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Qualitative Validation of Native Diazotrophs Through *nif*H gene and Whole Cell Protein Profiling

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The native diazotrophic bacterial isolates (ABA-1, ABA-10, ABA-14, ABA-2010, ACG-2 and ASA-1) and four reference strains (MTCC-446 and MTCC-124 (*Azotobacter chroococcum*), MTCC-1226 (*Acetobacter diazotrophicus*) and MTCC-2306(*Azospirillum lipofarum*) were used for the study. Bacterial culture were grow on their specific nitrogen free medium. Detection of *nif*H (Nitrogen fixation) gene by polymerase chain reaction (PCR) amplification using specific *nif*H primer and whole cell protein profiling by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Presence of *nif*H gene confirms the nitrogen fixing nature of native diazotrophic isolates thus native diazotrophic isolates and reference strains which have showed the best nitrogen fixation ability and further validation of the Nitrogenases enzyme (MoFe- 220 to 240 kDa or Fe- 60 kDa protein) which is responsible for biological nitrogen fixation in all native nitrogen fixing bacterial isolates as well as standard cultures viz., (MTCC-446 and 124), (MTCC-1226) and (MTCC-2306) at three different incubation hours 24, 48 and 96 hrs proving their diazotrophic nature. Detection of *nif*H gene and Fe- 60kDa protein validation, which gave authentication to all the native nitrogen fixer bacterial isolates belonging to *Azotobacter*, *Acetobacter* and *Azospirillum*.

Keywords: Diazotrophs, PCR, *nif*H gene, Nitrogenases, SDS-PAGE, Biological Nitrogen Fixation (BNF).

Molecular approaches have been developed and successfully applied to describe such diazotrophic communities in different cultivated and forest soils (Poly *et al*., 2001 and Shaffer *et al*., 2000), pasture, agricultural soils (Poly *et al*., 2001), wetland soils (Chelius, 1999) and rhizospheres (Hamelin *et al*., 2002). The molecular approaches for detection of diazotrophic organisms is primarily based on PCR amplification of a marker gene for nitrogen fixation (*nif*H).

The *nif* genes which encode the nitrogenase complex and other enzymes involved in nitrogen fixation has consensus sequences among nitrogen fixing bacteria. The structure of the *nif* genes is similar and the regulation of the *nif* genes varies between different diazotrophs (nitrogen fixing organisms), depending also upon the organism's evolutionary hierarchy. The nitrogenase complex is encoded by approximately 20 different *nif* genes (Pedrosa *et al.,* 2001).

Biological nitrogen fixation (BNF) is an important source of fixed nitrogen for the biosphere. Microorganisms catalyze biological nitrogen fixation with the enzyme nitrogenase, which has been highly conserved through evolution. Nitrogenases are composed of two proteins that can be purified separately: dinitrogenase and dinitrogenase reductase. Dinitrogenase, also referred to as the MoFe protein or component 1 is a 220- to 240-kDa tetramer of the *nif*D and *nif*K gene and Dinitrogenase reductase, also referred to as the Fe protein or component 2, is a 60- kDa dimer of the product of the *nif*H gene, which is responsible for nitrogen fixation. Biological nitrogen fixation can be represented by the following equation, in which two moles of

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ammonia are produced from one mole of nitrogen gas, at the expense of 16 moles of ATP and a supply of electrons and protons (hydrogen ions):

$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi$

This reaction is performed exclusively by prokaryotes (the bacteria and related organisms), using an enzyme complex termed nitrogenase. This enzyme consists of two proteins - an iron(Fe) protein(60 kDa) and a molybdenum-iron(MoFe) protein (230 kDa). The reactions occur while N_2 is bound to the nitrogenase enzyme complex. Electrons donated by ferredoxin first reduce the Fe protein. Then the reduced Fe protein binds ATP and reduces the molybdenum-iron protein, which donates electrons to N_2, producing HN=NH. In two further cycles of this process (each requiring electrons donated by foredooming) HN=NH is reduced to $H_2N\text{-}NH_2$ and this in turn is reduced to $2NH_3$. Depending on the type of microorganism, the reduced ferredoxin, which supplies electrons for this process, is generated by photosynthesis, respiration or fermentation.

This approach allowed a sound method for the unambiguous validation of the Nitrogenases enzyme which is responsible for biological nitrogen fixation, also in view of deepening knowledge on the ecology of these microorganisms and their biotechnological exploitations.

To achieve the maximum BNF there is a need to characterize root nodule and free living beneficial bacteria which are made commercially available for field application as biofertilizers. Few native diazotrophic bacterial cultures available at Department of Microbiology, BACA, AAU, Anand are studied for presence of *nif* gene by molecular methods to investigate and prove their ability to fix atmospheric nitrogen by confirming the presence of *nif*H gene and unambiguous validation of the Nitrogenases enzyme (MoFe or Fe protein) which is responsible for biological nitrogen fixation. As no information is available on molecular characterization of nitrogen fixation gene (*nif*H) from native diazotrophs isolates from Anand *viz*, *Azotobacter, Acetobacter* and *Azospirillum*, an attempt was made through research efforts.

MATERIALS AND METHODS

Bacterial strain and Cultures revival

Six native diazotrophic bacterial isolates isolates (ABA-1, ABA-10, ABA-14, ABA-2010, ACG-2 and ASA-1) and four reference strains (MTCC-446 and MTCC-124 (*Azotobacter chroococcum*), MTCC-1226 (*Acetobacter diazotrophicus*) and MTCC-2306(*Azospirillum lipofarum*) grown in respective nitrogen free media *viz*, Burk's medium for *Azotobacter*, diluted cane juice semi solid medium for *Acetobacter* and NFB (Nitrogen Free Bromothymol blue medium) for *Azospirillum* (Cavalcant and Dobereiner, 1988) (Table-1). No human or animal were harmed or taken for experimentation during present investigation.

Amplification of *nif*H gene

Genomic DNA of all native diazotrophic bacterial isolates and standard strains were isolated using the protocol described by Nour *et al*. (1995) and Sambrook *et al*. (1989). Fragments of *nif*H genes were amplified by two PCR reaction. PCR was performed in PCR reaction mixture (25 µl) containing 2.5 µl Taq Buffer (10 X), 0.5 µl dNTPs (2.5 mM each) mix, 2.0 µl Template DNA (25 ng/µl), 0.4 µl Taq polymerase (5U/µl), 17.8 µl Millipore Sterilized Water using degenerated following primers (Poly *et al*., 2001) 1.0 µl Primer 1 (Pol F- 5' TGCGAYCCSAARGCBGACTC 3') and 1.0 µl Primer 2 (Pol R- 5' ATSGCCATCATYTCRCCGGA 3') and the primers synthesized at MWG Bio-tech Pvt. Ltd., Germany. PCR was successful to amplify a 360 base pair (bp) *nif*H fragment from the 3 different native diazotrophic bacterial isolates.

PCR reaction mixture was prepared from the stock solutions of each individual components. The reagents were mixed thoroughly by a short spin using microfuge. The tubes were placed in Mastercycler personal (Eppendorf) and subjected to PCR, according to the following protocol. Denaturation at 94 C for 45 sec, annealing at 55 C for 45 sec, elongation at 72 °C for 45 sec. An initial denaturation step at 94 C for 3 min and a final extension step at 72 °C for 5 min were also performed. PCR reactions were run for 30 cycles. PCR products were analyzed by gel electrophoresis with molecular marker DNA (100 bp ladder) of known molecular weight on 1.8 % agarose gel at 80 V using 1 X TAE buffer and ethidium bromide (0.5 µg/ml). Gels were visualized under UV light and photographed using gel documentation system.

The secondary PCR was performed with a 25 µl reaction mixture same as above with modification containing 0.5 µl of the first PCR

product as the DNA template. PCR amplification condition used were, denaturation 30 sec. at 94°C; annealing 30 sec. at 55 °C and extension 30 sec. at 72°C.

PCR products were subjected to gel electrophoresis with molecular marker DNA (100 bp ladder) of known molecular weight on 1.5 % agarose gel at 80 V using 1 X TAE buffer and ethidium bromide (0.5 µg/ml). Gels were visualized under UV light and photographed using gel documentation system.

Whole cell protein profiling by SDS-PAGE

Quantification of protein

The cell sediments of these cultures were used for protein quantification by the Lowry method (Lowry *et al*. 1951). The cultures (10 ml) were centrifuged (10,000 rpm, 5 min), re-suspended in an equal volume of sterilized water and diluted (100 µl cell suspension: 400 µl water). Further the cell suspension (500 µl) was mixed with NaOH 1 M (500 µl) and boiled (100°C, 5 min) to disrupt the membranes and release the total cellular contents. The extracted cellular sap (500 µl) were mixed with 2.5 ml of fresh reagent mixture. After 10 min. of reaction 500 µl Folin–Ciocalteu's Phenol reagent (1 M) was added. After 30 min of incubation in the dark the protein content of each sample was measured at 750 nm using spectrophotometer (ELICO SL 150 UV-VIS Spectrophotometer). A standard curve of bovine serum albumin was used for calibration and protein quantification.

The whole cell protein profile was carried out by SDS-PAGE for all the native diazotrophic bacterial isolates with standard strains protocol described by Sambrook *et al*. (1989).

Silver staining protocol

The gel ready for staining is fixed in 40% methanol, 10% acetic acid and 50% deionized water for 1 hr or more, washed twice for 20 minutes with 30% methanol and 70% deionized water and washed for 20 minutes with deionized water. The gel is than ssensitized with 0.02% sodium thiosulfate for 1 minute. Wash thrice for 20 seconds with deionized water and passed through 0.2% silver nitrate and 0.02% formaldehyde (37%) for 20 minutes. After washing thrice for 20 seconds with deionized water, the gel was developed 3% sodium carbonate, 0.5% Formaldehyde (37%) and 0.0005% sodium thiosulfate for 2 to 15 minutes. It was then washed for 20 minutes with deionized water, placed in 0.5% glycine solution to stop further development for 5 minutes and washed thrice for 10 minutes with deionized water. The gel was kept overnight and the bands visualized and photographed with biotech gel documentation system or camera.

RESULTS AND DISCUSSION

Amplification of *nif*H gene

The presence of *nif*H gene was detected by PCR amplification (Fig. 1). This gene codes for Fe-protein (component II) the enzyme nitrogenase complex. Variable sized fragments were generated from diazotrophic isolates.

The isolated genomic DNA from diazotrophic native isolates and reference strains were amplified using two stage PCR approach. The PCR amplicon include primary and secondary products. The desired size of *nif*H gene amplicon should be 360 bp, provided primers were Pol F and Pol R (Poly *et al*., 2001). As shown in Fig. 1 resolved band at expected size of approximate 360-bp was observed in secondary PCR product only (Pandey *et al*., 2004, Raja *et al*., 2006, Fernando *et al*., 2006, Sato *et al*., 2009, Reghuvaran *et al*., 2012).

Two stage PCR approach has been adopted in several studies for detection of *nif*H (Choo, Samian & Najimudin, 2003; Yeager *et al*., 2004). This approach was favoured as two stage PCR is less affected by sample inhibition compared to single-stage PCR (Zani *et al*., 2000). In addition, Widmer *et al*. (1999) had proven in their findings that PCR approach based on conserved primer target improved the specificity and sensitivity of *nif*H amplification. One pair of degenerate (universal) primer was used to amplify a relatively short (~360bp) but phylogenetically informative segment of *nif*H gene (Zehr & McReynolds, 1989). Degenerate primer was used to amplify most of the diazotrophs from representative taxa as it compromised specificity during amplification (Zehr & McReynolds, 1989). For the primary amplification, primer (pol-F & pol-R) was used, smearing and non specific bands observed which attributed to the amplification of irrelevant sequences flanked within both the primers (pol-F & pol-R) and variation in the size of PCR products as well as presence of more than one fragment in *nif*H gene amplification is to be explained based

Table 1. Composition of different media used for strain isolation and revived of native diazotrophs

Ingredient	Burk's mediumgm/lit.	Nitrogen Free Bromothymol blue mediumgm/lit.	LGIP mediumgm/lit.
Malic acid	—-	5 g	—-
KOH	—-	4 g	—-
K_2HPO_4	0.8 g	—-	0.2 g
KH_2PO_4	0.2 g	0.5 g	0.6 g
$MgSO_4.7H_2O$	0.2 g	0.01 g	0.2 g
NaCl	0.2 g	0.02 g	—-
$CaSO_4$	0.1 g	—-	—-
$CaCl_3$	—-	—-	0.02 g
H_3BO_3	100 mg	—-	—-
$FeCl_3$	—-	—-	0.01 g
$ZnSO_4.7H_2O$	100 mg	—-	—-
$FeSO_4.7H_2O$		0.05 g	—-
$MnSO_4.4H_2O$	10 mg	0.01 g	—-
$CuSO_4.5H_2O$	3 mg	—-	—-
Na_2MoO_4	—-	0.002 g	0.002 g
Cane sugar	—-	—-	100 g
KI	1 mg	—-	—-
FeMo mixture	1 ml	—-	—-
Bromothymol blue (0.5% alc.soln)	—-	2 ml	5 ml
Distilled water	1000 ml	1000 ml	1000 ml
Agar	20 g	1.75 g	20 g
pH	7.3	6.6-7.0	6.0
FeMo mixture: $FeCl_3.6H_2O$-14.5 g $Na_2MoO_4.2H_2O$-2.53 g	—-	—-	—-

on earlier reports of Pandey *et al.* (2004) and Laguerre *et al.* (1996).

Our results suggested that specific and more resolved bands were observed only in target DNA fragment of an approximate 360 bp was predominantly amplified in diazotrophic isolates. Presence of *nif*H gene confirms the nitrogen fixing nature of diazotrophic isolates as in present study (Fig. 1) has conformed. The *nif*H gene is widely used as marker gene for screening nitrogen fixing prokaryotes in soil (Burgmann *et al.,* 2004). Thus native diazotrophic isolates and reference strains which have shown the presence of *nif*H gene thus conforming their best nitrogen fixation ability.

Whole cell protein profile by SDS-PAGE

Quantities of total protein (whole cell) present in native diazotrophic bacterial isolates were ranging from 141.78 to 984.91 µg/ml (Fig. 2). All native isolates were comparable with each group of reference strains for protein production. *Azotobacter* isolates *viz*, ABA-1 and ABA-10 showed average amount of protein quantity, but

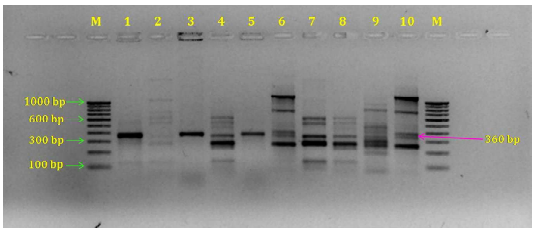

Fig. 1. Amplification of the nitrogen fixation (*nif*H) gene from diazotrophic native isolates and their reference strains using degenerated universal *nif*H gene primer. *Azotobacter* isolate, Line: 1. ABA-1, 2. ABA-10, 3. ABA-14, 4. ABA-2010 and reference strains 5. MTCC-446, 6. MTCC-124 (*A. chroococcum*). *Acetobacter* isolates, Line: 7. ACG-2 and reference strain 8. MTCC-1226 (*A. diazotrophicus*). *Azospirillum* isolate, Line: 9. ASA-1 and reference strain 10. MTCC-2306 (*A. lipoferum*)

ABA-14 and ABA-2010 showed higher amount of protein (979.78 µg/ml) and (984.91 µg/ml) respectively compared to the standard strain (863.14 µg/ml) at 96 hrs (Fig. 2). On other hand *Acetobacter* isolate ACG-2 produced lowest amount of protein (433.90 µg/ml) compared to the respective standard strain MTCC1226 (608.86 µg/ml) While *Azospirillum* standard strain MTCC-2306 showed better (655.65 µg/ml) amount of proteins compared to the native ASA-1 isolate (555.21 µg/ml) at 96 hrs (Fig. 2). To study the whole cell protein profile of native diazotrophic bacterial isolates were used for primary detection of protein (MoFe or Fe protein) (Peng *et al*., 2006) at different hours (0, 24, 48 and 96 hrs.) for checking the initial period of *nif*H gene product (Fe protein) and detecting quality protein produced by test isolates over different time interval (hrs.).

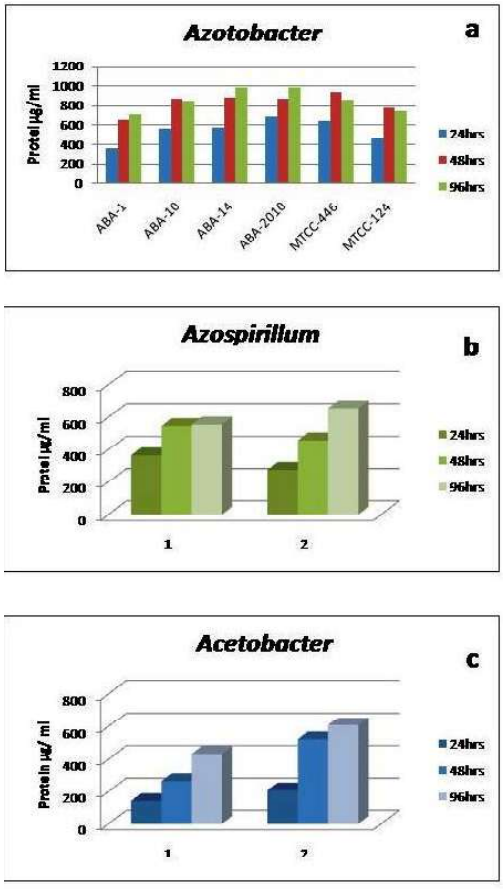

Fig. 2. Protein concentration (µg/ml) of diazotrophic isolates (a) *Azotobacter*, (b) *Acetobacter* and (c) *Azospirillum* at different growth hours (24, 48 and 96)

Azotobacter isolate ABA-1 &10, ABA-14 & 2010 and MTCC-446 & 124 (Fig. 3) (*A. chroococcum*) reference strains showed the whole cell protein profile at different growth hours (0, 24, 48 and 96 hrs.). The polypeptide band highlighted in yellow box on plates seems to be Fe protein band of *Azotobacter* isolates, which were matching to 60 kDa molecular marker. It means *Azotobacter* isolates and standard strains are producing Fe protein (*nif*H gene product) at 24 hrs, 48 hrs and 96 hrs responsible for nitrogen fixation on specific nitrogen free media.

For *nif*H gene, native diazotrophic isolates showed the 60 kDa polypeptide band was subjected to Fe protein. Thus, SDS-PAGE gives preliminary information about *nif*H gene expression product (Fe protein) according to may be a specific polypeptide band (60 kDa) present in isolates.

Whole cell protein profile of *Acetobacter* isolate ACG-2 (Fig. 3g) and respective reference strain MTCC-1226 (*A. diazotrophicus*) (Fig. 3h) at different growth hours (0, 24, 48 and 96) showed scanty protein banding patterns compared to other diazotrophic isolates (Fig. 3abcdefij). Protein banding pattern started from 48 hrs in MTCC-1226 (*A. diazotrophicus*) reference strain and at 96 hrs in ACG-2 *Acetobacter* isolate. Due to slow growth in nature polypeptide band (highlighted in yellow box) matching to Fe protein (60 kDa), however intensity was very low in both culture.

Azospirillum isolate ASA-1 (Fig. 3i) and reference strain MTCC-2306 (*A. lipoferum*) (Fig. 3j) represent the whole cell protein profile at different growth hours (0, 24, 48 and 96 hrs) showed clean separation of protein band on gel for both the cultures. Banding pattern of that cultures started from 24 hrs and intensity of protein bands were gradually increased with the increasing time that means when increasing the growth period of culture their specific nitrogen fixing protein intensity increases.

Microorganisms catalyze biological nitrogen fixation with the enzyme nitrogenase. Nitrogenases are composed of two proteins, dinitrogenase and dinitrogenase reductase. Dinitrogenase, also referred to be MoFe protein or component 1, which is a 220 to 240 kDa tetramer of the *nif*D and *nif*K gene products. Dinitrogenase reductase, also referred to as the Fe protein or component 2, is a 60 kDa dimer of the product of

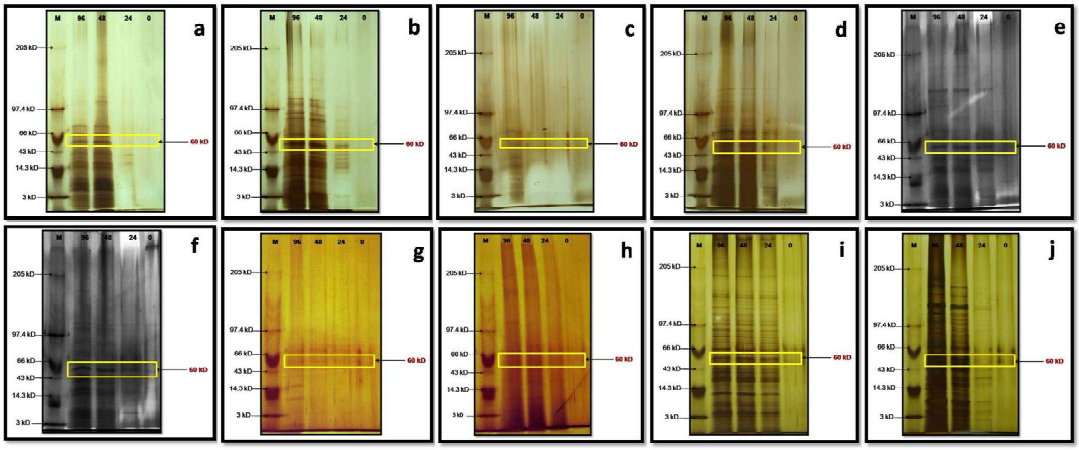

Fig. 3. Protein patterns of native diazotrophs (a) ABA-1, (b) ABA-10, (c) ABA-14, (d) ABA-2010, (e) MTCC-446, (f) MTCC-124, (g) ACG-2, (h) MTCC-1226, (i) ASA-1 and (j) MTCC-2306 at different growth hours; 0, 24, 48, 96 with M (Marker)

the *nif*H gene. In the present investigation whole cell protein profiling specific polypeptide Fe protein (60 kDa) responsible for nitrogenase enzyme in nitrogen fixation in diazotrophs were conformed three different growth hours (24, 48 and 96 hrs.) indicating that polypeptide Fe protein is quickly and profusely produced by all native diazotrophic isolates except *Acetobacter* where was induced little late due to slow growth of culture in vitro.

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