Medium Optimization for Production of *Monascus purpureus* Pigment through Solid-state Fermentation

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Abstract. Colorants are component that is commonly added to many kinds of product, including food products. Regardless of its effectiveness and lower price, the effects of synthetic colorants towards human’s health become a challenge for food colorant industries. Production of natural food colorant using microorganism as the producing agent is a promising prospect because microorganism has a high growth rate. *Monascus purpureus* can produce a set of natural pigments consist of yellow (monascin and ankaflavin), orange (rubropunctatin and monascorubrin) and red (rubropunctamine and monascorubramine) pigment.

The objective of this research is to optimize the micronutrient composition in the medium for the production of *Monascus* pigment through solid-state fermentation using the job’s tears (*Coix lacryma-jobi* L) as the substrate. Response surface method (RSM) was used to optimize the concentration of four substrate components: MSG, NaCl, KH$_2$PO$_4$, and MgSO$_4$. From the experiment, it was found that the maximum red pigment was produced in medium with additional of (w/w): MSG 1.496%, NaCl 1.0%, KH$_2$PO$_4$ 3.515% and MgSO$_4$ 0.206%. The yellow pigment was maximally produced in medium with additional of (w/w): MSG 1.5%, NaCl 1.0%, KH$_2$PO$_4$ 2% and MgSO$_4$ 0.2%. Biomass was maximally produced in the medium with additional of (w/w): MSG 1.41%, NaCl 1.01%, KH$_2$PO$_4$ 5.0% and MgSO$_4$ 0.2%. Among those nutrients, MSG gave the biggest impact on the increasing of pigment and biomass production.

1. Introduction
Food colorant is an important aspect in food as it is the one that first perceived by the consumer. Nowadays, the common coloring agent used is synthetic colorant. However, there are increasing concern in effect of synthetic coloring agent to human health as in potential of cancer and teratogenicity [1-2]. It is causing a shift in tendency to use natural food color over the synthetic one.

Fungi of *Monascus* genus has been well known to produce natural pigment. The pigment mainly consists of 3 colors: red (monascoruramine, rubropuntamine), yellow (monascin, ankaflavin), and orange (monascorubrin, rubropunctatin) [3]. *Monascus* pigment has been long used since ancient China as angkak to cure dengue fever. It also has good effect on promoting human health as it could lower cholesterol level, help to prevent osteoporosis and diabetes [4-6]. These pigments are secondary metabolite products, which are produced during stationary phase [7].

*Monascus* commonly utilized using rice as the substrate. There are already many researches to utilize *Monascus* using different substrates, such as jackfruit seed, soy bean waste, durian seed, and corn cob [8-11]. So far, there are no research to utilize Job’s tears as substrate for pigment production yet.

Job’s tears, which is also known as adlay, coix-seed, Chinese pearl barley, *hanjeli* (Indonesia) or *hato mugi* (Japan), is originated from China and now can be found worldwide. It is commonly mixed with rice and consumed to warm the body and stamina increasing. Job’s tears consist of 16.2% protein,
4.65% fat and 79.17% carbohydrate. It also has some amino acid such as leucine, lysine, arginine and tyrosine [12]. It is also relatively easy to plant like paddy rice that don’t need any special treatment.

In fermentation, micronutrients play an important role in microorganism growth and metabolite product formation. Micronutrients are nutrients that are added to fermentation medium in small amount (trace). Many of micronutrients are cofactor that play role in activating its respective enzyme, such as Mg$^{2+}$. The other examples of micronutrient are sulfur that is used as backbone for sulfur-containing amino acid and Na$^+$ that plays important role in regulating nutrient transport rate and osmotic pressure of the system [13-14]. Those micronutrients can support the microbial growth and production of metabolite product, but macronutrients such as carbon, nitrogen and oxygen are still essential for fermentation. Those macronutrients are the main backbone of cell composition. In case for pigment production, specific nitrogen sources also play role in promoting the growth and pigment production of the cell [15].

With the increasing trend of healthy lifestyle and concern about artificial substance, development of natural pigment sees an interesting prospect. Natural pigments can be obtained from plants and animals, but since both have long generation time, the quantity may be insufficient for industrial utilization, thus microbial-based pigment is more reliable and potential [16]. This study has main objective to utilize Job’s tears as substrate to produce Monascus pigment via solid state fermentation and also optimize the composition of micronutrient usually added to grow Monascus fungi by using Response Surface Methodology (RSM) approach. RSM method is a model commonly used for optimization in biological process. RSM will yield mathematical model that can show the effect of each independent factor and its interaction with each other independent factors [17].

2. Material and Method

2.1. Culture
Culture of Monascus purpureus ITB CC L61 was obtained from Laboratory of Microbiology and Bioprocess Technology Culture Collection, Institut Teknologi Bandung. The culture was maintained in Potato Dextrose Agar (PDA) medium, preserved at 4°C and was re-cultured every month.

2.2. Inoculum preparation
Monascus purpureus was grown on PDA medium at 30°C until fully sporulated (10 days). The spore then scrapped by adding 5 ml of sterile water under aseptic condition. This suspension then used as inoculum.

2.3. Solid-state fermentation
Job’s tears obtained from Punclut, West Java were used as substrate. Each variation used 35 grams of Job’s tears. Job’s tears were washed with clear water then filled into 250 mL Erlenmeyer flask. Micronutrients diluted in 105 mL of distilled water were then added into the medium with varied concentration. The medium was then cooked until the water content reached 65%. The flask was then covered with sterile fatty cotton and cooled until room temperature, and then inoculated with prepared inoculum. Fermentation took place at 30°C for 14 days.

2.4. Experimental design
Variation done in this research was the concentration of micronutrient and additional monosodium glutamate (MSG) as nitrogen source to find the optimal concentration. Optimization was done via RSM approach with full central composite design experiment, with detail shown in Table 1. In total, there were 31 runs done in this research.
Table 1. Experimental design for medium optimization.

| No | MSG (A) (%w/w) | NaCl (B) (%w/w) | KH$_2$PO$_4$ (C) (%w/w) | MgSO$_4$ (D) (%w/w) |
|----|----------------|-----------------|--------------------------|---------------------|
| 1  | 0.5            | 1               | 5                        | 0.2                 |
| 2  | 0.5            | 1               | 5                        | 1                   |
| 3  | 0.5            | 1               | 2                        | 1                   |
| 4  | 0.5            | 1               | 2                        | 0.2                 |
| 5  | 0.5            | 3               | 5                        | 0.2                 |
| 6  | 0.5            | 3               | 2                        | 1                   |
| 7  | 0.5            | 3               | 5                        | 1                   |
| 8  | 0.5            | 3               | 2                        | 0.6                 |
| 9  | 0.5            | 2               | 3.5                      | 0.6                 |
| 10 | 1              | 1               | 3.5                      | 0.6                 |
| 11 | 1              | 2               | 2                        | 0.6                 |
| 12 | 1              | 2               | 3.5                      | 0.6                 |
| 13 | 1              | 3               | 3.5                      | 0.6                 |
| 14 | 1              | 2               | 3.5                      | 0.6                 |
| 15 | 1              | 2               | 3.5                      | 0.6                 |
| 16 | 1              | 2               | 5                        | 0.6                 |
| 17 | 1              | 2               | 3.5                      | 0.6                 |
| 18 | 1              | 2               | 3.5                      | 0.6                 |
| 19 | 1              | 2               | 3.5                      | 0.6                 |
| 20 | 1              | 2               | 3.5                      | 0.6                 |
| 21 | 1              | 2               | 3.5                      | 0.6                 |
| 22 | 1              | 2               | 3.5                      | 0.2                 |
| 23 | 1.5            | 1               | 5                        | 0.2                 |
| 24 | 1.5            | 2               | 3.5                      | 0.6                 |
| 25 | 1.5            | 3               | 2                        | 0.2                 |
| 26 | 1.5            | 3               | 5                        | 1                   |
| 27 | 1.5            | 1               | 2                        | 1                   |
| 28 | 1.5            | 3               | 5                        | 0.2                 |
| 29 | 1.5            | 1               | 5                        | 1                   |
| 30 | 1.5            | 3               | 2                        | 1                   |
| 31 | 1.5            | 1               | 2                        | 0.2                 |

2.5. Pigment analysis

Pigment analysis was done by extracting pigment from 8 grams of fermented substrate. Pigment extraction was done by adding that fermented sample into a tube containing 40 mL ethanol 60%. The tube was then centrifuged with rotation speed of 200 rpm in room temperature. Extraction result was filtered through Whatman No. 1 filter paper and then analyzed by using spectrophotometry [8]. Blank used was the filtration result from unfermented substrate that undergone the same preparation. Measurement was done at wavelength of 510 nm to analyze the red pigment [15].

2.6. Biomass analysis

Biomass analysis was done by measuring the N-acetyl-D-glucosamine concentration formed due to acid hydrolysis of chitin contained in the fungi cell wall [18]. A tube containing 0.5 gram of acid hydrolysis product was added with 1 mL of acetyl acetone and then heated at 100°C for 20 minutes. Sample was then cooled to 65°C, and then 6 mL of concentrated ethanol and 1 mL of Ehrlich Reagent was added and then incubated for 10 minutes. Sample was then analyzed by using spectrophotometer at 530 nm. Unfermented substrate was undergone the same preparation and used as blank for analysis. The optical
density (OD) obtained from spectrophotometer analysis was then converted into mass by using standard curve prepared by doing the same treatment to some certain concentrations of N-acetyl-D-glucosamine.

3. Result and discussion

3.1. Pigment production

The result of analysis is shown in Table 2.

Table 2. Analysis result for each run.

| Variation | Pigment (OD) | Biomass (g/g) | Variation | Pigment (OD) | Biomass (g/g) |
|-----------|--------------|---------------|-----------|--------------|---------------|
| 1         | 5.6          | 0.532         | 17        | 5.0          | 0.250         |
| 2         | 8.6          | 0.622         | 18        | 5.7          | 0.272         |
| 3         | 12.2         | 0.394         | 19        | 6.7          | 0.262         |
| 4         | 8.0          | 0.304         | 20        | 7.7          | 0.224         |
| 5         | 3.6          | 0.528         | 21        | 6.6          | 0.275         |
| 6         | 1.6          | 0.158         | 22        | 8.7          | 0.258         |
| 7         | 9.5          | 0.192         | 23        | 3.7          | 0.374         |
| 8         | 7.9          | 0.116         | 24        | 4.4          | 0.452         |
| 9         | 2.6          | 0.278         | 25        | 8.0          | 0.452         |
| 10        | 6.9          | 0.258         | 26        | 7.9          | 0.452         |
| 11        | 4.6          | 0.128         | 27        | 7.8          | 0.482         |
| 12        | 4.3          | 0.082         | 28        | 8.1          | 0.344         |
| 13        | 8.0          | 0.19          | 29        | 8.2          | 0.452         |
| 14        | 8.8          | 0.258         | 30        | 11.0         | 0.43          |
| 15        | 1.6          | 0.258         | 31        | 13.2         | 0.472         |
| 16        | 8.0          | 0.498         |           |              |               |

The data obtained in Table 2 was then analyzed using Minitab® in order to determine the optimal concentration for pigment production. Based on the optimization result, it was found that red pigment was maximally produced by adding 1.5 % MSG (A), 1.788 % NaCl (B), 2.909 % KH₂PO₄ (C), and 0.2 % MgSO₄ (D). Pigment production can be estimated by using equation (1), with effect of all additional component can be seen in Figure 1

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Pigment = 7.70 + 2.29A - 1.05C - 0.56D + 2.1A^2 - 3.25B^2 - 2.35C^2 + 2.15D^2 - 0.68AD + 0.61BC + 1.04BD + 0.85CD\]  \hspace{1cm} (1)

The equation was the simplified version that has already discarded insignificant coefficients that have p-value lower than 0.005. Based on the pareto diagram and the equation, MSG positively affecting pigment production. Monascus pigment contains amine substances which may be enhanced by the additional MSG in the medium. Close value between singular and quadratic constant of MSG from the equation (1) also means that the optimal value is within the variation used, in this case, the optimal value for MSG is 1.5 %. However, excessive MSG addition is not recommended since it may lead to inhibition of red pigment production [19].

The other significant factors in pigment production are KH₂PO₄ and MgSO₄ concentration, and both are affecting the pigment production negatively. It may be due to the nature of pigment as secondary metabolite product, which is produced during stationary state. Both components are essential in cell growth as cofactor. However, often that cell growth is not linear to secondary metabolite production, as
secondary metabolite product is usually produced as “emergency measure” when the growth is inhibited. If the growth is promoted, cell will find secondary metabolite production as unnecessary, thus reducing the amount. As shown in Figure 2, both KH$_2$PO$_4$ and MgSO$_4$ have optimal value between the minimum and maximum value, highlighting its importance for cell in general but may inhibit the product yield if given in abundant.

![Main Effects Plot for Pigment (510 nm)](image)

**Figure 1.** Pareto diagram for pigment production.

**Figure 2.** Main effects plot for pigment production.

3.2. **Biomass production**

Similar to pigment analysis, data obtained in Table 2 was then analyzed using Minitab$^\text{®}$ in order to determine the optimal concentration for biomass growth. It was found that biomass was maximally produced by adding (w/w) 1.41 % MSG (A), 1.01 % NaCl (B), 5.0 % KH$_2$PO$_4$ (C), and 0.2 % MgSO$_4$ (D), and can be quantified by following equation (2), with the effect of all additional component is shown in Figure 3.
Based on the simplified equation, besides MgSO$_4$, additional nutrients have significant effect on biomass production, with only NaCl affect negatively towards cell growth. Positive effect from MSG and KH$_2$PO$_4$ can be understood as the additional of cofactor and nitrogen source will enhance the growth of microbial. Nitrogen is one of the cell’s backbones, while increasing the activity of the enzyme included in main growth metabolism pathway will accordingly enhance the growth of the cell. As shown in Figure 4, excessive addition of each component will negatively affect the cell growth.

The effect of salt is pretty interesting, since by itself, it will hinder the cell growth, but interaction between salt and MSG provide positive effect. It is well known that salt plays important rule in nutrient transport. However, excessive amount of salt will lead to hyperosmotic condition, in which, non-tolerant cell will be forced to adapt by changing the viscosity of cell membrane of the phospholipid composition.
of the cell wall [20]. However, the interaction between NaCl and MSG gives positive effect as additional nitrogen source will be transported into the cell more easily, thus somewhat offsetting the negative effect of salt as singular entity. However, further study may be required in order to confirm this hypothesis.

4. Conclusion

Adding MSG as additional nitrogen source can enhance the production of pigment and biomass for Monascus purpureus, however, excessive addition will instead inhibit the cell performance. Moreover, among all micronutrient added, only MgSO$_4$ shows no significant effect in both cell and pigment production. Based on the optimization result, maximum biomass that can be produced is 0.568 gr/gr by adding (w/w) MSG 1.41 %, NaCl 1.0 %, KH$_2$PO$_4$ 2.0 % and MgSO$_4$ 0.2 %. Maximum pigment that can be produced based on optimization result is 15.94 OD by adding (w/w) MSG 1.5 %, 1.788 %, 2.0 %, and 0.2 %. This study might be helpful for future development of Monascus fermentation, especially in the selection of additional micronutrient and helpful to avoid additional of components that may hinder the performance of each other.

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