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A novel 2,3-xylenol-utilizing *Pseudomonas* isolate capable of degrading multiple phenolic compounds

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ABSTRACT

This work characterized a novel 2,3-xylenol-utilizing *Pseudomonas* isolate XQ23. From 16S rRNA phylogenetic analysis, XQ23 was found to be a member of the *Pseudomonas putida* group. Most of its physiological characteristics also shared similarities to *P. putida*. Phenols were catabolized by the meta-cleavage pathway. The dependence of the specific growth rate on 2,3-xylenol concentration could be well fitted by the Haldane model, with the maximum occurring at the concentration around 180 mg l^{-1}. Kinetic parameters indicated that XQ23 was sensitive to 2,3-xylenol and had low affinity. Three patterns, i.e. constant, linear decline, and allometric decline, were proposed to describe the biomass yields of phenols during bacterial degradation and XQ23 under 2,3-xylenol culturing conditions followed the allometric pattern. In a mineral-salts medium supplemented with 180 mg l^{-1} of 2,3-xylenol as the sole carbon and energy source, over 40% of 2,3-xylenol was turned into CO_2 to provide energy by complete oxidization.

1. Introduction

Petroleum and coal, the two main sources of energy and raw chemical materials supporting modern human civilization, share similar origin and formation processes. A striking evidence perhaps comes from similar phenolic constituents in the effluents of oil refining (Tang et al., 1994) and coal coking (Broholm and Arvin, 2000), including phenol and its 9 derivatives: 3 cresols (o-, m-, and p-isomers) and 6 xylenols (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5 isomers).

Phenolic compounds, or simply phenols, are toxic, carcinogenic, mutagenic and teratogenic. They are growth inhibitors to microorganisms and 1 mg l^{-1} of phenol would cause noticeable influence to aquatic life (Veeresh et al., 2005). Because many of phenolic chemicals in effluents or from accidental leakages can be resistant to microbial degradation, they can accumulate in water and soil for months and even years (Ke et al., 2008). With the increasing awareness of environmental consequences and health implications caused by phenolic pollutants, their metabolic processes by microorganisms have received extensive investigations over the past few decades. To date, however, no known microbes are capable of simultaneously degrading all the 10 phenols.

Recently, a *Pseudomonas* strain named XQ23, was isolated from phenols-contaminated soil using 2,3-xylenol as the sole carbon source (Xiao et al., 2012c). To the best of our knowledge, *Pseudomonas* sp. XQ23 was the first 2,3-xylenol-utilizing bacterium capable of degrading all the 10 phenols except 2,6-xylenol (Xiao et al., 2012b). 2,6-Xylenol often exists in very low level in effluents and it could be photo-oxidized in natural water (Faust and Hoigne, 1987). Therefore, strain XQ23 would be of great interest, in particular to the industrial remediation of phenolic pollutants from petroleum and coal sources. On the other hand, growth kinetics is an essential and mandatory factor for the design of any bioreactors where microbial degradation is carried out (Banerjee and Ghoshal, 2010). Although the degradation processes of phenol by various microorganisms have been well documented, the current knowledge on the kinetics of xylenol biodegradation is rather limited. In this study, morphological, phylogenetical, physiological, enzymatic, and biokinetic experiments on this novel isolate were performed to provide useful clues on xylenol biodegradation.

2. Methods

2.1. Chemicals and bacterial strain

2,3-Xylenol was 99% pure from Acros Organics (USA). Other chemicals used in this study were all commonly available analytical reagents. The strain *Pseudomonas* sp. XQ23 was deposited in China Center for Type Culture Collection with deposition number M209175.

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2.2. Culture media and conditions

The media used for 2,3-xylenol degradation in this study was based on a previous mineral-salts (MS) medium (Xiao et al., 2012a). Different amount of 2,3-xylenol or phenol was supplemented as the sole carbon source. All the media were adjusted to pH 7 and sterilized by autoclaving for 20 min at 121 °C. The phenol-grown cells of XQ23, which was stored in sterile saline (0.85% NaCl) containing 40% (v/v) glycerol at −20 °C, were washed with sterile saline before inoculation. All the 2,3-xylenol degradation experiments except for TOC (total organic carbon) investigation were performed using Corning 24-well plates (Corning Life Sciences) added with 24 ml of medium (1 ml/well). The 24-well plates were placed in a MB100-4P rotary shaker (Thermo-Shaker for Microplates, Hangzhou Allsheng Instruments Co. Ltd.) controlled at 30 ± 0.5 °C, oscillation amplitude 2 mm, with desired shaking speeds. For TOC analysis, the bacterium was incubated in 500-ml Erlenmeyer flasks (shaken at 150 rpm under 30 °C) each containing 100 ml of MS medium supplemented with 180 mg l^{-1} of 2,3-xylenol as the sole carbon and energy source.

2.3. Morphological analysis

Live-cell observation was performed using a light microscope (DMI3000B Microscope, Leica). In order to gain further magnified images of cells, bacterial samples were washed twice with a phosphate buffer (50 mM, pH 7.0) and fixed with 2.5% glutaraldehyde. For scanning electron microscope (SEM) analysis, specimens were dehydrated using ethanol, critical point-dried with CO_2 and sputter coated with gold routinely before examination using JSM-6390LV (JEOL). For transmission electron microscopy (TEM) analysis, specimens were negatively-stained with phosphotungstic acid (1%) before examination using JEM-1200EX (JEOL).

2.4. Phylogenetic analysis

Partial 16S rRNA gene sequence of *Pseudomonas* sp. XQ23 (GenBank accession No. GQ451456) was obtained previously (Xiao et al., 2012c). To locate the position of strain XQ23 in the phylogenetic tree of the *Pseudomonas* genus, 16S rRNA sequences of authentic *Pseudomonas* species according to Anzai et al. (2000) were retrieved from GenBank. Phylogenetic tree and bootstrap values (1000 replicates) based on the 16S rRNA sequences were generated using the maximum composite likelihood method and the neighbor-joining method in the MEGA5 program.

2.5. Physiological and aromatic ring-cleavage pathway analysis

Physiological characterization of strain XQ23 was done using BoJian Gram-negative Bacteria Identification System (QingDao Hopebio-Technology Co. Ltd.) according to the manufacturer's instructions.

For benzene ring-cleavage pathway analysis, XQ23 cells grown in MS medium with 200 mg l^{-1} of phenol as the sole carbon source were collected by centrifugation at 8000 rpm for 15 min at 4 °C, washed with KH_2PO_4–K_2HPO_4 buffer (50 mM, pH 7.0), resuspended and sonicated in ice bath. The cell debris was then removed by centrifugation at 12,000 rpm for 30 min at 4 °C to prepare the crude extracts. Activities of catechol 1,2-dioxygenase (EC 1.13.11.1) and catechol 2,3-dioxygenase (EC 1.13.11.2) in the crude extracts were assayed spectrophotometrically with an ultraviolet/visible spectrophotometer (Shimadzu, UV-1700 PharmaSpec) by quantitating the formation of *cis,cis*-muconic acid (increase of absorbance at 260 nm) and 2-hydroxymuconic semialdehyde (2-HMSA, increase of absorbance at 375 nm), respectively.

2.6. Analytical methods

Bacterial growth in the 24-well plates was measured by optical density at 600 nm (OD_{600}) using SpectraMax M2e Multi-Mode Microplate Reader (Molecular Devices). Dry cell weight (DCW) was calculated from OD_{600} with a linear correlation factor (1 OD_{600} = 243 DCW mg l^{-1}). 2,3-Xylenol concentration in the media was measured periodically using 4-aminoantipyrine colorimetric method. For TOC analysis, samples were withdrawn every hour from Erlenmeyer flasks and measured using liquiTOC II (Elementar Analysensysteme GmbH). Metabolites in the media were analyzed with an Agilent 7890A gas chromatograph (GC) equipped with a flame ionization detector and a 30-m HP-5 capillary column (HP Agilent).

2.7. Kinetic and biomass yield analysis

Haldane's model is used for the determination of kinetic parameters because it is appropriate for dealing with inhibitory substrates such as phenolic compounds (Kurzbaum et al., 2010; Saravanan et al., 2008; Essam et al., 2010). The Haldane equation is:

$$\mu = \mu_{max} S/(K_s + S + S_2/K_i) \quad (1)$$

where μ is the specific growth rate (h^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), S is the initial substrate concentration (mg l^{-1}), K_s is the half-saturation constant of growth kinetics (substrate-affinity constant) (mg l^{-1}), and K_i is the substrate-inhibition constant (mg l^{-1}).

Batch cultures of strain XQ23 were grown in MS media supplemented with 2,3-xylenol ranging from 45 to 720 mg l^{-1}. Bacterial initial inocula (X_0) were set to 36.6 ± 0.3 mg l^{-1}. The shaker was controlled at 400 rpm. The value of μ was estimated by performing a linear least squares regression on the semi-logarithmic plot of the biomass concentration over cultivation time in the exponential growth phase (Li et al., 2010). From the values of μ vs. S (initial 2,3-xylenol concentration), the values of μ_{max}, K_s and K_i were obtained by nonlinear regression analysis using MATLAB (Release 2010b).

Dry-weight biomass yield Y (mg cell/mg 2,3-xylenol) can be calculated using the following equation:

$$Y = (X - X_0)/(S - S_s) \quad (2)$$

where X is the maximum cell concentration (DCW), S_s is the 2,3-xylenol concentration corresponding to X, X_0 and S are defined as above-mentioned.

3. Results and discussion

3.1. Morphology

Light microscopic imaging showed that XQ23 cells were gram negative short rods. Irregularly-shaped and loosely flocculated biomass objects of less than 100 μm in diameter could be observed when strain XQ23 was incubated in MS media with gentle shaking (150 rpm). Under the same incubation conditions, XQ23 cells tended to adhere to the cotton fibers added in the media. Thus the number of planktonic cells was sharply reduced and the cotton fibers "grew" thicker in diameter (from several to dozens of micrometers). However, autoaggregation or adhesion could not be observed when the shaking speed was increased to 400 rpm, indicating that such autoaggregation or adhesion ability was not strong enough to overcome intense agitation. To visualize the fine structure of XQ23 cells, SEM and TEM micrographs were taken. Whilst the SEM image clearly revealed formation of cell aggregates,