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Degradation of phenol by *Acinetobacter* strain isolated from aerobic granules

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Abstract

Aerobic granules effectively degrade phenol at high concentrations from which no *Acinetobacter* species, that can effectively degrade high concentrations of phenol, have ever been isolated from aerobic granules. The phenol-fed aerobic granule studied was made by merging several smaller granules, each with a core of proteins and nucleic acids surrounded by an outer layer enriched with polysaccharides. In the present study, a strain of *Acinetobacter* sp. was isolated from the phenol-fed aerobic granules and was identified using DNA sequencing. The fluorescent *in situ* hybridisation combined with the confocal laser scanning microscope test revealed that the isolated *Acinetobacter* strain was mainly distributed in the core regime of granule. Batch tests revealed that the suspended *Acinetobacter* strain could effectively degrade phenol at an initial phenol concentration of up to 1000 mg l^{-1} with no cell growth taking place at a phenol concentration of 1500 mg l^{-1}. The Haldane model describes the inhibitory kinetics of the phenol degradation data. The suspended *Acinetobacter* strain had a propensity to attach to the surface of sterilized polyurethane foam at a concentration of 12.3 mg dry cells mg^{-1} dry foam. The immobilized cells could not only degrade phenol at a rate similar to the suspended cells at phenol concentration of 500 mg l^{-1}, but also effectively degraded phenol at 1500 mg l^{-1}. The polysaccharides outer layer protected the *Acinetobacter* strain from phenol's toxicity; while the strain may also contribute to bioaggregation of the granule for its high propensity to attach to solid surface.

Keywords: Granule structure; Confocal laser scanning microscope; Phenol degradation; Cell immobilization

1. Introduction

The aerobic granule process, a self-immobilization of microorganisms, has been extensively investigated (Morgenroth et al., 1997; Beun et al., 1999; Tay et al., 2001; Yang et al., 2003; Liu and Tay, 2004; Chiu et al., 2006). Aerobic granules yield a very high biomass concentration (up to 15 g l^{-1}) (Di Iaconi et al., 2005) and have the ability to degrade high-strength wastewater (up to 15 kg COD m^{-3} d^{-1}) (Moy et al., 2002). These granules settle rapidly in water pools owing to their dense structure (Liu and Tay, 2004).

Phenol is toxic to particular aquatic species (Brown et al., 1967; Kibret et al., 2000; Chung et al., 2003) and adds odor to drinking and food-processing water (Rittmann and McCarty, 2001). Aerobic granules were applied to the degradation of phenol (Jiang et al., 2002, 2004a; Chou et al., 2004; Chou and Huang, 2005; Tay et al., 2005a,b). Tay et al. (2004) demonstrated that their granules degraded phenol at a specific rate exceeding 1 g phenol g^{-1} VSS d^{-1} at 500 mg l^{-1} of phenol, or at a reduced rate of 0.53 g phenol g^{-1} VSS d^{-1} at 1900 mg l^{-1} of phenol. One likely reason that accounts for the high phenol degradation efficiency by granules is the mass transfer barrier provided by the granule matrix that produces lower local phenol concentrations on cells than the bulk value (Liu and Tay, 2004).

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Various aerobic and anaerobic phenol degrading microorganisms have been isolated and characterized (Ryoo et al., 2000; Santos and Linardi, 2001; Chen et al., 2004). Hao et al. (2002) studied the degradation of phenol by *Acinetobacter* species at a concentration of 350 mg l^{-1}. Jiang et al. (2004b) isolated 10 bacterial strains from their aerobic phenol-degrading granules, including six β-*proteobacteria*, three *actinobacteria*, and one γ-*proteobacterium* and identified their potential for degrading phenol. Heinaru et al. (2000) isolated 39 strains from polluted river water (38 *Pseudomonas* spp. and 1 *Acinetobactor* sp.).

The *Acinetobacter* species have highly hydrophobic cell surface and are adhesive to solid surface (Ishii et al., 2004), and may play significant role on bioaggregation in activated sludge and aerobic granule processes. No *Acinetobactor* strains had been isolated and characterized from phenol-degrading aerobic granules. The only member belonging to γ-*proteobacteria* (*Xanthomonas axonopodis* strains 53) isolated by Jiang et al. (2004b) showed no growth in the phenol-containing medium.

Adav et al. (2006) cultivated aerobic granules using a column-type sequential reactor at 30 °C to degrade phenol with zero order kinetics at a rate of 49 mg-phenol g^{-1} VSS h^{-1} at 50–1000 mg l^{-1} of phenol. This work isolated and characterized a strain from the so-cultivated granule, identified as the bacterium *Acinetobacter*, with a high potential of phenol biodegradation. The growth and phenol degradation kinetics of this strain were examined and it was found that it had a high propensity to attach sterilized sponge to form immobilized biofilms that survived and degraded 1500 mg l^{-1} phenol in water.

2. Materials and methods

2.1. Granule staining and CLSM imaging

The collected granules using methods described in Adav et al. (2006) were maintained fully hydrated during staining. Fluorescein isothiocyanate (FITC) (Molecular Probes, Eugene, USA) was applied to stain the amine-reactive compound-like proteins and amino sugars. Fluorescently labelled lectin Concanavalin A (Con A, Molecular Probes, Eugene, USA) conjugated with tetramethylrhodamine were employed to bind the α-mannopyranosyl and α-glucopyranosyl sugar residues. SYTO 63 (Molecular Probes, Carlsbad, CA, USA), which is a cell-permeative nucleic acid stain, was utilized to differentiate extracellular polymeric substances from cells.

The SYTO 63 (20 μM, 100 μl) was first dripped onto the granule sample and placed on a shaker table for 30 min. Next, 0.1 M sodium bicarbonate buffer (100 μl) was added to the sample to retain the amine group in a non-protonated form; then, the FITC solution (10 g l^{-1}, 100 μl) was added to the sample for 1 h at room temperature. Subsequently, the Con A solution (0.2 g l^{-1}, 100 μl) was added and it was incubated for a further 30 min. After each of these four staining stages, the sample was washed twice by phosphate buffered saline (PBS, 0.1 M, pH 7.2) to remove excess stain. Some stained granules were frozen at −20 °C and sectioned into specimens 40-μm thick using a cryomicrotome for viewing from the side of the granules.

Confocal laser scanning microscopy (CLSM) (Leica TCS SP2 Confocal Spectral Microscope Imaging System, Gmbh, Germany) was used to investigate the internal structure of the granules. The FITC probe was detected via excitation at 488 nm and emission at 500–540 nm. Excitation at 543 nm and emission at 550–600 nm were utilized to detect Con A conjugates. The fluorescence of SYTO 63 was determined based on excitation at 633 nm and emission at 650–700 nm.

2.2. Strain cultivation and identification

Mature granules from the reactor fed with 400 mg l^{-1} phenol were placed in aseptically mixed sterilized tubes containing 10 ml MP medium (1000 mg l^{-1} $(NH_4)_2SO_4$, 200 mg l^{-1} $MgCl_2$, 100 mg l^{-1} NaCl, 20 mg l^{-1} $FeCl_3$, 10 mg l^{-1} $CaCl_2$), and phosphate buffer (pH 6.8) to break up the granules. The supernatant was serially diluted with medium (10^1–10^9 fold dilutions), and 1 ml of each 10^5–10^9 dilutions were spread onto agar plates containing MP medium supplemented with phenol (400 mg l^{-1}) solidified with 1.2% bacteriological agar. The plates were inverted and incubated at 30 °C in an incubator. Visible colonies appeared after one wk of incubation. Morphologically distinguished visible colonies of phenol degrading bacteria were isolated within one wk by several replating cycles onto MP-phenol medium. Purity was examined with a microscope and scanning electron microscope (SEM, Jeol JSM- 5310, Tokyo, Japan). A total of 20 morphologically different phenol-degrading isolates were selected and inoculated into liquid MP medium (without agar) containing higher concentrations of phenol (500 mg l^{-1}).

The DNA from the isolated strain was extracted via enzymatic lysis using extraction buffer (5 ml; 100 mM Tris–HCl at pH 8.0, 100 mM EDTA at pH 8.0, and 1.5 M NaCl) containing Proteinase K (100 μl; 10 mg ml^{-1}). Samples were incubated at 37 °C for 30 min and shaken at 150 rpm. The sodium dodecyl sulfate, was added (1 ml; 20%) to samples and incubated at 65 °C for 90 min. Samples were subjected to two cycles of freeze-thawing. The supernatant was collected after centrifugation at 6000 × *g* for 10 min, and the aqueous phase was extracted with phenol:chloroform:isoamyl alcohol (25:24:1, v:v:v). The DNA was precipitated utilizing isopropanol and pelleted by centrifugation (10 000 × *g*) for 10 min, then resuspended in water. The DNA amplification reaction was performed with primers PDF-5′-AGA GTT TGA TCM TGG CTC AG-3′ and PDR-5′-GGG TTG CGC TCG TTG-3′ (Bastos et al., 2000). The polymerase chain reaction (PCR) mixture consists of 5 μl PCR buffer (10×), 2.5 μl $MgCl_2$ (25 mM), 2.5 μl dNTP (2.5 mM), 0.25 μl (100 mM) each of the primer solutions, 0.5 μl Taq. Polymerase (Promega, Madison, Wisconsin) and 100 ng of template DNA.

The DNA was amplified using an eppendorf mastercycler (Eppendorf AG, Germany) by denaturation at 94 °C, 3 min, 35 cycles consisting of 94 °C for 30 s, 55 °C for 60 s, 72 °C for 90 s, and final extension at 72 °C for 7 min. The PCR-amplified 16 S rRNA was sequenced using the ABI Prism model 3730 (version 3.2) DNA sequencer.

2.3. Phenol degradation test

The experiment was started by inoculating medium containing phenol (400 mg l^{-1}) and the isolated strain (of biomass concentration 0.88 ± 0.04 mg l^{-1}) at 30 °C in a reactor shaken at 200 rpm. After 15 h of incubation, inoculum was added into batch reactors containing phenol of 100–1500 mg l^{-1}. Reactors were kept at 30 °C and shaken in an orbital shaker at 200 rpm. The pH, biomass amount, and phenol concentrations of samples in reactors were periodically measured in duplicate to ensure data quality.

To examine the propensity of the isolated strain to solid surface, sterilized polyurethane (PU) foam was added into a medium containing phenol (500 mg l^{-1}) and the isolated strain at a biomass concentration of 29.1 mg l^{-1} for 72 h with aeration. Then the phenol degradation capability of the immobilized cells on PU form was tested. The biomass content of attached cells was estimated by subtracting the weights of PU foam before and after test.

2.4. Analytical methods

Cell growth was determined spectrophotometrically by measuring absorbance at 600 nm using a UV-spectrophotometer (V530; Jasco Co., Tokyo, Japan). Phenol concentration in suspension was measured using a 4-aminoantipyrine colorimetric approach (APHA, 1998) on supernatant drawn from samples centrifuged at $6000 \times g$ for 10 min. Washed granules were prepared for SEM observation by fixing with 2.5% glutaraaldehyde for 2 h, suspended in osmium after washing with water, and dehydrated via successive passages through 30, 50, 75, 85, 90, 95 and 100% ethanol and subjected to critical drying. The oligonucleotide probe designed to target the 18S rRNA gene of *Acinetobacter* was generated after comparing the aligned sequence of *Acinetobacter* with other microbes. Granules were prepared for fluorescence *in situ* hybridization (FISH) by washing with PBS (130 mM NaCl, 10 mM sodium phosphate buffer pH 7.2) and fixing for 1 h in 4% paraformaldehyde. Following fixation, the granules were washed again with PBS and hybridized using hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl at pH 7.4, 0.01% sodium dodecyl sulfate) containing 5 ng μl^{-1} of probe (5′-CTC CCA GTC GAT ATC CAC G-3′ labeled at the 5′-end with Fluorescein phosphoramidite (FAM dye) for 2 h at 48 °C. This step was followed by a 20-min washing step at 48 °C in wash buffer prior to a final wash with Milli-Q water.

3. Results

3.1. Phenol-degrading granule

Fig. 1 presents the combined CLSM image with SYTO 63 (nucleic acids, in red), FITC (proteins, in green), and Con A (polysaccharides, in blue[1]), scanned at 360 μm from the outer surface of the phenol-degrading granule. The indicating line in Fig. 1 was about 611 μm in length. The large granule was made by merging several smaller granules, among which redundant polysaccharides were present (central regime in blue). Each small granule had a core mainly made of proteins and nucleic acid (in yellow), and a composite shell made of polysaccharides, proteins and cells.

A layer of thickness of approximately 10 μm existed at granule surface (point A), which was rich in proteins and nucleic acids. Very likely this layer corresponded to a cell layer with associated proteins on membrane. Beneath this layer was a polysaccharide-rich layer of thickness of about 60 μm (point B). Protein and nucleic acid contents were low in this polysaccharide-rich layer. The inside core was made of mainly proteins and cells (between point C and D). A common proposition as to why microorganisms form granules is that a granule provides a mass transfer barrier to toxic substances. The microorganisms in the core regime were located within a thick layer of polysaccharides and consequently might be protected from exposure to high-level concentrations of phenol, fully utilizing the mass transfer barrier generated by the granule matrix.

3.2. Strain identification and biochemical characteristics

Selected strains from the inoculated isolates from phenol granules were examined. The partial genome of 1059 bp was isolated from the strain and aligned utilizing National centre for biotechnology information blast, which had a 99% sequence similarity with *Acinetobacter* (strain ATCC 11171). The gene sequence was deposited in GeneBank under accession no. DQ 837531.

The SEM photographs of isolated, pure culture (Fig. 2) revealed interlinking pigments that connect cells, which might contribute to the adhering capability of *Acinetobacter* to solid surface (Ishii et al., 2004). The CLSM images of isolated *Acinetobacter*in granule with molecular probe labeled with FAM at 5′ are shown in Fig. 3. The FISH-CLSM results showed that the isolated strain mainly appeared as clusters and distributed at the core regimes of small granules. Certain amount of the *Acinetobacter* strain could be detected at the outer layer in the granules, but in a less amount than at core regimes.

[1] For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.

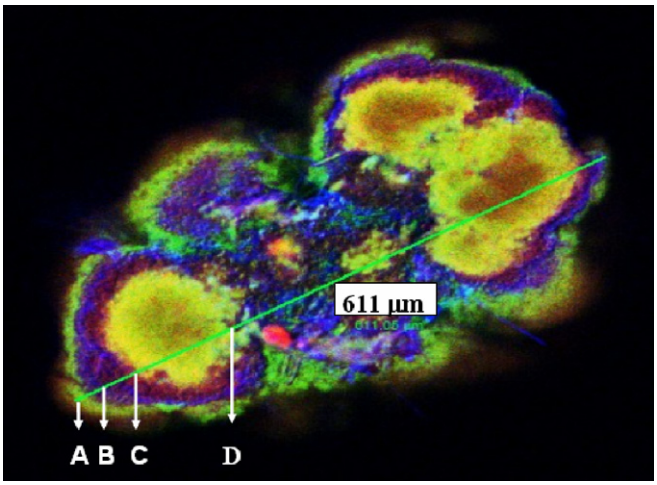

Fig. 1. The CLSM image of a phenol-degrading granule, cross-sectioned at 360 μm from top surface. Green (FITC): proteins; red (SYTO 63): nucleic acids; blue (ConA): polysaccharides; yellow: proteins + nucleic acid; purple (SYTOX blue): nucleic acids + polysaccarides; A – protein + nucleic acid layer, B – Polysaccharide layer, C – nucleic acid layer, D – protein + cells.

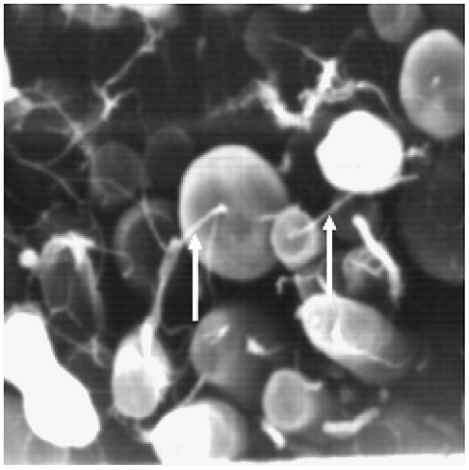

Fig. 2. The SEM image of isolated, *Acinetobacter* strain. The arrows indicate the interlinking pigments among cells.

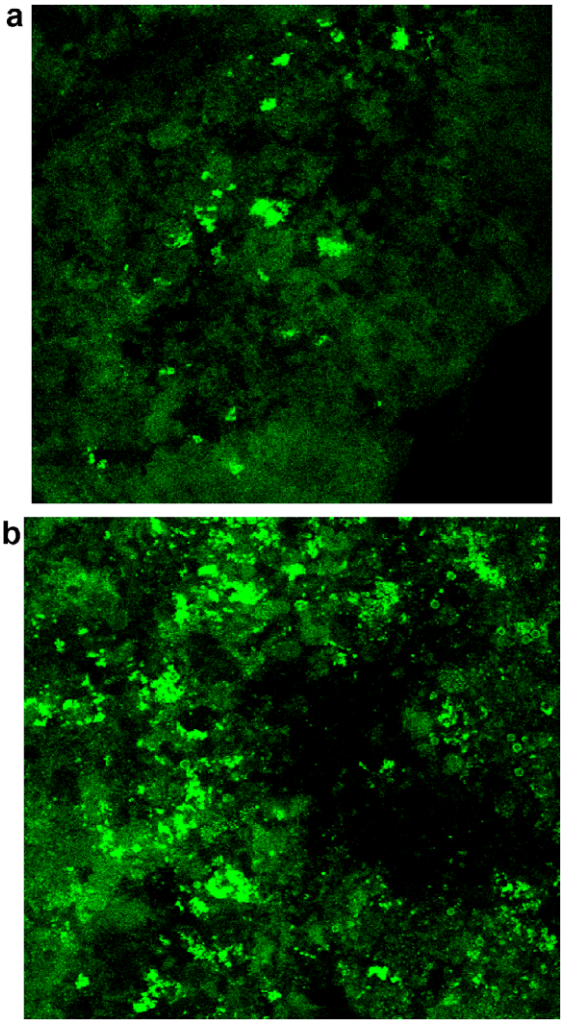

Fig. 3. The FISH-CLSM images for the isolated *Acinetobacter* in the phenol-fed granule. Size of scanned area = 2.25 mm^2: (a) scanned at outer layer of granule (points A–C in Fig. 1) and (b) scanned at core regime of small granule (Fig. 1).

3.3. Effects of initial phenol concentration

Batch tests for phenol degradation using the isolated, suspended *Acinetobacter* strain were conducted in the mineral medium with phenol concentrations of 100–1000 mg l^{-1} at pH 7.0 and 30 °C (Fig. 4a and b). Degradation could be completed if the reaction time increased with increasing phenol concentration. For instance, with initial phenol concentration of 200, 500 and 800 mg l^{-1}, all of the phenol could be degraded in 15, 24 and 39 h, respectively. Cell growth occurred accompanied with the degradation of phenol (Fig. 5a and b). No apparent inhibitory effects were noted for the suspended *Acinetobacter* strain at initial phenol concentration up to 1000 mg l^{-1}.

The average phenol degradation rates could be calculated based on data shown in Fig. 4. The strain could degrade phenol at a rate higher than 20 mg l^{-1} h^{-1} with phenol concentration between 400 and 1000 mg l^{-1}. Strong inhibitory effects were present with a phenol concentration of 1500 mg l^{-1}. Santos et al. (2003) reported a maximum phenol degradation rate of 20.13 mg l^{-1} h^{-1} for their *Graphium* sp. FIB4. The present *Acinetobacter* strain exhibited a maximum degradation of around 35–40 mg l^{-1} h^{-1} considering the maximum slopes noted in the tests in Fig. 4.

Although criticized by Allsop et al. (1993), the Haldane model describes the inhibitory kinetics of phenol degradation data at pH 7 and 30 °C as follows:

$$\frac{1}{X}\frac{dX}{dt} = \frac{\mu_{max} S}{K_s + S + S^2/K_i} \quad (1)$$

$$\frac{1}{X}\frac{dS}{dt} = -k\frac{\mu_{max} S}{K_s + S + S^2/K_i} \quad (2)$$

where μ is specific growth rate, μ_{max} is the maximum specific growth rate, X is cell density, K_s is the saturation

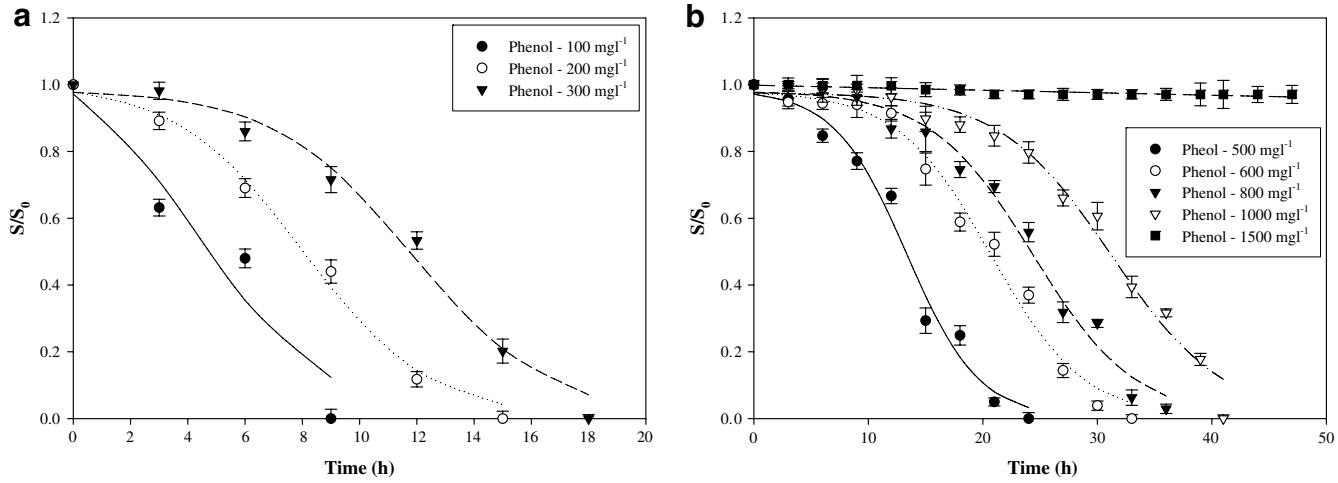

Fig. 4. Effect of initial phenol concentrations on phenol biodegradation by isolated, suspended *Acinetobacter* strain. [line-model fit, symbol-experimental data; S = phenol concentration (mg l^{-1}), S_0 = initial phenol concentration (mg l^{-1})].

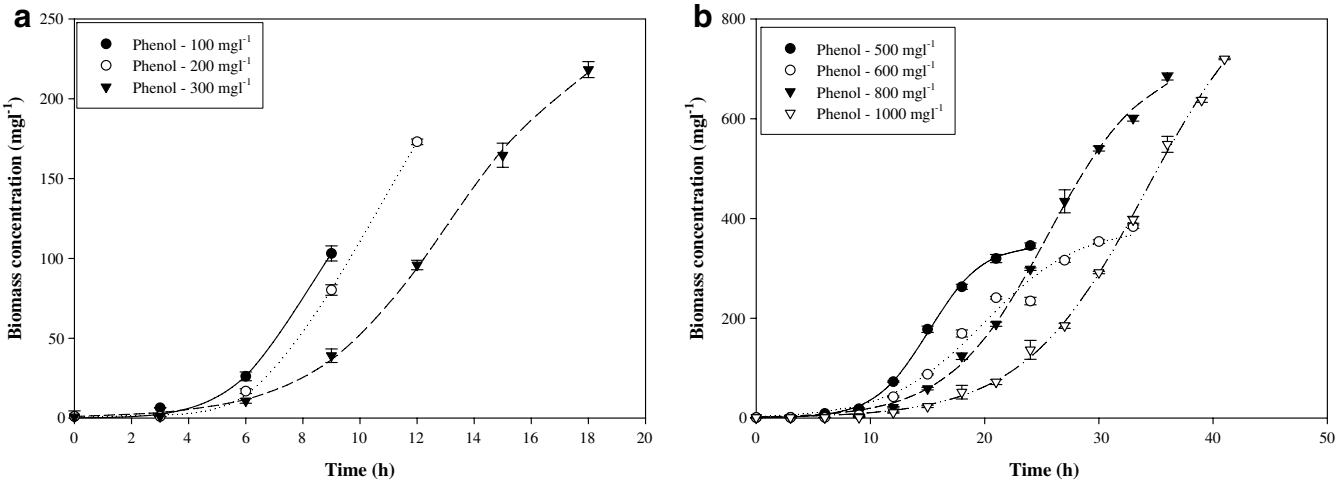

Fig. 5. Effect of initial phenol concentrations on biomass concentrations of the isolated, suspended *Acinetobacter* strain. The initial biomass content was low (close to zero as indicated in the figure).

coefficient, and K_i is the inhibition coefficient. The model's kinetic parameters for the isolated *Acinetobacter* strain were evaluated and listed in Table 1 at various initial phenol concentrations.

The parameter K_s increases with initial phenol concentration, reaching 740 mg l^{-1} at 1000 mg l^{-1} of phenol. Meanwhile, the corresponding μ_{max} was constant (0.31–0.33) over 100–1000 mg l^{-1} phenol. The specific growth rate and specific degradation rate declined with increasing phenol concentration, indicating the presence of inhibitory effects. With 100 mg l^{-1} of phenol the specific growth rate, specific degradation rate, and yield were 0.29 h^{-1}, 0.76 mg phenol mg^{-1} cell dry weight h^{-1}, and 1.30, respectively, while with 1000 mg l^{-1} of phenol, the corresponding values are 0.13, 0.21, and 0.79, respectively. At 1500 mg l^{-1} of phenol, no cell could grow in the medium.

Specific growth rate of *Acinetobacter* sp. was high at low phenol concentration and decreased with increasing phenol concentration (Table 1). Du Preez et al. (1981) reported a lower growth yield value of 0.51 for other *Acinetobacter* spp. on the more oxidized substrate like acetate, which has a lower carbon/oxygen ratio. The determined growth yield value noted herein was within the range of other pure culture studies with phenol (0.51–1.24) (Yang and Humphrey, 1975; Hill and Robinson, 1975; Saez and Rittmann, 1993).

3.4. Phenol degradation by suspended cells and immobilized cells

The propensity of the isolated *Acinetobacter* strain to sterilized PU foam was studied. The SEM image verifying the attachment of the *Acinetobacter* strain on the interior surface of the foam after 72 h contact (not shown). The immobilized biomass in the foam was estimated to be 12.3 mg dry cells mg^{-1} dry foam, by subtracting the dry

Table 1
Kinetic parameters for the isolated *Acinetobacter* strain using Haldane model

Initial phenol conc. (mg l^{-1})	μ_{max} (h^{-1})	K_s (mg l^{-1})	Yield (mg cell mg^{-1} phenol)	Specific growth (h^{-1})	Specific degradation rate (mg phenol mg^{-1} cell dry weight h^{-1})
100	0.31	47	1.30	0.29	0.76
200	0.32	140	1.10	0.28	0.96
300	0.33	230	0.98	0.22	0.49
400	0.33	280	0.97	0.20	0.38
500	0.33	330	0.75	0.16	0.31
600	0.34	420	0.66	0.14	0.23
800	0.33	540	0.91	0.13	0.23
1000	0.34	740	0.79	0.13	0.21
1500	–	–	–	NG	–

NG: no growth of cells in the medium.

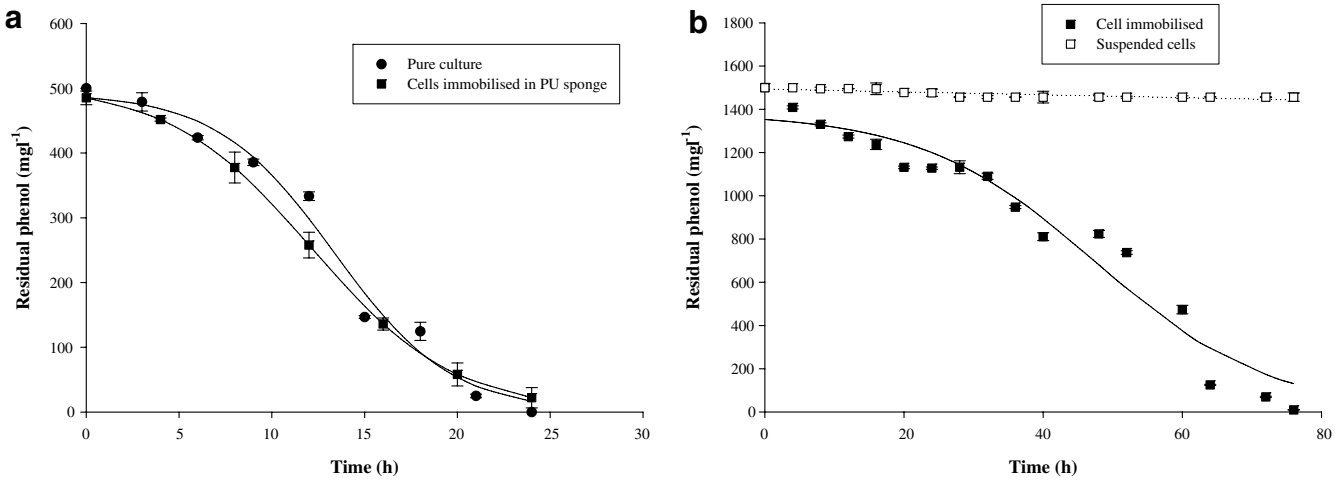

Fig. 6. Phenol degradation by suspended cells and immobilized cells (30 °C). Shaken at 200 rpm.

weights (at 102 °C) for the free and for the immobilized foam.

At 500 mg l^{-1} of phenol, the immobilized cells had a similar degradation rate as with the suspended cells (Fig. 6a). This level of phenol did not significantly inhibit the degradation rates of the suspended cells (Fig. 4). Apparently the external and internal mass transfer resistances of substrates (phenol and oxygen) in the PU foam were not the determinant factors controlling the degradation process. (Otherwise the phenol degradation rate for immobilized cells should be lower than the suspended ones.) After 24 h of testing, the biomass concentration increased to 370 mg l^{-1}, comparable to that noted for suspended cells (350 mg l^{-1}).

The suspended cells could not grow in 1500 mg l^{-1} phenol (Fig. 6b). The immobilized cells on the other hand could degrade 1500 mg l^{-1} phenol at a rate of 19.7 mg l^{-1} h^{-1}, comparable to those for suspended cells at 500–1000 mg l^{-1} of phenol (Fig. 4). Complete removal had reached in 76 h. At the end of the test the biomass concentration on foam surface had increased to 925 mg l^{-1}. Hence, the biofilm made by pure *Acinetobacter* strain could protect itself from the toxicity of high level phenol. The mass transfer barrier provided by the polysaccharide-rich surface layer of the aerobic granule (Fig. 1) may also protect the *Acinetobacter* strain inside to allow it to degrade high strength phenol in water. The *Acinetobacter* strain may also contribute to bioaggregation of phenol-fed aerobic granule for its high propensity to attach to solid surface, whose role may be overlooked in related research works on aerobic granules.

4. Conclusions

This work has isolated and characterized a strain from phenol-fed aerobic granule, identified as the bacterium *Acinetobacter* with 99% sequence similarity, with a high potential of phenol biodegradation and high propensity to attach to solid surface. The FISH combined with the CLSM revealed that the isolated *Acinetobacter* strain was mainly distributed in the core regime of granule.

Degradation tests for the *Acinetobacter* strain at various phenol concentrations suggested that the strain completely degraded phenol with no time lag at an initial phenol concentration 500 mg l^{-1}, and with a time lag of 10–15 h at an initial phenol concentration of 800–1000 mg l^{-1}.

Inhibitory effects became significant for the strain at >600 mg l^{-1}. The Haldane model describes the inhibitory kinetics of the phenol degradation data. No growth of the suspended cells was noted for the isolated strain.

The isolated *Acinetobacter* strain has a high propensity to the surface of sterilized PU foam. The immobilized cells could degrade phenol at a rate similar to the suspended cells at 500 mg l^{-1} of phenol. Meanwhile, the immobilized cells could degrade 1500 mg l^{-1} phenol at a rate of 19.7 mg l^{-1} h^{-1}, comparable to those for suspended cells at 500–1000 mg l^{-1} of phenol. Hence, the mass transfer barrier provided by the polysaccharide-rich surface layer of the aerobic granule may protect the *Acinetobacter* strain inside to allow it to degrade high strength phenol in water. Moreover, the high propensity of the *Acinetobacter* strain to PU foam suggests that it may significantly contribute to bioaggregation of the phenol-fed aerobic granule. The role of this has yet to be confirmed.

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