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Research Article

Fungal Spore Production, Biomass and Colony Diameter of *Penicillium* sect. *Chrysogenum* in the Oil Sludge Medium from Phytoremediation

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Abstract

Penicillium sect. chrysogenum has the ability to degrade hydrocarbon compounds in the oil sludge, by decreasing the Total Petroleum Hydrocarbon (TPH) and Polycyclic Aromatic Hidrocarbons (PAH) levels. The present work aims to obtain the optimum oil sludge concentration to maximize biomass and spore production of *Penicillium sect. chrysogenum*. Fungal biomass production was analyzed by measurement of cell dry weight, while the fungal spore production was analyzed by cell counting method haemocytometer. The scanning electron microscope (SEM) was performed to observe changes in fungal cell growth during observation. Determination of optimum concentration was analyzed by one-way ANOVA followed by Duncan test, and morphological observations were analyzed descriptively. The results showed that the addition of oil sludge into the growth medium was not significantly different compared to that of control (0.099 ± 0.014 g/L). The optimum concentration of oil sludge in the medium was found at 3% with highest fungal spore production (32.45 x 10^8± 5.60 x 10^8 cell/mL). Morphological observation demonstrated that there was an acceleration in hyphae and spores growth on the oil sludge-contained medium. This study can give valuable information on the medium optimization of *Penicillium sect. chrysogenum* as bioremediation agent in hydrocarbons contaminated soil.

Keywords: *Penicillium* sect. *Chrysogenum*; Oil sludge; Biomassa; Spora

Introduction

Oil and natural gas have an important role as the main source of energy for human use in various needs. Great demand in oil and gas production carries potential risk to the environment, due to the waste produced during the exploration and production in form of oil sludge containing polyhydrocarbons and heavy metals. Particulrly in Indonesia, these industrial wastes have not been treated and have only stockpiled in the storage warehouses of the national petroleum enterprise (PT Pertamina).

The oil-contaminated environments can be treated physically, chemically and biologically. According to Mangkoedihardjo (2005), physical and chemical remediation is considered as short-term and incomplete remediation (mass transfer between environmental medium), where only about 10-15% of pollutants can be removed. On the other hand, biological remediation (bioremediation) is considered as relatively effective, low cost and sustainable method. One of the application of bioremediation is to use microorganisms such as fungus that have the ability to degrade hydrocarbon compounds because they have extracellular enzymes such as hydrocarbons and hyphae that can penetrate the substrate in order to accumulate heavy metal elements.

Previous study from Rossiana., *et al*. [1-3] showed that a total of 9 indigenous fungal isolates from Tanjung Uban are the potential candidate for bioremediation agents. One of the fungal species was *Penicillium sect. chrysogenum* that produce biosurfactant and is able to reduce TPH level (with 23.55 ppm of biosurfactant) by 17.92% after 15 d of fermentation in the 10% oil sludge-contained medium. Another study from Dhar., *et al*. (2014) revealed that *Penicillium corylophilum* can degrade the kerosene by 41% and is considered as an effective bioremediation agent compared to *Cladosporium tenuissium*, *Fusarium moniliforme*, *Trichoderma koningii* and *Aspergillus niger*.

Elevated PAH concentration in the bioremediation process may affect the growth of hyphae radially and sporulation on several sungal species, thus altering the structure of cell membrane, inhibition of conidia formation and changing spore color from

green to yellow at 2 and 4% of PAH concentration [4]. Based on these findings, it is important to assess the optimum concentration of oil sludge in the medium to observed the fungal adaptation ability that will be measured with biomass production and spore productivity. The optimum oil sludge concentration can show the optimum medium condition that can be applied for bioremediation starter.

Materials and Methods

The present study was conducted with descriptive experimental method in two steps: (1) biomass profile analysis and spore production, and (2) determination of the optimum concentration from the variation of oil sludge concentration. The analyzed parameters were divided into main and supporting parameters. The main parameters observed were the fungal biomass production and spore production, while the supporting parameters were the observation of hyphae and spores using scanning electron microscope (SEM). Fungal biomass production was analyzed measuring dry weight of the cell and the number of spores produced was analyzed using counting chamber (haemocytometer). The observation time intervals were everyday for seven days to observe biomass, while observation for spore production was started from the fifth to tenth day.

Determination of optimum concentration was conducted experimentally with random complete design with oil sludge concentration as single factor (0, 1, 2, 3 and 4%) and five replicates for each concentration. The obtained data was then analyzed using one-way ANOVA with 95% of confidence level. When the significancy between treatments was present, the Duncan post-test was performed. All statistical analyses were carried out using SPSS version 20 statistical software. Additionally, data obtained from the morphology of hyphae and spores were analyzed descriptively. In the present work, images obtained from SEM observation was used to confirm changes in the growth of fungal cells during the observation.

Results and Discussion

Optimum concentration of Oil Sludge

It was found that different concentration of oil sludge in the fungal growth medium significantly affect the biomass production of *Penicillium sect. chrysogenum* (one-way ANOVA, $p < 0.05$). Furthermore, Duncan post-test analysis showed that the addition of oil sludge into the medium at all concentrations significantly difference to that of control (Table 1) Analisis data kemudian dilanjutkan dengan uji Duncan untuk mengetahui perlakuan yang paling berbeda nyata.

No	Type of treatment	Biomass production
1	Control	0.099 ± 0.014 [a]
2	1%	0.076 ± 0.004 [b]
3	2%	0.078 ± 0.004 [b]
4	3%	0.085 ± 0.011 [b]
5	4%	0.082 ± 0.007 [b]

Tabel 1: Duncan post-test analysis on the effect of different concentration of oil sludge in the medium on biomass production of *P. chrysogenum.* One-way ANOVA analysis was performed prior to Duncan test with 95% of confidence level. Values with different lower case letters are significantly different.

Duncan post-test revealed that the fungal biomass production in control medium was significantly different with that of oil sludge-contained medium at all types of concentration. This result indicates that *P. chrysogenum* that grow in the medium with the addition of oil sludge can not use this oil sludge optimally as the source of carbon. Therefore, in the present study, the ideal concentration of oil sludge for optimum fungal biomass production could not be obtained. However, oil sludge concentration at 3% resulting fungal biomass production as much as 0.078 ± 0.004, which is closed to the biomass produced in control (0.099 ± 0.014).

Effect of oil sludge in the medium on fungal spores production was done by using haemocytometer. It appeared that the oil sludge addition into the medium gave significant effect on the biomass production of *P. chrysogenum* (one-way ANOVA, $p < 0.05$). Additionally, post-test revealed that the control medium was not significantly different with 1 and 2% of oil sludge concentration in the medium, but it was significant as compared to that of 3 and 4% (Table 2). We found that the 3% of oil sludge in the medium was the optimum concentration due to the number of spores produced ($32.45 \times 10^8 \pm 5.6 \times 10^8$)

No	Type of treatment	Spores production
1	Control	$20.72 \times 10^8 \pm 4.81 \times 10^8$ [a]
2	1%	$22.18 \times 10^8 \pm 6.12 \times 10^8$ [ab]
3	2%	$27.47 \times 10^8 \pm 3.56 \times 10^8$ [abc]
4	3%	$32.45 \times 10^8 \pm 5.6 \times 10^8$ [c]
5	4%	$28.47 \times 10^8 \pm 6.99 \times 10^8$ [bc]

Table 2: Fungal spores production of P. chrysogenum at different concentration of oil sludge. One-way ANOVA analysis was performed prior to Duncan test with 95% of confidence level. Values with different lower case letters are significantly different.

Growth profile of *P. chrysogenum*

Profile of biomass and spores at control medium

Growth profile is the main indicator of the growth of the micro-organisms. Observation of growth profiles is the indicative of how microorganisms adapt on their life stages. The growth profile of *P. chrysogenum* that was cultivated in liquid culture is presented in Figure 1.

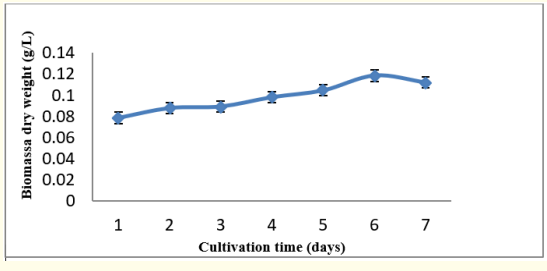

Figure 1: Growth profile of biomass production of P. Chrysogenum.

It appeared that *P. chrysogenum* can grow and adapt to culture medium and its environment. This fungi experienced an exponential growth phase until sixth day, then entered the stationary phase until the seventh day. The exponential growth of *P. chrysogenum* was occurred from 1 d to 6 d (Figure 1). During this period, *P. chrysogenum* actively grow and achieve its maximum growth with dry weight biomass as much as 0.11868 g/L. In the exponential phase, cells start to actively metabolize such as synthesizing acidic products, for instance, pyruvic aci, nucleic acids and other macromolecule compounds. In addition, during the exponential phase, cells are in a stable state. The constituent of new cell is formed at a constant rate thus the mass increases exponentially (Volk and Wheeler, 1993).

The dry weight of biomass decreased on day 7th due to the nutrient depletion by *P. chrysogenum* for growth and metabolism, consequently cells must compete to obtain the required nutrients. Cells that are unable to compete will experience mortality, thus increasing cell mortality will reduce cell's dry weight (Rachman., *et al*. 2016). According to Tarigan (1990), microfungi will experience an increase in growth which proportionally correlated with nutrient consumption and production of enzymes that can break down the substrate.

The main parameters of adaptation in fungi are the biomass production and productivity of spores. Indeed, spores are an early form of fungi, as result of both sexual and asexual reproduction. High number of spores produced means higher probability that the fungi can produce large amounts of cell biomass. Spores production of *P. chrysogenum* in culture medium is presented in Figure 2.

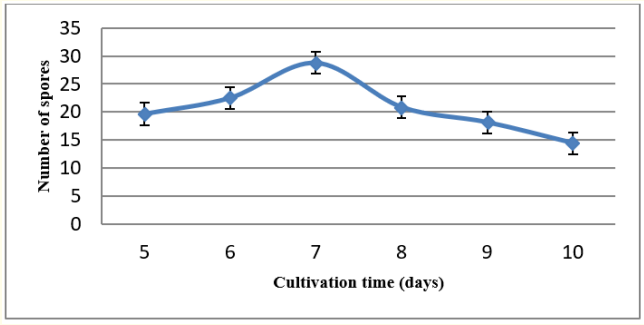

Figure 2: Growth profile of spores production in P. Chrysogenum.

Observation of the number of spores was carried out from 5 d to 10 d because new spores are formed after the growth of hyphae and conidia. Asexual reproduction in *P. chrysogenum* was started with the formation of a conidiophore, which is the branch of the hypha that raises toward the surface. Growht of aerial conidiophores occur when due to cytoplasmic flow that carries genetic material, including cell nuclei that are packed in the form of spores [5], the tip of conidiophores will expand because of the movement of protoplasmic contents to form conidia and continue to develop into conidium. After, conidium will break and produce new spores as a result of asexual reproduction [6].

In this study, the exponential phase of *P. chrysogenum* started from 5 d until 6 d. At 6 d, the spores of *P. chrysogenum* increased exponentially until 7 d with maximum number of spores as much as 28.78 x 10^8 cell/mL (Figure 2). Large number of spores produced is probably due to the time span of *P. chrysogenum* that is in the reproduction phase, hence conidia as an asexual reproduction device will grow and produce new spores. On 7 d, the number of spore of *P. chrysogenum* decreased (Figure 2). Reduction in the number of spores occurs because the formed spores need to adapt to the medium and grow into new hyphae that are confirmed in the morphological observation on 10 d (Figure 8). Afterwards, the population of *P. chrysogenum* that has been formed will decline from stationary to death phase where the nutrients content in the medium reduced significantly.

Profile of biomass and spores in oil sludge-contained medium

Optimum biomass production must be achieved through selection of culture medium for the bioremediation application. The addition of oil sludge in the culture medium was done to

obtain optimum biomass production. The growth profile of biomass production of *P. chrysogenum* at different concentrations is presented in Figure 3.

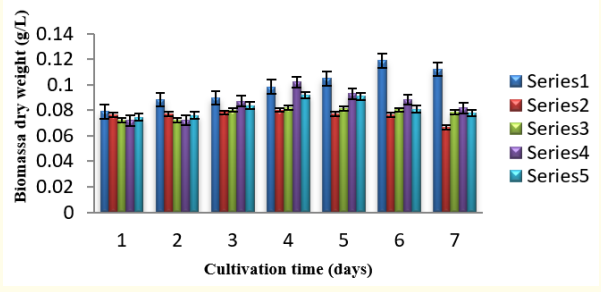

Figure 3: Growth profile of biomass production of P. chrysogenum at different concentration of oil sludge.

It was found that the optimum biomass production for the control medium occurred at 6 d. In contrast, biomass production in the culture medium containing oil sludge was not significantly different. The factors that may influence this insignificancy perhaps due to the agitation during the culture. Indeed, shakers must be used during cultivation, while at the time of cultivation, stirring is performed periodically hence it may affect the biomass production of *P. chrysogenum*. According to Rawn and Etten [7], the planting of mycelium fragments in liquid culture is influenced by agitation that can increase the growth of biomass in the culture. The shaker serves to homogenize the culture medium. Moreover, the speed of agitation has significant effect in influencing mycelium biomass [8].

The optimum biomass production in the medium containing 3% oil sludge was observed with dry weight biomass as much as 0.10212 g/L. According to Khan., *et al*. [9], *P. janthinellum* produces dry weight biomass as much as 0.38 g/L at 3% concentration of kerosene at 5 d. Dry biomass production at oil sludge concentration 1, 2 and 4% produced dry weight biomass that are not significantly different to that of dry weight biomass at 3% of oil sludge, because according to similar authors, biomass production decreases when kerosene concentrations exceed 3%. Indeed, at concentrations of 10 and 20% the biomass production are lower.

Culture medium with oil sludge produced optimum biomass at d 4. This indicates that the addition of oil sludge can accelerate cultivation time. Fungal growth on the control medium was slower than that of containing oil sludge (Figure 3) and confirmed by cell morphological observation on d 3 (Figure 5). This indicates that *P. chrysogenum* is able to use carbon sources from oil sludge for its growth. Generally, fungi can assimilate PAH as source of carbon and energy, although it is not the only source thus they still require co-metabolites to detoxify PAH [10].

PAH degradation by filamentous fungi is mediated by extracellular ligninolytic enzymes or by intracellular cytochrome P450 monooxygenase. Additionally, metabolism of PAH by *Penicillium* involves the cytochrome P450 monooxygenase enzyme system. The initial results of oxidation of PAH is the formation of monophenol, diphenol, dihydrodiol and quinone. After, the water soluble conjugates such as sulfate and O-methyl conjugate, which considered as detoxification products can be formed, yet both pathways produce quinon PAH as the main oxidation product. This metabolite has a higher solubility and water reactivity than PAH [10].

Oil sludge does not only affect biomass production but can also affect production of spores. The growth profile of *P. chrysogenum* based on spore production at different concentration of oil sludge in the culture medium is presented in Figure 4.

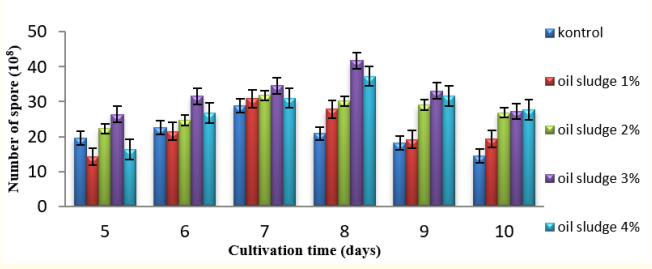

Figure 4: Growth profile of P. chrysogenum at different concentration of oil sludge based on spore production.

It was found that spore production of *P. chrysogenum* at control and at both oil sludge 1 and 2% undergo the adaptation phase from d 5 until d 6, then exponentially grew from d 6 until d 7. Eventually, number of spore decreased from d 8 until d 10. The culture medium with oil sludge addition at 3 and 4% experience longer adaptation phase, that occurred until d 7 and reached optimum production at d 8. The optimum spore production at 3% oil sludge medium was 41.8 x 10^8 cell/mL, indicating that *P. chrysogenum* can utilize the source of carbon in oil sludge efficiently. Observation of spore production of *P. notatum* on the oxalic acid added-medium was carried out by Frank., *et al*. [11]. The authors demonstrated that the optimum production was 2.94 x 10^8 cell/mL on the sixth day. Conversely, from the ninth day until tenth day the spore production decreased due to the breakdown of conidia without their regeneration.

Based on the profile of spore production, it appeared that the culture medium with concentration oil sludge 3% gave the highest number of spore. The optimum spore yield occurs because microfungi is cultured in solid medium to provide optimum results. Previous study on spore production confirmed that solid culture medium is the most ideal culture medium to obtain optimum spore produced by aerial hyphae [12].

Effect of oil sludge on cell morphology of *P. chrysogenum*

The biomass production of *P. chrysogenum* in the culture medium containing oil sludge was not significantly different to that of control. Morphological observation of *P. chrysogenum* (Figure 3) using SEM showed no morphological changes at different concentration of oil sludge in the medium either at the beginning or at the end of cultivation time.

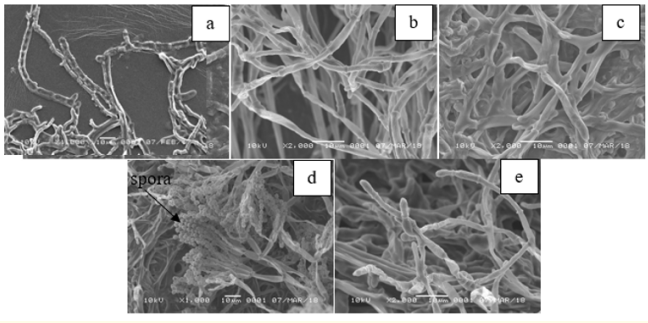

Figure 5: Morphology of P. chrysogenum on day 3: (a) control; (b) P1; (c) P2; (d) P3; (e) P4.

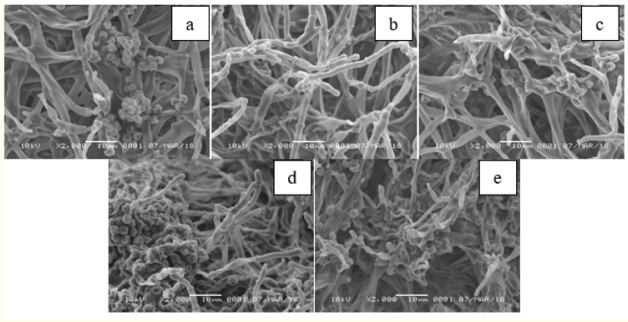

Figure 6: Morphologi of P. chrysogenum at day 7: (a) control; (b) P1; (c) P2; (d) P3; (e) P4.

Morphological observation revealed that *P. chrysogenum* has hyphae equipped with septate. At day 3, (Figure 6) it was found that hyphae have been formed and at concentration of oil sludge 3% spores already formed. Suggesting that the presence of oil sludge in the medium can accelerate the growth of *P. chrysogenum*. On the other hand, observation on day 7 showed that the hyphae have accumulated and spores have been formed in all treatmets. Overall, there was no distinct difference in morphological characteristic of fungi in the culture medium containing oil sludge as compared to control. This suggests that *P. chrysogenum* can adapt in the medium containing oil sludge up to 4%. Additionally, the oil sludge used is derived from 18 months of phytoremediation and has lower toxicity level (unpublished results) hence it has no effect on the morphology of fungal hyphae.

Effect of oil sludge on the spore morphology of *P. chrysogenum*

The concentration of oil sludge in the culture medium was significantly affect spores production, but not their morphology (Figure 7,8).

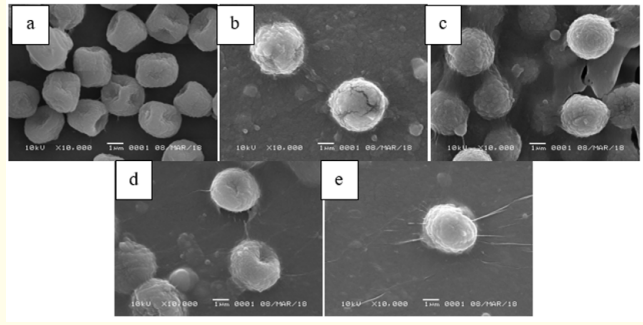

Figure 7: Spores morphology of P. chrysogenum at day 5: (a) control; (b) P1; (c) P2; (d) P3; (e) P4.

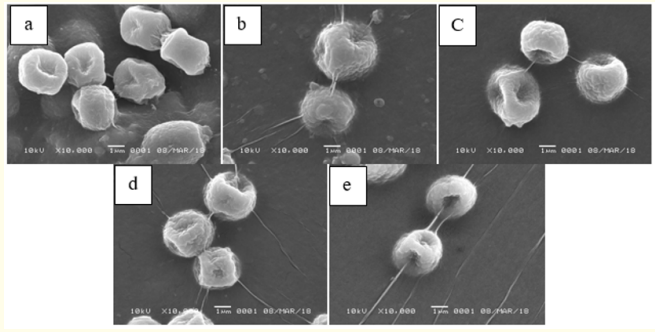

Figure 8: Spores morphology of P. chrysogenum at day 10: (a) control; (b) P1; (c) P2; (d) P3; (e) P4.

Morphologically, *P. chrysogenum* has a round shape spores with grooves inward. Spores in the control medium have a denser mass (Figure 8a) compared to that of oil-sludge treated medium. How-

ever, the size of spores in control medium was smaller than the oil sludge-contained medium. At day 10, the spores of *P. chrysogenum* in the oil sludge-contained medium appeared to have hyphae that were observed around the spores. This is related to the mass of spores that are sparse thus provokes faster growth of hyphae. Insignificant changes in morphological traits of spores in the culture medium with different concentration of oil sludge indicates that *P. chrysogenum* is able to adapat with the presence of oil sludge in the medium.

Effect of oil sludge on colony diameter of P. chrysogenum

We found that the addition of oil sludge into the culture medium affect pigmentation of conidia. The pigmentation of conidia occurred gradually from green to yellow at concentration of oil sludge 1, 2 and 3%. Conversely, no pigmentation observed at concentration 4%, but the fungal colonies were not filled with mycelium and at day 10, these colonies faded. As for control, pigmentation occurred on the tenth day, whereas at concentration 1%, 2%, dan 3% the pigmentation can be observed at day 5.

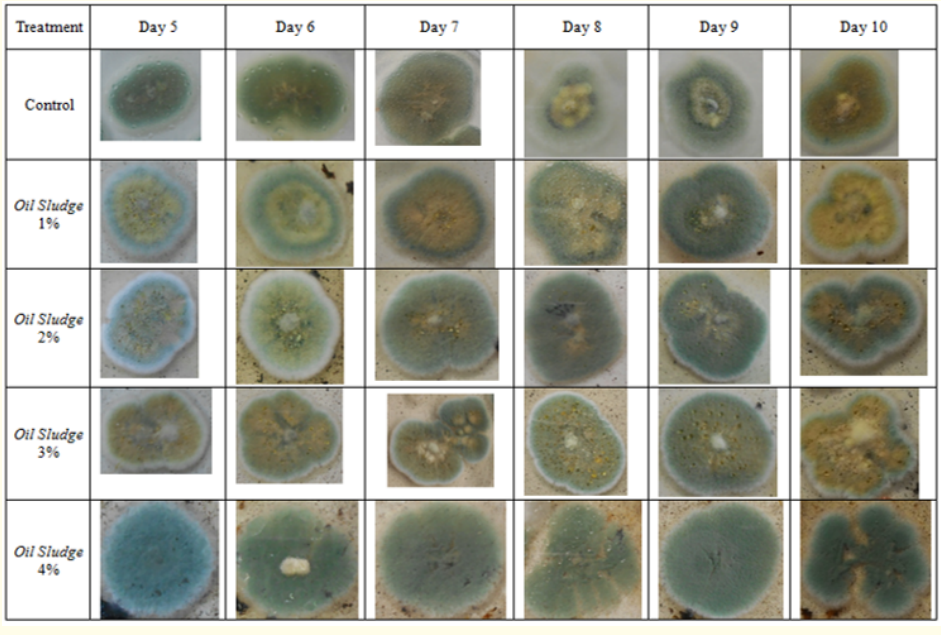

Figure 9: Matrix of colony diameter.

Observation on colony diameter of *P. chrysogenum* showed that no significant difference was observed between control and treated culture medium. Mean addition of colony diameter in control medium was 0.17 cm, while in the oil sludge-contained medium at 2% was 0.16 cm. On the other hand, the average of addition of colony diameter in 1, 3 and 4% of oil sludge-contained medium were equal with control (0.13 cm). Suggesting that the addition of oil sludge can affect the colony diameter, in accordance with the results of biomass production in oil sludge-contained culture medium which is lower than the control. The augmentation of colony diameter in control medium reached its highest point at day 10 (0.4 cm), whereas at 1% oil sludge-contained medium the maximum increase in colony diameter was occurred at day 7.

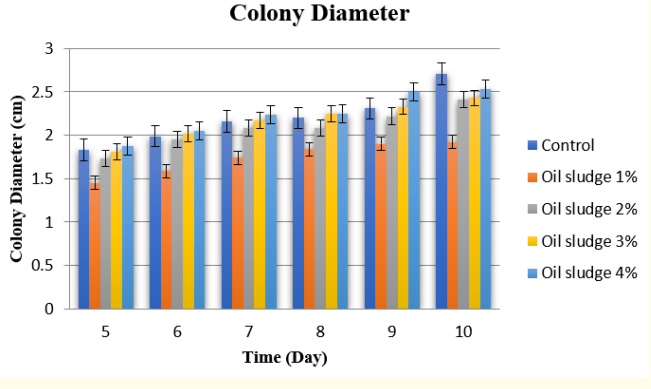

Figure 10: Mean of colony diameter.

Moreover, the colony with oil sludge-contained medium at 2 and 3% have the maximum increase in diameter at day 6 as much as 0.22 and 0.24 cm, respectively. In contrast, highest difference in colony diameter at 4% was obtained on day 9 (0.25 cm). These findings showed that the addition of oil sludge affects sporulation in *P. chrysogenum*, which is caused by the presence of PAH that can decelerate the addition of colony diameter. Indeed, according to Zafra., *et al.* [4] at concentration of PAH 1 and 2% can alter sporulation, whereas no sporulation are observed at 4 and 6% [13-16].

Conclusion

The present work explains that oil sludge concentration affect the spores production, where the production is significantly higher than control, but not in terms of biomass production. Concentration of oil sludge at 3% is considered as the optimum concentration to produce the spores (32.45 x 10^8 cell/mL), while the biomass production is higher in control medium without addition of oil sludge (0.099 g/L) as compared to concentration of oil sludge at 3% (0.078 g/L).

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