5B)**.

4.5.4. Effect of NifH Mutation on NifD and NifK Genes

NifD and *nifK* are the critical nitrogenase genes, and their relative expression levels were determined in the three bacterial strains by RT-qPCR. Significant variations were observed among the expression levels

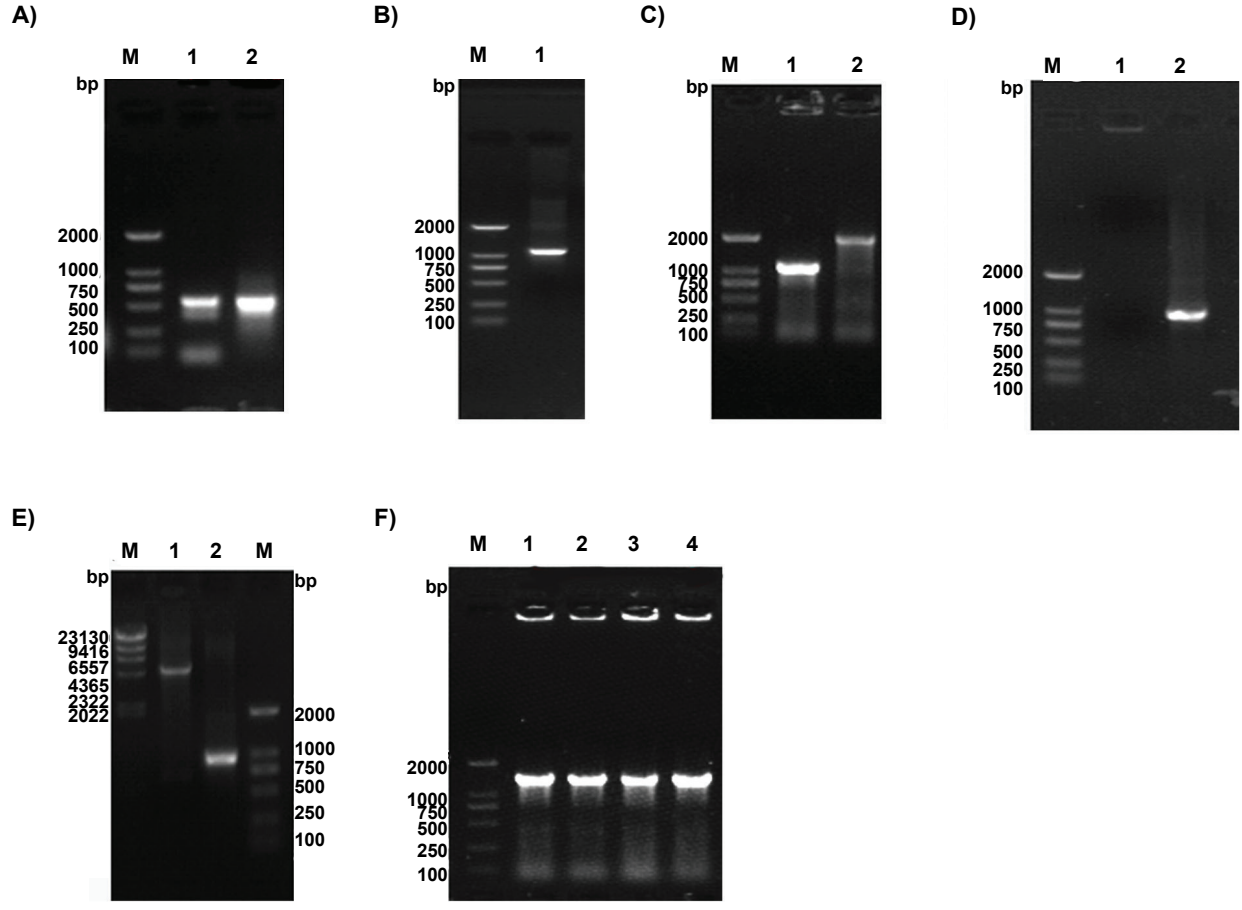


Figure 4. Construction of nifH gene deletion mutations and complementary strains. A) Upstream and downstream homology arm amplification. M: marker;1: *nifH* UP; 2: *nifH* Down. **B)** PCR amplification of homology arm fusion. M: marker; 1: Upstream and downstream fusion fragments, **C)** *nifH*-UP-F/*nifH* down R PCR identification. M: marker; 1: Δ*nifH*; 2: DX120E. **D)** *nifH*-ter-F/*nifH*-ter-R PCR identification. M: marker; 1: Δ*nifH*; 2: DX120E. **E)** *nifH* and plasmid frame amplification. M: marker; 1: *nifH*; 2: pBBR1MCS2. **F)** *nifH*-MCS-DX120E Δ*nifH* colony PCR identification. M: marker; 1-4: C (Δ*nifH*).

of *nifK* and *nifD* genes in wild-type, knockouted, and complementary strains. Relative expression of *nifD* and *nifK* genes were significantly higher in wild-type than mutant and complementary strains **(Fig. 5C)**.

4.5.5. Effects of NifH Gene Knockout on NifH Protein Expression

The Western blotting technique identified the effects of *nifH* gene knockout on NifH protein expression in the three strains of N-fixing bacteria. The result displayed that mutation of the *nifH gene* downregulated the expression of NifH protein in the strains. A comparison of NifH protein expression among these three strains showed that the expression level was higher in the complementary strain than the wild-type and knockouted strains **(Fig. 5D)**.

5. Discussion

The sugarcane microbiome consists of diverse plant growth-promoting microorganisms, including N-fixing bacteria (diazotrophs). A survey conducted in the sugarcane area of Guangxi, China, found the *Enterobacteriaceae* family, especially *Klebsiella*, is the most abundant plant-associated N-fixing bacteria (19). *Klebsiella variicola,* an N-fixing strain, is a member of the same family and colonizes sugarcane, promoting plant growth and has the potential to replace chemical fertilizer partially (10). Extensive analysis of N-fixing

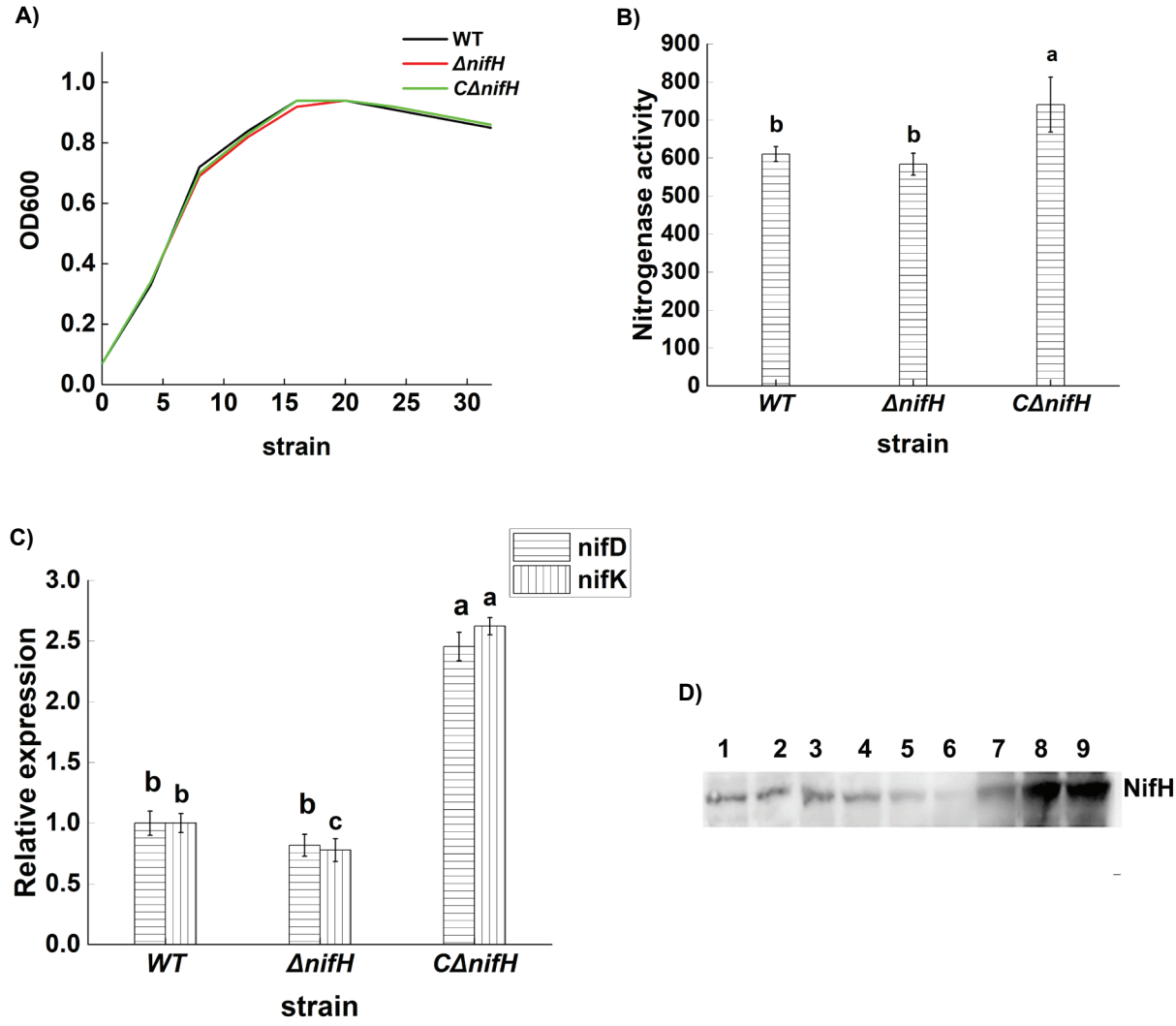


Figure 5. Effects of *nifH* gene deletion on the growth, N-fixing ability, and expression of N-fixing genes in different strains of N-fixing bacteria. A) Growth curve; **B)** Nitrogenase activity; **C)** Expression of nitrogenase gene; **D)** Western blot detection of NifH protein level in wild-type, Δ*nifH* and cΔ*nifH*. 1, 2, 3: wild-type; 4, 5, 6: Δ*nifH*; 7, 8, 9: cΔ*nifH*.

Note: wild-type, WT; mutant strains, Δ*nifH*; complement strain, cΔ*nifH*. Data are mean±standard error (n=3). The different lowercase letters above the bars indicate significant differences at p≤0.05 in Duncan's multiple range test.

genes has served as a base for developing molecular assays and bioinformatic methods to explore N fixation in the environment (20). Nitrogen fixation is carried by an enzyme complex called nitrogenase which consists of two main components: a dinitrogenase encoded by *nifD* and *nifK* and an iron-containing reductase, also called Fe protein which is encoded by *nifH*. However, the specific function of the *nifH* gene in the nitrogen-fixing bacterium DX120E and its application in assessment of nitrogen fixation capacity in microbial and sugarcane systems have not been systematically investigated.

In addition to the acetylene reduction method, biological N fixation could also be assessed through the nitrogen-fixing enzyme's expression by antibodies, and NifH is one of the most detected proteins (21, 22). In the current study, the expression of NifH protein was determined using a polyclonal antibody prepared in this experiment, and enhanced NifH protein expression was noticed in sugarcane seedlings co-cultured with *Klebsiella variicola* DX120E for 3 days. N-fixing microorganisms contain dinitrogenase, one of the subunits encoded by *nifH*. Detection of *nifH*

gene expression indicated the presence of N-fixing bacteria and N-fixation in plants. However, after 3 days, the expression level of NifH protein decreased. A possible reason for the reduction in the expression of NifH protein may be the gradual consumption of nutrients in the culture medium, which led to the nutritional competition between DX120E and plants. But this assumption needs further investigation. Li, *et al*. (23) reported an upregulation of nifH in the pre-inoculation period and a downregulation later. NifH proteins were also detected in control, which could be linked with the presence of endosymbiotic bacterial colonies in plants (24).

Gavini and Burgess (25) have shown that the *nifHDK* genes are transcribed as a single unit in many microorganisms, hence the mutant strains cannot synthesize the *nifH* polypeptide and FeMo cofactor. The absence of NifH protein, a central nitrogenase component, causes a reduction in N fixation capacity (26). In the *nifH* knockouted strain, the *nifD* and *nifK* gene transcript copies and nitrogenase activity decreased. Western blotting analysis showed a decrease in the content of Fe protein in the *nifH* knockouted strain and an improvement in the complementled strain. This result matched well with previous research that found the wild-type strain *Acetobacter diazotrophicus* PAL5 grew better and the plants inoculated with the wild-type strain accumulated more nitrogen with a lower $\delta^{15}N$ signature after 60 days than the plants inoculated with *nifH*-mutant (27). Enhanced expression of NifH protein was found in the replenishment strain, which might be due to the overexpression of the *nifH* gene in response to solid plasmid motion (28).

To our knowledge, *Klebsiella variicola* is a well-known species that contains diazotrophs. In the present study, one of the N-fixing enzyme genes, *nifH* has been cloned and expressed in *E. coli* BL21 (DE3). A polyclonal antibody prepared by immunizing rabbits with *nifH* protein was used to successfully detect the expression of endogenous and exogenous Fe proteins, which lay the foundation for assessing the nitrogen-fixing capacity of nitrogen-fixing bacteria by enzyme-linked immunity. Moreover, the *nifH* gene knockouted mutant of DX120E showed lower nitrogen fixation capacity in strains and microbial sugarcane systems.

6. Conclusion

Using the rabbit polyclonal antibody proved that the core fraction of N-fixing enzymes, Fe protein, is presented in nitrogen-fixing bacteria and endophytic microbial and sugarcane systems. The *nifH* deletion limited the nitrogen fixation enzyme activity of DX120E and reduced the expression levels of the nitrogen fixation-related genes *nifD*, *nifK* and Fe protein at molecular level. Although the immunological and CRISPR-Cas9 system gene editing approaches have laid the foundation for studying other nitrogen-fixing genes, the interaction mechanism between N-fixing genes and plants still needs exploration. So, it is emphasized to develop comprehensive strategies to use N-fixing bacteria to improve crop performance, particularly growth and yield.

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Human or animal participants

The current article does not possess any studies with humans. Rabbits bought from ABclonal Biotechnology co., Ltd. Rabbits were placed in a standard 12 h light-dark cycle with free access to food and water.

Declaration of competing interest

No conflicts of interest were declared.

References

1. Hoffman BM, Lukoyanov D, Yang Z-Y, Dean DR, Seefeldt LC. Mechanism of nitrogen fixation by nitrogenase: The next stage. *Chem Rev*. 2014;**114**(8):4041-4062. doi:10.1021/cr400641x
2. Pankievicz VCS, do Amaral FP, Ané J-M, Stacey G. Diazotrophic bacteria and their mechanisms to interact and benefit cereals. *Mol Plant-Microbe Interact*. 2021;**34**(5):491-498. doi:10.1094/mpmi-11-20-0316-fi
3. Boddey RM, Urquiaga S, Alves BJR, Reis V. Endophytic nitrogen fixation in sugarcane: present knowledge and future applications. *Plant Soil*. 2003;**252**(1):139-149. doi:10.1023/A:1024152126541
4. Gaby JC, Buckley DH. A comprehensive aligned *nifH* gene

database: a multipurpose tool for studies of nitrogen-fixing bacteria. *Database*. 2014;**2014**. doi:10.1093/database/bau001

5. Meiler S, Britten GL, Dutkiewicz S, Gradoville MR, Moisander PH, Jahn O, *et al*. Constraining uncertainties of diazotroph biogeography from *nifH* gene abundance. *Limnol Oceanogr*. 2022;**67**(4):816-829. doi:10.1002/lno.12036
6. Addo MA, Dos Santos PC. Distribution of nitrogen-fixation genes in prokaryotes containing alternative nitrogenases. *Chem Bio Chem*. 2020;**21**(12):1749-1759. doi:10.1002/cbic.202000022
7. Berman J, Gershoni JM, Zamir A. Expression of nitrogen fixation genes in foreign hosts. .*J Biol Chem*. 1985;**260**(9):5240-5243. doi:10.1016/S0021-9258(18)89011-1
8. Arnold W, Rump A, Klipp W, Priefer UB, Pühler A. Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. *J Mol Biol*. 1988;**203**(3):715-738. doi:10.1016/0022-2836(88)90205-7
9. Rosenblueth M, Martínez L, Silva J, Martínez-Romero E. *Klebsiella variicola*, a novel species with clinical and plant-associated isolates. *Syst Appl Microbiol*. 2004;**27**(1):27-35. doi:10.1078/0723-2020-00261
10. Lin L, Wei CY, Chen MY, Wang HC, Li YY, Li YR, *et al*. Complete genome sequence of endophytic nitrogen-fixing *Klebsiella variicola* strain DX120E. *Stand Genomic Sci*. 2015;**10**(1):22. doi:10.1186/s40793-015-0004-2
11. 11. Wei CY, Lin L, Luo LJ, Xing YX, Hu CJ, Yang LT, *et al*. Endophytic nitrogen-fixing *Klebsiella variicola* strain DX120E promotes sugarcane growth. *Biol Fertil Soils*. 2014;**50**(4):657-666. doi:10.1007/s00374-013-0878-3
12. Alhashem Y, Farid A, Al Mohaini M, Muzammal M, Khan M, Dadrasnia A, *et al*. Protein isolation and separation techniques of *Pasteurella multocidavia* one-and two-dimen-sional gel electrophoresis. 2022;**14**:1-8. doi:10.31782/IJCRR.2022.141208
13. Masood S, Zhao XQ, Shen RF. *Bacillus pumilus* promotes the growth and nitrogen uptake of tomato plants under nitrogen fertilization. *Sci Hortic*. 2020;**272**:109581. doi:10.1016/j.scienta.2020.109581
14. Wu W, Yin C, Yue A, Niu J, Du W, Liu D, *et al*. Rapid and visual detection of soybean mosaic virus SC7 with a loop-mediated isothermal amplification strategy. *Sens Actuators B*. 2022;**373**:132733. doi:10.1016/j.snb.2022.132733
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*. 2001;**25**(4):402-408. doi:10.1006/meth.2001.1262
16. Zhou G, Yang LT, Li YR, Zou CL, Huang LP, Qiu LH, *et al*. Proteomic analysis of osmotic stress-responsive proteins in sugarcane leaves. *Plant Mol Biol Rep*. 2012;**30**(2):349-359. doi:10.1007/s11105-011-0343-0
17. Taylor SC, Posch A. The design of a quantitative western blot experiment. *BioMed Res Int*. 2014;**2014**:361590. doi:10.1155/2014/361590
18. 18. Zhang KK, Niu JQ, Wei CY, Xing YX. Cloning and sequence analysis on *nifH* gene of *Klebsiella variicola* DX120E isolated from sugarcane. *J South Agric*. 2014;**45**(10):1719-1725.
19. Singh RK, Singh P, Sharma A, Guo DJ, Upadhyay SK, Song QQ, *et al*. Unraveling nitrogen fixing potential of endophytic diazotrophs of different *Saccharum* species for sustainable sugarcane growth. *Int J Mol Sci*. 2022;**23**(11):6242. doi:10.3390/ijms23116242
20. de Lima DRM, dos Santos IB, Oliveira JTC, da Costa DP, de Queiroz JVJ, Romagnoli EM, *et al*. Genetic diversity of N-fixing and plant growth-promoting bacterial community in different sugarcane genotypes, association habitat and phenological phase of the crop. *Arch Microbiol*. 2021;**203**(3):1089-1105. doi:10.1007/s00203-020-02103-7
21. Staples CR, Lahiri S, Raymond J, Herbulis LV, Mukhophadhyay B, Blankenship RE. Expression and association of group IV nitrogenase nifD and nifH homologs in the non-nitrogen-fixing archaeon *Methanocaldococcus jannaschii*. *J Bacteriol*. 2007;**189**(20):7392-7398. doi:10.1128/JB.00876-07
22. Li Q, He XJ, Liu PX, Zhang HW, Wang MY, Chen SF. Synthesis of nitrogenase by *Paenibacillus sabinae* T27 in presence of high levels of ammonia during anaerobic fermentation. *Appl Microbiol Biotechnol*. 2021;**105**(7):2889-2899. doi:10.1007/s00253-021-11231-z
23. Li H-B, Singh RK, Singh P, Song Q-Q, Xing Y-X, Yang L-T, *et al*. Genetic diversity of nitrogen-fixing and plant growth promoting *Pseudomonas* species isolated from sugarcane rhizosphere. *Front Microbiol*. 2017;**8**. doi:10.3389/fmicb.2017.01268
24. Hardoim PR, Overbeek LSv, Berg G, Pirttilä AM, Compant S, Campisano A, *et al*. The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol Mol Biol R*. 2015;**79**(3):293-320. doi:10.1128/MMBR.00050-14
25. Gavini N, Burgess BK. FeMo cofactor synthesis by a *nifH* mutant with altered MgATP reactivity. *J Biol Chem*. 1992;**267**(29):21179-21186. doi:10.1016/S0021-9258(19)36814-0
26. Chang CL, Davis LC, Rider M, Takemoto DJ. Characterization of *nifH* mutations of *Klebsiella pneumoniae*. *J Bacteriol*. 1988;**170**(9):4015-4022. doi:10.1128/jb.170.9.4015-4022.1988
27. Sevilla M, Burris RH, Gunapala N, Kennedy C. Comparison of benefit to sugarcane plant growth and $^{15}N_2$ incorporation following inoculation of sterile plants with *Acetobacter diazotrophicus* wild-type and *nif* mutant strains. *Mol Plant-Microbe Interact*. 2001;**14**(3):358-366. doi:10.1094/MPMI.2001.14.3.358
28. Chen M, Li Y, Li S, Tang L, Zheng J, An Q. Genomic identification of nitrogen-fixing *Klebsiella variicola*, *K. pneumoniae* and *K. quasipneumoniae*. *J Basic Microb*. 2016;**56**(1):78-84. doi:10.1002/jobm.201500415