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Enrichment, isolation, and characterization of 4-chlorophenol-degrading bacterium *Rhizobium* sp. 4-CP-20

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Abstract The objectives of this research were to monitor the variations of species in mixed cultures during the enrichment period, isolate species and identify and characterize the pure 4-chlorophenol (4-CP) degrading strains from enriched mixed cultures. Strain *Rhizobium* sp. 4-CP-20 was isolated from the acclimated mixed culture. The DGGE result indicated that strain *Rhizobium* sp. 4-CP-20 was undetectable at the beginning but detectable after 2 weeks of enrichment. The optimum growth temperatures for *Rhizobium* sp. 4-CP-20 were both 36°C using 350 mg l^{-1} glucose or sodium acetate as the substrate. The optimum pH range for degrading 100 mg l^{-1} 4-CP was between 6.89 and 8.20. Strain *Rhizobium* sp. 4-CP-20 could degrade 4-CP completely within 3.95 days, as the initial 4-CP concentration was 100 mg l^{-1}. If the initial 4-CP concentration was higher than 240 mg l^{-1}, the growth of bacterial cells and the activity of degrading 4-CP were both inhibited.

Keywords Biodegradation · 4-Chlorophenol · DGGE · *Rhizobium* sp. 4-CP-20 · BIOLOG

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Introduction

A large variety of chemicals are commercially produced and newly synthesized each year. Compounds are discharged into the environment during use and manufacture of these chemicals (Annachhatre and Gheewala 1996). Over the last decades, halogenated aromatic compounds have been used extensively as pesticides and herbicides (Zouari et al. 2002). Their widely use has caused great damages to the environment and to living organisms. These are among the most persistent environmental pollutants because of their physicochemical characteristics. Among chlorophenolic compounds, 4-chlorophenol (4-CP) is a toxic and recalcitrant compound which is formed from wastewater chlorination in pulp mills from the breakdown of herbicides and the anaerobic degradation of more highly chlorinated phenols (Westerberg et al. 2000). In treating chlorophenols, the biological method has attracted more attention than physical and chemical methods because of relative inexpensive cost and less secondary pollution (Kargi and Konya 2006). Moreover, many different types of microorganisms utilizing 4-CP as their sole carbon and energy source were isolated, such as *Arthrobacter ureafaciens* CPR706 (Bae et al. 1996a), *Arthrobacter chlorophenolicus* A6 (Backman and Jansson 2004), and *Comamonas testosteroni* JH5 (Hollender et al. 1994).

To obtain pure 4-CP-degrading strains, it is very important to enrich mixed cultures with 4-CP. The

reason is to increase the amount and activity of cells containing the ability to degrade 4-CP. The development of molecular biological techniques has provided numerous useful tools for studying microbial diversity and gave operators important information for modifying engineering processes and bioremediation system (Whiteley and Bailey 2000; Eichner et al. 1999). Moreover, molecular analysis demonstrates the presence of structured microbe communities evolved in response to differential selective pressures within the enrichment system. Thus, monitoring the organism variations in the mixed culture during the enrichment period may also produce useful information for isolating pure strains. However, few papers investigated the variations in mixed cultures during the enrichment period. The objectives of this research were: (1) to monitor the variation in species mixed cultures during the enrichment period with DGGE, and (2) isolate and characterize pure 4-CP-degrading strains from enriched mixed cultures.

Materials and methods

Mixed culture acclimation

All experimental procedures were performed in the inorganic culture media mentioned by Yang et al. (2006). The mixed cultures originated from the pentachlorophenol (PCP)-contaminated soils located at a PCP-contaminated site in Southern Taiwan. The unsieved soil (25 g) and inorganic culture media (300 ml) were mixed using a Waring blender for 2 min. The liquid from the Waring blender was then added into the 3 l flask containing 1 l LB medium (Miller 1972) to increase the number of cells. The flask was then shaken at 120 rpm in the dark at 30°C. After 2 days, the cells were harvested by centrifugation (6,000 × g at 4°C for 14 min) and washed twice with fresh inorganic culture medium. The pellet was resuspended in inorganic culture media and 50–275 mg l^{-1} 4-CP was added to serve as the sole carbon and energy source. The biodegradation of 4-CP in the flask was monitored periodically with HPLC with 4-CP repeatedly added into the flask until the degradation reached a stable level.

Isolation of pure strains and the determination of their 4-CP degradation potential

At stable 4-CP degradation, the mixed culture was diluted in the inorganic culture media and a series of the dilution were spread-plated on R2A (Reasoner and Geldreich 1985) to obtain single colonies (the plates were incubated at 30°C for 24 h). The loopfuls of single colony from each plate were streaked onto other fresh R2A plates to check for purity. After initial purification, each strain was checked for its ability to degrade 4-CP by inoculating them into separate flask containing the inorganic media and 75 mg l^{-1} 4-CP. The flasks were incubated and the degradation of 4-CP was monitored. Furthermore, some isolates were selected as the ladder markers for DGGE experiment.

4-chlorophenol degrading bacteria identification and substrate utilization

The DNA of 4-CP degrading bacteria was extracted from 1.5 ml of pure culture after pelleting using genomic DNA mini kit (Geneaid Biotech Ltd., Taoyuan, Taiwan). Then, the 16S ribosomal genes were amplified using the polymerase chain reaction (PCR) and then the amplified PCR product were sequenced and compared against the GenBank database using the NCBI Blast program. The detailed protocols were described as the research of Yang et al. (2006). Moreover, in order to test the ability of 4-CP-degrading bacterium to utilize (oxidize) various carbon sources in a short time, the BIOLOG bacterial identification test kit was selected (BIOLOG, Hayward, CA, USA), and the procedures were followed with commercial protocol.

4-chlorophenol removal using pure culture

These experiments were conducted using a series of 125 ml batch reactors. Each reactor contained 40 ml of inorganic culture media with a pure bacteria culture. The initial amount of pure bacteria started at a cell concentration yielding 0.1 OD units. Different 4-CP concentrations were then added and sealed with cotton stoppers. The reactors were shaken at 120 rpm in the dark at 30°C, and sampled periodically to analyze the variation of 4-CP concentration with

HPLC. Moreover, the pH and OD values and chloride concentration were also measured.

Optimum temperature for growth experiment

The optimum temperature for the growth of the bacterial isolates was determined following the procedure of Lee and Wang (2004) but using 350 mg l^{-1} of glucose or sodium acetate as the growth substrate under aerobic condition. Optical density at 600 nm was followed as a measurement of growth, and the mid-log growth data for strain was selected for calculating the specific growth rates. After calculating, the specific growth rate was then plotted versus incubation temperature to obtain the optimum temperature for growth.

Determination of optimum pH for 4-CP removal by pure bacterial culture

In order to find out the optimum pH for 4-CP removal, various pHs of inorganic culture media (pH = 4.97, 5.96, 6.89, 7.59, 8.2, and 9.18) were prepared. The pHs of the inorganic culture media were adjusted with NaOH/HCl. Each 125 ml batch reactor contained 40 ml inorganic culture media with cell suspensions containing 10^7 cells ml^{-1}, and then 100 mg l^{-1} of 4-CP was added to serve as the sole carbon and energy source. After sealing with cotton stoppers, the reactors were shaken at 120 rpm in the dark at 30°C to observe 4-CP removal under aerobic conditions.

16S rDNA amplification and DGGE analyses

During the enrichment period the acclimated mixed culture was sampled every week. DNA was extracted from 3 ml of mixed culture suspension using the genomic DNA mini kit (Geneaid Biotech Ltd.). Four hundred thirty-three bp of the 16S rDNA product was amplified from all nucleic acid samples using PCR using primers 968F-GC clamp and 1401R (Nubel et al. 1996). The PCR was performed in a GeneAmp PCR system 2700 thermal cycler with a hot start performed at 95°C for 3 min, followed by 30 cycles at 95°C for 45 s, 54°C for 45 s, and 72°C for 1 min, followed by a final extension performed at 72°C for 3 min.

DGGE analysis was performed by loading 30 µl of PCR-amplified DNA product onto an 8% (wt./vol.) acrylamide gel containing a denaturant gradient of 30–60% (100% denaturant consisted of 7 M urea and 40% [vol./vol.] formamide) parallel to the electrophoresis detection using the D-Code system (Bio-Rad, Hercules, CA, USA). Gels were electrophoresed at 60°C at a constant voltage of 200 V for 6.5 h prior to being stained using Ethidium Bromide (EtBr). After staining, gels were scanned digitally with a Kodak EDAS290 system (Eastman-Kodak, Rochester, NY, USA). All gels were standardized by the addition of a ladder generated by mixing PCR products of the 16S rDNA gene from a range of cultured isolates from the acclimated mixed culture.

Analytical methods

The cell suspensions were clarified by centrifugation at 8,000 rpm for 3 min. The concentration of 4-CP in the supernatant was analyzed using HPLC (Hitachi L7100 system, Tokyo, Japan) with a UV detector and the analysis condition was the same as that used in the study of Yang et al. (2005). The chloride concentration was analyzed using IC (Dionex 100, Sunnyvale, CA, USA). The pH and OD were measured using a pH meter (PHM82, standard pH meter, Radiometer, Copenhagen, Denmark) and Spectrophotometer (Beckman Du® 530, Irvine, CA, USA) at 600 nm, respectively.

Results and discussion

The isolation, identification, and substrate utilization of strain *Rhizobium* sp. 4-CP-20

After acclimation for 2 months, 23 strains were isolated from the mixed culture. The ability of individual bacterial strains to degrade 4-CP was tested. The test results indicated that only strain 4CP-20 expressed the ability to degrade 4-CP, and the other 22 strains could not decompose 4-CP. Molecular identification based on sequencing of the total 16S rDNA gene of strain 4CP-20 (accession no. EF659423) revealed that strain 4CP-20 belongs to the genus *Rhizobium* (alpha-Proteobacteria subdivision). Comparison of the complete 16S rDNA sequence

with data bank sequences (GenBank + EMBL + DDBJ + PDB) showed that strain 4CP-20 had identity levels of 99% with *Rhizobium* sp. MK15 (GenBank accession no. EF173319). Strain *Rhizobium* sp. 4-CP-20 was a Gram-negative and aerobic rod-shaped bacterium. Among 95 different types of carbon sources, *Rhizobium* sp. 4-CP-20 could utilize 74 types of substrate, and the pattern generated was a good match with strain *Rhizobium radiobacter* in the BIOLOG database.

The optimum growth temperature of 4-CP-degrading bacterium

The result reveals that *Rhizobium* sp. 4-CP-20 is a mesophilic bacterium. Figure 1 shows the specific growth rate of *Rhizobium* sp. 4-CP-20 with different temperatures. The optimum growth temperature of *Rhizobium* sp. 4-CP-20 was 36°C by utilizing 350 mg l^{-1} of glucose as substrate. However, complete growth inhibition of *Rhizobium* sp. 4-CP−20 occurred at 45°C. The same result was found while the substrate was 350 mg l^{-1} sodium acetate. Strain *A. chlorophenolicus* A6 had the ability to degrade 4-CP at low temperatures. When subjected to temperature fluctuations between 5 and 28°C, strain A6 continued to degrade 4-CP and remained active (Backman and Jansson 2004). According to the result of the optimum growth temperature for *Rhizobium* sp. 4-CP-20, although the tested C-source was not 4-CP, the temperature range throughout which growth occurred is 15–42°C.

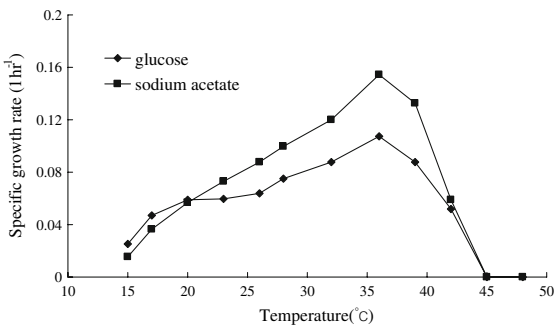

Fig. 1 Specific growth rate for strain *Rhizobium* sp. 4-CP-20 at different temperatures when 350 mg l^{-1} glucose and sodium acetate served as the carbon source, respectively

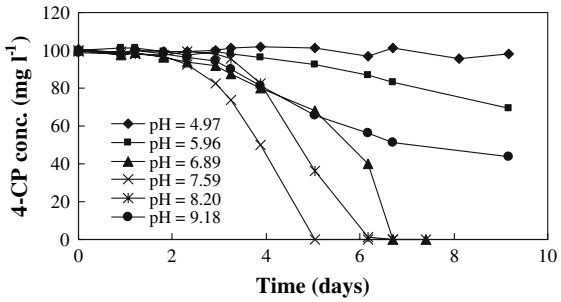

Fig. 2 Removal of 100 mg l^{-1} of 4-CP by *Rhizobium* sp. 4-CP-20 in a batch reactor with different initial pH value

The optimum pH for 4-CP removal by *Rhizobium* sp. 4-CP-20

The removal of 100 mg l^{-1} of 4-CP by *Rhizobium* sp. 4-CP-20 with different initial pH value is shown in Fig. 2. The result indicated that strain *Rhizobium* sp. 4-CP-20 could not remove the 4-CP, while the initial pH value was below 4.97. When the tested pHs were 5.96 and 9.18, 4-CP could not be decomposed entirely by strain *Rhizobium* sp. 4-CP-20 at the end of experiment (9.16 days) and the 4-CP removal efficiencies were 30 and 55.7%, respectively. As the initial pH value was 6.89, 7.59, and 8.20, *Rhizobium* sp. 4-CP-20 could remove the 4-CP completely within 6.69, 5.04, and 6.69 days, respectively. Besides, there was a significant lag phase occurred in the initial period at each pH value except for pH 4.97 before 4-CP started to be biodegraded. This phenomenon revealed the 4-CP degradation should be inducible. Nordin et al. (2005) investigated the biochemistry and genetics of 4-CP degradation in *A. chlorophenolicus* A6. Their results indicate that *A. chlorophenolicus* A6 degraded 4-CP via hydroxyquinol. Moreover, hydroxyquinol was removed from cell extracts derived from 4-CP-grown cells but not from extracts of cells grown on succinate. Therefore, they concluded that this phenomenon was clearly induction of the ability to remove hydroxyquinol when 4-CP is the growth substrate compared to when an alternative substrate is used. Balfanz and Rehm (1991) examined *Alcaligenes* sp. A 7–2 for degrading 4-CP in sandy soil, and determined of various parameters a model system for biodegradation of xenobiotics in soil. They observed all degradation kinetics for 4-CP could be divided into a lag phase and a degradation phase, and they conjectured this

effect was probably due to induction processes of the enzyme systems needed for degradation.

According to the results mentioned above, the optimum pH range for 4-CP removal by *Rhizobium* sp. 4-CP-20 was between 6.89 and 8.20. However, the most optimum pH value for 4-CP degradation should be 7.59 because of the shorter period of lag phase. Generally, most of enzymes in the microorganism can maintain their activities at the neutral pH. Thus, the whole activity of the cells was better so that the enzymes involved in 4-CP degradation were induced earlier and then the total period for 4-CP removal was shortened. This could be proven by the calculated specific growth rate and specific substrate utilization rate at each pH value condition. The specific growth rate of strain *Rhizobium* sp. 4-CP-20 at pH 7.59 was calculated to be 0.067 day^{-1} compared to the 0.0342 and 0.0537 day^{-1} at pH 6.89 and 8.20, respectively. Moreover, the specific substrate utilization rate at pH 7.59 was also higher than that at pH 6.89 or 8.20. Nevertheless, 4-CP also could be decomposed in the slight acidic and alkaline condition but the removal efficiency became quite lower. Besides, 4-CP biodegradation accompanied chloride release and biomass growth (data not shown).

Figure 3 shows the removal of different 4-CP concentrations by *Rhizobium* sp. 4-CP-20 in 125-ml flasks. In the batch culture, strain *Rhizobium* sp. 4-CP-20 was able to degrade 100 mg l^{-1} 4-CP completely within 3.95 days, and to release concentration 27.3 mg l^{-1} chloride at the same period of time. However, before 4-CP was utilized, a significant lag phase occurred in the initial period. Besides, 4-CP degradation accompanied obvious biomass growing and pH value decreasing. After calculating the dechlorination of 100 mg l^{-1} 4-CP, 4-CP was dechlorinated to ~100% by bacterial cells. However, as the 4-CP concentration was higher than 240 mg l^{-1}, the growth and activity of pure culture were completely inhibited.

Fava et al. (1995) isolated one pure strain *Pseudomonas pickettii* with the ability to degrade 2-CP, 3-CP, and 4-CP. This pure culture could remove 0.75 mM (96 mg l^{-1}) within 40 h. Bae et al. (1996b) isolated one pure 4-CP degrading strain CPW301 and it was identified as *C. testosteroni* CPW301. They studied the effect of the different initial 4-CP concentration on 4-CP degradation by strain CPW301. The result indicated while the initial 4-CP concentration was higher than 0.9 mM (115 mg l^{-1}), 4-CP could not be degraded within 500 h. Strain CPW301 required about 340 h to decompose 0.75 mM 4-CP completely. Strain *A. chlorophenolicus* was obtained from the soil and it could degrade 100 mg l^{-1} 4-CP within 1 day (Westerberg et al. 2000). Lima et al. (2004) studied the capacity of the microalgae consortium to degrade and grow on *p*-chlorophenol. Experiments carried out with the microbial community obtained directly from the waste discharge container resulted in the biodegradation of *p*-CP at 25, 50, and 100 mg l^{-1}. Complete removal of *p*-CP from the cultures supplied with 25 and 50 mg l^{-1} of *p*-CP was observed after 9 days, whereas 100 mg l^{-1} of *p*-CP were completely removed from the culture medium in 15 days.

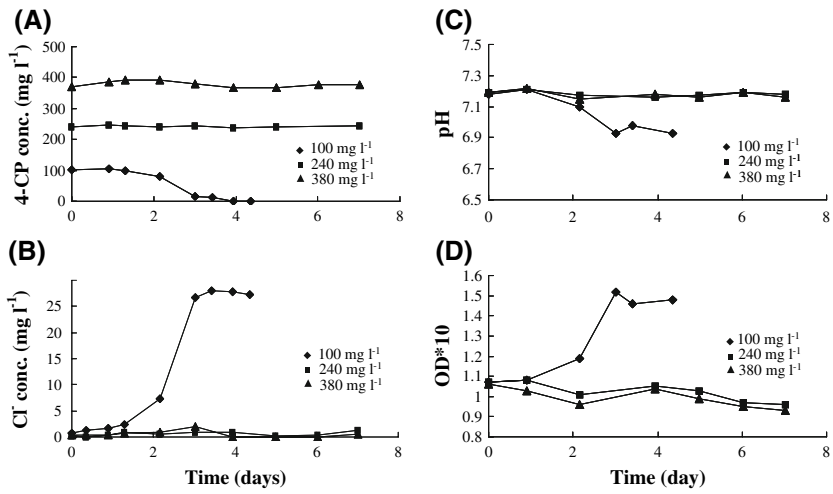

Fig. 3 Removal of different concentrations of 4-CP by *Rhizobium* sp. 4-CP-20 in a batch reactor. 4-CP concentration (**A**), chloride concentration (**B**), pH (**C**), and OD (**D**) variations

Comparing the results of strain *Rhizobium* sp. 4-CP−20 with other pure and mixed cultures mentioned in previous studies, the ability strain *Rhizobium* sp. 4-CP-20 to degrade 4-CP might not be the best. However, strain *Rhizobium* sp. 4-CP-20 may have potential to be applied for soil bioremediation. Suominen et al. (2000) evaluated the potential of combining a nitrogen-fixing leguminous plant, *Galega orientalis*, with its microsymbiont *Rhizobium galegae* for the bioremediation of BTX (benzene, toluene, xylene)-contaminated soils in microcosm and mesocosm scale. In the mesocosm assay the Galega plants showed good growth, nodulation and nitrogen fixation, and developed a strong rhizosphere in soils contaminated with oil or spiked with 2,000 mg l^{-1} *m*-toluate. Our isolate *Rhizobium* sp. 4-CP-20 belongs to the rhizobial species and has the ability of degrading 4-CP. Its ability to fix nitrogen and symbiosis with plants will be the next topic needed to be investigated.

DGGE 16S rDNA profile during enrichment period

Figure 4 shows the 4-CP concentration and the microbial community variation during the enrichment period. To obtain pure strain with better ability to degrade 4-CP, the 4-CP concentration increased gradually during the enrichment period. The first 4-CP concentration was 50 mg l^{-1}, and the mixed culture could remove the 4-CP within 2 weeks. Within 2 weeks, strain *Rhizobium* sp. 4-CP-20 became detectable, especially in the second week, and an obvious band meaning strain *Rhizobium* sp. 4-CP-20 was detected. After increasing the 4-CP concentration to about two times the initial concentration (88 mg l^{-1}), 88 mg l^{-1} 4-CP could be decomposed within another week. This indicated that the ability of a mixed culture to degrade 4-CP improved. The reason for increasing the 4-CP degradation ability might be the well-maintained 4-CP-degrading strain because of the significant presence of strain *Rhizobium* sp. 4-CP-20. After 5 weeks, the 4-CP concentration was adjusted to about four times the initial concentration (207 mg l^{-1}). The higher concentration might slightly inhibit the mixed culture. The mixed culture required another 3 weeks to completely degrade the 4-CP. The mixed culture community was also changed because strain

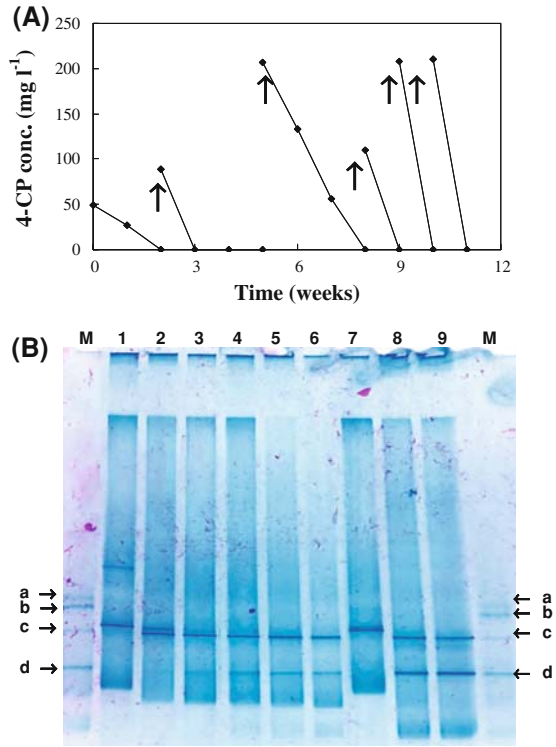

Fig. 4 The degradation of 4-CP (**A**) and the community variation of the mixed culture during enrichment period (**B**). *Arrows* show the time points of adding 4-CP. PCR-DGGE mixed culture community during enrichment, and individual lanes contain 16S rDNA fragments amplified by PCR from genomic DNA extracts. Lane M: Marker (from a to d: *Photorhabdus luminescens* 4CP-3, *Brevibacillus centrosporus* 4CP-8, *Pseudoxanthomonas mexicana* 4CP-1, and *Rhizobium* sp. 4CP-20), lanes 1–9: weeks 0–5 and 8–10

Rhizobium sp. 4-CP-20 was undetectable and the pattern of the mixed culture community was very different from the other mixed culture communities. Thus, the 4-CP concentration was reduced to two times the initial concentration (109 mg l^{-1}). The mixed culture recovered its' ability to degrade 4-CP. In the later enrichment period (weeks 8–11), 4-CP removal efficiency was well maintained and the 4-CP could be degraded within 1 week. The community variation in the mixed culture was not obvious.

One special phenomenon for discussion is shown in Fig. 4. Strain *Pseudoxanthomonas mexicana* 4CP-1 seemed to resist the toxicity of 4-CP because it could be detected in the mixed culture during the enrichment period, except for weeks 0 and 8. This strain was identified as *P. mexicana* (98% similar to the *P. mexicana* UR374_02). We are sure that strain

4CP-1 contains no ability to degrade 4-CP. The reasons of stable existence of this species during whole enrichmental period might be that a DNA fragment of strain 4CP-1 was easily amplified by the primers that we selected, or this strain was stronger in competition than the other strains, especially its advantage for tolerating 4-CP toxicity. Moreover, strain 4CP-1 might contain the ability of degrading the 4-CP intermediates so that this strain could be maintained well in the enriched culture. Besides, the whole DGGE profile did not show abundant bacterial variation. The first reason might be it was hard to amplify the 16S rDNA of some species. When maker ladder was prepared, not all 16S rDNA of all isolates could be amplified successfully. Only four strains could be amplified easily from the total eight candidates. The reason might also be the inappropriate staining method. In this study, Etbr was chose as stainer. However, some other staining methods such as silver staining can provide higher sensitivity. The most sensitive procedure is silver staining, although silver-stained gels cannot be used for hybridization experiments and single-strand DNA fragments are also detected (Ercolini 2004). Thus, inappropriate staining method might cause the absence of the bacterial variation.

From the results of 4-CP removal by mixed culture, the mixed culture could decompose about 200 mg l^{-1} 4-CP within 1 week. Comparing the abilities to degrade 4-CP by strain *Rhizobium* sp. 4-CP-20 and the enriched mixed culture (Figs. 3, 4), the later culture had better capability to degrade 4-CP than the former. There might be some microorganisms helping 4-CP removal in the mixed culture, but the evidence needs to be identified.

Conclusions

One 4-CP-degrading strain was isolated from a 4-CP-enriched mixed culture. This strain was identified as *Rhizobium* sp. 4-CP-20 using a method based on the 16S rDNA gene sequence. The optimum growth temperatures for *Rhizobium* sp. 4-CP-20 were 36°C utilizing glucose or sodium acetate as the substrates. The optimum pH range for degrading 100 mg l^{-1} 4-CP with strain *Rhizobium* sp. 4-CP-20 was between 6.89 and 8.20. However, 4-CP could also be decomposed in both slight acidic and alkaline conditions. The DGGE result indicated that strain *Rhizobium* sp. 4-CP-20 was undetectable at the beginning but was evident after 2 weeks of enrichment. The 4-CP removal result could reflect the community variation during the enrichment period. *Rhizobium* sp. 4-CP-20 could degrade 4-CP completely within 3.95 days, as the initial 4-CP concentration was 100 mg l^{-1}. If the initial 4-CP concentration was higher than 240 mg l^{-1}, the 4-CP degrading activity by *Rhizobium* sp. 4-CP-20 was completely inhibited.

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