Keywords: Soil; Dodecane; Slurry Sequencing Batch Reactor (SSBR); Microbial consortium; Co-metabolism

Introduction

Linear hydrocarbons are including alkanes, alkenes and alkynes, and alkanes with moderate length are the most important pollutants of soil [1]. Among Alkanes, N-Alkanes with medium chain have been identi ed as the most important contaminants of soil [2,3]. Dodecane ($C_{12}H_{26}$) with low solubility in water (0.0037 mg L^{-1} at 25°C) is one of medium-chain n-alkanes [4]. In some studies, Dodecane has been used as a representative hydrocarbon for liquid alkanes in various hydrocarbon mixtures [5]. Several studies have shown that a wide range of micro-organisms are capable of degenerating diesel fuel [6-8] and n-alkanes [9-11] without co-substrate. Petroleum hydrocarbons are decomposed by microorganisms like bacteria, yeast and fungi that can use crude oil as a source of carbon and energy [12-15]. Aerobic decomposition of alkanes with varying chain lengths has been widely studied and documented [16-19].

Biodegradation is an option capable of removing and destroying toxic contaminants using natural biological activities. By de nition, biodegradation is use of living organisms, primarily micro-organisms, to break down environmental pollutants to less toxic forms. Biodegradation use natural plants, fungi or bacteria to break down or detoxify substances hazardous to human health or the environment [20]. Micro-organisms may be native to the contaminated region or they may be taken from elsewhere to the contaminated sites. Polluting substances change shape by living organisms through reactions as part of the metabolic processes within them. Biodegradation of a

environmental conditions [35-37]. SBs are o en used to assess the feasibility and actual potential of biological strategies in the nal restoration of contaminated soil or sites [25,28,38]. e pollutant depletion rate under slurry conditions depends primarily on the degradation activity by microorganisms in the system [34]. Generally, the obtained results show the soil's actual potential in biological depuration [1,39]. e SB technology is an engineered complex that usually includes four stages: installations for handling and treating polluted soil, bioreactor battery, installations for handling and disposing treated soil, and auxiliary equipment for treatment of process by-streams [33,34]. In terms of operation, SBs are classi ed as batch, semi-continuous, and continuous. Another classi cation is based on the main electron acceptor that is used in the biodegradation process includes: aerobic (molecular oxygen), anoxic (nitrate and some metal cations), anaerobic (sulfate-reducing, methanogenic, fermentation) [2], and mixed or combined electron acceptors [40,41]. Aerobic SBs have been widely used and anaerobic SBs are ourishing in the area of research and development [36].

SBs have some remarkable and distinctive features. ey include the fact that oil is treated in aqueous suspension of 10 to 30% w/v and they provide mechanical or pneumatic mixing. Process advantages of these features include: (a) an increase in mass transfer rates and contact microorganisms, pollutant and nutrients; (b) an increase in pollutant biodegradation rate over in situ bioremediation or ad situ solid phase biotreatment; (c) shorter treatment times; (d) likelihood of using diverse electron acceptors (O2 SO4-2 ,CO2 ,NO3-) ; (e) e ective use of biostimulation and bioaugmentaion; (f) control and use of several environmental parameters such as temperature, pH, etc. and (g) increased desorption and availability of pollutant by adding surfactants and solvents [2,14,26,42].

For more than 20 years, Cometabolic bioremediation has been applied to some of recalcitrant contaminants like polychlorethylene, trichlorethylene, TNT, dioxins, Atrazine, aromatic hydrocarbons, chlorinated alkenes, halogenated aliphatic and etc. In many systems the essential nutrients needed for complete biodegradation are not available and therefore biodegradation is limited [43]. Several studies have shown that environmental compatibility of microorganisms and their potential for biodegradation can be increased by adding nutrients like yeast extract and glucose [20,25]. Also, in many studies, lactose [44], sucrose [45] and molasses [44,46,47] have been used as co-substrates. In some other studies, glucose has been used as co-substrate [44,48]. But, glucose as co-substrate has not been used for removal of medium-chain alkanes such as Dodecane. e aim of this study is to evaluate the use of glucose as an external carbon source (Co-substrate) to enhance the decomposition of organic contaminants, particularly medium-chain petroleum hydrocarbons (Dodecane). Experiments to study the e ect of glucose on aerobic decomposition of Dodecane in slurry sequencing batch reactor by measuring Dodecane concentrations with and without co-substrates.

Materials and Methods

Materials Speci cations

Chemical materials used in this study including Hexadecane, 1,2,4-trchlorobenzene, acetone, glucose, HCl, H_2SO_4, NaOH, NaN_3, Na_2SO_4, NH_4Cl, NaCl, $MgSO_4$, $FeCl_3.6H_2O$, $CaCl_2$ and $MnCl_2.4H_2O$. All chemicals material used in this study were 99.7% purity and purchased from Merck, Germany.

Preparing the soil

e soil used in this study was agricultural soil collected from Haskoohak region 40 Km from Shiraz, Iran. e physicochemical analysis of the soil has been presented in Table 1. In order to prepare the soil, rst the soil was sieved with a 10 mesh (2mm) sieve for screening the soil and reaching uniformity. en, it was soaked with distilled water and autoclaved for 15 minutes at 121°C. At the end, it was located in the oven at 160°C to make it sterile and dry and reach its primary weight. A er being dried, it was sieved with a 10-mesh (2 mm) sieve. At the end, it was transferred to a 1-liter container and contaminated in di erent concentrations. In order to arti cially contaminate the soil with dodecane at 1, 4, 7, and 10% concentrations, rst the necessary amount of dodecane was dissolved in 30 ml hexane. en, the obtained solution was added to the soil. In order for uniform distribution of dodecane in the soil, the soil was completely submerged in the solution. en, the soil was regularly mixed in short time intervals and it was permitted to dry completely under the vent at room's temperature. At the end, a one-week period was considered for absorption of dodecane by the soil.

Preparing the essential nutrients

In order for the SSBR to operate, in addition to contaminating the soil with dodecane, essential nutrients and water are also needed

Pseudomonas aeruginosa, was used. These bacteria were isolated from the soil in another study [49]. In order to increase the number of bacteria and add them to the reactor, the bacteria which had been cultured in the Agar-Agar medium were cultured in the nutrient broth medium. Afterwards, they were located on the mixer in the incubator at 37.5°C for 24 hours in order to be grown. Thereafter, the nutrient broth medium including the grown bacteria was transferred to the test tubes in order to be isolated completely and was then centrifuged at 4000 rpm for 5 minutes. Finally, the optical density of the bacteria was measured at the wave length of 600 nm to ascertain the uniformity and equal distribution of the bacteria in all the bioreactors. It should be noted that the optical concentration of the bacteria was reached to one using normal saline. After all, the bacteria were added to the reactor [50].

Measuring the number of active bacteria

To determine the number of the bacteria grown in the SSBR, samples were taken from the reactor at different times of operation and they were then cultured at three dilutions; i.e. 10^{1}, 10^{2}, and 10^{3}, on the nutrient agar medium. After that, the samples were located in the incubator at 37.5°C for 24 hours. After being assured about their growth, the colonies were counted by the colony counter and the number of bacteria was reported based on CFU/ml.

Dodecane extraction and analysis

In order to analyze the residual of dodecane from the soil, dodecane was extracted from the soil through USEPA method 3550c [51]. Briefly, the sample was taken from the deposited sediment after the process of sedimentation and was dried at 37.5°C. Afterwards, 0.5gr of the dry soil was mixed with 0.5gr anhydrous sodium sulfate as the dehumidifier factor. The content of the Balon Joje was reached to 5ml with 4ml normal hexane and it was completely mixed in order to mix the soil with normal hexane. The Balon Joje was then put in the ultrasonic bath at 30°C for 2 minutes in order to extract dodecane. At the end of the extraction time, the upper liquid of the Balon Joje was transferred to a test tube. In order to extract dodecane more efficiently, this operation was repeated twice. After that, the test tube was located in the centrifuge system at 4000 rpm for 5 minutes to isolate the soil and upper liquid completely. Then, 1ml of the upper liquid was taken by the sampler and was moved to the vial. Afterwards, 10 µl of the internal standard (1,2,4-trichlorobenzene) was added to the vial by Hamilton syringe. Finally, 2 µl was taken from vial content by the injection syringe and was injected to the GC-FID system. The recovery percentage of dodecane was averagely obtained as 72% at different concentrations through the extraction method.

In order to measure the residual of dodecane in the study samples, the GC-FID system was used. CP-SILSCB (silica, USA) column (30 m length × 0.025 mm id × 0.25 µm film thickness) was used at a temperature program of 80°C for 1 min, increased to 125°C at 10°C min^{-1}

such a way that increase of concentration led to a decrease in the amount of dissolved oxygen.

In order to determine the pH, some samples were taken from the reactor on the zero (one hour a er the reactor's beginning of working) rst, second, and third days. pH was measured using the pH-meter system. e results showed that in concentrations of 1, 4, 7, and 10 percent of dodecane, pH respectively decreased to 1.07, 1.08, 1.09, and 1.1 a er three days. Moreover, a signi cant strong, negative, linear relationship was found between dodecane concentration and pH on the zero, rst, second, and third days (0.96, P 0.05); such a way that pH decreased following the increase in the concentration.

Also, samples were collected from the reactor on the zero (one hour a er the reactor's beginning of working), rst, second, and third

Discussion

Various physical, chemical and biological contaminants have been used to clean oil contaminations. In the eld of biological cleanup, Shields et al. [6], Kim and Hao [16], Lee and Gibson [19] and Lee et al. [18] reported the positive e ect of the presence of Pseudomonas bacteria in removing organic pollutants [6,16,18,19]. Wackett et al. [7] reported the positive e ect of bacillus on decomposing organic pollutant compounds. Bossert and Bartha [8] reported the e ects of bacteria like pseudomonas, arthrobacter, Corynebacterium, Flavobacterium, Achromobacter, Micrococcus, Nocardia,

study, because the experiments were carried out in di erent seasons, the reactor encountered inappropriate temperature conditions which can disturb the reactor and a ect the biological removal of Dodecane. Another factor a ecting the biological removal of Dodecane is the possibility that reactor is not fully disinfected during the evacuation and preparation for the next cycle and thus degradation factors enter the reactor from the outdoors. Also, Dodecane may be evaporated due to low solubility in water. is fact has been noted in other studies [9].

In the present study, the growth of bacteria increased over time (Figure 4). at can be because bacterial consortium adapted to the conditions of the reactor. e growth rate of bacterial consortium in lower Dodecane concentrations was higher than in higher Dodecane concentrations. e reason can be that with the increase in Dodecane concentrations, bacteria were trapped in the oil layers and failed to have the functionality needed to remove Dodecane. In Boopathy's study [47] on bioremediation of tetryl-contaminated soil using SSBR, within 30 days of the reactor operation, count of bacteria under both aerobic and anaerobic conditions was high in reactor with molasses as the growth substrate. is shows the presence of aerobic and anoxic bacteria in the contaminated soil. In the reactor operated without molasses, the number of bacteria was signi cantly lower. is indicates that Tetryl was the sole carbon source not used for growth.

In this study the amount of dissolved oxygen was decreased with time (Figure 5). is could be due to higher activity of microorganisms and their higher growth and oxygen uptake rate (OUR) over time. On the other hand, given the direct relationship between temperature and dissolved oxygen, this reduction of dissolved oxygen can be because o increased temperature over time. Juneson et al. [10] used a bacteria consortium including Brevibacterium iodinum, Rhodococcus luteus and Bacillus brevis for studying biodegradation of bis (2-ethylhexyl) phthalate in a soil slurry-sequencing batch reactor. e Dissolved oxygen levels decreased with time. In other words, the activity of micro-organisms and the rate of oxygen uptake increased. Also, Venkata et al [48] studied bioslurry phase degradation of di-ethyl phthalate (DEP) contaminated soil in periodic discontinuous mode operation as well as the e ect of bioaugmentation using ETP micro ora on the degradation

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