Antifungal abilities of *Serratia plymuthica* UBCF_13 produced in different types of media and culture time

F Darmawan¹, R Fatiah, J Jamsari¹³*

¹Department of Agrotechnology, Agriculture Faculty, Andalas University, Padang, West Sumatera, 25163 Indonesia
²Agricultural Sciences Department, Doctoral Program, Andalas University, Padang, West Sumatera, 25163 Indonesia.
³Department of Biotechnology, Postgraduate Program, Andalas University, Padang, West Sumatera, 25163 Indonesia

E-mail: jamsari@agr.unand.ac.id

**Abstract.** *Serratia plymuthica* strain UBCF_13 is a potential biocontrol agent to inhibit pathogen causing anthracnose disease in plants, *Colletotrichum gloeosporioides*. The production of bacterial antifungal compounds is affected by nutrition and the growth phase of bacteria. This study is aimed to determine the optimal medium and duration of culture for the production of antifungal compounds. This study used three types of liquid media culture for UBCF_13 [King's B, Potato Dextrose, and tryptic Soy] and six variations in duration culture [8, 16, 24, 32, 40, and 48 hours]. Extracellular that has been produced was used for antifungal activity assay against *C. gloeosporioides*. The results showed that the extracellular produced in the PDB with 48 hours culture duration could inhibit 18% of *C. gloeosporioides*. Growth of *C. gloeosporioides* was affected by different nutritional compositions for UBCF_13 cultures. Different nutritional compositions for UBCF_13 cultures show affect the metabolism and compound produced as well. This was indicated by differences in protein profiles of the intracellular and extracellular. Further studies are necessary to be carried out, particularly to see the mechanism of the nutrient medium that could affect the metabolism of UBCF_13 in the synthesis of antifungal compounds.

**Keywords:** *Serratia plymuthica* UBCF_13, medium nutrition, antifungal, growth phase, protein profile

1. Introduction

*Serratia plymuthica* is a biocontrol agent that has been widely studied for its use to protect plants from various fungal pathogens such as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Phytophthora capsici* *Dickeya* sp. *Rhizociona solani* and *Colletotrichum gloeosporioides* [1–5]. *Serratia plymuthica* can control plant damage from pathogens with several mutually synergistic mechanisms such as Antibiosis, parasitism involving the production of lytic enzymes, competition for nutrients, induction of plant resistance, and the production of various secondary metabolites[6,7]

Some of the secondary metabolites produced by *S. plymuthica*, such as haterumalide, pyrrolnitrin, prodigiosin, antibiotic dipeptide CB-25-I, 1-acetyl-7-chloro1-H-indol, and 1-acetyl-7-chloro-1-hindole function to prevent germination. spores of some filamentous fungi[6,8]. The production of secondary metabolites is strongly influenced by environmental conditions. One of these environmental conditions is a source of nutrients that function structurally or functionally in the cell. In general,
bacteria need nutrients such as C, H, O, N, S, P, K, Mg, Fe, Ca, Mn, and other mineral elements [9]. Among the media commonly used for the production of secondary metabolites by bacteria are LB, King's B, NB, TSB, and PDB media. The duration of culture also influences the production of antifungals.[10] reported that the antifungal activity of the bacterium Comamonas acidovorans NB-10II was first detected after 15 hours, namely in the late exponential phase, and continues to increase during the stationary growth phase until it reaches maximum activity at 42 hours of incubation. Therefore, it is necessary to optimize the type of media and the duration of bacterial culture to increase the production of antifungal compounds by bacteria.

2. Materials and methods

2.1. Microorganism and growth condition
Serratia plymuthica strain UBCF_13 and Colletotrichum gloesporioides were obtained from the internal collection of the Biotechnology Laboratory Agriculture Faculty Andalas University. UBCF_13 has grown in Luria Bertani [LB] media for 16 h at room temperature in darkness. A 5 mm diameter fungal mycelium was cut out from the margin of a colony grown on Potato Dextrose Agar [PDA] medium and transferred to the center of PDA medium and incubated for 8 days at room temperature in darkness.

2.2. Production of Extracellular Antifungal Compound
The strain UBCF_13 was grown in LB medium until the Optical Density [OD$_{600}$] had reached 1.0 and 0.5 ml cell suspension [OD$_{600}$ = 1.0] was added into each 50 ml three types medium. They are King’s B, Potato Dextrose Broth [PDB], and Tryptic Soy Broth [TSB]. The cultures are grown in 6 variations of culture duration [8, 16, 24, 32, 30, 48 h] under shaking condition 150 rpm at room temperature. After being incubated according to the time variation set, the bacteria measured the OD$_{600}$ value. Furthermore, the culture was harvested by centrifugation [12,000 rpm for 10 min at 4°C] and then filtered by syringe filter 0.22 µm.

2.3. Antifungal activity assay of extracellular compounds produced by UBCF 13 against C. gloesporioides
Antifungal activity assay used food poison method[11]. A total of 40 mL of sterile supernatant was mixed with 60 mL of sterile PDA concentrate [temperature around 70°C]. So that the final concentration of bacterial supernatant was obtained at 40% [v/v]. As the control, the sterile supernatant was replaced by an additional bacterial culture medium of the same type to culture the bacteria. Then the mixture is poured into 4 pieces of Petri dish with a volume of 25 mL each. Once solid, the 0.5 cm diameter of mycelia is applied to the media. Each sample was incubated at room temperature for 8 days.

2.4. Protein extraction

2.4.1. Intracellular Protein Extraction.
A total of 10 ml of bacterial culture from each media for extracellular compound production was measured for OD$_{600}$ and was then harvested by centrifugation at a speed of 12,000 rpm at 4°C for 10 minutes. The excess medium was discarded to get the bacterium cells. Each of the bacterium cell pellet with OD$_{600}$ = 1 was added with 1000 µL of 1xFast Break Lysis [Promega] and incubated in a shaker incubator at room temperature for 20 minutes[12].
2.4.2. **Extracellular Protein Extraction.**

This extracellular protein extraction was based on [13] protocol with slight modification. A total of 20 mL of cell-free culture supernatant was precipitated with 20% cold TCA-Acetone for 1x supernatant volume, then incubated at -20°C for 18 hours. The suspension was then centrifuged at a speed of 15,000 rpm and a temperature of 4°C for 15 minutes until a precipitate was obtained. The precipitate was then washed with 80% cold acetone three times and was deposited again by centrifugation at a speed of 12,000 rpm and a temperature of 4°C for 15 minutes. The supernatant was removed, and the protein precipitate was dried, then the protein precipitate was dissolved with 50 µL rehydration buffer [1% SDS, 10% glycerol, 10 mM Tris-HCl [pH 6.8], 2 mM EDTA, 160 mM dithiothreitol [DTT]].

2.5. **SDS-PAGE**

The molecular mass of extracted protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] in a 12% polyacrylamide resolving gel. A total of 10 µL of protein samples were inserted into the gel well. As a marker, 5 µL prestained protein ladder was also loaded into the gel. Running SDS PAGE with a constant voltage of 200 volts for 40 minutes. Commassie Brilliant Blue was used for staining the gel and then decolorized to obtain the protein bands.

3. **Result**

3.1. **The density of bacterial culture on various types of media and culture duration**

The measurement of bacterial cell density in culture media was carried out at a wavelength of 600 nm using the spectrophotometric method [OD₆₀₀]. The results of bacterial density measurements on PDB, King's B, and TSB media for various culture durations are shown in figure 1.

![Figure 1](image_url)

**Figure 1.** The density of bacterial culture on various types of media and culture duration

The trend in these data shows that the optimal medium for bacterial growth was TSB media and low bacterial growth is in PDB. The optimal bacterial growth in TSB media was due to the dominant...
tryptone composition in TSB media [17 g/L]. This is following HiMediA technical data on TSB media which states that the combination of tryptone and soytone contains nitrogen, carbon, and amino acids for the growth of microorganisms. Also, TSB contains glucose dextrose as a source of carbohydrates. [14] stated that the nitrogen source is needed for cell growth while the carbon element is used to increase the biosynthetic energy of microorganisms.

Bacteria undergo four phases during their growth period, starting from the lag phase, the exponential phase, the stationary phase, and ending in the death phase. The lag phase is the phase the bacteria prepare for the next phase. The exponential phase is the condition in which the bacteria divide by increasing constantly. Furthermore, the bacteria enter the stationary phase, in this phase, the bacteria cannot divide anymore, due to unfavorable bacterial growing conditions [nutrients have been reduced] [15,16]. Furthermore, bacteria enter the death phase. In this phase, bacterial cells lose viability, but can still survive for years [17].

Based on the above statement, it is known that the UBCF-13 bacteria cultured in the three types of media experienced an exponential phase ranging from 0 hours of culture duration to 16 hours of culture duration. This can be seen from the bacterial density which increased drastically over that duration. Furthermore, bacterial growth enters a stationary phase which is characterized by a density of bacterial cultures which tends to be constant for up to 48 hours of culture duration.

[18] stated that S. plymuthica M24T3 bacteria entered an exponential phase when the culture density reached 0.6. The previous study of [19] stated that the exponential phase of E. coli bacteria is when the culture density is 0.6 A - 1.0 A. The S. plymuthica UBCR_12 bacteria enter a stationary phase when the bacterial culture density ranges from 1.0 A to 1.2 A. this occurs after the bacteria are cultured for 12 hours [20].

3.2. Optimal Inhibition Using Extracellular

The comparison of extracellular inhibition from UBCF_13 against C. gloesporioedess shows in table 1. They compared data was based on the highest inhibition from each treatment.

Based on the ANOVA test, there was an interaction between the type of media and the duration of culture on the percentage inhibition of C. gloesporioedess fungal growth by extracellular UBCF_13. PDB medium with a culture duration of 48 hours had the highest percentage of inhibition [18%] which was significantly different from the other treatments. Meanwhile, King’s B medium with a culture duration of 8 hours had the lowest percentage of power, namely 0.69%. From Table 1, it is also known that all culture durations in PDB media show higher inhibition than the duration of the same culture in other media. This shows that PDA media is the best medium for producing antifungal compounds.

Table 1. Inhibition percentage of extracellular compounds

| CultureDuration | PDB [1DAT*] | King’s B [8 DAT*] | TSB [8 DAT*] |
|----------------|-------------|------------------|-------------|
| 8 hours        | 11.00 c A   | 0.69 d B         | 1.32 c B    |
| 16 hours       | 10.00 c A   | 6.48 b B         | 3.51 b A    |
| 24 hours       | 15.00 ab A  | 4.40 bc B        | 6.80 ab B   |
| 32 hours       | 13.00 bc A  | 1.85 cd C        | 8.33 a B    |
| 40 hours       | 12.00 bc A  | 5.32 bc B        | 6.80 ab B   |
| 48 hours       | 18.00 a A   | 13.7 a B         | 1.97 c C    |

Figures followed by the same letter are not significantly different according to Duncan’s test at the 5% level. Alphabet capital is read horizontally [row] and lowercase is read in a vertical direction [column].

*DaysAfterTreatment

Potato extract and dextrose which the main component in PDB are rich sources of carbon. The high bacterial inhibition power on PDB media is thought to be because PDB has higher C elements than other media. This conjecture is supported by [21] which states that dextrose is a carbon source that
facilitates the growth of organisms. Simple sugars such as dextrose are easily used by Bacillus cells for growth, thus accelerating the increase in biomass and showing good cell growth. Previous studies of [22] stated that the antifungal activity increased with an increase in the concentration of potato extract by about 200 g/L [antifungal activity 6200 AU/mL].

3.3. UBCF_13 intracellular and extracellular antifungal protein profiles

The intracellular protein profile of bacteria UBCF-13 obtained in this study shows the visualization of the proteins involved in the mechanism of formation of antifungal compounds. The protein-coding genes were identified from the genomic data of S. plymuthica UBCF_13 [Fatiah, unpublished result]. The visualization results of the protein bands can be seen in figure 2.

![Figure 2. Profile of intracellular and extracellular proteins at 48h duration in each extracellular culture medium. (P: PDB, K: King's B, T: TSB)](image)

Bands with estimated sizes of 238 kDa and 106 kDa are up-regulated in the GDP medium. This band is a protein encoded by the oocR gene. The band with an estimated size of 106.7 kDa is the protein encoded by the admO gene. 35.4 kDa is the protein encoded by the prnB gene. The oocR, admO, and prnB proteins were seen in all media and were upregulated in the PDB and TSB media. The band estimated to be admI measuring 10.1 kDa was also seen in all media and up-regulated in King's B media. The genus Serratia reportedly produces antifungal secondary metabolites pyrrolnitrin, oocydin, and andrimide [23–25].

A band with an estimated size of 75 kDa is up-regulated on King's B media and a band with an estimated size of 130 up-regulated on TSB media. However, the protein types of the two bands with the estimated size are not known. A ribbon with an estimated size of 44.6 is visible in all media. Bands with an estimated size of 41.1 are up-regulated in King's media. Bands with an estimated size of 32.2 kDa are seen in King's B and TSB media. The three sizes of these bands are proteins encoded by the oocF, oocW and oocA genes, respectively. Research conducted by [26] identified that oocF, oocW and oocA are genes that code for proteins or enzymes involved in the biosynthesis of oocydinA. This
compound is one of the antifungal compounds. The gene clusters involved in the biosynthesis of oocydinA are also present in S. plymuthica strains A153, 4Rx5 and 4Rx13, and S. marcescens MSU97. The length of these gene clusters in the genomes of various S. plymuthica ranges from 77-80 kb consisting of 23 ORF [25].

The extracellular protein profile of bacteria UBCF_13 obtained in this study shows the visualization of the proteins secreted outside the cell. Bands with an estimated size of 46.8 kDa are up-regulated on the GDP medium. This band is a protein encoded by the ChitinasePut gene. Following a study conducted by [27] states that the protein band S. plymuthica strain UBCR_12 with a size of 47 kDa is estimated to be ChitinasePut-II.

Bands with an estimated size of 41.2 kDa are up-regulated in the GDP and TSB media. This band is a protein encoded by the prnD gene. [1] states that S. plymuthica bacterium IC14 has pyrrolnitrin, protease and chitinase antibiotics. This compound can protect cucumbers from S. sclerotiorum and B. cinerea. Meanwhile, chitinolytic activity in the IC14 strain did not play an important role in suppressing fungal pathogens.[28] also stated that S. plymuthica strain HRO-C48 produces pyrrolnitrin and has the potential to become an agricultural fungicide on a large scale. Prn is a secondary metabolite of L-Tryptophan derivatives that plays an important role in the biocontrol of plant diseases. The expression of the prn ABCD operon which is responsible for prn biosynthesis is regulated by RpoS[23,29]

A band with an estimated size of 14.8 kDa is up-regulated in TSB media and a band with an estimated size of 9.1 kDa is up-regulated in PDA media. The two bands are proteins, each of which is encoded by the admG and oocG genes. [25] state that oocydin is a natural product of an antifungal compound whose polyketides are regulated by N-acyl-L-homoserine.

UBCF-13 bacteria cultured in three different types of media showed different protein profiles in each medium. The difference in protein production makes the inhibitory power obtained also vary. The up-regulated proteins are thought to be very influential in the production of antifungal compounds by the UBCF-13 bacteria.

4. Conclusion

The optimal culture duration for extracellular antifungal production by UBCF_13 on PDB medium with antifungal inhibition 18%. UBCF_13 bacteria are cultured in three different types of media, showed different protein profiles in each medium. This difference proves that the type in nutrition affects the type of compound produced by the bacteria UBCF_13.

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