Mutation of *Candida tropicalis* by Irradiation with a He-Ne Laser To Increase Its Ability To Degrade Phenol[▽]

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Candida tropicalis isolated from acclimated activated sludge was used in this study. Cell suspensions with 5×10^7 cells ml⁻¹ were irradiated by using a He-Ne laser. After mutagenesis, the irradiated cell suspension was diluted and plated on yeast extract-peptone-dextrose (YEPD) medium. Plates with approximately 20 individual colonies were selected, and all individual colonies were harvested for phenol biodegradation. The phenol biodegradation stabilities for 70 phenol biodegradation-positive mutants, mutant strains CTM 1 to 70, ranked according to their original phenol biodegradation potentials, were tested continuously during transfers. Finally, mutant strain CTM 2, which degraded 2,600 mg liter⁻¹ phenol within 70.5 h, was obtained on the basis of its capacity and hereditary stability for phenol biodegradation. The phenol hydroxylase gene sequences were cloned in wild and mutant strains. The results showed that four amino acids were mutated by irradiation with a laser. In order to compare the activity of phenol hydroxylase in wild and mutant strains, their genes were expressed in *Escherichia coli* BL21(DE3) and enzyme activities were spectrophotometrically determined. It was clear that the activity of phenol hydroxylase was promoted after irradiation with a He-Ne laser. In addition, the cell growth and intrinsic phenol biodegradation kinetics of mutant strain CTM 2 in batch cultures were also described by Haldane's kinetic equation with a wide range of initial phenol concentrations from 0 to 2,600 mg liter⁻¹. The specific growth and degradation rates further demonstrated that the CTM 2 mutant strain possessed a higher capacity to resist phenol toxicity than wild *C. tropicalis* did.

Phenol is present in many industrial wastewaters, involving coke petroleum, pulp and paper, pharmaceutical, wood-processing chemicals, and paint solvents (18, 20, 23, 26). Wastewaters contaminated by phenol endanger fish life even at a relatively low concentration, e.g., 5 to 25 mg liter⁻¹ (2, 14). For drinking water, the phenol concentration should be no higher than 1 µg liter⁻¹ (31). Furthermore, the breakdown products of phenol may be more harmful when phenol is incompletely degraded through physical and chemical methods or natural oxidation. Therefore, the complete removal of phenol from industrial aqueous effluents is of great importance for environmental protection.

Compared with physical and chemical methods (28), biological methods of phenol removal are preferable in wastewater treatment processes due to their relatively low processing costs (5, 32). Besides, as phenol can be completely degraded to water and carbon dioxide, biodegradation resulted in very low possibility of secondary pollution (30). Presently, many microorganisms are isolated from activated sludge, and it has been demonstrated that they can utilize phenol as the sole carbon and energy source (4). Fialová et al. (9) reported that the yeast *Candida maltosa* can degrade phenol at concentrations up to 1,700 mg liter⁻¹. A wild strain was isolated from acclimated activated sludge in our lab with the potential to degrade 2,000 mg liter⁻¹ phenol within 66 h (12). To further promote phenol

biodegradation potential, improvement of microorganisms is of great importance. It was worth mentioning that Chang et al. (6) separately isolated auxotrophic mutants and phenol-degrading defective mutants in a phenol-utilizing strain of *Candida tropicalis* M4 which was pretreated by UV light irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). The mutants were then hybridized through protoplast fusion, and some fusants with phenol-degrading ability were obtained. It was reported that two of the fusants exhibited higher growth rates than the wild strain did when the cells were grown on phenol medium and that these two fusants possessed about 1.9- and 2.2-fold-higher phenol hydroxylase activity than the wild strain did. Alexievaa et al. (1) treated the parent strain cells with NTG for 1 h to a final concentration of 0.5 mg liter⁻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 enhancement in the activity of phenol hydroxylase, which is a key enzyme in the biodegradation of phenol to hydroxylate phenol to catechol (1, 17, 22).

Recently, the use of low-power laser irradiation technology to mutate the biological strains has been attracting attention. Kohli et al. (16) have reported that irradiation with a He-Ne laser (632.8 nm) could stimulate *Escherichia coli* strain KY706/pPL-1, which led to the induction of *phr* gene expression. The optimal irradiation parameters were also obtained. Karu et al. (13) determined the mechanisms of the effects of irradiation on *E. coli* with a He-Ne laser, and the quantity of viable cells changed in the irradiated culture. However, no effort has been made to apply this laser technology to environmental

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microorganisms such as those involved in phenol biodegradation. Furthermore, we do not know of any report expounding laser-induced mechanism using the gene sequence of a key enzyme.

The objectives of these studies are to acquire a positive mutant strain with high phenol-degrading potential using a He-Ne laser to stimulate wild-type *C. tropicalis*, to investigate the variety of phenol hydroxylase activities and the sequences of phenol hydroxylase genes, and to investigate the cell growth and intrinsic phenol degradation kinetics of the mutant strain with a focus on those factors not influenced by transport processes but only on those factors concerning the concentration of reactants, temperature, and the character of the solvent by Haldane's equation (3, 10, 12).

MATERIALS AND METHODS

Microorganism and culture conditions. Wild *C. tropicalis* was isolated from acclimated activated sludge taken from Tianjin Gasworks in China and identified by the Institute of Microbiology, Chinese Academy of Sciences. Both wild and mutant *C. tropicalis* grew on yeast extract-peptone-dextrose (YEPD) medium (peptone, 20 g liter⁻¹; yeast extract, 10 g liter⁻¹; glucose, 20 g liter⁻¹; agar, 18 g liter⁻¹), and the initial pH value was adjusted to 6.0 (25). A mineral medium was used for phenol biodegradation experiments with the following ingredients: 0.4 g liter⁻¹ K₂HPO₄, 0.2 g liter⁻¹ KH₂PO₄, 0.1 g liter⁻¹ NaCl, 0.1 g liter⁻¹ MgSO₄, 0.01 g liter⁻¹ MnSO₄ · H₂O, 0.01 g liter⁻¹ Fe₂(SO₄)₃ · H₂O, 0.01 g liter⁻¹ Na₂MoO₄ · 2H₂O, and 0.4 g liter⁻¹(NH₄)₂ SO₄, pH 6.0. In addition, *E. coli* DH5α and *E. coli* BL21(DE3) purchased from Novagen Company were maintained and grew in Luria-Bertani (LB) medium (tryptone, 10 g liter⁻¹; NaCl, 7.5 g liter⁻¹; yeast extract, 5 g liter⁻¹; agar, 18 g liter⁻¹) supplemented with ampicillin at 80 μg ml⁻¹ when necessary.

Mutagenesis. The wild strain of *C. tropicalis* was inoculated into fresh YEPD medium. Cells at the end of the exponential phase (optical density at 600 nm [OD₆₀₀] of 1.3) were centrifuged at 7,500 rpm for 10 min, washed twice with 0.1 M sodium phosphate buffer (pH 6.0), and resuspended in a tube with 5 ml sterile saline water of 0.9% (wt/vol) at a concentration of 1.0 × 10⁹ cells ml⁻¹ (21). Then a 100-μl cell suspension was transfused into the glass tubes (16 by 160 mm) containing 2 ml sterile saline water. The obtained suspension was irradiated using a He-Ne laser with an 800-μm optical fiber using an optical laser wavelength of 632.8 nm in the dark (14, 29). The optical fiber was fixed with a delivery arm that permitted precise positioning of the fiber tip 80 mm above the cell suspension.

After mutagenesis, the irradiated cell suspension was diluted 10⁴- to 10⁷-fold, and 100-μl samples of each of the diluted suspensions were plated on YEPD medium. After about 36 h of cultivation at 30 °C, all the individual colonies on each plate with approximately 20 individual colonies were inoculated on slants. The colonies from the slants were tested for the cell capacity to degrade phenol in a mineral medium with 1,000 mg liter⁻¹ phenol, and the wild strain was used as the control culture under the same conditions except this culture was not irradiated. Finally, the mutants that completely degraded 1,000 mg liter⁻¹ phenol within 34.5 h were considered positive mutants because their biodegradation capacities were higher than that of the wild strain. The survival rate was calculated as follows: (number of colonies that survived irradiation/total number of colonies in the control culture) × 100%. The positive mutation frequency was calculated as follows: (number of positive mutants/number of colonies that survived irradiation) × 100%.

Enzyme assay. Enzyme activities were spectrophotometrically determined in the cell extracts at room temperature using quartz cuvettes with a 1-cm path length.

After the cells (OD₆₀₀ of 1.3) were harvested and washed twice with 0.1 M phosphate sodium buffer (pH 6.0), they were resuspended (pH 6.8). The cell pellets were disrupted by sonication (four cycles of 30 s each with 1-min periods of cooling on ice between the cycles), and then the cell debris was removed by centrifugation of the homogenized cell suspension at 15,000 rpm for 20 min at 4°C. The cleared supernatant was immediately used for the assays of both enzyme and total protein. The total protein concentration in the cell extracts was monitored by the Lowry method (1, 19). The phenol hydroxylase (EC 1.14.13.7) activity in *C. tropicalis* strictly depends on the presence of NADPH (Sigma Corporation). Its activity was assayed spectrophotometrically according to NADPH absorbance at 340 nm (21). Quartz cuvettes had a total volume of 3 ml: 100 μM Tris-sulfate, 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

activity was defined as the amount of enzyme that caused the oxidation of 1 μM NADPH per min in the presence of phenol. Enzyme activity was expressed as units per gram of cells.

Cloning of phenol hydroxylase gene. *E. coli* DH5α was used in gene cloning. Wild *C. tropicalis* and its mutant strain were cultured and then harvested. Techniques for DNA manipulation, including plasmid preparations, chromosomal DNA isolation, restriction endonuclease digestions, DNA recovery, ligation, and transformation into *E. coli*, were performed according to established techniques and the standard protocols provided by manufacturers (11, 15, 24).

The phenol hydroxylase genes of wild and mutant strains were amplified by a PCR method. Oligonucleotide upstream primer 5'-ATGACAGTTAAACATTC TGAAAC-3' and downstream primer 5'-TTATACGGCTAATCTTGTTG-3' were designed according to the homology of the phenol hydroxylase genes of *Candida albicans* (GenBank accession numbers AACQ01000108, AACQ01000109, AACQ01000216, and AACQ01000217). The purified PCR products were cloned in pGEM-T Easy vector (Promega Corporation, Madison, WI) according to the manufacturer's instructions. After transformation into *E. coli*, the positive transformants were screened by ampicillin and α-complement. The recombinant plasmid (named pGMPH) was extracted by alkaline lysis and determined by both PCR and restriction enzyme digestion. The reaction system (10 μl) contained 1 μl of 10× reaction buffer (90 mM Tris, 10 mM MgCl₂, 50 mM NaCl, pH 7.5), 1 μl EcoRI, and 8 μl combined plasmid. The PCR profile was as follows: initial denaturation at 95°C for 1 min; 30 cycles, with 1 cycle consisting of 94°C for 45 s, 53°C for 45 s, and 72°C for 2 min; and a final extension at 72 °C for 15 min. Sequence analysis was performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. All biological reagents were purchased from Dalin TaKaRa Corporation, Promega, and Merck & Co., Inc.

Heterogenous expression of phenol hydroxylase. Phenol hydroxylase genes were amplified by PCR methods. PCR products and plasmid pET-28c (+) (Novagen, Madison, WI) were digested by restriction endonuclease BamHI and XhoI. After DNA recovery, ligation, and transformation into *E. coli* BL21(DE3), the BCT and BCTM strains containing recombinant plasmid pET-28-PH and pET-28-PHM were obtained, which were used to determine phenol hydroxylase activity. The BCT and BCTM strains were inoculated into 5 ml LB medium containing kanamycin at 25 μg ml⁻¹. After cultivation for 12 h at 37°C and 150 rpm, 200 μl culture was transferred into the shaking flasks containing LB medium with 20 ml kanamycin. When the OD₆₀₀ reached 0.6 to 0.8 (after approximately 2.5 h), 200 μl of 100 mM IPTG was added to the shaking flasks and left for 3 h. As a control, a culture was grown under the same conditions except that no IPTG was added. Finally, all the samples were used to determine enzyme activity by the method mentioned above.

Phenol biodegradation. The phenol biodegradation experiments were initiated by inoculating 10 ml YEPD medium with the mutant strain from nutrient agar slants. After 16 h of incubation at 30°C, 2 ml of this cell culture was added to 100 ml fresh YEPD medium. Cells (OD₆₀₀ of 1.3) were harvested and used as inoculum. A 5% subculture was inoculated into the mineral medium with various initial phenol concentrations ranging from 0 to 100 mg liter⁻¹ at 20-mg liter⁻¹ intervals and initial phenol concentrations ranging from 200 to 2,600 mg liter⁻¹ at 200-mg liter⁻¹ intervals. In the process of batch culture, samples were periodically taken for the measurements of biomass and phenol concentrations.

On the basis of the biodegradation experiments above, the intrinsic kinetics of the cell growth and phenol biodegradation were investigated. The growth process of mutant strain CTM 2 obtained from *C. tropicalis* was described by the Haldane's equation because of the inhibition of high phenol concentration on cell growth:

$$\mu_x = \frac{\mu_{x,\max} C_s}{K_s + C_s + \frac{C_s^2}{K_i}} \quad (1)$$

where C_s was the phenol concentration (mg liter⁻¹), $\mu_{x,\max}$ was the maximum specific growth rate (h⁻¹), K_s was the saturation coefficient for cell growth (mg liter⁻¹), and K_i was the inhibition coefficient for cell growth (mg liter⁻¹).

At each initial phenol concentration, the residual phenol concentrations and the cell concentrations were monitored during the experiments, and the specific growth rate of mutant strain CTM 2 at the exponential phase was calculated as follows:

$$\mu_x = \frac{\gamma_x}{C_x} = \left(\frac{dC_x}{dt} \right) \left(\frac{1}{C_x} \right) \quad (2)$$

where μ_x was the specific growth rate (h⁻¹), γ_x was the cell growth rate (h⁻¹), and C_x was the cell concentration (mg liter⁻¹).

Analyzing the utilization of the substrate in cells in more detail, the consumption of substrate for growth and maintenance and also for product formation if

FIG. 1. Mutagenic results of exposing a strain of *C. tropicalis* to irradiation with a He-Ne laser. In the irradiation experiments, an output power of 7.5 to 15 mW at 2.5-mW intervals and an irradiation time of 7.5 to 17.5 min at 2.5-min intervals were used.

possible, had to be considered (8). The substrate consumption rate of phenol biodegradation was calculated as follows (12):

$$\mu_s = A\mu_x + B \quad (3)$$

where A is the growth-associated constant for phenol consumption ($\text{mg phenol g}^{-1} \text{ cell}$) and B is the non-growth-associated constant for phenol consumption ($\text{mg phenol g}^{-1} \text{ cell h}^{-1}$). Both A and B are kinetic constants, and linear regression of these kinetic constants was performed on the basis of the calculated specific cell growth and phenol biodegradation rates upon the experimental data:

$$\mu_s = \frac{\gamma_s}{C_x} = \left(\frac{dC_s}{dt} \right) \left(\frac{1}{C_x} \right) \quad (4)$$

Phenol analysis procedures. To measure the concentration of residual phenol, samples of the suspended culture were centrifuged at 7,500 rpm for 10 min. The cell-free supernatants were used to determine the phenol concentration by high performance liquid chromatography using a Lab Alliance (model series III) system with a C_{18} column ($250 \times 4.6 \text{ mm}$; Lab Alliance). Elution was performed with 400/300 (vol/vol) methanol/water at a flow rate of 1.0 ml min^{-1} , and detection was realized with a UV detector (model 500; Lab Alliance) at 280 nm.

Statistics. All experiments were repeated three to five times. The data shown in the figures were the mean values of the experimental results, and the error bars indicate the standard deviations.

RESULTS AND DISCUSSION

Irradiation studies. To investigate the effect of any possible rise in temperature on the survival rate of cells irradiated with a laser, the temperature of the culture was measured by inserting a temperature probe into the culture before and after irradiation with a laser. There was a small variation in temperature during irradiation. For example, at the maximum output power of 25 mW and the longest irradiation time of 25 min, the increase in temperature was no more than 0.4°C . This minor change could not lead to cell death, which was also confirmed by Kohli et al.

(16). Thus, irradiation was conducted with different dose levels which were controlled by adjustment of the output power and irradiation time at room temperature (13).

The irradiation experiments were conducted with a wide range of doses of irradiation ranging from 0.75 to 18.75 J cm^{-2} . The irradiation experiments were performed by controlling the output power of the laser and exposure time. The output power ranged from 5 to 25 mW at 5-mW intervals, and irradiation exposure time ranged from 5 to 25 min at 5-min intervals.

The expected results were obtained from the irradiation experiments. The survival rate exhibited a great regularity. Once either one of the two parameters employed in mutagenesis was fixed, the cell survival rate decreased with increases in the other parameter. The maximum survival rate occurred at 5-mW output power. A very high survival rate clearly indicated that irradiation with low output power had little effect on *C. tropicalis*. No positive mutant was obtained, even with a long exposure time. The similar phenomenon occurred at 20 and 25 mW, where very low positive mutation frequencies were obtained. Although irradiation with high doses resulted in some cell death, a low survival rate did not ensure the ideal positive mutation frequency under such conditions. As far as the positive mutation frequencies were concerned, both low ($\leq 5\text{-mW}$) and high ($\geq 20\text{-mW}$) output powers were unfavorable to the mutagenesis of *C. tropicalis*. Satisfactory results were acquired at 10 mW for 10 and 15 min and 15 mW for 10 min, respectively, and the corresponding fluence varied from 3 to 4.5 J cm^{-2} .

On the basis of the investigations above, the following irradiation experiments were further performed under these conditions: the output power was adjusted from 7.5 to 15 mW at 2.5-mW intervals, and irradiation time was varied from 7.5 to

FIG. 2. Cell growth and phenol degradation of mutant strain CTM 2 and its parent strain in a mineral medium with 2,000 mg of phenol liter⁻¹ with a 5% starting inoculum.

17.5 min at 2.5-min intervals. The experiments were repeated five times.

The results of further mutagenesis can be seen in Fig. 1. It was noted that a positive mutation frequency of about 10% was obtained at 7.5 mW for 12.5 to 17.5 min. At 12.5 mW, a high positive mutation frequency was observed. The mutagenic results obtained under the original two irradiation parameters reoccurred. In general, high positive mutation frequencies were achieved under the following conditions: 10 mW and 12.5 mW for 10 to 15 min and 15 mW for 10 min, where the positive mutation frequencies could reach up to 20%. In contrast to the maximum positive mutation frequencies, the survival rate curves always varied regularly in these experiments: with an increase in the output power and irradiation time, the survival rate decreased.

Finally, a total of 70 positive mutants were tested in the five repeated irradiation experiments. They were named CTM 1 to 70 according to their original phenol biodegradation potentials. These positive mutants were continuously transferred through seven generations from one nutrient agar slant to the next, and the strains of each generation were tested for phenol biodegradation in the same culture. The results showed that the CTM 1 mutant strain had the highest original phenol degradation velocity among the tested mutants, yet it began to decline from the fourth generation culture. Thus, the CTM 1 mutant strain was discarded for its poor phenol biodegradation stability. Mutant strain CTM 2 not only had a high original phenol biodegradation rate but also was stable in successive transfers. Thus, the CTM 2 mutant strain was stored and used in the following studies.

Laser-induced mechanism. The present studies demonstrated that mutant strain CTM 2 was an efficient phenol-degrading microorganism. Hence, the improvement in phenol hydroxylase activity was of great importance, since it led to an increase of the phenol-degrading potential of mutant strain

CTM 2. Enzyme assay demonstrated that phenol hydroxylase activity increased from 46.49 to 62.96 U g⁻¹ cells. The experimental phenomenon might be attributed to the following three reasons. (i) During the course of irradiation with a laser, the enzyme protein in the wild strain was not mutagenized, but the promoter was mutated, which could promote the genetic transcription. (ii) Different levels of protein expression in the wild and mutant strains might lead to different activity of phenol hydroxylase. (iii) The mutation of the phenol hydroxylase gene resulted in the change in protein structure, which led to the different activity of phenol hydroxylase. Thus, the increase of enzyme activity needed further analysis. The heterogenous expression experiment could precisely account for the increase in enzyme activity.

The PCR method was used to clone the phenol hydroxylase gene, which eventually resulted in the successful cloning of hydroxylase gene sequences of the wild and mutant strains. The genes of both the wild and mutant strains were first submitted to GenBank (GenBank accession numbers DQ241990 and EF053058). The gene sequence analysis indicated that the phenol hydroxylase genes of *C. tropicalis* isolated in this lab showed 84.28% identity to that of *C. albicans* (accession number AACQ01000217). Through the comparison of the phenol hydroxylase amino acid sequences of wild *C. tropicalis* and mutant strain CTM 2, changes were found in four amino acids (172D→A, 233L→F, 268N→F, and 296V→D), which might lead to the increase in phenol hydroxylase activity.

In order to further understand the increase in biodegradation potential, the phenol hydroxylase genes obtained were used to conduct the heterogenous expression experiment in *E. coli* BL21(DE3). Experimental results clearly showed that phenol hydroxylase without IPTG induction were not observed either in the wild or the mutant strains, while in the IPTG-induced cells, phenol hydroxylase activity increased from 30.11 to 39.06 U g⁻¹ cells after mutagenesis.

FIG. 3. Cell growth and phenol degradation of mutant strain CTM 2 in medium containing phenol at initial concentrations ranging from 2,200 to 2,600 mg liter⁻¹ at 200-mg liter⁻¹ intervals with a 5% starting inoculum.

Phenol biodegradation. Figure 2 shows the cell concentrations and specific degradation rates of the parent strain and mutant strain CTM 2 at the initial phenol concentration of 2,000 mg liter⁻¹. It was very impressive that the CTM 2 mutant strain entirely degraded 2,000 mg of phenol liter⁻¹ within 54.5 h. This was 11.5 h less than its parent strain. Higher specific degradation rates indicated that the CTM 2 mutant strain possessed a very strong capacity to degrade phenol. It can still be seen from the semilog diagram of cell growth that the higher activity of mutant strain CTM 2 efficiently overcame the intense substrate inhibition, and the specific growth rate of the CTM 2 mutant strain was higher than that of its parent strain. It could be attributed to the fact that the CTM 2 mutant strain grew into an exponential growth phase more quickly to consume phenol, and more phenol was utilized to synthesize new cells (27).

Figure 3 illustrated the phenol biodegradation potential of the CTM 2 mutant strain at different high phenol concentrations. It could be noted that all 2,600 mg of phenol liter⁻¹ was utilized by CTM 2 within 70.5 h. With the step increase of initial phenol concentration, the specific growth and degradation rates declined, and longer time was consumed to completely degrade phenol in the medium. However, the biomass slightly increased.

Intrinsic kinetics. Haldane's equation was used to describe kinetic behavior of cell growth. The values of the unknown kinetic parameters in equation 1 were acquired by a nonlinear least-square regression analysis using the MATLAB software: $\mu_{X,\max} = 0.54 \text{ h}^{-1}$, $K_S = 6.7 \text{ mg liter}^{-1}$, and $K_I = 234 \text{ mg liter}^{-1}$.

After mutagenesis, K_S decreased by 42.7%, while $\mu_{X,\max}$ and K_I increased by 12.5% compared to the values for the wild strain (12), which was attributed to the increase of key enzyme

activity during phenol metabolism and the enhancement of the resistance to substrate inhibition, leading to the increase in the biodegradation capacity of the CTM 2 mutant strain. The values of the root mean square of the residuals at these parameters were small (0.035), indicating that the regression parameters agreed well with the experimental data.

Phenol biodegradation kinetics were described using equation 3. The parameters $A = 878 \text{ mg phenol g}^{-1} \text{ cell}$ and $B = 298 \text{ mg phenol g}^{-1} \text{ cell h}^{-1}$ were obtained through linear regression. The correlation coefficient, R^2 , was found to be 0.990. It showed that the model regression agreed well with the experimental data. The values of kinetic parameters of mutant strain CTM 2 were bigger than those of its parent strain (12), which indicated that mutant strain CTM2 possessed a higher potential to degrade phenol.

The He-Ne laser-induced technique was successfully applied in an environmental microorganism. A mutant strain, CTM 2, that had a higher phenol-degrading capacity than its parent strain was obtained. The gene cloning and heterogenous expression experiments indicated that four amino acids were mutated, and the activity of phenol hydroxylase increased after irradiation with a laser, which led to the higher capacity for phenol biodegradation.

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