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Isolation and screening of native polyhydroxyalkanoate producing bacteria from oil contaminated soils of Abadan refinery

Hossein Motamedi *
Associate Professor of Microbiology, Shahid Chamran University of Ahvaz, Iran, hhmotamedi@yahoo.com
Mohammad Roayaee Ardakani
Professor of Microbiology, Shahid Chamran University of Ahvaz, Iran, roayaei_m@yahoo.com
Nasim Mayeli
MSc of Microbiology, Shahid Chamran University of Ahvaz, Ahvaz, Iran, mayeli.nasim@yahoo.com

Abstract

Introduction: Environmental contaminations due to petrochemical plastic usage have forced researchers to search new biological methods for biodegradable polymer production. The aim of this study was to find native PHA producing bacteria from Abadan oil refinery in order to be used in biodegradable polymer production studies.

Materials and methods: For this purpose soil samples were harvested from oil sludge contaminated soil of Abadan refinery. After primary enrichment, screening of PHA producing bacteria was done by PHA- Detection agar and was confirmed by Sudan black and Nile Blue A staining methods. These isolates were identified based on phenotypic methods and sequencing of *16s rRNA*. Polymer extraction was performed and optimized using different concentrations of HClO and SDS.

Results: As a result of this study 26 different bacterial isolates were obtained from which 17 isolates were PHA producer with different potentiality. Based on the polymer accumulation 4 isolates were selected for further studies. The efficiency of PHA production in these isolates was 75.53±5.08, 82±19.05, 81.06±6.92 and 79.86±11.84%. Based on sequence analysis in NCBI database, these isolates were identified as *Bacillus cereus*.

Discussion and conclusion: With respect to the results of this study it can be suggested that oil contaminated soils due to high C/N and C/P ratios and also different carbohydrate contents are suitable candidates for PHA producer bacteria isolation. So the native strains in such habitats with high carbon content can be optimized for industrial polymer production.

Key words: Biodegradable, Oil sludge, Polyhydroxy alkanoate, Polymer

*Corresponding Author

Introduction

Today the industrial world is heavily dependent in many procedures to fossil fuels as a source of energy and this need will be increased till 2020 (1). Synthetic polymers derived from petroleum and are approximately used in all industries (2). Polyethylene, polyvinyl chloride and poly styrene are extensively used in plastic synthesis. The annual production of plastic is 140 million tons that need 750 million tons of fossil fuels. At least, half of the produced plastic is used for short term applications such as packaging or producing single use products that are disposed several weeks after their applications (3 and 4). The petrochemical plastics are produced from non- renewable petroleum resources and are incompatible with carbon cycles in environment. So, huge amounts of these polymers are accumulated as industrial and municipal wastes in ecosystems (5). It is estimated that more than 40% of produced plastics are introduced to soil and thousands tons of them are released to water resources. Most of synthetic polymers, due to high molecular weight (50000- 100000 D), presence of several aromatic rings, unique chemical bonds and halogenation are resistant to microbial degradation. Hence, in average these products are remained for 100 years in environment and through releasing of dioxin and hydrogen cyanide from their basic substances such as acrylonitrile and polyvinyl chloride cause serious air pollution (1, 5 and 6). Therefore, there is increasing demand to new methods for synthesis of biodegradable polymers (7).

Presently, biopolymers such as polyhydroxyalkanoates (PHAs), polysaccharides, polylactic and aliphatic polyesters are good candidates for petrochemical plastics substitution (8). From these, PHAs have been more considered by researchers because they have similar physicochemical properties with synthetic plastics and are completely degraded in environment (9 and 10). PHAs are a group of completely degradable and compatible polyesters that under unfavorable growth condition are produced as intracellular granules by many types of bacteria and act as carbon and energy source without producing any toxic compound during their degradation (5 and 11). Nutrition limitation activates metabolic pathways for PHAs synthesis and acetyl moieties from TCA are consumed for polymer production (12). Poly- 3 hydroxybutyrate, the best known polyhydroxyalkanoate, is the first intracellular polymer that was isolated from *Bacillus megaterium* by lemoigne (1926). Presently, more than 300 bacterial species are known that are able to produce these polymers (13). More than 150 different monomers as homo- or co- polyesters or a mix of different polyesters are form PHA and R side chain of these monomers determines the type of polymer. The most common monomers are 3- hydroxybutyrate and 3- hydroxyvalerate that have C3 structure with methyl and ethyl in their side chains, respectively. Commonly, PHA polymer is composed of 10^3- 10^4 monomers. This monomer can vary widely which determines the physicochemical structure of polymer (10).

Polyhydroxybutyrate is preferred than other degradable plastics due to having characteristics such as high resistance to high temperature, water insolubility and its impermeability to oxygen. In spite of synthetic polymers, PHAs are biodegradable and biocompatible polymers without any side effects on living organisms and are fully degraded by wide range of microorganisms. So, in this manner in aerobic condition they are degraded and produce H_2O and CO_2, while produce CH_4 under an aerobic environment (14).

Considering physical and mechanical characteristics of PHAs, they can be suitable substitutes for petrochemical plastics in order to produce bottles, films, packaging material, etc. Furthermore, the ability to control their degradation has introduced them as important biopolymers in medicine, nanobiotechnology as a surface for fixing biomolecules, biosensor development and also to be used in agriculture and packaging products. Using inexpensive carbon sources for PHAs production can increase their value (15).

The main aim of the present study is screening oil polluted soil from Abadan oil refinery in order to achieve native PHA producing bacteria. With regard to the long history of oil refinery in this region, high temperature and salinity of soil, the bacterial inhabitants of this habitat are good candidates for industrial production of this biodegradable polymer. There is no report at present on the presence and potential of biopolymer producing bacteria in this environment.

Materials and methods

Sampling

Five soil samples (each 100 gr) were collected from oil sludge contaminated soil in Abadan oil refinery. The samples were kept in sterile capped glassware till experiments.

Primary bacterial isolation

One gram of each sample was mixed with 10 ml saline (0.9%) and shaked with 130 rpm at 27°C for 1h. Then, 100µl of obtained supernatant was inoculated on nutrient agar (Merck, Germany) and incubated at 27°C for 48h. The appeared colonies were subcultured to obtain pure culture.

Screening of polymer producing bacteria

PHA Detection Agar (PDA) was used as screening medium. This was composed of glucose (20 gr/L), KH_2PO_4 (13.3 gr/L), $MgSO_4$ (1.3 gr/L), $(NH_4)SO4$ (2 gr/L), citric acid (1.7 gr/L), Trace elements solution (10 ml) and agar (15 gr/L) with final pH 7. Following bacterial culture on PDA and incubation at 27°C for 72 h, the polymer producing isolates were screened based on Sudan Black and Nile Blue A staining methods (16). In Sudan Black staining, the fixed bacterial smear was immersed for 15 min in 0.03% Sudan Black (in 60% ethanol), washed with tap water and air dried. Then it was for several times immersed in xylene and dried on whatman paper. Finally, the smear was washed (5-10s) with 0.5% safranin, then with tap water and dried. *Bacillus megaterium* and *Ralestonia eutropha* as positive controls and *Escherichia coli* as negative control were stained simultaneously. The polymer granules will be appeared as blue- black

granules in a pinkish cytoplasmic background (3).

In fluorescent staining, the prepared smear was immersed in 1% Nile Blue A solution (in sterile distilled water or ethanol) at 55°C for 10 min. Then washed with tap water and immersed for 1 min in 8% aqueous solution of acetic acid. Finally the smear was washed, air dried and studied by fluorescent microscope which the appearance of light orange fluorescent granules confirms PHA production (17).

Polymer extraction by chemical digestion

In order to polymer extraction and determine the production yield, NaClO and sodium dodecyle sulfate (SDS) were used as follow. 30 gr/L of lyophilized bacterial biomass was separately mixed with 4, 6, 8, 10 and 12 gr/L of SDS and remained for 20 min at 55°C. Following centrifugation (4000 rpm, 15 min), the precipitate was washed twice with distilled water and dissolved in 1 ml of 10, 20 and 30% of NaClO and incubated at 30°C for 3 min. Then centrifuged at 8000 rpm for 10 min and washed with distilled water. The final precipitate was dried and weighted. All experiments were done as triplicates (18). The production yield was calculated according to the following formula (19):

$$\frac{Polymer\ weight}{Biomass\ weight} \times 100 = \text{polymer\%}$$

Molecular identification

For genomic extraction, 1 ml of 48 h culture of isolate was centrifuged for 10 min at 7500 rpm and its genome was extracted by DNA extraction Kit (Cinnagen, Iran). To confirm genome extraction, 5µl of genome solution was electrophoresed in 1% agarose (Cinnagen, Iran) at 90 V for 50 min. Universal primers were used to amplify *16S rRNA* gene. The primers were synthesized by GenFanavaran (Iran) and prepared according to its recommendation. The sequences of primers are presented in table 1.

Table 1- The sequence of used primes for polymerase chain reaction (20)

Forward (FD1)	37 b	CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG
Reverse (RD1)	37 b	CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT

The PCR reaction was performed in final 25 µl reaction containing, $MgCl_2$ (2mM), dNTPs (0.2 mM), forward and reverse primers (10 ρmol/µl), PCR buffer (1x), Taq DNA polymerase (1.5 u) and template DNA (1 µl). The following program was used for amplification (icycler, Biorad, USA): initial denaturation (94°C, 1 min), then 35 repeated cycles each consisted of denaturation (94°C, 1 min), annealing (60°C, 40s) and extension (72 °C, 150s) and a final extension (72°C, 20 min). The PCR product was analyzed by electrophoresis in 1% agarose containing DNA safe stain (90V, 30 min) alongside of 1kb DNA ladder and documented (UViTec, UK). The 1500 pb product was sequenced (Macrogene, Korea) and the obtained data was compared with registered data in NCBI based on BLAST algorithm and its phylogenetic tree was designed using Mega 6 software.

Phenotypic identification

Colony morphology including color, shape, size, margin, surface, texture and its attachment to agar in nutrient agar medium and also enzymatic and biochemical tests were used for phenotypic identification (21).

Results

To obtain the most possible polymer producing bacteria from sludge oil polluted soil, at first bacterial isolation and then screening of isolates were done. From total collected samples, 26 bacterial isolates were obtained and isolated. Following screening of isolates on PDA and staining with Sudan Black and Nile Blue A, 17 isolates were identified that were able to produce polymer with different potentials. The results have been presented in Fig.s 1 and 2.

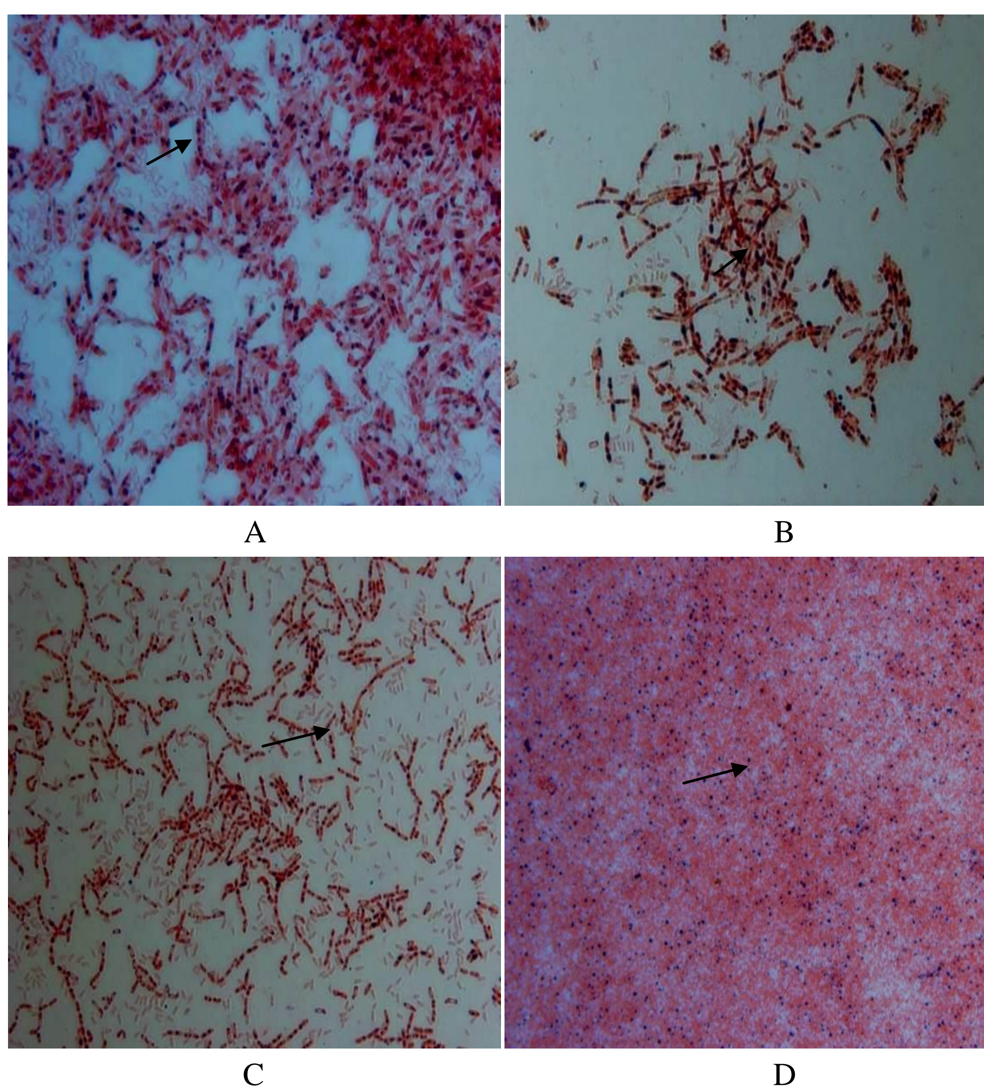

Fig.1- Sudan Black staining for identification of PHA producing isolates. PHA was stained as black granules in pink cytoplasm. The arrows show the location of PHA inside bacterial cell.A: SS4; B: SA2; C: SS2 and D: SS3

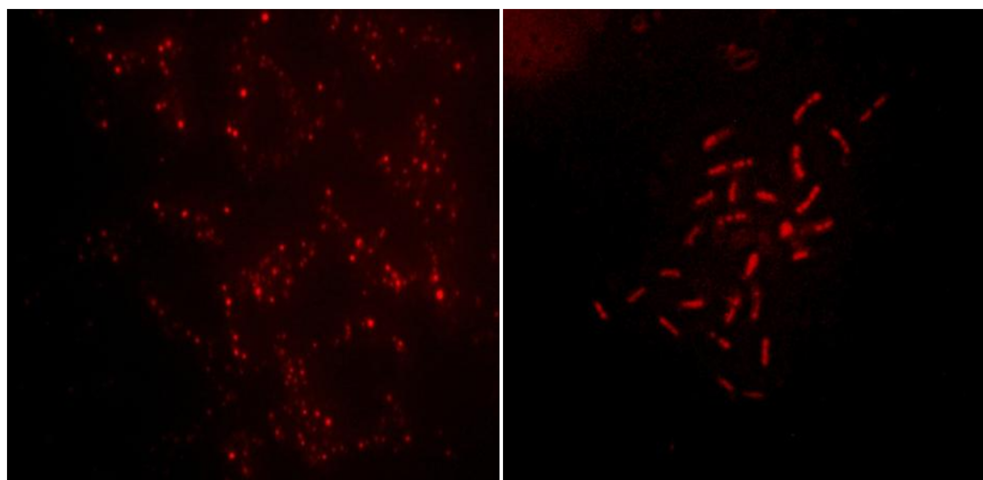

Fig.2- Nile Blue A staining for identification of PHA producing isolate. The polymer granules appear as orange fluorescent granules on black background

Table 2- SDS concentration optimized. Mean and standard deviation of triplicates extracted polymer from selected isolates

	SDS Concentration (gr/L) / 30% NaClO				
Isolate	4	6	8	10	12
SA_2	53.3 ± 29.59	65.53±13.45	48.85±16.75	49.96± 26	59.96±17.66
SS_2	77.76± 10.72	71.6±12.64	62.2±3.81	68.83±3.86	79.86±11.84
SS_3	74.3±11.83	63.67±13.93	65.53±5.08	78.86±16.42	82±19.05
SS_4	75.53±5.08	74.4±15.41	72.16±5.09	74.4±13.89	69.96±8.78

Table 3- Optimization of NaClO concentration. The Mean and standard deviation for three repeats of polymer extraction were presented

Isolate	Optimum SDS concentration (gr/L)	NaClO concentration			Maximum yield (%)
		10%	20%	30%	
SS_4	4	55.5±11.68	55.53±26.97	75.53±5.08	75.53±5.08
SS_3	12	72.2±10.17	77.63±14.87	82±19.05	82±19.05
SA_2	6	81.06±6.92	67.73±18.32	65.53±13.45	81.06±6.92
SS_2	12	69.96±3.35	67.73±9.64	79.86±11.84	79.86±11.84

From 17 isolates with PHA producing potential, 4 isolates were selected based on polymer content in bacterial cytoplasm. These isolates were named as SS2, SS3, SS4 and SA2. The mean results for 3 repeats of polymer extraction using different concentrations of SDS and NaClO have been presented in table 2 and 3.

As it can be found from obtained data the best concentration of SDS and the highest polymer extraction yield are respectively for SS4 as 4 gr/L and 75.53±5.08%, for SS3 as 12 gr/L and 82±19.05%, for SA2 as 6 gr/L and 81.06 ± 6.92% and for SS2 as 12 gr/L and 79.86 ± 11.84%. So the SS3 isolate has the highest production yield than other isolates.

The results of phenotypic identification revealed that all isolates are *Bacillus cereus*. In molecular identification, 1500 bp PCR product was successfully amplified for 4 selected isolates (Fig.3). Comparison of the obtained sequences with registered data in gene bank of NCBI was also confirmed that all isolates are *Bacillus cereus*. Fig. 4 presents the phylogenetic tree of these isolates.

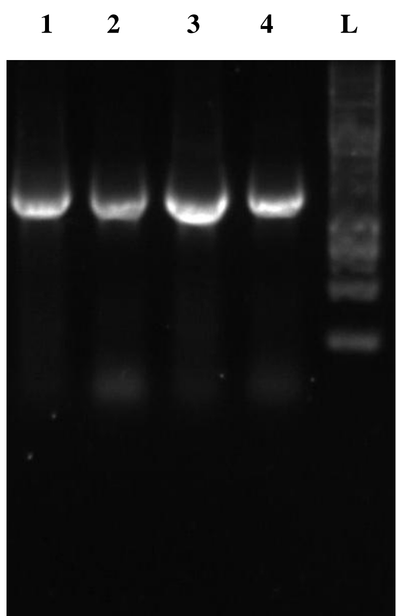

Fig.3- Electrophoresis of PCR products at 90V for 30 min. L: 1kb DNA ladder, 1: SS2, 2: SS3, 3: SS4 and 4: SA2

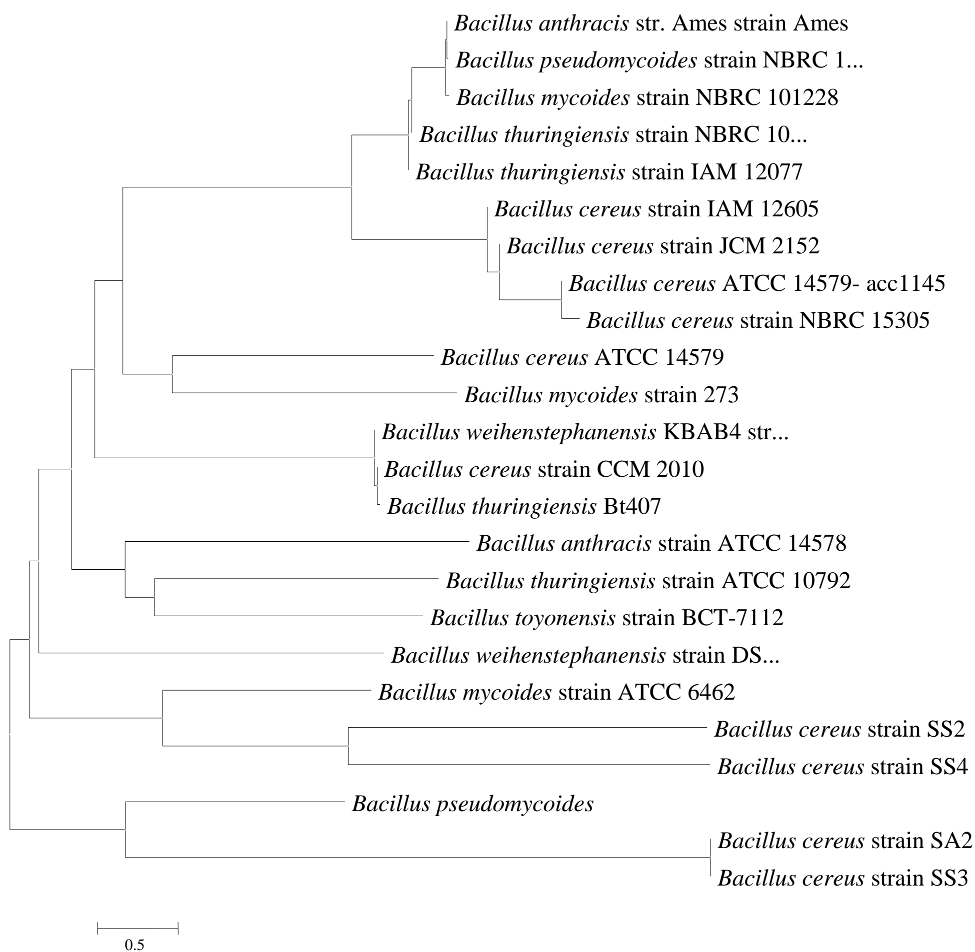

Fig.4- Phylogenetic tree of PHA producing isolates

Discussion and conclusion

During last decades synthetic polymers or plastics have been widely used by humans. These plastics are produced from petroleum resources that are incompatible with carbon cycles in environment. So, huge amounts of these polymers are returned to ecosystem as industrial and municipal wastes. With respect to the cautions of different environmental protection agencies on problems related to releasing these plastics in environment, researches have been done through the world in order to produce biodegradable polymers.Till now many types of biocompatible and degradable plastics have been produced. But limitations such as high cost of substrate, low yield and costs for preparing sterile environment caused that their large scale production be limited and they be unable to compete with synthetic plastics. Among the biodegradable polymers produced till present, PHA and its copolymers that are more than 40 different products have been paid more attention because they are fully degradable, flexible, water resistant, have low production cost and are easily produced (10).

Many studies have been done on isolation of polymer producing bacteria from different environments such as soil polluted with organic wastes, heavy metals, oil and munitions of war and also from sludge and water. Oil sludge contaminated soils were selected in this study due to their high carbon contents. Carbon to nitrogen imbalance in soil provides suitable conditions for enrichment of highly potential polymer producing bacteria in this environment. Few studies have been selected this habitat for finding PHA producing bacteria.

26 bacterial isolates were obtained in the present study from oil sludge contaminated soils. Then through culturing on PDA and specific staining for PHA, 17 isolates were confirmed as PHA producer. The results of phenotypic tests and also *16S rRNA* sequencing showed that all isolates belong to Bacillus genus. Bacillus members are most common bacterial inhabitants of environment and in studies done on isolation of polymer producing bacteria have been isolated with high frequency and also have high potential for biopolymer production. Furthermore, in order to polymer extraction the chemical digestion with NaClO and SDS was used and concentrations of these chemicals were optimized. However, polymer extraction using organic solvents such as chloroform leads to highly pure polymer but the extracted polymer with more than 5% (W/v) polymer is highly viscous and removing cell derbies from it is difficult. Furthermore, this method needs large amount of toxic and volatile solvents that not only are expensive and increase the final costs of polymer production, but also are hazardous for environment. In this study NaClO digestion was applied that is an alternative extraction method. In this method cellular contents except polymer are degraded and so digestion with NaClO in optimum condition yields most polymer extraction and least polymer degradation. But this method is not commonly used because it is necessary that NaClO and SDS concentrations be optimized for each bacterial isolate.

Liu *et al*, have isolated PHA producing bacteria from activated sludge under aerobic and anaerobic conditions. In their study 2 new bacterial isolates, namely *Dermatophilus* sp. and *Terrabacter* sp. were known that are able to produce PHA. Polymer extraction was done by chloroform and the highest extraction was 44% (22).

Tajima *et al,* using culture media similar to present study have isolated PHA producing bacteria from gasoline contaminated soils and introduced *Bacillus* sp. INT005. Polymer extraction was performed by chloroform and the highest polymer extraction was 35% (23).

In the study of Rehmn, different samples including oil contaminated samples, sewage and soil contaminated with molasses were screened for PHA producing isolates. As a result, 16 isolates were identified that some of them were from Bacillus genus. Extraction with chloroform was also used in this study and most polymer yield was 30% (1).

Arshad *et al* isolated bacterial strains with PHA producing ability from clean and contaminated environment. 16 bacterial strains were obtained in their study that was from Pseudomonas, Citrobacter, Entrobacter, Kelebsiella, Escherichia and Bacillus genus. Interestingly, isolated bacteria from clean environment did not have the ability to produce polymer. Polymer extraction by NaClO was done in this study and most polymer yield was 65%.

In Mizuno *et al* study on isolation of PHA producing bacteria from soil contaminated with munitions of war, similarly *Bacillus cereus* YB- 4 was reported with 44% polymer yield (2).

Ataee *et al* have isolated biodegradable polymer producing bacteria from date syrup waste and reported 6 isolates from Bacillus genus. Maximum polymer yield in their study was 71% while in our study was 82% (4).

Matias *et al* isolated Actinomycetes strains with the ability of PHA production from different soil samples. 34 isolates were obtained in their study and maximum polymer yield following chloroform extraction was 47% (24).

Arun *et al* isolated PHA producing isolates from different water ecosystems. Two *Vibrio* sp., *Bacillus cereus* and *Bacillus mycoides* were obtained as results of this study. In optimization experiment it was found that the two *Vibrio* sp. were able to produce more polymers (46%) than *Bacillus* Spp. in optimized conditions (7).

Termite gut ecosystem was screened for PHA producing bacterial species in the study of Tay *et al*. As a result, 3 PHA producers were isolated that was from Bacillus genus. Polymer extraction was performed by chloroform and maximum polymer yield was 34% (9).

Shirvasta *et al* screened soil and water environments for polymer producing bacteria. They used a byproduct of a biodiesel production process as a carbon source. *Halomonas hydrothermalis* and *Bacillus sonorensis* were the positive isolates with 71 and 75% polymer yield following NaClO extraction, respectively (25).

In present study 4 bacterial isolates with high potential of polymer production were isolated from oil sludge contaminated of Abadan oil refinery. These soils have long history of exposure with heavy chain oil hydrocarbons and also heavy metals. In spite of this fact that mentioned 4 isolates were identified as *Bacillus cereus*, they were significantly different in polymer production, so it is possible to introduce them as different biotypes of *Bacillus cereus*. This finding emphasizes that in screening producers, it is possible that several isolates which are identified as same species have different biological abilities and each isolate must be assessed and compared with others. Another important finding is that a habitat such as oil sludge contaminated soil that have high amounts of toxic substances and high temperature, provides a unique habitat that its bacterial inhabitants are preferred for industrial and large scale production of PHA than isolates from other environments. So, it is suggested that such environments be screened for the presence of microorganisms with different biological potentials.

Finally, with regard to the results of this study it can be explained that soils contaminated with petroleum hydrocarbons due to long storage of oil hydrocarbons and higher ratio of C/N/P are suitable environments for obtaining potent PHA producer bacteria (26). In this manner, strains adapted with high carbon content environments can be optimized and applied for industrial production of this biopolymer.

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غربال‌گری و جداسازی باکتری‌های بومی تولید کننده پلیمر پلی‌هیدروکسی‌آلکانوآت از خاک‌های آلوده به نفت پالایشگاه آبادان

حسین معتمدی *: دانشیار میکروبیولوژی، دانشگاه شهید چمران اهواز، ایران، hhmotamedi@yahoo.com
محمد رعایایی اردکانی: استاد میکروبیولوژی، دانشگاه شهید چمران اهواز، ایران، roayaei_m@yahoo.com
نسیم مایلی: دانشجوی کارشناسی ارشد میکروبیولوژی، دانشگاه شهید چمران اهواز، ایران، mayeli.nasim@yahoo.com

چکیده

مقدمه: افزایش آلودگی محیط زیست به واسطه مصرف پلاستیک‌های سنتزی موجب گرایش به سمت روش‌های نوین بیوتکنولوژی برای تولید پلیمرهای تجزیه‌پذیر شده است. بنابراین، تحقیق حاضر با هدف یافتن باکتری‌های بومی تولید کننده PHA به منظور استفاده از آن‌ها در تولید پلیمرهای زیست تخریب پذیر انجام شد.

مواد و روش‌ها: نمونه خاک آلوده به پسماندهای نفتی جمع‌آوری و پس از غنی سازی اولیه، غربال‌گری باکتری‌های تولید کننده پلیمر پلی‌هیدروکسی‌آلکانوآت در محیط PHA Detection Agar انجام و با رنگ‌آمیزی سودان سیاه و Nile Blue A تایید شد. جدایه‌ها با استفاده از روش‌های فنوتیپی و تعیین توالی *16S rRNA* تعیین هویت شدند. استخراج و تعیین بازده تولید پلیمر با استفاده از غلظت‌های مختلف هیپوکلریت سدیم و SDS انجام شد.

نتایج: از ۲۶ جدایه مختلف، ۱۷ جدایه دارای توانایی تولید پلیمر به میزان‌های متفاوت بودند که با توجه به میزان تجمع پلیمر درون سلول باکتریایی، ۴ جدایه به منظور مطالعات بیشتر انتخاب شدند. بیش‌ترین درصد بازده جدایه‌های منتخب ۵/۰۸± ۷۵/۵۳، ۱۹/۰۵±۸۲، ۶/۹۲± ۸۱/۰۶ و ۱۱/۸۴±۷۹/۸۶ درصد محاسبه شد. در تعیین هویت مشخص شد که تمامی جدایه‌ها به گونه *باسیلوس سرئوس* تعلق دارند. در واقع بیوتیپ‌های مختلف این گونه در خاک‌های آلوده به نفت توان متفاوتی در تولید پلیمر نشان دادند.

بحث و نتیجه‌گیری: با توجه به یافته‌های این تحقیق می‌توان بیان کرد که خاک‌های آلوده به ترکیبات نفتی به علت این‌که مقدار کربن بسیار بالاتری نسبت به نیتروژن و فسفر دارند و ترکیبات هیدروکربنی متفاوت طی زمان طولانی در این مناطق انباشته شده‌اند، محل‌های مناسبی برای جداسازی سویه‌های موثر در تولید پلیمر پلی‌هیدروکسی‌آلکانوآت هستند. به این ترتیب می‌توان سویه‌های سازش یافته با محیط با منبع کربن بالا را با بهینه‌سازی شرایط تولید در جهت تولید صنعتی این پلیمر به‌کار برد.

واژه‌های کلیدی: پلی‌هیدروکسی‌آلکانوآت، پلیمر، زیست تخریب پذیر، پسماند نفتی

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