

# Inducing Fungal Pelletization Using Affordable Microparticle

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**Abstract.** Filamentous fungi have been known as one of the potential microorganisms in various industries. One of the isolates with great potential is *Mucor irregularis* that offer substantial potential to their growth characteristics. In submerged cultures, these microorganisms often aggregate into mycelia, enabling high-density cultivation and enhanced productivity. *M. irregularis* have a high lipid content of 43.46% and a yield of 3.28 g/L. To further lipid-rich biomass production, pelletization is explored to involve the addition of microparticles like magnesium silicate and calcium carbonate. Microparticles have demonstrated the ability to control growth and enhance biomass in various strains. This study investigates the impact of microparticle addition on *M. irregularis* biomass production and pellet formation. Preliminary tests reveal that the addition of magnesium silicate microparticles (0, 1, 2, and 3 g/L) induces pellet formation, with the 2 g/L treatment yielding optimal results. Microscopic observations confirm that higher magnesium silicate concentrations result in more compact pellets. Biomass production peaks at 72 hours of incubation, reaching  $3.09 \pm 0.43$  g/L, while the largest pellet diameter of 1.27 mm occurs at 48 hours of incubation. This research offers insights into enhancing biomass production and pellet formation in *M. irregularis*, holding promise for diverse applications.

## 1 Introduction

Filamentous fungi have long been known as microorganisms for industrial purposes, for example in the production process of enzymes, antibiotics, and even biofuels [1]. In various submerged culture systems, fungi can assume diverse morphological structures, including suspended mycelia, agglomerates, or pellets. Numerous research investigations have thoroughly examined the merits and drawbacks associated with distinct growth morphologies in relation to various bioproducts [2]. The growth of fungi in pellet form is a very profitable alternative for the cultivation process of these microorganisms [3]. Pellet formation not only provides opportunities for repeatable production, but can also improve the rheology of the culture to provide more mass and oxygen transfer into the biomass. This provides lower energy consumption for aeration and agitation. [4].

Furthermore, the filamentous structure of fungi capable of forming pellets offers distinct advantages when it comes to simplifying the biomass harvesting process. In contrast to

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microalgae and yeast cells, which require a relatively complex and costly filtration procedure [5], filamentous growth typically concentrates itself in the form of pellets or granules during submerged cultivation. The adoption of fungal compartments in pellet form significantly enhances biomass production at high densities, leading to substantially increased productivity [6]. Moreover, these fungal pellets can be effortlessly harvested from the culture medium using a straightforward filtration method. This particular feature holds great promise, especially when considering its potential application in biomass accumulation for the production of valuable products, given the prohibitively expensive separation costs associated with current biotechnological processes [7].

Several recent studies show that the addition of microparticles in the fermentation process show positive effects for mold morphological growth. Research conducted by Gao et al. [7] on accumulation *Mortierella isabellina* lipid isolate added with magnesium microparticles silica or  $\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$  which is commonly marketed as talc or talcum with varying concentrations of 0 to 10 g/L. The research results show *M. isabellina* lipid production increases with increasing microparticle concentration and followed by a decrease in pellet size. Xia et al. [5] added that fungal pellets formed within around 12 hours of culture after  $\text{CaCO}_3$  (0.4 g/100 mL) was added into the cell cultivation.

These microparticles play a key role in pellet formation of several potential molds, such as *Mucor irregularis* which shows that it has quite high lipid content and has the potential to be developed into an attractive lipid producer. Haura & Ilmi [8] found that the lipid content of the *M. irregularis* isolate (JR 1.1) was successfully isolated from fruit was 43.46% with a yield of 3.28 g/L. Results obtained from a small-scale cultivation process in 50 ml of media for 6 days at a temperature of  $28 \pm 2$  °C and pH 5.5 in an incubator shaker with agitation of 200 rpm. This research describes a handy method by which to induce cell pelletization in submerged fungal cultivation. The type and concentration of microparticles can indicate that the effect is different for each mold strain. Therefore, further investigation is required to determine the appropriate microparticle concentration for growth and produce the resulting product. This research aimed to investigate the effect of adding microparticles on productivity biomass and pelletization of *M. irregularis*.

## 2 Materials and Methods

### 2.1 Strain and inoculum preparation

*M. irregularis* JR 1.1 provided by Microbiology Laboratory, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia was the microorganism used in this research. The research began by preparing a spore suspension to be used for inoculating flask cultures. The spores were obtained by preparing agar plates with a sporulation medium (24 g/L potato dextrose broth with 20 g/L agar) coated with spores and incubating for 6 days at 27°C [8]. Then the spores that grow on the surface of the media are harvested by adding 10 mL of sterilized Triton X-100 to the agar plate to release the air mycelium. Spores were counted using optical microscope.

### 2.2 Culture Medium

The flask cultures medium contained glucose (30 g/L) as carbon source, both yeast extract (5 g/L) (Merck, Cat No. 1.08337.1000) and  $\text{KNO}_3$  (1 g/L) as nitrogen source,  $\text{KH}_2\text{PO}_4$  (2.5 g/L),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.5 g/L),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.01 g/L),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0.002 g/L),  $\text{MnSO}_4$  (0.01 g/L),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.02 g/L), and  $\text{CaCl}_2$  (0.1 g/L) [9] also microparticles magnesium silicate ( $\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$ ) (1, 2, 3 g/L) [7] and calcium carbonate ( $\text{CaCO}_3$ ) (1, 2, 3 g/L) [5].

The composition of the culture medium can vary according to specific conditions for each culture, including key growth factors like the initial pH level and the temperature of the culture.

### **2.3 Cultivation Methods**

The factors on pellet formation were carried out by a completely randomized design with two replicates. Two different microparticles (magnesium silicate and calcium carbonate) were studied. The cultures of *M. irregularis* were accomplished in 250 mL Erlenmeyer flasks containing 50 mL of medium on a water bath shaker (Memmert WNB7L4 shaking Water bath) at 120 rpm for 48 hours [5,7]. Two fermentation runs per culture experiment were performed. The initial pH of the culture medium was 5.5 after sterilization with 28°C temperature, and the cultivation conditions were the same for all experiments [9]. Subsequently, optimal conditions were determined through the identification of the most suitable combination of microparticles with pellet diameter reaching 1500 µm (1.5 mm) [10]. Once these optimal conditions were established, cultivation was performed under identical conditions with time intervals of 0, 24, 48, and 72 hours to maximize pellet diameter and fungal biomass.

### **2.4 Determination of pellet and biomass**

Pellet morphology was observed by A Fujifilm XE-4 photograph (Fujifilm, Japan). To enable a clear visual examination, all the pellets from each flask were transferred to multiple petri dishes. The diameter of the pellets was meticulously gauged employing a digital vernier calliper (Deli Inc.) [11]. After obtaining pellet documentation, the sample was transferred into a previously weighed Falcon tube and washed twice with distilled water. The tubes were centrifuged after each washing step for 20 min at 5000 rpm and the supernatant was withdrawn. Biomass that has been washed in a Falcon tube is dried in an oven (Memmert) at 50°C and then the dry weight of the biomass is measured.

### **2.5 Analytical Methods**

The analysis of *M. irregularis* research data was carried out using Microsoft Office Excel to determine quantitative parameters such as total biomass and pellet diameter. Furthermore, statistical analysis was carried out using the SPSS V.23 and the one-way analysis of variance (ANOVA). This rigorous statistical methodology facilitated the evaluation of key study outcomes.

## **3 Results and Discussions**

### **3.1 Fungal pellets by addition of microparticles**

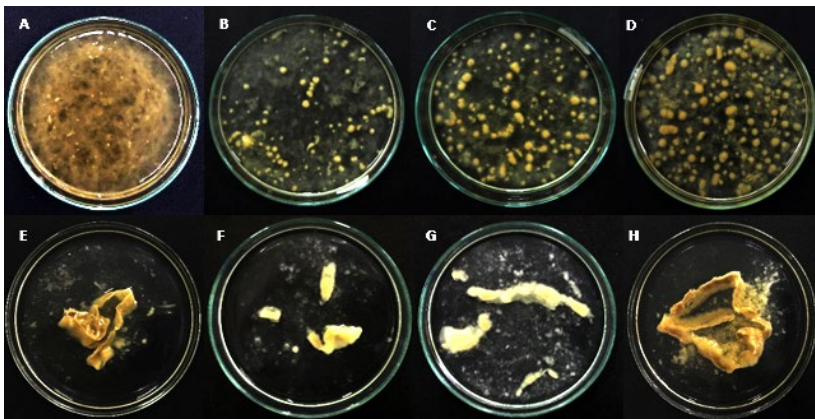
The addition of magnesium silicate and calcium carbonate yielded different results in pellet formation. Initially, large pellets with approximately 2 mm diameter formed in higher concentrations of microparticles [12]. However, as microparticles were gradually added, the pellets became smaller but more numerous, resulting in increased culture viscosity. According to Fig. 1, magnesium silicate induced mycelium growth into pellets, while calcium carbonate did not support pellet formation. Instead, biomass grew into clump form and dispersed more widely. Morphological analysis (Table 1) revealed that higher microparticle concentrations substantially increased the average size of pellets/mycelial aggregates. Sizes

ranged from 1.61 mm (1 g/L) to 1.66 mm (2 g/L) and 2.09 mm (3 g/L), respectively. At concentrations of 1 g/L microparticles and higher, mycelia formation was observed. The control group without microparticle addition exhibited only mycelium aggregates, whereas the addition of microparticles resulted in more uniform morphologies, as depicted in Fig. 2. Moreover, an escalation in microparticle concentration led to a reduction in circularity, indicating a tendency for the strain to form looser mycelial aggregates [7].

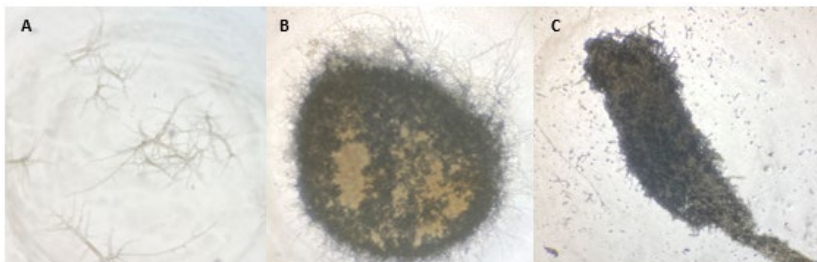
**Table 1.** Morphology analysis of pellets under different microparticle concentrations

Microparticle	Concentrations (g/L)	Pellet Size (mm)	Fungal Morphology
Magnesium silicate	0	-	Clump
	1	1.61	Non-uniform pellet
	2	1.66	Uniform pellet
	3	2.09	Non-uniform pellet
Calcium carbonate	0	-	Clump
	1	-	Clump
	2	-	Clump
	3	-	Clump

\*“-” means non-pellet.



**Fig. 1.** Morphological changes of *M. irregularis* with different concentrations of microparticles, From A to D, the concentrations of magnesium silicate are 0, 1, 2 and 3 g/L; From E to H, the concentrations of calcium carbonate are 0, 1, 2 and 3 g/L.



**Fig. 2.** Microscopical differences of *M. irregularis* with microparticles; A. Without microparticles, B. Addition of magnesium silicate, C. Addition of calcium carbonate.

The results indicate that incorporating microparticles can be a highly effective method for precise control over the morphology of the oleaginous fungus *M. irregularis*. Previous research has highlighted that the critical step in pellet formation involves the aggregation of

spores. Driouch et al. [12] demonstrated that microparticles, by physically interacting with spores during the initial and germination stages, either prevented spore aggregation or disrupted existing spore aggregates in the cultivation of *Aspergillus niger*. Considering that *M. irregularis* is a coagulating species, it possesses spores of a size range (5–10mm) similar to magnesium silicate microparticles (with an average size of 10 mm) [8]. Those views explain higher concentration of microparticles expanding the likelihood of physical collisions between microparticles and spore aggregates, ultimately reducing the size of pellet aggregates. In contrast to other methods for controlling morphology, such as pH adjustment, inoculum variation, and agitation control, which often affect morphology and productivity, the addition of microparticles has minimal direct impact on productivity [13]. Hence, it proves to be an effective method for establishing a distinct and independent connection between morphology and productivity. The utilization of 2 g/L of magnesium silicate in the cultivation process was observed to produce consistently uniform pellets. Consequently, this particular combination was chosen to track alterations in pellet formation over time [12].

**Table 2.** Effect of adding microparticles and incubation time on pellet diameter of *M. irregularis*

Incubation time (hour)	1	2	Average
0	0.00±0.00	0.00±0.00	0.00 ±0.00 <sup>a</sup>
24	0.95±0.12	1.12±0.12	1.03±0.12 <sup>b</sup>
48	1.27±0.07	1.28±0.07	1.27±0.07 <sup>c</sup>
72	1.23±0.02	1.27±0.02	1.25±0.02 <sup>c</sup>

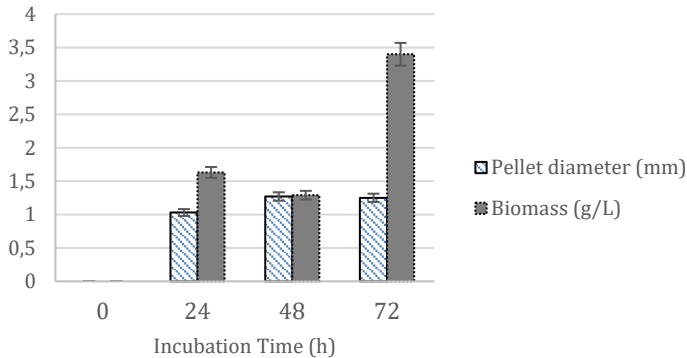
Description : Data presented in average form. Different superscript symbols indicate significantly different results for each treatment based on the one-way Anova test and Duncan test with significance  $p < 0.05$

The results of the ANOVA show that these fermentations have a significant effect on pellet diameter, with a p value of 0.05. According to Table 1, incubation time 0 produced statistically different results than the 24 hour, 48 hours, and 72 hours treatments. The 24 hours treatment produced considerably different results from the 0 hours, 48 hours, and 72 hours treatments. There was no significant difference in results between the 48 and 72 hours treatments. The average pellet diameter indicates that the precise pellet size created at 48 hours was 1.27 mm, followed by 1.25 mm at 72 hours and 1.03 mm after 24 hours of incubation. The average pellet diameter with a magnesium silicate addition of 2 g/L exhibits relatively constant results of 1 mm [12]. Microparticles can influence cell shape by avoiding mycelium aggregation into large pellets [14]. The microparticles will interact with the isolated hyphae and spores in the medium to create bonds based on electrostatic forces, hydrophobic forces, and Van der Waals forces, leading spore aggregation to be disrupted [15].

### 3.2 Microparticles addition effects on pellet size and biomass of *M. irregularis*

Incubation time has an effect on overall biomass production. *M. irregularis* biomass was highest at the longest incubation time, 72 hours, as compared to other incubation times (Fig. 3). Fluctuations in biomass output can be attributed to the substrate decomposition process's cell growth cycle, which causes the number of cells to rise as the incubation period increases [16]. The biomass production outcomes and the size of the pellets indicated a unique connection. Increases in biomass amount were not accompanied by an upsurge in pellet diameter. The size of the pellets formed did not demonstrate a significant variation in

incubation time and the amount of additional biomass obtained while reaching the maximal biomass production [17].



**Fig. 3.** Effect of microparticles on pellet size and biomass of *M. irregularis*

These findings contrast with previous research [7], where the introduction of microparticles impacted production performance by altering morphological characteristics. As the concentration of microparticles increased from 0 to 3 g/L, there was a corresponding decrease in the size of mycelium pellets or aggregates, coupled with an increase in cell biomass output [18]. The increased concentration of microparticles led to the rupture of hyphal fragments, which, in turn, was linked to a reduction in cell biomass production [19]. According to Driouch et al., the shift from large pellets to smaller pellets or free mycelium results in an augmented surface area and a decrease in pellet size. This, in turn, facilitates the transfer of oxygen and nutrients from the medium to the mycelia [20].

## 4 Conclusion

The addition of magnesium silicate into *M. irregularis* has demonstrated a reduction in pellet diameter. Notably, the precise mean pellet diameter recorded was  $1.27 \pm 0.07$  mm after 48 hours of incubation. In contrast, when magnesium silicate was added, biomass production exhibited an upward trend with increasing incubation time, reaching its peak at  $3.09 \pm 0.43$  g/L after 72 hours of incubation. These findings hold significant implications for the advancement of products reliant on mold as the primary producer. Consequently, further investigations are imperative to optimize biomass yield and pellet formation through varying magnesium silicate concentrations. Furthermore, it is crucial to conduct subsequent tests to assess lipid production and the lipid profile of *M. irregularis* in the presence of microparticles. This research paves the way for critical developments in the field to the future engineered cultivation of mold.

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