Lichen-Associated Bacterium, a Novel Bioresource of Polyhydroxyalkanoate (PHA) Production and Simultaneous Degradation of Naphthalene and Anthracene ^S

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Lichens are generally known as self-sufficient, symbiotic life-forms between fungi and algae/cyanobacteria, and they also provide shelter for a wide range of beneficial bacteria. Currently, bacterial-derived biodegradable polyhydroxyalkanoate (PHA) is grabbing the attention of many researchers as a promising alternative to non-degradable plastics. This study was conducted to develop a new method of PHA production using unexplored lichen-associated bacteria, which can simultaneously degrade two ubiquitous industrial toxins, anthracene and naphthalene. Here, 49 lichen-associated bacteria were isolated and tested for PHA synthesis. During the GC-MS analysis, a potential strain of EL19 was found to be a 3-hydroxyhexanoate (3-HHx) accumulator and identified as *Pseudomonas* sp. based on the 16S rRNA sequencing. GC analysis revealed that EL19 was capable of accumulating 30.62% and 19.63% of 3-HHx from naphthalene and anthracene, respectively, resulting in significant degradation of 98% and 96% of naphthalene and anthracene, respectively, within seven days. Moreover, the highly expressed *phaC* gene verified the genetic basis of PHA_{mcl} production under nitrogen starvation conditions. Thus, this study strongly supports the hypothesis that lichen-associated bacteria can detoxify naphthalene and anthracene, store energy for extreme conditions, and probably help the associated lichen to live in extreme conditions. So far, this is the first investigation of lichen-associated bacteria that might utilize harmful toxins as feasible supplements and convert anthracene and naphthalene into eco-friendly 3-HHx. Implementation of the developed method would reduce the production cost of PHA_{mcl} while removing harmful waste products from the environment.

Keywords: Biodegradation, biopolymers, bioproducts, hazardous waste, *Pseudomonas*

Introduction

Lichens are generally known as self-sufficient, symbiotic life-forms between fungi and algae/cyanobacteria [1, 2]. In hostile environmental conditions, lichen thalli also serve as a sanctuary for a wide range of bacteria including *Azotobacter*, *Pseudomonas*, *Beijerinckia*, *Bacillus*, *Clostridium*, *Paenibacillus*, *Actinomycetes*, and *Burkholderia* among others [3–7]. Bacteria associated with lichens offer several physiological benefits to the lichen. Moreover, lichen holds

a very unique biochemical relationship with its partners, a fact that has sparked an interest in many researchers to study these functions in detail. Grube *et al.* [8] proved that these bacteria are involved in supplying nutrients, degrading the older lichen parts, synthesizing vitamins and hormones, providing protection from toxicities, and so on. However, the bacterial diversity, stress tolerance and adaptive mechanisms, as well as their biochemical and physiological mechanisms still need to be discovered.

Moreover, synthetic plastics/polymers have become an

integral part of daily life because of their stable, durable, and suitable mechanical and thermal properties. Currently, approximately 127 million metric tons of synthetic plastic are produced annually throughout the world to meet consumer demand [9]. However, this results in the accumulation of solid wastes that are environmentally harmful and expensive to manage. Biodegradable plastics that degrade naturally in the environment are gaining attention because of their potential to overcome plastic oriented problems. Polyhydroxyalkanoate (PHA) is a biodegradable polyester produced directly in bacteria that has been identified as being valuable for industrial processes because of its potential environmental, electrical, pharmaceutical, cosmetic, and biomedical applications [10]. Depending on carbon monomers, this microbial polymer can be categorized as short-chain-length PHA (PHA_{scl}, 4-5 carbons) and medium-chain-length PHA (PHA_{mcl}, 6-16 carbons). PHA_{scl}, 3-hydroxybutyrate (3-HB), is crystalline and brittle whereas, those classified as PHA_{mcl} such as 3-hydroxyhexanoate (3-HHx, C₆) and 3-hydroxydecanoate (3-HD, C₁₀), are elastomers and rubbers [11]. These PHA_{mcl} have promising industrial and biomedical applications because they have better physical properties than PHA_{scl} [12, 13].

Although four to five genera of bacteria are known to be capable of producing this biopolymer [14–16], the cost of using this method of production is four to nine times higher than that of traditional production methods [17]. Previously, PHA_{mcl} was produced from a wide range of renewable and inexpensive carbon sources such as industrial by-products, waste sludge, BETX (benzene, toluene, ethylbenzene, and xylene) and even polycyclic aromatic hydrocarbons, in short PAHs (naphthalene, phenanthrene, etc.), to reduce costs [18–20]. The ubiquitous components of PAHs are found in the industrial (coke, gas, petroleum, and fuel- and coal-based) areas with particularly high concentrations [21]. The US EPA (Environmental Protection Agency) and ATSDR (the Agency for Toxic Substances and Disease Registry) have identified them as hazardous chemicals for aquatic ecosystems because of their photo-induced toxicities [22]. Microbial degradation of toxins is often a better way of removing these materials than costly physicochemical treatments. Although some studies have investigated the production of PHA using toxins, no studies have explored the amount of toxin degradation/utilization throughout the process. To combine these two microbial activities (PHA_{mcl} production and toxin degradation), a bi-functional stress tolerant bacterium is needed.

It is well recognized that lichen is a highly tolerant life-form. Microorganisms present in the lichen are also well

adapted to various stressful conditions as well as supporting the lichen in several ways. Cernava *et al.* [23] reported some bacteria with antagonistic potential, bearing genes involved in the biosynthesis of stress-reducing metabolites. Besides the elimination of stress, the lichen-associated microbiomes showed evidence in the detoxification of inorganic and organic matters such as arsenic, copper, and zinc as well as xenobiotic/PAH substances [24, 25]. Moreover, a comprehensive proteomic study on lung lichen *Lobaria pulmonaria* showed that bacterial enzymes identified from *L. pulmonaria* are responsible for the metabolism of methanol/C1-compounds and CO-detoxification [26]. On the other hand, it has been strongly believed that PHA synthesis is one of the survival mechanisms of the bacteria to live in stressful and unstable environments [27]. For example, Pham *et al.* [28] found that PHA production by *Pseudomonas aeruginosa* plays a very important role in biofilm formation, a surface-dependent complex bacterial aggression. Thus, a connection among lichen-associated bacteria, their tolerance against PAHs, and PHA production was assumed. Therefore, the present study focused on the stress tolerant mechanism of lichen-associated bacteria and enlightened a new source of PHA_{mcl} production for the first time. To this end, bacteria were isolated from a range of lichen thalli. Two common PAHs, naphthalene and anthracene, were used for the study. The effects of nitrogen concentration on PHA_{mcl} accumulation in lichen-associated bacteria were recorded. The detoxification/degradation of naphthalene and anthracene was also determined and evaluated in a time frame manner. Finally, the PHA_{mcl} synthase-encoding gene with the expression was also verified.

Materials and Methods

Lichen Collection and Isolation of Lichen-Associated Bacteria

Identified lichen samples were kindly provided by the Korean Lichen Research Institute (KoLRI), Sunchon National University, Korea for this study. Lichen samples were collected from several arid areas in Xining Shi, Qinghai Sheng, China (37°20'16.8"N, 101°23'53.1"E). Forty lichen samples of twenty-one different species were used in the present study. Lichen samples were washed thrice with sterile 0.85% NaCl solution before bacterial isolation. Aseptically ground lichen thalli were spread on nutrient agar (Difco, USA) plates at 22°C for bacterial growth. All observed single colonies were then purified by repeated streaking for isolation on nutrient agar plates.

Growth Media and Conditions for Initial Screening

The purified lichen-associated bacteria were screened for PHA production by subjecting the organisms to feast and famine

growth conditions. Isolates were first grown in nutrient broth (Difco) for 72 h to generate a large number of cells, after which they were transferred to nutrient-limited media (6.0 g Na_2HPO_4 , 3.0 g KH_2PO_4 , 0.5 g NaCl, 1.0 mM MgSO_4 , and 0.1 mM CaCl_2 per liter) at 22°C and 180 rpm for 7 d [29]. The nutrient-limited media contained no nitrogen source and either glucose (20 g/l), naphthalene (200 mg/l), or anthracene (200 mg/l) as the carbon source to induce production of PHA in bacterial cells under stressful conditions. Similar glucose concentration also has been considered in previous studies [30, 31]. Dimethyl sulfoxide (DMSO) was used to prepare a stock solution of naphthalene and anthracene. All steps were conducted aseptically in triplicate. All chemicals used in the procedure were purchased from Sigma-Aldrich Co. (USA).

Initial Screening of Isolates by Nile Blue A

Initially, the PHA accumulation in the cells under famine conditions was detected by staining as described by Ostle and Holt [32]. Briefly, heat-fixed thin smears of the cells on glass slides were stained with 1% Nile blue A solution and observed under a Nikon Eclipse Ti microscope (Nikon Instech Co., Ltd., Japan) for the presence of fluorescent PHA granules in the cells.

Final Screening through Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis was conducted to detect the type of PHA produced by the fluoresced isolates. Briefly, the cells that generated PHA were collected by centrifugation at $3,500 \times g$ for 20 min, washed twice with sterile distilled water, and lyophilized through a freeze drier (Hanil Science Industrial Co., Ltd., Korea) for 6 h. The dry cell weight (DCW) represents the total cell biomass generated in each 100 ml of media. The PHA from the dry pellets was extracted through sonication with chloroform (80 ml/g of DCW) at 60°C for 6 h. The sample was filtered (0.45 μl syringe filters) and methanolized by using equal volumes of acidified methanol solution (15% [v/v] H_2SO_4) and chloroform at 100°C for 2 h [33]. The chemicals used were purchased from Sigma-Aldrich Co. (USA).

The PHA composition of the methanolized samples was evaluated using a GC-2010 gas chromatograph coupled with a QP2010 mass spectrometer (Shimadzu, Japan) and a Rtx-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm). For each analysis, 1 μl of sample was injected in split mode (1:50). The temperature gradient program followed that described by Dib *et al.* [34]. An MS detector using electron ionization (EI) at 70 eV was operated and EI mass spectra were acquired within a range of m/z 35–350 da. The target compounds were identified using the National Institute of Standards and Technology (NIST02) database with their chemical formula, molecular weight, and NIST number.

Identification and Phylogenetic Analysis of the Bacteria Based on 16S rRNA Sequencing

Fresh cultures of interest were identified by PCR amplification

of the 16S rRNA region of the strains. The DNA of the isolates were extracted using a G-spin DNA extraction kit (iNtRON Biotechnology, Korea) to amplify them with 20 μl of Accupower PCR premix (Bioneer, Korea) and the universal primer set, 27F/1492R with the thermal conditions of 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 30 sec and then final extension at 72°C for 10 min [35]. Amplification was confirmed by electrophoresis on 1% agarose gel. PCR product was sequenced, and the generated sequence was aligned with the related strains obtained from the EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>) [36] using the Clustal_X program [37]. The obtained sequence was deposited in the NCBI database under the accession number KX442588. Phylogenetic analysis was carried out by neighbor-joining [38] method using Molecular Evolutionary Genetics Analysis software (MEGA7) [39]. Bootstrap analysis was performed based on 1000 replicates for the evaluation of tree topology.

Evaluation of Nitrogen Concentration with Other Environmental Conditions for Optimum PHA_{mc1} Production

The nitrogen concentration of the nutrient-limited media was evaluated using different amounts of NH_4Cl . For this study, the cells were first grown in nutrient broth, then transferred to nutrient-limited media containing 0 g, 0.25 g, 0.50 g, and 1.0 g/l of NH_4Cl with 200 mg/l of naphthalene. The samples were collected after five days of incubation at 22°C with 180 rpm and then prepared as described above for GC analysis. The monomer concentration was determined by comparison to the abundance peak of the standard and groups were compared by one-way ANOVA with Tukey's multiple test correction.

The optimum carbon concentration, temperature, and pH were determined for the PHA_{mc1} producing bacteria. Naphthalene and anthracene were added to nutrient-limited media at different concentrations (200, 400, 600, 800, 1,400, and 1,600 mg/l) to evaluate the tolerance of the strains at 22°C for 5 d. The optical density (OD) of the strains was determined by UV spectrometry (Optizen 3220UV, Mecasys, Korea). The optimum temperature and pH of the strains were determined based on the OD observed after cultivating the organisms at seven different temperatures (5°C, 15°C, 20°C, 25°C, 30°C, 37°C, and 40°C) and five different pHs (5, 6, 7, 8, and 9) in nutrient broth media for 24 h.

Yield Determination of the PHA_{mc1} Monomer

To determine the total yield, either naphthalene or anthracene was used for 7 d of famine stage culture. Additionally, glucose was used to compare the growth and PHA_{mc1} production. Samples and standards were prepared in the same manner as described above. Gas chromatography (GC) was conducted using an Agilent 7890A GC system equipped with an HP-20M capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) and a FID detector. GC conditions were maintained according to a procedure adapted from Goh and Tan [29]. The monomer was detected by comparison with a standard.

Analysis of Xenobiotic Degradation

The degradation patterns of naphthalene and anthracene were analyzed using GC-MS analysis. During sample preparation, the bacterial pellets were removed through repeated centrifugation (3,800 ×g for 20 min). The collected liquid part was sonicated (at 45°C for 90 min) using an equal volume (1:1, v/v) of n-hexane and the polar fraction was then collected. The filtered samples were analyzed by GC-MS while increasing the oven temperature from 60°C to 290°C at 6°C per min, after which the temperature was held at 290°C for 12 min. The sample was injected in split mode (1:30) with a purge flow rate of 3 ml/min⁻¹. The retention time (RT) and *m/z* of anthracene and naphthalene were determined using pure compounds (Sigma-Aldrich Co., USA). The percent of degraded xenobiotics was measured based on comparison with the absorbance area of the control. Acquired data were analyzed by one-way ANOVA with Tukey's multiple test correction.

Verification of the PHA_{mcl} Synthase-Encoding Gene

Amplification of the PHA_{mcl} synthase-encoding gene (*phaC*) fragment from the conserved region of the sample genomic DNA was conducted using the specific primer set, I-179L (5'-ACA GAT CAA CAA GTT CTA CAT CTT CGA C -3')/ I-179R (5'-GGT GTT GTC GTT GTT CCA GTA GAG GAT GTC -3') [40]. The procedure consisted of DNA denaturation at 94°C for 5 min followed by 30 cycles of amplification (30 sec at 94°C, 30 sec at 55°C, 1 min 30 sec at 72°C), after which the samples were subjected to a final extension at 72°C for 5 min. Amplified products were confirmed by electrophoresis in 1% agarose gel. The sequences were then used for BLAST searches of the NCBI database. The obtained sequence of *phaC* fragment was deposited in the NCBI database under accession numbers KY645972.

Expression Analysis of the PHA_{mcl} Synthase-Encoding Gene

To analyze the expression of *phaC* gene in vitro, bacteria were cultured in nutrient broth and then transferred to nutrient-limited media containing glucose (20 g/l), naphthalene (200 mg/l), or anthracene (200 mg/l) as described before. Bacteria were collected after 3 days of growth in triplicates for RNA extraction. Total

Table 1. Sequences of the primers used for Real-time PCR.

Primer	Nucleotide sequence (5' to 3')
rpoS-F	CTCCCCGGGCAACTCCAAAAG
rpoS-R	CGATCATCCGCTTCCGACCAG
EL19_phaC-F	GACAGCCAGATCGACAGC
EL19_phaC-R	GTAGTTCAGATCAGGTCGTTG

RNA of the sample was collected by using easy-spin Total RNA Extraction Kit (iNtRON Bio Technology, Korea) according to the manufacturer's instructions. The reverse transcription of mRNA to cDNA was performed using an ImProm-II Reverse Transcription System (Promega, USA) as stated in the manufacturer's protocol. Reactions were performed in a 10-μl volume containing 100 nM of each primer, 2 μl of cDNA (12.5 ng of input RNA) and 5 μl of iTaqTM Universal SYBR green one-step kit (Bio-Rad, USA). The primers used for this study were shown in Table 1. Real-time PCR was run on CFX96 real-time PCR detection system (Bio-Rad Laboratories). Normalization and comparison of mean threshold cycle (*C_t*) values were analyzed as described previously by Livak and Schmittgen [41]. To compare the relative abundance of the transcripts of target genes, the *C_t* of triplicate reactions was normalized by that of *rpoS* gene. Fold changes were compared to controls without anthracene and naphthalene.

Results

Isolation and Screening of Target Lichen-Associated Bacteria

A total of 49 lichen-associated bacterial strains were isolated from 40 lichen samples by repeated streaking. Initially, twenty-three Nile blue A-stained isolates were identified based on fluorescence emitted under the UV microscope (Fig. 1). However, only one isolate (EL19) from *Xanthoria* sp. was found to produce PHA_{mcl} from naphthalene and anthracene upon final screening. The detected monomer of PHA_{mcl} was identified as 3-hydroxyhexanoate

Fig. 1. Lichen-associated isolates under a UV microscope after staining with Nile blue A.

(A) Arrow pointing to the fluorescent PHA granules in the bacterial cell. (B) Bacterial cells with no PHA granules.

Fig. 2. Neighbor-joining phylogenetic tree of strain *Pseudomonas* sp. EL19 with all other *Pseudomonas* species based on the 16S rRNA gene sequences similarities.

Numbers at nodes represent the bootstrap values (based on 1,000 replications) and only >50% are shown. *Agitococcus lubricus* DSM 5822 has been selected from same order in different family and is used as the outgroup. Bar, 0.01 estimated nucleotide substitutions per site.

(3-HHx) by GC-MS analysis (Fig. S1) and further confirmed with the standard by GC analysis. The PHA_{mcl}-producing bacterium was identified by PCR amplification of the 16S rRNA region (~1,500 bp). A search of the aligned sequences in the EzTaxon database revealed the highest (99.5%) sequence similarity of the isolate with *Pseudomonas azotoformans* DSM 18862. Phylogenetic analysis exhibited that the strain formed a strong phyletic lineage with *Pseudomonas synxantha* DSM 18928, *Pseudomonas libanensis* CIP 105460, *Pseudomonas azotoformans* DSM 18862, *Pseudomonas cedrina* subsp. *cedrina* CFML 96-198, and *Pseudomonas cedrina* subsp. *fulgida* P515/12 within the genus *Pseudomonas* (Fig. 2). Therefore, the present isolate was further identified as *Pseudomonas* sp. strain EL19.

Evaluation of Growth Conditions for Maximum PHA_{mcl} Production

Four different concentrations of NH₄Cl (0, 0.25, 0.50, and 0.75 g/l) were used for the analysis. The study revealed that maximum 3-HHx could be produced under higher

nitrogen stress. Here, *Pseudomonas* sp. strain EL19 produced 82.5 mg/l of 3-HHx with the media containing no nitrogen, followed by 56.8 mg/l. In contrast, the lowest amount of 3-HHx (35.3 mg/l) was yielded from 0.75 mg/l of NH₄Cl after 7 d of famine condition (Fig. 3A). Moreover, the growth of *Pseudomonas* sp. strain EL19 was recorded for different concentrations of naphthalene and anthracene by observing the optical densities during incubation. *Pseudomonas* sp. strain EL19 showed growth with all concentrations of naphthalene, although the growth decreased as concentration increased (Fig. 3B). The strain showed no growth in response to higher concentrations of anthracene (1,400 and 1,600 mg/l) (Fig. 3B). The optimum temperature and pH for the growth of the *Pseudomonas* sp. strain EL19 were found to be 20°C–25°C and 7, respectively (Figs. 3C and 3D).

Yield Determination of 3-HHx

Pseudomonas sp. strain EL19 accumulated 3-HHx from naphthalene and anthracene during seven days of famine

Fig. 3. Different growth conditions of *Pseudomonas* sp. EL19 during 3-HHx production.

(A) Optimum nitrogen concentration for maximum 3-HHx production. Data are presented as the means $\pm$ SEM (3 replicates). Different symbols (*, **, ***) indicate significant differences among nitrogen concentrations for the highest 3-HHx production as determined by one-way ANOVA ($p < 0.05$). (B) Bacterial growth at different concentrations of naphthalene (EL19Naph) and anthracene (EL19Anth). (C) Optimum temperature for bacterial growth to produce PHA_{mcl}. (D) Optimum pH of the growth medium for PHA_{mcl} production.

conditions. The yield of 3-HHx was determined by GC analysis. In case of naphthalene as the carbon substrate, *Pseudomonas* sp. strain EL19 produced 88.43 mg/l of 3-HHx which was 30.62% of total dry cell weight of the bacterium (DCW) (Table 2). The bacterium produced 66.41 mg/l (19.63%) of 3-HHx from 333.66 mg/l of DCW when

anthracene was used as sole carbon source (Table 2). Although the maximum growth was achieved, the bacterium was unable to accumulate 3-HHx when glucose was supplied instead of naphthalene or anthracene as the carbon source (Table 2).

Degradation Analysis of Naphthalene and Anthracene

Significant differences were observed upon GC-MS analysis of the simultaneous degradation of naphthalene and anthracene during 3-HHx production. After five days of deprived condition with naphthalene, *Pseudomonas* sp. strain EL19 degraded 98.3% of naphthalene (Fig. 4A). Conversely, the bacterium was able to degrade 75.9% of anthracene after the fifth day of inoculation. However, *Pseudomonas* sp. strain EL19 degraded 96.4% of anthracene within seven days of famine condition (Fig. 4C). Data were compared using a GC-MS data comparison tool for strain EL19 during each day of degradation of naphthalene and

Table 2. Dry cell weight (DCW), amount of 3-HHx recovered and percent production by the lichen-associated bacteria, *Pseudomonas* sp. EL19.

Carbon source	DCW (mg/l)	3-HHx recovered (mg/l)	3-HHx production (% DCW)
Naphthalene	293.33 $\pm$ 12.68	88.43 $\pm$ 13.78	30.62 $\pm$ 6.04
Anthracene	333.66 $\pm$ 16.49	66.41 $\pm$ 18.29	19.63 $\pm$ 4.62
Glucose	696.67 $\pm$ 10.40	ND	ND

*Not detected: ND

Fig. 4. Degradation of naphthalene and anthracene over time.

(A) Percent (%) degradation of naphthalene (Naph.) by *Pseudomonas* sp. EL19 over time. (B) Chromatograms of each day comparing naphthalene degradation by *Pseudomonas* sp. EL19 to a control. (C) Percent (%) degradation of anthracene (Anth.) by *Pseudomonas* sp. EL19 over time. (D) Chromatograms of each day comparing anthracene degradation by *Pseudomonas* sp. EL19 to a control. Data are presented as the means $\pm$ SEM (three replicates). Values with different characters (a, b, c, d) indicate significant differences in daily degradation of anthracene and naphthalene by the isolates as determined by one-way ANOVA ($p < 0.05$).

anthracene. After the fifth day of incubation of *Pseudomonas* sp. strain EL19, no naphthalene was detected in the GC chromatograph (Fig. 4B). However, a lower absorbance of anthracene (compare to control) was still detected after the seventh day of incubation of the strain EL19 (Fig. 4D).

Verification and Expression of PHA_{mcl} Synthase-Encoding Gene

The PHA_{mcl} synthase-encoding gene (*phaC*) from the conserved region was amplified using a specific primer set and the expected 540 bp PCR product was obtained from gel electrophoresis (data not shown). The acquired sequence of the fragment was analyzed and compared with previously published sequences from the NCBI database. The results showed an 88% similarity with *Pseudomonas fluorescens* PhhR-like protein and PhaC (*phaC*) genes. Furthermore, the presence of *phaC* was confirmed by analyzing the expression of the gene (in vitro) through qRT-PCR. The relative abundance of the transcripts was

Fig. 5. Expression of *phaC* as determined by qRT-PCR.

Values shown representing the expression of *phaC* normalized against the expression of *rpoS*. Biological triplicates were assayed using technical replicates. Standard error bars are shown.

determined with both anthracene and naphthalene. *Pseudomonas* sp. strain EL19 showed higher expression of *phaC* with naphthalene than anthracene (Fig. 5). The normalized values for naphthalene and anthracene were 8.5 and 16.4, respectively.

Discussion

Although PHA_{mcl} is valuable for plastic industries, producers usually face several obstacles in PHA_{mcl} production, including selection of inexpensive carbon sources and suitable host bacteria. The present study was conducted to add several new aspects of research to PHA_{mcl} production from naphthalene and anthracene using lichen-associated bacteria. To accomplish this, PHA-producing bacteria were screened with Nile blue A stain followed by GC-MS. Twenty-three isolates showed positive results after staining with Nile blue A. However, only EL19 was found to be a PHA_{mcl} generator under stressful conditions by GC-MS analysis (Fig. 1). Different results between screening methods might occur because of the ability of lipophilic Nile blue A to stain neutral lipids along with the PHAs [42]. EL19 has been isolated from *Xanthoria* sp. and identified as a member of the genus *Pseudomonas* by the neighbor-joining tree topology based on 16S rRNA sequencing. *Pseudomonas* has been previously isolated from *Xanthoria elegans* with many other associated bacteria [43]. On the other hand, *Pseudomonas*, with *Stenotrophomonas*, *Micrococcus*, and *Burkholderia* have been characterized as bio-synthesizers of stress-reducing metabolites in the large epiphytic lichen, *Lobaria pulmonaria* [44]. Therefore, it can be assumed that the presence of *Pseudomonas* sp. EL19 in *Xanthoria* sp. may benefit in carbon storage, detoxification, and stress adaptation, which may help the lichen to live longer [45]. The metaproteome analysis of *L. pulmonaria* also supports the hypothesis. Eymann *et al.* [26] established that the functional diversities of the symbionts of *L. pulmonaria* assist the lichen in longevity.

The optimum nitrogen concentration for maximum production of 3-HHx was determined as bacteria are more likely to produce PHA when they are in stress, particularly under nitrogen-limited conditions [46, 47]. The results demonstrated that strain EL19 produced the maximum amount (82.5 mg/l) of 3-HHx with media containing 0 g/l of nitrogen, followed by 0.25 g/l of nitrogen (Fig. 3A). Hence, media with no nitrogen was optimal for higher productivity of 3-HHx. Mohan and Reddy [17] optimized critical factors for PHA production from a mixed culture system and found 0.10 g/l of ammonium chloride to be the

best-suited condition for maximum production. However, Thakor *et al.* [48] found that 0.20 g/l was the optimum concentration of ammonium nitrate for PHB production. Moreover, strain EL19 showed great tolerance to naphthalene in comparison with *P. fluorescens* 26K (600 mg/l), *Pseudomonas* sp. TN301 (12 mg/l), and *Bacillus* sp. CYR1 (100 mg/l) [19, 43, 49] at 20°C–25°C and pH 7 (Fig. 3). Panda *et al.* [50] found that neutral pH is also helpful for PHA-producing enzyme activation as well as PHA production. However, Antarctic *Pseudomonas* sp. UMAB-40 showed the highest growth at 15°C when cultured under nitrogen-limited conditions [29].

A number of stress tolerant bacteria were used for PHA_{mcl} synthesis from the related carbon substrate [29]. Here, the newly isolated lichen-associated bacteria were used for bioconversion of 3-HHx from two completely unrelated carbon substrates. Monoaromatic and polyaromatic hydrocarbons such as BTEX, styrene, phenol, 4-chlorophenol, naphthalene, and phenanthrene have previously been converted to PHA using *Pseudomonas*, *Aeromonas*, and *Bacillus* bacteria which were mainly comprised of 3-hydroxyhexanoate (3-HHx), 3-hydroxyoctanoate (3-HO), 3-hydroxydecanoate (3-HD), and 3-hydroxydodecanoate (3-HDD) [18, 22, 51]. Interestingly, the lichen-associated bacterium, *Pseudomonas* sp. strain EL19 was able to accumulate the single homopolymer, 3-HHx when naphthalene and anthracene were used as carbon substrates (Table 2). Typically, 3-HHx is strongly amorphous in nature, giving the polymer a suitable structure for biomedical applications, bio-implantation, and adhesiveness and also making it more useful [52]. Previously, Chung *et al.* [53], Elbahloul and Steinbüchel [54], and Gao *et al.* [55] reported 3-HDD and 3-HO production as the single homopolymer for better elastic and biodegradable properties than the highly studied PHA_{scl}.

In this study, *Pseudomonas* sp. strain EL19 showed higher 3-HHx production (30.62% DCW) with naphthalene (Table 2). Similarly, 19.63% DCW of 3-HHx was generated by strain EL19 from anthracene (Table 2). The total yield of 3-HHx from PAH compounds was higher than that of previous studies. Narancic *et al.* [19] found that *Pseudomonas* sp. TN301 produced 23% CDW PHA_{mcl} (C₆–C₁₂) with naphthalene and 3.5% CDW (C₈–C₁₂) with phenanthrene. Among total PHAs, 4% 3-HHx (C₆) was produced by the strain. Reddy *et al.* [22] reported 18% PHB production from phenanthrene by *Bacillus* sp. CYR1, while no PHB was generated by *Cupriavidus* sp. CY-1 [56]. However, *P. putida* KT2440 has been shown to produce higher amounts of PHA_{mcl} from octanoic acid, nonanoic acid, glucose, acrylic

acid, potassium octanoate, and potassium 10-undecenoate at levels ranging from 16.2 to 75.5% DCW [57–59]. In the current study, no 3-HHx was found in the glucose-containing medium.

Conversely, *Pseudomonads* are a well-known community of xenobiotic degraders [59, 60]. A number of bacterial isolates including *Pseudomonas* sp. are known to degrade polyaromatic hydrocarbons; however, very few of these can produce PHA compounds [61–63]. In the present study, we demonstrated that the EL19 strain significantly degraded 98% of the naphthalene in five days (Fig. 4A) and stored the carbon intracellularly as 3-HHx. Many studies have reported efficient degradation by *Pseudomonads*. For example, Filonov *et al.* [21] found that 95% of naphthalene was degraded by *Pseudomonas putida* G7 in the soil after 5 d. In batch cultures, *P. stutzeri* 19SMN4 was found to degrade 93% of naphthalene in sea-water [64], while 96.1% of naphthalene was removed by *P. aeruginosa* PTCC 1707 strain [65]. Jacques *et al.* [66] found that *P. aeruginosa* 312A, *P. citronellolis* 222A, and *P. aeruginosa* 332C could remove 71%, 51%, and 24.4% of anthracene, respectively. However, these strains are not able to act as bi-functional bacteria as the lichen-associated *Pseudomonas* sp. strain EL19 with the simultaneous production of 3-HHx.

Additionally, a genomic-confirmation of the PHA_{mcl}-producing capability of the strain EL19 had been conducted. The class II PHA synthase-encoded gene (*phaC*) which is responsible for PHA_{mcl} synthesis in *Pseudomonas* sp. and *Burkholderia caryophylli* [67, 68], was amplified and the expression of *phaC* (in-vitro) was analyzed by qRT-PCR. Previously, it has been used as a rapid tool for PHA-producing isolate identification [69, 70]. However, the current study focused on the genomic expression detected with both conditions (either naphthalene or anthracene) to verify the function of *phaC* gene. The result showed higher expression of *phaC* gene when naphthalene was used as sole carbon source (Fig. 5). Wang and Nomura [30] analyzed the expression level of PHA synthase gene encoding enzymes (*phaC1*, *phaC2*, *phaZ*, and *phaJ4*) and found higher expressions when *P. putida* was grown with lauric acid to produce 3-HO, which also agreed with our result. Therefore, it can be assumed that there is a direct correlation with *phaC* gene encoding enzymes in 3-HHx production by the lichen-associated *Pseudomonas* sp. EL19 when grown with PAHs.

The current study focused on a novel source of PHA_{mcl}-producing bacteria with a complete degradation of PAH compounds. The study shed light on how to utilize lichen-associated bacteria and their stress tolerant metabolic

system to accumulate 3-HHx using naphthalene and anthracene as a carbon source. The results suggest that *Pseudomonas* sp. EL19 is very efficient in the production of 3-HHx and simultaneous degradation of naphthalene and anthracene under nitrogen stress conditions. The highly expressed *phaC* gene also suggests correlation to class II PHA synthase enzymes with 3-HHx synthesis. Thus, this study strongly supports the hypothesis that lichen-associated bacteria can detoxify naphthalene and anthracene, store energy for extreme conditions, and probably help the associated lichen in self-defense. So far, this is the first investigation of utilizing lichen-associated bacteria in a bi-functional way. Implementation of the developed method would reduce the production cost of PHA_{mcl} while removing harmful waste products from the environment.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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