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Reduction of selenite to elemental Se(0) with simultaneous degradation of phenol by co-cultures of *Phanerochaete chrysosporium* and *Delftia lacustris*[§]

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The simultaneous removal of phenol and selenite from synthetic wastewater was investigated by adopting two different co-culturing techniques using the fungus *Phanerochaete chrysosporium* and the bacterium *Delftia lacustris*. Separately grown biomass of the fungus and the bacterium (suspended co-culture) was incubated with different concentrations of phenol (0–1,200 mg/L) and selenite (10 mg/L). The selenite ions were biologically reduced to extracellular Se(0) nanoparticles (3.58 nm diameter) with the simultaneous degradation of up to 800 mg/L of phenol. Upon growing the fungus and the bacterium together using an attached growth co-culture, the bacterium grew as a biofilm onto the fungus. The extracellularly produced Se(0) in the attached growth co-culture had a minimum diameter of 58.5 nm. This co-culture was able to degrade completely 50 mg/L phenol, but was completely inhibited at a phenol concentration of 200 mg/L.

Keywords: *Phanerochaete chrysosporium*, *Delftia lacustris*, phenol, selenite, Se(0) nanoparticles

Introduction

Petroleum and oil refineries discharge large quantities of phenol and polyaromatic hydrocarbons which have toxic, mutagenic, and carcinogenic effects on flora and fauna, even at very low concentrations (Abdelwahab *et al*., 2009). Rapid industrialization has led to the gradual accumulation of these recalcitrant phenolic compounds in water bodies (Pradeep *et al*., 2015), which are often found together with certain metalloids. For instance, selenium is a metalloid frequently found in the form of toxic selenite in oil refinery wastewaters (Lawson and Macy, 1995). There is a marginal difference in the selenium concentrations for being an essential nutrient or a toxic element (Tan *et al*., 2016). In some refinery wastewater, selenium concentrations ranging from 0.1 to 3.7 mg/L can be found (Nurdogan *et al*., 2012), while phenol concentrations can be as high as 230 mg/L (Almendariz *et al*., 2005; Hashemi *et al*., 2015). The toxic selenites in the presence of phenolic pollutants pose a great challenge, due to the high cumulative toxicity it exerts on all life-forms. Thus, the simultaneous biological removal of selenite and phenolic pollutants from petrochemical wastewater is of utmost importance.

Most of the previous studies have mainly focussed on bacterial anaerobic reduction, except few studies which have focused on aerobic bacterial reduction (Presentato *et al*., 2017). The detoxification mechanism of selenite ions and the selenite tolerance of fungi has also been reported in the literature (Gadd, 2007). The simultaneous reduction of selenite and degradation of phenol by fungal and bacterial cells has thus far, not been investigated. Fungal-bacterial co-cultures can mineralize the pollutants more efficiently because the substrates toxic to bacteria can be degraded by the fungus and vice versa (Cheng *et al*., 2017). Biological aerobic reduction of the selenium oxyanions coupled with the detoxification of these recalcitrant organic pollutants can lay the foundation of an inexpensive, energy-efficient, dual remediation process of hydrocarbons, and selenium oxyanions present in the effluents of the petrochemical industry.

P. chrysosporium is a well-studied white-rot fungus capable of detoxifying many polyaromatic hydrocarbons and phenolic compounds, mainly due to its ability to produce lignolytic enzymes during the secondary metabolic growth phase of the fungus. This white-rot fungus can reduce up to 10 mg/L selenite using glucose as the electron donor (Espinosa-Ortiz *et al*., 2015). The phenol degradation capacity in the presence of selenite by *P. chrysosporium* has been reported (Werkeneh *et al*., 2017). *Delftia lacustris* is a recently isolated bacterium which can reduce selenium oxyanions using lactate as the carbon source (Wadgaonkar *et al*., 2019).

A fungal-bacterial co-culture may thus stimulate the growth of the bacterium in the presence of phenol and enhance the reduction efficiency of selenite ions. The main objectives of this work were: (i) to investigate and compare the efficiencies of the simultaneous degradation of phenol and reduction of selenite ions by employing two different modes of growth of the fungal-bacterial co-culture, (ii) to identify the mechanisms involved in this type of reduction of selenium oxyanions coupled to the simultaneous degradation of phenol mediated by a fungal microbial consortium of *P. chrysosporium* and *D. lacustris*, and (iii) to characterize the Se(0) produced by both co-cultures.

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Materials and Methods

Microorganisms and growth of fungal-bacterial co-cultures

The white-rot fungus *P. chrysosporium* (MTCC187) was obtained from the Institute of Microbial Technology and was maintained on malt extract agar plates, at 37°C for 3 days and the spores were transferred to a glucose containing (10 g/L) medium (Espinosa-Ortiz *et al*., 2015) for the pelletization of the fungus. The fungal biomass was incubated at 30°C, for 2 days, at a pH of 4.5 under continous stirring at 150 rpm. The composition of the medium, except the carbon sources, was similar to Espinosa-Ortiz *et al*. (2015). Simultaneously, *D. lacustris* (NCBI MH158542) was separately grown in a lactate rich medium to obtain an actively growing bacterial biomass (Jørgensen *et al*., 2009). The fungal and bacterial biomass were thus grown separately. This type of co-culture is hereafter referred as "suspended co-culture." In order to determine the biodegradation capability of the fungus and bacterium separately, *D. lacustris* was incubated with phenol at concentrations ranging from 0 to 100 mg/L, in the presence of 0 and 10 mg/L of selenite.

When *D. lacustris and P. chrysosporium* were incubated together, the bacterium grew as a biofilm over the fungal cells and this type of co-culture is hereafter referred as "attached co-culture." Table 1 overviews the different carbon substrates used for establishing the co-culture. The 3-day old spore suspension of *P. chrysosporium* and the 2-day old active *D. lacustris* were mixed and allowed to grow together in glucose (5 g/L) and lactate (5 g/L) containing medium, for 2 days, at a pH of 6.5. The composition of the medium used for the growth of the attached co-culture, except the carbon sources, was similar to those reported by Espinosa-Ortiz *et al*. (2015).

Batch experiments

All experiments were carried out in airtight Erlenmeyer flasks (250 ml) with a working volume of 100 ml. In the suspended co-culture system, the fungal pellets were transferred to the phenol-rich medium after 2 days of incubation, wherein the phenol concentration was varied from 0 to 1,200 mg/L and 100 µl of the active *D. lactustris* culture was added to the medium and maintained at a pH of 6.5, temperature of 30°C and under agitation at 180 rpm. The selenite concentration was maintained constant at 10 mg/L. The control results for phenol degradation by *P. chrysosporium* solely was compared and standardised with respect to a previous work by Werkeneh *et al*. (2017).

In the attached co-culture system, the fungal-bacterial biomass was transferred aseptically to the medium wherein the phenol concentrations were varied from 0 to 200 mg/L, while the selenite concentration was maintained constant at 10 mg/L, at pH 6.5, 30°C, and agitation at 180 rpm agitation. About 1 ml of the sample from both co-culture systems were collected at an interval of 12 h to study the removal of phenol, growth rate of the microorganisms, the selenite reduction efficiency and the production of any detectable carbon compounds.

Analytical methods

Biomass analysis : In the suspended co-culture system, the biomass concentration in the liquid phase was determined gravimetrically by centrifuging 1 ml of the liquid broth and determining the dry weight of the pellet. It should be noted that the biomass in the liquid phase was primarily composed of bacterial biomass. Some mycelial fungal components could, nevertheless, have also been associated with the bacterial biomass during its estimation. The amount of fungal biomass present in the co-culture was estimated by measuring the dry weight of the fungal pellets. For the attached co-culture, the combined fungal-bacterial biomass and the biomass present in the suspension was measured on a dry weight basis.

Phenol and metabolite analysis

The concentration of phenol and volatile fatty acids were monitored by a gas chromatograph (GC) fitted with a flame ionization detector (FID) (Varian BV 430), according to the procedure described by Hashemi *et al*. (2015). 1 ml of liquid sample was withdrawn from the batch bottles, filtered and 1 µl of sample was injected to the GC. The samples were diluted 3 times for the measurement of phenol concentrations exceeding 400 mg/L. 50 µl of formic-isovaleric acid was added as an internal standard.

Scanning electron microscopy (SEM) - energy dispersive X-ray (EDAX) and transmission electron microscopy (TEM) analysis

A scanning electron microscope (Carl Zeiss, EVO 18) operating with an accelerating voltage of 15 kV was used to study the distribution of the nanoparticles in the biomass of both the suspended and attached co-culture systems. The samples were dried, fixed with 0.01% of glutaraldehyde solution on carbon coated plates, gold coated using a gold coater equipment (Hitachi, Model E1010) and subjected to SEM analysis. Later, EDAX was performed on the same samples to see the spatial distribution of the nanoparticles within the fungal-bacterial biomass. Approximately 1 ml of sample was collected after completion of the phenol degradation experiments from the fungal-bacterial suspended co-culture (120 h incubation) and the fungal-bacterial biofilm co-culture systems. About 10 µl of a dilute suspension of the culture medium containing the Se(0) was placed on a carbon coated Cu grid, which was then dried under a table lamp and stained subsequently for 3 min with Ruthenium vapor. A transmission electron microscope (JEOL JEM 2100 HR with EELS), with an accelerating voltage of 200 kV was used to analyze the particle size and shape of the Se(0) in the liquid medium.

Dynamic light scattering (DLS) for zeta potential and size distribution

The zeta potential and particle size distribution of the Se(0) produced in the two different co-culture systems were determined by the dynamic light scattering technique (DLS-MALVERN). Under identical conditions of the solution viscosity (0.8872 cp) and dispersant refractive index (1.330) in water as the dispersion medium, samples collected (after 120 h of incubation) from both the suspended and attached co-culture were analysed.

Fourier transform - infrared spectroscopy (FT-IR) analysis

The Se(0) and biomass of the co-culture system were finely ground and powdered by a fiber microtome and then blended with KBr. The mixture was converted into ultra-thin pellets by the application of pressure using a hydraulic pressure system (Atlas Manual Hydraulic Press 15T). The liquid samples of the degraded phenol medium were analysed in attenuated total reflection (ATR) mode of the FT-IR (Jasco FT-IR 6300), as described by Mandal and Chakrabarty (2011).

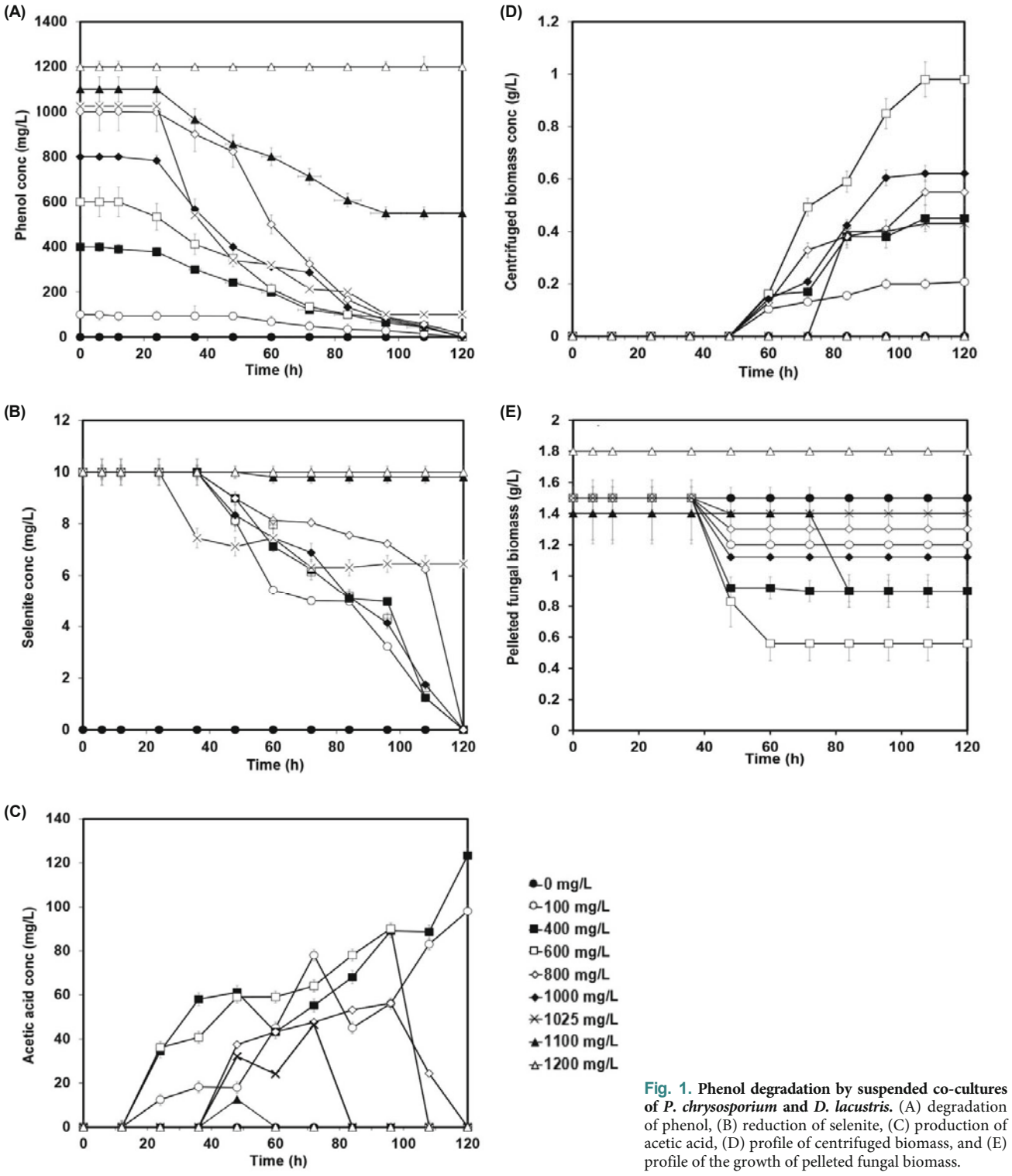

Fig. 1. Phenol degradation by suspended co-cultures of *P. chrysosporium* and *D. lacustris*. (A) degradation of phenol, (B) reduction of selenite, (C) production of acetic acid, (D) profile of centrifuged biomass, and (E) profile of the growth of pelleted fungal biomass.

Results

Suspended growth co-culture incubations

Biodegradation of phenol : The co-culture of *P. chrysosporium* and *D. lacustris* completely degraded up to 800 mg/L phenol with the simultaneous reduction of selenite to Se(0) at an intial pH of 6.5 within 120 h (Fig. 1A). The phenol degradation was almost negligible (7.2%) during the first 48 h of incubation. At concentrations of 1,000 and 1,025 mg/L of phenol, 987 and 800 mg/L of phenol were degraded, respectively, in 120 h and the residual phenol remained non-degraded. At the highest phenol concentration tested, i.e. 1,200 mg/L, the degradation was completely inhibited. The degradation rate varied from 1.33 mg/L/h (for an initial concentration of 100 mg/L) to 12.9 mg/L/h (for an initial concentration of 1,025 mg/L). However, it is noteworthy to mention that an active sole culture of *D. lacustris* was unable to degrade phenol even at low concentrations (10 mg/L).

Reduction of selenite ions

The suspended growth co-culture reduced about 10 mg/L of selenite ions to Se(0) and other selenium compounds in 72 h, with the simultaneous and complete degradation of 100–400 mg/L phenol (Fig. 1B). The selenite concentrations decreased from 8 to 0 mg/L in the presence of 600–1,100 mg/L phenol, respectively. Selenite was partially reduced to Se(0) by the fungus *P. chrysosporium*, as visualised by the orange-red colour of the originally white coloured fungus. *D. lacustris* was also found to reduce selenite to Se(0) as visualised by the orange-red colour of the bacterial biomass in the suspension. As *D. lacustris* and *P. chrysosporium* were growing in co-culture, the exact amount of selenite reduced by each of the two microorganisms could not be determined.

Acetic acid production

During phenol degradation by the co-culture, varying concentrations of acetic acid were produced in the batch incubations. The highest acetic acid concentration produced was 123 mg/L, in 120 h, by the suspended co-culture system containing an initial phenol concentration of 400 mg/L (Fig. 1C). No acetic acid production was observed during the first 48 h of co-culture incubation. Acetic acid was produced upon the initiation of the phenol degradation, at concentrations ranging between 100 and 1,100 mg/L. As no other carbon source was present in the medium, acetic acid was likely a metabolite of the phenol degradation.

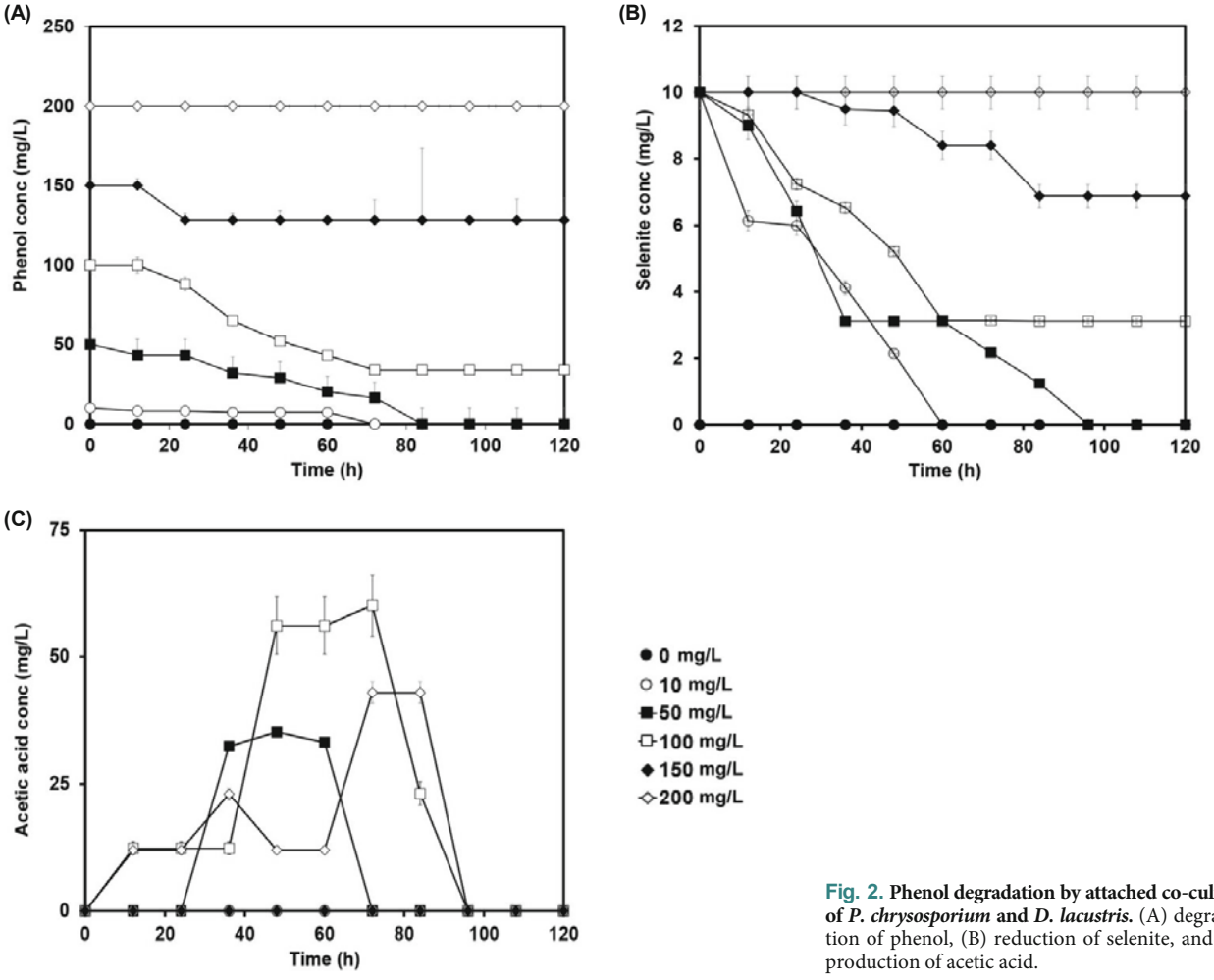

Fig. 2. Phenol degradation by attached co-culture of *P. chrysosporium* and *D. lacustris*. (A) degradation of phenol, (B) reduction of selenite, and (C) production of acetic acid.

Biomass concentration profiles

The biomass concentration in the liquid medium increased to ~0.2 g/L of dry weight after 48 h of incubation (Fig. 1D). The highest (~1 g/L dry weight) biomass concentration was achieved during the incubation with 600 mg/L of phenol. Thereafter, the final biomass concentration decreased to 0.55, 0.62, and 0.43 g/L in incubations with 800, 1,000, and 1,025 mg/L of phenol. The biomass consisted mainly of bacterial biomass, but still some fungal filaments were present. There was no significant change in the biomass of the fungal pellets until 48 h; however, afterwards, the fungal pellets began to lose some of the hairy filaments at phenol concentrations of 400 and 600 mg/L (Fig. 1E).

Attached growth co-culture system

Biodegradation of phenol : The attached co-culture biomass shows partial degradation of phenol, up to 100 mg/L (Fig. 2A) within 120 h (at the rate of 0.83 mg/L/h), whereas phenol degradation was completely inhibited at only 200 mg/L. The degradation decreased probably due to the presence of the layer of the bacterial biomass over the fungal biomass that presumably decreased the contact of the fungus to phenol. Thus, an increase in the phenol concentrations from 100 to 200 mg/L inhibited the degradation process.

Reduction of selenite ions

The attached fungal-bacterial biomass culture was found to reduce up to 6.88 mg/L of selenite ions within 72 h (Fig. 2B). The Se(0) was found to be sequestered by the fungal biomass of the attached growth co-culture system and the colour was orange-red. In contrast to suspended growth co-culture, no orange-red coloration was observed in the bacterial biomass attached to the fungus. Concerning biomass growth, there was no significant increase in the biomass content in the liquid medium and the fungal-bacterial pellets.

Acetic acid production

Acetic acid was still produced despite the much lower amount of phenol degraded (Fig. 2C). At an initial concentration of 10 mg/L phenol, no acetic acid was produced. However, at an initial phenol concentration of 50 mg/L, 33 mg/L of acetic acid was produced after 60 h of incubation of the co-culture. For an initial phenol concentration of 100 mg/L, the acetic acid concentration amounted to 56 mg/L after 60 h incubation, after that, the acetic acid concentration was almost constant.

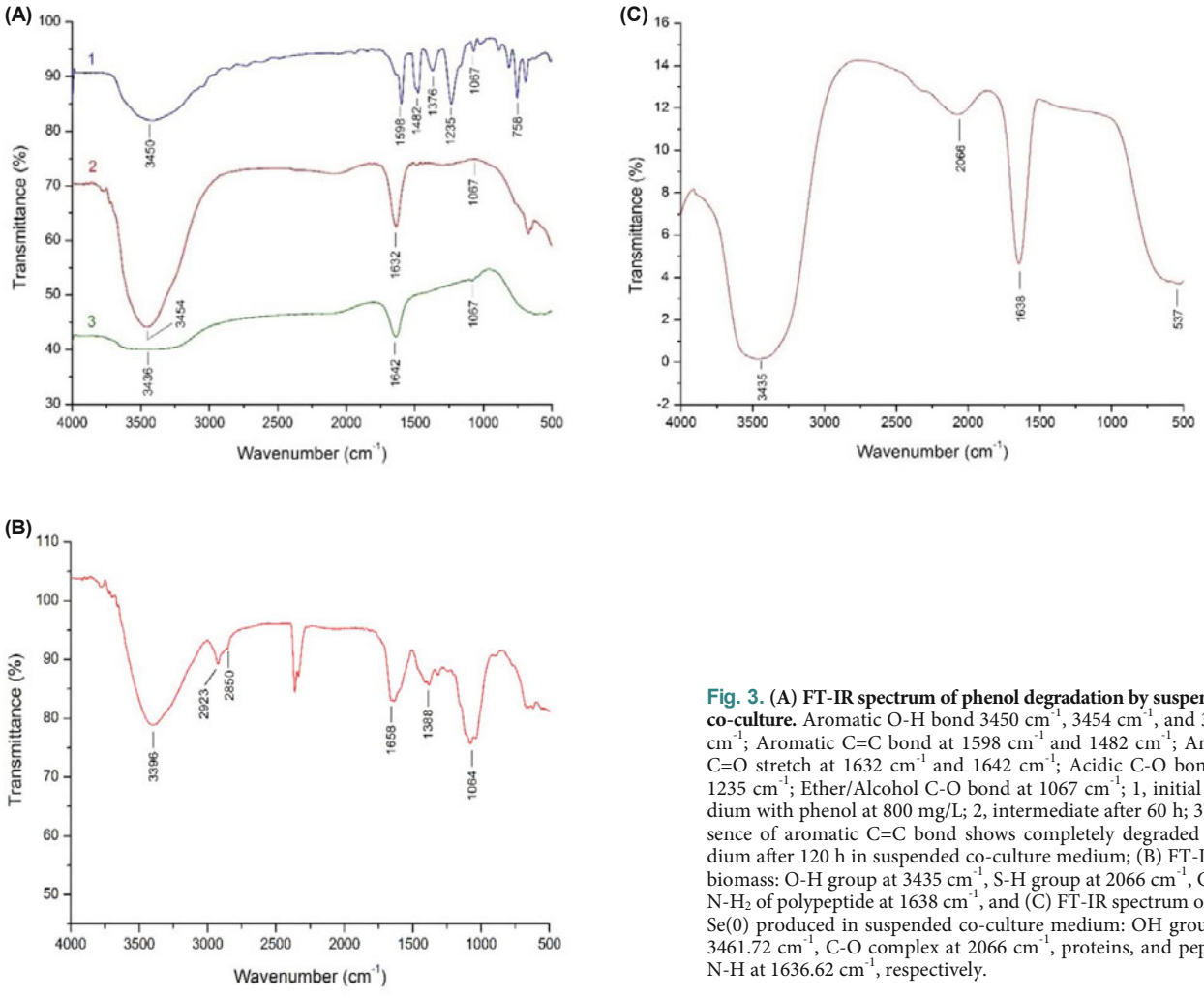

Fig. 3. (A) FT-IR spectrum of phenol degradation by suspended co-culture. Aromatic O-H bond 3450 cm^{-1}, 3454 cm^{-1}, and 3436 cm^{-1}; Aromatic C=C bond at 1598 cm^{-1} and 1482 cm^{-1}; Amide C=O stretch at 1632 cm^{-1} and 1642 cm^{-1}; Acidic C-O bond at 1235 cm^{-1}; Ether/Alcohol C-O bond at 1067 cm^{-1}; 1, initial medium with phenol at 800 mg/L; 2, intermediate after 60 h; 3, absence of aromatic C=C bond shows completely degraded medium after 120 h in suspended co-culture medium; (B) FT-IR of biomass: O-H group at 3435 cm^{-1}, S-H group at 2066 cm^{-1}, C-O-N-H_2 of polypeptide at 1638 cm^{-1}, and (C) FT-IR spectrum of the Se(0) produced in suspended co-culture medium: OH group at 3461.72 cm^{-1}, C-O complex at 2066 cm^{-1}, proteins, and peptide N-H at 1636.62 cm^{-1}, respectively.

FT-IR analysis

The phenol degradation was evident from the disappearance and appearance of related functional groups. Figure 3A shows the IR spectrum of the initial co-culture system containing mostly phenol. The spectrum resembles that of phenol with the following characteristic peaks: (i) a broad band in the region of 3,489 cm^{-1} representing the intermolecular H-bonded O-H. The O-H stretching of water and its intermolecular H bonding overlaps in this region (observed only if water is present in the system), and ii) the aromatic ring stretching of C-H, indicated by the small peak at 3,045 cm^{-1}, whereas the C ring stretching is given by the presence of multiple peaks at 1,596.43, 1,499.97, and 1,474 cm^{-1}, respectively. The OH bonding is represented by the peak at 1,368.23 cm^{-1}, while the C-O stretching is given by 1,234.66 cm^{-1}. The peaks at 811.18 cm^{-1} and 753.66 cm^{-1} represented the C-H bending, respectively.

The FT-IR spectra of the co-culture system of the liquid medium after biodegradation were different from the initial co-culture medium. The aromatic C-H stretching as observed previously at 3,054 cm^{-1} was not present in the co-culture system of the liquid medium. Besides, C=C ring stretching at 1,495 cm^{-1} and 1,468 cm^{-1}, which usually appear for phenol, was also not present. C-O stretching for phenol observed at 1,220 cm^{-1} was absent. In-plane O-H bending of phenol at 1,360 cm^{-1} does not appear in the system. On the other hand, a new peak appeared in the region of 1,725–1,720 cm^{-1}, indicating the stretching due to the C=O bond in acids (e.g. cis-cis muconic acids). The sharp peak in the region of 2305 cm^{-1} indicates the presence of C=C bonds of non-aromatic compounds which might be an intermediate compound that formed during phenol degradation. The large peak appearing in the region 3,400–3,300 cm^{-1} might be indicating hydrogen bound O-H stretching. This peak of phenol overlaps with the O-H peak of water present in the medium. These shifts in peaks confirm that phenol degradation has occurred in the incubations.

The FT-IR spectra of the co-culture also shows that thiols, i.e. S-H groups (Fig. 3B) are present, typically represented by the peak at 2850 cm^{-1} (Banwell and McCash, 1983). The S-H group may be related to glutathione and the glutathione peroxidase enzyme helps in the detoxification of selenite ions to Se(0) according to Eq. (1) (Kessi and Hanselmann, 2004):

$$\text{S-H-(Glutathione)} + \text{Selenite} \rightarrow \text{Se(0)} \quad (1)$$

The FT-IR spectra of the Se(0) produced as a result of the suspended co-culture system contains a sharp peak at 2066 cm^{-1} (Fig. 3C), indicating the presence of carbonyl complexes attached to the nanoparticles. A small band or peak near 2066 cm^{-1} was observed and based on the information available in the literature (Vessieres *et al.*, 1999), this indicates that some carbonyl complexes of various transition elements are present in this region. In the present case, with only selenite present in the incubation system, Se can form a complex with the carboxylic compounds derived from the phenol degradation.

Zeta potential analysis

The suspended fungal-bacterial co-culture had a zeta potential of -28.7 mV (Supplementary data Fig. S1A), while the attached fungal bacterial co-culture had a zeta potential of -12.9 mV (Supplementary data Fig. S1B), respectively. The conductivity of the suspended co-culture system was 0.207 mS/cm. In the suspended co-culture system, all the selenium particles had nanometric dimensions, which was interpreted from the curve size of the plot between diameter and intensity (%), with the highest at ~75 nm. A small width of the distribution curve indicates uniformity of the particle size,

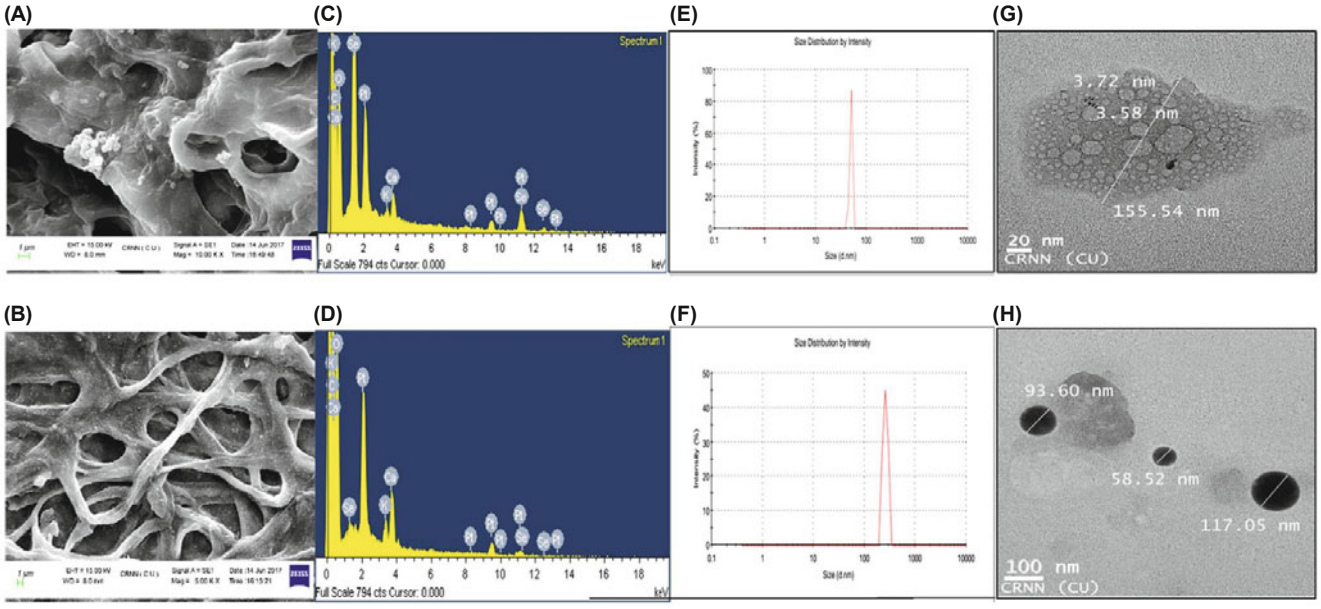

Fig. 4. Electron microscope analysis of the fungal-bacterial biomass: (A) suspended and (B) attached growth co-culture. EDAX of fungal and fungal-bacterial biomass: (C) suspended and (D) attached co-culture, size distribution of Se(0) in (E) suspended, and (F) attached co-culture. TEM of Se(0) produced in (G) suspended and (H) attached co-culture system.

i.e. the lower end fractions are on an average close to the higher end fragments in the statistical distribution of particles (Fig. 4D). This was further supported by the polydispersity index, which was found to be less than unity. In attached co-culture system, Se(0) had an average particle size of ~250 nm in diameter. Nevertheless, the visible particles has sizes >100 nm.

SEM-EDAX analysis of the biomass

The SEM analysis (Fig. 4A and B) reveals mainly changes in the structural morphology of the fungus predominating the fungal-bacterial co-culture. The suspended co-culture exhibited a spongy biomass and has a non-layered structure. In contrast, the attached growth system exhibited a layered structure where the reduced Se(0) particles spread in a non-uniform manner over each of the layers. The suspended co-culture also shows a non-uniform distribution of the reduced selenium, which was present not only on the surface but also deposited on the cell walls. EDAX analysis showed less sequestration of Se(0) in the visible predominant fungal biomass (Fig. 4C and D).

TEM analysis of the liquid phase

TEM analysis after the biodegradation tests reveals the fact that the Se(0) were formed mostly as nanospheres in the liquid medium. Table 2 shows the size distribution of Se(0) produced. For the suspended co-culture system, the Se(0) produced had the smallest diameter of 3.58 nm (Fig. 4G), while in the attached co-culture system, the smallest size of the Se(0) produced was 58.52 nm (Fig. 4H). The observed Se(0) particles were mostly nanospheres. The Se(0) produced in the medium was more likely expected be produced by *D. lacustris*, because the Se(0) produced by the fungus is usually entrapped in the fungal biomass (Espinosa-Ortiz *et al.*, 2015).

Discussion

Phenol removal by suspended and attached co-cultures of *P. chrysosporium* and *D. lacustris*

This study showed that the initial pH played an important role in the development of the fungal bacterial co-culture and subsequent degradation of phenol. The pH of 6.5 was found to be suitable for sustaining the metabolism of both the bacterium and the fungus. The initial lag period of 48 h during the uptake of phenol may be attributed to the acclimatization period of the fungal-bacterial system to the medium conditions and viability of the bacterial cells only increased with the adaptation of the fungus to the incubation conditions. However, *P. chrysosporium* was observed to degrade up to 400 mg/L of phenol at an initial pH of 4.5 (Werkeneh *et al.*, 2017). The suspended co-culture of *P. chrysosporium* and *D. lacustris* degraded phenol at a concentration of 1,025 mg/L at a maximum rate of 12.9 mg/L/h. Attached co-culture degraded phenol at an initial concentration of 100 mg/L at a maximum rate of 0.83 mg/L/h, but phenol degradation is completely inhibited at 200 mg/L. This decrease may be due to the presence of bacterial biomass on the fungus On the other hand, *D. lacustris* could not grow on phenol and reduce selenite simultaneously at pH ranging from 5.0–7.0 (data not shown). Therefore, the bacterium grew on the intermediates produced by the fungus, e.g. aromatic acid like cis-cis muconic acid, detected from FT-IR analysis (Fig. 3A).

Analysis of the mechanism of selenite reduction

The reduction of selenite to Se(0) followed the same pattern as the phenol degradation (Fig. 1A and B), emphasizing the simultaneous detoxification of selenite and phenol. The FT-IR analysis of the biomass reveals the generation of S-H groups bound to glutathione that facilitated the detoxification of se-

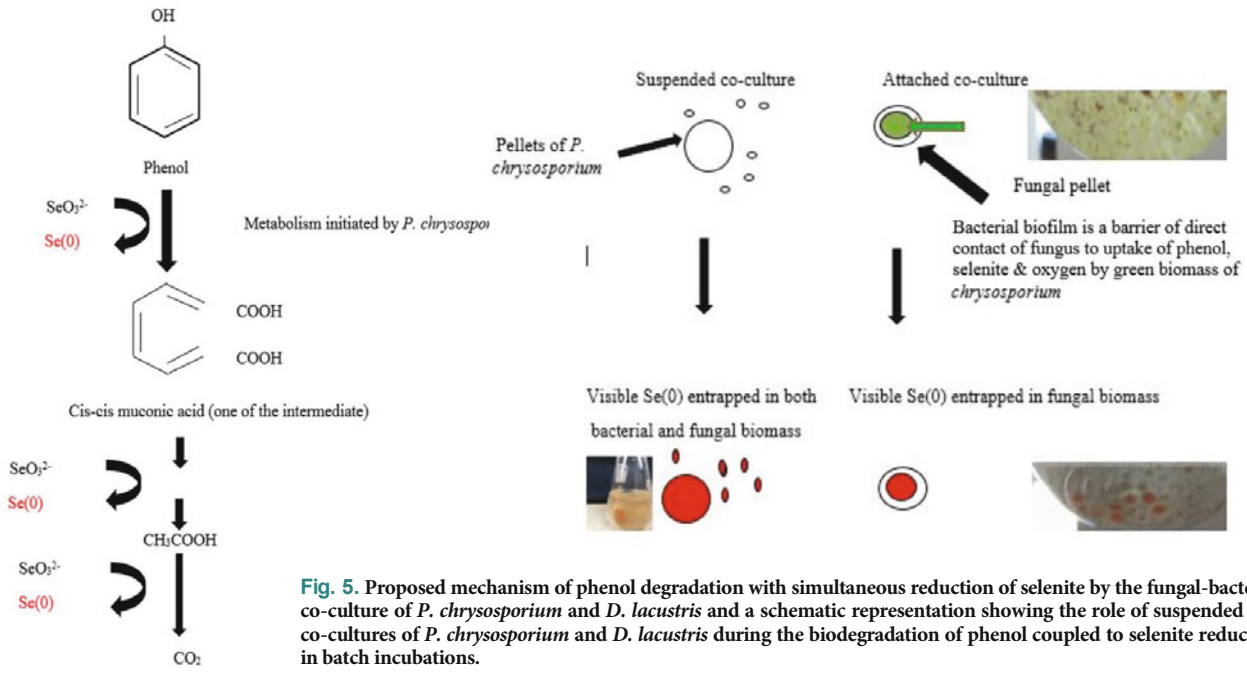

Fig. 5. Proposed mechanism of phenol degradation with simultaneous reduction of selenite by the fungal-bacterial co-culture of *P. chrysosporium* and *D. lacustris* and a schematic representation showing the role of suspended and co-cultures of *P. chrysosporium* and *D. lacustris* during the biodegradation of phenol coupled to selenite reduction in batch incubations.

lenite ions (Kessi and Hanselmann, 2004). The Se(0) appeared in the medium only after 48 h of incubation indicating the fact that, after acclimatization and initiation of the degradation of phenol, the intermediates of phenol degradation were utilised by the bacterium for the reduction of selenite to Se(0). After 60 h, however, the fungal pellets turned orange-red in colour (Fig. 6), indicating the bioconversion of selenite to Se(0).

The selenite reduction by the attached-growth incubation was more efficient compared to the suspended growth culture, i.e. 6.88 mg/L of selenite was degraded in the presence of 150 mg/L phenol. On the contrary, the suspended co-culture incubation was more efficient for the degradation of phenol. In the latter system, the layer of the bacterium surrounding the fungal pellets hindered the availability of oxygen and growth of the fungus, its metabolism, and subsequently, the uptake of phenol and selenite.

Proposed mechanism for the degradation of phenol coupled to selenite reduction

The fungus likely initiated phenol degradation with the production of an intermediate metabolite, which was consumed by the bacterium (Fig. 5). The presence of the metabolite stimulated phenol degradation and increased the degradation capacity of the fungus. Production of acetic acid upon initiation of the phenol degradation and the depletion of acetic acid later, indicates its consumption. Hence, it is more likely that an intermediate metabolite formed was utilised by the bacterium. The bacterial layer outside the fungal structure in the attached co-culture system most likely reduces the availability of phenol to the fungus, resulting in decreased degradation and hence the reduced concentration of the metabolite. Thus, the bacterial biofilm poses a major mass transfer limitation to the substrate uptake. Moreover, the respiratory activity of *P. chrysosporium* can also be negatively affected or inhibited by the multi-layered bacterial growth over the fungus, the presence of selenite ions and anoxic zones, which has also been reported in *P. chrysosporium* growing in drip-flow bioreactor configuration (Espinosa-Ortiz *et al*., 2016).

Biomass growth

In the suspended co-culture system, although a slight reduction in the biomass content was observed after 48 h of incubation, the dry weight remained constant till 120 h (Fig. 1E) indicating spongy and increased filamentous growth. After 48 h of incubation, the bacterial biomass was found to increase in weight (Fig. 1D). The delayed growth of the bacterial cells can be attributed to the adaptation of the bacterial cells to the phenol containing medium and the bioconversion of phenol to comparatively more bioavailable and less toxic intermediate compounds. Fungal biomass has also been reported to adsorb phenol from the medium (Farkas *et al*., 2013). Pelletization of fungal cells decreases with an increase in the phenol concentrations and the fungus becomes more filamentous (Figs. 3A and 5). This filamentous growth is due to its metabolic drive to consume the nutrients and make the necessary nutrients available for the bacterium to consume (Klein and Paschke, 2004).

In the attached growth system, the selenite was found to be mostly reduced and localized inside the fungal biomass with a white sheath of the bacterium surrounding the fungal biomass. Compared to the suspended co-culture system, red Se(0) was less prevalent in the medium. According to Espinosa-Ortiz *et al*. (2016), in a drip-flow bioreactor treating selenite, *P. chrysosporium* was more dense and less filamentous as observed in the attached biofilm co-culture system of the present study. All previous studies involving selenite have only focused on mono-cultures of fungal or yeast biofilms like *Candida albicans* and *Candida topicalis* (Harrison *et al*., 2007; Espinosa-Ortiz *et al*., 2016; Werkeneh *et al*., 2017). These studies have reported that the presence of selenite had inhibited hyphae formation, contamination with other bacteria and change in the biofilm structure and composition after prolonged treatment. Hence, further morphological studies are required to ascertain the effect of selenite on the co-culture composition and growth pattern.

Zeta potential and size distribution of the Se(0) particles

Table 2 compares the dimensions of the Se(0) particles produced by different biocatalysts. The suspended growth co-culture system had a zeta potential of -28.7 mV and an average particle size of 70 nm (Fig. 4E). In the facultative anaerobe *Citrobacter freundii* Y9, the average zeta potential value was -20.61 (± 1.29) mV for EPS (extra polymeric substance) in groundwater and -28.02 (± 0.08) mV for EPS in soil solution, respectively. These negative values of zeta potential have shown to stabilise the Se(0) particles (Wang *et al*., 2018). On comparing the two different co-culture systems, the suspended co-culture system was found to produce more stable Se(0) particles as evidenced from the relatively lower negative zeta potential of -28.7 mV in relation to -12.9 mV observed with the attached fungal-bacterial co-culture.

For the suspended co-culture system, the Se(0) produced had the smallest diameter of 3.58 nm (Fig. 4G). In this system, there could be a layer of EPS surrounding the nanoparticles, which possibly provided the necessary zeta potential and hence the stability. However, this was not so much pronounced in the attached co-culture system and hence the Se(0) particles exhibited a tendency to coalesce and form aggregates. This also led to the formation of relatively larger sized Se(0) particles in the attached co-culture system, wherein the smallest size of the Se(0) produced was 58.52 nm (Fig. 4H). In order to elucidate the factors that govern the size distribution of the produced Se(0) particles in co-culture systems, more in-depth studies are required to characterise the nature of EPS produced during the simultaneous removal of phenol and selenite.

Practical applications

P. chrysosporium is an efficient polyaromatic hydrocarbon (PAH) degrading white-rot fungus (Paszczynski and Crawford, 1995; Huang *et al*., 2017). In a recent study, the fungus improved the degradation capacity of an activated sludge reactor treating coking wastewater (Hailei *et al*., 2017). In another study involving mixed biocatalysts, the fungus reduced the toxicity of Pb^{2+} to the native microorganisms of a Pb-contaminated agricultural waste composting site. The fun-

gus is also a selenite reducing organism, capable of degrading phenol (400 mg/L) in the presence of selenite ions (15 mg/L) (Espinosa-Ortiz *et al*., 2015; Werkeneh *et al*., 2017). In a continuously operated upflow bioreactor inoculated with *P. chrysosporium*, removal efficiencies ranging from 50–75% (at a critical loading rate of 12 mg/L/h) and 75–90.8% (at a critical loading rate of 4.3 mg/L/h) was achieved (Werkeneh *et al*., 2017).

The present study focussed on the capability of *P. chrysosporium* to stimulate the activity of a Se-reducing bacterium, i.e. *D. lacustris*, which is not capable of degrading phenol (Fig. 1D). Refinery effluents have a neutral to alkaline pH (7.0–8.0), where the co-culture of *P. chrysosporium* and *D. lacustris* can sustain, whereas the single culture of *P. chrysosporium* was only able to degrade 67.3% of 400 mg/L phenol at an acidic pH of 4.5. A pure culture of *P. chrysosporium* solely was inhibited at phenol concentrations of 600 mg/L (Werkeneh *et al*., 2017). In the suspended co-culture, the inhibitory concentration of phenol was 1,200 mg/L (Fig. 1A). The co-metabolic degradation of phenol thus significantly enhanced the degradation efficiency. Additionally, the reduction of selenite in the co-culture system produces Se(0) nanoparticles, which has potential anticancer effect, antimicrobial activity and has several applications in the field of pharmaceutical, medical and environmental sciences (Guleria *et al*., 2018). Poly aromatic hydrocarbon (PAH) containing wastewater also contains toxic metal ions like Cr^{2+}, Cd^{2+}, Zn^{2+}, As^{3+}, As^{5+}, and Hg^{2+} (Liu *et al*., 2017), which may affect the selenite reducing capacity of the co-culture. More experiments with different concentrations and combinations of heavy metals are required to elucidate the effects of these metal ions on the co-culture.

The capacity of the suspended growth co-culture system to degrade phenol was found to be higher compared to the attached growth co-culture system. Surprisingly, the two different co-culture methods produced Se(0) of different size ranges by reducing selenite in the presence of phenol as the electron donor. In the suspended growth co-culture system, a filamentous and sponge-like fungal morphology was apparent, whereas a rigid and layered fungal-bacterial morphology was conspicuous in the attached growth co-culture system.

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Conflict of Interest

Competing financial interests: S.C. declares no competing financial interests. E.R.R. declares no competing financial interests. P.N.L.L. declares no competing financial interests.

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