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GENETIC RECOMBINATION AND GENE AMPLIFICATION OF *nif* H FROM FREE NITROGEN FIXER *Azotobacter chroococcum* TO *Escherichia coli* BY TRANSFORMATION

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ABSTRACT

The said research work on molecular level was carried out in the month of August and September, 2021, at the P.G. and U.G. Department of Microbiology, Miraj Mahavidyalaya, Miraj, Dist.: Sangli, affiliated to Shivaji University, Kolhapur. Maharashtra, India. Biological nitrogen fixation by nitrogenase has fascinated interest as an option toward chemical nitrogen fixation. The chemical nitrogen fixation requires high amount of fossil fuels. The *Escherichia coli* is fast growing bacterium than the *Azotobacter chroococcum* but lack the *nif* gene as like *Azotobacter chroococcum* . The present molecular study focused to transfer *nif* gene from *Azotobacter* to *Escherichia coli* by the transformation technique. *Azotobacter chroococcum* and *Escherichia coli* were isolated from rhizosphere soil and sewage sample respectively. Transformation technique was used to transfer of *nif* H gene from *Azotobacter chroococcum* to transformant *Escherichia coli*. Colonies of *Escherichia coli* were obtained on Ashbys mannitol agar plates, indicating successful transformation. With the transformation process, the *Nif* H genes were transferred from *Azotobacter chroococcum* to *Escherichia coli.* Extraction of the genomic DNA from transformant *Escherichia coli* was carried out to detect the presence of *nif* H genes by PCR and gel electrophoresis techniques, it was showed that transformant *Escherichia coli* contained *nif* H. Moreover, the new *Escherichia coli* as acquired this gene successfully, can be used as bio-fertilizer for nitrogen fixation and as *Escherichia coli* is a fast growing bacterium can fix the nitrogen fast as compared to *Azotobacter chroococcum* and be provided the nitrogen source to crops by such recombinant fast growing cell. Diazotrophs (Nitrogen fixer) has been taken in more consideration in the last little decades. The focus is on *nif* H because it is significant and does important role in agricultural and environment field. As the *nif* H is significant the molecular study will arise to make it more significant in new coming scenarios.

(Keywords: *Azotobacter chroococcum, Escherichia coli, nif* H, Tranformation, PCR)

INTRODUCTION

Free N_2 fixation by nitrogen fixer is generally expected to be the good source of biological nitrogen. In recent years, at certain conditions, gain a significant amount of nitrogen from associated N_2 fixing bacteria in the plant rhizosphere (Chalk, 1991 and Mark and Craswell, 1992). In case of non-legume, rice fields are good example for biological nitrogen fixation and is recognized that a significant variety of N_2 fixing bacteria are naturally connected with field where the rice grown (Balandreau, 1986). Variety of bacteria along with extremophile plays the different role in environment like degradation, different actinomycetes were isolated from deep and partial saline soils showed their biotechnological applications like degradation (Chougule and Deshmukh, 2009), antagonisms (Chougule *et al*., 2007). The free living heterotrophic N_2 fixing microorganisms are a significantly important source of N_2 fixation in fields of rice (Boddey *et al*., 1995 and Mahadevappa and Shenoy, 2000). In the study of several grasses, endophytic nitrogen fixing bacteria represents a moving phase in the biological nitrogen fixation (Baldani *et al*., 1997). Nitrogenase enzyme is extremely necessary which helps for reducing molecular nitrogen to ammonia. Nitrogenase enzyme is made of Fe (dinitrogenase) and Mo-Fe protein (dinitrogenase reductase) and determined by nif gene.

A large molecular variety of N_2 fixing bacteria (Nitrogen fixer) has been observed in the field of growing rice supported on retrieval of *nif* H or *nif* D gene fragments (Da Rocha *et al*., 1986). The nif H gene only observed in nitrogen fixing microorganisms, and monitored the diazotrophs nitrogen fixing microorgnaisms (Frank *et al*., 1998). As like soil actinomycetes (Chougule and Deshmukh, 2007) the bacterial cell also applicable in different ways. The bacterial cell like Azotobacter associated with a wide variety of soil microorganisms present in the soil rhizospheric area of crops and the interactions between them are significant where they promote the growth of the host in the occurrence of other microorganisms (Kennedy and Islam, 2001 and Nosheen *et al.*, 2011), Azotobacter are excellent non-symbiotic nitrogen

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fixer (Siddiqui *et al*., 2014). The biological nitrogen fixation in Azotobacter, can occur even at low levels of existing nitrogen with the help of different genes such as *nod*, *nif*, *fix*, (Shamseldin, 2013).

Free-living and symbiotic nitrogen fixation are by *nif* genes, and the *nif* genes can transcript in free-living and symbiotic nitrogen fixers (Dixon and Kahn, 2004). The nif gene expression and regulation be different from one nitrogen fixer to another (Spaink *et al*., 1998). In *nif* gene, the structural *gene* nif H, is important, and function to form Fe-protein complex (Cocking, 2003), it encodes the iron protein which is a subunit of nitrogenase enzyme, so *nif* H is highly preserved in all nitrogen-fixing groups, it provides as an ideal molecular marker for nitrogen fixing microorganisms (Deslippe and Egger, 2006).

It is well known that *Escherichia coli* is a fast growing bacteria and grown easily in environment. The Escheri*chia coli* do not possess *nif* gene to lack the nitrogen fixation in *Escherichia coli*. if they became and capable to adapt the *nif* gene by transformation, then because of fast growing ability of *Escherichia coli* these cells will fix the nitrogen fast and sufficient perhaps increasing the crops yield.

In between the fragment of chromosome of nitrogen fixers, it should contain *nif* K, *nif* D, *nif* M, *nif* A, *nif* N, *nif* B, *nif* Q, *nif* Z, *nif* P, *nif* F, *nif* W, *nif* B, *nif* L and *nif* Y genes which also contain the sub genes as *nif* H1, *nif* H2, *nif* H3 and on the fragment *nif* V, *nif* S and *nif* U are located (Hamilton *et al*., 2011).

In case of bacteria, DNA should be transferable from one bacteria to other. The DNA thus transferred from donor and can incorporate in the recipient, and firms it in the recipient permanently. Three well known mechanisms arbitrated resourceful movement of DNA between two bacterial cells viz, transformation, conjugation, and transduction (Acharya, 2013), Bacterial plasmid also inserted into other bacterial cell. Although transformation does arise naturally, numbers of different methods are ensuring the DNA uptake by bacterial cells (Yoo, 2010). In genetic transformation bacteria takes gene fragment inside and expressed it, and change organism's traits, Transformation takes place to a limited amount in many bacteria. But the rate of DNA uptake increases through laboratory techniques like preparation of competent transformant cell. In bacterial transformation, the process involves incorporation and expression the overseas genetic material in recipient from the environment or surrounding or other bacteria. As DNA is an extremely hydrophobic molecule, normally it does not pass across bacterial cell membrane.

Before uptake of overseas (foreign) DNA, the bacterial cells primary made competent. Competence is a one factor has to make the capability recipient cell to catch the foreign DNA from its environment or donor cell. In contrast to eukaryotic genomes like in plants and animals, bacteria, contain haploid genome, which is single circular chromosome and not diploid arranged in linear. Plasmids that are additional small, circular DNA are present in bacteria. Plasmid is little amount of genes and, significant for its origin of replication (ori) able to transfer between different species and kingdoms (Slonczewski, 2006). To transfer the plasmid in bacterial competent recipient cells is one of major method involved for cloning in molecular biology (Tu *et al*., 2005 and Das *et al*., 2017).

Transformation process holds in a very precise method by transforming suitable and proper DNA piece, so transformation is widely used in genetic engineering (Pimda and Bunnag, 2010). The objectives of gene transfer in recipient from donor to obtain constant inheritance and expression of new characteristics in recipient, which one a part of study of gene regulation (Moses, 1987).

MATERIALS AND METHODS

Isolation and confirmation of Azotobacter chroococcum and Escherichia coli.

The *nif* gene containing bacteria like Azotobacter chroococcum and fast growing bacteria Escherichia coli were isolated from soil and sewage sample respectively. Azotobacter chroococcum bacterium was isolated with the help of nitrogen free mannitol agar medium (Ashbys mannitol agar plates) , the medium is selective for the isolation of Azotobacter chroococcum . The 1 ml 1:10 diluted black rhizosperic soil was inoculated in the nitrogen free mannitol agar medium and plate was allowed to incubated at room temperature for 48 to 72 hrs to got the growth and brown colored colonies indicated the presence of Azotobacter chroococcum followed with the gram staining (Upadhyay *et al.*, 2015) .

Isolation of Escherichia coli was carried out using the MacConkeys agar medium, the sewage sample directly streaked on MacConkeys agar medium, plate was incubated at 37OC for 24 hrs. hours. The pink colored colonies on medium were primary indicated as positive for Escherichia coli and confirmed with gram staining and growth on endo agar plate. The colony with the metallic sheen on endo agar plate was confirmed as Escherichia coli. (Boer , 2004).

Outline of isolation and identification of microorganisms was taken by (Chougule and Deshmukh, 2006), where different techniques were described for the isolation of identification of actinomycetes.

Preparation of competent transformant cells of Escherichia coli

According to (Sambrook *et al*., 1989 and Dagert and Ehrlich, 1979) , a modified method was applied for the preparation of Escherichia coli as a competent cells used for transformation. 24 hours old culture of Escherichia coli was prepared in 100 ml of Sperber's broth medium. The culture was incubated on a mechanical shaker rotated at 100 rpm for 24 h at 28 °C, Process was repeated until OD approximately 0.3-0.6 was attained at 600 nm, Immediately at 4 °C, 10 ml aliquots was centrifuged at 5000 rpm for 15 min to pellet the cells. Discarded the supernatant and pellet cells were sus-

pended in 10 ml of 0.1 M CaCl2 over 1 hours on ice, and centrifuged at 5000 rpm for 15 minutes followed with washing using 5 ml 0.1 M CaCl2. Again cells were centrifuged at same rpm for 15 minutes and re-suspended in 5 : 5 ml mixture of ice-cold 0.1 M CaCl2 and 20% glycerol. The cells were ready to use for transformation while stored at -20 °C.

Bacterial transformation

(Olsen et al., 1992; Hanahan, 1983; Sinha and Iyer, 1971 and Lorenz and Wackernagel, 1994) described a method of natural transformation. DNA was observed to be spontaneously released out from the cells into the culture liquid.

A single colony of *A. chroococcum* acted as donor cell was inoculated in 10 ml of Ashby's broth medium, and 1 ml of competent cells of *Escherichia coli* acted as recipient cell inoculated in 10 ml of nutrient broth medium both were incubated on mechanical shaker rotated at 100 rpm at 28 °C for 48 hours.

Then 0.8 ml of the *A. chroococcum* (donor cells) were mixed with 0.2 ml of transformant *Escherichia coli* (recipient cells) and added in 1 ml of nutrient broth, the it was incubated at 28 °C for 5 hours under aerobic condition.

Then 0.1 ml mixture was spreaded on ashbys mannitol agar medium plates and control plates were prepared by separately spreading 0.1 ml culture of donor and recipient cells on ashbys mannitol agar plates all plates were incubated at 28 °C and then were checked for colonies.

Colonies of *Escherichia coli* were obtained on Ashbys mannitol agar plates, indicating successful transformation. Depending on colony morphology and PCR amplification to check the transformant colonies of *Escherichia coli*. Enough the presence of colony of *Escherichia coli* on ashbys mannitol agar medium plate (selective for nitrogen fixer) indicated the transformant colonies of *Escherichia coli* adapting nif gene and then these transformant colonies of *Escherichia coli* were screened to verify the transfer of nitrogen fixation genes (nif genes) from *A. chroococcum* to transformant *Escherichia coli* depending on molecular study. The molecular study was performed using PCR and gel electrophoresis techniques to detect transferred nif H gene that was transferred by transformation process, in transformant *Escherichia coli*.

Identification of the *nif* genes using *nif* H primer

Using the method of Chen and Kuo (1993) the total genomic DNA of *A. chroococcum*, *Escherichia coli*, and transformant *Escherichia coli* was isolated separately in 1 ml of distilled water. All cultures were kept in log phase and cell was harvested by centrifugation at 10,000 rpm for 10 min at 4°C separately. 100 ?l of buffer solution was added in each pallet. The buffer solution was prepared using 7.5 ml of 0.15 M NaCl, 10 ml of 0.1 M EDTA made to final volume 50 ml the buffer solution containing pallet was centrifuged it at 10,000 rpm for 10 min at 4°C. 100 ?l of buffer solution without pallet acted as a negative control.

25 % SDS was then added to each pallet mixed it thoroughly, again cell debris was removed by centrifugation which was carried out at 10,000 rpm for 10 min at 4°C. Top aqueous layer was collected and mixed with 1/10 volume of 3 M sodium Acetate, 2.5 ml of cold ethanol then the contents were precipitated at -25°C overnight.

All content was centrifuged at 15,000 rpm for 10 min at 4°C. With 100 ?l of 70% ethanol the pellet was washed and again centrifuged at 15,000 rpm for 10 min at 4°C to get the washed pallet. The pallet was then suspended in the 50 ?l TE buffer. it was made through the combination of 2 ml of 10 mM Tris HCL and 0.4 ml of 1 mM EDTA to made final volume 100 ml. The content was stored at 4°C. DNA was confirmed through 0.5% agarose gel loaded with 5 ?l DNA, and observed it in UV transilluminator.

DNA with the quantity of 3 ?l was used template for nif H amplification by PCR with applied primers as (5'GTTTTACGGCAAGGGCGGTATCGGCA3' and 5'-TCCTCCAGCTCTCCATGGTGATCsG-3'). Programmable PTC-100 thermo cycler was used for PCR initial denatured it at 94°C for 5 min, then 35 cycles followed included 1 min at 94°C, 1 min of 50°C, 2 min at 72°C and a finally extended for 10 min at 72°C, the content was then stored 4°C.

RESULTS AND DISCUSSION

The brown colored pigmented growth on nitrogen free mannitol agar medium indicated the presence of *Azotobacter chroococcum* shown in fig. 1 and 2 with the appearance of gram negative rods by staining shown in fig. 4.

Escherichia coli cells were isolated on MacConkey's agar (fig. 3) and was confirmed with gram negative short rod shaped nature by gram staining (fig. 5) and endo agar plate. The colony with the metallic sheen was confirmed as presence of *Escherichia coli.*

Transferring of chromosomal genes *nif* H from *A. chroococcum* to transformant *Escherichia coli* by transformation.

Transformation was done between *A. chroococcum* as donor cells and transformant *Escherichia coli* as recipient cells to examine the transference ability of nitrogen fixing *nif* H gene from isolated *A. chroococcum* to *Escherichia coli.* Colonies of only *A. chroococcum* and transformant *Escherichia coli* were obtained on ashbys mannitol agar medium plates was primary confirmation of transformant *Escherichia coli* containing *nif* H gene and also confirmed by PCR amplification (used for confirmation of transformant *Escherichia coli).*

Moreover, to confirm nitrogen fixing *nif* H gene transfer from donor to recipient, a molecular study was done by using PCR technique and gel electrophoresis for detecting *nif* H in each of *A. chroococcum, Escherichia coli,* and transformant *Escherichia coli.*

PCR amplification of *nif* H

After extraction and purification of genomic DNA

from *A. chroococcum, Escherichia coli* and transformant *Escherichia coli.* According to the manufacturer's protocol of PrestoTM Mini gDNA Bacteria Kit, PCR was performed for amplification of *nif* H by using specified primers of oligo -nucleotide that to be flanked the amplified DNA sequence, and in order to check for the presence of *nif* H in the chromosomal DNA of *A. chroococcum, Escherichia coli,* and transformant *Escherichia coli* using gel electrophoresis.

Lane 1 used for the DNA markers and matched results indicated that samples of *A. chroococcum* and transformant *Escherichia coli* showed the *nif* H (5' and 3') primers reveal positive PCR products of *nif* H on the gel at lanes 1 and 3 respectively with band of 130 bp. and negative control were shown negative PCR products at lane 2 and at lane 4. Lane 2 showed negative control of wild type *Escherichia coli* and Lane 4 showed negative control without cell or DNA as they did not create a bands, while the markers of DNA bands which was ranged from 100-1000bp shown in Figure 6.

Specific band or replicon showed 130 bp molecular weight that was indication of *nif* H gene for transformant *Escherichia coli.* it was showed that 98% homology in between primer, *nif* of *A. chroococcum* and transformant *Escherichia coli.* Often exchange of genetic materials happened in many bacteria from different species by a cell-to-cell contact through conjugation process, It has been widely studied for molecular aspects (Fernandez-Lopez *et al.,* 2005). PCR technology is more easy and fast to recognized nitrogen fixer by gene identification in contrast to other assay techniques , these techniques are low reliable and taken much and more time (Rennie and Rennie, 1983).

With extracting the genomic DNA followed to, PCR and gel electrophoresis using specific complementary primers to amplify them confirms the presence of *nod* D in *R. leguminosarium*, and also detect the *nif* H2 and *nif* H3 in the *A. chroococcum* (Rossen *et al.,*1985). Perrett and Broughton, (1998), were used *nif* H and *rec* A primers for the identification and distinguished *Rhizobium* species like as NGR 234 and *R. fredii* USDA 257. Some time different bands or replicons were appeared because of possible to slightly variations in amount of purity of template DNA (Berg *et al.,*1994).

Nif H responsible for dinitrogenase reductase enzyme, acted as obligate electron donor for dinitrogenase during process and it required for the biosynthesis of FeMo-C and maturation of apodinitrogenase (Shamseldin , 2013). Past research carried out by Khider (2012), Abid (2013) and Mohamed (2017) showed the survival of various *nif* H genes in *A. chroococcum* in several area belongs to Kurdistan. Iraq. Studied carried out by Talabani *et al.* (2019) successfully transferred *nod* D2, *Nod* D3 genes from *Rhizobium leguminosarum, Nif* H2 And *Nif* H3 genes from *Azotobacter chroococcum* to *Bacillus megaterium* by conjugation and transformation technology as single bacterium *Bacillus megaterium* fix nitrogen and solubilize phosphorus at a time. Davis *et al.* (2000) allow to observed the expression of different *nif* genes in transconjugant bacteria when number of *nif* genes were transferred from *A. chroococcum* to *Klebsiella pneumonia.* Khider (2011) transferred *nif* genes from *A. chroococcum* to *Lactobacillus planetarium* by conjugation.

Diazotrophs (Nitrogen fixer) has been taken in more consideration in the last little decades. The focus is on *nif* H because it is significant and does important role in agricultural and environment field. As the *nif* H is significant the molecular study will arise to make it more significant in new coming scenarios.

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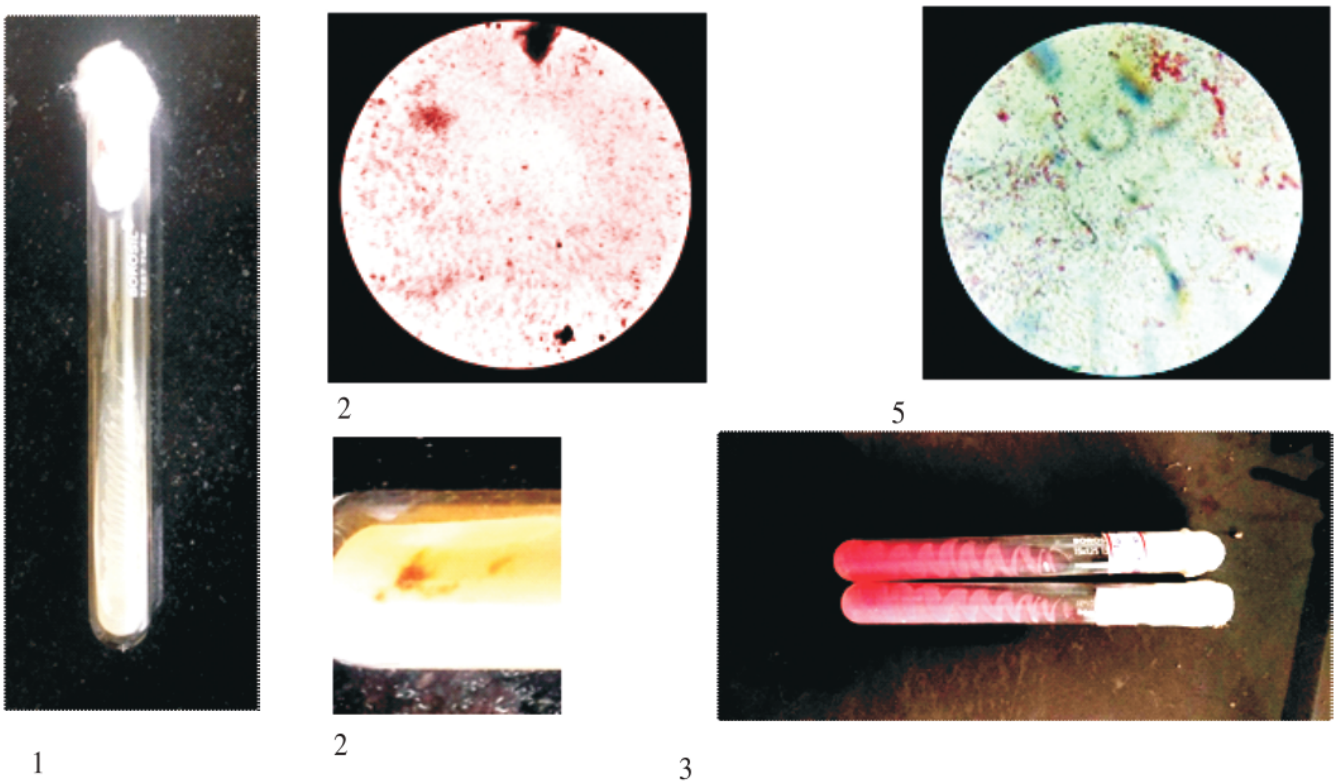

Fig. 1. Initial growth (without pigmentation) and 2. Late growth (with pigmentation) of *Azotobacter chroococcum*on ashbysmannitol agar medium and 3. *Escherichia coli* on MacConkey's agar 4. Gram staining of *Azotobacter chroococcum* and 5. Gram staining of *Escherichia coli*

6.

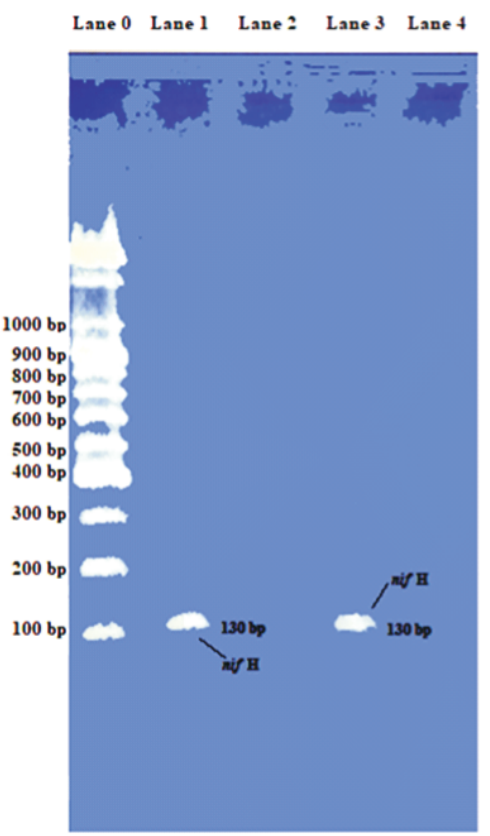

Fig. 6.Agarose gel electrophoresis sheet, the PCR amplified of 130 bp *nif* H gene with Lane 0: DNA marker, Lane 1: *nif* H gene with 130 bp of *A. chroococcum* (Positive Control), Lane 2: Wild *Escherichia coli* (negative control), Lane 3: *nif* H gene with 130 bp of transformant *Escherichia coli* and Lane 4: Negative control without cell or DNA

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