The Culture Medium Optimization Of *Serratia plymuthica* UBCF_13 To Produce Antifungal Compounds

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**Abstract.** Bacteria can produce secondary metabolites compounds that act as antifungal agents. But in natural conditions, the production of secondary metabolites is low and requires certain growth conditions. One of the methods to increase secondary metabolites production is the modification of the culture medium. In this study, four environmental factors combination was used to optimize the optimal culture medium for *S. plymuthica* UBCF_13 to produce antifungal compounds. The environmental factors were pH 8, 2 % peptone, 1 mM MnSO₄, and 8 hours culture duration. In inhibitory activity test to *C. gloeosporioides* fungi, cell-free supernatant and cell culture of *S. plymuthica* UBCF_13 were applied. The results showed that the highest inhibitory activity of cell-free supernatant application (17.80%) was obtained from a medium that adjusted with pH 8, 1 mM MnSO₄, and 8 hours duration. While the highest inhibitory activity of cell culture application (44.53%) was obtained from the medium with a combination of pH 8, 2% peptone, 1 mM MnSO₄, and 8 hours culture duration. In the SDS-PAGE visualization, 4 protein bands appeared which were thought to be closely related to the inhibitory activity of antifungal compounds of *S. plymuthica* UBCF_13. The optimum medium was found to be a medium with a combination of pH 8, 2% peptone, 1mM MnSO₄, and 8 hours of culture duration.

**Keywords:** cell culture, cell-free supernatant, secondary metabolites, SDS-PAGE, *Serratia plymuthica* UBCF_13

1. Introduction

Secondary metabolite compounds that control pathogens produced by microorganisms are an alternative that can be used as biocontrol agents. However, the production of metabolite compounds from microorganisms in some cases still needs to be optimized so that they can be utilized. Some of the main factors that influence the production of metabolite compounds are environmental factors, including nutrient source, pH, metal ions, and culture duration. Therefore, the influence of environmental factors must be considered to obtain the expected secondary metabolite compounds [1]. Aisyah et al. [2] report that *S. plymuthica* UBCF_13 is a candidate to control phytopathogenic fungi.

The antifungal activity of *S. plymuthica* UBCF_13 in each environmental factor as a single variable resulted in various antifungal activities. The single variable test that has been applied is the addition of metal ions, duration of bacterial culture, variations in pH levels, as well as carbon and nitrogen sources in the culture medium against the fungi *C. gloeosporioides* [3]. The addition of Mn²⁺ metal ion produced the highest inhibitory activity against *C. gloeosporioides* with a percentage of 17.78% [4]. The use of
bacterial cell culture with a culture duration of 8 hours resulted in the highest inhibition of 42.18%. Meanwhile, the application of free cell supernatant for 32 hours had an inhibition percentage of 19.15% [5]. The best antagonistic activity was obtained in bacterial culture media at pH 8 of 27.63% [6]. Sulastri 2019 personal communication, the addition of peptone as a nitrogen source resulted in the highest inhibition of 16.94%.

This confirms that each component of environmental factors has a role in the regulation of antifungal activity. This principle then becomes the basis for the importance of developing a special media that combines various environmental factors at once. This study aims to obtain the optimal composition of the S. plymuthica UBCF_13 bacterial culture media from the combination of various environmental factors on its antifungal activity in suppressing the growth of C. gloeosporioides.

2. Methods

2.1. Culture preparation of C. gloeosporioides fungi and S. plymuthica UBCF_13 bacteria

Fungi and bacteria used in this study were obtained from the collection of Agricultural Biotechnology Laboratory, Andalas University. Fungi were cultured on PDA medium (pH 7) for 7 days, while the bacteria were cultured on King’s agar medium for 18 hours.

2.2. Production of antifungal compound

Bacteria were cultured in King’s liquid media which was cultured with a combination of various environmental factors based on the results of previous studies. The four factors are combined (Table 1).

In this study, 2 types of applications were conducted, namely cell culture and free cell supernatant. The use of cultured bacteria immediately after incubation is called cell culture, while the bacterial culture media after incubation is transferred to a 500 ml falcon tube then centrifuged and filtered using a syringe filter is called a cell-free supernatant.

| Code | Treatment                                      |
|------|-----------------------------------------------|
| A    | pH 8 + (24 hours)                             |
| B    | (pH 7) + peptonee + (24 hours)                |
| C    | (pH 7) + 8 hours                             |
| D    | (pH 7 + 24 hours) + Mn                        |

2.3. Antifungal activity test

Testing is done using the method of Baurer et al. [7]. Fungi that have been cultured for 48 hours are then applied 5 µl for the type of cell culture application and 50 µl for the type of cell-free supernatant.
application. Subsequently incubated for 8 days and the resulting inhibitory power for 8 days was calculated using the formula:

\[
\text{Growth inhibition} = \frac{\text{DK} - \text{DP}}{\text{DK}} \times 100\%
\]

where DK is the diameter of control fungi (without cell culture application and cell-free supernatant) and DP for the diameter of fungi that are given cell culture application and cell-free supernatant.

2.4. Analysis of bacterial proteome profiles

The proteome profile analysis consisted of two stages, namely total proteome isolation and SDS-PAGE. Total extracellular proteome isolation was carried out using the protocol from Zhang et al. [8]. Furthermore, the extracted protein concentration was measured using the Bradford method [9] using BSA (Bovine Serum Albumin). Visualization of protein bands was obtained by separating protein from 12% SDS-PAGE gel.

2.5. Data analysis

The analysis used one-way analysis of variance using SPSS version 16.0 software [10]. Treatments that show a significant value will be further tested using the DMRT advanced test at the 5% level.

3. Results and Discussion

3.1. Inhibition of *S. plymuthica* UBCF_13 post optimization of media with various variable environmental factors

This study used two types of applications, namely cell-free supernatant and bacterial cell culture of *S. plymuthica* UBCF_13. The two types of applications are expected to determine the optimal conditions for producing the expected secondary metabolites.

Based on the results obtained, the suppression of inhibition for 8 days after application fluctuates. The best cell culture application on the 8th day post-application in this study was obtained from a combination of media with pH 8, 2% peptone, 1 mM MnSO₄, and 8 hours duration with a percentage of 44.53% inhibition, followed by media with a combination of pH 8 and the 8 hours incubation duration was 40.49%. Meanwhile, the lowest inhibitory power was obtained from media with a combination of pH 8, 1 mM MnSO₄, and incubation duration 24 hours with a percentage of 20% (Figure 1b). In table 2, based on the advanced DMRT test, it shows that the post-optimization media treatment of environmental factors gives different results on the inhibition power. The addition of peptone treatment and culture duration of 8 hours gave a higher inhibitory effect than the media without optimization.

Table 2. Antifungal activity of cell culture *S. plymuthica* UBCF_13 against *C. gloeosporioides* on day 8 post application

| Treatment                        | Growth Inhibition (%) |
|----------------------------------|------------------------|
| pH 8 + 24 hours + Mn              | 20.00 a                |
| pH 8 + Peptone + (24 hours)       | 22.56 ab               |
| (pH 7) + 8 hours                  | 24.08 ab               |
| pH 8 + Peptone + (24 hours) + Mn  | 24.58 abc              |
| pH 8 + 8 hours + Mn               | 24.84 abc              |
| pH 8 + Peptone + 8 hours          | 25.35 abc              |
| (pH 7 + 24 hours) + Mn            | 26.11 abc              |
| (pH 7) + peptone + (24 hours)     | 26.53 abc              |
The culture duration factor has a considerable influence on the expected production of antifungal compounds. In general, the inhibition of culture at 8 hours was higher than that of 24 hours. This is because at the duration of 8 hours secondary metabolite compounds have begun to form. Herliana [5] stated that S. plymuthica UBCF_13 entered an exponential phase with a duration of 8 hours starting at 0 hours and entered a stationary phase at a duration of 24 and 32 hours. In the stationary phase bacteria can carry out cell division if environmental conditions are still favorable.
Through the addition of a source of nutrients, metal ions, and the duration of the culture given, the bacteria are still able to carry out cell division.

The variation in inhibition showed that the effectiveness of fungal suppression was strongly influenced by the suitability of environmental factors. So it is important to find the optimal environmental conditions in the bacterial culture media to optimize the expected production of antifungal compounds.

3.2. SDS-PAGE

The protein profiles obtained with a molecular weight range of ± 38 to ± 125 kDa can be seen in Figure 2. Based on the results obtained, it is suspected that there are 4 protein bands with molecular weights of 38, 45, 54, and 63 kDa which are associated with inhibitory activity.

Some of the studies suggest that Serratia species are capable of producing chitinase with a range of molecular weight sizes varying from 42 to 60 kDa. The chitinase molecular weights of *S. marcescens* QM13 1466, *S. marcescens* B4A, and *S. plymuthica* HRO_C 48 were 58 kDa, 54 kDa and 60.5 kDa respectively. Meanwhile, from *Enterobacter* sp. G-1, *Enterobacter* sp. NRG4, and *E. aerogenes* have chitinase molecular weights, namely 60 kDa, 60 kDa, and 42.5 kDa [11] [12] [13] [14] [15]. These results are almost similar to those obtained in this study. This activity functions in a biocontrol role in most gram-negative bacteria, including Serratia.

![Figure 2](image)

**Figure 2.** Extracellular proteome profile of *S. plymuthica* UBCF_13 after media optimization for (a) 24 hours, and (b) 8 hours with a combination of various environmental factors variables, A-O = Treatment, K = Control, Ladder = prestained protein ladder.

4. Conclusion
Efforts to optimize bacterial culture media through a combination of environmental factors can increase inhibition against pathogenic fungi and besides that thought to stimulate the production of chitinase which acts as a biocontrol agent.
Figure 1. Effect of inhibitory activity by cell-free supernatant compound (a) and cell culture (b) Data are shown as an average of 5 replicates. Standard deviation (SD) are indicated by error bars. Notations indicate significant differences based on DMRT at a 5% level.
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References

[1] Masurekar, P. S. 2008. Nutritional and Engineering Aspects of Microbial Process Development. *Natural Compounds as Drugs Volume I. Birkhauser Baser.* Springer. **65** 293-328.

[2] Aisyah, S. N., S. Sulastri, R. Retmi, R. H. Yani, E. Syafriani, L. Syukriani, F. Fatchiyah, A. Bakhtiar, dan J. Hourssari. 2017. Suppression of *Colletotrichum gloeosporioides* by Indigenous Phyllobacterium and its Compatibility with Rhizobacteria. *Asian Journal of Plant Pathology.* **11** 139-147.

[3] Aisyah, S. N., J. Maldoni, I. Sulastri, W. Suryati, Y. Marlisa, L. Herliana, L. Syukriani, R. Renfyyeni, dan J. Hourssari. 2019. Unraveling the Optimal Culture Condition for the Antifungal Activity and IAA Production of Phylloplane *Serratia plymuthica*. *Plant Pathology Journal.* **18** 31-38.

[4] Suryati, W. 2018. Pengaruh Penambahan Ion Logam terhadap Efektivitas Senyawa Ekstraseluler Bakteri *Serratia plymuthica* Strain UBCF_13 dalam Menghambat Pertumbuhan Tiga Spesies Hoursur Fitopatogen. Skripsi: Fakultas Pertanian, Universitas Andalas, Padang. 74 hal.

[5] Herliana, L. 2019. Pengaruh Durasi Kultur terhadap Aktivitas Bakteri *Serratia plymuthica* Strain UBCF_13/-R_36/-R_36 terhadap Berbagai Spesies Hoursur Fitopatogen. Skripsi: Fakultas Pertanian, Universitas Andalas, Padang. 79 hal.

[6] Marlisa, Y. 2019. Pengaruh pH Media Kultur terhadap Aktivitas Antifungal Senyawa Ekstraseluler bakteri *Serratia plymuthica* Strain UBCF_13. Skripsi: Fakultas Pertanian, Universitas Andalas, Padang. 49 hal

[7] Baurer, A., W. Kirby., J. Sherris., and M. Turck. 1996. Antibiotic Susceptibility Testing by Standardizing Single Disc Method. *Am J Clin Pathol.* **40** 493-496.

[8] Zhang, C., X. Zhang, dan S. Shen. 2014. Proteome Analysis for Antifungal Effects of *Bacillus subtilis* KB-1122 on *Magnaporthe grisea* P131. *World Journal of Microbiology and Biotechnology.* **30** 1763-1774.

[9] Bradford, M. M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry.* **72** 248-254.

[10] Gaur, A. S. dan S. S. Gaur. 2006. *Statistical Methods for Practice and Research: A guide to data analysis using SPSS*, Sage.

[11] Frankowski, J., M. Lorito, F. Scala, R. Schmid, G. Berg, dan H. Bahl. 2001. Purification and Properties of Two Chitinolytic Enzymes of *Serratia plymuthica* HRO-C48. *Archives of microbiology.* **176** 421-426.

[12] Dahiya, N., R. Tewari, R. P. Tiwari, dan H. G. Singh. 2005. Chitinase from *Enterobacter* sp. NRG4: Its Purification, Characterization and Reaction Pattern. *Electronic Journal of Biotechnology.* **8** 14-25.

[13] Zarei, M., S. Aminzadeh, H. Zolgharnein, A. Safahihe, M. Daliri, K. A. Noghabi, A. Ghorighi, dan A. Motallebi. 2011. Characterization of a Chitinase with Antifungal Activity from a Native *Serratia marcescens* B4A. *Brazilian Journal of Microbiology.* **42** 1017-1029.

[14] Babashpour, S., S. Aminzadeh, N. Farrokh, A. Karkhane, dan K. Haghbeen. 2012. Characterization of a Chitinase (Chit62) from *Serratia marcescens* B4A and its Efficacy as a Bioshield Against Plant Fungal Pathogens. *Biochemical Genetics.* **50** 722-735.

[15] Fadhil, L., A. Kadim, dan A. Mahdi. 2014. Production of Chitinase by *Serratia marcescens* from Soil and its Antifungal Activity. *J Nat Sci Res.* **4** 80-6.