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Characterization of phenol degradation by *Rhizobium* sp. CCNWTB 701 isolated from *Astragalus chrysopteru* in mining tailing region

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Abstract

To screen high strength phenol degrading bacteria, we selected 108 rhizobial strains isolated from nodules of eight wild legumes species in the mining tailing region of Shaanxi province, northwest of China, and cultivated them in a basal salt (BS) medium supplemented with different phenol concentrations as a sole carbon source. The results showed that some of the strains could use phenol as sole carbon source. In order to study the characteristics of phenol degradation, the strain CCNWTB701 isolated from *Astragalus chrysopteru* was used as well, due to the fact that it was very efficient in phenol degradation. The phenol degradation was around 99.5 and 78.3%, with an initial concentration of 900 and 1000 mg/l phenol in 62 and 66 h, respectively. Kinetic studies indicated that the strain had a high K_S (743.1 μM) and an extremely high K_{SI} (10,469 μM) in Haldane's model. The phylogenetic analysis based on 16S rRNA gene sequences showed that CCNWTB701 belonged to the *Rhizobium* genus, and it was closely related to *Rhizobium mongolense* and *Rhizobium gallicum*.
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Keywords: *Rhizobium*; Phenol; Biodegradation; Kinetic; Phylogeny

1. Introduction

Phenol and phenolic compounds are important for many industries, which are involved in pesticide, tincture, bakelite, and medicine extensively. Therefore, a large amount of phenol and phenolic compounds are effused into stream and soil, and have badly polluted the environment. Phenol is now one of the most common toxic environmental pollutants, which mainly originates from industrial processes, such as oil refineries, cooking plants, industrial resin manufacturing, petroleum-based processing plants, pharmaceuticals, plastic, and varnish industries, etc. [1,2]. Phenol is hazardous to aquatic life, plants, and many other organisms, and acts as a substrate inhibitor in the biotransformation [3]. Thus, elimination of phenol effectively is necessary to preserve the environment and the health of human beings.

Now, different treatment methods are available for the degradation of phenol, and compared with physico-chemical methods, the biodegradation methods of phenol reduction is universally preferred, because of lower costs and the possibility of complete mineralization [4]. Applying microorganism to degrade phenol is the most efficient and prevalent way. Several bacterial strains belonging to the species of *Pseudomonas*, *Bacilli*, *Klebsiella*, *Ochrobactrum*, *Rhodococcus*, etc. were reported for phenol degradation [5,6]. In addition, some bacteria belonged to rhizobia were also reported to utilize phenol and aromatic compounds [7–9]. Employing rhizobia to phenol polluted soil firstly might degrade phenol via legume-rhizobia symbiosis, and may also increase the soil nitrogen level which will have an impact on promoting indigenous ecology rebuilding.

In this paper, we study phenol degradation ability and kinetics of strain CCNWTB701 in order to determine the quantitative and qualitative characterization of phenol degradation, and the degradation capability of aromatic substrate. Besides, physiological and biochemical features were used to characterize CCNWTB701, and phylogenetic analysis based on 16S rRNA gene was used to reveal genetic relationship of the isolate with other rhizobial strains.

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2. Materials and methods

2.1. Isolation of strains and culture conditions

Rhizobial strains were isolated from fresh nodules of *Medicago lupulina*, *Astragalus chrysopteru*, *Vicia unijuga*, *Vicia cracce*, *Lespedeza cuneata*, *Indigofera pseudotinctoria*, *Campylotropis macrocarpa*, *Pueraria lebata*, which grow in the mining tailing region of Shaanxi province in north-western China. Hundred and eight rhizobial strains were obtained by a standard method and using yeast-mannitol agar medium (YMA) [10]. Single colonies were picked and checked for purity by repeated streaking and microscopic examination. All strains were incubated at 28 °C and maintained on YMA slants at 4 °C, or in 20% (v/v) glycerol at −70 °C. The ability of each isolate to nodulate the original host plants was checked in glass tubes filled with vermiculite as described [10]. The temperature in the greenhouse was kept at 25 °C during the day and at 18 °C during the night with illumination of 10,000–20,000 lx for 14 h a day. Nodulation was observed after one month.

2.2. Phenol utilization of tested strains

Hundred and eight rhizobial strains were tested for their ability to utilize phenol as the sole carbon source in a basal salt solid medium (BS) supplemented with phenol at a concentration of 400, 500, 600, 700, 800, 900 mg/l, respectively. BS (per liter) contained 6.8 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.5 g of NaCl and NH_4Cl, respectively. The pH was adjusted to pH 6.8 ± 0.2. Strains grew at a temperature of 28 °C for three days. In order to prove the test result, the strains utilizing high concentration phenol were transferred into BS liquid medium supplemented with phenol at a concentration of 400, 500, 600, 700, 800, 900, and 1000 mg/l phenol, and were incubated at 28 °C with shaking at 200 rpm for 72 h. The growth of strains was determined by absorbance at 600 nm (OD_{600}). The OD_{600} >0.3 was considered as the standard of utilizing phenol [9]. The residual phenol concentration was determined by 4-aminoantipyrene colorimetric assay [11]. All tests were done in triplicate.

2.3. Kinetics of phenol degradation

The culture of CCNWTB701 growing in TY (5 g of tryptone, 3 g of yeast extract, and 0.7 g of $CaCl_2 \cdot 2H_2O$ per liter) liquid medium overnight, from late exponential phase was harvested and rinsed with 50 mM phosphate buffer (pH 6.8) twice, then 1 ml of the culture was inoculated into 100 ml BS medium which containing 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 mg/l, respectively. The incubations were grown at 28 °C with shaking at 200 rpm, cell growth biomass and residual phenol concentrations were determined at 2 h intervals. Cell growth biomass was monitored using Perkin–Elmer UV–vis spectrometer at 600 nm and phenol concentration was determined according to Folsom et al. [11]. All tests were done in triplicate, hence the results are the means of three independent experiments. The initial specific phenol degradation rate ($v_{0,\text{phenol}}$) was determined by Eq. (1):

$$v_{0,\text{phenol}} = \frac{1}{X}\frac{\mathrm{d}C_{\text{phenol}}}{\mathrm{d}t}\Big|_{t\to 0} \qquad (1)$$

where X denotes the cell concentration (g/l) or in absorbance unit at 600 nm (OD); C_{phenol} denotes the concentration of phenol (μM) and t denotes the cultivation time (h). The relationship between $v_{0,\text{phenol}}$ *versus* C_{phenol} represents the kinetics of phenol degradation by the strain CCNWTB701. To describe the kinetics of phenol-inhibition cell growth and phenol degradation, Haldane's substrate-inhibition model was used, as it has a widespread acceptance for phenol degrading and simplicity.

2.4. Multi-aromatic substrates utilization by CCNWTB701

To determine the inhibition of aromatic compounds to the growth of CCNWTB701, cultures of CCNWTB701 growing in TY liquid medium overnight to late exponential phase were harvested and twice rinsed with 50 mM phosphate buffer (pH 6.8). Then 1 ml of the culture was inoculated into 100 ml BS medium, which contained 4 mM of phenol, benzophenone, resorcin, cinnamic acid, and 4-hydroxybenzoic acid as the sole carbon source, respectively. The incubations were shaking in 200 rpm at 28 °C. The absorbance values were determined every 4 h at 600 nm by Perkin–Elmer UV–vis spectrometer.

2.5. Physiological and biochemical characteristics of CCNWTB701

Sixty-seven physiological and biochemical features were used to characterize the strain CCNWTB701. The analysis covered the growth range of temperature and pH; resistance to NaCl, antibiotics, dyes, chemicals; utilization of sugars and organic acids as sole carbon source; utilization of amino acids as sole nitrogen source; litmus milk reaction; reduction of methylene blue and nitrate; production of urease and catalase; acid or alkali production [12]. All tests were done in triplicate.

2.6. 16S rDNA amplification and phylogeny analysis of 16S rRNA gene

Strain CCNWTB701 was incubated in TY and the genomic DNA was extracted using a phenol-chloroform extraction protocol [13]. The 16S rRNA gene was amplified using primers P1 (5′-CGggatccAGAGTTTGATCCTGGTCAGAACGCT-3′) and P6 (5′-CGggatccTACGGCTACCTTGTTACGACTTCACCCC-3′), which corresponded to the nucleotide position 8–37 and 1479–1506 of *E. coli* 16S rRNA gene [12]. Fifty microliters of PCR volume consisted of 1 μg of total DNA, 5 μl 10× PCR buffer, 2.5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM P1, and P6, 2.5 U DNA polymerase and adding ddH_2O making the volume up to 50 μl. The PCR was conducted at 94 °C predenaturing for 5 min and the 30 cycles (94 °C denaturing 1 min, 56 °C annealing 1 min and 72 °C extending 2 min), finally 72 °C extending 5 min again. The PCR amplification product was purified using the PCR purification kit (geneworks). After purification, the PCR product was sequenced directly using an ABI Prism 377

DNA sequencer (Perkin–Elmer applied biosystems). Nucleotide sequences of each 16S rRNA gene were aligned and phylogenetic tree was constructed with the software ClustalX Version 1.81 by using the neighbor-joining method.

The generated 16S rDNA sequence (EF370483) and the sequences for reference strains obtained from the GenBank data library were used to construct the phylogenetic relationship tree.

3. Results

3.1. Screening of phenol-utilizing rhizobial strains

Hundred and eight rhizobial strains isolated from nodules of eight wild legumes species in the mining tailing region of Shaanxi province in northwest of China were incubated in BS medium with 400–900 mg/l phenol as sole carbon source to identify the degree of utilization for different concentrations. Among all of the strains, CCNWTB021, CCNWTB041, CCNWTB351, CCNWTB701, and CCNWTB1012 could grow on media containing 900 mg/l phenol. To study further into the characteristics of phenol utilization, we selected CCNWTB701 isolated from *A. chrysopteru* as a test strain, as it had the best growth ability (OD_{600}) after two days. CCNWTB701 was capable of nodulation in its host plant, and the nodules were pink, indicating a functioning symbiosis.

3.2. Kinetics of cell growth and phenol degradation by CCNWTB701

Fig. 1 showed the cell biomass of strain CCNWTB701 in 100–1000 mg/l phenol. From the curve, it was demonstrated that the lag time of CCNWTB701 was prolonged and biomass of the cell was hardly antiblastic, as the phenol concentration increased. The kinetic trends in specific growth rate *versus* different initial phenol concentration appeared to Andrews's substrate inhibition model (Eq. (2)) [14]. Its relevant constant values were listed in Table 1. From the model, the specific growth rate also increased at the beginning with phenol concentration increasing. While the phenol concentration exceeded 2858 μM, the specific growth rate decreased (Fig. 2). According to our data, the strain CCNWTB701 can grow in different concentration of phenol as the sole carbon source, and with the concentration increase, the lag time of growth was extended too. With a phenol concentration of 800, 900, and 1000 mg/l, the lag time of growth was 40, 44, and 56 h, respectively.

$$\mu = \frac{\mu_{max} C_{phenol}}{K_{S,G} + C_{phenol} + C_{phenol}^2 / K_{SI,G}} \quad (2)$$

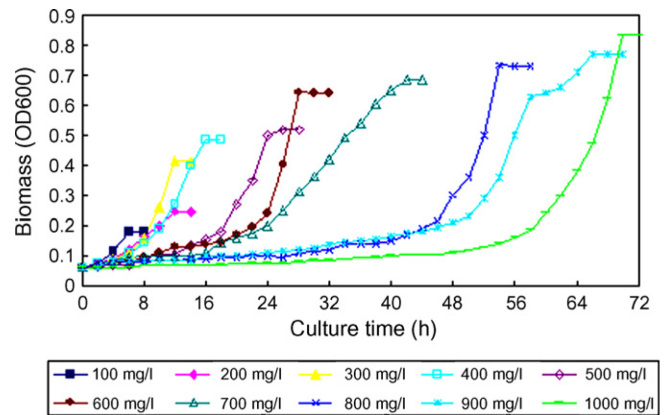

Fig. 1. Growth of CCNWTB701 in BS medium containing different initial phenol concentration.

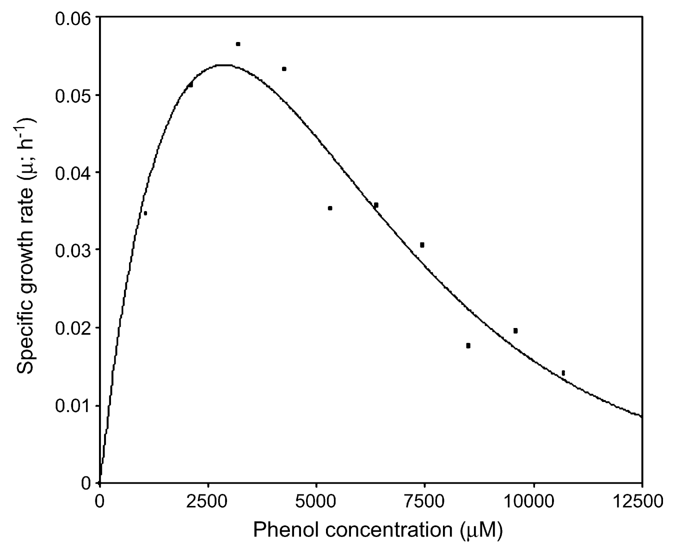

Fig. 2. The specific growth rate of CCNWTB701 on initial phenol concentration.

where the μ denotes the specific growth rate of strain CCNWTB701 on BS medium with phenol as the sole carbon source (h^{-1}); μ_{max} denotes the maximum specific growth rate of strain CCNWTB701 on BS medium with phenol as the sole carbon source (h^{-1}); C_{phenol} denotes the initial phenol concentration (μM); $K_{S,G}$ and $K_{SI,G}$ denote the half-saturation and inhibition constant for growth kinetics respectively.

At the same time, phenol degradation and the specific phenol degradation rate of strain CCNWTB701, corresponding to different initial phenol concentrations was demonstrated in Figs. 3 and 4. The time for phenol degradation increased in high phenol concentration, the strain CCNWTB701 degraded

Table 1
Kinetic parameters estimated from numerical simulations with the Andrews's substrate inhibitory model and Haldane's model

Parameters index	Phenol-limited growth kinetics (Andews's model)		Phenol degradation kinetics (Haldane's model)	
Maximum rate	μ_{max} (h^{-1})	0.054	$v_{max,phenol}$ (μM/min/g cell)	41.5
Half-saturation constant	$K_{S,G}$ (μM)	673.4	K_S (μM)	743.1
Inhibition constant	$K_{SI,G}$ (μM)	7818	K_{SI} (μM)	10469
Coefficient of determination	R^2	0.963	R^2	0.947

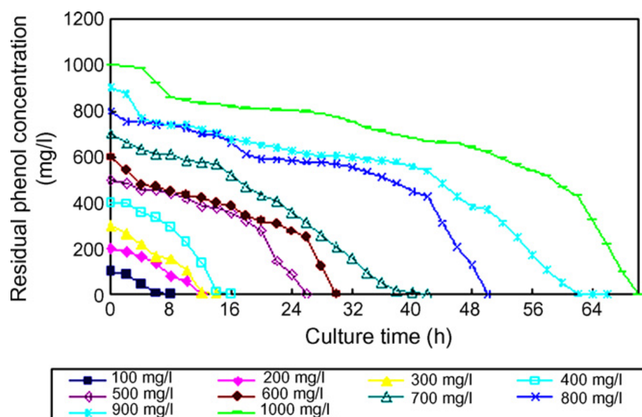

Fig. 3. The biodegradation of phenol by CCNWTB701 in BS medium containing different initial phenol concentration.

about 99.5 and 78.3% of phenol in 62 and 66 h, correspondingly, starting with an initial concentration of 900 and 1000 mg/l. The specific phenol degradation rate was firstly increased and then reduced whilst the concentration of phenol was up to 3248 μM. In order to simulate the data of phenol degradation by CCNWTB701, the Haldane's mathematical model was used (Eq. (3)) [14], and its kinetic parameters were showed in Table 1.

$$v_{\text{phenol}} = \frac{v_{\text{max,phenol}} C_{\text{phenol}}}{K_{\text{S}} + C_{\text{phenol}} + C_{\text{phenol}}^2 / K_{\text{SI}}} \tag{3}$$

3.3. Aromatic compounds-utilizing by CCNWTB701

The strain CCNWTB701 had an excellent ability for the decompounding of phenol, however, the growth biomass was greatly inhibited in high concentrations of phenol. To evaluate the influence of aromatic compounds on CCNWTB701, we selected phenol, benzophenone, resorcin, cinnamic acid, and 4-hydroxybenzoic acid as the sole carbon source on BS liquid medium. Fig. 5 was the growth biomass curve compare to the culture time. From Fig. 5, we knew

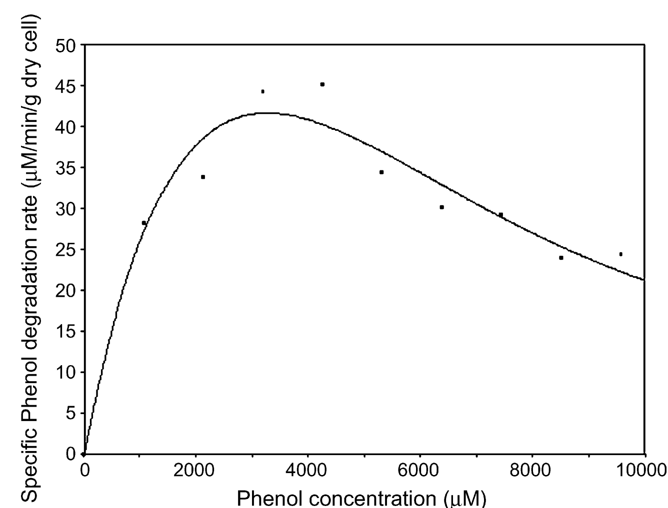

Fig. 4. The specific phenol degradation rate of CCNWTB701 on initial phenol concentration.

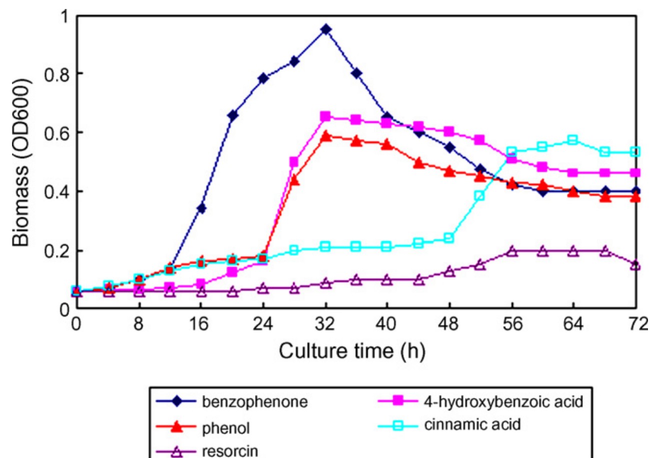

Fig. 5. Growth of CCNWTB 701 in BS medium with the five aromatic compounds each as the sole carbon source.

CCNWTB701 had good utilizing behavior towards benzophenone in which the OD_{600} was increasing up to 0.955 after 30 h. The ranking of the five aromatic compounds used by CCNWTB701 is as follows: benzophenone > 4-hydroxybenzoic acid > phenol > cinnamic acid > resorcin.

3.4. Physiological and biochemical characteristics of CCNWTB701

The physiological, biochemical characteristics of CCNWTB701 were given in Table 2. CCNWTB701 had an effective growth at 28 °C, a wide range pH tolerance of 9.0–11.0 and salt tolerance up to 3% of NaCl. CCNWTB701 could use the following materials (mg/ml): sucrose, erythritol, arabinose, rhamnose, galactose, D-xylose, sodium pyruvic acid, glucosamine hydrochloride, amylomaltose, galactosylglucose, and D-fructose as sole carbon sources, and L-norleucine, L-leucine, L-aspartate, L-cysteine, L-glutamate and L-tryptophan as sole nitrogen sources. CCNWTB701 was resistant to the following antibiotics (μg/ml): penicillin (5–100), fosfomycin (5–300), lincomycin (5–300), and chloromycetin (5–50). It could grow in a medium supplemented with congo red (0.1%) and thymol blue (0.2%). CCNWTB701 produced acid in YMA and catalase, and it was a nitrate-reducer.

3.5. 16S rRNA gene phylogeny of CCNWTB701

16S rRNA sequences analysis is a fast and accurate method to identify CCNWTB701 phylogeny position. Full-length (about 1500 bp) 16S rRNA genes were sequenced and used to construct phylogenetic tree (Fig. 6). We found that the tested strain CCNWTB701 was classified in the *Rhizobium* genera, the similarities between CCNWTB701 and *Rhizobium mongolense* USDA1844, *Rhizobium gallicum* R602, *R. yanglingense* CCBAU71623, and *R. sullae* IS123 were 99.0, 99.0, 98.5, and 98.4%, respectively. So, CCNWTB701 was closely related to *R. mongolense* and *R. gallicum*.

Table 2
The physiological and biochemical characteristics of CCNWTB701

Physiological characteristics		Antibiotics and dyes resistance		Sole carbon and nitrogen sources (1 mg/ml)		Biochemical characteristics	
Growth at temperature (°C)		Penicillin (5, 50, 100 μg/ml)	+	Sucrose	+	Litmus milk alkali production	–
4	–	Penicillin (300 μg/ml)	–	Sodium hippurate	–	Litmus milk acid production	–
10	–	Streptomycin (5, 50, 100, 300 μg/ml)	–	Sodium citric acid	–	Litmus milk peptonization	–
28	+	Fosfomycin (5, 50, 100, 300 μg/ml)	+	Sodium oxalic acid	–	Litmus milk reduction	+
40	–	Lincomycin (5, 50, 100, 300 μg/ml)	+	Erythritol	+	Reduction of methylene blue	+
60	–	Chloromycetin (5, 50 μg/ml)	+	Arabinose	+	Reduction of nitrate	+
Growth at pH		Chloromycetin (100, 300 μg/ml)	–	Sorbose	–	Production of urease	–
4	–	Neutral red (0.1, 0.2%)	–	Rhamnose	+	Production of catalase	+
5	–	Methylene blue (0.1, 0.2%)	–	Glucogen	–	Acid production	+
9	+	Congo red (0.1%)	+	Galactose	+	Alkali production	–
10	+	Methyl green (0.1, 0.2%)	–	D-xylose	+		
11	+	Methyl red (0.1, 0.2%)	–	Sodium pyruvic acid	+		
12	–	Methyl orange (0.1, 0.2%)	–	Glucosamine hydrochloride	+		
Growth on NaCl (%)		Thymol blue (0.1, 0.2%)	+	Amylomaltose	+		
1	+			Galactosylglucose	+		
2	+			D-fructose	+		
3	+			Alanine	–		
4	–			L-norleucine	+		
5	–			L-leucine	+		
6	–			L-tyrosine	–		
				L-aspartate	+		
				L-cysteine	+		
				Glycine	–		
				L-glutamate	+		
				L-tryptophan	+		
				DL-histidine	–		

4. Discussion

Bacterial genera capable of degrading phenolic compounds in the environment are important soil bacteria and may play a role in degrading phenolic compounds of toxic organic pollutants. Currently, a number of bacteria were discovered to have excellent capability of phenol degradation. Identification of these bacteria showed the dominance of genus *Pesudomonas*, especially *Pesudomonas putida* mainly because of its spread distribution in soils. Besides, many other genera of bacteria were described as degrading strains of phenolic compounds, including *Agrobacterium*, *Burkholderia*, *Acinetobacter*, *Ralstonia*, *Klebsiella*, *Bacillus*, *Rhodococcus* [15]. In the genera of rhizobial bacteria, phenolic waste degradation was also covered with *Bradyrhizobium*, *Rhizobium*, except for *Ralstonia*, which belonged to β-proteobactreria rhizobia [7–9]. From our study, the *Rhizobium* could utilize phenol as the carbon source, which suggests a metabolic pathway maybe through an *ortho*- or *meta*-oxidation typically [16]. In our study, CCNWTB701 could grow well in 900 mg/l phenol and degrade 99.5% in 64 h. It also degrades 78.3% in 66 h in 1000 mg/l of phenol. This demonstrated that this strain had excellent phenol degrading ability.

Cell growth on phenol has been observed to display substrate inhibition phenomena at high phenol concentration, and the Haldane's equation is often used to describe cell growth on phenol either by pure or mixed cultures [17]. To represent the growth kinetics of phenol inhibition, several kinetic models were fitted to the experimental data for selecting the best models. Out of the models, Haldane's model was used due to its mathematical simplicity and wide acceptance for representing the growth kinetics of inhibitory substrates [18]. Watanabe applied the Haldane's model to describe the kinetics of phenol degradation for a number of bacteria and classified the phenol-degrading bacteria into three kinetically different groups according to the K_S and K_{SI} values [14]. Our research of phenol degradation kinetics showed CCNWTB701 possessed a high K_S (743.1 μM) and an extremely high K_{SI} (10469 μM), which appeared to belong to none of the three general kinetic groups suggested by Watanabe et al. [14]. The result was different with *Ralstonia taiwanensis* TJ86 isolated from *Mimosa pudica,* which possessed a low K_S value [9], because the lag time of growth for CCNWTB701 was more prolonged than *R. taiwanensis* TJ86. The unique kinetic characteristics of CCNWTB701 with a high K_S and an extremely high K_{SI} give better phenol degradation activity. The *Rhizobium* sp. CCNWTB701 reported here seems to be the first documented rhizobial bacterium isolated from *Astragalus* that has the nature of strength degrading phenol. In cell growth kinetic model and degradation kinetic model of CCNWTB701 in phenol, R^2-values were 96.3 and 94.7%, respectively. This result showed Haldane's model was effective for describe the dynamics of phenol degradation for CCNWTB701 [19].

The genus *Astragalus*, including 1500–2000 species, is one of the largest genera in the family leguminosae. Many species of *Astragalus* form nitrogen-fixing symbioses in association with root-nodule bacteria [20], and many species have ecological uses, for example, as green manure, protection for soil ero-

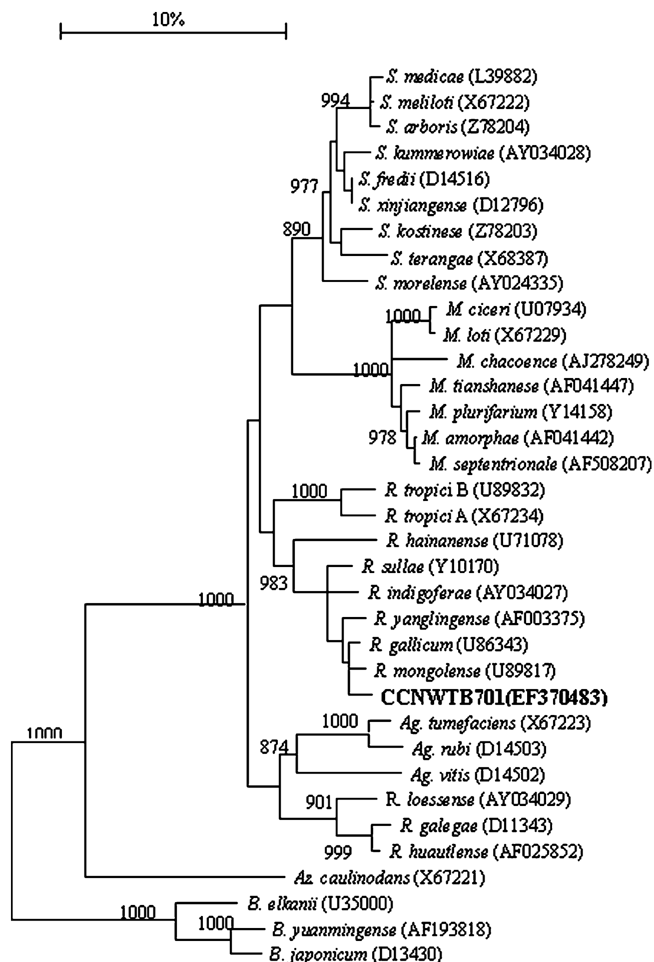

Fig. 6. Phylogenetic tree of rhizobia based on 16S rRNA gene sequence according to the method of neighbor-joining. Numbers at the branch nodes are bootstrap values (per 1000 trials). Only values greater than 800 are indicated. The bar represents 10% base substitution. Abbreviations of Genus: *Ag*, *Agrobacterium*; *Az*, *Azorhizobium*; *B*, *Bradyrhizobium*; *M*, *Mesorhizobium*; *R*, *Rhizobium*; *S*, *Sinorhizobium*.

sion and bioremediation [21]. Rhizoremediation has recently been recognized to be of interest because it is a low-cost *in situ* treatment method for pollutants in soils. Plants and their rhizomicrobes have been shown that they could enhance degradation of toxic compounds in the rhizosphere [21]. Rhizobia are known for their capacity to fix atmospheric nitrogen in a symbiotic relationship with the root of their specific host leguminous plants. This symbiotic capacity can assist the growth of their host plants in nutrient-poor soils and increase nitrogen input into soils. Thus, there exists the potential for application of pollutant degrading rhizobial strains along with host plants so as to establish a rhizosphere clean-up system for remediation of polluted soils.

5. Conclusion

The rhizobial strain CCNWTB701 isolated from *A. chrysopteru* had high strength phenol degrading ability, and the phenol degradation was 99.5 and 78.3%, with an initial concentration of 900 and 1000 mg/l phenol in 62 and 66 h, respectively. The strain CCNWTB701 had a high K_S (743.1 μM) and an extremely high K_{SI} (10469 μM) in Haldane's model. CCNWTB701 belonged to the *Rhizobium* genus according to phylogenetic tree based on 16S rRNA gene, and it was closely related to *R. mongolense* and *R. gallicum*.

Acknowledgements

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