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Original Paper

A comparative study for petroleum removal capacities of the bacterial consortia entrapped in sodium alginate, sodium alginate/poly(vinyl alcohol), and bushnell haas agar

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ABSTRACT

The purpose of this study was to identify and compare the degradation efficiencies of free and entrapped bacterial consortia (*Staphylococcus capitis* CP053957.1 and *Achromobacter marplatensis* MT078618.1) to different polymers such as Sodium Alginate (SA), Sodium Alginate/Poly (Vinyl Alcohol) (SA/PVA), and Bushnell Haas Agar (BHA). In addition to SA and SA/PVA, which are cost-effective, non-toxic and have different functional groups, BHA, which is frequently encountered in laboratory-scale studies but has not been used as an entrapment material until now. Based on these, the polymers with different surface morphologies and chemical compositions were analyzed by SEM and FT-IR. While the petroleum removal efficiency was higher with the entrapped bacterial consortia than with the free one, BHA-entrapped bacterial consortium enhanced the petroleum removal more than SA and SA/PVA. Accordingly, the degradation rate of bacterial consortia entrapped with BHA was 2.039 day^{-1}, SA/PVA was 1.560, SA was 0.993, the half-life period of BHA-entrapped bacterial consortia is quite low ($t_{1/2}$ = 0.339) compared with SA ($t_{1/2}$ = 0.444) and SA/PVA ($t_{1/2}$ = 0.697). The effects of the four main factors such as: amount of BHA (0.5, 1, 1.5, 2, 2.5, 3 g), disc size (4, 5, 6, 7, 8 mm), inoculum concentration (1, 2.5, 5, 7.5, 10 mL), and incubation period on petroleum removal were also investigated. The maximum petroleum removal (94.5%) was obtained at ≥ 2.5 mL of bacterial consortium entrapped in 2 g BHA with a 7 mm disc size at 168 h and the results were also confirmed by statistical analysis. Although a decrease was observed during the reuse of bacterial consortium entrapped in BHA, the petroleum removal was still above 50% at 10th cycle. Based on GC-MS analysis, the removal capacity of BHA-entrapped consortium was over 90% for short-chain *n*-alkanes and 80% for medium-chain *n*-alkanes. Overall, the obtained data are expected to provide a potential guideline in cleaning up the large-scale oil pollution in the future. Since there has been no similar study investigating petroleum removal with the bacterial consortia entrapped with BHA, this novel entrapment material can potentially be used in the treatment of petroleum pollution in advanced remediation studies.

1. Introduction

Energy, which is at the center of achieving sustainable development goals, is and will continue to be primary for economic development. While many of the countries need energy for sustainable socio-economic development, the usage of energy comes in with its cost of increasing environmental pollution (Akpan and Akpan, 2012). Petroleum, one of the main energy sources of modern industry, is unfortunately one of the most common organic pollutants and most harmful to the environment (Adipah, 2019; Haider et al., 2021). Due to increased exploration and development in the offshore oil and transportation industries, there is an increase in oil spills from offshore platforms as well as oil tankers and offshore facilities. Unsuitable safety measures and sudden accidents in the extraction, transportation, use, storage, and oil leakage processes cause the spread of large amounts of oily wastewater and environmental pollution (Chen et al., 2017). The environmental pollution caused by petroleum hydrocarbons is the main problem worldwide. Petroleum hydrocarbons have devastating and harmful effects not only on human health, but also to nature. It changes the

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functionality of the ecosystem, especially with its inhibitory effect on microbial species. In addition, plants exposed to petroleum hydrocarbons have limited access to oxygen, water, and essential nutrients due to the restricted movement of plants in the soil matrix. Thus, plant growth and productivity significantly reduce (Agarwal et al., 2009; Ahmed and Fakhruddin, 2018; Dutta et al., 2017; Hung et al., 2020; Truskewycz et al., 2019; Ukalska-Jaruga et al., 2019). Additionally, one of the most important environmental concerns of the last decade is global climate change, also known as global warming or the greenhouse effect. Fossil-fuel combustion is the major human contribution to the greenhouse effect resulting from emissions of environmental pollutants such as carbon monoxide, hydrocarbon compounds, sulfur and nitrogen oxides, and methane (Akpan and Akpan, 2012; Perera, 2017).

The development of environmentally friendly improvement methods for the removal of petroleum hydrocarbons draws attention all over the world (Lei et al., 2019; Lim et al., 2016). Among these methods, bioremediation is frequently preferred with its advantages such as green, reliable, low cost, absence of secondary pollution, and easy to scale up (Achife et al., 2021; Ahmed et al., 2018; Dai et al., 2020; Fang et al., 2022; Ghorbannezhad et al., 2018; Gielnik et al., 2019; Luo et al., 2020; Rocha et al., 2007). Bioremediation is a process in which target pollutants are degraded or detoxified by living microorganisms (Abdulkarim et al., 2019; Pongsilp and Nimnoi, 2022). Due to the synergistic effect of the consortia formed with various bacterial strains with different enzymatic capacities, biodegradation is expected to increase significantly compared to a single microbial strain. The role of bacterial consortium on bio removal has been reported in similar studies (Lu et al., 2014; Luo et al., 2020). As an alternative approach to the direct application of microbial consortia to the contaminated water, microbial cells should be immobilized/encapsulated to a permeable polymeric gel matrix and indirectly exposed to the contaminated area (Mehrotra et al., 2021).

Immobilization is a reusable technique that provides mechanical, biological, and chemical stability, inhibition of cell loss, high tolerance to adverse conditions, high activity, low-cost and ease of storage and use (Chavan and Mukherji, 2008; Jeon et al., 2019; Luo et al., 2020). This technique has a significant role in maintaining the long-term viability of microorganisms (Ezebuiro et al., 2019). Furthermore, it not only increases the concentration and purity of microorganisms in the bioreactor, but also provides a great advantage in controlling the reaction (Chen et al., 2017). Similar studies reported that immobilized microorganisms increase the degradation of petroleum hydrocarbons more than free-living cells (Carabajal et al., 2015; Moreno-Medina et al., 2014). Apart from bioremediation, immobilized cells are widely used in pharmaceutical and food industries, chemical engineering, and biosensor applications. Adsorption and covalent bonding on solid matrices, cross-linking, encapsulation by membranes, and entrapment in a porous matrix are frequently used as immobilization methods (Krasňan et al., 2016; Luo et al., 2020; Martins et al., 2013; Mehrotra et al., 2021). However, entrapment and encapsulation of the microorganisms in a polymeric matrix or attachment to a solid support is one of the most used. As the immobilization of microorganisms on suitable materials provides an easy recovery, reuse of bacteria, and high tolerance to toxic organic compounds, this is an effective way to the remediation of organic pollutants (Berillo et al., 2021; Partovinia and Rasekh, 2018). But it is expected that the entrapment matrices should be light-weight, flexible, cost-effective, partially insoluble in water, easily available, non-toxic, non-polluting, inert, non-biodegradable, mechanically and chemically stable, high diffusivity with high biomass retention, and reusable. Inorganic materials, natural organic polymers, and synthetic organic polymers can be used as entrapment/carrier/support/materials in immobilization processes (Ezebuiro et al., 2019; Mehrotra et al., 2021). Silica- and metal-oxide-based matrices such as zeolites, mesoporous silica, alumina, ceramics, porous glasses, magnetic nanoparticles with a wide range of mechanical properties can be used as inorganic support materials (Ezebuiro et al., 2019; Hartmann and Kostrov, 2013; Zucca and Sanjust, 2014). However, organic materials are more preferred than inorganic ones for whole-cell immobilization, as they are inexpensive, readily available, biodegradable, non-toxic, and generally have higher absorptivity. Natural (dextran, agar, agarose, alginate, chitosan, collagen, seaweed, dead mushroom biomass, rice hulls, lignin, bagasse, corn cob, saw dust, charcoal, plant fibers) and synthetic polymers (polyacrylamide, polyvinyl alcohol, polyethylene glycol, polycarbamoyl sulphonate, polypropylene, polyethylene, polyvinylchloride, polyurethane, polyacrylonitrile) can be used for the preparation of support materials with inexpensive, non-toxic and reactive functional groups (Berillo et al., 2021; Eweida et al., 2022; Ezebuiro et al., 2019; Jeon et al., 2019; Khandelwal et al., 2022; Mehrotra et al., 2021; Mohamad et al., 2015; Nan et al., 2009; Nwankwegu and Onwosi, 2017; Partovinia and Rasekh, 2018; Zucca et al., 2016).

Sodium Alginate (SA), a natural polysaccharide derived from brown seaweed, has been identified as a safe material by the Food and Drug Administration (FDA) (Abdelghany et al., 2020; Hemalatha et al., 2014; Yadav and Maji, 2019). SA is one of the most preferred organic macromolecules for immobilization process with its low cost, high performance in mass transfer, non-biological toxicity, and availability. Bacteria can grow well in natural polymers such as alginate, which is permeable and does not undergo significant physicochemical changes during the immobilization. They are also quite useful for the detoxification of the various hydrocarbons, organic and inorganic dyes, aromatic compounds, and heavy metals. Despite the widespread use of alginate in the immobilization process, there are still some major disadvantages (Mehrotra et al., 2021). Although the entrapment with SA offers an environment in which damage to living cells is minimized, the technique should be developed to improve its limited gel structure and poor mechanical strength. Additionally, limiting the mass transfer of immobilized cells is the major problem which can reduce the degradation efficiency (Ailijiang et al., 2016; Ghorbannezhad et al., 2018; Jiang et al., 2015). To tolerate the disadvantages of the single immobilization methods, the degree of immobilization and microbial activity can be improved by combining two or more entrapment materials. The combination of entrapment materials such as poly (vinyl alcohol) (PVA) and SA provides biocompatibility, stability, and high permeability (Chen et al., 2019; Luo et al., 2020; Sun et al., 2015). PVA, a synthetic linear polymer prepared by hydrolysis or alcohol lysis of polyvinyl acetate under alkaline or acidic conditions, is characterized by its film forming ability, unique physical properties, and high chemical resistance (Abdelghany et al., 2020; Gaaz et al., 2015; Yadav and Maji, 2019). Each of them has low removal capacity, difficult to prepare and recover from the environment, so their use is impractical (Mollaei et al., 2010; Sakdapetsiri et al., 2021). Polysaccharide agar, which is a non-toxic biological carrier besides being cheap and easily available, is widely used for cell entrapment with its properties (melting at 85–95 °C, gelling at 33–45 °C). Although agar is a stable material with high gelling capacity, it has some inherent disadvantages such as poor mechanical strength and poor interactions between bacteria and agar. Therefore, it can be combined with carrageenan, which has high mechanical stability (Alba and Kontogiorgos, 2019; Sagir et al., 2018; Shamsuri et al., 2012). Considering the whole properties of these materials, the choice of the most suitable entrapment material for cell immobilization emerges as a major criterion (Mehrotra et al., 2021). There

is a need for a new entrapment material with high chemical and mechanical stability that overcomes the disadvantages of these support materials, high removal capacities, easy to use, reusable to minimize the bacterial loss and prolongs the survival time of the microorganism against the toxic effect of petroleum. In this context, we have introduced a new approach to petroleum removal with a bacterial consortium entrapped with BHA in addition to SA and SA/PVA. Further, no similar study has been found investigating the petroleum removal capacity of a bacterial consortium entrapped in BHA and characterizing the structure and chemical bonds of BHA as an entrapment material. So, it is anticipated that the current outputs of this study can be used to clean up the large-scale oil pollution. Considering all these, this study brings a different perspective than similar studies in the literature.

The current study aims to (i) compare the degradation efficiencies of free and entrapped bacterial consortia into SA, SA/PVA, and BHA, (ii) obtain the main factors (amount of entrapment material, disc size, inoculum concentration, incubation period) affecting the petroleum removal and the reusability of the gel-entrapped consortium in the removal of petroleum, (iii) characterize the surface morphologies of SA, SA/PVA, and BHA, (iv) analyze the existing functional groups and bond types of SA, SA/PVA, and BHA, (v) detect the removal efficiencies of short-, medium- and long-chain *n*-alkanes with potent gel-entrapped bacterial consortia.

2. Materials and methods

2.1. Chemicals

Bushnell Haas Agar (Himedia, #M349), Bushnell Haas Broth (BHB) (Himedia, #M350), nutrient broth (NB), glucose, yeast extract, Triton X:100, NaCl, $CaCl_2$, sodium alginate (synthetic, powder form, #W201502), and poly (vinyl alcohol) $(C_2H_4O)_n$ (powder form, fully hydrolyzed (Mw approx. 30000) for synthesis, #821039) were obtained from Merck (Germany)/Sigma Aldrich (USA). The crude oil with API gravity = 37 and specific gravity = 0.840 obtained from an oil field in Turkey.

2.2. Bacterial strains

Staphylococcus capitis CP053957.1 (S1) and *Achromobacter marplatensis* MT078618.1 (A1) strains were collected from Hacettepe University Culture Collection Laboratory.

2.3. Preparation of inoculum

2.3.1. Preparation of individual bacterial strains

The bacterial inoculum to be used in the petroleum removal assay was prepared according to Soyuer and Bilen Ozyurek (2023). Accordingly, the individual bacterial strain was inoculated in NB and incubation period was carried out at 150 rpm at 30 °C for overnight in a rotatory incubator (Miprolab, Turkey). Following the incubation period, the individual bacterial strain was centrifuged at 4650×*g* for 10 min with Eppendorf 5810R (Germany), then the culture-supernatants were discarded and the whole cell pellets were washed twice with sterile 0.9% NaCl at pH = 7.0. The bacterial suspensions were adjusted to 0.8 at a wavelength of 600 nm with UV spectrophotometer (Shimadzu-UV1700, Kyoto, Japan) for petroleum removal assay.

2.3.2. Preparation of bacterial consortium

2.5 mL of each bacterial strain which were adjusted to equal growth density, was combined at 1:1 ratio for the preparation of bacterial consortia (Bilen Ozyurek and Seyis Bilkay, 2020; Luo et al., 2020).

2.3.3. Entrapment of the bacterial consortia

Bacterial consortia were entrapped in SA, SA/PVA and BHA according to the immobilization procedures (Ghorbannezhad et al., 2018; Jeon et al., 2019; Luo et al., 2020).

2.4. Preparation of the entrapment materials: SA, SA/PVA, BHA

1g of SA was dissolved into 45 mL of NaCl (0.9%, pH = 7.0) and heated until it was completely dissolved. Following the sterilization at 121 °C for 15 min, the gel was cooled to 45 °C. 5 mL of bacterial consortium was added into the gel and was completely mixed, then 10 mL of the liquid was injected to the sterile syringe and dropped into 0.2 M $CaCl_2$ solution for cross-linking effect using a magnetic stirrer (IKA, Germany). Then, SA alginate beads were washed twice with sterile NaCl solution (0.9%, pH = 7.0) and were used for petroleum removal assay.

1g of SA and 0.6 g of PVA were dissolved into 45 mL of NaCl (0.9%, pH = 7.0) and heated until it was completely dissolved. Following the sterilization at 121 °C for 15 min, the gel was cooled to 45 °C. 5 mL of consortium was added into the gel and was completely mixed, then 10 mL of the liquid was injected to the sterile syringe and dropped into saturated H_3BO_3 + 0.2 M $CaCl_2$ solution for cross-linking effect using a magnetic stirrer. Then, SA/PVA beads were washed twice with sterile NaCl (0.9%, pH = 7.0) solution and were used for petroleum removal assay.

1g of BH agar was dissolved into 45 mL of NaCl (0.9%, pH = 7.0) and heated until it was completely dissolved. Following the sterilization at 121 °C for 15 min, the gel was cooled to 45 °C. 5 mL of consortium was added into the gel and was completely mixed, then 10 mL of the liquid was added into sterile Petri dish then it was allowed to solidify at 4 °C in the refrigerator for overnight. Agar beads obtained with a 7 mm diameter perforator dropped into 0.2 M $CaCl_2$ solution for cross-linking effect. Then, agar beads were washed twice with sterile NaCl (0.9%, pH = 7.0) solution and were used for petroleum removal assay.

2.5. Petroleum removal

The assay was carried out with Erlenmeyer flask containing 50 mL BHB (g/L): $MgSO_4$ (0.2), $CaCl_2$ (0.02), KH_2PO_4 (1.0), K_2HPO_4 (1.0), NH_4NO_3 (1.0), $FeCl_3$ (0.05) supplemented with petroleum (1%, *v*/*v*) sterilized with 0.22 μm syringe filter (Millipore, Sartorius), glucose (0.1%, *v*/*v*), yeast extract (1%, *v*/*v*), Triton X:100 (1%, *v*/*v*) and trace elements (0.1%, *v*/*v*) were added to enhance petroleum removal. 1% (*v*/*v*) petroleum concentration was used in laboratory scale biodegradation studies (Chen et al., 2017; Koolivand et al., 2022). The pH was adjusted to 7.0. 10 mL of immobilized beads and 1 mL of free bacterial consortia were inoculated into the BHB medium, separately. The flasks were incubated in a rotary incubator at 30 °C and 150 rpm for 7 days (Miprolab, Turkey) (Ezebuiro et al., 2019). At the end of the incubation period, petroleum removal was calculated gravimetrically.

The degradation of the petroleum was calculated through Eq. (1) (Bilen Ozyurek et al., 2021; Fu et al., 2020).

$$D = \frac{(p_0 - p_1 - p_2)}{p_0} \times 100\% \quad (1)$$

2.6. Petroleum degradation kinetics

The degradation kinetics of petroleum and half-life period of microorganisms immobilized by SA, SA/PVA, and BHA were calculated through Eqs. (2) and (3) (Bilen Ozyurek and Seyis Bilkay, 2020).

$$\ln ct = \ln c_0 - K_t \quad (2)$$

$$t_{1/2} = \ln \frac{2}{K} \quad (3)$$

where ct is the residual concentration of petroleum; c_0 is the initial concentration of petroleum; K is the rate constant for petroleum biodegradation (day^{-1}); and t is the time (day).

2.7. The effect of the main factors on petroleum removal with entrapped bacterial consortia

The effect of the amount of BHA (0.5, 1, 1.5, 2, 2.5, 3 g), disc size (4, 5, 6, 7, 8 mm), inoculum concentration (1, 2.5, 5, 7.5, 10 mL), incubation period (12, 24, 48, 72, 96, 120, 144, 168 h) on petroleum removal and reusability of gel-entrapped bacterial consortia in the removal process of petroleum were also investigated.

2.8. Characterization of the entrapment materials

The surface morphologies of SA, SA/PVA, and BHA were visualized by Scanning Electron Microscopy (SEM) (Zeiss EVO 50 SEM, Germany). The existing functional groups and bond types of the entrapment materials were also analyzed by FT-IR that was performed by Hacettepe University Advanced Technologies Application and Research Center (HUNITEK) with Thermo Fisher Nicolet iS50 (USA) (Elazzazy et al., 2015).

2.9. Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis was carried out as described before by Bilen Ozyurek et al. (2021) using TRB-1 GCMS-QP-2020 (Shimadzu, Tokyo, Japan). The extracted petroleum sample was dehydrated by anhydrous sodium sulfate, filtered by 0.22 μm filter membrane, blow-dried with nitrogen, then dissolved in 1 mL of dichloromethane (DCM). The mass of various compounds in the petroleum were analyzed by GC-MS with injecting 1 mL of the sample (Luo et al., 2020). With this analysis, while the *n*-alkane fractions of petroleum were obtained, the decrease in *n*-alkane hydrocarbons could be detected at the level of ppm (parts per million).

2.10. Statistical analysis

The petroleum removal experiments were carried out in triplicate with BAH-entrapped bacterial consortia and the control groups. The obtained data were evaluated using SPSS (the Statistical Package for the Social Sciences) 23 program (Chicago, IL, USA). The mean, standard deviation and range were given on petroleum removal efficiencies of bacterial consortium entrapped into BHA. The effect of the amount of BHA, disc size, inoculum concentration, and incubation period on petroleum removal by entrapped bacterial consortia were compared using the Kruskal-Wallis H test. Post hoc range and pairwise multiple comparison tests were also performed. The results were evaluated at the 5% significance level.

3. Results and discussion

3.1. The characterization of the entrapment materials: SA, SA/PVA, and BHA

3.1.1. SEM

The surface morphologies of SA, SA/PVA, and BHA were shown in Fig. 1(a), (b) and (c). Regarding the topologies of SA, SA/PVA and BHA beads, they showed varying degrees of porosity. While SA has a porous structure required for the attachment of bacteria, the porosity of SA/PVA is quite high (Fig. 1(a) and (b)). The indented structure of the BH agar was clearly shown in Fig. 1(c).

SEM micrographs of cell entrapped materials (SA, SA/PVA, and BHA) showed different surface morphology due to the entrapment of the bacterial consortia (Fig. 2(a), (b) and (c)). When SA, SA/PVA and BHA beads (Fig. 1(a), (b) and (c) are compared with SA, SA/PVA and BHA-entrapped with bacterial consortia (Fig. 2(a), (b) and (c)), it is clearly stated that SA, SA/PVA and BHA can entrap the bacterial consortium and that the bacterial consortia adhere to the inner surface of these entrapment materials. However, the loading capacity of cell entrapped SA, SA/PVA, and BHA beads cannot be detected clearly (Fig. 2(a), (b) and (c)). The surface morphologies of entrapment materials were interpreted according to the SEM micrographs of similar materials (Acosta et al., 2019; Badita et al., 2020; Chen et al., 2017; Helmiyati Aprilliza, 2017; Jeon et al., 2019; Luo et al., 2022).

Although each support material with different physical and chemical advantages can be used in the entrapment technique, BHA with its indented structure facilitating the attachment of bacteria, excellent mechanical strength in each cycle, good chemical stability led to extensive petroleum degradation (Fig. 1(c) and Fig. 2(c)). High porosity is required for a large contact area between the entrapment material and bacterial cell (Bayat et al., 2015; Ezebuiro et al., 2019). Furthermore, the density of the entrapped bacterial cell depends on nature of the support material (hydrophobicity, charge, etc.) and ionic strength, as well as the structure, pore size, and surface area of the material, and environmental conditions (Berillo et al., 2021). While SA has a porous structure required for the attachment of bacteria, it has low resistance to 7 days incubation period due to its low mechanical strength (Fig. 1(a), Fig. 2(a)). Although SA is used as a support material in various industrial studies, a long storage period is needed as it is less resistant to degradation processes (Wang et al., 2011). Therefore, SA should be combined with a synthetic polymer such as PVA with desired mechanical properties to have enhanced the mechanical stability and biocompatibility (Eghbalifam et al., 2015). Similarly, the porosity of SA/PVA is quite high, its durability during the incubation period is higher than immobilized SA but lower than the BHA (Fig. 1(b), Fig. 2(b)). SA and PVA were cross-linked with each other and showed a porous network structure, providing the microenvironment for good growth of consortia inside. The sodium alginate increases the interaction by improving surface properties such as diffusion and porosity, while PVA increases durability. Accordingly, their use as an entrapment material in the removal processes of petroleum has been reported in various studies (Idris et al., 2008; Partovinia and Naeimpoor, 2014; Takei et al., 2011). However, in some cases higher porosity can result in lower density and crosslinking (Jadbabaei et al., 2021). Besides, the activity of microorganisms decreases significantly after immobilization with PVA due to the agglomeration effect and the toxicity of the boric acid solution used in crosslinking. Hence, BHA was found to be a more potent entrapment material in the removal of petroleum than SA

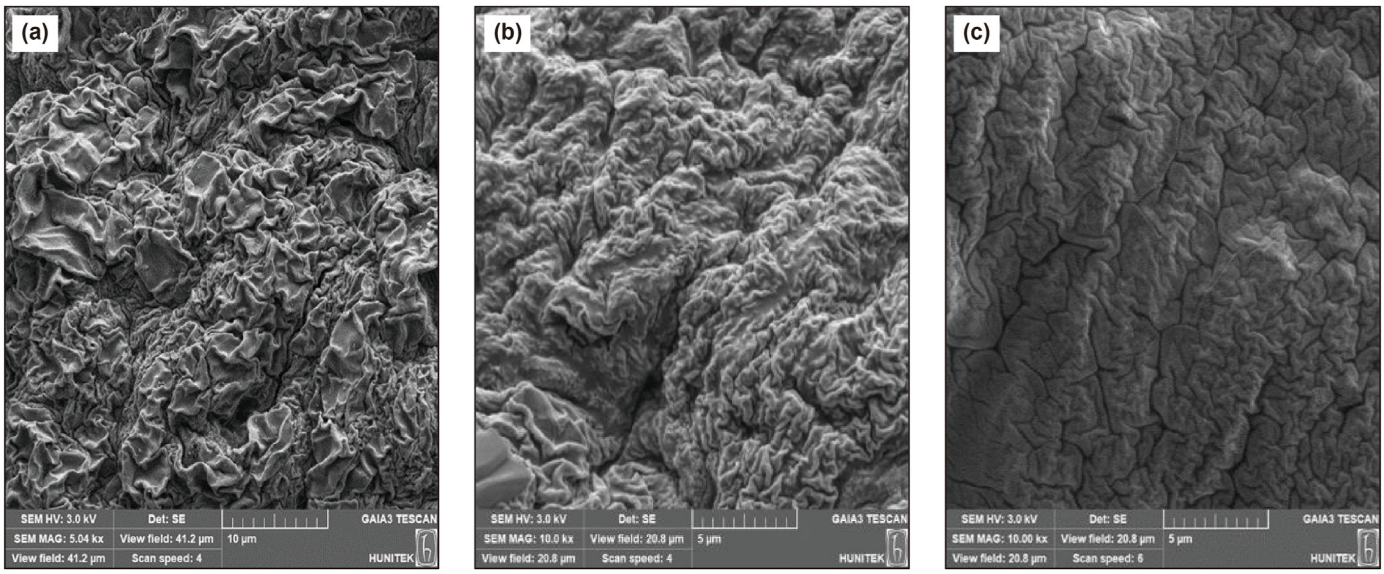

Fig. 1. SEM micrographs of (**a**) SA, (**b**) SA/PVA and (**c**) BHA.

and SA/PVA with its mechanical strength and chemical stability.

3.1.2. FT-IR

Fig. 3(a), (b) and (c) show the FT-IR spectrum of SA, SA/PVA, and BHA. The existing functional groups and bond types of the entrapment materials were interpreted in Table 1 according to the similar studies in the literature (Abdelghany et al., 2020; Badita et al., 2020; Helmiyati Aprilliza, 2017; Qari and Haider, 2021; Shahnaz et al., 2019).

3.2. Comparison of the petroleum removal capacities of free and gel-entrapped bacterial consortia

The use of free bacterial strains in petroleum remediation process has limitations due to the lack of reproducibility, reduced activity with cell damage, and need for optimal conditions. However, the use of entrapment materials has great advantages on ensuring long-term use and reusability of potent microorganisms (Jeon et al., 2019). Accordingly, the petroleum removal capacities of free and gel-entrapped bacterial consortium were shown in Fig. 4. In this context, it was found that the bacterial consortium entrapped in SA, SA/PVA, and BHA was more effective compared to free-living consortium after 7 days of incubation period. Moreover, the half-life period of the entrapped bacterial cells decreased compared to the free ones. The immobilization provides suitable microenvironment for bacterial consortia, which enhances the tolerance to toxic effects of petroleum hydrocarbons. The bacterial cells were coated with entrapment materials, creating a small internal space for them that was separated from the external environment. Moreover, the entrapment method increases the survival and bioremoval capacities of microorganisms (Ezebuiro et al., 2019). It also contributes significantly to the petroleum-water interface interaction, the transition of petroleum from the solid phase to the aqueous phase, the coalescence of small particle droplets and adsorption, as well as the acceleration of microbial degradation (Luo et al., 2020). Similar studies have been reported that immobilized bacteria are more successful in the removal of petroleum hydrocarbons (Fang et al., 2022; Obuekwe and Al-Muttawa, 2001; Quek and Ting Tan, 2006; Sakdapetsiri et al., 2021). Unlike our study, Jeon et al. (2019) showed that free cell V2 and D9 strains were more effective in the degradation of waste oil than immobilized cells. However, it was clearly emphasized in this study that adsorbing organic compounds on the surface or pores of gel-entrapped bacteria increases the possibility of contact between bacteria and petroleum, thereby increasing the rate of degradation (Fu et al., 2020).

3.3. Comparison of the petroleum removal capacities of the bacterial consortia entrapped in SA, SA/PVA and BHA

The entrapment technique, which is one of the leading immobilization methods, provides more efficient bioremoval than absorption. Although this method is efficient, fast, durable, and cost-effective, it has limitations such as deactivation during immobilization, low loading capacity, erosion of the support material, and restriction in mass transfer of substrate to the enzyme active site (Jeon et al., 2019; Mohamad et al., 2015). The use of suitable entrapment materials in this process not only reduces competition with indigenous microorganisms, but also provides a protective micro-environment that preserves the viability and activity of microorganisms (Sakdapetsiri et al., 2021). Accordingly, the petroleum removal capacities of the bacterial consortia entrapped in SA, SA/PVA, and BHA were compared in Fig. 4. Although the removal capacities of entrapped cells with SA/PVA and BHA were close to each other, the removal efficiency of BHA was found to be higher (87.2% ± 0.84%) than SA/PVA (79.7% ± 4.50%). The bioremoval capacities of entrapped cells with SA (63.4% ± 5.3%) was found to be quite low compared to SA/PVA and BHA. The higher removal rate observed with the bacterial consortium entrapped into BHA can be explained by the high affinity between the entrapped bacterial cells and petroleum (Barreto et al., 2010; Luo et al., 2022; Radwan et al., 2002). The removal efficiencies of the entrapment materials may also vary depending on the permeability of the entrapped bacteria. However, the relationship between permeability and petroleum-entrapment material in the removal mechanism of immobilized bacteria is not clearly known (Fu et al., 2020).

According to the first-order kinetics model equation, the degradation rate of bacterial consortium entrapped in BHA was 2.039 day^{-1}, SA/PVA was 1.560, SA is 0.993. While the half-life period of entrapped microorganisms with BHA was quite low ($t_{1/2}$ = 0.339) compared with SA ($t_{1/2}$ = 0.444) and SA/PVA ($t_{1/2}$ = 0.697). Accordingly, the lower half-life of microorganisms

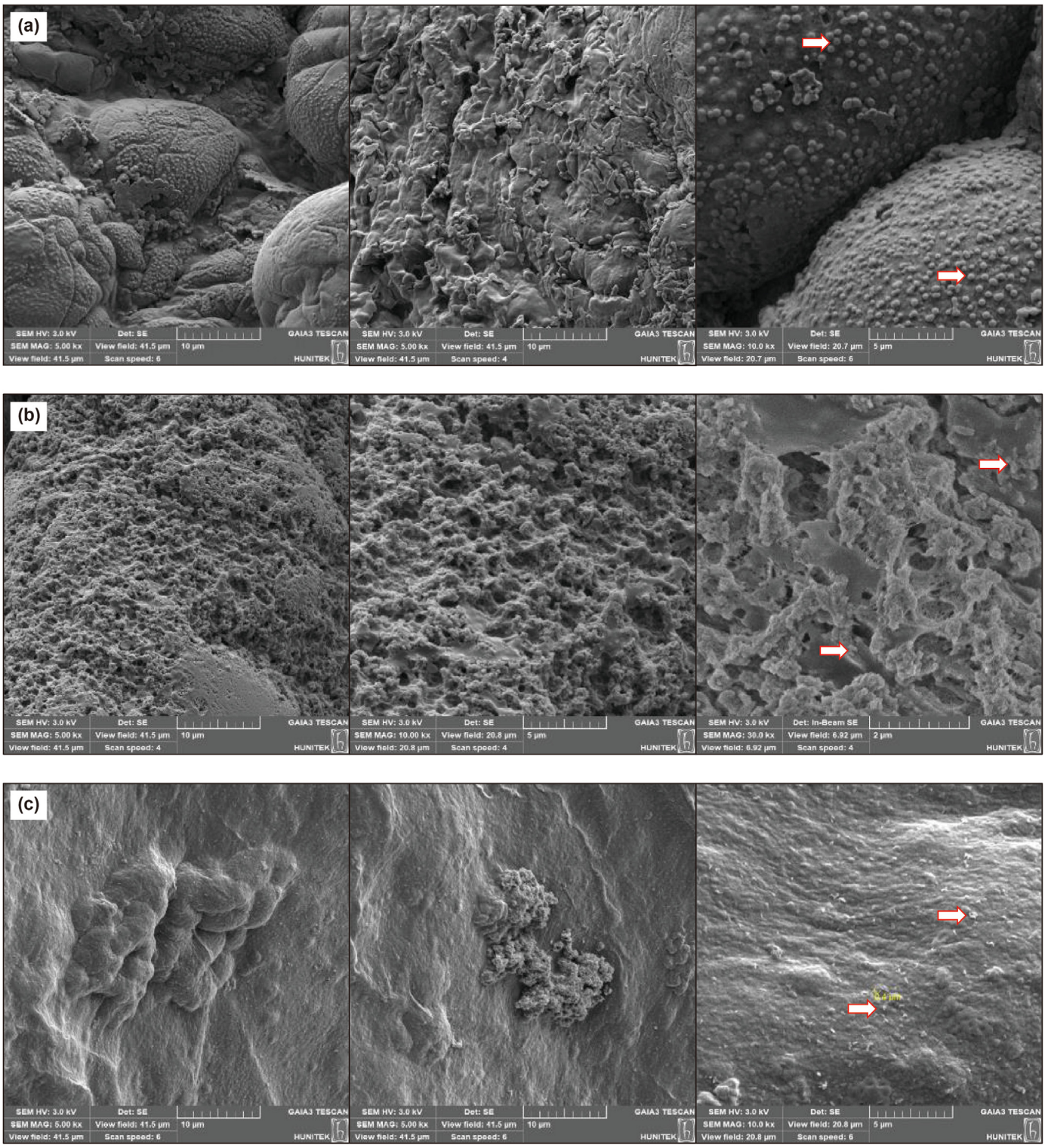

Fig. 2. SEM micrographs of the bacterial consortium entrapped in (**a**) SA, (**b**) SA/PVA and (**c**) BHA.

entrapped in BHA increases petroleum removal as well as microbial activity (Manghabati and Pazuki, 2014; Zhao et al., 2011). The petroleum-water interface is the main area of microbial degradation. Based on this, microorganisms immobilized with BHA are more effective in accelerating degradation by increasing the growth of microorganisms with using petroleum more effectively than SA and SA/PVA. Similarly, Pongsilp and Nimnoi (2022) demonstrated that *Paenibacillus* sp. strain OL15 strain has more degradation ability of waste lubricating oil than the alginate-immobilized bacterial strain. Similar studies have been reported that various entrapment materials were used for the immobilization of bacteria to enhance the removal of petroleum (Jeon et al., 2019; Li et al., 2021; Nhi-Cong et al., 2021). The choice of the entrapment material plays an important role on the remediation of organic

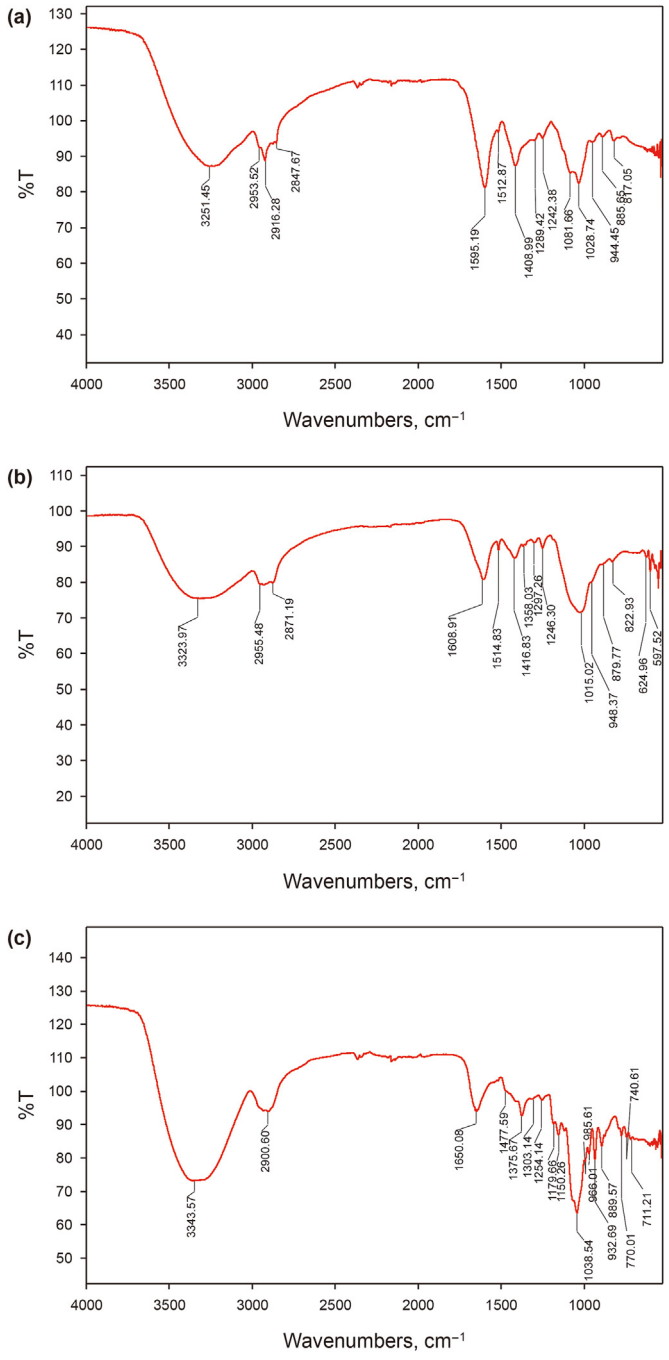

Fig. 3. FT-IR spectrum of (**a**) SA, (**b**) SA/PVA and (**c**) BHA.

pollutants as the degradation efficiency of immobilized bacteria varies greatly depending on their preparation on different support materials (Fu et al., 2020). The support materials have also been used in various applications besides petroleum remediation. Accordingly, *Klebsiella pneumoniae* entrapped in agar/carrageenan-Fe_3O_4 was used for phenol degradation, bacteria entrapped in calcium alginate was used for diesel degradation, agar-entrapped *Pseudomonas plecoglossicida strain* (TA3) was also used for *N*-methylated carbamates (synthetic pesticide) degradation (Fang et al., 2022; Fareed et al., 2019; Liu et al., 2015). However, the usability of the bacterial consortium entrapped in BHA agar in bioremediation processes or other industrial applications has not yet been reported.

Table 1
IR spectrums and bond types of SA, SA/PVA, and BHA.

Entrapment material	IR frequency, cm^{-1}	Bond type
SA	3251	–OH stretching
	2916	–CH vibration
	1595	C=O symmetric stretching
	1408	C=O asymmetric stretching
	1028	C–C stretching
SA/PVA	3323	–OH stretching
	2955	–CH vibration
	1608	C=O stretching
	1015	C–C, C–O– stretching
BHA	3343	–OH, N–H stretching
	2900	–CH asymmetric stretching
	1650	C=O symmetric stretching
	1375	CH_3 vibration
	1179, 1150	S=O
	1038	C–C, C–O–, C–O–S stretching

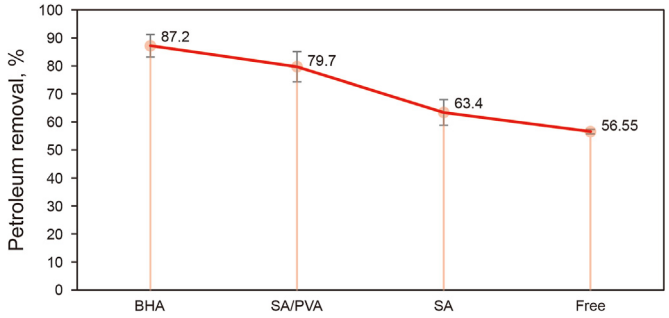

Fig. 4. The comparison of the petroleum removal capacities of free and bacterial consortia entrapped in SA, SA/PVA and BHA.

3.4. Statistical design to determine the main factors affecting the petroleum removal by BHA-entrapped consortia

The use of microorganisms that can degrade petroleum hydrocarbons even at extreme temperature, pH and NaCl concentrations provides cost effective remediation of petrochemical and petroleum refinery wastewater (Bastos et al., 2000). Therefore, the petroleum adsorption and degradation capacities of immobilized cells should be increased by optimization (Sakdapetsiri et al., 2021). Accordingly, the four factors: the amount of BHA (0.5, 1, 1.5, 2, 2.5, 3 g), disc size (4, 5, 6, 7, 8 mm), inoculum concentration of consortia (1, 2.5, 5, 7.5, 10 mL), and incubation period (12, 24, 48, 72, 96, 120, 144, 168 h) were investigated in terms of their effects on petroleum removal. The main factors affecting the petroleum degradation by BHA-entrapped bacterial consortia were evaluated according to similar studies in the literature (Chen et al., 2017; Sakdapetsiri et al., 2021; Elkemary et al., 2023). While a stable structure was not formed at values < 2 g, a rather rigid structure was formed at values > 2 g preventing the diffusion of substrate and enzymes throughout the matrix. According to the statistical analysis, a significant difference was found between the different amounts of BHA ($p = 0.001$; Table S1), disc sizes ($p = 0.002$; Table S2), inoculum concentrations ($p = 0.008$; Table S3), and incubation periods ($p < 0.001$; Table S4) in terms of petroleum removal efficiencies. While the highest removal value was observed at 2 g BHA with a 7 mm disc size, the lowest value with a 2 mm disc size (Fig. 5(a) and (b)). While 2.5–10 mL inoculum concentration of bacterial consortia was found to be similar in terms of petroleum removal ($p = 0.067$), the lowest removal was obtained with 1 mL inoculum concentration of bacterial consortium (Fig. 5(c)). While the petroleum removal increased up to 72nd h, the removal remained

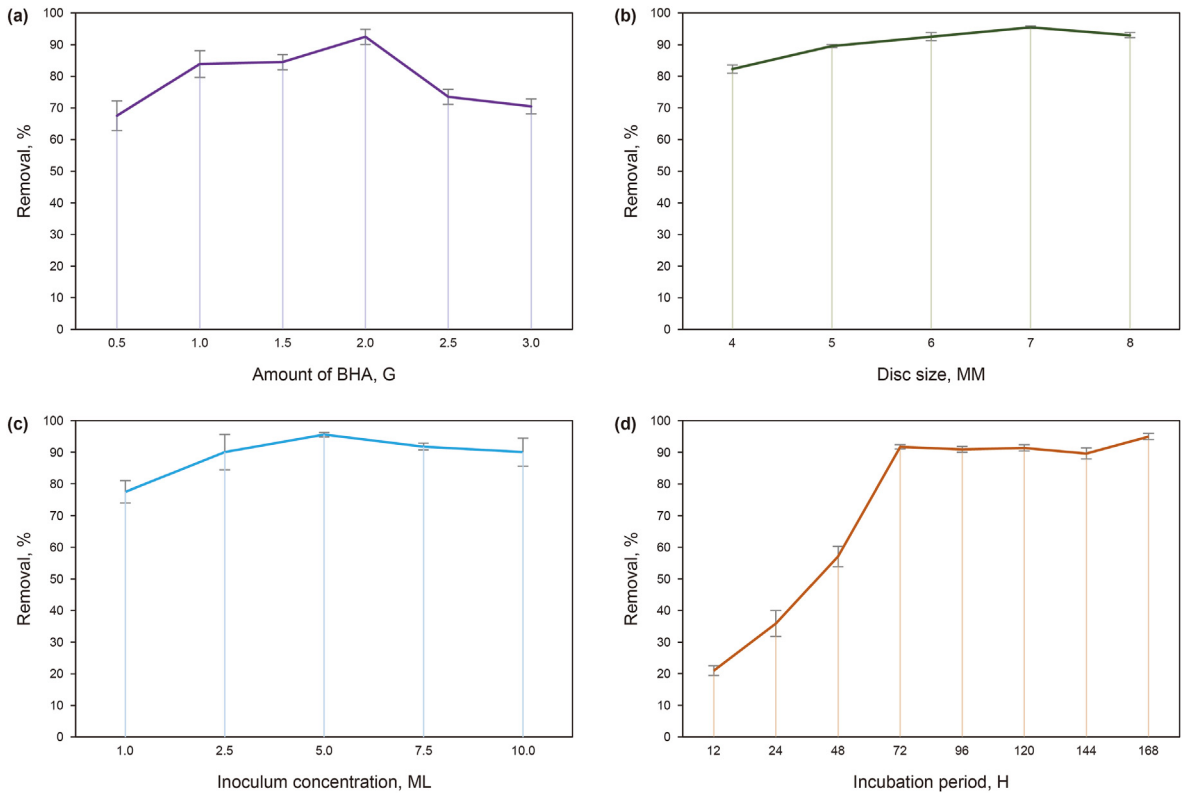

Fig. 5. The main factors affecting the petroleum removal of BHA-entrapped bacterial consortium (**a**) amount of BHA, (**b**) disc size, (**c**) inoculum concentration and (**d**) incubation period.

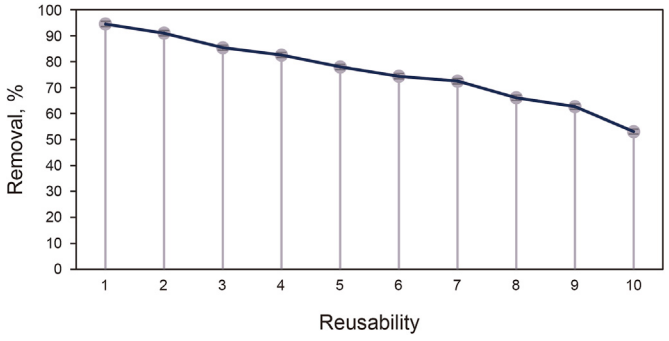

Fig. 6. The reusability of BHA-entrapped bacterial consortium in the removal process of petroleum.

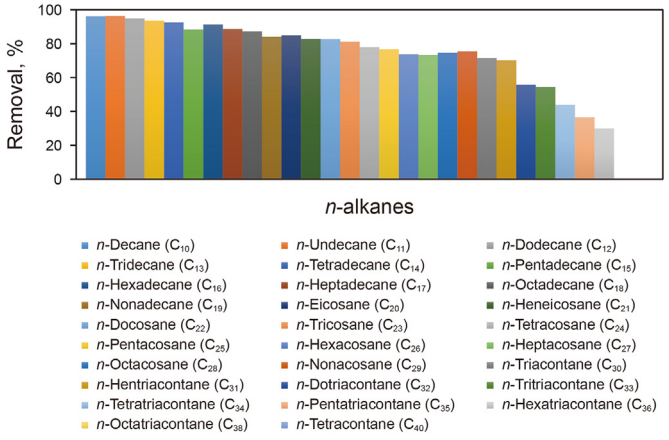

Fig. 7. The removal efficiencies of the *n*-alkane fractions of petroleum with BHA-entrapped bacterial consortium.

constant from the 72nd to the 144th h, a slight increase was observed at 168 h (Fig. 5(d)). The degradation efficiency of petroleum increases initially and stabilizes over time (Chen et al., 2016; Wu et al., 2009). Similarly, Luo et al. (2022) reported that 86% of diesel oil was degraded after 3 days of incubation by an efficient oil-degrading bacterial consortium (Y9, F9, W3 and X1) immobilized with puffed rhubarb rice (PRR)/calcium alginate. Chen et al. (2017) reported that degradation of 1% (*w*/*v*) crude oil could reached 86.1% with 5% (*v*/*v*) immobilized bacterial consortium after 7 days of incubation period. Similarly, various factors were investigated for the maximum petroleum removal with immobilized cells (Ghorbannezhad et al., 2018; Sakdapetsiri et al., 2021).

To sum up, the results of the statistical analysis clearly showed that the four main factors were effective on petroleum removal with bacterial consortia entrapped in BHA (Fig. 5(a)–(d)). The parameters were designed as main factor and the petroleum removal as dependent variable. It is of great importance to obtain the best factors for the maximum petroleum removal with immobilized microbial consortia for industrial applications. Therefore, more parameters such as pH, temperature, petroleum content & concentration, and salinity should be investigated for large-scale bioremediation studies. In this context, a simulation study for ex-situ oil spill removal should be carried out according to the similar studies in the literature (Elkemary et al., 2023; Farag et al., 2018; Imarhiagbe and Amaechi, 2017; Liang et al., 2021). For this, it is recommended to create an open system with the aquarium motor placed on both sides of the glass vessel containing seawater and petroleum.

3.5. The reusability of the BHA-entrapped bacterial consortium in petroleum removal

The recycling and reuse of biocatalysts in biological processes

carried out on an industrial scale is important in terms of determining their effectiveness (Jiang et al., 2015). Accordingly, it was clearly observed that BHA-entrapped bacterial consortium could be actively used in the removal process of petroleum for at least 10 cycles (Fig. 6). Although a decrease in petroleum removal was observed during the reuse of entrapment material, the removal capacity of BHA-entrapped bacterial consortium was still above 50% at 10th cycle. It was concluded that the film layer formed by BHA provides better protection for the entrapped consortium and thus its reusability. This preserves the integrity of the enzyme produced by the bacterial consortium and increases its reusability compared to SA and SA/PVA. Varjani (2017) reported that the recollected and reuse of the support material have a great significance for its use in industrial processes. Similarly, Sakdapetsiri et al. (2021) showed that the immobilized *Exiguobacterium* sp. AO-11 cells can be reused for at least 5 cycles without any loss of microbial activity. However, since breakage begins after the 5th cycle, agar beads are not suitable for use as support material (Ghorbannezhad et al., 2018). Unlike, Jeon et al. (2019) reported that SA/PVA and SA/CHI beads-entrapped cell can be reused up to 10 cycles. In reusability, the immobilized cells retain their biodegradation activities, making them suitable for bioremediation.

3.6. GC-MS analysis

Fig. 7 shows the removal efficiencies of *n*-alkane fractions of petroleum with bacterial consortium entrapped in BHA. According to the results of GC-MS analysis, while the *n*-alkane fractions of petroleum in the range of C_{10}–C_{14}, C_{16} were removed above 90%, C_{15}, C_{17}–C_{23} *n*-alkanes were removed above 80% by bacterial consortium entrapped in BHA. It was also detected that BHA-entrapped bacterial consortium also showed the ability to remove long-chain *n*-alkanes in a range of 30%–70%. The results clearly showed that short chain *n*-alkanes in the petroleum were removed over 90% by BHA-entrapped bacterial consortium. This is because microorganisms use short chains faster than medium *n*-alkanes. Moreover, the degradation of medium-chain *n*-alkanes as C_{19} to C_{24} in the petroleum was close to long-chain *n*-alkanes in the range of C_{25} to C_{31}. This can be due to the accumulation of medium chain *n*-alkanes after the degradation of long chains' (Li et al., 2016). Fu et al. (2020) reported that immobilized bacteria degraded the majority of C_{11}–C_{25} and highest degradation was found for C_{15}. Luo et al. (2020) also suggested that SA/PVA was slightly better in the removal of short-chain *n*-alkanes, while SA/PMA was highly effective in the removal of medium- and long-chain *n*-alkanes. Besides, most components of diesel oil were degraded by immobilized consortium. Chen et al. (2017) found that the degradation efficiency of *n*-alkanes with immobilized bacteria increased by 31.9% compared to free cells, and the degradation capacity was 85.4%. Besides, they suggested that the degradation efficiency of *n*-C_{17} to C_{24} was less than that of mostly short chains (< *n*-C_{17}) and long chains (> *n*-C_{24}). To sum up, this study suggests that BHA-entrapped bacterial consortium, which has a great removal ability in a wide range of *n*-alkanes, could potentially be used in the treatment of petroleum hydrocarbon pollution in aquatic environment.

4. Conclusion

Immobilized microbial technology, which has several advantages such as high microbial density, stable biological activity, less biomass loss, resistance to toxic chemicals and strong environmental tolerance, is frequently encountered in petroleum bioremediation studies. However, this technology requires the development of new entrapment materials that are non-toxic, environmentally friendly, low cost, reusable, have high cell mass loading, and petroleum adsorption capacity. Accordingly, the main conclusions of this comparative study are as follows:

(1) The petroleum removal efficiencies of SA-, SA/PVA- and BHA-entrapped bacterial consortia were higher than that of the free bacterial consortium (*Staphylococcus capitis* CP053957.1 and *Achromobacter marplatensis* MT078618.1).
(2) The entrapment of the bacterial consortium with BHA enhanced microbial activity and efficiency of the petroleum removal, but SA and SA/PVA-entrapped bacterial consortia was not as successful as BHA-entrapped consortia in terms of petroleum removal.
(3) The extensive removal capacity of BHA with its indented structure facilitating the attachment of bacteria and excellent mechanical strength in each cycle, was emphasized. Although a decrease was observed in the removal capacity during the reuse of BHA-entrapped bacterial consortium, the removal capacity of this entrapment material was still above 50% at 10th cycle.
(4) The highest petroleum removal was observed at $\geq$ 2.5 mL of bacterial consortium entrapped in 2 g BHA with a 7 mm disc size at 168 h of incubation period. The impact of the main factors in increasing the petroleum removal capacity (94.5%) of the BHA-entrapped consortium was also highlighted.
(5) The efficient degradation capacity of the BHA-entrapped bacterial consortium on short-, medium- and some long-chain *n*-alkanes were clearly demonstrated.
(6) To the best of our knowledge, this is the first report in which the possibility of using BHA as a potent entrapment material in the removal of petroleum. The results of this study provide an important data regarding the applications of BHA-entrapped bacterial consortium for the remediation of oil pollution caused by human or industrial activities.
(7) The findings of this study will be a useful guide for the development of new entrapment materials for further bioremediation processes. However, further studies that will develop new cosmopolitan matrices with higher removal capacity by combining BHA and natural/synthetic materials are needed to optimize the potent entrapment material for use in the clean-up of large-scale oil pollution.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.petsci.2023.11.003.

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