ANALYSIS OF *Pseudomonas* sp. POLYCYCLIC AROMATIC HYDROCARBONS DEGRADATION PROTEINS BY LC-MS/MS

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Abstract. Uncontrolled emission of polycyclic aromatic hydrocarbons (PAHs) has raised concerns about potential environmental consequences. A bioremediation strategy that involves introducing PAH-degrading bacteria into a polluted region has several drawbacks, including strain survival and potential violations of international legislation. Biocatalysis of PAHs as alternatives to bioremediation was detected from biostimulated-PAHs degrading enzymes (PDEs) from *Pseudomonas putida* strain B4. Extracellular PDEs were determined by the production of 9,10 anthraquinone from PAH biodegradation assay. Biostimulated and non-stimulated proteins were analysed by 2D electrophoresis. A spot was detected as upregulated protein while three spots were found to be downregulated proteins. These spots underwent LC-MS analysis for protein identification utilizing SEQUEST HT software against the most recent Uniprot database. The upregulated proteins were chaperonin GroEL protein (WP_010952474.1), ATP synthase subunit beta (WP_003253197.1), ATP synthase subunit alpha (WP_010955887.1) and two dehydrogenase proteins, dihydrolipoyl dehydrogenase (WP_010954949.1) and malate dehydrogenase (WP_004575488.1), respectively. The downregulated proteins identified were azurin (WP_003249580.1) and cold-shock protein (WP_003250656.1). This research aimed to screen and discover proteins that can be used as PAHs enzyme degraders. In the future, these proteins will be genetically modified, expressed and tested for their ability to work together to achieve the best enzyme consortium for biocatalysis remediation.

Keywords: polycyclic aromatic hydrocarbons, biodegradation, laccase, *pseudomonas*, LC-MS.

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Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are carbon and hydrogen atom-based organic compounds. They are all around us and are harmful to the environment and people's health by causing a carcinogenic effect to the lungs, cervix, bladder, breast, and prostate, as well as causing bone loss, eye irritation, and skin sensitivity [1]. Incomplete combustion from natural (forest and brush fires) and man-made combustion sources (automobile emissions and cigarette smoke) produce the PAHs [2]. PAHs can be found in the linear, cluster, or angular patterns of two or more benzene rings [3-4]. Higher hydrophobicity and resistance to microbial degradation are associated with the increased number of fused rings [5].

PAHs in the environment can be degraded through various processes such as photolysis, volatilization, chemical degradation and bioremediation (utilizing plants and microorganisms) [1, 6]. Scientists have found PAHs degrading enzymes from fungus and bacteria, such as laccase producer from *Trichoderma* sp., *Bacillus* sp., *Streptomyces* sp., and *Pseudomonas* sp., as another treatment for PAH pollution [1, 7-8]. Most of the bacteria species that were isolated from petroleum leakage, and PAHs contaminated area was from *Pseudomonas* species, including *Pseudomonas putida*. Safahieh et al. (2010) reported that *Pseudomonas aeruginos*a and *Pseudomonas putida* were identified as bacteria with great capacity in naphthalene biodegradation in Khoor Moosa, with rates of naphthalene degradation by these bacteria after 120 hours of 96% and 91%, respectively [9-10].

To produce early functioning products, the PAHs breakdown process may include more than two enzymes. For example, naphthalene dihydrodiol dehydrogenase, 1,2-dihydronaphthalene dioxygenase, 2-hydroxy-2H-chromene-2-carboxylate isomerase, trans-o-hydroxybenzylidenepyruvic hydratase-aldolase, salicylaldehyde dehydrogenase, and salicylate hydroxylase are enzymes involved in the breakdown of naphthalene to catechol [11]. The degradation of PAHs for example naphthalene usually begins with the breakdown of aromatic hydrocarbons to form catechol. The catechol will be further degraded to form acetyl-CoA and succinyl-CoA, which subsequently be incorporated into the citric acid cycle. The series of degradation processes will finally degrade the hydrocarbons to produce carbon dioxide and water, which are the safest end products [1, 11-12]. This study, biostimulated *Pseudomonas putida* strain B4 to produce PDEs by exposing the cell to petroleum crude and identified the produced enzymes by utilizing a combination of proteomic study and Liquid Chromatography-Mass Spectrometry (LC-MS) analysis.

Material and Methods

Bacterial strain and culture conditions for biostimulation. The bacteria used throughout this project is *Pseudomonas putida* strain B4 (PPB4) as shown in Figure 1 [13]. PPB4 was treated with 0, 5, 10, 15, 20, 25 and 30% of petroleum crude in Bushnell Haas media with 1% peptone. The bacteria cultures were incubated for 3 days at 30 °C, 150 rpm.

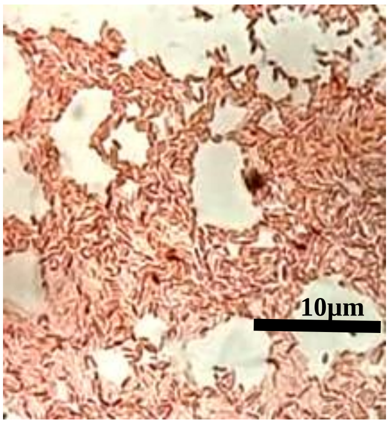

Figure 1. Gram staining for *Pseudomonas putida* strain B4

PAH biodegradation screening. PAHs catalysis activity was examined *via* PAH biodegradation assay [14] with 100 µM of anthracene as substrate in 100 mM acetate buffer pH 5.0. The assay was run overnight (16 hours) at room temperature, in a dark room. The production of 1 µmole of 9, 10 anthraquinone in 1 minute is the unit activity for PAHs degradation. Oxidation of anthracene to 9, 10 anthraquinone was colorimetrically measured at 419 nm and the amount of 9, 10 anthraquinone produced was further determined by using 9, 10 anthraquinone standard curve. For identification purposes, PPB4 was treated with 0 and 5% petroleum crude. PPB4 without petroleum crude (0%) was used as the control, whereas PPB4 with 5% petroleum crude was used as the treatment. The significant value $p<0.05$ were statistically measured by using the ANOVA test.

Collection of protein samples. PAHs was collected from two parts of bacteria culture, which are intracellular and extracellular. To separate the intracellular and extracellular PAHs, the 50 mL bacteria culture was centrifuged at 10,000 rpm for 5 mins. Extracellular PAHs were extracted from the supernatant, whereas intracellular PAHs were extracted from bacteria pellets that had been ultrasonicated prior to enzyme testing.

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Two-Dimensional Gel Electrophoresis (2DE) and image analysis. In the 2DE step, the extracellular proteins were used for the subsequent protein identification process, in view of higher extracellular activity compared to the intracellular activity. The extracellular proteins were precipitated using 10% TCA and solubilized in 0.2 M NaOH and standard sample solubilization buffer SSSB [15]. The soluble proteins were used for 2DE electrophoresis. The 2DE protocol was applied according to the manufacturer's instructions (Biorad, United States) and the Protean IEF Focusing PROTEAN® i12 IEF System was used for the isoelectric focusing step.

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis and protein identification. The 2D gels (control and treated) were then submitted to ABI-National Institutes of Biotechnology Malaysia, for spots analysis and spot picking services by using PROGENESIS analysis. Two gels overlap each other to see the spot difference between 0% and 5% gel. The spots that were analysed as significant upregulated and downregulated proteins were sent to MPOB-Malaysian Palm Oil Board for Liquid Chromatography-Mass Spectrometry (LC-MS) services for protein identification purposes. The protocols were applied by Lau and friends (2020) [16]. The identification process will involve three major steps which are in-gel digestion, LC-MS and data processing. The identification process involves three primary processes which are in-gel digestion, LC-MS and data processing

Briefly, proteins were digested with 0.1 µg trypsin (Promega, Wisconsin, USA), before LC-MS analysis. Following incubation for 16 hours at 37 °C, the peptides were eluted in several concentrations of acetonitrile (ACN) and subsequently centrifuged at 14,000 rpm, 15 mins. The peptide extract was then vacuum-dried. A total volume of peptide mixture containing 20 µL of 0.1% formic acid (FA) and 5% (ACN) and 2 µL of digests was loaded onto an Acclaim PepMap 100 C18 column (Thermo Scientific, MA, USA) with 95% of the FA and 5% of the ACN as mobile phases A and B, respectively. Mobile phase B gradient of 5-35% was applied in 75 mins at a flow rate of 300 nL/min for peptide elution. A precursor ion scan with a resolution of 70,000 and a mass range of *m/z* 310-1800 was performed. At 7,500, 0.7 Da, and 60 ms were set as the resolution, isolation window, and ion injection period, respectively. The mass range of the scanned precursors was set to *m/z*110-1800. For data processing, software such as Tune (Ver. 2.11 QF1 Build 3006) (Thermo Scientific, MA, USA) Proteome Discoverer (Ver. 2.4) (Thermo Scientific, MA, USA) were used to generate peptide mass spectra and mass list, SEQUEST HT search engine and Proteome Discoverer were used to compare the produced mass list to *Pseudomonas putida* FASTA sequences acquired from Uniprot (122,144 sequences) and Percolator algorithm (Version 2.04) to further evaluate the spectra that matched the sequences, using the *q*-value at a 1% false discovery rate.

Results and Discussion

Biostimulation and PAH biodegradation screening of Pseudomonas putida strain B4 (PPB4). Biodegradations of extracellular and intracellular proteins were enhanced when PPB4 was treated with 5 to 30% petroleum crude, as shown in Figure 2. However, when petroleum crude increased to higher than 10%, extracellular proteins show higher biodegradation activities compared to intracellular. This indicates that laccase was expressed extracellularly compared to intracellular. This finding contradicts previous reports by Mandic et al. (2019) that higher laccase activities were found in the cell-free extracts than in supernatants of all tested *Pseudomonas* sp. cultures [17].

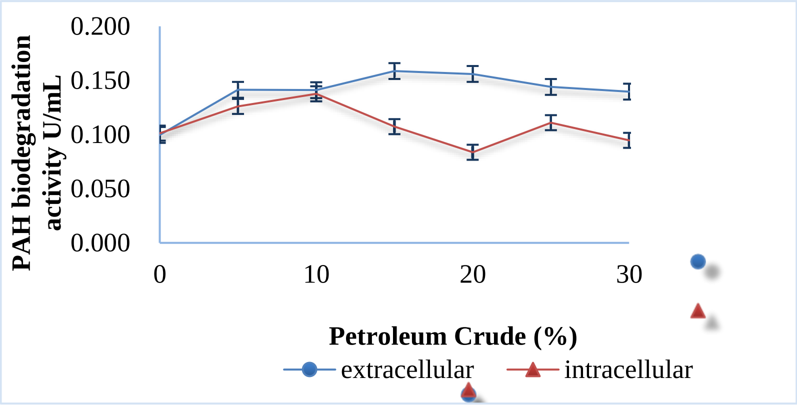

Figure 2. PAH biodegradation activity from PPB4 intracellular and extracellular proteins. Statistical analysis (ANOVA multiple comparisons) for PDEs activity from PPB4 cultures treated with 5, 10, 15, 20, 25 and 30% (v/v) against 0% petroleum crude (control) had shown no significant difference $p>0.05$ for intracellular activity but significant difference $p<0.05$ for extracellular activity.

DE analysis of Pseudomonas putida strain B4 (PPB4). To determine the changes in microbial function and protein expression towards petroleum crude, proteomic approaches were utilized (crude data can be seen in Figure 3). There are four spots detected and picked from the 2D gel by using PROGENESIS software analysis, coded as 21, 78, 105 and 106 as shown in Figure 4.

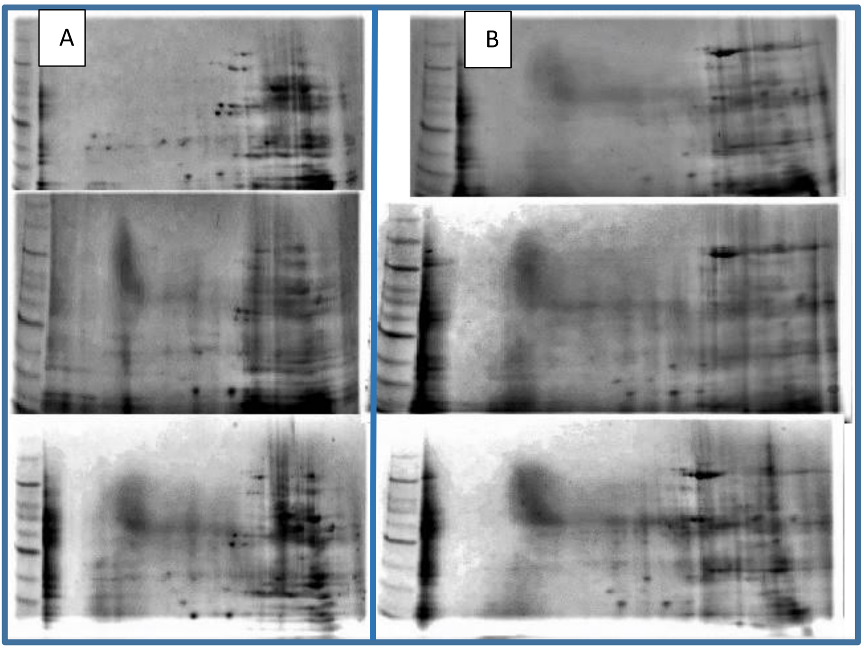

Figure 3. Spots comparison analysis between three replicates of 2DE gel for control (0% petroleum crude) in box A, and treatment (5% petroleum crude) in box B.

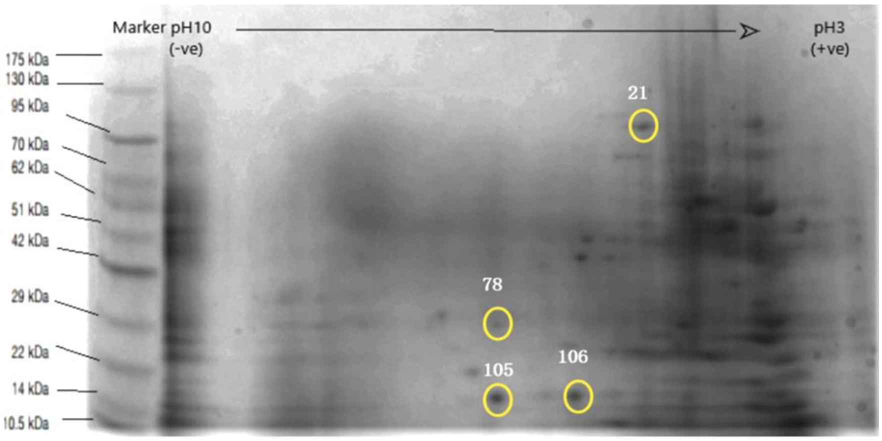

Figure 4. Two-dimensional electrophoresis of PPB4 extracellular proteins by using IPG strips (pH3-10) Non-Liner. Numbers (21, 78, 105 and 106) indicates the spots where the significant difference of protein expression detected from treatment (5% petroleum crude) and control (0% petroleum crude) gel.

Table 1 shows the comparative analysis of protein expression fold between control and treated samples whereby spot 21 was upregulated (protein expression was 2.2-fold higher in the treated sample compared to control) while spot 78, 105 and 106 were downregulated (protein expression was 2.2-, 1.7- and 1.5-fold lower in the treated sample compared to control). These four spots were cut and proceeded with LC-MS analysis for protein identification.

Table 1. Protein expression comparison between control and treated sample by using PROGENESIS analysis software

Spot no.	ANOVA (p)	Expression Fold	Average Normalised Volumes	
			0% (v/v)	5% (v/v)
21	0.008	2.2*	1.09E+04	2.42E+04
78	0.009	2.2	1.04E+04	4759.293
105	0.023	1.7	1.31E+04	7806.617
106	0.001	1.5	1.71E+04	1.13E+04

Note: Expression fold with ‘*’ symbol indicates that protein expression in 5% (v/v) (treatment) was higher than 0% (v/v) (control) while expression fold without * symbol indicates lower protein expression in treatment compared to control.

Protein identification by LC-MS analysis. According to the results, shown in Table 2, the five highest SEQUEST HT scores came from upregulated proteins spot 21, with identified protein name chaperonin GroEL (coverage 80%), F0F1 ATP synthase subunit beta (coverage 94%), dihydrolipoyl dehydrogenase (coverage 53%), F0F1 ATP synthase subunit alpha (coverage 47%) and malate dehydrogenase (coverage 72%) with SEQUEST HT score 1963.59, 1760.76, 791.69, 684.89 and 288.96 respectively. Meanwhile, for downregulated proteins, spot 78, the identified protein name is azurin (coverage 49%), with SEQUEST HT 243.31, while for both spots 105 and 106, the identified protein is a cold shock protein with SEQUEST HT score 238.1 and 139.45, and the peptide coverage at 66 and 50% respectively.

Table 2. Protein identification from LC-MS analysis

Spot No.	Accession No.	Protein Name	Coverage [%]	Amino Acids	MW [kDa]	pI	Score SEQUEST HT
21	WP_010952474.1	chaperonin GroEL [Pseudomonas]	80	546	56.7	5.06	1963.59
	WP_003253197.1	F0F1 ATP synthase subunit beta [Pseudomonas]	94	458	49.3	5.01	1760.76
	WP_010954949.1	dihydrolipoyl dehydrogenase [Pseudomonas]	53	478	49.9	6.32	791.69
	WP_010955887.1	F0F1 ATP synthase subunit alpha [Pseudomonas]	47	514	55.3	5.52	684.89
	WP_004575488.1	malate dehydrogenase [Pseudomonas]	72	422	45.1	5.24	288.96
78	WP_003249580.1	azurin [Pseudomonas]	49	149	16	6.92	243.31
105	WP_003250656.1	cold-shock protein [Gammaproteobacteria]	66	70	7.7	7.25	238.1
106	WP_003250656.1	cold-shock protein [Gammaproteobacteria]	50	70	7.7	7.25	139.45

Note: These proteins were selected based on their highest protein score (Sequest HT)

To explore potential PDEs from PPB4 and to improve current bioremediation strategies, we managed to screen PAH biodegradation activity PPB4 and identified five possible proteins that might work together in degrading PAHs. Our target proteins were laccase or laccase-like proteins, for instance, proteins from the oxidase family. According to a previous study, laccase induction in certain fungal species is caused by anthracene [18] and lead to the production of new isoforms of these enzymes that promotes laccase activity [19-22]. By using 5% of petroleum crude as biostimulants, PPB4 showed a significant difference in PAH biodegradation activity [$p < 0.05$]. Dai et al. (2021) reported that the addition of laccase may increase the heavy oil biodegradation efficiency ($p<0.05$) and biodegradation rate of the bacterial consortia [23]. Proteomics strategies are crucial for evaluating changes in microbial function and protein expressions as a result of environmental conditions [24-25]. LC-MS analysis showed five possible proteins that expressed in wildtype PPB4 when the cell was induced with 5% petroleum crude. These five proteins might work together in increasing PAH degradation based on each protein function. They are chaperonin GroEL (WP_010952474.1), F0F1 ATP synthase subunit beta (WP_003253197.1), dihydrolipoyl dehydrogenase (WP_010954949.1), F0F1 ATP synthase subunit alpha (WP_010955887.1) and malate dehydrogenase (WP_004575488.1). The proteins mostly function in protein folding and electron transfer which might help to increase the PAH degradation activity as the first step in the PAHs degradation. This involves proton attacks from the oxygen. For instance, under stress conditions, the chaperonin GroEL protein prevents misfolding and induces refolding and proper assembly of unfolded polypeptides. F0F1 ATP synthase subunit beta and alpha are involved in proton transfer, an important subunit for monooxygenase and dioxygenase enzyme. While proteins like dihydrolipoyl dehydrogenase and malate dehydrogenase are involved in oxidoreductase activities, might be implicated in the PAH biodegradation. Meanwhile, the identified proteins from spots 78, 105 and 106 are not related to the PAH biodegradation activities.

Conclusion

In this preliminary study of PAHs degradation enzymes, the researchers discovered five proteins that were substantially expressed after exposure to 5% petroleum crude and had strong PAHs degradation activity when compared to controls. Molecular work applications will investigate on the effectiveness of these proteins in collaborating with laccase or laccase-like enzymes.

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Author Contributions

All authors contributed toward data analysis, drafting and critically reviewing the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict to Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards (section 3, Biosafety Act 2007).

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