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Degradation of Aniline by *Delftia tsuruhatensis* 14S in Batch and Continuous Processes

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Abstract—A *Delftia tsuruhatensis* strain capable of consuming aniline as the sole source of carbon, nitrogen, and energy at concentrations of up to 3200 mg/l was isolated from activated sludge of the sewage disposal plants of OAO Volzhskii Orgsintez. The strain grew on catechol and *p*-hydroxybenzoic acid but did not consume phenol, 2-aminophenol, 3-chloroaniline, 4-chloroaniline, 2,3-dichloroaniline, 2,4-dichloroaniline, 3,4-dichloroaniline, 2-nitroaniline, 2-chlorophenol, or aminobenzoate. Aniline is degraded by cleavage of the catechol aromatic ring at the *ortho* position. Cells were immobilized on polycaproamide fiber. It was shown that the strain degraded aniline at 1000 mg/l in a continuous process over a long period of time.

Aniline and its derivatives are common environmental pollutants formed by incomplete degradation of some pesticides (linuron and propanid) in soil. Another source of aniline and its derivatives is the waste of oil, pulp, coal, and chemical industries, which is weakly susceptible to biodegradation.

The importance of the problem of detoxification of aniline-polluted sewage is determined by the fact that aniline concentration in industrial sewage is, as a rule, broadly variable, often exceeding the permissible limit. This hampers normal operation of biological purification works. A promising way to overcome this difficulty is to develop methods of local biological purification of aniline-containing sewage.

At present, a number of strains capable of degrading aniline under laboratory conditions are known [1–7]. However, few studies have been dedicated to their industrial application [8, 9].

In the present work, we sought to identify and isolate a strain that would be capable of intense aniline degradation and resistant to its high concentrations in continuous processes, with a view for studying its potential in a continuous-flow bioreactor.

MATERIALS AND METHODS

Enrichment cultures, isolation of the active strain, and cultivation conditions. Samples of activated sludge, taken from biological purification works of Volzhskii Orgsintez enterprise (20 ml), were inoculated into 250 ml of mineral medium containing (g/l) Na_2HPO_4 (0.73), KH_2PO_4 (0.35), $MgSO_4 \cdot 7H_2O$ (0.10), NH_4NO_3 (0.75), $NaHCO_3$ (0.25), $MnSO_4$ (0.002), and $FeSO_4$(0.02), supplemented with aniline (0.06). The cultures were shaken in Erlenmeyer flasks at 29°C and 180 rpm and replated at 14-day intervals. During cultivation of the enrichment cultures, they were inoculated into petri dishes with beef-extract agar (BEA). Individual colonies were tested for the ability to consume aniline by cultivation under the same conditions in a liquid mineral medium with 60 mg/l aniline. Growth of the strain on various aromatic substrates was tested in the medium of the same mineral composition, supplemented with 50 mg/l substrate.

Strain identification. The taxonomic position of the strain was determined by its morphological, physiological, and biochemical traits and the 16S rRNA gene sequence. The cells sampled during the log phase were examined by phase-contrast microscopy. Gram staining was performed as described in [10]. Physiological and biochemical tests were performed according to [11]. DNA was isolated by the method described in [12]. The genes of 16S rRNA were amplified using the primers 27f and 1525r on a GeneAmp 2700 PCR System (Applied Biosystems, USA). The amplicons were sequenced on a CEQ2000XL automated DNA sequencer (Beckman–Coulter, USA). Nucleotide sequences were compared using BLASTn software (http://www.ncbi.nlm.nih.gov/BLAST). Alignment of sequences and construction of a 16S rRNA similarity matrix were performed with ClustalX software.

Methods of analysis. For the assay of aniline and metabolites, the culture liquid was acidified with 1M H_2SO_4 to pH 2.0 and extracted with ethyl acetate. The extracts were evaporated, and dry matter was dissolved in methanol. Qualitative analysis of the extracts was performed by TLC on Kieselgel 60 F245 plates (Merck, Germany) in the system benzene–dioxane–acetic acid (90 : 10 : 2 v/v/v). The spots were visualized under UV

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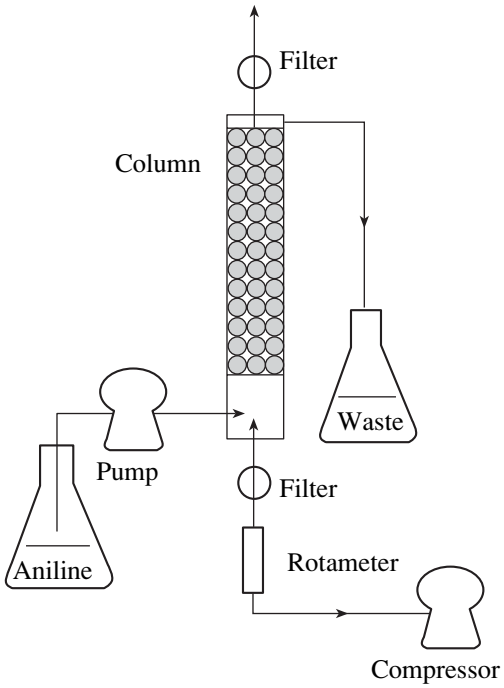

Fig. 1. Pilot biofilter for detoxification of aniline-containing sewage.

illumination. Quantitative assay of aniline was performed by GLC on a Pye Unicam 104 chromatograph (Philips, England) equipped with a flame ionization detector. Conditions: 1.5 mm × 1 m column with 3% SE-30 Chromosorb G-AW-DMSC; injector temperature, 125°C; column temperature, 80°C; and detector temperature, 290°C. Mass spectrometry was performed using an ITD-700 chromatograph–mass spectrometer (Finnigan, England): ionization energy, 70 eV; multiplier voltage, 1750 V; ionization chamber temperature, 220°C.

Enzyme activity assay. To prepare a cell-free extract, the cells were harvested by centrifugation at 10000 *g* for 10 min, washed in 50 mM Tris–HCl, pH 7.2, and disrupted with an IBFM press at an operation pressure of 3200 kg/cm^2. After addition of 5 mg/l DNase, the material was centrifuged at 18000 *g* for 30 min. The activities of the enzymes were determined in the supernatant: catechol 1,2-dioxygenase (EC 1.13.11.1) [13], catechol 2,3-dioxygenase (EC 1.13.11.1) [14], and muconate cycloisomerase (EC 5.5.1.1) [15]. Total protein was assayed by the Bradford method, with modifications [16].

Experimental continuous process. Cells were immobilized on polycaproamide fiber by a method based on cell adhesion [17]. The fiber was boiled, washed in distilled water, and sterilized at 1 atm for 30 min. The sterile fiber (18 g) was placed into a flask with 300 mg/l cell suspension. The suspension was obtained by cultivation of the cells in a liquid mineral medium with a starting aniline concentration of 500 mg/l for two days, until the optical density of the culture (D_{545}) was no lower than 0.5. Immobilization was performed by agitating the flask for 48 h. The fiber was placed into a pilot continuous biofilter, schematically depicted in Fig. 1. The volume of the biofilter column was 500 ml. Model sewage (1000 mg/l aniline in mineral medium without NH_4NO_3) was continuously supplied with a peristaltic pump (LKB, Sweden). Aeration was performed through a glass filter at the bottom of the column using a Resun microcompressor (China) at 50–80 cm^3/min. The air was sterilized by passing it through a cotton filter.

RESULTS AND DISCUSSION

A strain capable of complete consumption of aniline was isolated from enrichment cultures. The strain consisted of motile Gram-negative rods of the size 0.5–1.0 × 1.0–4.0 μm. When grown on BEA, it formed small white turbid mucous colonies. Flat gray colonies formed on mineral agar medium with 300 mg/l aniline as the source of carbon, nitrogen, and energy. According to biochemical, morphological, and physiological evidence, the strain was closest to the genus *Comamonas*. Comparison of the nucleotide sequence of a 618 bp fragment of 16S rRNA gene showed a close similarity to the corresponding fragments of *Delftia* spp (this bacterial genus was recently separated into a different taxon from Nomamonadaceae) (table). Thus, the cumulative morphological, physiological, and genetic data indicate that the strain is most similar to the species *Delftia tsuruhatensis*. We designated it *Delftia tsuruhatensis* 14S.

The strain consumed aniline, *p*-hydroxybenzoate, and catechol, but not phenol, 2-aminophenol, 3-chloroaniline, 4-chloroaniline, 2,3-dichloroaniline, 2,4-dichloroaniline, 3,4-dichloroaniline, 2-nitroaniline, 2-chlorophenol, 2,4-dichlorophenoxyacetate, benzoate, terephthalate, *o*-phthalate, *iso*-phthalate, or aminobenzoate.

Analysis of the growth of the strain (in the medium containing aniline as the sole source of carbon, nitrogen, and energy) and the dynamics of the content of aniline in the medium showed that the strain consumed aniline at high initial concentrations. Degradation of aniline present in the medium at concentrations below 1000 mg/ml started immediately after inoculation. The growth of the strain was observed after a short (~24 h) lag phase. Higher initial concentrations of aniline (up to 3200 mg/l) prolonged the lag phase and the period preceding the start of aniline degradation to 7 days. Nevertheless, even at that high concentration, the substrate was degraded completely. In all experiments, the fastest aniline degradation matched the maximum growth rate of the culture.

No products of aniline degradation were found in the culture liquid after cultivation at initial aniline concentrations of up to 1400 mg/l. After 3 days of cultiva-

Similarity (%) of the nucleotide sequences of the 16S rRNA genes of *Delftia* sp to closely related strains of the genus *Delftia*

Strain	*D. tsuruhatensis* 14S	*D. sp.* AN3	*D. tsuruhatensis* IFO 16741T	*D. acidovorans* WDL 34	*D. acidovorans* IAM 12409T
D. tsuruhatensis 14S					
D. sp. AN3	100.0				
D. tsuruhatensis IFO 16741T	100.0	100.0			
D. acidovorans WDL34	99.2	99.2	99.2		
D. acidovorans IAM 12409T	98.1	98.1	98.1	98.9	
D. acidovorans ACM 489	98.1	98.1	98.1	98.9	97.7

tion at initial aniline concentrations in excess of 1400 mg/l, compounds with R_f 0.21 and 0.28 were detected in the culture liquid at insignificant quantities, which did not disappear until the end of cultivation (17 days). According to mass spectrometry, these compounds had M^+ values of 135 and 121, and their fragmentation patterns corresponded to acetanilide and formanilide. These compounds are known as products of the detoxification process widely occurring among microorganisms [18]. At aniline concentrations above 1400 mg/l, biomass growth was nonlinearly dependent on aniline consumption. As a rule, this phenomenon is observed with substrates that either fail to support growth or are toxic [19]. Thus, high aniline concentrations exerted weak toxic effects on *D. tsuruhatensis* 14S.

The growth rate (μ) of the strain, determined at the initial concentration of 1000 mg/l, was 0.2 h^{-1}, and the yield coefficient (Y) was equal to 36%.

Cells of *D. tsuruhatensis* 14S can grow on catechol. The cell-free extract of the strain grown on aniline had high catechol 1,2-dioxygenase (0.21 U/ml) and muconate cycloisomerase (0.176 U/mg) activities. The latter enzyme converts the product of *ortho* cleavage of the aromatic ring. No catechol 2,3-dioxygenase or protocatechuate 3,4-dioxygenase activities were detected. Thus, it is reasonable to suggest that the pathway of aniline degradation involves hydroxylation of the aromatic ring at the *ortho* position followed by deamination to form catechol, whose aromatic ring is cleaved by catechol 1,2-dioxygenase at the *ortho* position.

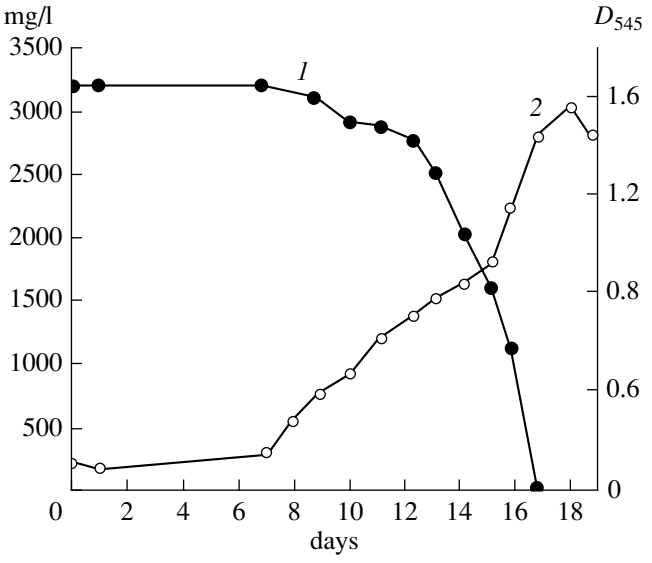

Fig. 2. Aniline degradation by *D. tsuruhatensis* 14S: (*1*) aniline consumption, (*2*) biomass growth.

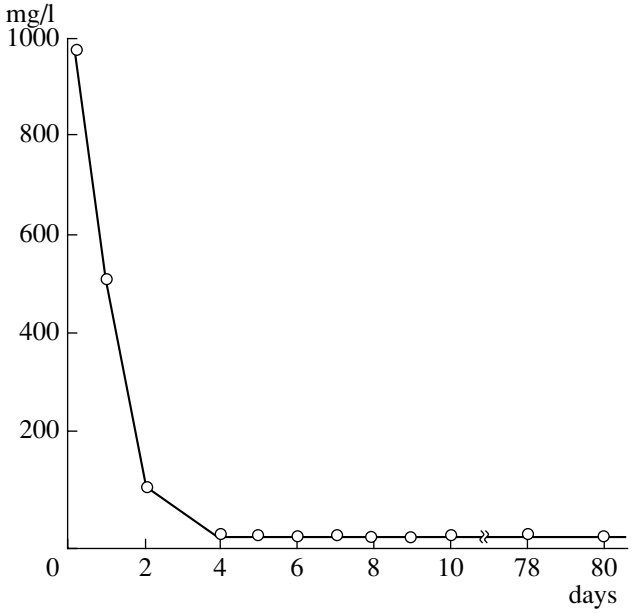

Fig. 3. Aniline concentration (mg/l) in model sewage at the output of the pilot biofilter.

The kinetics of aniline degradation in a continuous process was studied in a chemostat mode with controlled supply of aniline as the source of carbon and nitrogen. Figure 3 shows the dynamics of aniline consumption in a pilot biofilter. At 0.12 h^{-1} dilution rate and 1000 mg/l aniline, it took about 96 hours for the system to adapt to the conditions and reach stable performance, at which aniline output was not recorded. After the adaptation phase, the system showed stable operation throughout the experiment (about 6 weeks).

Thus, strain *D. tsuruhatensis* 14S, isolated from biological purification work, is capable of consuming aniline at high concentrations. Laboratory modeling of a continuous process for purification of sewage from aniline based on the use of immobilized cells showed that the strain could be advantageous in industrial biological purification.

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REFERENCES

1. Surovtseva, E.G. and Vol'nova, A.I., *Mikrobiologiya*, 1980, vol. 49, no. 1, pp. 49–52.
2. Aoki, K., Ohtsuka, K., Shinke, R., and Nishira, H., *Agric. Biol. Chem.*, 1984, vol. 48, no. 4, pp. 865–872.
3. Surovtseva, E.G., *Mikrobiologiya*, 1992, vol. 61, no. 2, pp. 165–175.
4. Loidl, M., Hinteregger, Ditzelmuller, G., Ferschl, A., and Streichsbier, F., *Arch. Microbiol.*, 1990, vol. 155, no. 1, pp. 56–61.
5. Hyung-Yeel, Kahng, Jerome J. Kukor, and Kye-Heon Oh, *FEMS Microbiol. Lett.*, 2000, vol. 190, no. 2, pp. 215–221.
6. Boon, N., Goris, J., Vos, P., Verstraete, W., and Top, E.M., *Appl. Environ. Microbiol.*, 2001, vol. 67, no. 3, pp. 1107–1115.
7. Liu, Z., Yang, H., Huang, Z., and Liu, S.-J., *Appl. Microbiol. Biotechnol.*, 2002, vol. 58, no. 5, pp. 679–682.
8. Nikov, I., Nikolov, V., and Dimitrov, D., *Bioproc. Eng.*, 1999, vol. 21, no. 6, pp. 547–552.
9. O'Nell, T.J., Bromley-Challenor, K.C.A., Greenwood, R.J., and Knapp, J.S., *Wat. Res.*, 2000, vol. 34, no. 18, pp. 4397–4409.
10. Gerhart, P., Murray, R.G., Wood, W.A., and Kreig, N.R., *Methods for General and Molecular Bacteriology*, Washington, DC: Amer. Soc. Microbiol., 1994.
11. *Bergey's Manual of Determinative Bacteriology*, Holt, J.G., Krieg, N.,R., Sneath, P.H.A., Staley, J.T., and Williams, S.T., Eds., Baltimore: Williams and Wilkins, 1994.
12. *Molecular Cloning: a Laboratory Manual*, Sambrook, J., Fritsch, E.F., and Maniatis, T., Eds., New York: Cold Spring Harbor, 1989.
13. Aoki, K., Konohana, T., Shinke, R., and Nishira, H., *Agric. Biol. Chem.*, 1984, vol. 48, no. 8, pp. 2087–2095.
14. Bayly, R.C. and Wigmore, G.J., *J. Bacteriol.*, 1973, vol. 113, no. 3, pp. 522–525.
15. Schmidt, E. and Knackmuss, H.Y., *Biochem. J.*, 1980, vol. 192, pp. 339–347.
16. Schloemann, M., Schmidt, E., and Knackmuss, H-J., *J. Bacteriol.*, 1990, vol. 172, no. 9, pp. 5112–5118.
17. Zaborina, O.E., Degradation of Chlorophenols by *Strertomyses rochei* 303 Culture, *Cand. Sci. (Biol.) Dissertation*, Pushchino: IBFM RAN, 1993.
18. Lyons, C.D., Katz, S., and Bartha, R., *Appl. Environ. Microbiol.*, 1984, vol. 48, no. 3, pp. 491–496.
19. Karasevich, Yu.N., *Osnovy selektsii mikroorganizmov, utiliziruyushchikh sinteticheskie organicheskie soedineniya* (Essentials of Selection of Microorganisms Utilizing Synthetic Organic Compounds), Moscow: Nauka, 1982.