

Full Length Research Paper

# Characterization of p-nitrophenol degrading actinomycetes from Hundung limestone deposits in Manipur, India

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**p-Nitrophenol (PNP)**, a major nitroaromatic xenobiotic is released into the environment as a result of its widespread use and as a breakdown product of organophosphate (OP) agricultural pesticides such as parathion and methyl parathion. It is highly toxic to soil microflora and other non-target organisms. Of several Microbial Biotechnology Research Laboratory (MBRL) strains isolated from limestone deposits in Ukhrul, Manipur, India, two strains, (HS4-2 and HS6-1) were found to be promising PNP biodegraders, degrading up to 30 mg/L PNP in neutral basal salts medium (BSM, pH 7.0). Higher strength alkaline BSM (5X BSM, pH 8.0) was found to be more favorable for PNP degradation by these strains. Under these more optimal conditions, HS4-2 and HS6-1 could degrade up to 270 and 350 mg/L PNP respectively, remarkably high concentrations as PNP is quite toxic even at low concentrations. Only a few bacterial strains degrading such high concentrations of PNP have been reported. As these strains seem promising, they were subjected to phenotypic and genotypic characterization. The phenotypic characteristics of these strains were typical of actinomycetes. 16S recombinant deoxyribonucleic acid (rDNA) sequence analyses indicated strain HS6-1 to have the highest level of sequence similarity with *Rhodococcus triatoniae* IMMIB RIV-085(T)/AJ854055 (98.28%) and HS4-2 with *Brevibacterium casei* NCDO2048 (T)/X76564 (99.37%). Strain HS6-1 may possibly be a novel species of *Rhodococcus*. Further studies on optimization, kinetics and pathways of biodegradation can improve the biodegrading capacities of these actinomycete strains.

**Key words:** Nitroaromatics, p-nitrophenol, limestone deposits, Hundung, *Brevibacterium*, *Rhodococcus*.

## INTRODUCTION

Nitroaromatic compounds are major pollutants produced during industrial production of dyes, explosives, solvents, pesticides, herbicides and drugs. Among them, p-nitrophenol (PNP) is used as a major raw material and is also a breakdown product of organophosphate (OP) agricultural pesticides such as parathion and methyl parathion.

PNP can be highly toxic to soil microflora and other non-target organisms. p-Nitrophenol (PNP) is probably the most important among the mononitrophenols in terms

of quantities used and potential environmental contamination (Karin and Gupta, 2002). They are environmentally significant nitroaromatic compounds and the U.S. Environmental Protection Agency (EPA) listed mononitrophenols on its priority pollutant list (<http://www.epa.gov/waterscience/methods/pollutants.htm>) and EPA recommends restricting PNP concentrations in natural waters to <10 ng/L (Gemini et al., 2005). However, despite their hazardous nature, some microorganisms that are able to transform or degrade nitrophenols have been found to exist in nature (Dua et al., 2002; Furukawa, 2003; Loris et al., 2005).

Microbial bioremediation has become more attractive than traditional methods for decontamination due to convenient processes, lower costs and minimal impacts

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on the environment (Labana et al., 2005). Taking into account the high toxicity of PNP to microorganisms, biodegradation of PNP has been mostly studied at lower concentrations (Bhushan et al., 2000). Several bacterial strains are able to utilize PNP as their sole source of carbon and energy have been documented. These include species of *Arthrobacter* (Chauhan et al., 2000; Jain et al., 1994; Li et al., 2008; Qiu et al., 2009), *Bacillus* (Kadiyala and Spain, 1998), *Brevibacterium* (Ningthoujam, 2005), *Burkholderia* (Bhushan et al., 2000; Chauhan et al., 2000, 2010), *Ochrobacterum* (Qiu et al., 2007), *Pseudomonas* (Ningthoujam and Shovarani, 2008; Kulkarni and Chaudhari, 2006; Liu et al., 2005; Prakash et al., 1996; Shinozake et al., 2002; Zaidi and Mehta, 1996), *Rhodobacter* (Roldan et al., 1998), *Rhodococcus* (Shinozake et al., 2002; Ghosh et al., 2010) and *Stenotrophomonas* (Liu et al., 2007).

The present paper reports characterization of robust actinomycete strains biodegrading high concentrations of PNP, isolated from an unusual biotope, Hundung limestone deposits in Hundung, Ukhrul district, Manipur, India. This is an interesting habitat as the environment is alkaline due to natural calcium carbonate (CaCO<sub>3</sub>) deposits. Hence, we surveyed this biotope for biotechnologically interesting actinomycetes as the latter are more prevalent in such alkaline ecosystems.

## EXPERIMENTAL

### Acclimation for p-nitrophenol degradation

Strains showing potential for PNP degradation in preliminary plate screening in nutrient agar (NA) containing 10 mg/L PNP were initially acclimatized in Tryptone Soya Broth (TSB) medium containing 10 mg/L PNP under shaking conditions for biomass buildup (150 rpm, 48 h). Cells were then harvested and pellets were washed with 0.9% saline solution. After washing, cells were inoculated in basal salts medium (BSM) (Ningthoujam, 2005) containing 10 mg/L PNP and shaken in an orbital shaker at 150 rpm. After a week of incubation, the cells were pelleted down and washed twice with saline, and resuspended in BSM containing 10 mg/L PNP. Such transfers were repeated for two cycles. The PNP-induced cells were then used for degradation studies.

### p-Nitrophenol biodegradation studies

Degradation was monitored by visible turbidity and/or disappearance of characteristic yellow colour of PNP. PNP depletion was also monitored by following the absorbance of alkalinized culture supernatants at nm (data not shown). Once degraded, the isolate was streaked on nutrient agar plates supplemented with 10 mg/L PNP from the previous degradation flask and incubated at 30 °C for 48 to 72 h. The freshly grown culture were then inoculated in TSB media supplemented with 10 mg/L PNP for increasing the biomass and shaken in an orbital shaker at 150 rpm. After 48 h of incubation, the cells were harvested and pellets were then collected. The pellets were washed with 0.9% saline solution and inoculated in BSM supplemented with 10 mg/L PNP, shaken in an orbital shaker at 150 rpm till degradation occurred. Pellets were then collected and washed with 0.9% saline solution and further suspended in BSM and adjusted to O.D. of 1.5 at nm and used as inoculums (6%) in BSM supplemented with different

concentrations of PNP for degradation studies. When higher concentrations of PNP were used, higher strength BSM (5× BSM) adjusted to pH 8 was used. The biodegrading strains were maintained on NA and BSM slants with or without PNP. Microbial biomass was monitored by absorbance at 600 nm and PNP depletion by measuring its absorbance peak at 400 nm.

### Phenotypic characterization

Various morphological, physiological and biochemical characterization tests were carried out using the standard procedures (Cappuccino and Sherman, 2004; Gunasekaran, 2000; MTCC Actinomycetes Manual, 1998; Shirling and Gottlieb, 1966; Kuster and Williams, 1964).

### Phylogenetic analysis

16S rDNA sequencing of the biodegrading strains were performed with the help of IMTECH, Chandigarh. The complete 16S rDNA sequence was submitted to EzTaxon server (Chun et al., 2007) which contains manually curated databases of type strains of prokaryotes. Related strains were selected for alignment by CLUSTAL W program and phylogenetic analyses were done according to the neighbor-joining method (Saitou and Nei, 1987) using the MEGA version 4.1 (Tamura et al., 2004, 2007). To determine the support of each clade, bootstrap analysis was performed with 1000 replications (Felsenstein, 1985).

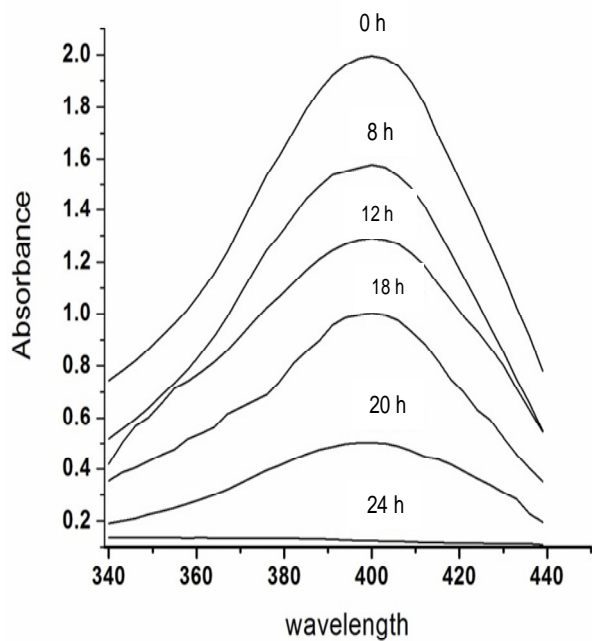
## RESULTS

### p-Nitrophenol biodegradation studies

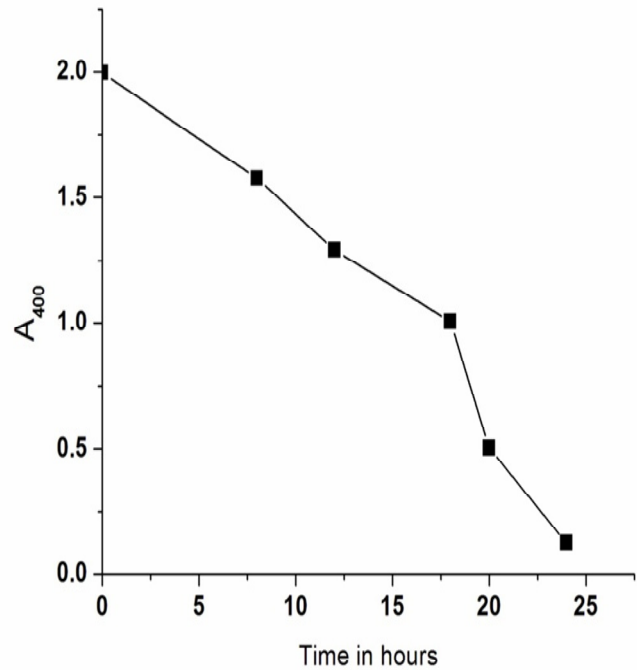
Initial biodegradation studies on NA medium containing PNP (10 mg/L) indicated that two limestone actinomycete strains have the potential to degrade PNP. The biodegrading capacities of these strains were confirmed by shake flask studies. Hence, these two strains, HS4-2 and HS6-1, were chosen for further shake flask studies in neutral BSM (pH 7.0). Initially, they were able to degrade up to 30 mg/L of PNP. The ultraviolet (UV)-Visible spectra for monitoring PNP degradation and time course of degradation of 30 mg/L of PNP by HS4-2 and HS6-1 are shown in Figures 1 and 2, respectively. Results of PNP degradation at various pH values suggested that a slightly alkaline pH is favorable for PNP degradation by these PNP degrader strains (Table 1). Hence, studies on various concentrations of PNP were performed at higher strength alkaline BSM (5× BSM, pH 8.0). For further acclimation under these conditions, HS4-2 and HS6-1 were able to degrade up to 270 and 350 mg/L of PNP respectively (Table 2); remarkably high concentrations as compared with most PNP degrading strains reported in the literature. As these strains seem to be promising robust PNP degraders, they were subjected to phenotypic and genotypic characterization.

### Phenotypic characterization

Phenotypic characteristics of the biodegrading strains are

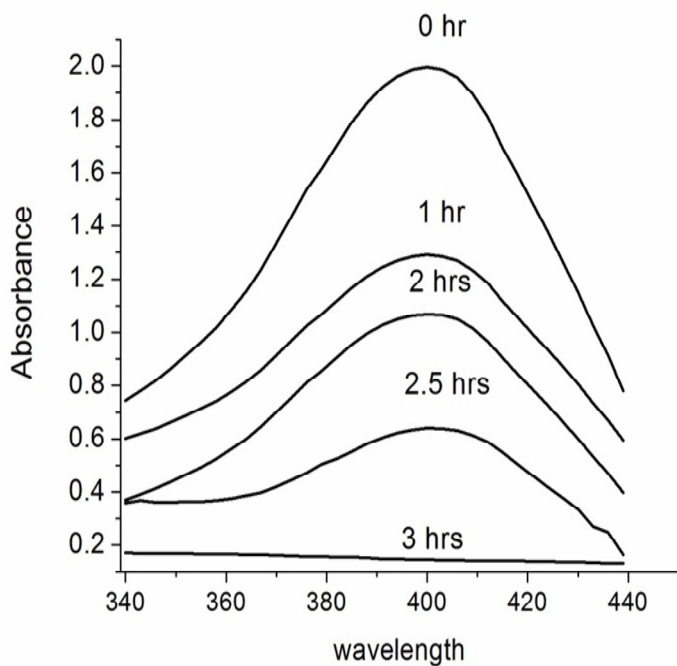


a)

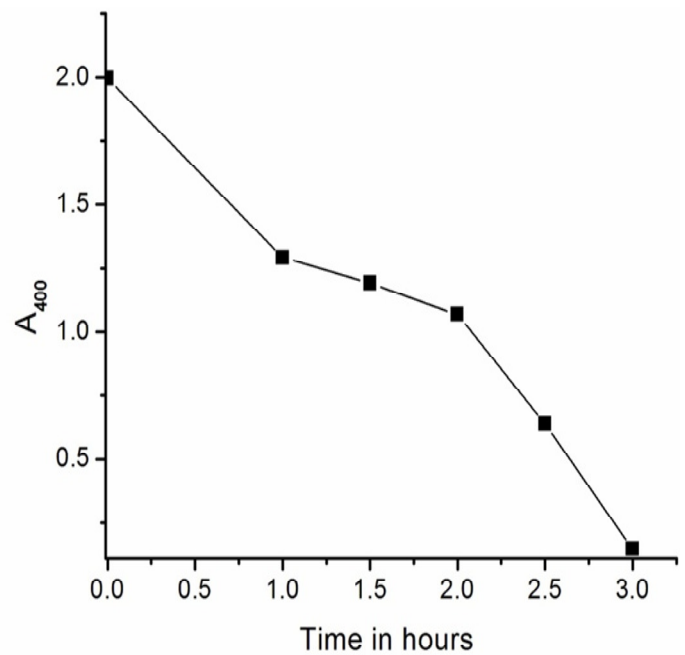


b)

**Figure 1.** a) UV-VIS spectra for degradation PNP (30 mg/L) by strain HS4-2 and b) Time course for degradation of PNP (30 mg/L) by strain HS4-2.



a)



b)

**Figure 2.** a) UV-VIS spectra for degradation of PNP (30 mg/L) by strain HS6-1 and b) time course for degradation of PNP (30 mg/L) by strain HS6-1.

**Table 1.** Degradation of PNP (30 mg/L) at different pH values.

pH	HS4-2 (h)	HS6-1 (h)
5	28.58	6.00
5.5	28.42	5.30
6	26.50	5.00
7	25.42	4.17
8	24.50	3.00
9	25.00	3.17
10	25.17	3.33

**Table 2.** Degradation time of various concentrations of PNP (h).

PNP concentration (mg/L)	HS4-2	HS6-1
30	24.50	3.00
50	30.00	4.42
100	42.50	6.67
150	49.00	16.50
200	59.42	19.00
250	107.50	38.00
270	113.00	43.00
300	No degradation	46.50
350	-do-	87.00
400	-do-	No degradation

listed in Tables 3 and 4, respectively. The growth morphologies of HS4-2 and HS6-1 on various ISP and other actinomycete specific media are shown in Table 5. The gross morphologies of these two strains grown on SCNA media and their micromorphologies (Carl Zeiss Microscope, Axio Scope A.1, Germany) are shown in Figure 3 (magnification, 600).

### Phylogenetic analysis

The phylogenetic trees of these two strains are shown in Figures 4 and 5, respectively. HS4-2 was found to be most closely related to *B. casei* (similarity index 99.37%) and HS6-1 was found to be closely related to *R. triatomae* (similarity index 97.28%). HS6-1 is likely to be a novel species of *Rhodococcus*. Further analyses including deoxyribonucleic acid (DNA)-DNA hybridization with the respective type strains and other polyphasic studies will be necessary for definitive taxonomic placements of the above strains which are beyond the scope of the present study.

### Discussion

This study reports PNP degrading actinomycete strains

from an unusual biotope, limestone deposits in Hundung, Ukhrul district, Manipur, India. Upon characterization, the two strains were identified as strains of *Brevibacterium* and *Rhodococcus* sp. However, based on the phylogenetic analysis alone, their taxonomic positions could not definitely ascertained the species level. Hence, these biodegrading strains have been designated as *Brevibacterium* sp. HS4-2 and *Rhodococcus* sp. HS6-1. There are only a few reports on PNP degradation by *Rhodococcus* species (Shinozake et al., 2002; Ghosh et al., 2010). Only one report exist in the literature for degradation of PNP by *Brevibacterium* (Ningthoujam, 2005). Our findings show both strains to be promising additions to the repertoire of PNP degrading microbial isolates.

HS4-2 and HS6-1 could degrade up to 270 and 350 mg/L respectively; quite high concentrations considering that PNP can be highly toxic even at much lower concentrations (Qiu et al., 2009). The strain HS6-1 took less than 4 days (87 h) for complete degradation of 350 mg/L PNP while HS4-2 took less than 5 days (113 h) for complete degradation of 270 mg/L PNP. Interestingly, both the strains were obtained from limestone deposit sites and not from the usual contaminated sites. PNP biodegradation by these limestone actinomycetes was faster at higher pH. Similar reports have also been reported by several workers (Kulkarni and Chaudhari, 2006; Labana et al., 2005; Qiu et al., 2009; Unell et al., 2008). This may be due to increased bioavailability and decreased toxicity of PNP, and optimal metabolic activity of the bacterial cells at higher pH (Wan et al., 2007). Further studies are now being undertaken to ascertain optimization, kinetics and pathway(s) of PNP biodegradation. As the strain HS6-1 could possibly be a novel species of *Rhodococcus*, DNA-DNA hybridization and other molecular studies to establish its exact taxonomic position are also targets of our further investigations.

### Conclusion

The biodegrading strains HS4-2 and HS6-1 seem to be promising additions to the repertoire of PNP degrading microbial isolates and may be potential agents for the biodegradation of nitroaromatic xenobiotics and for possible production of value-added compounds of biotechnological significance (for example, catechols or siderophores).

### Acknowledgements

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**Table 3.** Phenotypic characterization of the Hundung strain HS4-2.

<b>Characteristic</b>	<b>Reaction</b>
<b>Colony morphology</b>	
Shape	Round
Margin	Smooth
Elevation	Convex
Gram's staining	+
Production of diffusible pigment	-
<b>Growth at</b>	
4°C	-
15°C	-
25°C	+
30°C	+
37°C	+
42°C	+
60°C	-
<b>Growth at (pH)</b>	
5.2	+
7.0	+
8.0	+
9.0	-
10.0	-
<b>Growth in the presence of</b>	
2% NaCl	+
5% NaCl	+
7% NaCl	+
10% NaCl	+
<b>Degradation of</b>	
Adenine 0.5%	+
Guanine 0.05%	-
Tyrosine 0.5%	+
Xanthine 0.4%	-
<b>Hydrolysis of</b>	
Casein	+
Starch	+
Urea	-
Catalase	+
Oxidase	-
Methyl Red (MR)	-
Voges Proskauer (VP)	-
Citrate utilization	-
Indole production	-
Nitrate reduction	-
Gelatin liquefaction	-
H <sub>2</sub> S production	-
<b>Utilization of C sources</b>	

**Table 3.** Contd.

L-Arabinose	-
D-Fructose	+
D-Galactose	-
Meso-inositol	-
Mannitol	-
D-Mannose	-
L-Rhamnose	-
Salicin	-
Sucrose	-
D-Xylose	+
<b>Utilization of N sources</b>	
Arginine	+
Cysteine	-
Histidine	-
Hydroxyproline	+
Methionine	+
Phenylalanine	+
Potassium nitrate	+
Serine	+
Threonine	-
Valine	+

Obs. For degree use <sup>o</sup>**Table 4.** Phenotypic characterization of the Hundung strain HS6-1.

<b>Characteristic</b>	<b>Reaction</b>
<b>Colony morphology</b>	
Shape	Round
Margin	Smooth
Elevation	Convex
Gram's staining	+
Production of diffusible pigment	-
<b>Growth at</b>	
4°C	-
15°C	-
25°C	+
30°C	+
37°C	+
42°C	-
60°C	-
<b>Growth at (pH)</b>	
5.2	+
7.0	+
8.0	+
9.0	+
10.0	+

Table 4. Contd.

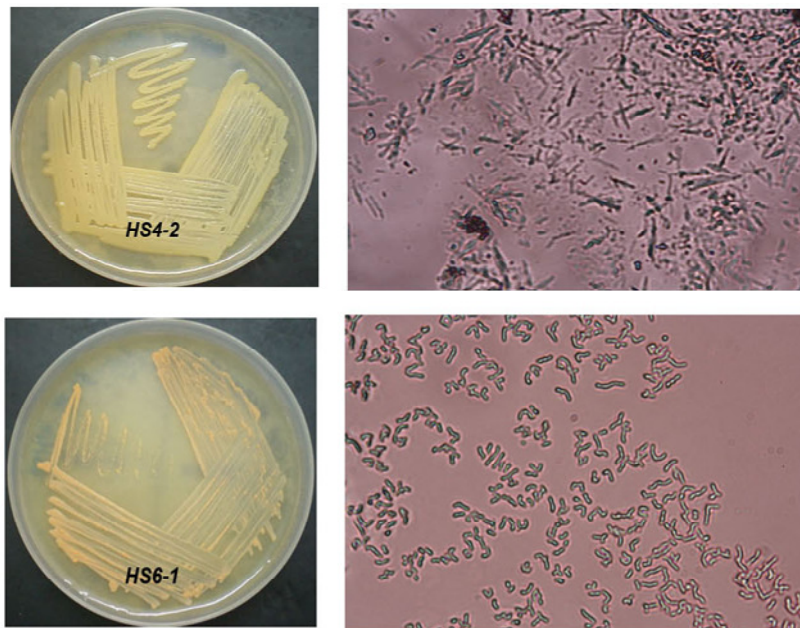
<b>Growth in the presence of</b>	
2% NaCl	+
5% NaCl	+
7% NaCl	+
10% NaCl	-
<b>Degradation of</b>	
Adenine 0.5%	+
Guanine 0.05%	-
Tyrosine 0.5%	-
Xanthine 0.4%	-
<b>Hydrolysis of</b>	
Casein	-
Starch	+
Urea	-
Catalase	+
Oxidase	-
Methyl Red (MR)	-
Voges Proskauer (VP)	+
Citrate utilization	-
Indole production	-
Nitrate reduction	-
Gelatin liquefaction	-
H <sub>2</sub> S production	-
<b>Utilization of C sources</b>	
L-Arabinose	-
D-Fructose	-
D-Galactose	-
Meso-Inositol	-
Mannitol	-
D-Mannose	-
L-Rhamnose	-
Salicin	-
Sucrose	-
D-Xylose	+
<b>Utilization of N sources</b>	
Arginine	-
Cysteine	-
Histidine	+
Hydroxyproline	-
Methionine	-
Phenylalanine	+
Potassium nitrate	+
Serine	-
Threonine	-
Valine	-

Obs. For degree use <sup>o</sup>

**Table 5.** Growth morphologies of HS4-2 and HS6-1 on different ISP and other actinomycete specific media.

Media	Isolate →	HS4-2	HS6-1
ISP1	AM	Light yellow	Pitch
	SM	Light yellow	Cream
ISP2	AM	Light yellow	Pitch
	SM	Light yellow	Light yellow
ISP3	AM	Light orange	P.G
	SM	Cream	P.G
ISP4	AM	Light yellow	P.G
	SM	White	P.G
ISP5	AM	Light orange	Pitch
	SM	Light orange (P.G.)	Cream (P.G.)
ISP6	AM	Pale cream	Pale cream
	SM	Cream	Cream
ISP7	AM	Light orange	Cream
	SM	Light orange	Cream
SCNA	AM	Light orange	Pale cream
	SM	Cream	Cream
SA	AM	Pale cream	Pitch
	SM	Cream	Cream
TSA	AM	Cream	Pale cream
	SM	Light yellow	Cream

ISP, International Streptomyces Project;SA; streptomycetes agar; TSA, tryptone soya agar; AM, aerial mycelium; SM, substrate mycelium; P.G, poor growth.



**Figure 3.** Gross Morphology and micromorphologies of the strains, HS4-2 and HS6-1 (magnification, 600x).



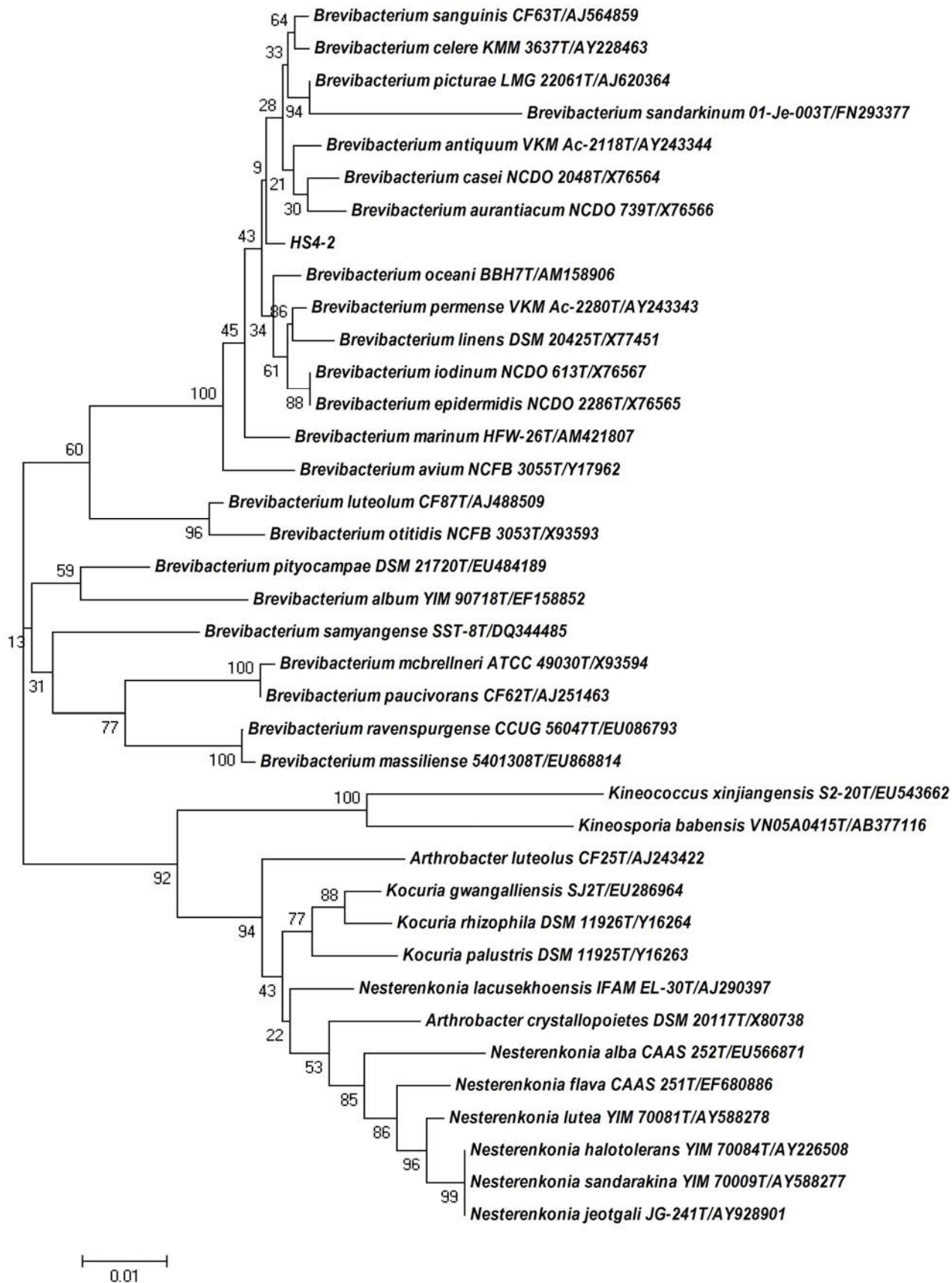
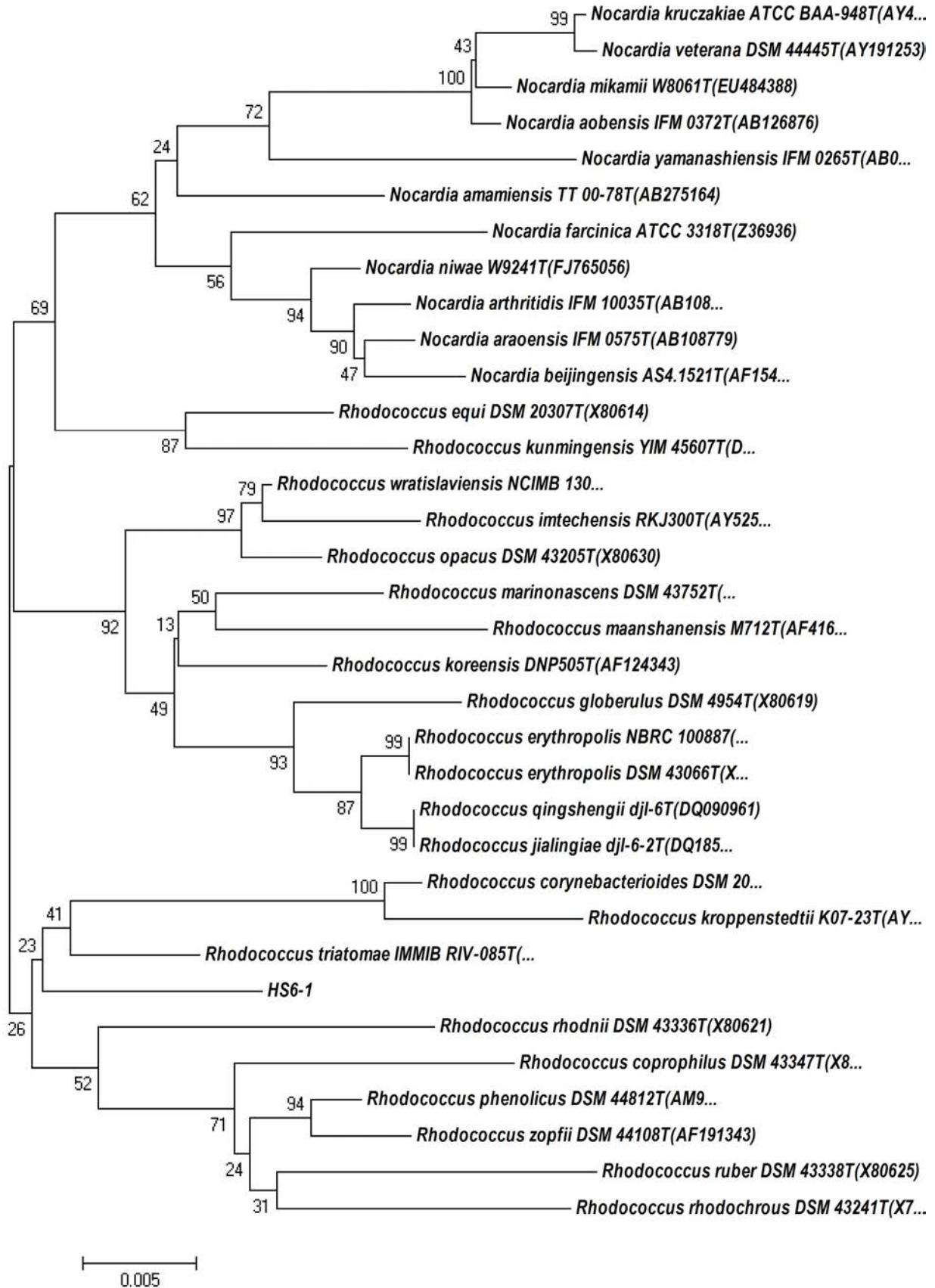


Figure 4. Phylogenetic relationship of HS4-2 with its closest homologs.



**Figure 5.** Phylogenetic relationship of HS6-1 with its closest homologs.

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