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Technical Note

Mutant AFM 2 of *Alcaligenes faecalis* for phenol biodegradation using He–Ne laser irradiation

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Abstract

He–Ne laser technology was utilized in this study to investigate the response of *Alcaligenes faecalis* to laser stimulation. The irradiation experiments were conducted by the adjustment of the output power from 5 to 25 mW and the exposure time from 5 to 25 min. The results showed that the survival rate changed regularly with the variety of irradiation dose, and high positive mutation frequency was determined by both the energy density and the output power. The mutant strain AFM 2 was obtained. Phenol biodegradation assay demonstrated that AFM 2 possessed a more prominent phenol-degrading potential than its parent strain, which presumably attributed to the improvements of phenol hydroxylase and catechol 1,2-dioxygenase activities. The phenol of 2000 mg l^{-1} was completely degraded by AFM 2 within 85.5 h at 30 °C. In addition, the cell growth and phenol degradation kinetics of the mutant strain AFM 2 and its parent strain in batch cultures were also investigated at the wide initial phenol concentration ranging from 0 to 2000 mg l^{-1} by Haldane model. The results of these experiments further demonstrated that the mutant strain AFM 2 possessed a higher capacity to resist phenol.

Keywords: *Alcaligenes faecalis*; He–Ne laser irradiation; Kinetics; Mutagenesis; Phenol biodegradation

1. Introduction

Phenol at high concentration is widely distributed as an environmental pollutant due to its common presence in the effluents of many industries, including oil refineries, ceramic plants, steel plants and coal conversion (Bandhyopadhyay et al., 2001; Aleksieva et al., 2002; Kavitha and Palanivelu, 2004). Once wastewater containing phenol is discharged into the receiving body of water, it endangers fish life, even at a relatively low concentration e.g. 5–25 mg l^{-1} (Chung et al., 2003). For drinking water, a guideline concentration of 1 μg l^{-1} (WHO, 1994) has been prescribed. Therefore, the removal of phenol from industrial aqueous effluents is of great practical significance for environmental protection.

By contrast with physical and chemical methods, biological methods of phenol removal are preferable in wastewater treatment process because of their relatively low processing costs and a low possibility of a secondary pollution (Zumriye et al., 1999; Wang et al., 2000). Presently, many microorganisms for phenol biodegradation are isolated from activated sludge with the potential to utilize phenol as a sole carbon and energy source. Claußen and Schmidt (1998), González et al. (2001) and Annadurai et al. (2002) have isolated and characterized some bacteria from industrial effluents for mineralizing 100–1000 mg l^{-1} phenol. However, little has been known about phenol biodegradation at initial concentration above 1200 mg l^{-1} by free cells. That is mainly attributed to poor control and activity of the microorganisms involved in the biodegradation, which limited the application of biological technology in practice. To further promote phenol biodegradation potential, modification of microorganisms is very important and necessary. Chang et al. (1995) obtained the

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mutants of *Candida tropicalis* M4 through the protoplast fusion technique. It was found that two of the fusants exhibited higher rates of growth than that of the wild strain when the cells were grown on phenol medium, and they possessed about 1.9 and 2.2 times higher phenol hydroxylase activity than the wild strain. Aleksieva et al. (2004) treated the parent strain with *N*-methyl-*N*-nitro-*N*-nitrosognanidine for 1 h to the final concentration of 0.5 mg l^{-1} and nistatine at a concentration of 25 U ml^{-1} for 1 h to obtain the mutants of *Trichosporon cutaneum* R57. It should be pointed out that this mutant possessed higher specific growth and degradation rates than those of the wild strain because of the increase in the phenol hydroxylase activity. Recently, it has been more attractive for low-power laser irradiation technology to mutate the biological strains. Kohli et al. (2001) have reported that He–Ne laser (632.8 nm) irradiation can stimulate *Escherichia coli* strain KY706/Ppl-1, leading to the induction of *phr* gene expression. And the optimum irradiation parameters were also obtained. Karu et al. (1994) determined the mechanisms of He–Ne laser irradiation on *E. coli*, and the quantity of viable cells changed in the irradiated culture. However, no study was reported about the application of laser-induced technology to the environmental microorganisms such as phenol biodegradation.

Objectives of the present study are to acquire a positive mutant strain with the higher phenol-degrading potential by He–Ne laser to stimulate wild-type *Alcaligenes faecalis*, and to investigate the cell growth and phenol degradation kinetics of the wild and mutant strain using Haldane's equation (Hao et al., 2002).

2. Materials and methods

2.1. Microorganism and culture conditions

Wild-type *A. faecalis* was isolated in this lab from acclimated activated sludge collected from a municipal gasworks in China and identified based on physiological and biochemical tests and 16S rDNA by the Institute of Microbiology, Chinese Academy of Sciences.

The wild and mutant strains were all grown and maintained in LB medium (Byrne and Kropinski, 2005). A mineral medium supplemented with phenol was used for phenol biodegradation studies. The media other than phenol were autoclaved at 121 °C. Phenol was separately filter-sterilized through membranes (pore size of 0.2 μm) and added to other components before inoculation (Léonard et al., 1999).

2.2. Mutagenesis

Strain *A. faecalis* was inoculated into fresh LB medium. Cells in the end phase of the exponential stage ($OD_{600} = 1.2$) were centrifuged at 7500 rpm for 10 min, washed twice with 0.1 M sodium phosphate buffer (pH = 7.2) and then resuspended in a tube with 5 ml sterile saline water at a concentration of 1.0×10^9 cells ml^{-1}. Then a 100 μl cell suspension was transfused into the glass tubes (diameter 16 mm) with 2 ml sterile saline water. The suspension was irradiated by a He–Ne laser with 800-micron optical fiber using the optical maser wavelength of 632.8 nm in the dark (Kohli et al., 2001).

To investigate the effect of any possible rise in temperature on survival rate in laser irradiation, the temperature of the culture was measured by inserting a temperature probe into the culture before and after the laser irradiation. For example, at the maximum output power of 25 mW and the longest irradiation time of 25 min which corresponds to a fluence of 18.75 J cm^{-2}, the increase in temperature was no more than 0.4 °C. The minor change cannot lead to cell death, which was also confirmed by Kohli et al. (2001).

After mutagenesis, exposed cell suspension was diluted 10^4–10^7-fold, and was plated on LB medium. Then all the individual colonies with quantities of about 20 on each plate were inoculated on the slants. Activated in LB medium twice, these individuals were determined for the phenol-degrading potential. The control culture was also kept under the same conditions, but was not irradiated. After each inducing experiment, positive mutants were continuously transferred for seven generations from one nutrient agar slant to the next, and the strain of each generation was tested for phenol biodegradation under the same conditions. Survival rate and positive mutation frequency were respectively calculated as:

$$\text{Survival rate} = \frac{\text{Survival colonies}}{\text{Total colonies under the control culture}} \times 100\%$$

$$\text{Positive mutation frequency} = \frac{\text{Positive mutants}}{\text{Survival colonies}} \times 100\%$$

2.3. Determination of enzyme activities

Enzyme activities were spectrophotometrically determined in cell-free extracts at room temperature using quartz cuvettes of 1 cm path length. Cells were harvested by centrifugation. After being washed twice with 0.1 M phosphate buffer (pH 7.2) and resuspended in the same buffer, the cell pellet was disrupted by sonication for 5 min, and then the cell debris was removed by centrifugation at 15000 rpm for 20 min at 4 °C. The cleared supernatant was immediately used both for enzyme assays and total protein assays. Phenol hydroxylase (EC 1.14.13.7) activity was assayed spectrophotometrically, according to NADPH absorbance at 340 nm (Neujahr and Gaal, 1970). Quartz cuvettes contained 3 ml total volume: 100 μM Tris–sulphate pH 7.6, 0.5 μM NADPH, 0.02 mg protein, and 0.5 μM phenol were added to 0.1 ml distilled water to initiate the reaction. One unit of hydroxylase activity was defined as the amount of enzyme which caused the oxidation of 1 μmol NADPH per min in the presence of phenol. Catechol 1,2-dioxygenase