

Biodegradation of pyrene by *Candida* sp. S1 under high salinity conditions

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Abstract Polycyclic aromatic hydrocarbon is a toxic recalcitrant environmental pollutant and its removal from the environment is very essential. In this study, a novel S1 strain isolated from the tropical rain forest was identified as *Candida* species based on 18S rRNA. The pyrene biodegradation was performed by *Candida* sp. S1. Pyrene was 35% degraded in 15 days. The percentage of pyrene biodegradation increased up to 75% with 24 g L⁻¹ of sodium chloride and decreased along with increasing salinity. Under the acidic condition, the biodegradation was increased up to 60% at pH 5. It was also found that the increasing glucose concentration of more than 10 g L⁻¹ had no significant effect on pyrene biodegradation, while agitation proved to have greater influence. There was a positive relationship between biomass growth and biodegradation rate of pyrene. One pyrene metabolite was identified from the extract solution and analyzed by a thin-layer chromatography, UV–visible absorption and gas chromatography–mass spectrometry. The metabolite found in the pyrene degradation was benzoic acid.

Suitable conditions must be found to promote a successful microbial augmentation in liquid culture.

Keywords *Candida* sp. S1 · Pyrene biodegradation · Metabolite identification · Salinity

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds composed of two or more benzene rings fused together. The arrangement of the rings could be straight linear or linear with angular or cluster. Their existence in environment could be natural from volcanic eruptions and forest fire or anthropogenically, i.e., traffic fumes, incomplete combustion of fossil fuel, industries, organic waste, petroleum seepage and cigarette smoke [1]. Due to PAHs' carcinogenic and mutagenic effects [2], the Environmental Protection Agencies identified 16 compounds of PAHs on the priority contaminant list [3, 4]. The removal of organic pollutants can be achieved by adsorption, photo-degradation, phytoremediation and biodegradation [5–7]. Among these, biodegradation is favorable considering the ecological and economical aspects. In the biodegradation process, the chemical structure of molecules that are introduced to the environment can be changed by microorganisms' enzymes [8]. Higher molecular weight (HMW) PAHs show more resistance to degradation by microorganisms, which indicates their potential as environmental bio-remediators. Pyrene, a four-ring PAH that has low biodegradability and high persistence in the environment, had been listed as a priority pollutant by the United States Environmental Protection Agency (US EPA). Pyrene can easily enter the human body through skin contact and inhalation. It is toxic toward our organs, including kidney and liver. Due to its

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toxic characteristic and xenobiotic property, its very important to remove PAHs from environment [9].

Various filamentous fungi had been recognized for their ability to eliminate a wide range of PAHs. Filamentous fungi was chosen for as degrader in this research because of fast growing and resistance to various conditions in the environment. The capability of these fungi in the degradation of persistent pollutants mainly depends on its capacity to secrete the specific enzymes [10, 11]. Many factors such as temperature, pH, agitation, initial concentration of PAH, salinity concentration and carbon source concentration can influence the biodegradation of PAHs by microorganisms. Increasing the temperature would increase the solubility and bioavailability of pollutants to microorganisms [3, 12]. Salinity is one of the important factors considering saline soil and sea water salinity. At high salinity concentration, biological treatment performance is very low due to the loss of cell activity and plasmolysis of microorganisms, which cause most microorganisms to die or live at an attenuated level. On the other hand, some microorganisms are tolerant and resistant to a high salinity and these microorganisms are potential for removing the pollutants [13]. In this study, *Candida* sp. S1, isolated fungus from the tropical rain forest of Malaysia, is used to transfer pyrene, a four-ring PAH. The most important parameters that influence the biodegradation process, such as temperature, pH, agitation, salinity, carbon source, and the initial concentration of PAH, were investigated to accelerate pyrene degradation.

Materials and methods

Chemicals

Pyrene was procured from TCI chemical industry, Japan (Table 1). Ethyl acetate, dichloromethane, *N,N*-

dimethylmethanamide, and glucose were purchased from QREC (Asia). Reactive Black 5 and benzoic acid (purity 98%) were purchased from Sigma-Aldrich. Chloramphenicol was obtained from ACROS Organic.

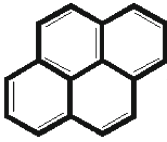
Isolation of fungi

Reactive Black 5 (RB5) dye has a chemical structure similar to PAHs. It has been used as an indicator to screen dye-decolorizing fungi. In preliminary screening, the fungus was inoculated into MEA amended with 200 mg L⁻¹ of RB5 to measure the decolorization ability of isolated fungal strains. The growth and decolorization rate were monitored daily and the efficient decolorizing fungal strain was selected and stored in a refrigerator prior to further use.

Identification of the fungus by 18S rRNA analysis

Genomic DNA of the selected fungal strain was extracted according to the previous method [18]. The 18S rRNA gene fragment was amplified by polymerase chain reaction (PCR) using a set of universal primers: ITS1 (TCCG TAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTAT TGATATGC). The PCR conditions (initial denaturation at 95 °C for 5 min; 25 cycles of 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C; and final extension at 72 °C for 10 min) were performed in a PCR thermal cycler (Mastecycler Gradient, Eppendorf). The PCR product of 18S rRNA gene was sent to the First BASE Laboratories Sdn. Bhd., Malaysia, for DNA sequencing. The full length sequence of 18S rRNA genes was submitted to BLAST from NCBI to compare with the nucleotide sequences from Genbank database, and phylogenetic trees were constructed using MEGA4 software.

Table 1 Physical and chemical characterization of pyrene

Structure	
	
Molecular formula	C ₁₆ H ₁₀
Appearance	Colorless solid
Molecular weight	202.25
Melting point	145–148 °C
Boiling point	404 °C
Aqueous solubility (15 °C)	0.135 mg/L

Microorganisms and culture medium

Our preliminary experiment revealed that *Candida* sp. S1 was not able to consume pyrene as the sole carbon source or nutrient. Additional nutrient was needed to support the initial degradation of pyrene. *Candida* sp. S1 was cultured into 20 mL liquid culture and incubated at room temperature. Various liquid media were tested to check the degradation performance as shown in Table 2. Pyrene was completely degraded within 25 days in mineral extract broth (MEB) medium. The composition of growth medium MEB is shown in Table 3. The ability of this isolate to utilize various sugars as co-substrate in pyrene degradation was examined (Table 4). The stock solution of pyrene with a concentration of 20 mg L⁻¹ was prepared by dissolving pyrene in 10 mL *N,N*-dimethylmethanamide. To prevent bacterial growth, 200 mg L⁻¹ of chloramphenicol was added to the medium. A small piece (~5 mm diameter) of fungus mycelia taken from the solid cultured media was subsequently inoculated into 100 mL flasks containing pyrene solution. All experiments were performed in duplicate and evaluations for each parameter were summarized as shown in Table 5.

Sample extraction

20 mL of ethyl acetate was added to each sample and shaken at 100 rpm for 24 h to extract pyrene from the liquid medium. The liquid portion was separated from the fungal biomass using a filter paper (4A, 7 cm diameter) and the biomass was rinsed with 20 mL of ethyl acetate. The

Table 2 Pyrene degradation performance of *Candida* sp. S1 in various media

Media	Time for degradation (days)
Malt extract broth	55
Nutrient broth	38
Czapek Dox broth	33
Peptone extract broth	45

Table 3 Concentrations of constituents of mineral extract broth (MEB)

MEB constituents	Concentration (g/L)	Trace element	Concentration (mg/L)
Malt extract	12	FeSO ₄ ·7H ₂ O	12
Glucose	10	MnSO ₄ ·7H ₂ O	3
Yeast extract	4	ZnSO ₄ ·7H ₂ O	3
KH ₂ PO ₄	2	CoSO ₄ ·7H ₂ O	1
MgSO ₄ ·7H ₂ O	1	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	1
CaCl ₂ ·2H ₂ O	0.1		
Ammonium tartrate	0.1		
Ammonium nitrate	0.1		
Trace element	10 mL		

fungal biomass were dried in oven (80 °C) for 24 h, then extracted with 20 mL of ethyl acetate. The extracted pyrene in ethyl acetate was purified by 100 mL of dichloromethane in silica gel and the volume was reduced to 10 mL.

Instrumental analysis

The samples were injected into the Agilent Technology 7820A GC system with the following conditions: the injection volume, 1 µL; the oven temperature heated to 100 °C and held for 2 min, increasing to 260 °C in 30 °C min⁻¹; the column type, HP5; length, 25 m; the inside diameter, 0.1 mm. Derivatization of carboxylic acids for gas chromatography–mass spectrometry (GC–MS) analysis was performed with a trimethylsilyl method [14, 15]. The suspected compound was compared with pyrene in terms of retention time. The retention time for pyrene is 6.625 ± 0.006 min. The MS of the sample was compared with the corresponding authentic standards and the mass spectra library of Wiley 275L.

Results and discussion

Screening of fungi strains

The collected fungal strains (AH3 and S1) showed different ability to decolorize RB5, respectively (data not shown). This result shows that tropical fungi species are different in terms of their ability to decolorize dyes. A similar result was noticed by Nor et al. [16].

Microscopic features of isolate fungal strain S1

The macroscopic features of fungal isolate S1 are smooth and pasty appearance, glistening or dry, wrinkled and dull, and creamish to yellowish color. The microscopic appearance of fungal isolates is shown in Fig. 1. The

Table 4 Utilization of various carbon sources by *Candida* sp. S1

Carbon source	Result
Starch	–
Cellulose	–
Glucose	++
Fructose	–
Galactose	+
Xylose	–
Cellobiose	+
Sucrose	+
Maltose	–
Lactose	–

++ very good; + good; – poor

blastoconidia appeared as small clusters with elongation and the pseudohyphae are long and branched.

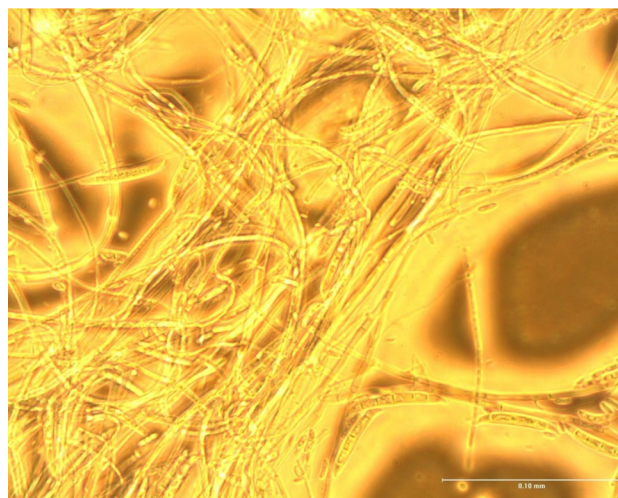
Phylogenetic tree analysis

The phylogenetic tree was established using BLAST-Webpage and MEGA4 Software to determine the phylogenetic relatedness of operational taxonomic units (OTUs) [17]. The identifiable homologous characters (nucleic acid bases) of the S1 18s rRNA gene sequence were aligned and only 11 homologous sequences (include S1 sequence) were used for the phylogenetic tree construction in MEGA4. The phylogenetic tree that has been depicted using BLAST-Webpage using neighbor-joining method showed that S1 was closely related to the *Candida* sp. This is a confirmation that the S1 belonged to Ascomycota yeast, class Saccharomycete, genus *Candida* as shown in Fig. 2.

Influence of culture conditions on pyrene biodegradation

Effect of temperature and pH on pyrene degradation by *Candida* sp. S1

The biodegradation of pyrene and fungal biomass growth at 20, 28 and 37 °C in 15 days were 19, 33 and 10% and

**Fig. 1** Microscopic appearance of *Candida* sp. S1

2.4, 3.5 and 1.9 g L⁻¹, respectively, as shown in Fig. 3a. This result clearly shows that the optimum temperature for biodegradation and growth was 28 °C. The biodegradation of RB5 and growth of fungi was decreased in temperature higher and lower than 28 °C. This may be because *Candida* sp. S1 is considered to be a mesophilic yeast, which prefers 28 °C for best growth and biodegradation. This result is consistent with biodegradation of organic pollutants such as dye, phenol and pyrene by other species of *Candida* [11, 18–20]. The optimum temperature was 30 °C for the growth and biodegradation of many PAHs including four rings by *Phanerochaete chrysosporium* [3]. The temperature has great effect on the biodegradation of compounds in the aqueous phase. Increasing the temperature would increase the solubility and bioavailability of pollutants to microorganisms. On the other hand, increase of temperature to some level would reduce the solubility of oxygen, which affects the microorganisms' growth and PAH biodegradation rate. From Fig. 3, it can be concluded that there is a positive relationship between *Candida* sp. S1 biomass growth and biodegradation rate of pyrene.

The influence of pH on the biodegradation of pyrene and biomass growth was investigated at a range of pH from 3 to

Table 5 Summary for all experiments of batch culture

Parameters	Initial pH	Temperature (°C)	NaCl concentration (g/L)	Glucose concentration (g/L)	Agitation (rpm)	Pyrene concentration (mg/L)
Effect of initial pH	3–6	28	0	2.5	0	20
Effect of temperature	4	20, 28, 37	0	2.5	0	20
Effect of contact time	4	28	0–72	2.5	0	20
Effect of initial concentration	4	28	0	2.5–30	0	20
Effect of agitation	4	28	0	2.5	0 and 80	20
Effect of pyrene concentration	4	28	0	2.5	0	10–40

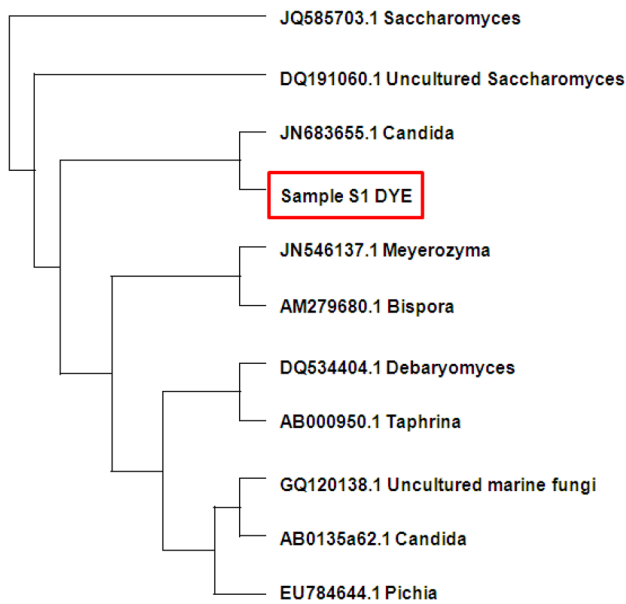


Fig. 2 Phylogenetic tree of *Candida* sp. S1

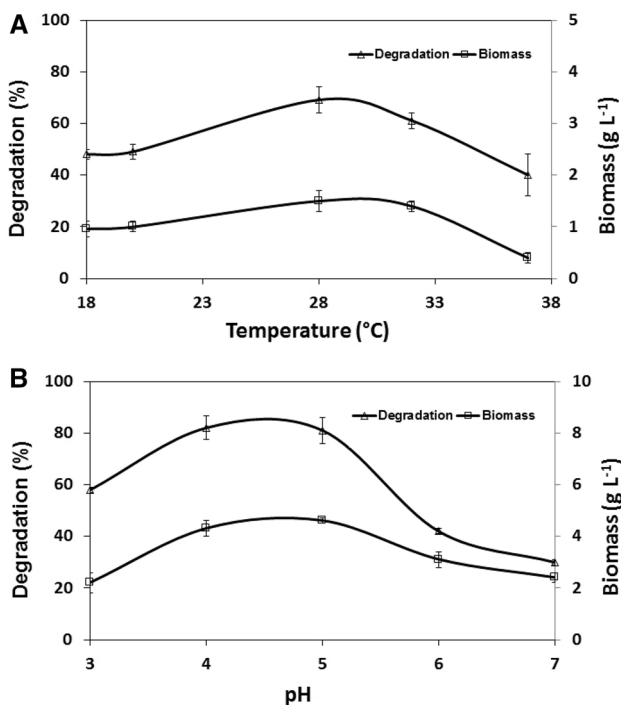


Fig. 3 Effect of temperature and pH on pyrene degradation by *Candida* sp. S1

6. The maximum rate of biodegradation (46%) was achieved at pH 5 with 8 g L⁻¹ biomass growth as shown in Fig. 3b, whereas it was decreased at pH values 6 and 3. This result matches with the previous researches [20, 21]. Sood et al. reported that *Candida digboiensis* can transfer pyrene at pH 3, which may be due to the development of the mechanism of adaptation of this fungus [12]. In general, fungi grow faster under acidic condition than neutral

condition, while in alkaline condition the growth was slow due to the inhabitation of fungal growth [22]. Many members of the *Candida* genus could produce organic acids and decrease the pH of the medium. Therefore, the pH of the medium was checked during the growth of the organism in liquid medium. The result showed that the pH of the medium was slightly decreased; however, it will not affect the biodegradation process.

Effect of agitation and glucose concentration on pyrene degradation by Candida sp. S1

The biodegradation rate of pyrene was 31 and 46% in 15 days in the stationary and agitation phase as shown in Fig. 4a. In the agitation phase, the growth increased from 3.5 to 6.5 g L⁻¹. Agitation has two main roles in microorganisms' growth and biodegradation rate. Firstly, it increases the contact between the reagents (substrate, oxygen, and biomass) which enhances the mass transfer between microorganisms' cells and the medium [14]. Secondly, it increases the dissolved oxygen in the liquid medium which is responsible for pyrene oxidation. This result is similar to the biodegradation of pyrene by *Armillaria* [15].

The biodegradation rate reached 25, 30, 32, 34 and 35% at 2.5, 5, 10, 20 and 30 g L⁻¹ glucose, respectively, while the biomass growth reached 2.5, 3.7, 4.1 and 4.3 g L⁻¹, respectively, as shown in Fig. 4b. In non-glucose addition, there was no biomass growth, and biodegradation of pyrene was observed. This may be because *Candida* sp. S1 could not utilize pyrene as a sole source of carbon and energy at the beginning of the culture. The microorganism needs glucose for the initial growth; then they consume pyrene as a source of carbon or energy. According to Peng et al., there is no absolute evidence that fungi can utilize PAH as growth substrate [23]. On the other hand, the addition of carbon sources (such as glucose, cellulose and sawdust) can induce a rapid increase of fungal biomass. The addition of glucose enhanced the biodegradation of pyrene up to 42% comparative to 28% without glucose [24]. The relatively fixed biodegradation rate and biomass growth on increasing the amount of glucose additive may be because the glucose amount was enough for fungi growth of *Candida* sp. S1.

Effect of salinity and pyrene concentration on pyrene degradation by Candida sp. S1

Salinity is one of the important factors that affect the biodegradation of pollutants in industrial saline wastewater, saline soil and sea water. The performance of biological treatment in saline condition is usually low due to the adverse effect of salt on the microbial flora. In choosing a

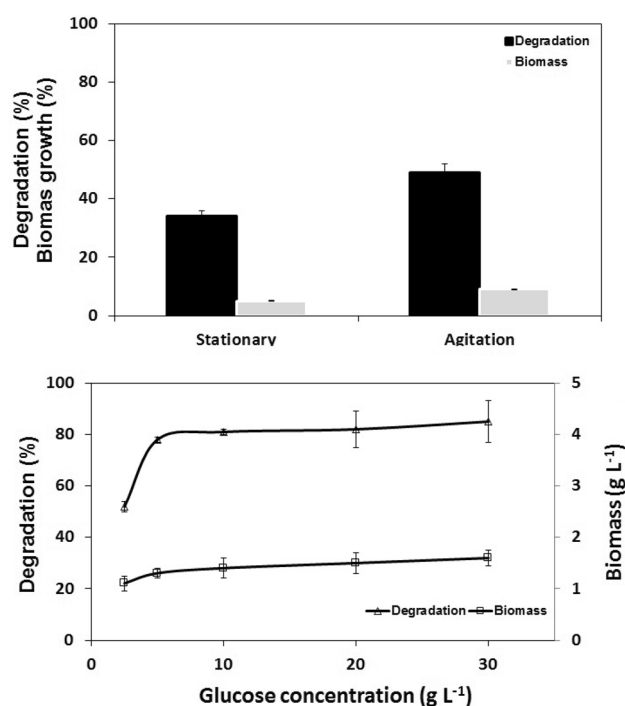


Fig. 4 Effect of agitation and glucose concentration on pyrene degradation by *Candida* sp. S1

suitable fungal species in the remediation of beaches, marshes and saline soils contaminated with PAH, it is important to assess the inhibitory effect of salinity on the biodegradation process. The transformation of pyrene in 15 days with different concentrations of sodium chloride 0, 24, 48 and 72 g L⁻¹ reached 32, 42, 29 and 30%, while the biomass reached 4.1, 6.3, 9.5 and 8.9 g L⁻¹, respectively, as shown in Fig. 5a. The best biodegradation of pyrene was reached when sodium chloride concentration was 24 g L⁻¹; increasing the salinity concentration can enhance the growth rate but decrease the biodegradation. It was noticed that the biomass growth rate was slow at the beginning of the incubation period <7 days (data not shown) and a small amount of biomass was detected; however, it increased sharply after 7 days compared to zero salinity which has fixed biomass growth rate. This may be due to the adaptation ability of *Candida* sp. S1 to high salinity. This result was similar with previous result that some indigenous microbial communities have the ability to adapt to high salinity concentration and transfer PAH [25]. Mastuda et al. studied the response to sodium chloride variation among different fungal species and that growth of some fungal species was not affected by increasing salinity due to their tolerance [26]. These results indicate that *Candida* sp. S1 can be a good candidate for the bioremediation of PAH in water containing high salinity. The different concentrations of pyrene (10, 20, 30 and

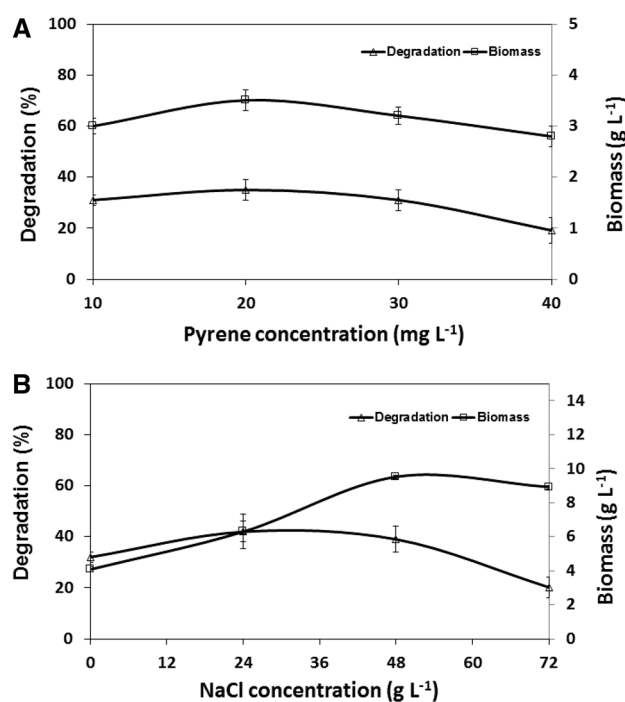


Fig. 5 Effect of salinity and pyrene concentration on pyrene degradation by *Candida* sp. S1

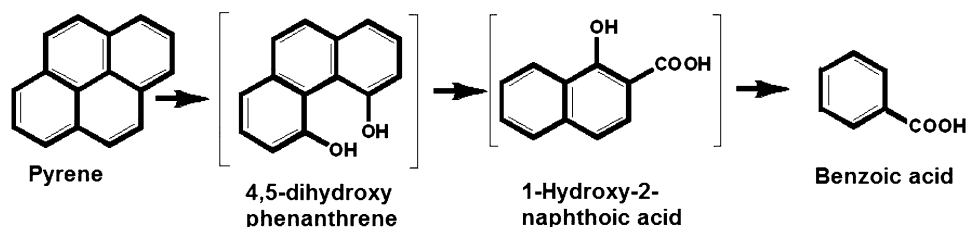
40 mg L⁻¹) showed the biodegradation of pyrene at 31, 35, 31 and 19% in 15 days, while the biomass reached 3, 3.5, 3.2 and 1.8 g L⁻¹, respectively, as shown in Fig. 5b. Increasing the concentration of pyrene can cause the reduction of biodegradation rate and biomass growth due to toxicity.

Analysis of metabolites

The metabolite has a R_f value of 0.29 and UV characteristics (λ_{max} , 226 and 275 nm), similar with the benzoic acid standard. The retention time (t_R) of metabolite was at 8.2 min, had M^+ at m/z 282 and fragmentation ions at m/z 267 (M^+-15), sequential loss of methyl ($-CH_3$), and 193 (M^+-89), sequential loss of $-OSi(CH_3)_3$, as well as the expected fragment ions at 147, 209, and 73 [$(CH_3)_3Si$] (Table 6). Based on the result obtained above, we determined the metabolite of pyrene by *Candida* sp. S1 as shown in Fig. 6. *Candida* sp. S1 is grown on pyrene as the source of carbon and energy with contribution of additional nutrient at the initial degradation; the initial attack occurs at the fourth and fifth position, by dioxygenase, to form a hydrodiol, which is further converted to 4,5-dihydroxyphenanthrene. 4,5-Dihydroxyphenanthrene can be further degraded into a major product, 1-hydroxy-2-naphthoic acid. 1-Hydroxy-2-naphthoic acid can be further transformed to form benzoic acid.

Table 6 Mass spectral analysis of the metabolites identified during the degradation of pyrene by *Candida* sp. S1

Metabolites	Retention time (min)	<i>m/z</i> of fragment ions (% relative abundance)	Structure identified
Benzoic acid	8.2	367 (95, M ⁺ -15), 73 (71), 91 (23), 147 (27), 149 (11), 193 (8), 209 (33), 268 (37)	Benzoic acid–TMS derivative (confirmed with a standard)

Fig. 6 The proposed pathway for the degradation of pyrene by *Candida* sp. S1. Compounds between brackets were not identified in our culture extract

Conclusions

Candida sp. S1, screened from the tropical forest in Malaysia, was selected based on its ability to decolorize RB5. It was used to transfer the pyrene, a four-ring PAH. Through this research, parameters such as temperature, agitation, pH, glucose concentration, salinity and initial pyrene concentration were studied to investigate their influence on pyrene biodegradation. The optimum temperature for biodegradation and growth was 28 °C, whereas agitation at 80 rpm had a positive influence on the biodegradation and growth. The pyrene biodegradation and *Candida* sp. S1 growth were enhanced under acidic conditions. *Candida* sp. S1 could not utilize pyrene as a sole source of carbon and energy; thus, adding glucose would enhance the growth and biodegradation rate. *Candida* sp. S1 growth increased with increase in the salinity, while the maximum pyrene biodegradation reached 24 g L⁻¹. In final conclusion, *Candida* sp. S1 is a potential candidate for pyrene bioremediation under saline and acidic environment. The metabolites showed that the initial attack occurred at the fourth and fifth positions of pyrene by dioxygenase to form a hydrodiol and it was further converted to dihydroxypyrene. Dihydroxypyrene can be further degraded into a major product, 1-hydroxy-2-naphthoic acid. 1-Hydroxy-2-naphthoic acid can be further transformed to form benzoic acid.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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