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RESEARCH ARTICLE

Comparative Study of PAH Degradation Using Bacteria Grown on Agar and Gellan Gum Media

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Abstract

A mean high concentration of high molecular weight (HMW) polycyclic aromatic hydrocarbons (PAHs) compounds was detected in sediment collected from Tanoura Bay on the Yatsushiro Sea in Japan with concentration 30,200 ng/g (dry weight). The indigenous bacteria applied were *Burkholderia fungorum* isolate no 1 and *Mycobacterium gilvum* isolate no 13. A culture preparation approach was carried out using the bacteria grown on two media. *B. fungorum* isolate no 1 grown on the agar (*Bf*-A) and the strain grown on the gellan gum (*Bf*-G) can degrade fluoranthene and pyrene at the level of 56.1 % $\pm$ 8.1; 62.4 % $\pm$ 7.7 at 20-day and 96.2 $\pm$ 0.5; 96.3 $\pm$ 0.2 at 16-day incubation of a period, respectively. Furthermore, fluoranthene and pyrene could be reduced by *M. gilvum* isolate no 13 grown on the agar media (*Mg*-A) and those grown on the gellan gum (*Mg*-G) at the concentration of 70.9 $\pm$ 6.2; 78.6 $\pm$ 0.4 after 24 days and 86.4; 98.5 $\pm$ 2.5 after 12 days. Almost the entire fluoranthene and pyrene degradation required 24 - 28 days and 16 - 20-day incubation of a period, respectively. Compared with bacteria grown on agar, bacteria grown on gellan gum had an easier PAHs degradation process than other.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are one class of organic pollutants comprising of about 100 different non-polar compounds that have accumulated in the environment [1]; [2]. These organic pollutants belong to the most hazardous environmental pollutants because of their possible toxic, recalcitrance, mutagenic, carcinogenic and teratogenic properties. Furthermore, the pollutants not only can produce tumor but also have adverse effects on reproduction, development and immunity [3]; [4]. PAHs can be categorized into two groups : low-molecular-weight (LMW) compounds consist of two or three rings, and high-molecular-weight (HMW) compounds, more than three rings [5].

A mean high concentration of HMW PAH compounds at a concentration level of 30,200 ng/g (dry weight) was found in sediment collected from the Tanoura Bay, Yatsushiro Sea, Japan. The presence of fluoranthene and pyrene are detected in this area as the two highest of PAHs with number concentration in bivalve samples of 110 and 74 (ng/g wet wt.), respectively [6]. For cleaning up the pollutants, bioremediation using selected bacteria, which have an ability to degrade the PAHs is urgently required. Bioremediation is one of the most widely used as a promising technology considered to be economical, effective and environmentally friendly for reducing the pollutant

[7]. However, there is a well-known limitation for the investigation of the cultures preparation approaches obtaining the optimum process of bioremediation.

Agar and gellan gum were two media considered to provide the preparation of microbiological cultures. Agar is a gel-forming polysaccharide identified its origin in Japan in 1658. It is purified from certain seaweeds belonging to the red algae family, such as Gelidiaceae or Gracilariadeae. This polysaccharide is composed of β-D-Galactose and 3,6 anhydro-L-galactose disaccharide [8]; [9]. The galactose is defined as the basic skeleton; one is based on the neutral polysaccharide (agarose) and the other is the acidic polysaccharide (agaropectin) [10]. The agarose is the essential part of agar determining the gelling ability. It has molecular weight above 100,000 and often exceeds 150,000 Daltons as well as a low sulfate content (below 0.15 %). Moreover, the agaropectin is the agar rest fraction which has a lower molecular weight of 14,000 - 20,000 Daltons and is including ionic groups such as sulfate, pyruvate and methoxy groups [8]; [10]. Agar can supply an excellent reversibility with gelling and melting temperature (45^{o} C). Because of its gel characteristic, agar is necessary applied especially as media in the microbiological culture preparation such as yeast, moulds and bacteria [8]. Meanwhile, gellan gum is a high molecular mass polysaccharide gum, approximately 500,000 Daltons, discovered in the USA in 1978. It consists of glucuronic acid, rhamnose and glucose produced as a fermentation product by a pure culture, *Pseudomas elodea* [11]; [12] and is not affected by natural condition that influence the grade of agar-based media [13]. This gum is a suitable gelling agent in bacteriological media, which can be used to substitute agar for isolation, identification, enumeration [11]; 12] and preparation of microbiological culture.

For the conventional culture preparation, agar is usually used as bacteria growth media. Previously published studies on the grade of agar revealed that it was not consistent, due to agar extracted from seaweed and detected containing toxic matter which influences to susceptive organism. On the other hand, some researchers reported that gellan gum purified from a fermentation process of pure strain might be a suitable substitution for agar. Therefore, we carried out a comparative study of the microbiological culture preparation using the isolated bacteria that grow on agar and gellan gum media for PAHs degradation. An investigation of growth and activity rate of the bacteria was conducted. This aimed to probe the influence of media used in microbiological culture preparation for the PAHs degradation.

MATERIALS AND METHODS

PAHs degrading bacteria

The PAHs degrading bacteria applied in this study, close to *Burkholderia fungorum* (GenBank Accession Number: NR_025058.1) and *Mycobacterium gilvum* (GenBank Accession Number: NR_074644.1). They were successfully isolated from soil collected on Tanoura Bay, Yatsushiro Sea, Japan and identified through the analysis of the partial 16S rRNA gene sequences with similarity of 99 % using the Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) database [14].

Fig. 1 *B. fungorum* isolate no 1 grown on (a) agar and (b) gellan gum sprayed with pyrene
Fig. 2 *M. gilvum* isolate no 13 grown on (a) agar and (b) gellan gum sprayed with pyrene

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Chemicals

Agar and gellan gum were acquired from Wako Pure Chemical Industries Co., Ltd. (Japan) and Phyto Technology Laboratories (USA), respectively. Two PAHs, fluoranthene and pyrene, obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Nacalai Tesque, Inc. (Kyoto, Japan), respectively. All chemicals used in this study were laboratory grade with > 98.0% analytical standards.

Culture preparation of bacteria grown on two media

After the PAHs degrading bacteria were successfully isolated, they were transferred into two media plates with concentration at 2% w/v of each, i.e., agar and gellan gum containing the minimum medium (MM) sprayed pyrene of 100 mg/L. Then, they were incubated at 30 ^{o}C for ten days. The bacteria grown on the two medium plates had the clearing zones as depicted in Fig. 1-2. Each bacterium was picked into the sterile Erlenmeyer flask containing 50 mL of MM and 100 mg/L of pyrene and was shaken on an orbital shaker at a speed of 180 rpm at 30 ^{o}C in the dark condition for two days. Subsequently, it was concentrated using a centrifuge at 10,000 rpm for 10 minutes. The inoculum was prepared by culturing the concentrated strains within 0.05 LB medium at 30 °C for 24 hours. Afterward, by centrifugation at 10,000 rpm for 10 minutes, the cells were harvested and then were washed twice with a NaCl solution of 0.85 %. By using a UV-visible spectrophotometer (UV-1700 Model TCC-240A, Shimadzu, Japan), the optical density (OD) of cell was adjusted at 600 nm to OD of 0.1 [15]. According to [16], the bacteria could grow on soil and break down the PAHs such as anthracene, phenanthrene, fluoranthene or pyrene at CFU/g of soil dry weight of 10^3 - 10^5. In the experiment, the initial number of colonies was adapted equal or more

than 1 x 10^5 in which the cell number of colonies on agar and gellan gum were 7 x 10^6 and 6 x 10^5, respectively for *B. fungorum* isolate no 1 and 1 x 10^6 and 5 x 10^5 for *M. gilvum* isolate no 13, respectively.

Degradation of Fluoranthene and Pyrene Experiment

Fluoranthene and pyrene were applied as sole carbon and energy sources in the degradation experiments with the initial concentration of each at 100 mg/L using test tube containing 2 mL MM. The cell inoculum obtained from both medium plates (agar and gellan gum) were each taken 1 mL, then added separately to 50 mL of MM then divided to each test tube of 2 mL by shaking at 180 rpm for 1 hour previously. The culture was shaken and incubated under aerobic condition in the dark on a shaker at a room temperature of 30 ℃, at a speed of 180 rpm with an incubation period of 24 days for fluoranthene and of 20 days for pyrene. Every 4 days, the samples were taken and investigated, the remaining amount of PAHs and the growth cell rates through colony forming unit per mL (CFU/mL). The remaining PAHs concentration was analyzed by using HPLC method, and the CFU value was measured by plating on solid media plate.

Activity and growth cell test

The activity cell rate was examined by observing the number of cultivation of bacteria, which grow on the media test plates. The media were used such as 0.1 Luria Bertani-agar (0.1LB-A), and minimum medium-agar sprayed with pyrene (MM-AP). Meanwhile, growth cell rate test was performed by counting the cell grown on the 0.1LB and MM-AP plates. The growth cells shown were derived from the 10^{-4} - 10^{-5} dilution. The activity and growth cell test were conducted every 4 days during PAHs degradation experiment.

RESULTS AND DISCUSSION

Degradation of Fluoranthene and Pyrene

In the PAHs degradation, the used bacteria were *B. fungorum* isolate no 1 grown on agar (*Bf*-A) and those grown on gellan gum (*Bf*-G); *M. gilvum* isolate no 13 grown on agar (*Mg*-A) and the same strain grown on gellan gum (*Mg*-G). The whole pyrene degradation required 16 - 20-days and the fluoranthene degradation needed 24 - 28-days incubation for reducing almost completely.The precentage of PAHs degradation profiles using the bacteria are shown in Fig. 3 - 4.

Fig.3 Percentage degradation of (a) fluoranthene and (b) pyrene with *B. fungorum* isolate no 1

Fig.4 Percentage degradation of (a) fluoranthene and (b) pyrene with *M. gilvum* isolate no 13

In the initial condition, the CFU number of bacteria grown from agar was higher than those from gellan gum. The results indicated that both *B. fungorum* isolate no 1 and *M. gilvum* isolate no 13 obtained from coastal can actively grow on the agar plate due to the presence of consumable mineral compounds [17]. Agar compounds extracted from wall component of seaweeds belonging to the red algae family such as Gelidiaceae or Gracilariadeae [10]. The *Bf*-A and *Mg*-A bacteria can utilize both carbon sources from agar compounds and pyrene on its metabolism so it can have an effect on the microorganism growth. Meanwhile, the *Bf*-G and *Mg*-G bacteria can only consume a carbon source from pyrene sprayed onto the gellan gum purified from heterpolysaccharide secreted by *Sphingomonas elodea* or formerly known as *Pseudomonas elodea* [18]. Therefore, there is no other energy source compound in the gellan gum plate except from pyrene.

The fluoranthene degradation using the *Bf*-A and *Bf*-G bacteria at 24-day incubation were achieved at 99.61 % $\pm$ 0.68 and 99.83 % $\pm$ 0.30, respectively. For the pyrene degradations were reached at 98.61 % $\pm$ 1.86 and 98.70 % $\pm$ 0.45, respectively after 20-day. Meanwhile, the whole of fluoranthene concentration can be degraded by *M. gilvum* isolate no 13 grown on two media at 28-day. Furthermore, for pyrene degradations were obtained at a level of 99.9 % $\pm$ 0.1 and 100 % after 16-day by *Mg*-A and *Mg*-G, respectively. The PAHs degradation with both the bacteria grown on gellan gum were better than those on agar, except in the beginning degradation and in the end incubation. The PAHs degradation using *Bf*-A and *Mg*-A during the initial incubation was more effective than *Bf*-G and *Mg*-G. These results might be caused by the cell number (CFU/mL) of the bacteria grown on the agar plates were higher than those grown on the gellan gum plates. Moreover, it was required the appreciable energy when the bacteria break down the fluoranthene and pyrene since initiating the oxidation of PAHs [19]. The consumable mineral compounds of agar can supply a sufficient strength reserve therefore the bacteria can consume fluoranthene and pyrene more.

On the other hand, the PAHs degradation with the bacteria grown on the gellan gum were preferable than those grown on the agar in the intermediate incubation. There was a difference between the percentage of PAHs degradation by using the bacteria grown on the variant media. The results indicated that the PAHs degradation processes using bacteria grown on gellan gum were easier than those grown on agar media. It might be caused by the medium plate only containing pyrene can be better inducing some proteins involved in PAHs metabolism than the

plate containing pyrene and mineral compounds from agar as shown in Fig. 1-2. The presence of pyrene sprayed onto the solid medium plate could induce some proteins involved in PAHs pathway [20]. Interestingly, there were similar PAHs degradation percentages using both bacteria grown on two media in the end incubation. The results showed that the PAHs degradation processes were almost same. The condition might be caused by the CFU number of bacteria grown on gellan gum were lower than those grown on agar, except for pyrene degradation by *Mg*-G (Fig. 4b). Further analysis, the similar percentages happened between using two strains grown on the media was a probability due to their enzymatic activities.

Activity and growth cell

Activity and growth cell were carried out using two types of medium tests with PAHs as carbon and energy sources represented in Table 1-2.

Table 1 Investigation of cultivation number for PAHs degradation using *Bf-A* and *Bf-G*
Table 2 Investigation of cultivation number for PAHs degradation using *Mg-A* and *Mg-G*

In the beginning experiment, the growth bacteria coming from agar were faster than those from the gellan gum in two plates test, i.e., 0.1LB-A and MM-AP plates. It revealed that the bacteria grown on agar had a higher cell activity than those grown on other media. Then, the table showed that there had been a decrease in the number of cultivation in both strains. *B. fungorum* isolate no 1 can be easy consuming the mineral compound from the 0.1LB-A, while it takes a longer time for utilizing the component from MM-AP plate. Conversely, there was an extended growth lag when the *M. gilvum* isolate no 13 transferred into 0.1LB-A plate than MM-AP plate. The different cell activity might be caused by substrate specificity which influences enzymatic activity [21]. In the end experiment, the cultivation amount of the bacteria from gellan gum was increased. It might be the remaining pyrene or fluoranthene with bacteria grown on gellan gum was less than those with other. Moreover, there was a prolongation period that occurred between pyrene and fluoranthene degradation, where it was a probability due to the difference of specificity activity. The bacteria which degrade pyrene had a greater specificity activity than those that consume fluoranthene. Even so, the cultivation condition showed that the activity cell between the same strain grown on agar and gellan gum was similar in the PAHs degradation.

Meanwhile, the growth cell investigation was also performed using the same media test. In the starting experiment, there was the difference of the cell growth number, approximately 0.5 - 1 order of CFU (1/mL) between bacteria grown on agar and gellan gum, where the bacteria grown on agar had a higher number of growth cell. However, the bacteria grown on gellan gum had a greater growth cell rate than those grown on agar after initial incubation. These results might be caused by the gellan gum properties, such as being wet enough for assisting the growth cell and coarse enough for approving the diffusion of macromolecular carbon sources or other nutrients [22]. Furthermore, the condition occurred was a possibility because gellan gum purified from fermentation product, so it contains no contaminating matters, such as those detected in agar (e.g., phenolic compounds), which are toxic to certain sensitive organisms [22]. In the previous study, most of the viable cell counts (33 of 50 isolates) were higher on gellan gum than on agar. It would appear that based on bacterial growth, the gellan gum is less inhibitory than other [12]. Therefore, it is easier to consume the carbon sources and other nutrient's compound by microorganism grown on gellan gum. These factors may explain the relatively good correlation between the use of gellan gum as a gelling agent for the culture preparation and the performance of PAH degradation.

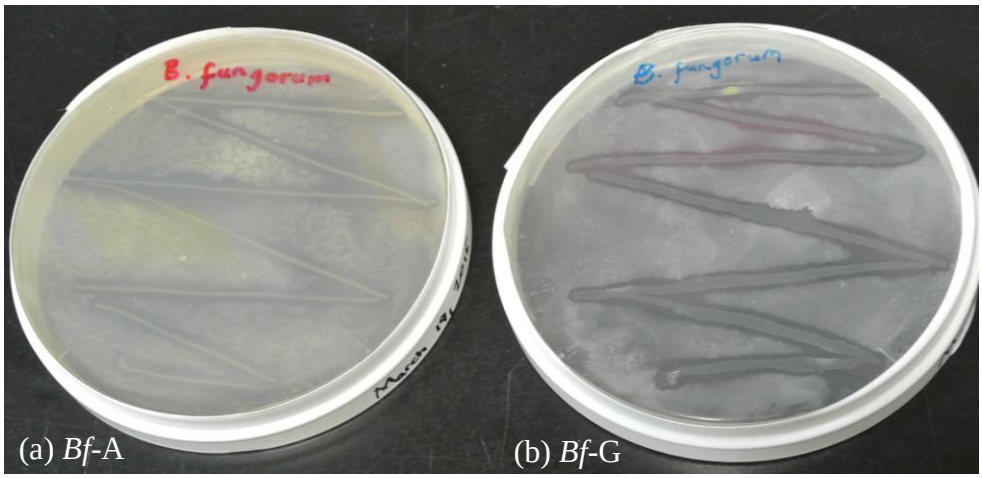


Fig.1 *B. fungorum* isolate no 1 grown on (a) agar and (b) gellan gum sprayed with pyrene

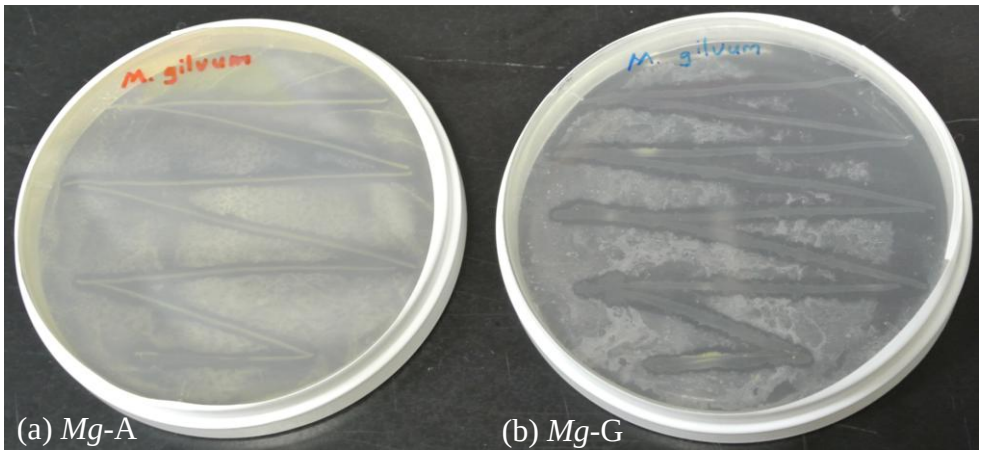
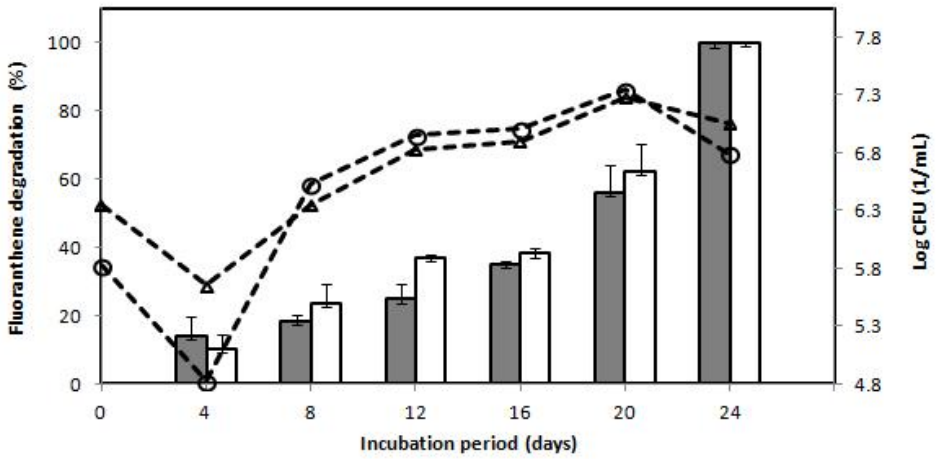


Fig.2 *M. gilvum* isolate no 13 grown on (a) agar and (b) gellan gum sprayed with pyrene

(a)



(b)

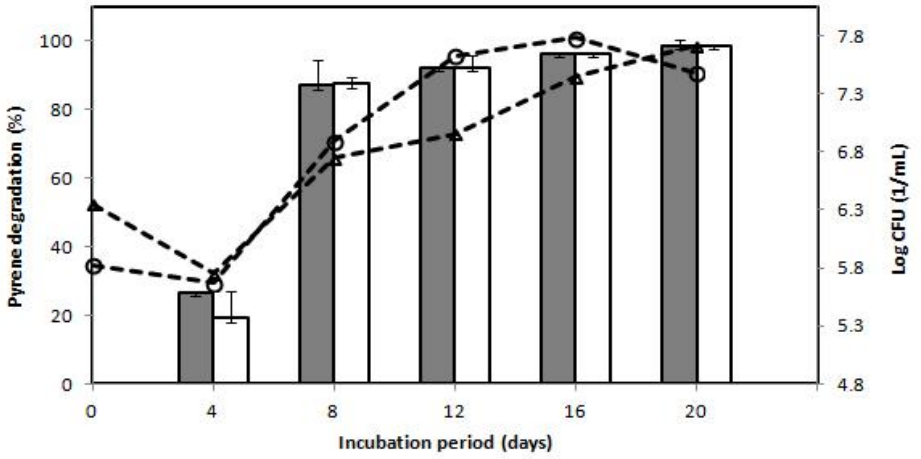
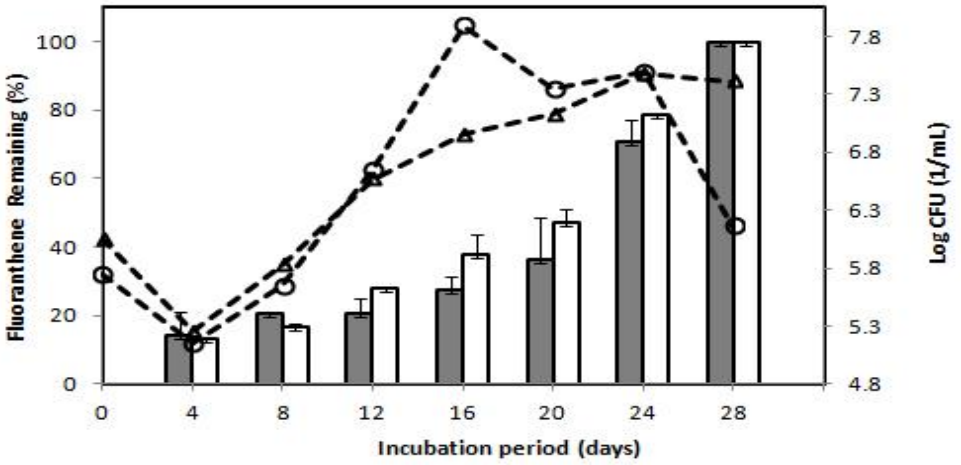


Fig.3 Percentage degradation of (a) fluoranthene and (b) pyrene with *B. fungorum* isolate no 1
Black crossbar, fluoranthene degradation (%) using the strain grown on agar *(Bf*-A*)*; white crossbar, pyrene degradation (%) using the strain grown on gellan gum *(Bf*-G*)*

Open triangle, CFU of *Bf*-A (*dotted line*); open circle, CFU of *Bf*-G (*dotted line*)

(a)



(b)

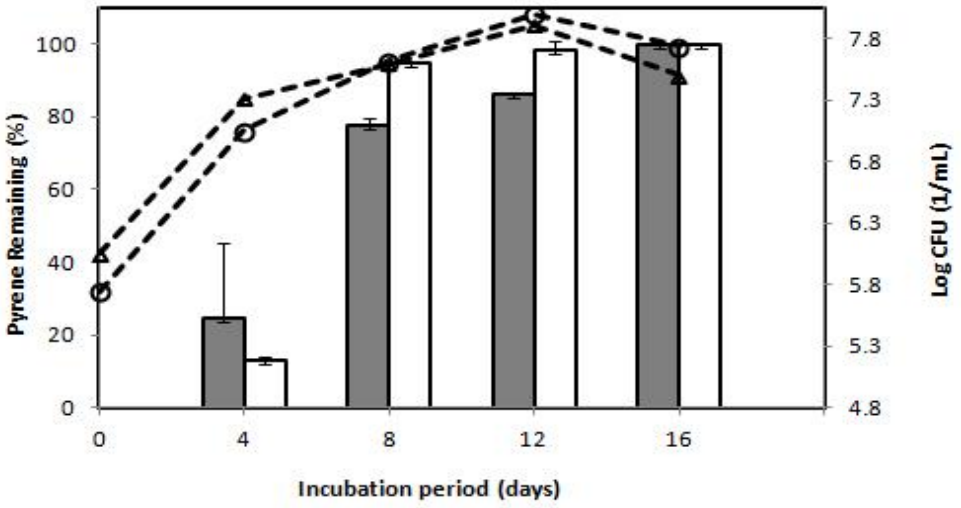


Fig.4 Percentage degradation of (a) fluoranthene and (b) pyrene with *M. gilvum* isolate no 13
Black crossbar, fluoranthene degradation (%) using the strain grown on agar (*Mg*-A); white crossbar, pyrene degradation (%) using the strain grown on gellan gum (*Mg*-G)
Open triangle, CFU of *Mg*-A (*dotted line*); open circle, CFU of *Mg*-G (*dotted line*)

Table 1. Investigation of cultivation number for PAHs degradation using *B. fungorum* isolate no 1

Media used for test	Strain	Number of cultivation during PAHs degradation (day)						
		0	4	8	12	16	20	24
0.1LB-A[(a)]	*Bf*-A[(c)]	4	4	2	2	2	2	3
	Bf-G[(d)]	5	4	2	2	2	4	5
MM-AP[(b)]	*Bf*-A	5	4	4	4	4	4	5
	Bf-G	7	4	4	4	4	5	6

a, Luria Bertani-agar; b, minimum medium-agar sprayed pyrene; c, *B. fungorum* isolate no 1 grown on agar; d, *B. fungorum* isolate no 1 grown on gellan gum

Table 2. Investigation of cultivation number for PAHs degradation using *M. gilvum* isolate no 13

Media used for test	Strain	Number of cultivation during PAHs degradation (day)							
		0	4	8	12	16	20	24	28
0.1LB-A	*Mg*-A[(e)]	6	6	6	5	5	4	4	5
	Mg-G[(f)]	7	6	6	5	5	4	4	5
MM-AP	*Mg*-A	5	5	5	5	5	5	6	6
	Mg-G	6	5	5	5	5	6	6	6

e, *M.gilvum* isolate no 13 grown on agar; d, *M.gilvum* isolate no 13 grown on gellan gum

CONCLUSION

There are three tendencies result from the degradation of PAHs by using bacteria that grow on agar and gellan gum. In the initial experiment, the abilities of PAHs degradation from bacteria grown on the agar were bigger than those grown on the gellan gum. The contrast capability occurred when bacteria were incubated during the intermediate period. The PAHs degradation percentages of bacteria grown on the gellan gum were achieved as well as those on the agar at the end of the experiment. Nevertheless, generally, the bacteria grown on the gellan gum media had higher the PAHs degradation rate than those grown on the agar plate. The gellan gum features might be able to support the diffusion of macromolecular carbon sources or other nutrients and the bacteria growth well. The presence of pyrene sprayed onto the gellan gum plate might be able to be better inducing some proteins required in PAHs metabolism than other. These results propose that gellan gum might be an appropriate successor of agar for the microbiological culture preparation in the PAHs degradation.

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