Isolation, Screening and Characterization of Nitrogen-Fixing Bacterial Strain *Brucella anthropi* from Rhizospheric Roots of Black pepper(*Piper nigrum*L.),Kolli hills, Tamil Nadu, India

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AbstractBiological nitrogen fixation is the most promising process which plays a significant role in agriculture. Diazotrophs are recognized as efficient soil microbes which fix the atmospheric nitrogen. Anegative gram-staining, aerobic, rod-shaped, non-sporeforming bacterial strain Nfb-6, was isolated from the rhizosphere of *Piper nigrum* L. growing in different areas of Kolli hills, Tamil Nadu, India. Morphological and biochemical tests were conducted for identification and the strain was identified as *Brucella*. This was further confirmed by sequence analysis of the PCR-amplification of 16S rRNA gene and Nfb-6, and was identified as *Brucella anthropi*.

Keywords: *Nitrogen fixing bacteria, Brucellaanthropic, Piper nigrum*

1. Introduction

In the past decades, agricultural practices have focused on maximizing yields by increasing fertilization, mainly N and P fertilizations. However,an excessive use of these compounds causes leaching, pollution of water resources, and gaseous emissions to the atmosphere, with irrepairable consequences to the environment and human health [1]. Nowadays, scientists of the world in the field of agriculture are very much concerned about the fixation of atmospheric nitrogen associated with crops.

Biological nitrogen fixation is one of the most important biological processes in nature. Diazotrophic-associated bacteria plays a key role in plant growth, development and nutrition by numerous mechanisms[2]. Application of biofertilizers can decrease the use of chemical fertilizersand also can increase the yield of crops [3, 4].Considering the adverse effects of chemical fertilizer application, more sustainable, cost-effective and eco-friendly techniques are being explored for improving crop productivity [5-7]. A potential solution to this challenge is the use of beneficial microbes such as bacteria, fungi and blue-green algae as a method for improving agricultural productivity. Microbial-based formulations such as biofertilizers are now widely employed in sustainable agriculture [8].

The aim of the present study was to isolate and characterize the strain Nfb-6, isolated from the rhizosphere of *P. nigrum* and to identify the strain with 16S rRNA. 16S rRNA genesequence similarities placed the strain in the genus *Ochrobactrum*, sharing highest similarity with *Ochrobactrumanthropi (*100 %).

The genus *Ochrobactrum* is affiliated with the family *Brucellaceae*[9].16S rRNAgene and recA sequence-based phylogenies both suggest that the genera *Brucella* and *Ochrobactrum* could be unified in a single genus [10-12]. The genus comprises 13 species: *O.anthropi*[13], *O. intermedium*[14], *O. tritici* and *O. grignonense*[15], *O. gallinifaecis*[16],*O. oryzae*[17], *O. lupini*[18], *O. pseudintermedium*[19], *O. cytisi*[20], *O. haematophilum* and *O.pseudogrignonense*[21], *O. rhizosphaerae* and *O. thiophenivorans*[10].

Thegenus*Ochrobactrum*, class Alphaproteobacteria, was originally described by Holmes *et al*., 1988 [13] and currentlycomprises species isolated from different environmentincluding soil and industrial environment [15,10,22]. *O. anthropi* is considered as a pathogenic bacteria by[24,25].The present work is not only to isolate and identify the Nfb-6 strain but also to study the carbon, aminoacid and antibiotic utilization by the strain and compare it with other related strains.

2. Materials and methods

2.1.Sample collection

Rhizospheric root samples were collected from rhizosphere of pepper plant (*P. nigrum*) grown in the different pepper cultivating regions of Namakkal (Kolli hills), India. It lies west of the Pacchaimalai, with a total

area of 503 sq.km. The altitude ranges from 180m to 1415m above sealevel. Pepper plants are grown in natural conditions on sloppy terrains, rich organic soils under the cover of tree plantation crops like coconut (*Cocosnucifera* L.), coffee (*Coffea arabica* L.), areca (*Areca catechu* L, tea (*Camellia sinensis*L. Kuntze), and Sesbania (*Sesbaniagrandiflora*L.) [26] and they were irrigated artificially, the rhizospheric rootswerebrought to the laboratory in polythene bags. These soils were mixed together and triplicates were taken for further analysis. The soil samples were processed immediately or stored at 4-8℃ for the isolation of microorganisms.

2.2.Preparation of root samples

One gram of root sample was macerated under aseptic conditions with a sterile mortar and pestle in 9ml of sterile water. Then it was serially diluted upto 10^{-5}cfug/l^{-1} and each dilution was transferred (1ml) to nitrogen free semisolid medium with three replications and incubated for4-8 days at 27℃. Nitrogen free medium wassterilized byautoclaving at 121℃ for 20 min and cooled to room temperature. The bacterial isolates were purified by streak plate method.

2.3.Media preparation

Nitrogen free media was used for the isolation of nitrogen fixing bacteria.

2.3.1.Nitrogen-free malate medium (NFb)(Table1)

pH was adjusted to 6.8 with diluted NaOH. For semisolid media, 1.8g agar per liter was added. For solid medium 15g of agar was added with 20mg of yeast extract[27].

2.3.2.JNFb medium(Table 2)

pH was adjusted to 5.8 with diluted sulphuric acid. 1.9g agar per liter was used for making semisolid media and 20g agar with 20mg yeast extract for solid media.

2.3.3.LGIP medium(Table 3)

The medium was made up to 1000ml with distilled water. pH was adjusted to 5.5 using diluted acetic acid. Solid medium was prepared with 20g agar and 20mg yeast. 2g agar was used for semisolid medium[28].

2.4.Isolation and purification of nitrogen fixing bacteria

The isolation of nitrogen fixing bacteria was done according to the procedure by Bacon and White, 2002 [29]. The samples were uprooted manually and washed in running tap water. The root sections of 2cm length were excised using flame sterilized scalpel from 1cm to 2cm above the soil line. All the samples were blotted dry with filter paper and then weighed to have finalsample of 0.5g. The surface sterilization of the root pieces was done

Table1.Nitrogen-free malate medium (NFb)

Ingredients	g/l
Malic acid	5.0
K2HPO4	0.5
MgS047H20	0.2
NaCl 0.1 CaCl2.2H20	0.02
Fe EDTA (1.64% sol)	4.0 ml
Bromothymol blue	2.0 ml (0.5% in 0.2N KOH)
KOH	4.5
Micro nutrient solution	2.0 ml
Vitamin solution	1.0 ml
Water	1000 ml
Micro nutrient solution	
Na2Mo04.2H20	0.2
MnSo H2O	0.235
H3B03	0.28
CUSO4H2O	0.008
ZnS047H20	0.024
water	200ml
Vitamin solution	
Biotin	10 mg
Pyridoxin HCl	20 mg
Water	100 ml

Table2.JNFb medium

Ingredients	g/l
Malic acid	5
K2HPO4	0.6
KH2PO4	1.8
$MgSO_4$. H20	0.2
NaCl	0.1

CaCl2.2H20	0.02
Fe EDTA (1.64% sol)	4.0 ml
KOH	4.5
Bromothymol Blue	2.0 ml (0.5% in 0.2 N KOH)
Micro nutrients solution	2.0 ml
Vitamin solution	1.0 ml
Water	1000 ml

Table3.LGIP medium

Ingredients	g/l
Cane sugar	100
K2HPO4	0.2
KH2PO4	0.6
$MgSO_4$.7H20	0.2
CaCl2.2H20	0.02
Na2Mo04.2H20	0.002
FeCIg. H20	0.01
Bromothymol blue	5.0 ml (0.5% solution in 0.2N KOH)
Vitamin solution	1.0 ml

with the following immersion sequence: 70% ethanol for 1 min, 3% sodium hypochlorite for 5 min followed by 70% ethanol wash for 1 min. The plates were incubated for 4-8 days at 27℃.The isolates were purified by sub-culturingrepeatedly on nitrogen free agar plates.

2.5.Characterization of nitrogen fixing bacteria

Macroscopy was done by observing morphology and cultural characteristics of the isolates on the nutrient agar plates.

2.5.1.Morphological tests

The following morphological tests *viz*., cell shape, gram staining reaction, and motility were carried out to characterize the tentatively identified as nfb as per the standard procedures. The isolate were identified by conducting the test according to specific characters described in Bergey's manual of systematic bacteriology. Cultural characters of the pure isolates were studied on the basis of colony elevation, margin, form, texture and opacity.

2.5.1.1. Gram staining

Gram staining was carried out as per modified method[30]. The slides were viewed with the light microscope under oil-immersion. Gram-positive bacteria appear violet and gram-negative bacteria appear pinkish red.

2.5.2.Biochemical characterization

The biochemical characterization of the isolates was done as per the procedures outlined by Cappuccino *et al*.,1996 [31].

2.5.2.1.Oxidase test

The isolates were streaked on Trypticase soy agar medium and incubated at 30℃ in an inverted position for 48h. After the incubation period, 2-3 drops of para-aminodimethyl aniline oxalatesolution were added on the streaked area and the plates were observed for the color change from pink to maroon and finally to purple within 30 sec indicated a positive reaction.

2.5.2.2.Catalase test

Nutrient agar slants were inoculated with overnight growth of test organisms and were incubated at 300℃ for 24h. After incubation, the tubes were flooded with 1ml of 3% hydrogen peroxide and observed for gas bubbles. The occurrence of gas bubbles was scored positive for catalase.

2.5.2.3.Methyl red test

Test tubes containing MR-VP broth were sterilized and inoculated with the test cultures. The tubes were incubated at 28±2℃ for 48h. After incubation, 5 drops of methyl red indicator was added to each tube and gently shaken. The production of red colour was taken as positive for the test andproduction of yellow colour was taken as negative for the test.

2.5.2.4.Voges – Proskauer test

To the pre-sterilized tubes containing MR-VP broth the test cultures were inoculated. The tubes were incubated for 48h at 28±2℃. After incubation, 10 drops of Barrit's reagent–A was added and gently shaken followed by addition of 10 drops of Barrit's reagent-B. The development of rose colour in the broth was taken as positive for the test.

2.5.2.5. Indole production

To the pre-sterilized SIM agar tubes, the test cultures were inoculated. The tubes were incubated for 48h at 28±2℃. After incubation, each tube was added with 10 drops of Kovac's reagent. The production of red colour was taken as positive for the indole production.

2.5.2.6. Nitrate reduction test

The endophytic isolates were inoculated into 10ml of nitrate brothtaken in test tubes and the tubes were inoculated at 30℃. After 14 days,2ml of the broth was tested by adding equal amounts of sulfanilicacidand alpha naphthylamine. Development of red color indicated thatnitrate had been reduced to nitrite.

2.5.2.7.Gelatin hydrolysis

The activity of the enzyme gelatinase for hydrolyzinggelatinwastested by gelatin liquefaction. The test cultures were stab inoculated intonutrientgelatin deep tubes, incubated at refrigerated condition for 48h and observed for gelatine liquefication[32].

2.5.2.8. Starch hydrolysis

Starch hydrolysis test was done to study the activity of amylase.The isolates were streaked on nutrient agar plates containing 2% insoluble starch and incubated at room temperature.Hydrolysis of starch was tested by flooding with iodine solution and theplates were observed for the presence of clear zones surrounding thecolonies and considered for positive reaction.

2.5.2.9. Cellulose degradation

The activity of the enzyme cellulase for hydrolysis of cellulose wastested. Theendophytic isolates were streaked on cellulose agar minimal mediumcontaining cellulose and incubated at 300℃ in inverted position for 2-5days. After incubation plates were flooded with 0.2% aqueousCongo red and destained with 1M NaCl for 15 minutes. The plates wereobserved for the presence of clear zones surrounding the colonies after30 min. Clear zone surrounding the colony indicated cellulose activity.

2.5.2.10. Pectin degradation

The production of the enzyme pectinase by the endophyticbacterial isolates was tested by using Hankin's medium. The autoclavedmedium was poured into sterile petri plates and allowed to solidify. Theendophytic isolates were aseptically inoculated on one each of pH 7 andpH 5 by streaking and the plates were incubated at 30℃ for 48-72h. All the plates were flooded with 1% aqueous solution of hexadecyltrimethyl ammonium bromide. The formation of a clear zone around the growth on both the media indicated positive result forpectinase.

2.5.2.11. Carbon utilization

Growth and acid production were tested using specific medium in which carbon source was replaced by individual carbon substrates (5g/l) such as D-glucose, sorbitol, meso-inositol, mannose, glycerol, L-rhamnose, lactose, fructose, L-arabinose, trehalose, L-raffinose, meso-erythritol, galactose, mannitol, cellobiose, xylose, sucrose, starch, sodium acetate, maltose and organic acids (0.5% V/V) *viz*.,adipic, malonic, succinic, oxalic, valeric, fumaric, hippuric, malic, tartaric, ketoglutaric, citricacids were also tested[33].

2.5.2.12. Aminoacid utilization

Growth on aminoacids were performedwithL-cysteine, L-glutamic, L-proline, L-tryptophane, L-leusine, L-histidine, L-lysine, L-tyrosine and L-valinein the presence of sorbitol as carbon source.

2.5.2.13. Antibiotics resistance

The bacterial isolates resistant to different concentrations of antibiotics (streptomycin, rifampicin and tetracycline) were subjected to filter paper sensi-discs. Filter paper discs were being dipped in different concentrations of antibiotics and placed on Muller Hinton agar medium. 1 ml of 48h old respective liquid cultures were used for inoculation. The level of resistance was observed after 72h of growth. When there was no inhibition zone around the filter paper discs, the strain was considered as resistant tothat particular concentration of antibiotics [34].

2.6.Detection and estimation of indole acetic acid (IAA) in the cultures

2.6.1. Production of IAA

The IAA produced by the cultures was detected by using few drops of Kovac's reagent (Para-dimethylaminobenzaldehyde) to 1ml of the broth culture. The percentage of IAA in the cultures was estimated using Salkowski's reagent. According to the method of Gordon and Weber, 1951[35], when chlorine is added to indole acetic acid in the presence of a mineral acid, oxidation of IAA results in the production of a red color product, nitrosoindole, which can be estimated by colorimetry.

2.6.2.Estimation of IAA

Standard IAA solution was prepared in the concentration of 500µg/ml different aliquots of the standard solution were prepared using distilled water. A blank with 1ml of distilled water and 1ml of the sample was prepared. 2ml of Salkowski's reagent was added into each of the test aliquots and incubated at room temperature for 15 min. A stable red colour developed immediately which was read using UV-Vis spectrophotometer (Elico. UV- VIS). Optical density of the tubes was measured at 530nm.

2.7.Detection of ammonia production

Qualitative detection of ammonia production was done by the method given by Bakker *et al*., 1987 [36].

2.7.1. Method

Nessler's reagent: 100g Mercuric iodide and 70g potassium iodide were dissolved in a small quantity of distilled water and this mixture was added slowly with stirring, to a 500ml cooled solution of sodium hydroxide (160g sodium hydroxide dissolved in 500ml distilled water) and finally, this solution was diluted to 1l with distilled water. Reagent was stored in rubber-stopperedborosilicate glassware in darkness to maintain its stability for up to a year under normallaboratory conditions. Bacterial isolates were grown in peptone water for 2-3 days at optimum growth temperature. After incubation, 1ml of Nessler's reagent was added in each tube. Tubes showing faint yellow color indicated small amount ofammonia, and deep yellow to brownish color indicated maximum amount of ammonia.

2.8.Molecular characterization of the nitrogen fixing bacteria

Molecular characterization of the nitrogen fixing bacterial isolates was done by sequencing their 16S rRNA gene.

2.8.1.Isolation of genomic DNA

Bacterial genomic DNA was isolated using the genomic DNA isolation kit. An isolated bacterial colony was picked and suspend in 1ml of sterile water in a microfuge tube. Centrifuge it for 1 min at 10,000–12,000rpm to remove the supernatant. Add 200µl of Insta Gene matrix to the pellet and incubate at 56℃ for 15 min.Vortex at high speed for 10 sec and place the tube in a 100℃ in heat block or boiling water bath for 8 min. Finally, vortex the content at high speed for 10 sec and spin at 10,000–12,000rpm for 2 min. In result, 20µl ofthe supernatant was used per 50µl PCR reaction.The amount of DNA was quantified by recording the absorbance at 260nm wavelength using UV-Vis spectrophotometer (Bio Rad, SmartSpec 3000). DNA was stored at -20℃ for further use.

2.8.2.PCR amplification

Using below 16S rRNA Universal primers gene fragment (Table 4) was amplified using Thermal Cycler**.**

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

Table 4. 16S rRNA primer sequence

Reaction Mixture	Quantity(µl)
Buffer 10 X	2.5
MgCl2 (25mM)	1.5
dNTPs mix (10 mM each)	2.0
Taq DNA polymerase (5U/µl),(Life Technologies India, Pvt. Ltd)	0.2
Primer forward 20 pmol	1.0
Primer reverse 20 pmol	1.0
Water (SDW)	14.8
DNA (10ng/ µl)	2.0
Total Volume	25.0

Table 5. PCR reaction mixture

Table 6. PCR condition for 16S rRNA amplification

Steps	Temperature (℃)	Time
1. Initial denaturation	94	5.00 min
2. Denaturation	94	45sec
3. Annealing	53	45sec
4. Elongation	72	30sec

Table 7. Sequencing Primer

Primer Name	Sequence Details	Number of Base
785F	GGATTAGATACCCTGGTA	18
907R	CCGTCAATTCMTTTRAGTTT	20

The PCR amplification was carried out in 0.2ml PCR tubes with 25µl reaction volume consisting of above components (Table 5). Reaction mixture was vortexed and centrifuged in a microfuge (Bangalore Genei, India). Amplifications were performed using thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) with the PCR condition given in Table 6.

The thermal cycler was programmed for 30 cycles with 1 cycle of initial denaturation and steps 2-4 were repeated 30 times and a final extension at 72℃ for 30 sec using fastest ramp time between the temperature transitions.

2.8.3.Purification of PCR products

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit .The PCR product was sequenced using the 518F and 800R primers (Table 7). Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

2.8.3.Nucleotide sequence analysis

Single-pass sequencing was performed on each template using above mentioned 16S rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled waterand subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).Sequence data was aligned and analyzed for Identifying the Sample.

The 16S rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.The program MUSCLE 3.7 was used for multiple alignments of sequences [37]. The resulting aligned sequences were cured using the program G blocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) [38,39].Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be atleast as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML wasshown to be atleast as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering[40].

3. Results

The isolate which was inoculated in the NFb medium clearly showed the pellicle formation thus proving the presence of nitrogen fixing bacteria. This was sub-cultured to obtain pure isolates and stored at 4℃ for further morphological, physiological and biochemical studies. Enumeration of bacterial number was $4x10^{-5}g^{-1}$. The colony characterization showed the diameter ranging 0.4cm,milky white abundant growth on NFb plates and microscopic observation revealed oval shaped, gram negative bacterial cells, motile, hilly opaque, lobate, milky white, continuous chain with raised margin (Table 8).

The biochemical reactions of the bacteria showed the presence of oxidase enzyme by the change of oxidase disc to blue colour. Addition of hydrogen peroxide on the culture drop showed the effervescence thus showing the presence of catalyze enzyme in the bacteria. The bacteria also showed the production of ammonia with Nessler's reagent. The isolate showed the positive results for indole, citrate utilization and urease, nitrate reduction and negative result for methyl red,vp,starchhydrolysis,gelatine,pectinase and cellulose (Table 9). The isolatenitrogen fixing bacteria was treated with various antibiotics indicated that this bacterium was susceptible to ciprofloxacin(30mcg)and resistant to amikacin(20mcg), ampicillin(10mcg), chloramphenicol(18mcg),gentamicin(20mcg), streptomycin(18mcg), tetracycline(20mcg) (Table 12). This isolate was not able to grow in the medium supplemented with amino acids as N source such as histidine but good growth was observed in the NFb medium supplemented with the aminoacids such as

alanine, amino butyric acid, cysteine, arginine, valine, leucine, phenyl alanine, serine, tryptophan, lycine, methionine (Table10). The study isolate not utilized azelic acid and oxoglutaric acids as carbon sources for its growth and utilized organic acids like malic, malonic and sugars like fructose, galactose, glucose, lactose, mannitol, meso-erythritol, meso-inositol, maltose, mannose, rhamnose, sorbitol, sucrose, xylose as carbon substrates (Table 11).

Table 8. Comparitive study of morphological characteristics of *O. anthropi*

Morphology	Nfb-6	*O. ciceri*	*O. oryzae*	*O. daejeonense*	*O. anthropi*
Growth	abundant	abundant	abundant	abundant	abundant
Color	milky white	offwhite-mucoid	milky white	shiny viscous pale yellow	milky white
Colony Size/ Diameter	medium/0.4cm	2-3mm	medium	2-5mm	medium/ 0.4cm
Colony Shape	oval	circular	circular	circular	circular
Edge/Margin	lobate	entire	–	–	–
Elevation	hilly	–	–	–	–
Appearance	opaque	–	–	–	–
Texture	continuous chain like with raised margin	–	–	–	–
Configuration	complex	–	–	–	–
Gram's Stain	gram –ve	gram –ve	gram –ve	gram –ve	gram –ve
Cell Shape	rod	rod	rod	rod	Rod
Motility	motile	motile	motile	motile	motile

Table 9. Comparitive study of biochemical characteristics of *O. anthropi*

Biochemical	Nfb-6	*O. ciceri*	*O. oryzae*	*O. daejeonense*	*O. anthropi*
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
MR Test	-	-	-	-	-
VP Test	-	-	-	-	-
Indole	+	+	+	+	+
Citrate Utilization	+	+	+	+	+
Starch Hydrolysis	-	-	-	-	-
GelatinLigufaction	-	+	-	-	-
Urease	+	+	+	+	+
Nitrate reduction	+	+	+	+	+
Pectinase	-	-	-	-	-
Cellulose	-	-	-	-	-

+ Positive Growth – No Growth

Table 10. Comparitive study of amino acid utilizationof *O. anthropi*

Aminoacids	Nfb-6	*O. ciceri*	*O. oryzae*	*O. daejeonense*	*O. anthropi*
Alanine	+++	+++	+++	+++	+++
amino butyric acid	+++	+++	+++	+++	+++
arginine hydrochloride	+++	+++	+++	+++	+++
histidine	+++	+++	+++	+++	+++
tryptophan	+++	+++	+++	+++	+++
lycine	–	–	–	–	–
valine	+++	+++	+++	+++	+++
leucin	+++	+++	+++	+++	+++
methionine	+++	+++	+++	+++	+++
phenyl alanine	+++	+++	+++	+++	+++
ornithine	+++	+++	+++	+++	+++
cysteine	+++	+++	+++	+++	+++

+++ Abundant Growth – No Growth

Table 11. Comparitive study of carbon utilization of *O.anthropi*

Carbon utilization	Nfb-6	*O. ciceri*	*O. oryzae*	*O. daejeonense*	*O. anthropic*
Adiphic acid	+++	+++	+++	+++	+++
Arabinose	+++	+++	+++	+++	+++

Azelic acid	–	–	–	–	–
4.Cellulose	+++	+++	+++	+++	+++
Dextrose	+++	+++	+++	+++	+++
. Galactose	+++	+++	+++	+++	+++
Glucose	+++	+++	+++	+++	+++
glutamic acid	+++	+++	+++	+++	+++
.glycerol	+++	+++	+++	+++	+++
Inulin	+++	+++	+++	+++	+++
keto glycerol	+++	+++	+++	+++	+++
Lactose	+++	+++	+++	+++	+++
Mannitol	+++	+++	+++	+++	+++
malonic acid	+++	+++	+++	+++	+++
.meso-erythriol	+++	+++	+++	+++	+++
meso-inositol	+++	+++	+++	+++	+++
malic acid	+++	+++	+++	+++	+++
Maltose	+++	+++	+++	+++	+++
Mannose	+++	+++	+++	+++	+++
N-acetyl glucosamine.	+++	+++	+++	+++	+++
oxalic acid	+++	+++	+++	+++	+++
oxo- glutaric acid	–	–	–	–	–
Pectin	+++	+++	+++	+++	+++
Raffinose	+++	–	–	–	–
Rhamnose	+++	+++	+++	–	–
Sucrose	+++	+++	+++	+++	+++
Sorbitol	+++	+++	+++	+++	+++
Starch	+++	+++	+++	+++	+++
Xylose	+++	+++	+++	+++	+++

+++ Abundant Growth – No Growth

Table 12. Comparitive study of antibiotic utilizationof *O.anthropi*

Antibiotics test	Nfb-6	*O. ciceri*	*O. oryzae*	*O. daejeonense*	*O. anthropi*
Amikacin	S	S	S	S	S
Ampicillin	-	-	-	-	-
Chloramphenicol	IN	IN	IN	IN	IN
Ciprofloxacin	S	S	S	S	S
Gentamicin	S	S	S	IN	S
Penicillin G	-	-	-	-	-
Steptomycin	IN	IN	IN	IN	IN
Tetracycline	S	S	S	S	S

S-Sensitive, IN- Intermediate, - No Growth

4. Discussion

The isolate which was inoculated in the NFb medium clearly showed the pellicle formation thus proving the presence of nitrogen fixing bacteria.The biochemical analyses of the strain Nfb-6 showing the carbon utilization, aminoacid production and atibiotics resistance of the bacterium, played a significant role in identifying the strain at the genus level as *Ochrabactrum.* However, 16S rRNA sequence analysis indicated that this isolate showed 100% sequence similarity with *B. anthropi* (Accession No KY848518.1)(Figure 1&2). The other closely related organismswiththisisolateare *Ochrabacterum* (95.24%), *B. haematophila* (95.30%). The biochemical studies of the Nfb-6 strain *B. anthropii* was compared with other *Brucella* species, and found to be almost similar in its morphological and biochemical characters.However the *B. anthropi* is identified by many workers and are treated as novel species. Recent reports have described the isolation of *Ochrobactrum* strains from nodules of *Acacia mangium*[23], and *Lupinus albus*[18].Comparison of the 16S rRNA gene sequences of *Ochrobactrum* isolates from *A. mangium* showed 98% sequence similarity with *O.intermedium* and *O.anthropi* [23], whereas the 16S rRNAgene sequence of the proposed type strain of '*O.lupini*' showed 100%similarity with that of *O. anthropi*, and further investigations are necessary to confirm the status of '*O. lupini'* as a distinct species [18].Three nodulating species have been described that form nodules on *Acacia*[23], *Lupinus*[18], and *Cytisus*[20].Based on these above reports, the isolated strain Nfb-6 can be considered as a distinct species as it is isolated from *P. nigrum*, which has not been reported so far. Though our strain Nfb-6 showed 100% similarity with *O. anthropi*, after further investigations by DNA-DNA hybridization, it can be considered as a new species of *Ochrobactrum*. However our study is the first report to show that it is a nitrogen fixing diazotroph.

Figure 1: Phylogenetic tree based on 16S rRNA gene sequences constructed using neighbor joining method

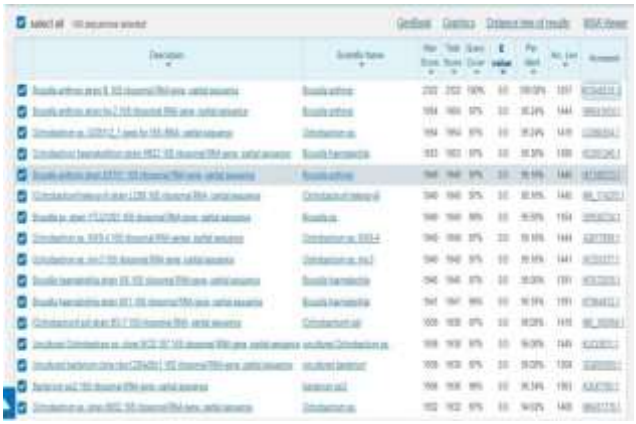

Figure 2: Blast analysis of the sequences

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