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Polycyclic aromatic hydrocarbons (PAHs) degradation potential, surfactant production, metal resistance and enzymatic activity of two novel cellulose-degrading bacteria isolated from koala faeces

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Short running title: PAH degradation by novel bacteria isolated from koala faeces

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Abstract

Cellulose-degrading bacterial strains, KC3 and 5, capable also of degrading both low and high molecular weight PAHs were isolated from koala faeces for the first time. KC3 and 5 belong to the genera *Bacillus* and *Pseudomonas*, respectively. These strains completely degraded the model 3-(Phenanthrene), 4-(Pyrene) and 5-(Benzo-a-Pyrene) ring PAHs at 6, 7 and 40-50 days, correspondingly. They were shown to be able to produce a rhamnolipid type of biosurfactant during PAH degradation. The biosurfactants produced from both the strains showed good pH (2-12) as well as thermal (up to 80°C) stability and were able to tolerate up to 20 g/L salinity. The strains also had resistance towards heavy-metals, attributed to the amount of biosurfactant produced. The *Bacillus* strain in particular showed excellent metal resistance; the minimum inhibitory concentrations were 5 (Cd^{2+} , Cu^{2+}) and 7 (Pb^{2+} , Zn^{2+}) mg/L of relatively bioavailable metal ions but >15 mg/L metal concentrations were lethal to the microbe. Additionally, both strains possessed activity of more than one extracellular enzyme (cellulase, lipase and protease). The limiting factors in PAH biodegradation are low PAH bioavailability and microbial intolerance towards HMW PAHs and co-contaminants (heavy metals). The novel strains identified thus had (a) potential to biodegrade both LMW and HMW PAHs, b) pH, thermal and saline-tolerant biosurfactant production that aids PAH solubility enhancement, and importantly, c) heavy metal resistance. Both *Bacillus* and *Pseudomonas* strains are appropriate candidates in field-scale PAH bioremediation at mixed contamination sites and for several industrial applications due to their enzymatic activities.

Keywords: Bioremediation; Polycyclic aromatic hydrocarbon degradation; Koala faeces; Metal resistance; Biosurfactants

1. Introduction

PAHs are a group of the most widespread organic pollutants, being widely distributed in soils, sediments, air, marine organisms and plants (Wilcke, 2007). Currently the fate of PAHs in nature is of great environmental concern due to their potential toxicity, mutagenicity and carcinogenicity. During the last 20 years, various techniques have been introduced to remediate PAH-contaminated soils, including physical, chemical and biological methods. Chemical and physical treatments, such as solvent flushing or thermal desorption, are the most efficient technologies to remove PAH from soils. However, these treatments remain expensive and are non-specific as the entire soil organic content is destroyed (Venkata Mohan et al., 2009). Only bioremediation is widely recognized as an economically-viable green approach to remediate PAH-contaminated sites (Nnamchi et al., 2006). Microbial biodegradation is the one and only effective and inexpensive approach, as long as the correct population of microorganisms is employed and the environmental conditions are conducive to the biodegradation of PAHs. To date, the biological clean-up of HMW PAH-contaminated zones, particularly at mixed contaminated sites (sites contaminated with both PAHs and heavy metals), remains a major challenge as there is very little data on the microbial degradation process. One promising solution to the existing limitations is the selection of a potent HMW PAH degrader with metal tolerance and surfactant production abilities (surfactants enhance the contaminant solubility). Only the findings of Thavamani et al. (2015) have indicated an ability of metal-tolerant HMW PAH-degrading bacterial consortia to remediate mixed contaminated soils. However, these authors did not consider ability to produce biosurfactants.

Digestive tracts of herbivorous animals have always attracted researchers as potential sources of bacteria with unique properties (Zhang et al., 2009). Many new promising microorganisms have been isolated from animal faeces. The koala (*Phascolarctos cinereus*) is an arboreal herbivorous marsupial native to Australia and feeds almost exclusively on eucalyptus leaves which are low in protein but high in recalcitrant substances such as lignin, cellulose, tannin and essential oils. Both aerobic and anaerobic bacteria are the main degraders of eucalyptus leaves in the hindgut of koala and this process requires the activity of cellulases, hemicellulases, ligninases, tannase and other enzymes that are of high biotechnological interest (Peterson et al., 2011). Recently, Peterson et al. (2009) isolated a number of fungi from koala faeces with diverse enzymatic activities against recalcitrant substrates such as lignin and cellulose. Cellulose-degrading bacteria play an important role in the enzymatic degradation of cellulose present in eucalyptus leaves for releasing the nutrients

for energy. Reports concerning the microflora of koala intestines are available only for tannin-protein-lignin degraders; there is still a paucity of data on the predominant cellulose-degrading microbes of koala.

The few studies published to date reveal that bacteria isolated from uncontaminated environments possess both enzymatic activity and hydrocarbon degradation potential (Margesin et al., 2003). There thus exists a possibility for the intestinal gut bacteria of koala to possess both enzymatic and biodegradation abilities. No attempt has so far been made to explore the cellulose degraders of koala faeces for their bioremedial (i.e. if the strains possess hydrocarbon degradation potential) and even industrial application. The study we report here was therefore established to: (a) isolate and characterize cellulose-degrading bacteria from koala faeces, (b) analyze the ability of the strains to degrade LMW and HMW PAHs, (c) identify the microbes by phylogenetic analysis, (d) determine the biosurfactant production potential of the strains during PAH utilization, (e) assay the stability of the biosurfactant produced, (f) characterize the microbial heavy metal tolerances and (d) evaluate the enzymatic activities of the microbes identified.

2. Materials and Methods

2.1. Sample collection

Koala faeces were obtained from Adelaide Zoo, South Australia. The collected faecal pellets were lightly brushed to remove any adhering soil and their outer layer removed by scraping with a sterile razor to detach any other foreign material before use in experiments.

2.2. Isolation and enumeration of cellulose-degrading bacteria with bioremediation potential

Bacteria were extracted from the faeces samples by blending 1 g wet weight with 9 ml of Tween buffer (1% Tween 80 and 0.9% NaCl) for 1 min before serial dilution. Bacterial strains were grown and isolated by plating on CMC-Congo red agar for enumeration of cellulose degraders by using a modified Hendricks method (Hendricks et al., 1995). Morphologically different colonies with a halo zone were selected and purified. To evaluate their bioremediation potential, cultures were grown at 25°C for 7 days in M9 medium (Thavamani et al., 2015) supplemented with 100 mg/L Phenanthrene (Phe) with shaking (120 rpm). Any increase in OD₆₀₀ was taken as an indication of a strain's ability to utilize PAH as its sole C-source (Ling et al., 2011). PAH-degrading strains were then subcultured and maintained in M9 broth with Phe (100 mg/L) as the C-source. Once the cells had reached the

exponential phase, they were harvested by centrifugation (4000 rpm for 20 min at 4°C), washed twice with sterile distilled water and resuspended in 5 mL of M9 medium. The resulting suspension served as the inoculum for further studies.

2.3. Molecular identification

Genomic DNA was extracted using a microbial DNA isolation kit (Mo-Bio, USA), following the manufacturer's instructions. The extracted DNA was used as the template in PCR amplification of 16S rRNA gene sequences with the forward 5'-AGTTTGATCCTGGCTCAG-3' and reverse 5'-AAGGAGGTGATCCANCCRCA-3' primers. The amplified PCR products were purified using an Ultraclean® PCR clean-up kit (Mo-Bio, USA) and sequenced at Flinders Sequencing Facility, Adelaide. BlastN was used to carry out the initial sequence analysis. Phylogenetic analysis was performed with MEGA 5 using neighbour-joining test. The nucleotide sequence of 16S rRNA gene of strains KC3 and 5 were deposited in GenBank under the accession numbers JN897277 and JN897279.

2.4. PAH degradation experiment

A batch experiment was conducted to determine the degradation potential of LMW and HMW PAHs by strains KC3 and 5. Phenanthrene (3-ring), Pyrene (4-ring) and Bnzo-a-Pyrene (5-ring) were chosen as the model PAH compounds. The PAH degradation study in liquid culture was conducted using 10 mL of broth in sterile 40 mL amber glass vials sealed with Teflon-lined screw caps. One percent of the concentrated inoculum was inoculated into the medium containing individual PAHs (200 mg/L Phe; 200 mg/L Pyr; 50 mg/L BaP) to provide an initial cell density (OD₆₀₀) of 0.5. The inoculated tubes were incubated on a rotary shaker (120 rpm) at 25°C in a walk-in incubator room. Controls without inoculations were also incubated under the same conditions. Samples were collected at different time intervals to determine the PAH concentrations as well as bacterial growth assayed by protein content. All experiments were performed in triplicate including the controls.

2.5. Analytical methods

All chemicals used in the experiments were of Analytical Grade, obtained from Sigma-Aldrich, Australia. All solutions were prepared with Milli-Q water (18 Ω/cm, Milli-Q, ELGA Labwater, UK). The PAH stock solutions were prepared in dimethylformamide and stored in the dark at 4°C. USEPA method 3550B was used for extraction of PAHs. The liquid cultures (10 mL) were extracted into 10 mL of hexane/acetone (1:1 v/v) and ultrasonicated

for 15 min (12 kHz sweep bandwidth, Soniclean Pulse Swept® Power Ultrasonicator, Soniclean Pty. Ltd., Australia). The extracts were then concentrated to 1 mL under a stream of nitrogen gas and analyzed by high-performance liquid chromatography (Agilent HPLC 1200, Japan, equipped with a UV detector set at 254 nm) according to EPA method 8310. A PAH reverse phase column (4.6 × 150 mm with 5 µm packing, Agilent Technologies Pty. Ltd., Australia) was used with a methanol gradient from 80 to 100% at a flow rate of 1 mL min⁻¹. The column temperature was maintained at 30°C. External calibration was performed for individual PAHs using PAH standards (Supelco, Bellefonte, PA, USA). For quality control, experiments on recovery were carried out by spiking a known concentration of PAH standards (5 and 10 mg/L) in liquid cultures. The results showed a good recovery of 90 ± 4.2%. Accuracy and precision of the calibration were checked periodically by injecting a known standard and a blank solvent into the column for every ten samples analyzed. The detection limit of the HPLC system was 50 µg/L. Subsequently, cell growth was monitored at designated time intervals by measuring the total cell protein content using the Lowry method (Classics Lowry et al., 1951) with Bovine serum albumin as the standard (1 – 100 µg/mL). Experimental culture media were centrifuged at 10,000 rpm for 10 min using a microcentrifuge (Sigma, Germany). The cell pellets were suspended in 1 mL of 1M NaOH at 70°C for 5 min then vortexed until the cell pellets disappeared and the solution became transparent. 500 µL cell lysate was incubated in the dark for 20 min with 700 µL Lowry's reagent, followed by 30 min incubation at 25°C with 100 µL Folin–Ciocalteu's reagent (diluted 1:1 by volume with MQ-water). The intensity of the resulting blue colour was measured at 660 nm using a Microplate Reader (Synergy™ HT, Bio-Tek).

2.6. Determination of biosurfactant production during PAH degradation

Excellent emulsification activity with hydrocarbons or vegetable oils is a direct implication of a bacterial strain's ability to produce surfactants (Xia et al., 2011). Supernatants remaining after collection of the cell pellets for protein estimation were assayed for their emulsification activity index using a method described by Cooper and Goldenberg (1987). Briefly, 4 mL of hexadecane was added to an equal volume of cell free supernatant. After homogenization in a vortex for 2 min, the solution was allowed to stand for 24 h. The emulsification index (E24) was then calculated using the expression,

$$E24 (\%) = \frac{\text{Height of the emulsified layer}}{\text{Total height of the aqueous solution}} \times 100$$

2.7. Biosurfactant stability analysis

In order to investigate the effects of temperature, pH and salinity on emulsification activity, surfactant containing supernatants were exposed to (a) different temperatures ranging from 18-80°C by incubation in a temperature-controlled room or water bath for 24 h, (b) varying pH by altering the solution pH from 2-12 using 1 M HCl and 1 M NaOH, and (c) varying amounts of NaCl up to 20 g/L. Supernatants acclimatized to (b) and (c) were incubated for 2 days at room temperature. Changes in the emulsification activity for all the treatment supernatants (from a, b and c) were then measured.

2.8. Detection of the type of surfactant produced – CTAB agar test

To identify the type of biosurfactant produced, shallow wells were cut on the agar plate surface containing media prepared by adding 20 g glucose, 0.7 g KH_2PO_4 , 0.9 g Na_2HPO_4 , 2 g NaNO_3 , 0.4 MgSO_4 , 0.1 g CaCl_2 , 2 mL trace element solution, 0.2 g CTAB and 0.005 g/L methylene blue solution. Fifty microlitres of the cell-free culture supernatants were added into each well. The plates were incubated for 48 h at 34°C and then stored in the refrigerator for at least 24 h. Glycolipids were detected as dark blue halos around the well, the diameter of halo zone being dependent upon the concentration of glycolipids produced by the strain (Walter et al., 2010).

2.9. Heavy metal resistance test

The metal resistances of the strains was determined for all the four test metals, viz., Pb, Zn, Cd and Cu, during PAH degradation by using Phe (200 mg/L) as the test PAH. The tests were conducted in triplicate using 48-well IWAKI well plates. Stock solutions of the metals were prepared in MQ-water. Cells of KC3 and 5 at an optical density (OD_{600}) of 0.5 were exposed to different concentrations of a metal in the range 0-50 mg/L for a week in an optimized mineral medium (OMM) designed by Thavamani et al. (2015). Well-plates were incubated at a constant room temperature of 25°C under agitation (120 rpm). Growth of the cultures was measured periodically in terms of turbidity (OD_{600}) using a microplate reader (BioTek, USA) and minimum inhibitory concentrations of the metals (metal concentration that inhibits 50% of the bacterial growth) were interpolated.

2.10. Qualitative screening of strains for enzyme production

Strains were screened for production of each enzyme on 1.8% (w/v) agar plates containing one of the following substrates (all supplied by Sigma- Aldrich, USA, unless

otherwise stated): casein (0.5% w/v) for protease; Tween 80 (1% w/v) for lipase; starch (1% w/v) for amylase and carboxymethyl cellulose for cellulase (Mazzucotelli et al., 2013). All plates contained minimal medium salts consisting of: KH_2PO_4 (1.5% w/v), $(\text{NH}_4)_2\text{SO}_4$ (0.5% w/v), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.06% w/v) and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.06% w/v). Enzymatic index (EI) was calculated by dividing the diameter of the clear zone around the colony with bacterial colony diameter (Carrim et al., 2006).

3. Results

3.1. Isolation and phylogenetic characterization

The population of cellulose degraders was 4.4×10^8 cfu per gram of koala faeces. A total of 10 morphologically different colonies was selected from the highest dilution for further studies. Of those, two strains KC3 and 5 showed an increase in growth measured in terms of turbidity (optical density) when Phe (100 mg/L), a LMW PAH model compound employed was supplied as the sole carbon source highlighting their potential to biodegrade PAHs. These two strains were characterized as aerobic and gram-negative. The BLASTN search result of the near full-length 16S rRNA gene sequence (1100-1200 bp) showed that KC3 and 5 belonged to the genera *Bacillus* (JN897279) and *Pseudomonas* (KJ541832), respectively. Both KC3 and 5 possessed a similarity of >97% to their related genera. Notably, the phylum Gammaproteobacteria (*Pseudomonas*) and Firmicutes (*Bacillus*) are among the most commonly isolated bacteria from faecal and intestinal samples of various animals (Jia et al., 2011).

3.2. Biodegradation of LMW and HMW PAHs

Bacillus strain KC3 and *Pseudomonas* strain 5 were initially screened in liquid culture for their ability to degrade the most widely used model LMW PAHs, Phe and HMW PAHs, Pyr. The study was then extended to BaP, a representative HMW PAH of environmental concern due to its demonstrable carcinogenicity and bioaccumulation potential. Both *Pseudomonas* and *Bacillus* strains were capable of utilizing LMW and HMW PAHs as their sole source of carbon and energy (Figs. 1a, b). It is clear from Fig. 1 that the degradation time is dependent upon the molecular weight and hydrophobicity of the PAH. For example, in order to degrade 200 mg/L of Phe, the cultures took about 6 days, but with 200 mg/L Pyr they took 7-15 days for complete degradation. In the case of the 5-ring BaP with low solubility, the degradation time was prolonged even more (50-60 days). The *Bacillus* strain was capable of degrading the entire three model PAHs with a proportional increase in its protein values

faster than did the *Pseudomonas* strain. Notably, the degradation of the HMW model compounds, Pyr (in 7 days) and BaP (in 50 days) by the *Bacillus* strain was 1.5- to 2-fold faster and greater in comparison to the *Pseudomonas* strain, suggesting its potential to achieve the bioremedial objective in a shorter time frame, thereby potentially reducing cost. No PAH oxidation was observed in the controls that received no cells. The PAH degradation was found to be growth-dependent (the decrease in PAH concentrations is directly proportional to the increase in cell protein) indicating that the degradation occurred as a result of microbial respiration or energy derivation. Furthermore, no PAH mineralization was observed in the blanks (sterile medium alone) during the incubation period, which indicates that the enzymatic reactions associated with the cells of *Bacillus* and *Pseudomonas* strains are solely responsible for PAH degradation.

3.3. Biosurfactant production during growth on PAHs

Figs. 1 and 2 indicate that the biosurfactant produced by the strains shows great potential for emulsification activity. For both the *Pseudomonas* and *Bacillus* strains, the production of biosurfactant (measured as emulsification activity) was proportional to the cell growth representing biosurfactant as a growth associated product. Notably, the amount of biosurfactant produced was found to be dependent upon the molecular weight of the PAHs. High surfactant production was observed when the microbes received LMW PAHs as the C source. As the molecular weight increased (in case of Pyr and Bap), the amount of biosurfactant produced decreased. Of the two strains, maximum emulsification activity reached about 53% during Phe degradation with the *Bacillus* strain. However, the *Pseudomonas* strain was capable of emulsifying 47% hexadecane under the same conditions. The *Bacillus* strain was therefore the best candidate for biosurfactant production as it produced the highest emulsification activity even in the presence of Pyr and BaP, whilst the *Pseudomonas* strain produced the lowest (up to 1.5-fold). On the basis of the emulsification activity it can be concluded that the biosurfactant properties of the two microbes makes them both appropriate candidates for enhanced PAH degradation, the *Bacillus* strain being the preferred one.

3.4. Anionic surfactant production

A preliminary investigation using CTAB agar plate method was carried out in order to confirm the type of surfactant product. This method developed by Siegmund and Wagner (1991) is a qualitative assay for the detection of extracellular glycolipids or other anionic

surfactants. Fig. 3 shows the formation of dark blue halos around the wells confirming the secretion of anionic surfactants, most commonly rhamnolipids (Walter et al., 2010) by the strains *Bacillus* and *Pseudomonas*. The dark blue halos are due to the formation of an insoluble ion pair of the secreted anionic surfactants with the cationic surfactant CTAB and the basic dye methylene blue.

3.5. Study of biosurfactant stability

As seen in Fig. 4, studies on the effect of heat treatment, pH and salinity all show that some changes in biosurfactants occurred and there were some differences in emulsification activity before and after treatment. Fig. 4a shows a decrease in the emulsification activity from 51 to 46% in *Bacillus* and 48 to 41% in *Pseudomonas* strains, with an increase (by 13°C) and a decrease (by 6°C) in temperature. This might be because of a decrease in hydration with fluctuating temperatures (Xia et al., 2011). However, the emulsification activity of the two strains did not decrease significantly with further increases in temperature (up to 80°C). It could therefore be interpreted that the biosurfactants produced by both the strains are heat-resistant and these could be used in extreme temperature conditions during PAH degradation. As demonstrated in Fig. 4b, the biosurfactants belonging to the *Bacillus* and *Pseudomonas* strains are more tolerant of alkaline conditions (up to pH 12). However, when the pH of the biosurfactants decreased, the emulsification activity decreased (1.2-fold). This could be the result of biosurfactant precipitation in acidic condition. In spite of a decrease in the emulsification activity, the relatively stable and effective activity from pH 2 to 12 indicates the applicability of these biosurfactants for remediation of PAH-contaminated soils over a wide range pHs. The data summarized in Fig. 4c show that the biosurfactants produced by these strains are resistant to salt concentrations up to 20 g/L.

3.6. Cd²⁺, Pb²⁺, Cu²⁺ and Zn²⁺ resistance in OMM media

Any metal resistance of a PAH degrader is significant for mitigating the metal inhibition of PAH biodegradation during the clean-up of mixed contaminant sites. Meaningful quantitative data on the responses to metals by biosurfactant-producing pollutant degraders is still lacking. Of the most toxic metals, Pb, Cd, Zn and Cu have emerged to have greater negative impact on the biodegradation of hydrocarbons (Amor et al. 2001; Sandrin and Maier, 2003). Hence the resistances of *Bacillus* and *Pseudomonas* strains to Cd²⁺, Pb²⁺, Cu²⁺ and Zn²⁺ were tested for minimum inhibitory concentrations (MIC) in a newly developed OMM medium (Thavamani et al., 2015) during a model PAH (Phe) degradation.

Variability in the degree of metal inhibition is dependent upon metal bioavailability. This medium has >60% metals as free ions at pH 6.5 as measured by an ion-selective electrode (Orion model 9448) and the visual MINTEQ model. *Bacillus* strain showed the highest resistance to heavy metals with a MIC of 5 mg/L for Cd^{2+} and Cu^{2+} , and 7 mg/L for Pb^{2+} and Zn^{2+} (Fig. 5a). Further increases in metal concentrations resulted in a significant decrease in growth. A concentration >15 mg/L for Cd^{2+} and Pb^{2+} , 12 mg/L for Zn^{2+} and 7 mg/L for Cu^{2+} appeared to be completely toxic and inhibited all growth in the OMM medium. The *Pseudomonas* strain (Fig. 5b) exhibited lower metal resistances and was sensitive to Cd^{2+} . It had a MIC of 1 mg/L for Cu^{2+} . Growth declined and reached a plateau at concentration >5 mg/L Cd^{2+} and Cu^{2+} . However, the MICs for Zn^{2+} and Pb^{2+} were 3 and 5 mg/L, respectively. Beyond 10-20 mg/L, both Zn^{2+} and Pb^{2+} completely inhibited the growth of the *Pseudomonas* strain.

3.7. Enzymatic activity

Though our principal focus was to assess the PAH degradation potential of the isolated strains, since *Bacillus* and *Pseudomonas* strains were both capable of degrading cellulose, in addition to the degradation studies, a preliminary evaluation of the enzymatic activities of the strains were accomplished. Enzymatic activity used in the study provided a semi-quantitative measure of enzyme production by the microbes (Table 1). Both the strains showed more than one extracellular enzymatic activity. Along with cellulolytic activity, both demonstrated proteolytic and lipolytic activities with a maximum enzymatic index (EI) for cellulose degradation observed in the *Bacillus* strain (0.68), and lipase followed by protease activities in the *Pseudomonas* strain (2.33 for lipase and 0.66 for protease).

4. Discussion

Presumably, the microbes in the digestive tract of koala are capable of degrading the recalcitrant compounds (cellulose, lignin) in eucalyptus leaves to gain energy. Such intrinsic potential to cleave organic compounds might be reasonable for the observed PAH degradation. Strains of this study have superior capacity for LMW and HMW PAH degradation over other *Pseudomonas* (Ben Said et al., 2008; Kumar et al., 2008; Zhang et al., 2011) and *Bacillus* strains (Feitkenhauer et al., 2003; Su et al., 2006; Reddy et al., 2010) reported so far. In the case of LMW PAH (Phe) degradation, both *Bacillus* and *Pseudomonas* possessed a similar degradation potential in par with previous reports available (Zhao et al., 2009; Reddy et al., 2010). Relatively rapid HMW PAH degradation was observed than the

other reported genera. For example, Thavamani et al. (2012) reported that the consortia of metal-resistant, PAH-degrading *Pseudomonas*, *Alcaligenes*, *Pandorea* and *Paenibacillus* strains isolated from a long-term mixed contamination soil could degrade around 85% BaP (50 mg/L) in 60 days. In other studies, *Stenotrophomonas maltophilia* (VUN10003) from a contaminated environment degraded 22% BaP in 42 days (Juhasz et al., 2000); *Klebsiella pneumonia* PL1 isolated from an agricultural soil utilized only 63.4% Pyr and 50% BaP of very low concentration (20 mg/L) in 10 days (Ping et al., 2014) and *Burkholderia* strain (VUN10013) isolated from PAH-contaminated soil degraded 42% Pyr over 21 days (Somtrakoon et al., 2008).

One of the most commonly cited and generally accepted drawbacks of PAH bioremediation considered to be the most important factor involved in the slow degradation of PAHs is the low solubility of these compounds resulting in low bioavailability to the pollutant-degrading organisms (Makkar and Rockne, 2003). Over the past decade, widely ranging research has focused on limitations to PAH bioavailability and the ways in which these may be overcome. A more promising solution for enhancement of PAH solubility is the use of surfactants (surface-active agents) as mobilizing agents in order to increase the microbial accessibility to insoluble substrates. The use of biosurfactants has several advantages over synthetic surfactants making them superior candidates in a bioremediation scheme viz., they are cheap, biodegradable and ecofriendly (no threat to the environment, non-toxic to microbes and do not inhibit degradation processes). Several biosurfactants have found applications in PAH remediation; for example, Phe removal by the rhamnolipids produced by *Pseudomonas aeruginosa* ATCC 9027 (Dean et al., 2001). Aliphatic and aromatic hydrocarbon degradation has also been demonstrated by crude surfactin from *Bacillus subtilis* O9 isolated from seawater (Moran et al., 2000). In some cases, the presence of biosurfactants has also been shown to reduce heavy metal toxicity and enhance PAH degradation (Sandrin et al., 2000). Thus, a pollutant degrader with biosurfactant production potential can always be considered as a promising tool in environmental clean-up. Our results are in agreement with the observations made by Bordoloi and Konwar (2009) who reported that PAH- (Phe and Pyr) utilizing strains of *Pseudomonas aeruginosa* (MTTCC7814 and MTTCC7815) were capable of producing biosurfactants (rhamnolipid type) and biosurfactant produced thus exhibited higher solubilization of HMW PAHs (Pyr) and enhanced their metabolism resulting in sustained growth. Accelerated HMW PAH degradation might be due to the capacity of the biosurfactant to entrap PAHs inside the micelle structure so facilitating microbial uptake and bioremediation. Few other studies have also confirmed enhanced PAH

degradation by the use of anionic surfactant-producing PAH degraders (glycolipid-like) as in our present study (Kumar et al., 2006, 2008; Reddy et al., 2010).

One of the important factors that determines the wider applicability of a biosurfactant is its stability. In our case, the strains isolated from koala faeces were thermal, salt and pH tolerant. Likewise, the biosurfactant produced by *Oleomonas sagaranensis* AT18 isolated from a mangrove sediment in south Thailand was shown to have thermal (up to 100°C) and pH (2-14) stability with tolerance to a high level of salt concentration (up to an 18% increase). Strain AT18 increased the bioavailability of PAHs for bacterial metabolism (Saimmai et al., 2012). In yet another study, the biosurfactants produced by *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Rhodococcus erythropolis* that were isolated from the formation water of a Chinese petroleum reservoir exhibited an emulsion index of 80% for crude oil and showed a good stability above pH 5, up to 120°C and the presence of a 20 g/L salt concentration. All the three strains enhanced oil recovery with a preference for the biosurfactant of *Pseudomonas aeruginosa*. The potential for biosurfactant production and the stability of the anionic surfactant produced by *Pseudomonas* and *Bacillus* sp. have been demonstrated before. However, the strains identified are novel as they have been isolated from a less unexplored environment, koala faeces, and are primarily cellulose degraders with biosurfactant production and more than three-ring PAH degradation abilities.

Metal resistance of both *Bacillus* and *Pseudomonas* were analogous and in some cases in disagreement with the previous studies. The MIC value of Cd for a PAH degrading bacterial consortium observed by Thavamani et al. (2015) was in par with our study. In case of Pb our study recorded a higher MIC value than reported for a population of indigenous PAH degraders by Maslin et al. (2000). However, MIC values for Zn and Cu recorded by Springael et al. (1993) and Irha et al. (2003) were 3x higher than the current study. Metal resistances of both the strains may be attributed to the biosurfactants produced. The mechanism by which biosurfactants reduces metal toxicity might involve a combination of anionic biosurfactant complexation of heavy metals and interaction with the cell surface so altering metal uptake, resulting in enhanced rates of bioremediation (Sandrin et al., 2000). It is evident that the *Bacillus* strain which produced more surfactants (as measured by emulsification activity), exhibited higher metal resistances than the *Pseudomonas* strain. Hence, a coupled PAH degradation with metal resistance is likely due to the biosurfactant production potential. Sandrin et al. (2000) observed reduced Cd toxicity with the addition of a 10-fold higher concentration of biosurfactant that allowed enhanced naphthalene biodegradation. Other research has also shown that metals such as Pb and Cd have stronger

affinities for anionic surfactants like rhamnolipids (Frazer, 2000). The selective complexation of cationic metal species such as Cd, Pb, Cu and Zn by anionic biosurfactant (rhamnolipid) produced by *Pseudomonas aeruginosa* and *Bacillus subtilis* has also been demonstrated (Singh and Cameotra, 2004).

As that of our study, previously Margesin et al. (2003) found that bacterial strains isolated from uncontaminated habitats possessed the potential to degrade hydrocarbons and produce enzymes such as lipase, amylase, protease, etc. Carrim et al. (2006) presented a similar EI of 2.4 for lipase by *Pseudomonas stutzeri* strain CAR8. But, either amylase or protease activities were not observed for the same strain. The isolate *Bacillus* strain CAR 10 of the same study did not present any enzymatic activities. Ryttersgaard et al. (2004) mentioned pectinase activity by *Bacillus* sp. The enzymatic activities in *Bacillus* and *Pseudomonas* strains suggest scope for various industrial applications (Kirk et al., 2002). Proteases and lipases can be employed in the textile, dairy and leather industries. Similarly, as researchers are looking for new strains with resilient cellulase enzyme systems that can reduce the cost of saccharification involved in cheap biofuel production, our strains could be used for bioethanol production (Cannella and Jorgensen, 2014).

5. Conclusion

This paper presents the first evidence for cellulose-degrading bacteria from koala faeces with enhanced low and HMW PAH degradation coupled with biosurfactant production. The added unique traits of relatively high heavy metal resistances and enzymatic activities (amylase, lipase and protease) thus suggest that these new strains of *Bacillus* and *Pseudomonas*, could be excellent candidates for application in the PAH remediation processes and in various industries. One of the best approaches for bioremediation of a PAH-contaminated soil is to apply these strains in the form of microbial inocula or as rhizobacteria in phytoremediation. On the other hand, they could be used in industrial fermentation processes for the production of enzymes using low-cost growth substrates. Koala faeces can therefore be regarded as a valuable resource for harnessing multi-functional microbial communities for environmental safety and commercial utility.

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Mater. 164, 863-869.

627 **Table 1.** Qualitative screening of the biosurfactant-producing PAH degrader for its enzymatic
 628 activity

	Enzymatic index (EI)	
	<i>Pseudomonas</i> sp.	<i>Bacillus</i> sp.
Amylase	-	-
Protease	0.66 ± 0.02	0.57 ± 0.1
Lipase	2.33 ± 0.5	2.14 ± 0.4
Cellulase	0.51 ± 0.07	0.68 ± 0.2

629

630

Fig. 1. Time courses of growth vs PAH degradation potential of *Bacillus* (a) and *Pseudomonas* (b) strains isolated from koala faeces. In the Figure, solid lines represent the PAH degradation and dotted lines the protein concentration. (Source of carbon = 200 mg/L Phe and Pyr, 50 mg/L BaP).

Fig. 2. Time courses of biosurfactant production during PAH utilization of *Bacillus* (a) and *Pseudomonas* (b) strains isolated from koala faeces. Biosurfactant production ability of the strains is expressed as the emulsification activity of the culture supernatants.

Fig. 3. Detection of rhamnolipid (anionic surfactant) production by the PAH degraders using agar plates containing methylene blue and cetyl trimethylammonium bromide. The blue halos formed around the wells each added with 50 μ L of culture supernatant represent the type of surfactant produced by the strains as rhamnolipids.

Fig. 4. Influence of temperature (A), pH (B) and salinity (C) on emulsification activity of the culture supernatant containing biosurfactant that was collected after 10 days when the strains had effectively utilized 200 mg/L phenanthrene as the sole carbon source.

688 **Fig. 5** Metal resistance of the surfactant-producing PAH degraders (Growth media = OMM
 689 with 200 mg/L Phe as C source + metal). (a) = *Bacillus* and (b) *Pseudomonas*.