CLONING, PROKARYOTIC EXPRESSION AND PROTEIN PURIFICATION OF SUGARCANE ENDOGENOUS AZOTOBACTER KLEBSIELLA VARIICOLA DX120E NIFK GENE

Kun-Kun Zhang^{1#}, Yong-Xiu Xing^{1, 2#}, Min Shao¹, Chao-Xing Wu¹, Li-Tao Yang^{1, 2*}, Yang-Rui Li^{1, 2*}

¹ State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, College of Agriculture, Guangxi University, Nanning 530004, China.

² Guangxi Key Laboratory of Sugarcane Genetic Improvement, Key Laboratory of Sugarcane Biotechnology and Genetic Improvement (Guangxi), Ministry of Agriculture, P.R. China, Sugarcane Research Center, Chinese Academy of Agricultural Sciences-Guangxi Academy of Agricultural Sciences, Nanning 530007, China.

ABSTRACT

NifK gene is a key nitrogenase and plays an important role in the nitrogen fixation process. It is necessary to obtain the nifK gene for exploring its relationship with nitrogen fixation. This study aimed to clone nifK gene, predict the secondary structure, the characters and functions of the nifK protein, and conduct prokaryotic expression. According to other nifK sequences registered in the NCBI, specific primers were designed. Klebsiella variicola DX120E nifK gene opening reading frame (ORF) was cloned by PCR amplification, and its nucleotide sequence, amino acid sequence and protein structure were analyzed by combining bioinformatics methods. Prokaryotic expression vector pET30a (+) was used to construct the recombinant expression vector pET30a-nifK. After PCR, double enzyme digestion and DNA sequencing, the recombinant plasmid was transformed into BL21 (DE3), and 1.0 mmol/L IPTG was used to induce the gene expression at 28 °C. The fusion protein expression was detected through SDS-PAGE electrophoresis. The nifK gene ORF cloned from Klebsiella

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variicola DX120E in this study was 1563 bp, encoding 520 amino acids. The prokaryotic expression protein by induction was detected by mass spectrometry, which had an isoelectric point of 5.54, and molecular weight of 58.12 kDa. The gene has been registered in GenBank with accession number KF732647. Amino acid sequence comparison and phylogenetic tree analysis showed that the nifK gene had the highest similarity with Klebsiella. The nifK gene was cloned and the nifK protein was successfully expressed in a prokaryotic expression system, which laid the foundation for further studying the function of DX120E nifK gene in sugarcane.

Key words: Sugarcane; *Klebsiella variicola* DX120E; clone of *nifK* gene; prokaryotic expression; protein purification

Introduction

Sugarcane (*Saccharum officinarum* L) is one of the most important sugar and energy crops. It has great economic importance due to its application in the food industry, and is also particularly valuable for its use in the production of ethanol (Menossi *et al.*, 2008). Originated in Asia, sugarcane is highly productive in the tropical and subtropical areas of the world. However, many factors affect the growth and development of sugarcane plants (Lisson *et al.*, 2005; Inman-Bamber *et al.*, 2005). For example, sugarcane production in China is facing many serious impediments, such as high nitrogen (N) fertilization and degeneration of the main cultivars (Wei *et al.*, 2014a). More than 60 % of the sugarcane fields in China are fertilized with over 500-700 kg N ha⁻¹ year⁻¹ (Li, 2010) whereas sugarcane fields in Brazil are fertilized with 60–70 kg N ha⁻¹ year⁻¹ (Urquiaga *et al.*, 2012). The much higher amount of nitrogen fertilizer input not only caused high sugarcane production costs but also produced serious adverse impact on the environment. Therefore, in China, it is imperative to reduce the N-fertilization. Biological nitrogen fixation is responsible for supplying more than 60% of the world's annual resources of new ammonia (Schlesinger *et al.*, 1991). This process is performed by nitrogenase,

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which is composed of two metalloproteins: MoFe protein (ecoded by nifD and nifK) and Fe protein (ecoded by nifH) (Rubio et al., 2005). The nucleotide sequence for coding regions of nifHDK genes and their respective genes are remarkably highly conserved among all nitrogen-fixing organisms (Dean et al., 1992). These genes have been cloned and sequenced from many different N₂-fixing microorganisms. For example, The complete nucleotide sequence of the *nifK* open reading frame (ORF) from *Cyanothece* sp. ATCC 51142 is 1533 bp in length and encodes a polypeptide of 511 amino acids (Colón-López *et al.*, 1999). The β subunit of Azotobacter vinelandii MoFe protein has 522 amino acids (Brigle et al., 1985). Strain DX120E was isolated from surface-sterilized roots of the main sugarcane cultivar ROC22 grown in Daxin, Guangxi, China. It is a Gram-negnative bacterium and can able to fix N₂, produce siderophores and indole-3-acetic acids, and solubilize Ca₃(PO₄)₂ (Lin *et al.*, 2012). Ar present, there are only few reports about molecular aspect in this bacteria, more about inoculation with sugarcane. For example, under greenhouse condition, inoculation of strain DX120E in sugarcane cultivar GT21 for 7 days showed improved dry weight, and N, P and K contents obviously (Wei et al., 2014a). Inoculation of bacteria DX120E in sugarcane cultivars B8 and GT21 effectively promoted the plant growth and nutrient uptake, significantly improved the nitrate reductase (NR) and glutamine systhetase (GS) activities, and the nitrate concentration in certain degree in leaves (Wei et al., 2014b). Inoculation of strain DX120E in ROC22 and B8 showed increases in chlorophyll, N and P contents (Xing et al, 2014). In this study, *nifK* gene was cloned and the protein was induced to lay foundation for further studying the molecular mechanism of strain DX120E promoting sugarcane growth, antibody preparation, enzyme activity in vitro and western blot analysis.

Materials and methods

Materials

Experimental materials

The tested bacterial strain Klebsiella variicola DX120E was isolated from

surface-sterilized roots of ROC22 sugarcane planting in Daxin county, Guangxi, China.

Experiment reagents

EcoRI and XhoI restriction enzymes were bought from Fermentas Company (Shanghai); DNA Ligation Kit Ver. 2.0 and pMD18-T vector were from TaKaRa Company (Dalian); Biospin Gel Extraction Kit was purchased from BioFlux Company (Beijing); DH5α and BL21 (DE3) were purchased from Beijing Gold Biotechnology Company; Prokaryotic expression vector pET-30a(+) were kept by the laboratory. Other conventional reagents were analytical grade reagents.

Experimental methods

Primer Design

According to *nifK* gene nucleotide sequences of other nitrogen-fixing species registered in GenBank, online software program Vector NTI Advance 11.0 was used to analyze the homology. The gene upstream primer 5'- AC<u>GAATTC</u>ATGAGCCAAACGATTGATAA-3' (underline parts are restriction sites EcoRI), and downstream primer 5'- ATA <u>CTCGAGTTAACGGACGAGATCGAAGCTG-3'</u> (underline parts are restriction sites XhoI) were designed. The primers were synthesized by Sangon Corporation (Shanghai).

nifK gene cloning

Klebsiella variicola DX120E was used as the template for PCR amplification. The total volume of the PCR reaction system for amplification of *nifK* gene was 25 μ L, operation was done following the EsTaq polymerase instructions. The PCR reaction parameters included pre-denaturation at 95°C for 5 min; denaturation for 50 s at 94°C; annealing for 30 s at 68°C, and extending for 1 min at 72°C, 5 cycles; followed by 15 cycles of denaturation for 50 s at 94°C; annealing at 63°C for 30 s, and extension for 1 min at 72°C, then 15 cycles of denaturation for 50 s at 94°C; annealing at 63°C for 30 s, and extension for 1 min at 72°C, then 15 cycles of denaturation for 50 s at 94°C; annealing for 30 s at 58°C, and extension for 1 min at 72°C, and the final extension for 10 min at 72°C. When the reaction finished, the PCR products were analyzed with 1.0% agarose gel electrophoresis. Then *nifK* gene was connected with pMD18-T vector at 16°C for 8

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h, and then transformed into $DH5\alpha$ competent cells by heat shock. Positive clones were screened out, and sequenced by Shanghai Sangon Company, China.

Bioinformatic analysis of nifK gene

The amino acid sequence of the gene was predicted by BioXM2.6, and basic physical and chemical properties of the speculated protein sequence of the gene were analyzed by ExPASy (http://expasy.org/tools/). The *nifK* gene and the homology with other species were analyzed by (http://blast.ncbi.nlm.nih.gov) GenBank Blast online analysis software. Http://www.cbs.dtu.dk/services/SignalP/ was used for protein signal peptide analysis. Online software http://www.cbs.dtu.dk/services/TMHMM/ was used for membrane protein analysis. Protein blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Motif Scan software were used to analyze the functional domains of the nifK protein. The phylogenetic tree of nifKgene amino acid sequences of DX120E with other species were constructed by MEGA6.0 software. The Vector NTI Advance 11.0 was used for amino acid sequence homology analysis. Construction of nifK prokaryotic expression vector

The PCR products of *Klebsiella variicola* DX120E *nifK* gene were recovered and purified. The plasmids of expression vector pET-30a(+) were extracted, then double digestion was done by using EcoRI and XhoI, and the digestion products were recovered. The target gene and expression vector were connected by DNA Ligation Kit, and the ligation products were transformed into the expression strain DH5 α by heat shock. The correct recombinant plasmids bacteria were detected and sent to Shanghai Biological Engineering Company for sequencing, and the recombinant plasmids were verified by double digestion.

Prokaryotic expression of recombinant plasmid pET30a-nifK

The recombinant strain and the empty pET-30a strain were both transformed into the liquid LB medium with Kan 100 mg \cdot L⁻¹ to induce gene expression separately at 28°C 200 r/min. When OD₆₀₀ reached 0.6, the medium was added with 1.0 mmol/L IPTG for induction, and 2.0 mL medium were collected at 0, 1, 2, 3, 4 and 5 h, respectively. After completion of the induction, the product was centrifuged at 12000×g for 20 min, the supernatant and precipitate

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were collected respectively, then 8μ L 3×1000 suffer were added and kept in boiling water bath for 5 min, and 25 μ L samples were taken for SDS-PAGE electrophoresis after 12000×g centrifugation for 5 min. The concentrations of spacer gel and separation gel for SDS-PAGE was 4% and 12.5%, respectively. After electrophoresis, the gel was stained with Brilliant blue R-250 for imaging analysis.

Results

nifK gene cloning and sequence analysis

Klebsiella variicola DX120E was used as the template with specific primers for PCR amplification. After recycling, ligation and transformation of target gene, a 1563 bp sequence was obtained, which matches the expected size (Fig1).

Compared the obtained sequence with those in GenBank Blast, the result showed the 1563 bp fragment was the open reading frame (ORF) sequence of the *nifK* gene encoding 520 amino acids (Fig. 2), which was registered in GenBenk with the accession number KF732647.

Bioinformatic analysis of *nifK* gene

ExPASy was used to analyze the nifK protein and it showed the molecular weight of nifK protein was 58.16 kDa with pI of 5.48, which was consistent with the mass spectrometric result. The nifK protein was an acidic protein. SOPMA was used to predict the secondary structure of the nifK protein, and the results showed that the protein contained α -helix, β strand, β folding and curl, and α -helix accounted for 42.88%, curl accounted for 38.46%, β folding accounted for 13.27%, while β strand accounted for 5.38% only (Fig. 3). SigalP 4.1 predictions showed that the protein had no signal peptide. The transmembrane helix of the protein was predicted using TMHMM online software and showed that it was an outer membrane protein (Tab. 1). NifK protein functional domains were analyzed through Motif Scan software, and the results showed that the sites 29-32, 47-50, 116-119, 194-197, 225-228, 260-263, 273-276 and 280-283 were casein kinase II phosphorylation sites; 73-78, 90-95,

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125-130, 133-138, 190-195, 318-323, 384-389 and 441-446 were N-myristoylation sites; 298-300 and 333-335 were protein kinase C phosphorylation sites; 404-412 were Tyrosine kinase phosphorylation sites, 136-139 were asparagines glycosylation binding sites, 87-94 and 150-164 were nitrogenase component alpha/beta subunits (Fig. 3). The data in Fig. 4 showed conserved domains of *nifK* gene which revealed *nifK* was a highly conserved gene in the functional region. MEGA6.0 was used to construct a phylogenetic tree (Fig. 5), and the Vector NTI Advance 11.0 was used to make amino acid sequence homology analysis (Fig. 6). Both results showed that nifK protein has higher homology with *Klebsiella*, but relatively lower with non-*Klebsiella* species.



Fig. 1. PCR product of *nifK* gene. M: 2000bp; 1: *nifK* gene

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1	ATGAGCCAAACGATTGATAAAATTCACAGCTGTTATCCGCTGTTTGAACAGGATGAATAC
61	CAGACCCTGTTCCAGAATAAAAAGACCCTTGAAGAGGCGCACGACGCGCAGCGTGTGCAG
121	Q T L F Q N K K T L E E A H D A Q R V Q GAGGTTTTTGCCTGGACCACCACCGCCGAGTATGAAGCGCTGAACTTCCAGCGCGAAGCG
1.01	EVFAWTTTAEYEALNFQREA
181	L T V D P A K A C Q P L G A V L C A L G
241	TTCGCCGGCACCCTGCCCTACGTGCACGGCTCCCAGGGCTGCGTCGCCTATTTTCGCACC
301	TACTTTAĂCCĢCCĂTTTTAĂAGĂĢCĊTĢTCĢCCTĞCĢTCTČCĢÁCTĊCAŢĢAĆCĢÃĢĢÁC
361	Y F N R H F L E P V A C V S D S M I E D GCGGCGGGTGTTCGGCGGCAACAACAACAACAATGAATCTGGGCCTGCAGAATGCCAGCGCGCGC
421	A A V F G G N N N M N L G L Q N A S A L TATAAACCCCGAGATTATCGCCGTCTCCACCACCTGTATGGCCGAGGTGATCGGTGACGAT
481	Y K P E I I A V S T T C M A E V I G D D CTGCAGGGGTTTATCGCCAACGCCAAAAAAGAGGGATTTGTTGACGACAGCATCGCCATT
	L Q A F I A N A K K E G F V D D S I A I
541	CCTTACGCCCATACCCCCAGCTTTATCGGCAGCCATGTCACCGGCTGGGACAATATGTTC PYAHTPSFIGSFIGSHVTGWDNMF
601	GAAGGGTTCGCGAAGACCTTTACTGCTGACTACGCCGGGCAGCCGGGCAAACAGCAAAAAG
661	cŢĊĂĂŢĊŢĠĠŢĠĂĊĊĠĠĂŢŢŢĠĂĠĂĊĊŢĂŢĊŢĊĠĠĊĂĂĊŢŢĊĊĠĊĠŢĠĊŢĠĂĂĠĊĞĠĂŢĠ
721	ATGGCGCGAGATGGATGTCCCGTGCAGCCTGCTCCCGACCCATCAGAGGTGCTCGACACC
781	M A Q M D V P C S L L S D P S E V L D I CCCGCCGACGGCCATTACCGGATGTACGCCGGCGGCACCAGCAGGAGGAGATCAAAACC
841	P A D G H Y R M Y A G G T S Q E E I K T GCGCCGGACGCCATTGACACCCTGCTGCTGCAGCCGTGGCAGCTGGTGAAAAGCAAAAAG
901	A P D A I D T L L L Q P W Q L V K S K K GTGGTTCAGGAGATGTGGAACCAGCCGCCACCGAGGTGGCCGTTCCGCTGGCCCTGGCC
061	V V Q E M W N Q P A T E V A V P L G L A
901	A T D A L L M T V S Q L T G K P I A D A
1021	LTLERGCGCGGCCGGCTGGTCGACATGATGCTGGATTCCCACACCTGGCTGCAT
1081	GGCAAAAAAATTCGGCCTCTACGGCGATCCGGGATTTCGTGATGGGGCTGACGCGCTTCCTG
1141	CTGGAGCTGGGCTGCGACCGACGGTGATCCTCAGCCATAACGCCAATAAACGCTGGCAA
1201	LELGCEPIVILSHNANKRWQ AAAGCGATGAAGAAATGCTTGATGCCTCGCCGTACGGTCAGGAAAGCGAAGTGTTCATC
	K A M K K M L D A S P Y G Q E S E V F I
1261	AACTGCGACCTGTGGCACTTCCGGTCGCTGATGTTCACCCGTCAGCCGGACTTTATGATC
1321	GTAĂCTČCTĂCGĞCAĂGTŤTAŤCCĂGCĞCGĂTAĆCCŤGGČAAĂGGĊCĂAGĊCTŤCGĂA GNSVGKFTQRDTLAKGKAFE
1381	GTGCCGCTGATCCGTCTGGGCTTTTCCGCTGTTCGACCGCCATCATCTGCACCGCCAGACC
1441	ACCTGGGGCTATGAAGGCGCAATGAACATCGTCACGACGCTGGTGAACGCCGTGCTGGAA
1501	AAACTGGACCACGACACCAGCCAGTTGGGCAAAACCGATTACAGCTTCGATCTCGTCCGT
1561	KLDHDTSQLGKTDYSFDLVR TAA
1001	1nn *

Fig. 2. cDNA sequence and deduced amino acid sequence of *Klebsiella variicola* DX120E *nifK* gene

Table 1. The analysis outer membrane helix of *nifK* gene

0
85
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Sequence Exp n	umber, first	60 AAs	26	-05			
Sequence Total	n		0.09190				
Sequence TMH	ide	1-	520				
10	00	20	40	50	60	70	
10	20	30	40	50	60	10	
	I						
MSQTIDKIHSCYPL	FEQDEYQTLF	QNKKTLEEAH	DAQRVQEVF <i>I</i>	WTTTAEYEAL	NFQREAL TVDI	PAKACQ	
cccchhccccccc	ccchhhhhhh	hteccecce	chhhhhhhh	փհիհիհիհի	hhocheeeco	cccccc	
PLGAVLCALGFAGT	LPYVHG SQGC	VAYFRTYFNR	HFKEPVACVS	DSMTEDAAVF	GGNNNMNLGL(QNASAL	
chhhhhhhht cccc	ceeecccttc	hhhhhhhhh	heeccechhł	ihhhhhhhee	tcchhhhhhh	hhhhh	
YKPEIIAVSTTCMA	EVIGDDLQAF	IANAKKEGFV.	DDSIAIPYAH	ITPSFIGSHVT	GWDNMFEGFAI	KTFTAD	
cccteeeeehhhhh	ihhht cchhhh	hhhhhht ccc	cccceeeee		hhhhhhhhhh	hhhcc	
YAGOPGKOOKLNLV	TGFETYLGNF	RVI.KRMMAQM	DVPCSLLSDF	SEVLDTPADG	HYRMYAGGIS	QEEIKT	
ccccccccceeee	ecceccech	hhhhhhhh	toceeecoor		cceeccocco	hhhhh	
	U WKSKKWWOF	MUNIOPATEVIA	VPI CI AATDA	LI MTVSOL TG	KPTADAI TI FI	RCRIND	
ht ohbbooocco	aaabbbbbbbb	###WQI AILYA ++ aaaaaaaaaa	occoccoch	hhhhhhhh	aaaabbbbbbbb	hhhhh	
		CICCCCCEECC CITERIIRIC	CECCECCE			ruuuuu Repurt	
MMLDSHIWLHGAAP	GLIGDPDFVM	GLIKFLLELG	CEPIVILSH	AWAAQWAANA	NMLDASP16Q	ESEVFI	
hhhhhhhettcee	eeecccthhh	hhhhhhhhtt	cceeeeecco	cccchhhhhh	hhhhccccc	cceeec	
NCDLWHFRSLMFTR	QPDFMIGNSY	GKFIQRDTLA	KGKAFEVPLI	RLGFPLFDRH	HLHRQTTWGYI	EGAMNI	
cchhhhhhhhhht c	cceeeeectt	chhhhhttc	ccc <mark>hheeee</mark> e	eecccccccc	ccccceeecc	cthhhh	
VTTLVNAVLEKLDH	DTSQLGKTDY	SFDLVR					
hhhhhhhhhhhh	tocccccce	eeeeeh					

Fig. 3. Secondary structure prediction of nifK protein

	1	75	150	225	300	375	450	5
luery seq. Beta subunit P clus tein beta/alpha sub HoFe prote	ter binding residu unit interactions in beta subunit/Fe	s A All	A A A		MC	MoFe protein dimer/ dim	er interactions	<u> </u>
pecific hits	DUF3364			Nitroger	nase_MoFe	_beta		
uperfamilies	DUF3364 superfa		Oxidoreductase_nitrogenase superfamily					
ulti-domains				ni	£V.			

Fig. 4 Conserved domains of nifK



Fig. 5. Phylogenetic tree of *Klebsiellavariicola* DX120E nifK protein and other species nifK

proteins

1	(1)	MSQ TIDKIHSCYPLFEQDEYQTLFQN <mark>KK</mark> T- <mark>le</mark> rahdaq <mark>r</mark> iqevFawititaeyealnfqrealtvdPakacqplgavlCalgrag tlpyvhgsqgcvayfrtyfnrhf <mark>ke</mark> p
2	(1)	MSQ TIDKIHSCYPLFEQDEYQTLFQN <mark>KK</mark> T- <mark>le</mark> rahdaq <mark>r</mark> iqevFawititaeyealnfqrealtvdPakacqplgavlCalgrag tlpyvhgsqgcvayfrtyfnrhfkep
3	(1)	MSQ TIDKIHSCYPLFEQDEYQTLFQNKKT- <mark>le</mark> rahdaq <mark>r</mark> iqe <mark>i</mark> fawttiaeyealnfqrealtydpakacoplg <mark>aylcalgr</mark> ag t <mark>lpyyhgsqgcyayfrtyfnrhfkep</mark>
4	(1)	MSQTIDKINSCYPLFEQDEYQELFRNKRQ-LE <mark>EAHDAQR</mark> VQEVFAWITTAEYEALNPQREALIVDPAKACQPLGAVLC <mark>S</mark> LGFANIL PYVHGSQGCVAYFRIYFNRHFKEP
5	(1)	MSQ TIDK INSCYPLFEQDEYQELFRNKRQ-LE <mark>EAHDAQ R</mark> VQEVFAWTT TAEYEALINFQREAL TVDPAKACQPLG AVLC <mark>S</mark> LGFANTL PYVHGSQGCVAYFRTYFNRHFKEP
6	(1)	MSQ TAEK TQPCYPLFEQEEYQTLFRAKKG-MEEAHDEQRVRDVFEWTT TQEYQDLANFQREAL TIDPAK ACQPLG AVLCALGF AN TL PYVHGSQGCVAYFRTYFARHFKEP
7	(1)	MSQNTENTKSCYPLFEQDEY (NLF AD KHAK YEEAHG DE KVREVFEWTT IQ EYKDLINFSREAL TID PAK ACQPLG AVLCSLGFEK TL PYVHGSQGCVAYFR TYFNRHFREP
8	(1)	MSQDVENTQP SYPLER NEEYKK VIGDKRAK VEEMEPAEKIREVFEWIT IK EYQELINFQREAL IVNPAK ACQPLG AVLCALGVEK IMPYVHGSQGCVAYFR TYFNRHFK EP
9	(1)	-MQIVDDIKPCYPLEGEDAYKATLARKREEFECHPEDKIAETFLWTTSEEYKELNEKREALTVNPGKACQPLGAVLCALGFEKTLPYVHGSQGCVAYFRTYFNRHEKEP
10	(1)	MSQ TVDK I KPGYPLFQQPEYQELFAK KRGEHEDOFDEARVKEYFEWTT TEEYKELNFARKQMALDPAK ACQPLO SVLCALOFEK TL PYVHGSQGCVAYFR TYFNRHFKEP
11	(1)	MTQX VDDIKPG <mark>PFLENDEDYQSML AFKRSNFEELASPEKVKEVFDWTTSEEY</mark> AKLINFERKH <mark>TTIDPAKACQPLG SVLC<mark>G</mark>LGFENTL PYVHGSQGCVAYFRTYFNRHFKEP</mark>
12	(1)	MTQX WEDIKUGVALEK OPEYOELFAS KHATFEEAVEEAKVKEVFDWTTIK EYOEINFNRKH <mark>WIID</mark> PAKACOPLSAVLCALGFEKIL PYVHGSOGCVAYFRTYFNRHFKEP
1	(110)	VAC VSD SMTEDAAVFGGNNNINLGLANA SALYKPETTAVSTTCMAEVIGDDLAAFTANAKKEGFVDDSTATPYAHTPSFTGSHVTGVDNIFEGFAKTFTADYAG— OPGK
2	(110)	VACVSDSMTEDAAVPGGNNNINLGLANASALYKPELTAVSTTCMAEVIGDDLAAFIANAKKEGFVDDRIATPYAHTPSFIGSHVTGVDNMFEGFAKTFTADVAG— OPGK
3	(110)	VACVSDSMTEDAAVPGGNNUNULGLANASALYKPETTAVSTTCMAEVIGDDLAAFTANAKKEGFVDDSTATPYAHTPSPTGSHVTGVDNNFEGFAKTFTADVAG— OPGK
4	(110)	I AC VSD SMTEDA AVPGGNINDINL GLA NA SALYKPETTA VSTTCIMAEVIGDDLAAFTA NAKKDOFVDSSTA VPHAHTPSPIGSHVTGVD NINFEGFAKTPTA DYQO— OPOK
5	(110)	IACVSDSMTEDAAVPGGNNUNUGLQNASALYKPEITAVSTTCMAEVIGDDLQAFIANAKKDGFVDSSTANPHAHTPSFIGSHVTGVDNNFEGFAKTFTADYQQ—QPGK
6	(110)	I AC VSD SMTEDA AVPGGNINTINSGLQNASALYQPENTAVSTTCMAEVIGDDLQAFIANAKKDGFVATDIPTPYAHTPSFIGSHITGWDNNFEGFARTFTTGEGKNYQPGS
7	(111)	VACVSDSMTEDAAVPGGHAMMIDGLANALALYKPENTAVSTTCMAEVIGDDLAAFTNNAKKDGFVPKDFPVPVAHTPSFVGSHTTGVDNNFEGFCNTFTADKAD-YKVGS
8	(111)	IACVSDSMTEDAAVPGG <mark>Q</mark> KNIFDGLENAKALYKP DNI AVSTICINAEVIGDDL NAFINNS KKEGHIPQEYPYPFAHTPSFYGSHTTGWDNINQEGILRYFTLMINEDKQWGA
9	(110)	I ACVSD SMTEDA AVFGG <mark>o</mark> knif Sole naralykpennavs ticmævigddi. Nafignakkeghipgeppppahtpsføgshvigvdnifeg i i Ryftinemtgkopos
10	(111)	<mark>vacvsdsmtedaavpgggknifdglanalalykpevlavsticmævigddlinafignakaegvidkdfptpfahtpsføgshttgwdgmfegfmryftlnemedkevgs</mark>
11	(111)	<mark>vacvsdsmtedaavpggqdnifyglqnayalykpevlavsticmaevigddlnafignak</mark> dk <mark>gfipaevp</mark> tpfahtpsfwgshingwdamfegniryftladkgeykv <mark>a</mark> s
12	(111)	<mark>wacysd smtedaavpggqknnf aglqna yalykpen tavs ticmaevigddlnaf ignak <mark>kegyl</mark>pes<mark>y</mark>p tpe ahtpspngshvtg wdnnfeg ilr yf tlnemd skev<mark>gs</mark></mark>

1	(218)	
2	(218)	ONT MUNICIPALITY CHERYLIKE WANNER AND THE STREET AND THE ADDRESS OF TAXANDA THE DATE OF THE STREET AND THE STREET A
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5	(218)	I PKI NI VICERTVI CHERVI VRVIROVAVPCSI I SDPSTVI DIPADCHVRVYSCITIOCEN RAPDATI – AAPOPIOI I KISK KUNDERIMOPATEVATELCI AA TIPICI V
6	(220)	OPELMI VTCERTVI CHERVINKEMIAONDVPCSLI SDESEVI DTPADCHVRIVACCT SOCEMERAPNATOTI I LOPHELVKTK KINODVMOPATAN PUPTCI AATOKI I V
7	(220)	NCKI NTVI CRETVI CNVRVIKKU TENCVPRANI SDESEVI DTPŠOGOVRIVACCITODENKDAPNATOTI NI OPVELVKIKKUVKET VCOPATDI STEVICI EVIDELI V
8	(221)	NGKI NYYEGETYI GRENTIKA MEENYETINI SPETEVI DIPADGERIYAGGI TADENKAAPNAEDI ELOPAGSIKIRAYAONI WHEAPKI NI PAGI EVIDEN A
g	(220)	NCKTNTVPCRETVI CHERVITKRMI TENCINYSET SDEERVID TENDOT VRIVACCT SOARUKDAENST TILLI OPI OLIVKITK VAVEAT VOHD TEKUDI PIKU FUTDOVI V
10	(221)	NGKTNTVPGFETYLGNYRVTHSM. OF IG VEYSLLCDPS EVID TPÅDGE VRIVAGGTPLAFIK DAPNALTTVLLOPDOLVKTK KETDUT VKHEVPALINT PIGLDYTDEFLM
11	(221)	ENKLATVPGFETYLGAVRVTKK M.DONGVDYSVLCDPSEVLDTPÅDGOVRNVÅGGTPISEVKSAPNÅKATLFI OPEHSVKSRKFTATT VEGETPDTKTPNGLDWTDEFVM
12	(221)	NGKTNTVPGFETYLGNYRVIGRILKE ID VDYSFLCDPSEVLDTPÅDGE VRIVAGGT KVDEVKDAPNATAT TILLOPDONTKST KFVK DT WVDE VPALINT PIGLEVTDDFI M
	,	
1	(328)	
2	(328)	
3	(328)	
4	(328)	TYSOL SCRETADALTI FROM UTIMIT DENTITI HERKRELYCOPOPTYWCI TRELL FLOCEPTYTI, SHNANKRWOKANKKM DA SPYCROSENETICDI WERRELMETROPO
5	(327)	TVSOLSCRPTADALTLERGRUNDTULDSH-WURCKKEGLYGDEDEVIGUTER LLELGCEPTVILSHNGODTLDKAINKMUDASRYGRDSEVETINRDUWERSLIFTRSAG
6	(330)	TWSELTGKPTGEALTLERGRLVDMULDSHTWLHGKRPGLYGDPDFVMGLTOPLLELGCEPTVTLCHNGSKRWLKAMKALLEASPYGQDSDVWTINCDLWHFRSLNFTQKPD
7	(330)	KWSALTGKATPASLELERGRLVDMITIGSHTWLHJKKPGWGDPDFVBGLVKPLLELGCEPNVILCNNGSKKWKKSMEKMLADSPYGQGSEVWGHDLWHPRSLMFTNKPD
8	(331)	KWSE <mark>I</mark> T GKPIP <mark>ES</mark> LIKERGRLVDM II DSHAWLH (K <mark>RYA</mark> LVGDPDFVMG <mark>MIK</mark> LLLELGAEP VHILAN <mark>NANKRWKKAMDKI</mark> LDD SPYGIGCEVHIGRDLWHLRSL <mark>W</mark> FIDKPD
9	(330)	K WSE IT OKPIPD SLTKERGRLVD MMTDSHAWLHCKRYAVWCOPDFT NC IT OF LLELGAEPLHVLCHNOCKRWEKAMKKMLENSPYNOMTRLVSKHDLWHMRSLVFTEKPD
10	(331)	K lae is g keif <mark>es</mark> lak a <mark>rgrlvd mut</mark> dshi vlhvisfs vygdpd vllg i vkflq elg cevkhil on <mark>gak kwkkka</mark> ealtae <mark>a</mark> pgtadae tvfkqdlwhfrslvftnkpd
11	(331)	K <mark>w selt g</mark> op <mark>tedelakergrlyd mut</mark> dsht wlhowsislygded yllg hv <mark>ke</mark> lt elgedikh <mark>vlehnankrwrkkh</mark> eal cae <mark>spy</mark> san av <mark>wev</mark> okdlwherslaftered
12	(331)	K <mark>wseltg kptpasltke</mark> rgrlud <mark>nut</mark> dsht wlhgvt is <mark>tegded vllg noklitelgceikhvlchnonkkwrkkm</mark> eaqlae <mark>te vgadaq ve v</mark> gkdlwhfrslmfteked
1	(43)	8) FMIGNSYGKFIQRDTLRKGKAFEVPLIRLGPPLFDRHHLHRQTTWGYEGAMNINTTLVNAVLEKIDHDTSQLGKTDYSFDLVR
2	(43)	8) FMIGNSYGKFIQRDTLRKGKAFEVPLIRLGPPLFDRHHLHRQTTWGYEGAMNINTTLVNAVLEKIDHDTSQLGKTDYSFDLVR
3	(43)	8) FMIGNSYGKFIQRDTLAKGKAFEVPLIRLGPPLFDRHHLHRQTTWGYEGANNINTTLVNAVLEKLDHDTSQLGKTDYSFDLVR
4	(43)	8) FMIGNSYGKFIQRDTLAKGKAFEVPLIRIGPPLFDRHHLHRQTTWGYEGANNINTTLVNAVLEKIDSDTSOLGKTDYSFDLVR
5	(43)	6) I MIGNSYGKF IQED TLAKGKAFEVPLIRIGFPLFDRHHLHRQTT SGYEGANNINTTI VNVVLEKID SDTSPAGK TDYSFDLVR
6	(44)	D) FMIGNSYCKFIQEDTLAKGEQFEVPLIRLGFPLFDRHHLHRQTTWGYEGANSILTTLWNAVLEKLDHDTMKLGQTDYNFDLIR
7	(44)	D) FMIGNSYGKFIQRDTLAKGKAFEVPLIRLGFPIFDRHHLHRMITLGYEGAN <mark>YMLITLVNAVMEKI</mark> DSETMELGKTDYNFDLVR
8	(44)	1) FLIGNSYGKFIQRDTLHKGEAFEVPLIRFGFPIFDRHHLHRDTTLGYEGANHWLKTLVNEVLARLDDDTRGMGTTDYNYDLIR
9	(44)	D) FMIGNSYGKFIQRDTRHK <mark>G</mark> KPFEVPLIRNGPPIFDRHHLHRMTTIGYEGA <mark>nvilttivnavleridde</mark> tnivn <mark>gk tdynvdli</mark> r
10	(44)	1) F <mark>i</mark> ignsygkfiqrdtkakgeefevplirlgfpifdrhhlhrnatigyega <mark>nymlittlynevlakidid isemgr</mark> idyg <mark>ydlyr</mark>
11	(44)	1) I <mark>mignsygkfierd tlakgaefevplyri</mark> gfp i fdrhhlhre <mark>ttigyeggnyilktivne il tkidrd inklig</mark> itdygf <mark>dlyr</mark>
12	(44)	1) L <mark>mignsygkfierd Tkakgaefevpl<mark>wri</mark>gpp<mark>i</mark>fdrhhlhrsa<mark>tlgyegamyvlttwnewlakl</mark>dr<mark>e tsdlgk tdya</mark>fdlwr</mark>

Fig. 6. Aligment of the deduced *nifK* amino acid sequence and its homologus amino acid sequences. 1. *Klebsiella variicola* DX120E; 2. *Klebsiella variicola*; 3. *Klebsiella pneumoniae*;
4. *Klebsiella oxytoca*; 5. *Klebsiella pneumoniae*; 6. *Dickeya solani*; 7. *Tolumonas auensis*; 8. *Thiobacillus prosperus*; 9. *Thioploca ingrica*; 10. *Aliagarivorans marinus*; 11. *Vibrio* sp.; 12. *Shewanella* sp.

Construction of prokaryotic expression plasmid and identification of recombinant

The n*ifK* gene was recovered and purified, and pET-30a (+) plasmid was extracted at same time. EcoRI and XhoI restriction enzymes were applied for double digestion. The recovered target fragment was connected with expression vector pET-30a (+), and then transformed into DH5 α competent cells. The positive colonies were selected for PCR detection. The agarose gel electrophoresis showed the fragment was about 1563 bp. The obtained recombinant pET30a-nifK was doublly digested by EcoRI and XhoI and then sequencing. The sequencing results showed that *nifK* gene had inserted to vector plasmid. The DX120E *nifK* protein prokaryotic expression vector pET30a-nifK had successfully constructed. The correct recombinant was transformed into competent cells BL21 (DE3).



Fig. 7. Double enzyme digesting identification of restructured plasmid. M: 10 000 bp; 1: pET30a-nifK; 2: double digestion of pET30a-nifK

SDS-PAGE analysis

The empty pET-30a strain and recombinant strain were both transformed into the liquid LB medium with Kan 100 mg \cdot L⁻¹ to induce gene expression. When OD₆₀₀ reached 0.6, the medium was added with 1.0 mM IPTG for induction and 2.0 mL medium was collected at 0, 1, 2, 3, 4 and 5 h, respectively, for SDS-PAGE. The empty pET-30a strain and recombinant strain without IPTG as control (Fig. 8). As seen in Fig. 8, the target protein started expression at 1 h, reached the maximum expression level at 3 h, and then the expression

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amount was not obvious.

Fig. 8 showed that, compared with the empty vector pET-30a (+), pET30a- nifK existed a fusion protein band in the molecular weight of approximately 58 kDa position, which was consistent with the expected size and the mass spectrometric nifK gene.



Fig. 8. SDS-PAGE analysis of restructured nifK protein. M: protein marker: 1: pET-30a without induction; 2: 1.0 mmol/L IPTG induced pET-30a (+)for 3 h; 3: pET-nifK without induction; 4-8: 1.0mmol/L IPTG induced pET-nifK for 1h-5h

Solubility analysis of fusion protein

The recombinant bacteria pET30a-nifK after IPTG induction was broken by ultrasonic fragmentation. After analysis, the recombinant protein could express both in the supernatant and precipitate (Fig. 9).



Fig. 9. Soluble analysis of pET-nifK protein. M: protein marker; 1: ITPG induced 3h precipitation of pET-nifK; 2: ITPG induced 3h supernatant of pET-nifK; 3: ITPG induced 3h bacteria of pET-nifK

Discussion

The abundant but inert N_2 in the atmosphere is converted to useful NH_3 by the metallonzyme nitrogenase. The Fe-protein is a dimer of identical subunits encoded by the *nifH* gene, with a molecular weight in the range of 58-72 kDa, both subunits are bridged by one [4Fe-4S] metal center and contain two nucleotide [MgATP or MgADP] binding sites (Peters et al, 1995; Kim et al, 1994; Schindelin et al, 1997). The MoFe-protein is a tetramer and is composed of two identical halves, containing α subunit and β subunit encoded by *nifD* and nifK genes, respectively, with a molecular weight in the range of 200-240 kDa (Howard et al, 1996; Burgess et al., 1996). The MoFe-protein contains metal clusters: the P-cluster and the iron-molybdenum cofactor or FeMo-co which participates in electron transfer from the Fe protein to the FeMo-cofactor (Chan et al., 1993). NifH and NifDK perform the reduction of N₂ into NH₃ by coupling the reduction process to the free energy liberated from the hydrolysis of MgATP (Bulen et al., 1996). In this study, the nifK gene was cloned from the nitrogen fixation strain *Klebsiella variicola* DX120E. The ORF is 1563 bp and has 520 amino acids. Comparisons of the amino acid sequence with other known *nifK* gene sequences suggested that Klebsiella variicola DX120E was most similar to *Klebsiella* spp. Phylogenetic tree also implied that nifK protein had the highest level of conservation with different species, which met the evolutionary relationships. It was predicted that nifK protein molecular weight of Klebsiella variicola DX120E is 58.12 kDa, and the pI is 5.54. The prokaryotic expression with system using pET30a (+) T7bacteriophage promoter could integrated poly-histidine-tagged into the protein. This method has many advantages such as overexpression, being easy to operate, and purification (Shan et al., 2008). T7 promoter on pET30a expression vector can specifically bind with T7 RNA polymerase within Escherichia coli BL21 (DE3), and then start downstream target gene expression of the T7 promoter. This study construct prokaryotic expression vector pET30a-nifK, after PCR, restriction enzyme digestion and sequencing, the correct recombinant strain was induced by IPTG out of a treated

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58.12 kDa fusion protein. The obtained recombinant protein laid foundation for further studying the *nifK* gene function, antibody preparation on nitrogen fixation. **Conclusions**

This study cloned *Klebsiella variicola* DX120E *nifK* gene from sugarcane and registered in GenBank with accession number KF732647. The ORF of *nifK* gene is 1563 bp, which encodes 520 amino acids. The protein molecular weight is 51.1 kDa, and the pI is 5.54. Bioinformatics analysis showed that the gene is highly conserved in the functional region. Phylogenetic analysis showed that the gene had the highest homology with that of *Klebsiella*. Prokaryotic expression results showed that the gene was expressed in fusion protein which relative molecular weight is 58.12 kDa approximately. The results laid molecular mechanism foundation for further studying *Klebsiella variicola* DX120E *nifK* gene interaction with sugarcane.

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