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Article

Proteomic Analysis of Diesel Oil Biodegradation by *Bacillus* sp. with High Phosphorus Removal Capacity Isolated from Industrial Wastewater

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Abstract

This study was initiated to evaluate the phosphorus (P) removal and diesel oil degradation by bacteria isolated from industrial wastewater. The bacteria isolated were identified as *Bacillus* sp. The P removal efficiencies by *Bacillus* sp. were 99% at the initial 20 mg/L P concentration. The diesel degradation efficiencies by *Bacillus* sp. were 86.4% at an initial 1% diesel concentration. Lipophilicity by bacteria was the highest in the log phase, whereas it was the lowest in the death phase. As the diesel was used as a carbon source, P removal efficiencies by *Bacillus* sp. were 68%. When glucose, acetate, and a mixture of glucose and acetate as second carbon sources were added, the diesel degradation efficiencies were 69.22%, 65.46%, and 51.46%, respectively. The diesel degradation efficiency was higher in the individual additions of glucose or acetate than in the mixture of glucose and acetate. When P concentration increased from 20 mg/L to 30 mg/L, the diesel degradation efficiency was increased by 7% from 65% to 72%, whereas when P concentration was increased from 30 mg/L to 40 mg/L, there was no increase in diesel degradation. One of the five proteins identified by proteome analysis in the 0.5% diesel-treated samples may be involved in alkane degradation and is known as the cytochrome P450 system. Also, two of the sixteen proteins identified in the 1.5% diesel-treated samples may be implicated in the fatty acid transport system and alcohol dehydrogenation.

Keywords: *Bacillus sp., P removal, Diesel degradation, Proteomics, Industrial wastewater*

1. Introduction

During the production, transportation, and storage of industrial chemicals, many xenobiotics have been released into the natural environment, including soil, and water, atmosphere[1,2]. As the pollutants are gradually exposed to the environment, the activities of biological organisms are decreased and their biodegradation activities are also suppressed and toxic chemicals are accumulated in the environment[3,4].

The methods to treat environmental pollutants include physical, chemical, and biological methods. Among them, biological treatment methods are favored because they are inexpensive and cause no second pollution problem[5,6]. Recently, the biological treatment method is increasingly important, because it provides the ability to alleviate the environmental pollution problems[7,8]. Among them, the microorganism is the most frequently used in biological treatment. First, the microorganisms have a highly diverse metabolic activity adapting to the environment through the use of the minimum energy resulting from small size and high specific surface area[5]. In the second, it has gene replication and transfer activity[9]. Third, it has a biologically stable cell structure[5]. Fourth, it forms an endo spore when the nutrient is depleted, but as it is returned to favorable conditions, it multiplies[10]. Finally, it has a critical role in the life cycle in the soil environment where it catalyzes the degradation of organic compounds into inorganic ones[6,11-13]. Bioremediation technology can degrade the toxic chemicals in the polluted soil and wastewater into nontoxic chemicals by microorganisms and plants into innocuous products. Especially, it is known as effective in remediating polluted areas which are low cost [5,8,14]. In 1989, a biological treatment method using microorganisms was used to remove oil spilled in the ocean and offshore. The Exxon Valdez accident in Alaska, USA, occurred and it was successful [15-17].

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Biological treatment methods have been used in removing phosphorus as a critical element causing eutrophication[18-21] and degrading the organic pollutants into inorganic compounds[22,23], respectively. They are cost-effective, are expected to be sustainable, and are extensively used for the remediation of contaminated wastewater. Biodegradation of pollutants using microorganisms which is the most important technology in bioremediation is controlled by their bioavailability to microorganisms through their reactions and transport between environmental matrix[24,25]. To accomplish this, the microorganisms possessing genes capable of degrading the pollutants must exist in the polluted site and adapt to environmental conditions such as temperature, pH, nutrient balance, etc[17,24,25]. The indigenous microorganisms used in the bioremediation of wastewater are exposed to a variety of pollutants. Therefore, they can adapt to the pollutants for their existence and survival by their genetic information[9,26,27]. The capability to adapt to the environment is developed from the degradation, uptake, and accumulation of pollutants in the cells of microorganisms[28].

Extensive research has been accomplished on the removal of separate single pollutants by single microorganisms or microbial consortiums[29-33].

Recently, according to the survey by the second soil conservation basic plan of the Korean Ministry of Environment during 2020-2029 years, it was reported that the soil pollutants found in the agricultural soil and wastewater of Korea were identified as mainly TPH and BTEX-derived from gas station, industrial site, and military base[34]. Gas stations and military bases are located near Korea's livestock and agricultural land[35]. In addition, they were contaminated with a mixture of high concentrations of P and petroleum oil resulting from the use of agricultural fertilizer and military base, respectively. However, the research has been focused on the individual single contaminant rather than the mixture. It is difficult to remove them together rather than separate single pollutants[36-40]. It was reported that microorganisms possessing the good adaptation capability to remove more than one pollutant simultaneously occur in the environment[41]. It was reported that *Gordonia* sp. *SD8* promoting plant growth in the rhizosphere is also degrading hydrocarbon oil[42] and heavy metal removal [43]. In addition, Khalifa[44] demonstrated that the newly isolated bacteria, DDK6, capable of degrading diesel oil can use many other organic compounds as carbon sources, which indicates its metabolic versatility. This can be applied to soil polluted with heavy metals and petroleum oil and also be used to enhance the removal of heavy metals by elevated plant metabolic activity. Also, proteome analysis can provide information on proteins involved in diesel oil degradation. However, very little information is still available about the simultaneous removal of P and diesel from the agricultural land and wastewater and further proteome analysis involved in the degradation of diesel which is the first report about it.

Therefore, the purpose of this study was to isolate the bacteria both capable of removing phosphorus and degrading diesel oil from the wastewater, to evaluate the simultaneous P removal and diesel degradation by the isolated bacteria, and finally to identify the proteins involved in the degradation of diesel by the isolated bacteria through proteomic analysis.

2. Experimental procedures

2.1. Isolation and cultivation of P-removing bacteria

The bacteria to remove P was isolated from the wastewater treatment plant of A company located in Daejeon City in Korea. The modified Zafiri media[20] was used for the isolation of P-removing bacteria. After the isolated bacteria colony was transferred to the liquid media, the growth of bacteria was measured and initial P concentration was measured periodically by UV (SHIMADZU, UV mini-1240) and the bacteria high in the P removal capacity was selected for the degradation of diesel oil. The P concentration was measured by the modified ascorbic molybdenum blue method[45]. The P concentrations in liquid media were measured after liquid sampling and filtering through a membrane filter (pore size: 0.20 μm, Diameter: 25 mm). The selected bacteria were identified by 16S rRNA gene sequencing.

2.2. Degradation of diesel oil as a sole source of carbon by the *Bacillus* sp. isolated

Bacillus sp. with high P-removal capacity was inoculated into the liquid media containing diesel oil as a sole source of carbon in each Erlenmyer flask and incubated at 30 °C for 7 days. During the experiment, the Erlenmyer flasks were sealed to prevent the volatilization of diesel oil, and a proper amount of media was added to the flask to use the air in the headspace. Periodically, the degradation capacity of diesel oil was confirmed by measuring the growth of bacteria and diesel oil degradation during the 7 days of incubation. Also, during the degradation of diesel oil by *Bacillus* sp., the P concentration and lipophilicity in the culture media were measured.

2.3. Degradation of diesel oil by *Bacillus* sp. according to the second carbon sources

Bacillus sp. was inoculated into liquid media in the screw-capped flask containing glucose, acetate, and glucose plus acetate as second carbon sources and mixed well and incubated during the 7 days at 30 °C, respectively. Periodically, P and diesel oil concentrations were measured during incubation.

2.4. Degradation of diesel oil by *Bacillus* sp. according to the initial P concentrations

According to P concentration, degradation of diesel oil and P removal by *Bacillus* sp. were measured. At 20, 30, and 40 mg/L of initial P concentrations, the diesel oil degradation by *Bacillus* sp. was measured in the liquid media during 7 days of incubation at 30 °C, respectively.

2.5. Analyses of P removal and diesel oil degradation

Periodically, P and diesel oil concentrations were measured during incubation. To measure the removal of P by *Bacillus* sp., P concentration was measured according to the certified P analytical method

[45]. To measure the degradation of diesel oil by *Bacillus* sp., TPH (total petroleum hydrocarbon) was used in the experiment. TPH concentration in the liquid media was measured by N-hexane extraction and GC-FID according to the certified TPH analytical methods[46]. The column name and its dimension for TPH analysis by GC were DB-5 and 30 m × 0.53 mm, 0.32 μm, respectively. The operating conditions for the gas chromatography used for TPH analysis were as follows: injection volume, 2 μL; Inlet temperature, 280 °C; Initial oven temperature, 40 °C; detector, FID)

2.6. Biosurfactant activity measurement

Biosurfactant activity was measured to indirectly evaluate the potential to degrade the diesel oil by surfactant which was produced by bacteria isolated in the study. For biosurfactant activity measurement, at first 2 mL of aliquots were taken from the culture flask by syringe and transferred to an e-tube, then centrifuged (10.000 × g, 15 min.), After removing hydrophobic oil phase and biomass, after filtering by filter (pore size: 0.20 μm, diameter: 25 mm), the 0.5 mL of filtrate was transferred to the test tube and 5 mL was taken and transferred to the test tube and 50 mM potassium phosphate buffer (pH 7.0) 2.5 mL was added and n-hexadecane 0.1 mL was added and mixed strongly for one minute[47]. The hexane concentration of hexane in the water phase after 10 minutes was measured at 610 nm by UV spectrophotometer (SHIMADZU, UV mini-1240). The n-hexadecane was used as a control.

2.7. Proteomic analysis

The flasks were treated with 0.5% and 1.5% of diesel oil at 20 mg/L of P concentration after *Bacillus* sp. was inoculated and shaken for 2 weeks (30 °C and 150 rpm). Each liquid culture from each flask was streaked on the agar plate and after culture growth of the colony, it was transferred to the tube containing autoclaved ultrapure water and centrifuged for 20 minutes (autoclaved ultrapure water) and 1 ml of the ultra-pure water was added to the cell pellet and centrifuged for 20 minutes and washed cells (8,000 rpm, 4 °C). By repeating this three times, one mL of pro-prep buffer was added to the washed cell, reacted on ice for 30 minutes, and sonicated three times for 30 seconds. The cell was broken completely and put on ice for 30 minutes. This tube was centrifuged for 30 minutes (14,000 rpm, 4 °C) and the sample was quantified by Bradford's[48] method and freeze-dried. 300 μL of 8M of rehydration buffer was added to the freeze-dried sample dissolved for 1 hour and sonicated for 10 seconds. After sonication, the samples were centrifuged for 30 minutes and their protein amounts were quantified by the Bradford method and used for electrophoresis. After that, those proteins were maintained at − 20 °C were used for 2-dimensional electrophoresis (2-DE). After 2-D was performed, silver staining was used for staining the gel. After decolorization, the supernatant was removed and dried in a centrifuge. 55 mM IAA (Indol-3-acetic acid) and 100 mM ABC was added to the tube with the supernatant removed and reacted for 40 minutes at dark and centrifuged (14,000 rpm, 4 °C) for 20 minutes and the supernatant was removed. The ultra-pure water with three times of IAA (Indole-3-acetic Acid) was added to the tube and centrifuged for 20 minutes (14,000 rpm, 4 °C) and the supernatant was removed and dried at centrifuged for 10 minutes. 0.1% TFA (TriFluoro Acetate) was added and dissolved the sample in the dried tube which was used for analysis by MALDI-TOF/MS. The peptide sample was salted out and purified after in-gel digestion, The purified peptide was mass analyzed by MALDI-TOF/MS (AXIMA CFR$^+$ Plus, Shimadzu, Japan) and reflection mode[49].

2.8. Statistical Analysis

The results were analyzed using SAS software (Statistical Analysis System, version 9.1, SAS Institute, Inc., Cary, NC, USA). A *t*-test, analysis of variance and Tukey's honestly significant difference test were performed to detect significant differences in the experimental results[50,51]. The confidence interval was set to 95%, and all experiments were performed in triplicate.

3. Results

3.1. Isolation of *Bacillus* sp. with high P removal efficiency isolated from industrial wastewater

The pure colony isolated from industrial wastewater was identified as *Bacillus* sp. (Figure 1). *Bacillus* sp. was added and incubated to the liquid media containing 20 mg/L of phosphorus to measure the P concentration remaining in the media at a predetermined interval (Figure 2). At predetermined intervals, samples were taken out and their P concentrations were measured. Figure 2 shows the growth curve of *Bacillus* sp. in which its growth was accomplished within 200 hrs *Bacillus* sp. showed the high P removal efficiency as its growth progressed.

3.2. Growth curves of and P removal efficiencies by *Bacillus* sp. at different temperatures

The growth of and P removal efficiencies by *Bacillus* sp. at temperatures of 15, 25, and 30 was measured to evaluate the effect of temperature on the P removal capacity of *Bacillus* sp. (Figure 3). At 15 °C, its growth was not measured, but at 25 and 30, its growth was measured, especially at 30 °C, its growth was the best. As it was grown, P was removed completely.

3.3. Growth curves of and P removal efficiencies by *Bacillus* sp. at different P concentrations

The growth of and P removal efficiencies by *Bacillus* sp. at initial P concentrations of 20, 30, and 40 mg/L were measured to evaluate the effect of initial P concentrations on the P removal capacity of *Bacillus* sp. (Figure 4). As a result, its growth was similar at 20 and 30 mg/L of P but was increased at 40 mg/L of P (Figure 4a). In addition, the P removal efficiencies at 20, 30, and 40 mg/L were 99.9%, 75.4%, and 59.6%, respectively (Figure 4b). It indicated that its P removal efficiency decreased as the P concentration was increased.

3.4. Degradation of diesel oil by the isolated *Bacillus* sp. and biosurfactant activity

Figure 5 shows a chromatogram by GC analysis of standard TPH (a), initially injected TPH (b), and TPH degraded after 7 days of

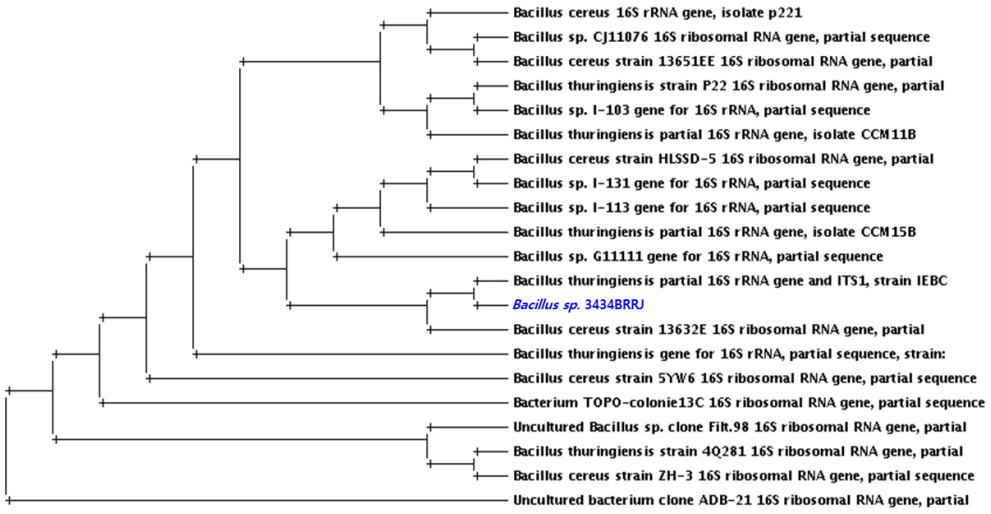

Figure 1. Phylogenetic tree of *Bacillus* sp.

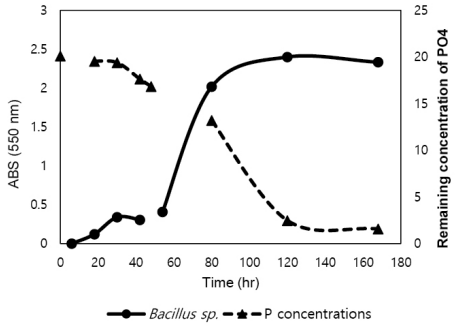

Figure 2. Growth curve (solid line) of and P concentrations (dotted line) removed by *Bacillus* sp. as a function of time.

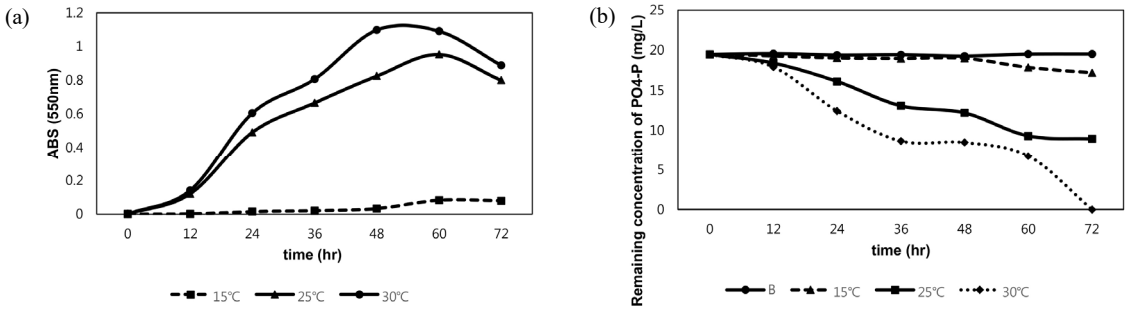

Figure 3. Growth curve and P concentrations removed by *Bacillus* sp. as a function of time in relation to temperature (15, 25, and 30 ℃). (a): Growth of *Bacillus* sp. (b): P concentrations remaining in liquid media after P was removed by the inoculation of *Bacillus* sp.

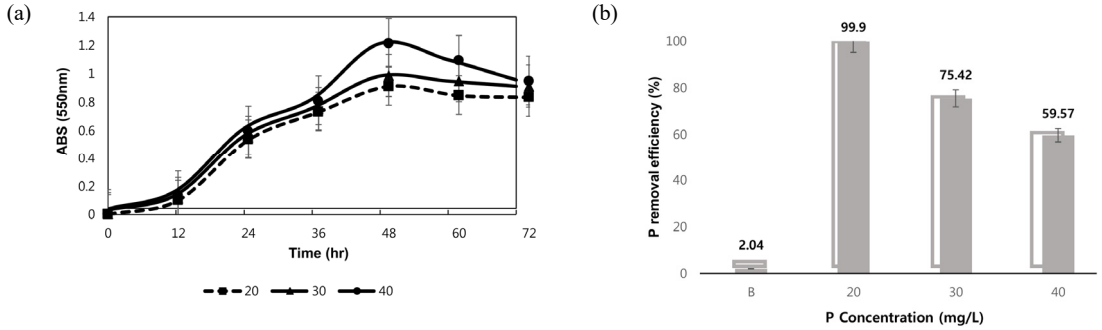

Figure 4. Growth curve(a) of *Bacillus* sp. as a function of time and its removal efficiencies of P(b) about P concentrations (20, 30, and 40 mg/L).

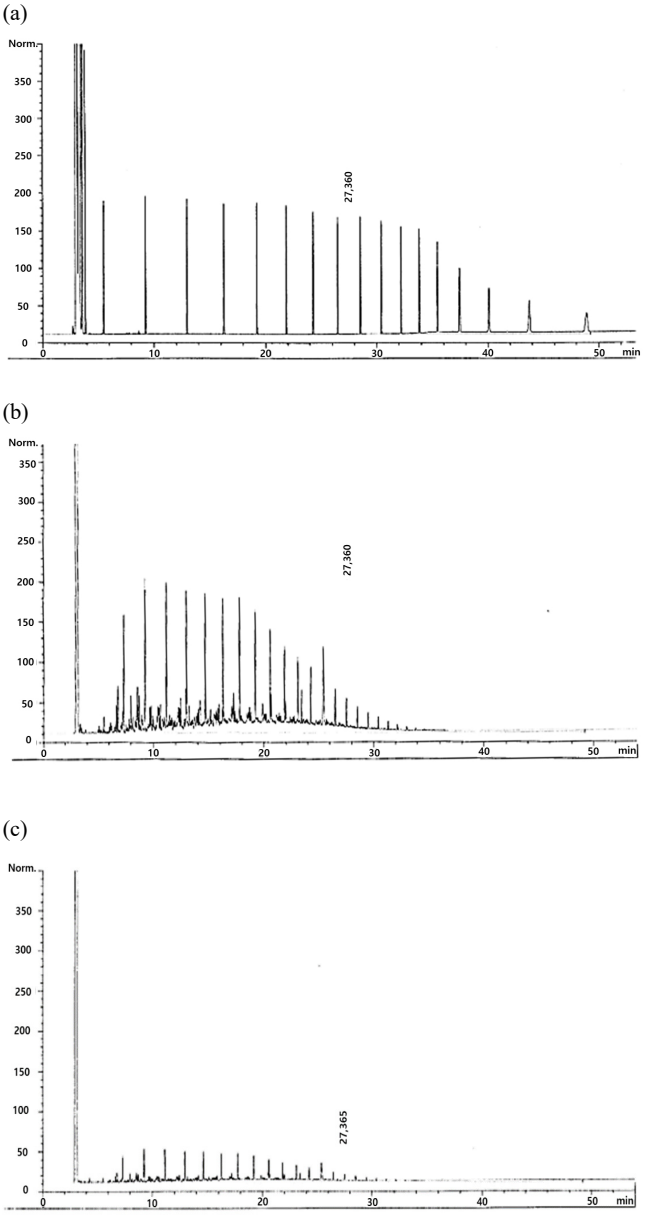

Figure 5. TPH chromatogram analyzed by gas chromatography. (a) TPH standard chromatogram, (b) initial TPH chromatogram, (c) after seven days, TPH chromatogram degraded by *Bacillus* sp.

Bacillus sp. incubation (c). When 1% (v/v) of *Bacillus* sp. capable of removing P was inoculated into the liquid media containing 1% of diesel as a carbon source, as its growth increased, TPH was correspondingly degraded by *Bacillus* sp. during the 7 days of incubation (Figure 6). In addition, biosurfactant activity was measured to indirectly evaluate the solubilization of the diesel oil by *Bacillus* sp.'s biosurfactant production (Figure 7). Their biosurfactant activity was increased during the 3 days of growth and then decreased after 3 days (Figure 7).

3.5. Effect of second carbon on degradation of diesel oil by *Bacillus* sp.

The effect of second carbon sources on diesel degradation by

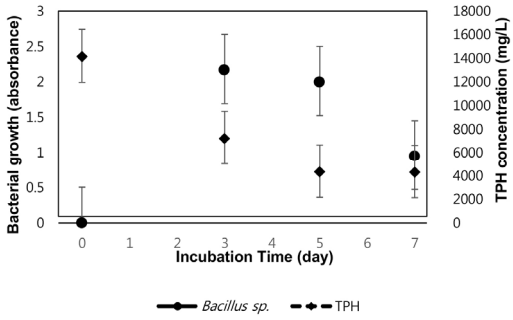

Figure 6. Growth of and TPH degradation by *Bacillus* sp. in liquid media during the 7 days of incubation.

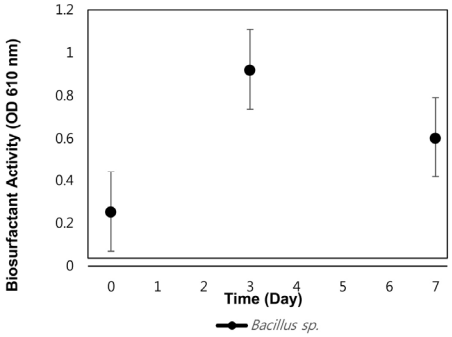

Figure 7. Biosurfactant activity by *Bacillus* sp. as a function of time.

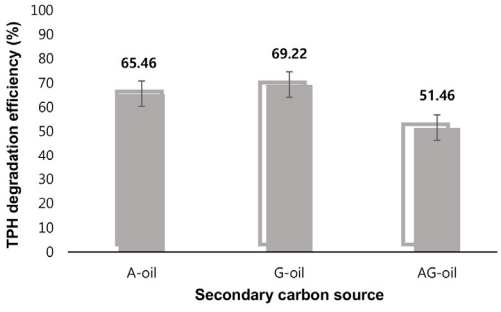

Figure 8. TPH degradation efficiencies by the inoculation of *Bacillus* sp. according to second carbon sources (A, acetate; G, glucose; AG, mixture of acetate and glucose).

Bacillus sp. was investigated (Figure 8). The diesel degradation efficiency by *Bacillus* sp. was the greatest in the treatment of glucose (69%), then acetate (66%), and then in the binary treatment of glucose plus acetate (51%) (Figure 8). Therefore, the degradation of diesel by *Bacillus* sp. was higher in the individual single treatment of second carbon sources than in the binary treatments.

3.6. Effect initial P concentration on the growth and P removal efficiencies of and degradation of diesel oil by *Bacillus* sp.

The growth of *Bacillus* sp., its P removal efficiency, and diesel degradation efficiencies were investigated in the liquid media containing both 1% of diesel and *Bacillus* sp. together with respective additions of 20, 30, and 40 mg/L of P (Figure 9). The result showed that the

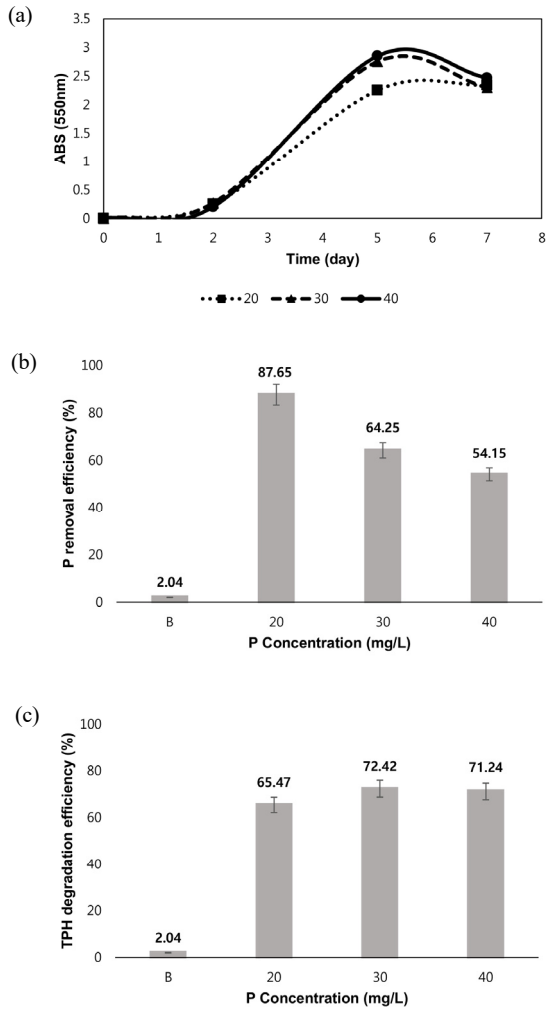

Figure 9. Growth curve (a) of *Bacillus* sp. as a function of time, P removal efficiencies (b), and TPH degradation efficiencies (c) at 7 days of incubation by its inoculation about initial P concentrations.

growth of *Bacillus* sp. was higher in the 30 and 40 mg/L than 20 mg/L (Figure 9 (a)) and its P removal efficiency was decreased as P concentration increased: the 87.7% in the 20 mg/L, 64.3% in 30 mg/L, then 54.2% in 50 mg/L in order (Figure 9 (b)). Also, the degradation efficiency of diesel was increased by 7% as the P concentration was increased from 20 to 30 mg/L, whereas it was not increased as the P concentration was increased from 30 mg/L to 40 mg/L (Figure 9 (c)).

3.7. Proteomic analysis for the expression of proteins involved in the degradation of diesel oil by *Bacillus* sp. according to the diesel oil treatments

The protein was extracted from the 0.5% and 1,5% diesel treatments

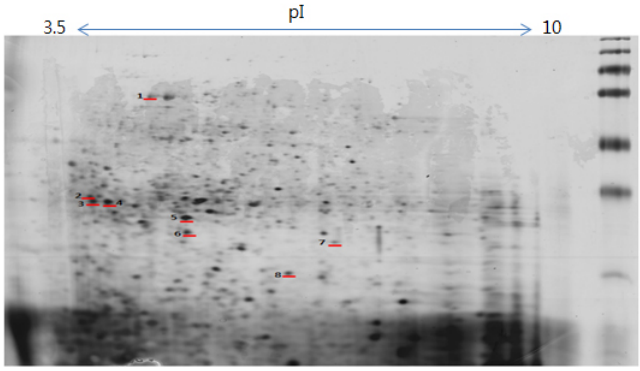

Figure 10. Two-dimensional electrophoresis pattern for proteins extracted from *Bacillus* sp. treated with the diesel 0.5%.

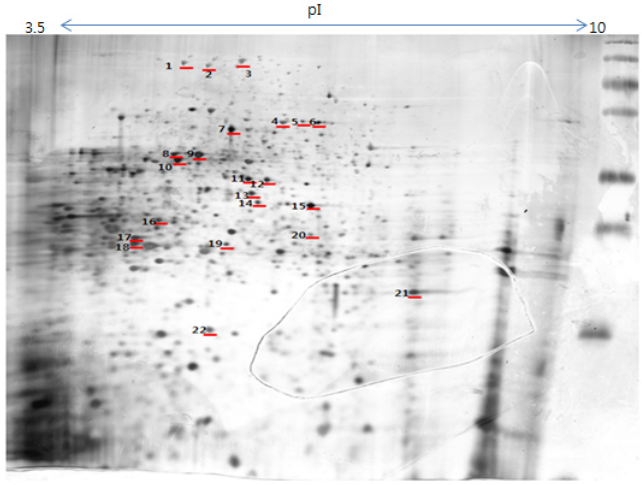

Figure 11. Two-dimensional electrophoresis pattern for proteins extracted from *Bacillus* sp. samples treated with the diesel 1.5%.

to *Bacillus* sp. cultures in the liquid media. After 2 DE analyses for the proteins extracted from two treatments, a total of 7 different proteins were expressed in the 0.5% diesel treatment compared to the 1.5% diesel treatment (Figure 10). In contrast, after 2-DE analysis for the proteins extracted from two treatments, a total of 22 different proteins were expressed in the 1.5% diesel treatment compared to the 0.5% diesel treatment (Figure 11). Therefore, according to a mass analysis by MALDI-TOF/MS for the protein spot removed from the gel and after BLAST searching by NCBI, the following proteins were identified as follows: a total of 5 proteins from 7 spots of the gel in 0.5% diesel treatment were identified in Table 2. Also, a total of 16 proteins from the 22 protein spots in 1.5% diesel treatment were identified in Table 3.

Table 1. Identification of Bacterial Strains by 16S rRNA Gene Sequences

Strain	Number of nucleotide (bp)	Homologous microorganism	Similarity
Isolated colony	*1111*	*Bacillus sp. 3434BRRJ*	937/937 (100%)

Table 2. List of the Protein Spots Extracted from *Bacillus* sp. 3 Samples Treated with 0.5% of Diesel and Characterized by Two-Dimensional Electrophoresis Analysis and MALDI-TOF/MS

Treat	Spot No	Accession No	Protein Name	Molecular Weight (Da)
0.5%	3	gi|190575647	Putative fructose-bisphosphate aldolase, cytoplasmic isozyme 1	36661
	4	gi|334136010	Amidohydrolase, EF_0837/AHA_3915 family	40387
	5	gi|70729882	Cytochrome P450 oxidoreductase	-
	6	gi|71905947	Elongation factor Tu	43419
	7	gi|37520288	Thioredoxin reductase	34445

Table 3. List of the Protein Spots Extracted from *Bacillus* sp. Samples Treated with 1.5% of Diesel and Characterized by Two-dimensional Electrophoresis Analysis and MALDI-TOF/MS

Treat	Spot No	Accession No	Protein Name	Molecular Weight (Da)
1.5%	1	gi|190575504	Putative TonB-dependent receptor protein	106801
	2	gi|190575504	Putative TonB-dependent receptor protein	106801
	3	gi|296162478	Conserved hypothetical protein	13398
	4	gi|332296991	Methyl-accepting chemotaxis sensory transducer with Cache sensor	71217
	6	gi|190572491	Putative fatty acid transport system	49950
		gi|149197333	Glycogen/starch/alpha-glucan phosphorylase	92933
	7	gi|254447956	S-(hydroxymethyl)glutathione dehydrogenase/class III alcohol dehydrogenase	39914
	8	gi|15677170	Alcohol dehydrogenase class-III	41080
	10	gi|190572491	Putative fatty acid transport system, membrane protein	49950
		gi|149197333	Glycogen/starch/alpha-glucan phosphorylase	92933
	14	gi|190575605	Succinyl-CoA synthetase subunit alpha	29881
	15	gi|194364001	3-hydroxybutyrate dehydrogenase	30002
	16	gi|21232524	Succinyl-CoA synthetase subunit beta	41575
	17	gi|254523634	Translation elongation factor G	78323
	18	gi|335042058	Seryl-tRNA synthetase	47423
	19	gi|190576160	Putative pyruvate dehydrogenase E1 component subunit alpha	39844
	20	gi|21230350	Elongation factor Tu	43386
	22	gi|15836844	Polynucleotide phosphorylase/polyadenylated	78268
		gi|75911215	Cysteine synthase	34092

4. Discussion

It was known that microorganisms possess more than one function in nature. It was reported that *Gordonia* sp *SD8* was capable of degrading oil and promoting plant growth[42]. Based on the report by Eweis *et al*.[52] that the addition of nutrients can stimulate biodegradation, we proposed the hypothesis that the P-accumulating microorganisms (PAO) from the wastewater may effectively degrade diesel oil.

4.1. Diesel degradation by P-removal *Bacillus* sp.

In this study, we tried to evaluate the degradation of diesel oil-polluting soil and wastewater by PAO. One percent of *Bacillus* sp. was added to the liquid media containing 1% of diesel oil as the sole carbon source and diesel degradation was measured. The diesel degradation efficiency and P removal efficiency by the bacteria were 86.4% and 68%, respectively. Based on the result, it was found that *Bacillus* sp. can both remove P and degrade diesel oil. As the diesel was added to the media as a carbon source, the P removal efficiency by *Bacillus* sp. was decreased to 68%. This may be because the diesel added to the media as the sole carbon source of *Bacillus* sp. decreased the luxury uptake of P. It was known that the type of carbon source can affect P release rather than P uptake. Unlike the easily available acetate as a carbon source, diesel is not immediately available. As a result, it seems that the P removal efficiency is decreased.

4.2. Biosurfactant production and activity by *Bacillus* sp.

Banat *et al*.[53] reported that biosurfactants can be used for industrial production. Rahman *et al*.[54] and Al-Hawash *et al*.[55] demonstrated that biosurfactants can be used in the degradation of hydrocarbon in the environment. Ko *et al*.[56] reported that the bacteria isolated excreted the biosurfactant which solubilized the hydrophobic hydrocarbon oils into small micelles and degraded them. Therefore, the biosurfactant activity was measured to indirectly evaluate the potential

to degrade the diesel oil by surfactant which was produced by bacteria isolated in the study. In this study, we measured the biosurfactant activity of *Bacillus* sp. As a result of biosurfactant activity measurement, it was relatively high, especially in the log phase even though low in the death phase (Figure 7). A similar pattern was found in the result for the biosurfactant activity of *Y. lipolytic* CL180 by No *et al*.[57]. This suggested that *Bacillus* sp.'s production and activity of surfactant was closely related to its growth. As a result, the biosurfactant activity measurement indicated that the diesel oil could be well degraded in the log phase of growth (Figure 7). The biodegradation of xenobiotic compounds was determined by their accessibility and bioavailability to microorganisms[15,58]. Biodegradability can usually be measured in water solution in controlled laboratory conditions. It indicated the susceptibility of chemicals to microorganisms rather than actual degradability in natural conditions. Therefore, the mobility and transport of chemicals are more involved in the bioavailability of chemicals[58]. More specifically, the biodegradability of chemicals is not changing but their bioavailability can be changed by types of chemicals, environmental matrix, and the interactions of microorganisms. Especially, biodegradation of organic chemicals in nature was affected by the kinds and characteristics of the environmental matrix by which their bioavailability can be determined[15,25]. Therefore, microorganisms degrading diesel oil use it as the sole source of carbon or with second carbon sources added. Some microorganisms produce surfactants and make diesel solubilized into the water and available to microorganisms and degrade using it as a carbon source for their growth. Therefore, the study of surfactants produced by microorganisms is needed to remove diesel oil from pollution sites[59,60]. *Bacillus* sp. may degrade the majority of diesel oil by increased bioavailability through its excretion of surfactants. Microorganisms degrading hydrocarbon oil were classified into 4 categories according to their uptake mode[61]. In the first, microorganisms excrete surfactant and make hydrocarbon oil smaller than its size (pseudo-solubilized oils), and uptake into the cell. The hydrophobicity of their cell surface is low and their lipophilicity is high. In the second type, the microorganisms attach to the surface of the hydrocarbon oil and uptake them, The cell surface of these microorganisms is hydrophobic and very low lipophilic. During the degradation of hydrocarbon oil, they are coagulated. The third type of uptake is that microorganisms uptake only the hydrocarbon oils solubilized in the water phase and the microorganisms' growth is slow compared to those in other types. The final type includes the above three types of uptake [61]. *Bacillus* sp. excrete surfactant and make hydrocarbon oil smaller than its own size (pseudo-solubilized oils), and uptake into the cell, according to the first type. Diesel oil degradation was compared as it was used as a sole carbon source and a second carbon source was used in addition to it.

4.3. Effect of second carbon source on diesel degradation

The effect of the second carbon source on the degradation of diesel by *Bacillus* sp. was evaluated in this study. As the glucose and acetate as second carbon sources were equally added to the media, the growth of *Bacillus* sp. was highest and then as glucose and acetate were added, respectively, their growths were similar to each other. The degradation efficiency of diesel by *Bacillus* sp. in glucose and acetate as second carbon sources was 69.2% and 65.5%, respectively. These degradation efficiencies were lower in glucose and acetate as the second carbon source than in only diesel as the sole carbon source. Namely, in the presence of second carbon sources, the degradation efficiency of diesel by *Bacillus* sp. was decreased. This indicated that *Bacillus* sp. used diesel as a carbon source and if a second carbon source was added, it favored and used the second carbon source added and easily degraded rather than the diesel not degraded well.

4.4. Effect of initial P concentrations on the growth, P removal efficiencies, and diesel degradation of *Bacillus* sp.

The effect of initial P concentrations on the growth, P removal efficiencies, and diesel degradation of *Bacillus* sp. were also evaluated in this study. Growth of *Bacillus* sp. was greater in initial P concentration of 30 to 40 mg/L than in 20 mg/L. whereas, the P removal efficiency by *Bacillus* sp. decreased from 87.7%, 64.5%, and 54.2% gradually decreased as initial P concentrations were increased from 20 to 30 to 40 mg/L. Also, diesel degradation efficiency by *Bacillus* sp. ranged from 65% to 73%, according to initial P concentrations. As the initial P concentrations were increased from 20 mg/L to 30 mg/L, diesel degradation efficiency was increased by 7%. This result was consistent with the report that the addition of nutrients caused the biodegradation of diesel[52] and the addition of nitrogen/phosphorus fertilizers increased the degradation efficiency of TPH[62]. Also, it was reported that as phosphate concentrations increased, emulsifying activity by microorganisms increased Therefore, it is assumed that as the concentrations of phosphate were increased, the degradation of diesel by *Bacillus* sp. was increased. However, as the P concentrations were increased from 30 mg/L to 40 mg/L, degradation efficiency was not correspondingly increased. This indicated that at 30 mg/L of P concentration, the maximum growth of *Bacillus* sp. was accomplished and diesel degradation efficiency was maximum.

4.5. Proteomic analysis for the expression of proteins involved in the degradation of diesel oil by *Bacillus* sp. according to the diesel oil treatments

Proteomics is the large-scale study of proteins[63-65]. Proteins are critical parts of living organisms and many functions include the formation of structural fibers of muscle tissue, enzymatic digestion of food, or synthesis and replication of DNA. Additionally, other proteins are antibodies protecting an organism from infection, and hormones that send important signals into the body. The proteome is the entire set of proteins produced and modified by an organism or system. Proteomics enables the identification of ever-increasing numbers of proteins. This varies with time and distinct requirements, or stresses, that a cell or organism undergoes[66]. It covers the exploration of proteomes from the overall level of protein composition, structure, and activity, and is an important component of functional genomics. Therefore, proteomics is useful in elucidating the kinds and functions of proteins involved in the degradation of the hydrocarbon diesel oil.

In this study, the differential expression of proteins according to the different treatments of diesel concentrations was evaluated, after two weeks of *Bacillus* sp. cultivation in the liquid media with 0.5% diesel and 1.5% diesel added, respectively. After proteomic analysis, compared to the 1.5% diesel treatment gel spot, in 0.5% treatment gel spot, a total of 7 protein spots were found and of those, a total of 5 proteins were identified by MALDI-TOF/MS and NCBI BLAST searching. Of these identified proteins, the protein from the No 5 spot was the cytochrome P_{450} system (Table 2), which was known as involved in the degradation of alkane[29,30]. In contrast, compared to the 0.5% diesel treatment gel spot, in the 1.5% treatment gel spot, a total of 22 protein spots were found and of those, a total of 16 proteins were identified by MALDI-TOF/MS and NCBI BLAST searching. Of these, the proteins from No. 6 and No 10 were assumed to be involved in the fatty acid transport system and the No 8 protein was probably to be alcohol dehydrogenase family (Table 3). The proteins identified belong to the enzymes involved in the production of intermediates of the microbial metabolic processes. It was assumed that as the concentrations of diesel were increased, their expression was correspondingly increased. In other words, as the concentrations of diesel were increased, the proteins in the fatty acid transport system and alcohol dehydrogenases of *Bacillus* sp. were activated and enhanced in the degradation processes of diesel[67]. This study investigated P removal and diesel degradation by *Bacillus* sp. As the growth of *Bacillus* sp. was increased, P removal efficiency increased, and diesel degradation efficiency increased. The most important point in removing environmental pollutants is to provide the best optimal conditions to improve microbial activity[25]. Therefore, studies to establish the best optimal conditions of microorganisms utilized in bioremediation have been continued[62,68]. Since the best optimal environmental conditions for the growth of microorganisms were different from each other, it is very difficult to uniformly adjust the conditions for the different microorganisms to remove many respective pollutants. Through this study, it is expected that finding one microorganism to remove more than one environmental pollutant can save time and effort in adapting to actual field conditions. The metabolic process for the P removal process and diesel degradation by *Bacillus* sp. needs to be studied in the future. In the P removal process by *Bacillus* sp. the study about whether it is uptaken into the cell or attached to the cell surface is needed. Finally, it should be confirmed that *Bacillus* sp.'s P removal and diesel degradation are simultaneously accomplished in the field conditions.

5. Conclusions

P removal and diesel degradation by *Bacillus* sp. isolated from industrial wastewater were investigated in this study. As a result of the experiment, it was found that as the growth of *Bacillus* sp. was increased, the P removal efficiency correspondingly increased. The P removal efficiencies by *Bacillus sp.* was 99%. The diesel degradation efficiencies by *Bacillus sp.* were 86.4%. Lipophilicity by bacteria was the highest in the log phase, whereas, it was the lowest in the death phase. As the diesel was used as a carbon source, P removal efficiencies by *Bacillus sp.* was 68%. When glucose, acetate, and a mixture of glucose and acetate as second carbon sources were added, the diesel degradation efficiencies were 69.22%, 65.46%, and 51.46%, respectively. The diesel degradation efficiency was higher in the individual additions of glucose or acetate than in the mixture of glucose and acetate. When P concentration was increased from 20 mg/L to 30 mg/L, the diesel degradation efficiency was increased by 7% from 65% to 72. By using proteomic analysis, one protein identified in the 0.5% diesel-treated samples may be involved in the degradation of alkane and is known as the cytochrome P450 system. Also, two proteins identified in the 1.5% diesel-treated samples may be involved in the fatty acid transport system and alcohol dehydrogenation, respectively. In conclusion, it appears that the most important thing in removing environmental pollutants is to provide the best optimal environmental conditions to increase microbial activity. However, since the microorganisms in the natural environment may have different optimal growth conditions, it may be difficult to adjust their growth conditions to simultaneously remove the individual pollutants by different microorganisms. Therefore, it appears that our study can contribute to saving time and effort to find the best optimal conditions to apply the microorganisms to remediate the polluted site.

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