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RESEARCH PAPER

Exploring the potential of halotolerant bacteria for biodegradation of polycyclic aromatic hydrocarbon

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Abstract
The present study aimed to determine the degradation and transformation of three-ring PAHs phenanthrene and anthracene by *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 under halophilic conditions. The growth progress of *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 on anthracene and phenanthrene was monitored by colony-forming unit (CFU) technique. The growth of the bacteria was maintained at a maximum concentration of 200 mg/L of all tested hydrocarbon, indicating that *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 significantly perform in the removal of the PAH-contaminated medium at low concentrations. The fit model to represent the biodegradation kinetics of both PAHs was first-order rate equation The extract prepared from cells supplemented with three different substrates exhibited some enzymes such as hydroxylase, dioxygenase, laccase and peroxidase. The results suggest that both strains had an impressive ability in the degradation of aromatic and aliphatic hydrocarbon but also could tolerate in the extreme salinity condition.

Keywords Halotolerant bacterial · *Cryptococcus* sp. MR22 · *Halomonas* sp. BR04 · Metabolites · Phenanthrene

Introduction

PAHs are environmentally recalcitrant pollutants produced from naturally or incomplete combustion of organic materials. PAHs are tough to degrade because they are hydrophobic and reported as mutagenic, carcinogenic and teratogenic compounds. Their toxicity and persistence also increase with their size and number of aromatic rings. The saline aquatic ecosystem was regularly polluted with organic compounds due to massive industrial activity [1]. Effective remediation strategies are required to eliminate PAHs in the environment, especially saline aquatic ecosystems, due to their increased production from anthropogenic sources [2]. Many physical and chemical treatment has been recommended to eliminate organic pollutant from the environment, such as oxidation, chemical decomposition, ion exchange, ozone, landfilling, solvent extraction and incineration. However, these treatments are expensive, commercially unattractive and no environmentally-friendly [3–7]. Bioremediation is one promising and prospective method for PAHs removal in comparison with existing treatment because it is relatively low-cost, low-technology procedures, which commonly have high public acceptance and environmentally friendly [8–10].

Previous studies have described the ability of microorganisms to degrade, biotransform, biosorption and cometabolite PAHs [11, 12]. However, the utilization of halophilic bacteria for bioremediation was not extensively employed [13]. Many documentations for implementation of halophilic and halotolerant bacteria as mediators of fish decay, preserving meat with solar salt and fermenting some foods. Recently, halophilic microorganisms have received great attention due

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to their adaptation in a high concentration of salt, with modification methodology for culturing and genetic engineering. However, microorganisms generally showed low removal ability due to extremely salty environment and often exposed to low-level and non-toxic contaminants [14]. Halotolerant bacteria have received significant attention for their ability in the biotechnological implementation as well as their role in the bioremediation mechanism of various recalcitrant pollutants in the environments. Previous reports have shown that halophilic bacteria have potential and promising applications in the degradation of hazardous pollutants than formerly expected [14]. Another study revealed that the best growth of some microorganisms could be considered as follows: non-halophilic bacteria (< 0.2 M NaCl), halotolerant bacteria (non-halophilic tolerating high salinity environment), low halophilic bacteria (0.2–0.5 M NaCl), moderately halophilic bacteria (0.5–2.5 M NaCl) and extreme halophilic bacteria (2.5–5.5 M NaCl). Most bacteria found in nature are categorized as non-halophilic or non-halotolerant that unable to live and survive in extreme salinity situations. An attempt to find newly halotolerant bacteria was conducted in different seawater, but those bacteria were indicated in the same species or genus as previously found [15]. Although a variety of bacteria have been studied for biodegradation of PAHs, only a few studies about halotolerant bacteria have been reported. The study aimed to determine the degradation and transformation of three-ring PAHs phenanthrene and anthracene by *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 under halophilic conditions. The kinetic and enzyme involved in the degradation process was also investigated.

Materials and methods

Chemical

Anthracene, phenanthrene and other suspected metabolites were obtained from SigmaAldrich (St Louis, MO, USA) and were of the highest purity available. Silica gel 60 (0.2–0.5 mm), ethyl acetate, chloroform, dichloromethane and other high-grade reagents were purchased from Across (German).

Batch study

The Bushnell–Haas (BH) liquid media in a 200-mL Erlenmeyer flask was prepared for the cultivation of the bacteria. BH media (40-mL) was used in this experiment because given a significant growth for halotolerant bacteria in removing hydrocarbons. *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04, the halotolerant bacteria were collected from high salinity soil contaminated with crude oil. Three plugs of the bacteria were punched out with cork borer (5 mm) and augmented in a liquid medium until it reaches optimal growth prior to the addition of the solution of aliphatic hydrocarbon and PAHs (100–2000 mg/L; 0.1% Tween 80). The flask was incubated at room temperature (24–28 °C in the dark condition). The low-temperature centrifugation (4000*g*) of media was carried out to obtain the supernatant and followed by resuspension with a new BH medium with varying concentrations of NaCl as such 100–2000 mg/L. The growth (log CFU/mL) progress of *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 on anthracene and phenanthrene was monitored by colony-forming unit (CFU) technique. Degradation of anthracene and phenanthrene was analyzed at intervals of 5 h to 30 h using a gas chromatography–mass spectrometry (GC–MS). Cells separation was conducted by using low-temperature centrifugation (8000*g*) for 12 min and followed by liquid–liquid extraction using ethyl acetate three times. The anhydrous sodium sulphate was added into the ethyl acetate extract to remove the trace of water. Column chromatography was conducted to purifying the targeted compounds from the extract. The autoclaved bacterial culture at 105 °C for 1 h was prepared as the abiotic control. All analysis was conducted in triplicate at each time point. The tolerance of bacteria in an optimum salinity medium was conducted by monitoring the highest growth in the specific NaCl concentration. Positive control for degradation was performed by culturing bacteria without contaminant while one flask without fungi was prepared as the negative control for the determination of any volatilization of the contaminant. The removal of PAHs by the biosorption mechanism was ignored in order to focusing the role of extracellular enzyme in degradation and transformation.

Quantification of residual PAHs and metabolites

The quantification of residual PAHs was calculated based on the data obtained from GCMS analysis. The condition of GCMS used in the experiment is as follows: Agilent 5975E GCMS equipped with a DB-1 column and flame ionization detector were set for the main instrument of analysis. DB-1 is a non-polar and low-bleed column that widely applied for general purpose in a wide variety of applications due to its precision, solvent rinseable and stable in high-temperature limits. The FID is the most commonly used detector for the detection and quantification of aromatic compounds due to its high sensitivity and relatively inexpensive to operate and acquire. Helium was set as the carrier gas at a fixed flow rate of 1 mL/min. The condition of GC temperature: the temperature was initially started at 70 °C, held for 2 min, increased to 150 °C at 18 °C/min, then gradually escalated to 330 °C at 20 °C/min. The temperature of the injector and detector was kept at 260 °C. To determine metabolic compounds, the bacteria were cultured under its ideal condition for 15

and 30 h. The metabolite was extracted from the bacterial culture by using liquid–liquid partition with ethyl acetate as a solvent. A rotary evaporator was used to concentrate the extract, and then derivatization was conducted by silylation using trimethylchlorosilane and pyridine. The identification of metabolites was performed by comparing the retention times and ion mass fragments (*m/z*) of sample and standard compounds. The data of Wiley 275L were also used when necessary.

Enzyme assays

A UV–visible spectrophotometer determined the activity of the enzyme during the degradation process. The crude enzyme was obtained by centrifugation (8000*g*) of liquid media and stabilization with a buffer comprising lysozyme and potassium phosphate. The solution was maintained in warm condition by putting in a water bath (37 °C; 45 min). The supernatant was produced by sonication of crude enzyme and mixing with chloric acid on a magnetic stirrer. One unit (U) is the number of enzymes needed to catalyze the substrate in 1 µmol per minute. The lignin peroxidase was determined by a reaction of veratryl alcohol oxidation to form veratryl aldehyde in sodium tartrate buffer. Manganese peroxidase was assayed by the reacting of phenol red with manganese ions. Dioxygenase was analyzed by reacting the catechol with phosphate buffer. Laccase was determined by observing the reaction of ABTS stock solution in sodium acetate buffer. Epoxide hydrolase was assayed by spectrophotometric using *p*-nitrostyrene oxide as a substrate [16].

Statistical analysis

All the treatments were performed in triplicate. The results were expressed as mean ± standard error.

Results and discussion

The adaptability threshold of bacteria to aromatic and aliphatic hydrocarbons

The simulation test of bacteria for their adaptability to the petroleum hydrocarbons was conducted under exposure to a varied concentration of different aromatic (3–4 rings) and aliphatic hydrocarbon (> C20) (Table 1). All bacteria could exploit all hydrocarbons as carbon and energy source for their metabolism at a maximum concentration of 200 mg/L, indicating that *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 were very effective in the removal of the PAH-contaminated medium at low concentrations. However, *Cryptococcus* sp. MR22 could grow in culture containing anthracene, phenanthrene and eicosane until the concentration of 2000 mg/L. This strain was inhibited and responsive to hydrocarbon characteristic, especially with the shape and molecular weight of the hydrocarbon (pyrene, chrysene, pentosane and triacontane). *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 could utilize anthracene, phenanthrene, pyrene and eicosane at high concentrations for their growth because these bacteria were originally isolated from soil contaminated with crude oil. The bacteria growth is susceptible to the changes in the surrounding environment, and the biodegradation process is influenced

Table 1 Bacteria growth at different concentrations of hydrocarbons

Bacteria	Hydrocarbon	Bacteria growth at different concentrations of hydrocarbons (mg/L)						
		100	200	300	500	1000	1500	2000
Cryptococcus sp. MR22	Anthracene	++++	++++	++++	++++	+++	++	++
	Phenanthrene	++++	++++	++++	++++	+++	+++	++
	Pyrene	++++	++++	++++	+++	+++	++	–
	Chrysene	++++	++++	+++	++	++	+	–
	Eicosane	++++	++++	++++	++++	+++	+++	+++
	Pentosane	++++	++++	++++	+++	+++	++	–
	Triacontane	++++	++++	++++	+++	++	+	–
Halomonas sp. BR04	Anthracene	++++	++++	++++	++++	+++	+++	++
	Phenanthrene	++++	++++	++++	++++	+++	+++	++
	Pyrene	++++	++++	++++	+++	+++	+++	++
	Chrysene	++++	++++	+++	++	++	–	–
	Eicosane	++++	++++	++++	++++	+++	+++	++
	Pentosane	++++	++++	++++	+++	++	++	–
	Triacontane	++++	++++	++++	+++	++	–	–

Growth < 30% (+); growth 30–60% (++); growth 60–90% (+++); growth > 90% (++++)

by many factors such as the type, length and availability of the pollutant. The environmental fate of PAHs was also dependent on the chemical properties such as the molecular size and molecule arrangement. Commonly, the persistence of HMW PAHs was affected by the number of aromatic ring and carbon atom of PAHs due to a concomitant increase in the molecule stability and hydrophobicity [17]. Another study showed that the persistence of PAHs in environments was also depended by its molecular weight and was closely related to their degradation rate by microorganisms [18]. Previous studies have shown the other bacteria showing a significant result in PAHs degradation such as *Mycobacteria* sp. CH1 and *Pseudomonas* sp. LGM2 [19, 20]. Unfortunately, these strains might not be proper for the degradation of PAHs in the environment with high salinity. While in the present study, *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 not only had an impressive ability in the degradation of aromatic and aliphatic hydrocarbon but also could tolerate in the extreme salinity condition.

Removal and kinetics of PAHs

The number of aromatic rings and molecular topology are some of the parameters that influence the percentage of PAHs degradation. Our study revealed that *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 showed their significant role and effective in the degradation of three-ring PAHs such as anthracene and phenanthrene. The positive exponential trend of degradation occurred in the initial 15 h of incubation; then, the process was slow-down after reaching the maximum level (Fig. 1). Anthracene and phenanthrene were eliminated by *Cryptococcus* sp. MR22 after 30 h of incubation, while *Halomonas* sp. BR04 reach > 95% degradation level, respectively. In all these experiments, *Cryptococcus* sp. MR22 resulted in being more active than *Halomonas* sp. BR04. Based on the data obtained that the fit model and generally practical to represent the biodegradation kinetics of anthracene and phenanthrene was first-order rate equation, as shown in Fig. 2, due to the attained R^2 rate, ranged from 0.93 to 0.98. The positive exponential phase of biodegradation by the bacteria was exhibited when a high quantity of substrate was readily available at the beginning of the experiment. Then, the degradation process was reduced when microbial metabolism did not impact the kinetics [21, 22]. Our results are similar to the previous study that the rate of removal of PAH was influenced by the type and habitat of bacteria that are able to adapt to their environments with high pollution. Also, since the bacteria were originated from a soil contaminated with oil, they are more resistant and tolerant to the high-concentrated PAHs medium and easy to manipulate the catabolic pathways of organic pollutants [8, 14]. *Halomonas* species was commonly known as hydrocarbon-degrading organisms and documented regarding their role in the environmental restoration. The previous study showed

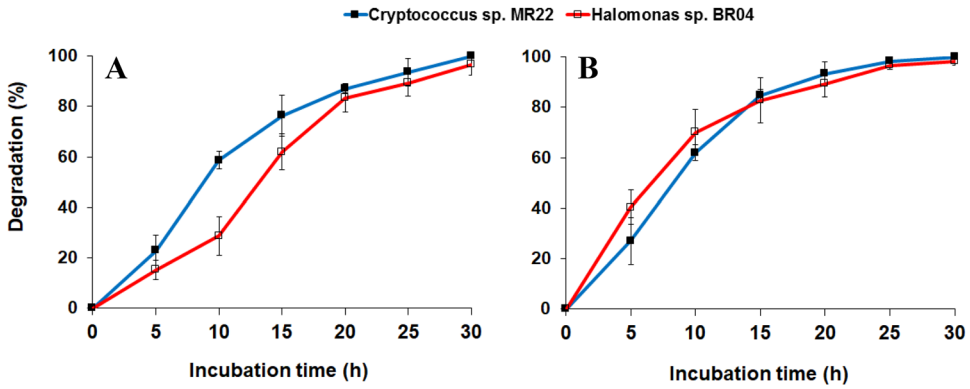

Fig. 1 Degradation of anthracene (**a**) and phenanthrene (**b**) by *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04

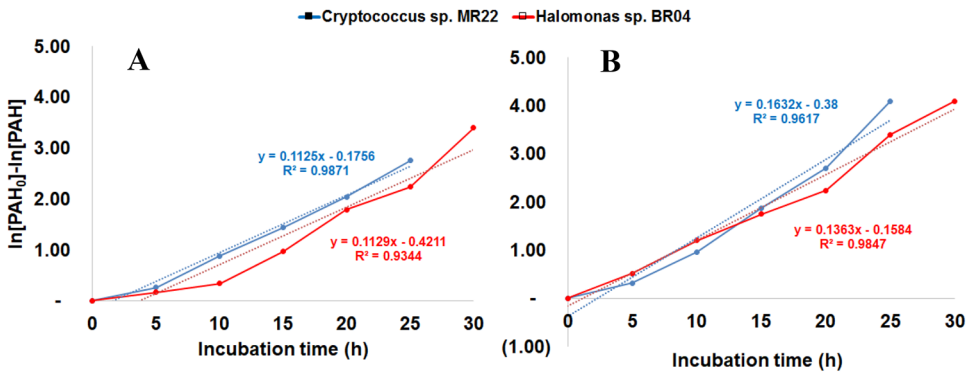

Fig. 2 First order of anthracene (**a**) and phenanthrene (**b**) degradation by *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04

that *Halomonas* sp. RM efficiently degraded 100 mg/L of phenanthrene (67.0%), pyrene (63.0%), naphthalene (60.0%) and benzo [a]pyrene (58.0%) after 7 days of incubation [23]. Our results exhibited that both bacteria were more resistant in very high concentrations (2000 mg/L) and without inhibition at 500 mg/L of phenanthrene or anthracene. Halomonas and cryptococcus were well known for their ability to survive and live in a various contaminated environment. Further, both bacteria can exploit various organic compounds as a source of carbon and energy for their metabolism. However, it was very few reports for Cryptococcus used for biodegradation processes for aromatic hydrocarbons.

Enzyme activities

The extract prepared from cells supplemented with three different substrates exhibited some enzymes such as hydroxylase, dioxygenase, laccase and peroxidase in the *Cryptococcus* sp. MR22 culture, all substrates show a positive reaction for all enzyme activity except glucose for hydroxylase (Table 2). Dioxygenase has shown the highest enzyme activity of all substrates, 2-hydroxybenzoic acid (1.88 U/mL), glucose (1.51 U/mL) and starch (1.21 U/mL). In the *Halomonas* sp. BR04 culture, the activity of hydroxylase and peroxidase were inhibited by the addition of starch as a substrate. The absence of hydroxylase and peroxidase in the extracts showed the negative effect of starch to produce certain enzymes in the metabolic process of bacteria. Addition of starch also showed the reduction of dioxygenase (1.25 U/mL) and laccase (0.98 U/mL). The highest production of enzyme was revealed by dioxygenase of *Cryptococcus* sp. MR22 (1.82 U/mL) in the culture supplemented by salicylic acid. The effect of different carbon sources in PAHs removal and their relationship with the enzyme activity is shown in Table 3. The degradation of anthracene and phenanthrene was correlated with the enzyme produced by both bacteria. Addition of salicylic acid showed a highest degradation of phenanthrene and anthracene (97–99%), while starch gave a lowest degradation (80–83%). This phenomenon can be assumed by the activity of enzyme during the degradation process. The activity of two enzymes (dioxygenase and laccase) was highly detected in the culture supplemented with salicylic acid that showed the highest degradation of phenanthrene and anthracene. On the contrary, in the culture supplemented by starch showed the lower activity of both enzymes. It was suggested that dioxygenase and laccase were playing an important role in degradation of phenanthrene and anthracene. The positive response for growth and enzyme production was shown in the culture supplemented by phenolic compounds such as salicylic acid. The aromatic acid as a redox mediator stimulated the enzyme to stabilize radical and catalyze other substances [24–26].

Table 2 Specific activity of various enzymes in the cell-free extract of halophilic strain *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 grown on different substrates

Bacteria	Enzymes	Specific activity (U/mL)		
		Glucose	Starch	Salicylic acid
Cryptococcus sp. MR22	Hydroxylase	–	0.04	0.82
	Dioxygenase	1.51	1.21	1.88
	Laccase	0.83	0.77	1.25
	Mn peroxidase	0.25	0.29	0.66
	Lignin peroxidase	0.19	0.08	0.21
Halomonas sp. BR04	Hydroxylase	0.12	–	0.22
	Dioxygenase	1.80	1.25	1.82
	Laccase	1.23	0.98	0.25
	Mn peroxidase	0.54	–	0.04
	Lignin peroxidase	0.51	–	0.12

Table 3 The effect of different carbon sources in PAHs removal and enzyme activity

Bacteria	Glucose		Starch		Salicylic acid	
	Degradation (%)	Enzyme (U/mL)	Degradation (%)	Enzyme (U/mL)	Degradation (%)	Enzyme (U/mL)
Cryptococcus sp. MR22	Anthracene (89); Phenanthrene (92)	Dioxygenase (1.48); Laccase (0.81)	Anthracene (80); Phenanthrene (83)	Dioxygenase (1.10); Laccase (0.62)	Anthracene (98); Phenanthrene (99)	Dioxygenase (1.78); Laccase (1.22)
Halomonas sp. BR04	Anthracene (97); Phenanthrene (97)	Dioxygenase (1.48); Laccase (0.81)	Anthracene (81); Phenanthrene (83)	Dioxygenase (1.13); Laccase (0.88)	Anthracene (97); Phenanthrene (97)	Dioxygenase (1.83); Laccase (0.29)

Metabolic of PAHs

The biotransformation of anthracene by *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 cultures was monitored and identified by GCMS (Fig. 3). After 15 days of incubation, 50% of anthracene was degraded into some metabolites, designated *Cryptococcus* sp. MR22 pathway (Ia and IIa) and *Halomonas* sp. BR04 pathway (Ib and IIb). Anthracene was converted by *Cryptococcus* sp. MR22 metabolism into β-hydroxynaphthoic acid(Ia) via 1,2-anthracenediol and 6,7-benzocoumarin. However, both metabolites were not found in the samples. Finally, β-hydroxynaphthoic acid was transformed into coumarin by comparing a query of mass spectra with reference to mass spectra in a Wiley library (Table 4). Metabolite Ia had a retention time at 7.2 min, M^+ at *m/z* 170, an ionic fragment at *m/z* 142 (M^+-28), indicating the sequential loss –CO, and fragment *m/z* 115 (M^+-55), conforming to the loss of $-CO_2H$. The molecular formula of second metabolite (IIa) was determined as $C_9H_6O_2$ and identified as coumarin based on mass spectroscopy analysis. The compound Ib has retention time at 5.8 min and M^+ at *m/z* 146, M^+-28 at *m/z* 118, as representative ion loss of –CO, and M^+-56 at *m/z* 90, in conjunction with the loss of CO + CO. On the other hand, a different pathway of anthracene was exhibited by *Halomonas* sp. BR04. The enzymes produced by *Halomonas* sp. BR04 catalyze

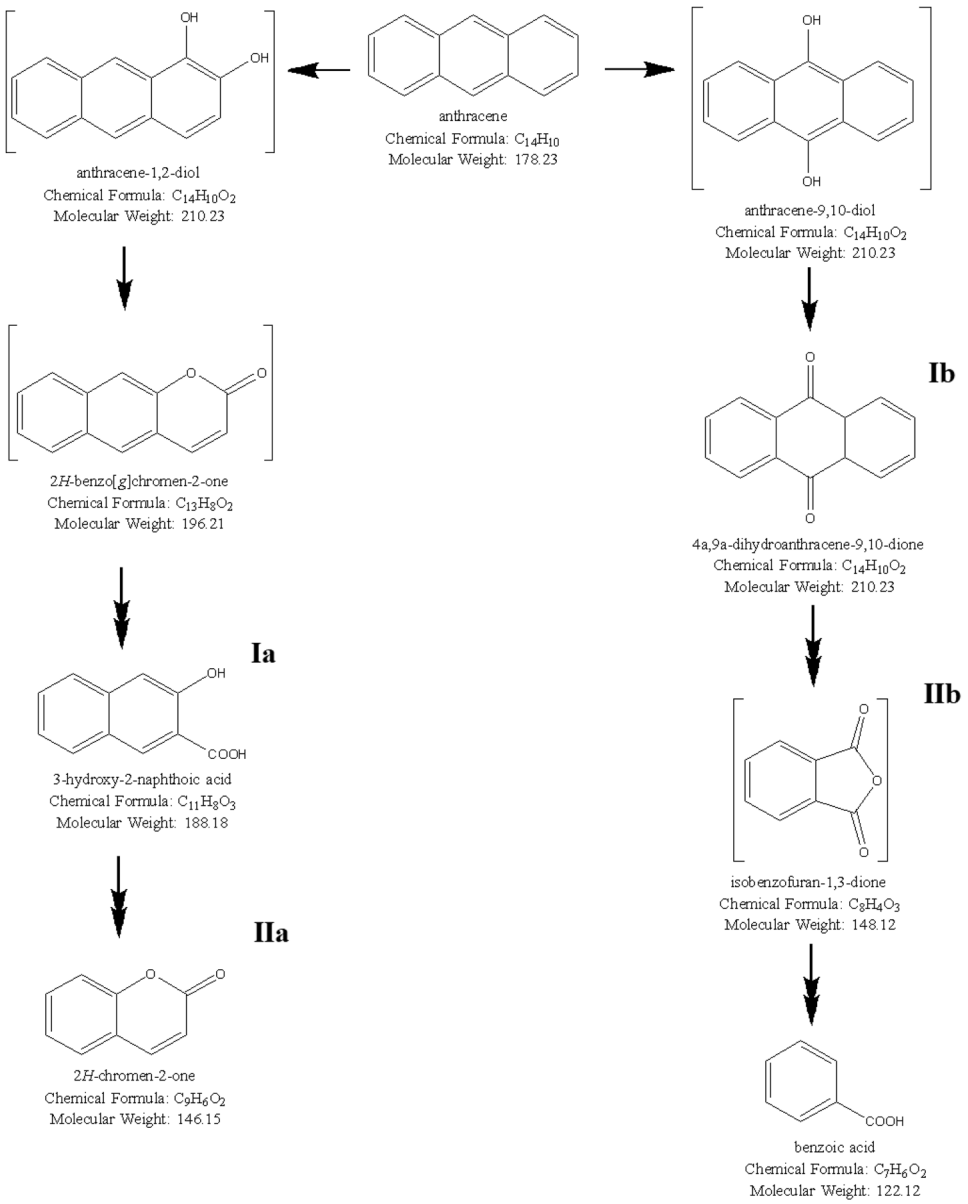

Fig. 3 Proposed pathway for the degradation of anthracene by the halophilic strain *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04. Compounds in brackets are hypothetical intermediates, but not detected

Table 4 Metabolites detected in degradation of anthracene and phenanthrene by *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04

Bacteria	Initial compound	Metabolite	Retention time (min)	*m/z* of fragment ions (% relative intensity)	Possible structure
Cryptococcus sp. MR22	Anthracene	Ia	7.2	57 (20), 63 (12), 71 (30), 113 (14), 114 (51), 115 (20), 142 (55), 143 (10), 170 (100), 171 (9), 188 (40), 189 (10)	β-hydroxynaphthoic acid
		IIa	5.8	146 (50, M^+), 39 (20), 62 (19), 63 (29), 89 (31), 90 (40), 118 (49), 146 (100)	Coumarin
	Phenanthrene	Ia	7.6	332 (12, M^+), 73 (100), 114 (44), 147 (45), 185 (22), 244 (20), 317 (39)	α-hydroxynaphthoic acid
		IIa	13.8	310 (10, M^+), 147 (100), 73 (55), 148 (65), 140 (24), 59 (19), 149 (33), 295 (30), 75 (12), 76 (12)	Phthalic acid
		IIIa	6.5	370 (82, M^+), 73 (100), 193 (93), 355 (39), 311 (40), 194 (31), 371 (30), 74 (27), 165 (9), 223 (25)	Protocatechuic acid
Halomonas sp. BR04	Anthracene	Ia	13.6	208 (28, M^+), 76 (55), 150 (14), 151 (30), 152 (42), 180 (100), 181 (28)	9,10-Anthraquinone
		IIa	8.3	367 (97, M^+-15), 73 (80), 74 (11), 75 (13), 91 (25), 135 (13), 147 (23), 149 (20), 193 (22), 209 (13), 293 (22)	Benzoic acid
	Phenanthrene	Ib	14.5	386 (12, M^+), 73 (100), 117 (19), 147 (18), 269 (21), 371 (9)	Diphenic acid
		IIb	8.6	367 (97, M^+-15), 73 (78), 74 (21), 75 (26), 91 (21), 135(11), 147 (19), 149 (15), 193 (21), 209 (20), 268 (37)	2-Hydroxybenzoic acid

the C9–C10 positions of anthracene in the initial attack to form 9,10-anthracenediol and 9,10-anthraquinone (Ib). The retention time (13.6 min) and mass spectra of the compound were confirmed by the mass pattern of its standard compound. The mass spectra had some important fragment ions that matched with 9,10-anthraquinone such as *m/z* 208 (M^+) and 180 (M^+-28), that consecutive loss of –CO, as well as other fragment ions at 151, 152. 9,10-Anthraquinone was then converted to final product *cis, cis*-muconic acid via benzoic acid; however, only benzoic acid was detected in the extract. The mass spectrum of compound IIb, eluting at t_R 8.3 min, the mass pattern at *m/z* 382 (M^+), 367 (M^+-15) as the subsequent of methyl loss, and 293 (M^+-89), a reflection of trimethylsiloxy group loss. In the first pathway, the production of *cis*-dihydrodiol occurred as a result of the fundamental catalysis to both C1 and C2 atoms of anthracene by multicomponent dioxygenases, followed by conversion to β-hydroxynaphthoic acid via 6,7-benzocoumarin (note detected). However, 6,7-benzocoumarin was detected as one of the anthracene metabolites by some bacteria such as *Mycobacterium* sp. PYR-1 [27], *Martelella* sp. AD-3 [28] and *Rhodococcus* species [29], and showed that 6,7-benzo coumarin is a common metabolite for the first reaction of anthracene transformation in most bacteria. Our result was similar to the previous study that anthracene was transformed into β-hydroxynaphthoic acid as the common intermediate, and it could be converted into salicylate and catechol by enzymes [28]. Our study also revealed that the transformation of anthracene was ended on coumarin. The second pathway was confirmed by a cleavage reaction of enzymatic attack at the C9–C10 positions to produce 9,10-anthraquinone via 9,10-anthracenediol. The previous report showed that *Martelella* sp. AD-3 and *Mycobacterium* sp. PYR-1 attacked the C9 and C10 of anthracene and oxidized it to form 9,10-anthraquinone through 9,10-dihydroxyanthracene [27, 28]. Our study suggested that the initial attack of both aromatic hydrocarbons was catalyzed by multiple enzymes system (dioxygenases, laccase and peroxidase). The presence of *cis*-dihydrodiols isomer indicated the role of several dioxygenases and ortho-ring cleavage reaction in the PAHs transformation [30]. Bioremediation of PAHs in saline–alkaline soils might provide economically and environmentally sustainable treatment strategies if the bacteria used to offer the complete degradation so that possibly hazardous metabolites was not accumulated in the environment [31].

The pathway of phenanthrene metabolites by strains *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 is listed in Fig. 4. Three metabolites, assigned as Ia, IIa and IIIa, were produced in the biodegradation process by *Cryptococcus* sp. MR22 (Table 3). Metabolite Ia had retention time at 7.6 min, M^+ at *m/z* 332, 73 which are characteristics of a silylation, and

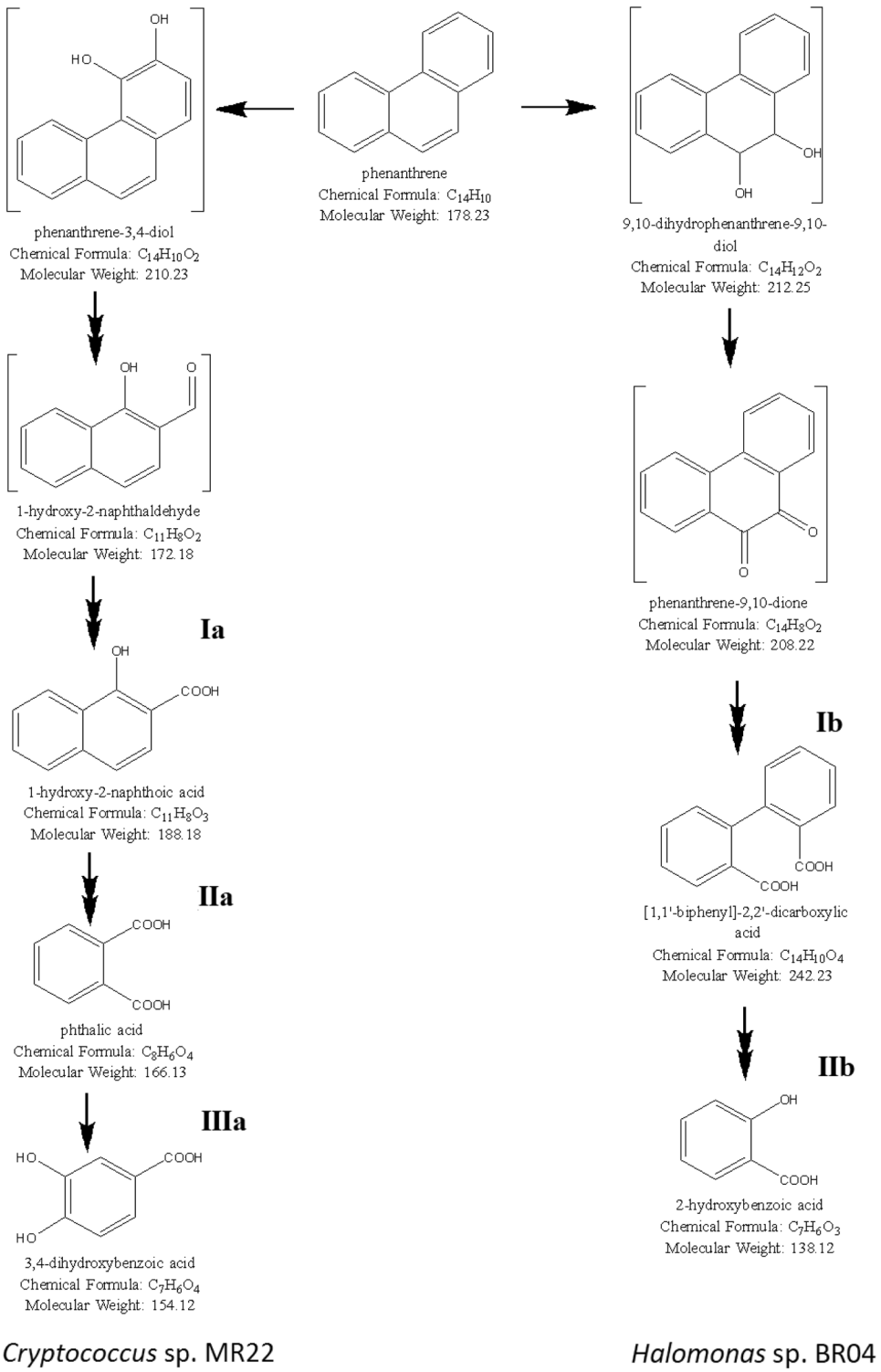

Fig. 4 Proposed pathway for the degradation of phenanthrene by the halophilic strain *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04. Compounds in brackets are hypothetical intermediates, but not detected

317 (M^+-15) corresponding to methyl loss and identified as α-hydroxynaphthoic acid. This metabolite was also found in the phenanthrene degradation process by *Nocardioides* species [32]. The compound IIa had a significant peak at 13.8 min, M^+ at *m/z* 310, 73 as silylation reflection, 295 (M^+-15; methyl loss) and confirmed as phthalic acid. Phthalic acid was also found during phenanthrene degradation by *Stenotrophomonas* species [33]. The identification of protocatechuic acid (IIIa) was performed by evaluation of its retention time (6.5 min) and some mass patterns such as M^+ at *m/z* 370 and 355 (M^+-15) as well as silylation pattern (73), which was also reported as a metabolite by *Arthrobacter sulphureus* RKJ4 [34]. Based

on the above analysis, it can be found that *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 have a different route, reflected by detected metabolites. In this study, phenanthrene was initially attacked by either dioxygenase or peroxidase at the 3, 4C positions to form α-hydroxynaphthoic acid, a metabolite that commonly found by halophilic or non-halophilic bacteria [35, 36]. On the other hand, *Cryptococcus* sp. MR22 had a distinct phenanthrene pathway because the enzymatic reaction of the strain initiated a ring-hydroxylating to form α-hydroxynaphthoic acid via 1-hydroxy-2-naphtaldehyde (not detected). The previous study reported that *Pseudomonas* sp. strain PPD usually metabolize the α-hydroxynaphthoic acid through two separate directions, the phthalic acid pathway or the naphthalene pathway [37]. Therefore, *Cryptococcus* sp. MR22 catalyzed α-hydroxynaphthoic acid into the phthalic acid pathway because only protocatechuic acid was found in enzyme extract. The enzyme peroxidase and dioxygenase were responsible for the conversion of α-hydroxynaphthoic acid that finally form tricarboxylic acid (not detected). The second pathway of phenanthrene by *Halomonas* sp. BR04 differed with *Cryptococcus* sp. MR22 since diphenic acid (Ib) and 2-hydroxybenzoic acid (IIb) were identified in the extract. TMS derivatives and significant fragment ion were matched with the Wiley library, i.e., t_R at 14.9 min, M^+ at *m/z* 386, 73 for the trimethylsiloxy group loss, 269 (M^+-117), subsequent loss of $-COOSi(CH_3)_3$ and 371 (M^+-15) for methyl loss. 2,2′-Diphenic acid was converted by *Halomonas* sp. BR04 to form 2-hydroxybenzoic acid that had M^+ at *m/z* 282, 267 (M^+-15; methyl loss), 193 (M^+-89; $-OSi(CH_3)_3$ loss) and 73 [$(CH_3)_3Si$]. Both metabolites have also been found in phenanthrene degradation by *Ensifer meliloti* [38], *Brevibacterium* species [34], *Nocardia otitidiscaviarum* TSH1 [39], proving that diphenic acid and 2-hydroxybenzoic acid were the common metabolites found in the degradation of phenanthrene through oxidation and cleavage mechanism in aromatic rings. It was assumed that the production of 9,10-phenanthrenequinone via oxidation of 9,10C-position and aromatic cleavage was the main pathway of phenanthrene in the *Halomonas* sp. BR04 metabolism. Dehydrogenation of 9,10-phenanthrenequinone, followed by ortho-cleavage of the ring oxidation, would produce diphenic acid. Laccases and dioxygenase are generally recognized for their role in the oxidation of various aromatic hydrocarbons through the conversion of methyl bond and stable formation of quinone.

Conclusion

Cryptococcus sp. MR22 and *Halomonas* sp. BR04 utilized all tested hydrocarbon for their growth at a concentration of 200 mg/L and could grow in culture containing anthracene, phenanthrene and eicosane until the concentration of 2000 mg/L. More than 60% of anthracene and phenanthrene were degraded within the early 15 h. Anthracene and phenanthrene were eliminated by *Cryptococcus* sp. MR22 after 30 h of incubation, while *Halomonas* sp. BR04 reach > 95% degradation level. The fit model to represent the biodegradation kinetics of both PAHs was first-order rate equation, due to the attained R^2 rate ranged from 0.93 to 0.98. Dioxygenase has shown the highest enzyme activity of all substrates, 2-hydroxybenzoic acid (1.88 U/mL), glucose (1.51 U/mL) and starch (1.21 U/mL). In the *Halomonas* sp. BR04 culture, the activity of hydroxylase and peroxidase was inhibited by the addition of starch as a substrate. The positive response for growth and enzyme production was shown in the culture supplemented by phenolic compounds such as salicylic acid. The pathway of phenanthrene metabolites by strains *Cryptococcus* sp. MR22 and *Halomonas* sp. BR04 was phthalic acid route (2-hydroxybenzoic acid, phthalic acid and protocatechuic acid) and 2-hydroxybenzoic acid route (diphenic acid and 2-hydroxybenzoic acid route). The results suggest that both strains had an impressive ability in the degradation of aromatic and aliphatic hydrocarbon but also could tolerate in the extreme salinity condition may be recommended for environmentally sustainable treatment for PAH in the saline condition.

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Compliance with ethical Standards

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

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