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Phenol biodegradation by halophilic archaea

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ABSTRACT

Phenol is a toxic aromatic compound produced as a by-product of industrial activities. Biological treatment of highly saline wastewaters containing phenol can be performed through halophilic microorganisms. In this study, the ability of halophilic archaeal isolates to degrade phenol was investigated. Among 103 tested isolates, the strain designated A235 was identified as having the highest phenol degradation capacity on solid and liquid media containing 20% (w/v) NaCl and phenol as the sole carbon and energy source. The strain was adapted sequentially to increasing phenol concentrations. The removal of phenol via cross-toluene adaptation was increased by 14% in the medium. The growth kinetics of strain A235 during growth on phenol was found to fit the Monod model. The values of μmax and Ks were calculated to be 0.015 h^{-1} and 71.4 g l^{-1}, respectively. For an initial phenol concentration of 100 ppm, the biodegradation by A235 was found to be optimal at pH 7.5, 37 °C and 200 rpm when the culture contained 20% (w/v) NaCl, 0.025% yeast extract and the inoculum size was set at 10%. A preliminary enzyme screening indicated that the degradation of phenol was achieved through a *meta*-cleavage pathway involving a catechol 2,3-dioxygenase. Catechol 2,3-dioxygenase displayed its highest catalytic activity at 42 °C, 2 M KCl, and pH 8. To the best of our knowledge, this is the first report showing the ability an extremely halophilic archaeon to metabolize phenol at higher salt concentrations.

1. Introduction

Phenolic compounds derived from industrial activities are ubiquitous in the environment. Since they are toxic to many organisms, they must be removed. It is estimated that 5% of industrial effluents are saline or hypersaline (Borgne et al., 2008). Phenol removal from hypersaline wastewaters is frequently achieved by physico–chemical approaches such as precipitation, coagulation, ion exchange, ultra-filtration or evaporation. These processes are usually energy-consuming and lead to secondary effluent problems. As an alternative, the implementation of biological processes increasingly gains interest, especially in the case of phenol since microorganisms can use it as sole source of carbon and energy. On the other hand, saline conditions may limit microbial degradation of phenol and make conventional biodegradation processes ineffective. Conventional microorganisms do not survive under these saline conditions. However, halophilic microorganisms have adapted to grow optimally in a very wide range of salt concentrations (from 3 to 35%) (Ventosa et al., 1998; Bastos et al., 2000; Margesin and Schinner, 2001).

These halophiles are good candidates for the bioremediation of hypersaline environments including saline effluents. The ability of several halophilic and halotolerant bacteria to biodegrade phenol has been demonstrated (Bastos et al., 2000; Alva and Peyton, 2003). For instance, biodegradation of phenol in hypersaline wastewaters was achieved by employing moderate halophiles (up to 15% salt) as reported by Woolard and Irvine (1994). Haddadi and Shavandi (2013) reported a *Halomonas* sp. strain that exhibited the ability to grow on phenol as the sole source for carbon and energy in the presence of 18% (w/v) NaCl. Although the biodegradation of phenol by halophilic and halotolerant bacteria has been well demonstrated, the information on phenol biodegradation by halophilic archaea has not been recorded yet. On the other hand, the capacity of halophilic archaea to degrade some other organic substrates (*p*-hydroxybenzoic acid, naphthalene, phenanthrene, and pyrene) has been documented (Erdogmus et al., 2013).

Since phenols are toxic to many microorganisms, different approaches, such as adapting the cells to higher phenol concentrations, immobilizing the microbial cells or adding a co-substrate such as yeast extract, glucose, carbohydrates and fatty acids have

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been suggested to overcome the phenol toxicity (Masque et al., 1987; Lob and Tar, 2000). Among these approaches, adaptation to higher phenol concentrations is the most effective to overcome substrate inhibition (Masque et al., 1987). In addition, pre-adaptation to phenol may have significant effects on the degradation patterns such as shorter degradation time or higher degradation rates (Kwon and Yeom, 2009). The mechanism of microbial adaptation to phenol degradation was explained on the basis intracellular enzyme induction (Yeom et al., 1997). Microbial degradation of phenol under aerobic conditions usually proceeds via an initial hydroxylation to generate catechol which is cleaved through the *ortho* or *meta* fission pathways (Bonfa et al., 2013). The *ortho* cleavage catalysed by the catechol 1,2-dioxygenase (EC 1.13.11.1) leads to the formation of *cis*,*cis*-muconic acid and the *meta*-cleavage catalyzed by the catechol 2,3-dioxygenase (EC 1.11.13.2) leads to the formation of 2-hydroxymuconic semialdehyde (Gurujeyalakshmi and Oriel, 1989).

In this work, we have investigated the ability of the halophilic archaeon strain A235 to use phenol a sole carbon source at high salt concentrations and we have obtained preliminary evidence that in this strain, phenol degradation proceeds through the *meta*-cleavage pathway. We also determined its growth kinetics during the degradation of phenol in batch culture. The effects of phenol and toluene adaptation as well as the effects of various growth conditions on phenol degradation were also investigated.

2. Materials and methods

2.1. Screening for phenol-degrading archaeal strains

A total of 103 halophilic *Archaea* isolated from different parts of Turkey were routinely cultured in Sehgal–Gibbons (SG) medium as explained before (Ozcan et al., 2006). The agar plates contained 2% (w/v) agar. The liquid cultures were incubated at 120 rpm and at 37 °C.

The halophilic archaeal isolates were screened for their ability to degrade phenol at 37 °C on a solid or liquid mineral salt medium containing phenol (PMS). The PMS medium was modified from Halohandbook online protocol (Dyall-Smith, 2009). The medium contained (g l^{-1}): NaCl 200; $MgSO_4.7H_2O$ 25; $MgCl_2.6H_2O$ 10; $CaCl_2.2H_2O$ 1.25; KCl 5; K_2HPO_4 8.7; KH_2PO_4 6.8; trisma baze 6; NH_4Cl 0.2; phenol and trace element 2 ml l^{-1} containing ((g/100 ml) $MnCl_2.4H_2O$ 0.03; $ZnSO_4.7H_2O$ 0.1; $FeCl_3$ 0.25; $CuSO_4.5H_2O$ 0.01; $Na_2MoO_4.2H_2O$ 0.03; H_3BO_4 0.3; NH_4NO_3 0.2). The pH of the medium was adjusted to 7.35. Preliminary screening was carried out by inoculating archaeal cell suspensions on PMS-agar plates containing 50 ppm (0.5 mM) phenol as sole carbon source. Cells capable of degrading phenol were successively transferred to an identical medium containing increasing concentrations of phenol ranging from 100 to 200 ppm. The highest phenol tolerant strains were inoculated in liquid PSM containing 100, 150, 200 and 250 ppm (1.1, 1.6, 2.1 and 2.7 mM, respectively) phenol for quantitative screening. The cultures were incubated at 120 rpm. The most efficient phenol degrading strain (strain A235) was selected by monitoring the optical density (OD_{600}) of the cultures after 15 days of incubation. Cells employed for phenol degradation were obtained from the late exponential phase cultures (OD_{600}~3) by centrifugation at 5000 rpm for 30 min at 4 °C and washing twice with 50 mM Tris–HCl buffer (pH 7.35). All cultures were incubated at 37 °C.

2.2. Analysis of archaeal growth and phenol degradation

Archaeal growth was carried out with various initial phenol concentrations ranging from 50 to 200 mg l^{-1}. Cell concentration was calculated by plotting the colony forming units (CFUs) of the culture vs. optical density measured at 600 nm.

Phenol concentration was determined spectrophotometrically at 500 nm by monitoring the production of 4-aminoantipyrine (Yang and Humphrey, 1975). This method is based on rapid condensation with 4-aminoantipyrine followed by oxidation with alkaline potassium ferricyanide. All samples were analysed three times to calculate an average value. The specific growth rates for various initial phenol concentrations were calculated from the growth kinetic slope.

2.3. Adaptation to phenol and toluene

Adaptation of isolate A235 to phenol was achieved by gradually increasing the phenol concentration of the medium, from 50 to 250 ppm and simultaneously decreasing the yeast extract concentration from 0.075 to 0%. Similarly, toluene adaptation was achieved by increasing the concentration of toluene. The initial toluene concentration was set at 10 ppm and it was changed stepwise to 20, 30, 40 and 50 ppm. The adapted cells were then tested for their phenol degradation capacities.

2.4. Effects of growth conditions on phenol degradation

The optimal cultural conditions for phenol biodegradation by strain A235 were determined by using different concentrations of phenol (50, 75, 100, 150 and 200 mg l^{-1}), NaCl (10, 15, 20 and 25 M) and yeast extract (0.3, 0.1, 0.005, 0.025%), and different inoculum sizes (2.5, 5,10, 15% v/v), pH (6, 7, 7.5, 8 and 8.5), temperatures (25, 30, 37, 45 and 50 °C) and agitation rates (120, 140, 170 and 200 rpm). For each variable, optical density and phenol concentrations were monitored during the cultivation.

2.5. Rothera test for the detection of ortho- and meta-cleavage products

The ring cleavage pattern of catechol by isolate A235 was determined by the Rothera's test (Ottow and Zolg, 1974). The test was performed as follows: the cell pellet was suspended in 2 ml 0.02 M Tris buffer (pH 8), then 0.5 ml of toluene and 0.2 ml of 4 mM catechol were added. Following vigorous shaking of the tube, the development of a yellow colour within 5 min was considered as an indication of *meta*-cleavage. In the absence of yellow colour, ammonium sulphate (1 g) was added and the mixture was further incubated at 30 °C for 1h. The addition of freshly prepared sodium nitroprusside and 2.0 ml of concentrated ammonium hydroxide develops a strong violet colour when the degradation proceeds through the *ortho*-clevage pathway.

2.6. Enzyme assays

2.6.1. Preparation of cell extracts

Cells grown to exponential phase under optimal degradation conditions were harvested by centrifugation at 15,000 rpm for 15 min at 4 °C and washed twice with 50 mM Tris–HCl buffer (pH 7.35). The pellet was suspended in the same buffer containing KCl and the cells were disrupted by sonication (Bandelin Sonopuls GM 200) on ice for a total time of 6 min (30 s on/30 s off). Cell debris was removed by centrifugation at 15,000 rpm for 30 min at 4 °C and the supernatant was used to assay the enzymes activities.

2.6.2. Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities

Catechol 1,2-dioxygenase (EC 1.14.13.1) and catechol 2,3-dioxygenase (EC 1.13.11.2) activities were measured