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Isolation, Identification and Degradation Characterization of a *p*-Chloroaniline Degrading Strain

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Abstract A bacterium (strain ycsd02) was isolated from rhizosphere soil in phragmites wetland using the enrichment culture contaminated with *p*-Chloroaniline (*p*-CA). Morphology, physio-biochemical characteristics and phylogenetic analysis based on 16S rDNA sequence indicated that the shape of the single cell is pole, which clearly belongs to *Bacillus licheniformis*. The optimal conditions for satisfactory degradation of *p*-CA were also studied. A dynamic test indicated that the biodegradation had no lag phase at concentrations from 0 to 20 mg/L in minimal medium culture fluids at vaccination of 15%, pH 7.0 and 32°C, and the growth of strain ycsd02 cells met the first-order kinetics.

Keywords *p*-Chloroaniline · Identification · Degradation · 16S rDNA

As members of chlorinated aromatic compounds, chlorinated aniline compounds are widely used in synthesis of pesticides, dyes, plastics and drugs (Yu and Zhang 2000) and cause extensive environmental pollution due to the lack of natural microorganism enzymes or enzyme systems to degrade them. They threaten ecological environment and human health for their lasting retention in the environment and bioaccumulation easy. As a result, such toxic compounds that are difficult to be degraded directly have been controlled by the EU legislation and recommended to be one of priority pollutants in the US EPA (Boon et al. 2001). In addition, being a kind of substance of environmental heterogeneous resource as, it is the common intermediate metabolite of a chlorinated aromatic nitro compounds and herbicides (phenylurea, aniline formic acid lipid and anilide) (Ren et al. 2005).

Being the richest owner of coastal reed wetlands in China, Yancheng has unique resource advantages for the research and development of coastal wetland ecosystem (Li 2007). "Reed bed" type constructed wetlands are good at removing COD, BOD, suspended solids (SS) and, heavy metals (Wang 2005). The aim of this work was to develop a method for isolating facultative aerobic *p*-Chloroaniline (*p*-CA) biodegrading microorganism from the reed rhizosphere soil in Yancheng wetlands, to probe into the morphological and physiological characteristics and information about the degradation characterization of the tested strain in terms of the ability of *p*-CA biodegradation.

Material and Methods

A wet soil was collected as the strain source in Match of 2008 using iron hoe from the upper 5–20 cm layer of a bulrush wetland at East Campus of Yancheng institute of technology, Yancheng, China, where no *p*-CA had been previously used. Soil samples (10 g) were placed onto 500 mL sterilized conical flasks and then washed by sterile water. The pH were adjusted to 7.4 with the addition of filter-sterilized 1 mol/L H_2SO_4 or NaOH on rotating shaker at around 150 r·min^{-1} for about 60 min. After 3–4 times repetition, all the washing water was collected into a 500 mL sterilized volumetric flask to get the cell suspension, stored at 4°C and evenly mixed for measurement before use.

Pure isolates were obtained by Plate Separation Methods under aseptic condition. The compositions of the enrichment

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liquid medium for the enrichment and domestication of the isolates from the cell suspension were as follows: 3.0 g/L glucose, 1.0 g/L peptone and 0.5 g/L yeast extract, pH value 7.0–7.2, sterilized for 25 min (Shen et al. 1999), added with *p*-CA concentration gradient of 20, 40, 80, 120, 160 and 200 mg/L in turn for every 4 d of domestication. The resulting isolates of bacteria at each concentration gradient were screened for their capability of reducing *p*-CA by degrading spots, carried out by agar block method (Shen et al. 1999), on the differential solid medium, the compositions of which were as follows: 0.12 g/L *p*-CA, 3.0 g/L beef extract, 10.0 g/L peptone, 5.0 g/L sodium chloride and 20.0 g/L agar, pH value 7.0–7.2, sterilized for 25 min. A few larger spots proved positive for *p*-CA degradation were picked out and transferred into a fresh higher concentration gradient enrichment medium. This procedure was repeated when the spot tests proved more positive. Finally, all the screened spots of the last test were transferred into a fresh differential liquid medium and cultured at 30°C on rotating bed with 150 r/min for 4 d to compare the capability of reducing *p*-CA by measuring the *p*-CA concentration. The strain with the highest degradation rate was then isolated and purified by plate streaking on fresh differential solid medium and stored at 4°Con inclined plane before use.

The stored and purified test strains were cultured at 32°C for 24 h on LB solid medium and in liquid medium at logarithmic phase in the bio-incubator for the further identification, respectively. The medium compositions were as follows: 5.0 g/L yeast extract, 10.0 g/L peptone, 5.0 g/L sodium chloride and 20.0 g/L agar (in solid medium), pH value 7.0–7.2, sterilized for 25 min (Shen et al. 1999).

Morphological observation included colony morphology and size on LB solid medium and cell morphology and size by SEM (Hitachi H7650) examination. Physiological-biochemical identifications referred to Manual of Bacteria Preservation (IMCAC 1980) and Berge's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974).

PCR amplification and sequencing of 16S rDNA: the logarithmic phase strains were transferred to an tube containing 100 μL aseptic ddH_2O. The mixture was centrifuged at 12000 r/min for 5 min after it was kept in boiling water for 7 min, the supernatant of which was directly used for amplification as template DNA. PCR amplification was done with primers 27F: 5c-AGAGTTTGATCCTGGCT CAG-3c and 1492r: 5c-GGTTACCTTGTTACGACTT-3c (Devereux and Willis 1995). PCR products were purified and sequenced at Shanghai Shenggong Bioengineering Technology Service Co., Ltd., China.

The tracking studies for the characterization of purified test strain cultured in minimal liquid medium, with the compositions as follows: 0.12 g/L *p*-CA, 4.35 g/L K_2HPO_4, 2.1 g/L NH_4Cl, 1.7 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.05 g/L $MnSO_4$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.03 g/L $CaCl_2 \cdot 2H_2O$, pH value 6.8–7.0, sterilized for 25 min (SEPAC 2002), included the initial concentration of *p*-CA, the degradation time, the inoculation amount, the degradation pH value, the degradation temperature and oxygen content as the influence factors on the *p*-CA degradation.

Cell concentration was determined by Visible Spectrophotometry (Shen et al. 1999). *p*-CA concentration was determined by Gas Chromatography (GC, Agilen 6890 N) with the dichloromethane as extractan (Thompson et al. 2007). The GC conditions was as follows: chromatographic column was A HP-INNOWax from Agilent (30 m × 0.32 mm × 0.25 μm) used with temperature programmed control. The temperature of the column was maintained at 70°C for 3 min, and then increased temperature at a rate of 30°C·min^{-1} to 180°C, maintain at 180°C for 5 min. The temperature of the injection port and gasification were both at 260°C and the temperature of the FID detector was 280°C; the flow rate of carrier gas N_2 was 45 mL/min, H_2 40 mL/min, air 450 mL/min, and the injection port diffluent ratio was 20:1 with the detection time 18 min.

All the experiments were conducted in triplicate. Excel 2007 and Origin 7.0 analysis software for windows were used for statistical tests.

Result and Discussion

After separation and enrichment, the bacteria with the highest degradation rate named strain ycsd02 was isolated from soil. Strain ycsd02 was identified as oil hydrolysis negative and gram-positive. Starch hydrolysis, gelatin hydrolysis, methylred test, V-P reaction and citric acid were all positive. The organism was aerobic with single cell of pole shape, 0.6–0.8 μm × 1.6–3.2 μm and single spore of oval, 0.7–0.9 μm × 1.0–1.2 μm, budding from the middle or subend of the strain, clearly belongs to *Bacillus licheniformis*. The single cell of strain ycsd02 with SEM was shown in Fig. 1.

The 16S rDNA gene sequence (GenBank Accession number is JF458450) of strain ycsd02 aligned with the sequences available from the GenBank by BLAST and all sequences were retrieved from GenBank individually and aligned using ClustalX 1.8 with default settings. Phylogenetic analysis was performed by MEGA version 2.1 (Kumar et al. 2001) software using Neighbor-joining method and selecting Kimura 2-parameter distance mode. The 16S rDNA sequences included in the phylogenetic analysis are shown in Fig. 2, in which a phylogenetic tree including most published representatives of validly described degrading organochlorine species and other correlative species was given. *Bacillus licheniformis* Constituted one big cluster on the phylogenetic tree. The 16S rDNA gene of strain ycsd02 showed high sequence

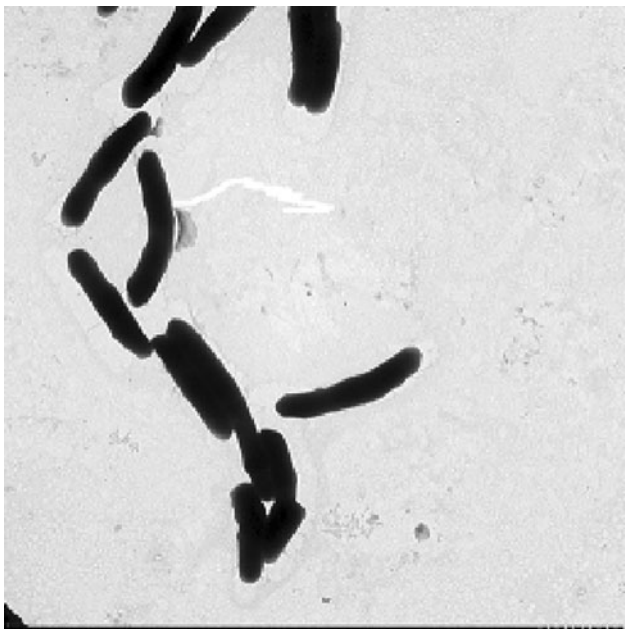

Fig. 1 SEM of strain ycsd02 (×1200)

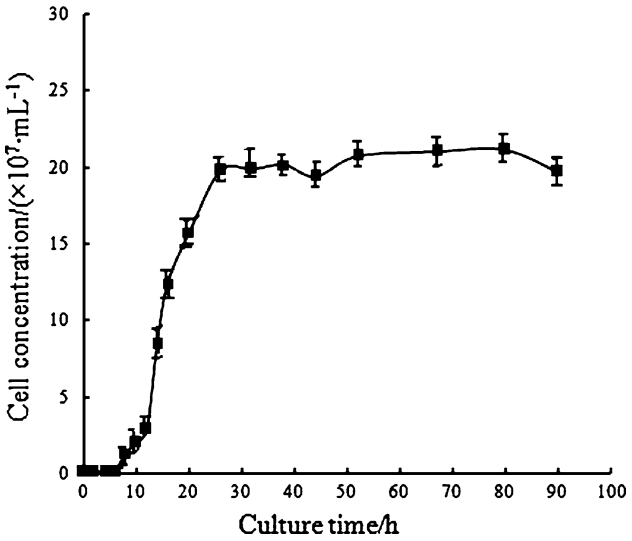

Fig. 3 Growth curve of strain ycsd02. The error bar at each data point represents the standard error (n = 3)

similarity (more than 99%) to the 16S rDNA genes of strains in *Bacillus licheniformis*.

The growth curve obtained during the growth of the strain ycsd02 in LB liquid medium was similar to "S' shape in Fig. 3. The lag phase was from 0 to 15 h; the log phase was from 15 to 35 h; the stationary phase was from 35 to 80 h; and the death phase begins from 80 h. From these we conclude that the inoculating time for identification and degradation was about 30 h.

Strain ycsd02 of 10% inoculation amount was cultured at 30°C in different minimal liquid mediums containing different *p*-CA concentrations as shown in Fig. 4a with the pH adjusted to 7.0 by the addition of filter-sterilized 1 mol/L H_2SO_4 or NaOH on rotating shaker at around 150 r/min for 4 d. The degradation rate changed regularly the same as the cell concentration and maintained 50–80% on the whole. The optimal initial *p*-CA concentration was about 120 mg/L.

Strain ycsd02 of 10% inoculation amount was cultured at 30°C in minimal liquid mediums containing *p*-CA concentration of 120 mg/L with the pH adjusted to 7.0 by the addition of filter-sterilized 1 mol/L H_2SO_4 or NaOH on rotating shaker at around 150 r min^{-1} for different degradation time as shown in Fig. 4b. Both the degradation

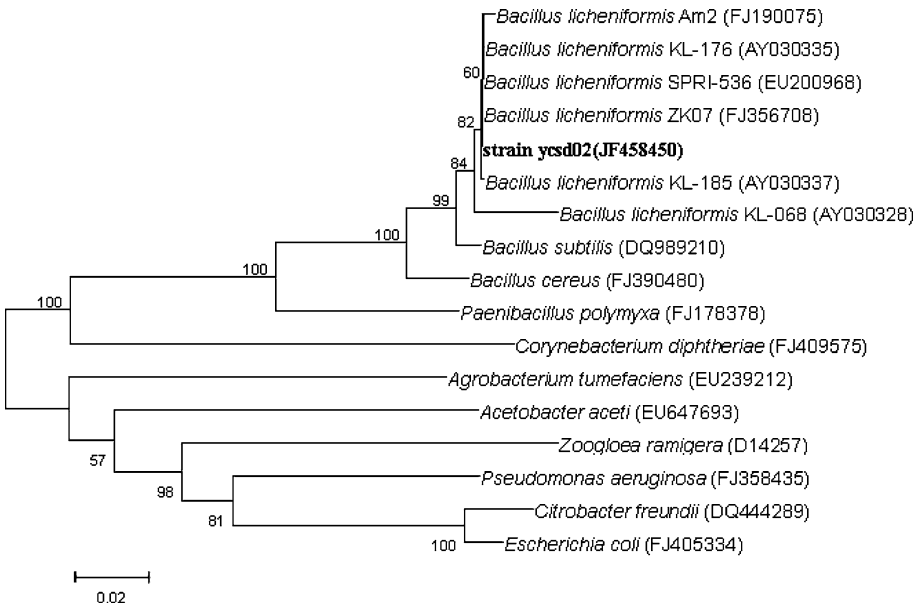

Fig. 2 The 16S rDNA phylogenetic tree of strain ycsd02

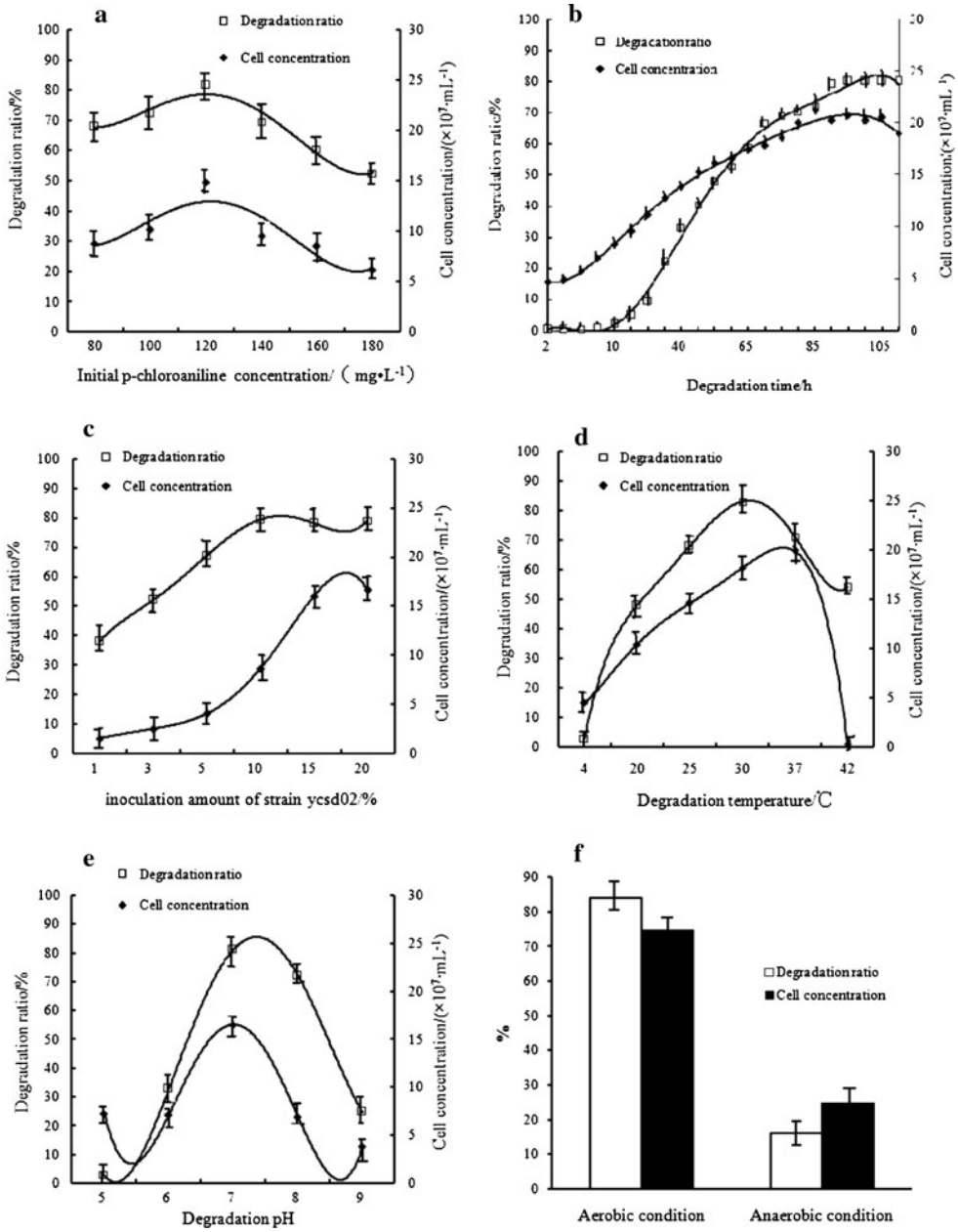

Fig. 4 Effects of different conditions on degradation characterization by Strain ycsd02. The *error bar* at each data point represents the standard error (n = 3). **a** initial *p*-chloroaniline concentrations; **b** degradation time; **c** inoculation amount; **d** degradation temperature; **e** degradation pH; **f** dissolved oxygen

rate and cell concentration were significantly increased in 15–90 h. The degradation rate kept on increasing but the cell concentration clearly decreased after 90 h. To achieve the ideal effect, the optimal degradation time was selected at about 4 d.

Strain ycsd02 of different inoculation amount as shown in Fig. 4c was cultured at 30°C in minimal liquid mediums containing *p*-CA concentration of 120 mg/L with the pH adjusted to 7.0 by the addition of filter-sterilized 1 mol/L H_2SO_4 or NaOH on rotating shaker at around 150 r/min for 4 h. Curves of the degradation rate and cell concentration were both similar to "S' shape. Both the degradation rate and cell concentration were rapidly increased of 3–15% inoculation amount, while slightly increased after 15%. So the optimal inoculation amount was selected at 15%.

Strain ycsd02 of 15% inoculation amount was cultured at different degradation temperature as shown in Fig. 4d in minimal liquid mediums containing *p*-CA concentration of 120 $mg \cdot L^{-1}$ with the pH adjusted to 7.0 by the addition of filter-sterilized 1 mol/L H_2SO_4 or NaOH on rotating shaker

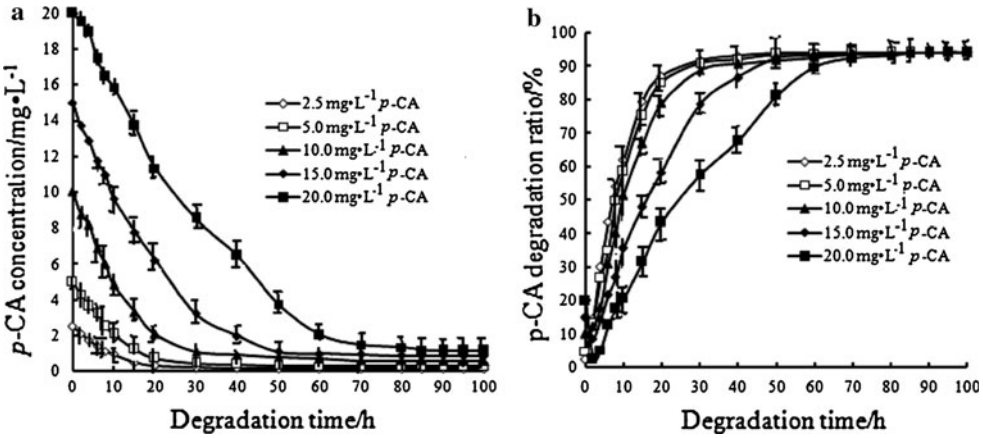

Fig. 5 Degradation dynamic curves of *p*-CA at different initial concentration by Strain ycsd02. The *error bar* at each data point represents the standard error (n = 3). **a** Residual concentration of *p*-CA at different initial concentration by Strain ycsd02; **b** Degradation ratio of *p*-CA at different initial concentration by Strain ycsd02

Table 1 The dynamic equation of different degradation concentration by Strain ycsd02

p-CA concentration (mg L^{-1})	Dynamic equation	R^{2*}
2.5	y = 1.1394e−0.0265x	0.7507
5	y = 2.517e−0.0275x	0.7865
10	y = 5.9606e−0.0288x	0.8503
15	y = 11.675e−0.0315x	0.9110
20	y = 20.756e−0.0324x	0.9782

* Significant at 0.05 probability level

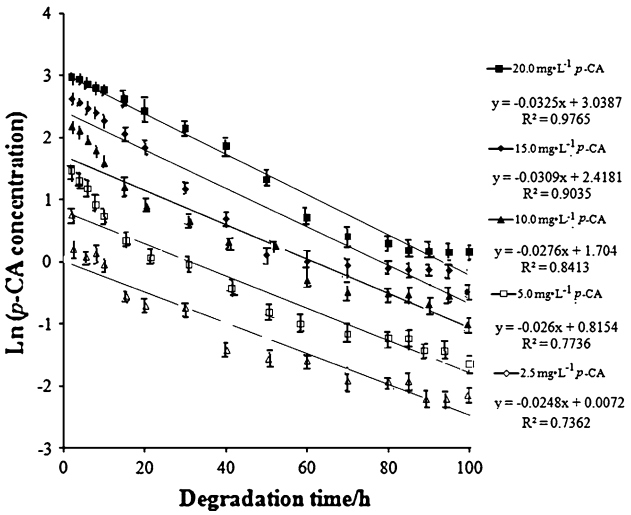

Fig. 6 Ln (*p*-CA concentration)-*t* curves of *p*-CA at different initial concentration by Strain ycsd02. The *error bar* at each data point represents the standard error (n = 3)

at around 150 r/min for 4 h. Curves of the degradation rate and cell concentration were both similar to inverse "V' shape. The optimal degradation temperature was about 30–32°C. Strain growth rapidly cut down when the temperature higher than 37°C or lower than 20°C.

Strain ycsd02 of 15% inoculation amount was cultured at 32°C in minimal liquid mediums containing *p*-CA concentration of 120 mg/L with different pH as shown in Fig. 4e adjusted by the addition of filter-sterilized 1 mol/L H_2SO_4 or NaOH on rotating shaker at ~150 r/min for 4 h. Both the degradation rate and cell concentration had the same "S' shapes. The optimal degradation pH was about pH 7.

Strain ycsd02 of 15% inoculation amount was cultured at 32°C in minimal liquid mediums containing *p*-CA concentration of 120 mg/L with pH 7 adjusted by the addition of filter-sterilized 1 mol/L H_2SO_4 or NaOH on rotating shaker at around 150 r/min (aerobic condition) for 4 h, while the control group (anaerobic condition) of liquid seal of atoleine was cultured under the same other conditions, as shown in Fig. 4f. It was concluded that strain ycsd02 was an aerobe because both the degradation rate and cell concentration were significantly higher under aerobic condition than those under anaerobic condition.

To study the biodegradation dynamic characterization, strain ycsd02 was cultured at 32°C and pH 7.0 with 15% inoculation for 100 h in different minimal liquid mediums containing *p*-CA concentrations of 2.5–20 mg/L as shown in Fig. 5. Degradation rate decreased immediately at the first, and then stabilized as a whole. Higher concentrations of *p*-CA led to higher stimulation and degradation period because the increasing of *p*-CA concentrations provided an ascending supply of the readily available substance for bacteria. The degradation ratio reached above 90% within 30 h when the *p*-CA concentrations was 2.5–10 mg/L while within 40–60 h when the *p*-CA concentrations was from 15 mg/L to 20 mg/L. The highest degradation ratio was about 94.2%. Result indicated that all the *p*-CA leftovers for 40 h' degradation met the GB3544-2008 (Discharge standard of water pollutants for pulp and paper industry) in China. Table 1 and Fig. 6 showed that when the *p*-CA concentration varied from 0 mg/L to 20 mg/L, the degradation of *p*-CA at different initial concentration

by Strain ycsd02 met the first-order kinetics equation as a whole.

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