Profiling bioactive compounds in secondary metabolites from co-cultivation between actinomycetes and pathogenic bacteria

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Abstract. Co-cultivation is one of the ways to induce the discovery of new compounds by activating the cryptic genes in a microorganism’s genome. Actinomycetes is an order of bacteria with many clusters of gene coding for bioactive compounds that has not yet been expressed in the genome. In this study, co-cultivation between Actinomycetes strain DHE5-1 with pathogenic bacteria are conducted. Activity assay was done with disk diffusion method and the bioactive compounds in secondary metabolites are identified using HPLC UV-Vis. The results showed the activity of secondary metabolites from co-cultivation as an antimicrobial agent against the pathogenic microorganisms tested. The bioactive metabolite compounds showed different profiles produced between the mono and co-culture based on the models of the chromatogram. Finally, co-cultivation has the potential to induce the expression of covert genes in the organisms’ genome. This finding can promote the discovery of novel compounds with enhanced antimicrobial activity against resistant bacteria such as MDR-SA.

1. Introduction

In the year 1940 to 1960, there have been many discoveries of antibiotics in the Actinobacteria group, mainly from the Streptomyces genus[1]. The discovery of known compounds is increasing, while the discovery of novel antibiotic is declining as time goes [2]. Moreover, the pathogenic bacteria are becoming resistant to the present antibiotics, therefore the search for novel antibiotics that have enhanced abilities is needed. Based on the study for Streptomyces’ genome, many cryptic genes that code for novel compounds are not expressed [3,4]. Implementing the Co-cultivation method, those silent genes can be expressed with the quorum sensing mechanism due to the signal transduction from the chemicals secreted by the inducer microorganisms [5].

Co-cultivation or mixed fermentation is one of the methods to culture two or more microorganisms under equal condition. This method is practiced with the objective to optimize the production of compounds which are not produced in monoculture [6,7,4]. This mixed fermentation can induce the discovery of novel compounds that can only be produced while these two or more microorganisms live together [8]. In addition, to escalate the expression, signal transduction can also decrease some compounds production as a response to the survival mechanism [5].

With the release of chemical substances, this survival mechanism can also happen when the nutrition in the cultivation medium depleted. The limited concentration of nutrition can induce a microorganism to adapt by utilizing other present sources of nutrition. For example, Bifidobacterium breve did a cross-feeding with degraded mucin from B. bifidum[9]. Furthermore, another adaptation mechanism is evolution. The short lifespan of microorganism causes them to undergo a faster evolution from the adaptation mechanism compared to other higher taxa organisms [10]. The microorganism can evolve to be resistant to the symbionts that are antagonistic for themselves. This co-culture technique can be used to increase the production of certain compounds, i.e. the lipid production from co-cultivation between microalgae with Aspergillus species [11] and T. spathulate fungi with the Chlorella vulgaris [12]. Depends on the compound produced, the compound can be an antibiotics, antifungals, or anti parasites [13,14]. The objective of this research is to analyze and
characterize profiling bioactive compound in the co-cultivation investigate between the Actinomycetes strain DHE5-1 with pathogenic bacteria using HPLC UV-Vis.

2. Materials and Method

2.1. Materials

Actinomycetes strain DHE5-1, Escherichia coli and Staphylococcus aureus are taken from the culture stock in the Laboratory of Applied Genetic Engineering and Protein Design at LIPI Cibinong. Bacillus subtilis and Salmonella typhimurium are collected from the Laboratory of Biology Department from Surya University Tangerang. The absorbances are analyzed with the spectrophotometry in Laboratory of Natural Product Chemistry at LIPI Cibinong. Profiling of the secondary metabolites are done using the High-Performance Liquid Chromatography (HPLC UV-Vis) in the Laboratory of Biopharma at Bogor Institute of Agriculture (IPB).

2.2. Methods

2.2.1. Microorganisms cultivation.

Actinomycetes strain DHE5-1, pathogenic bacteria (Escherichia coli, Staphylococcus aureus, Salmonella typhimurium dan Bacillus subtilis) are collected from the stock culture. Actinomycetes are cultivated in ISP2 agar medium [15] for 3 to 5 days at room temperature. The pathogenic bacteria are incubated in NA [16] medium for 1 to 3 days at room temperature.

2.2.2. Growth curve of pathogenic bacteria.

Four samples of pathogenic bacteria are cultivated in NB medium for 22 hours with a shaker at room temperature. Aseptically, 2 mL of each sample are isolated at 2, 6, 8, 10, and 22 hours after inoculation into a cuvette. The absorbance of each sample is analyzed using a spectrophotometer with OD at 600 [17].

2.2.3. Mono and co-cultivation of samples.

Actinomycetes strain DHE5-1 was incubated in a 15 mL seed medium ISP2 for 48 hours. Pathogenic bacteria colony were incubated in 15 mL seed medium NB for 24 hours [5]. All the cultures were incubated using a shaker at room temperature. Each sample were diluted until 10^-3 using NaCl 0.85% [18]. Aseptically, total plate counting (TPC) was done by injection of 100 µL diluted samples into ISP2 agar in the petri dish for Actinomycetes and NA for pathogenic bacteria. The samples were incubated at room temperature (30°C) for 1 to 2 days.

For the monoculture, 1 mL Actinomycetes 10^-1 was inoculated aseptically into 100 mL ISP2 production medium. For the co-cultivation, after 24 hours, the medium containing Actinomycetes was inoculated with 1 mL pathogenic bacteria at 10^-1 [5]. All of the mono and co-culture were incubated with a shaker for 8 days at 30°C temperature [13]. Each culture was inoculated into an object glass to analyze their morphology at 1, 5, and 8 days after inoculation. The samples then observed under a light microscope with 1000 times magnification.

2.2.4. Secondary metabolites’ extraction and bioactivity testing.

Each culture was filtered using filter paper aseptically. The filtrates were added with EtOAc (1:1 ratio) into a separatory funnel and shaken until 2 phases is formed. The ethyl acetate extracts then evaporated with an evaporator at 50°C and pressure at 60 to 70 kPa [7]. Aseptically, the dried extract of mono and co-cultures were weighed and eluted with methanol to a concentration of 0.3 mg/mL. Each disk paper was dripped with 20 µL eluted extract and air-dried. Filter paper then inoculated onto a medium agar containing the tested bacteria. Amoxicillin and methanol were used as positive and negative control, respectively [19].

2.2.5. Secondary metabolites’ profiling.

Ethyl acetate extracts were added with methanol 1mL/5mg dried extracts [4]. 20 µL aliquoted extracts then injected into HPLC UV-Vis with parameters explained below:C_{18} column, solvent MeOH 10% for 10 minutes, 10%-100% MeOH for 40 minutes,
and 100% MeOH for 10 minutes, using UV detector, and flow rate at 0.3 mL/minute [8] at 30°C temperature. The wavelength was set at 210 to 380 nm [4].

3. Results and Discussion

3.1. Growth curve of Pathogenic Bacteria analysis

In this study, Actinomycetes strain DHE5-1 were cultivated with 4 other pathogenic bacteria including *E. coli*, *S. aureus*, *B. subtilis*, and *S. typhi* murium*. These pathogenic bacteria act as the inducer of secondary metabolites production from Actinomycetes. The growth curve analysis of each pathogenic bacteria are conducted to find out the time each bacteria enter their growth phase (lag, log, stationary phase). Pathogens in the log phase will be inoculated to the medium containing 24 hours old Actinomycetes. This step is done to induce the competition between bacteria with depleting nutrition. Based on figure 1, all the bacteria were entering the log phase 6 hours after cultivation and stationary phase at 10 hours after inoculation. The production of metabolites can happen as a reaction for adaptation mechanism or stress response in the fermentation medium [5,9]. Other than adaptation, microorganisms can also evolve as time goes by to protect themselves from pathogenic bacteria bad effects[20,21]. Those secondary metabolites can be an active compound to fight against the pathogens [13].

![Growth curve of Pathogens](image)

**Figure 1.** The absorbance of pathogens at tested hours.

3.2. Mono and co-cultivation of samples

To understand the influence of the co-cultivation system for the secondary metabolites production, the mixed fermentation was done for 8 days. The interaction result between species affects the color change, viscosity, and turbidity in the mediums (figure 2). Presence of growing microorganisms and interaction in the co-cultivation medium were evaluated from morphology analysis of each culture using a microscope (figure 3). All the morphology analysis in day 1 to day 8 showed that the pathogenic bacteria are dominating the medium compared to Actinomycetes. Generally, the growth rate of Actinomycetes are slower [22,23] than the pathogenic bacteria, thus the Actinobacteria presence was unstable. Despite that, the Actinobacteria’s presence was monitored in the co-cultivation with *S. aureus* (figure3), with a shape of aggregate of hypha [23]. Besides that, the medium’s color are changing to a darker colour because of the secretion of metabolites from microorganisms cultivated [24]. From the DHE5-1 monoculture’s morphology analysis, aggregates were seen to be less dense. Other than that, in the co-culture’s morphology analysis, the bacteria’s presence was harder to be seen with the function of time. These two phenomena were estimated caused by the apoptosis mechanisms from each bacteria cell [25,26]. Pathogens’ cells were seen to have similar characteristics with each general morphology in the literature [27-30].
Figure 2. Mediums for co-culture with **E. coli** (A), **S. aureus** (B), **B. subtilis** (C), **S. typhimurium** (D), and monoculture (E) every 1, 5, and 8 days (left to right).
Figure 3. Morphology analysis of DHE5-1 co-culture for 1, 5, and 8 days (left to right) with *E. coli* (A), *S. aureus* (B), *B. subtilis* (C), *S. typhimurium* (D), and DHE5-1 monoculture (E) magnified 1000 times with a light microscope.
3.3. Secondary metabolites extraction and bioactivity testing

For the profiling of secondary metabolites, the cultures were extracted with a semi-polar solvent, ethyl acetate which can bind the bioactive compounds with a low molecular weight [31]. The dry extracts then eluted with methanol until 0.3 mg/mL concentration. Based on the data in figure 4 and table 1, each extract can variably inhibit the pathogens’ growth. The ability to inhibits by forming the clear zones depends on the extracts’ ability. The results showed that there is induction of bioactive compounds production from Actinomycetes against pathogens [5,9]. Results in table 1 showed that extract with the highest activity against all pathogens tested were from co-cultivation between Actinomycetes and E. coli (table 1, column 2). There is an increase of activity from co-culture (DHE5-1 with E. coli) compared to monoculture (DHE5-1), especially against S. aureus and E. coli itself. On the contrary against S. typhimurium and B. subtilis the inhibition was not as optimum as the monoculture (table 1, column 6). This incident can happen because of the difference of spectrum from each bioactive extract from mono and co-culture. The difference of spectrum from these two extracts can inhibit certain species of bacteria but not optimum for other certain bacteria [32]. More study about spectrum and ability of the extract is needed by using the other pathogenic bacteria as comparison. Some factors can affect the inhibition of extracts, including active compounds’ concentration, depth of agar’s concentration, or the growth rate of bacteria tested [33].

Different to all the activity testing, co-culture of DHE5-1 with S. typhimurium have the weakest activity against all the pathogens tested compared to the other 3 co-culture extracts (table 1, column 5). The bioactivities observed from S. typhimurium co-culture extract showed no discernible increase of bioactivity compared to the DHE5-1 monoculture. The two other co-culture extracts (S. aureus and B. subtilis) showed promising bioactivity, despite lower inhibition zone compared to the monoculture. The activities from these two co-culture extracts can be enhanced by genetic modification or in silico modelling to enhance the inhibition mechanism, thus they are also promising candidates [34,35].

Based on the data presented in table 1, all the extracts (co-culture and monoculture) are very optimum to inhibit the S. typhimurium growth (table 1, row 3), compared to the other pathogens generally.

![Figure 4. Disk diffusion assay on mono and co-culture for 24 hours at room temperature against E. coli (A), S. aureus (B), B. subtilis (C), dan S. typhimurium (D).](image-url)

| Pathogens tested | Clear zones from mono and co-culture extracts (mm) |
|------------------|--------------------------------------------------|
|                  | E. coli  | S. aureus | B. subtilis | S. typhimurium | Mono DHE5-1 |
| S. typhimurium   | 12       | 11        | 10          | 8              | 13          |
| B. subtilis      | 8        | 8         | 7           | 7              | 9           |
| E. coli          | 11       | 7         | 1           | 1              | 8           |
3.4. Secondary metabolites profiling

Extracts with significant inhibition activity that potential to produce bioactive compounds against pathogens was used. Three extracts with average the higher activity, including DHE5-1 monoculture, co-culture with *E. coli* and *S. aureus*’s secondary metabolites were characterized using HPLC UV-Vis. Analysis of the profiles was done to discover the variety of metabolites produced from different treatment.

Based on HPLC UV-Vis analysis, each sample has a different absorbance unit in equal time and wavelength (figure 5). Both co-culture (straight and dotted line) showed the presence of spectrum that are not appear in DHE5-1 monoculture at approximately 35 to 60 minutes. Despite that, monoculture extract has lower peaks in that area (35 to 60 minutes) indicating a presence of compounds but in low concentration (less of induction). This finding is concordant with the literature that co-cultivation technique can induce the production of novel compounds [4,5,7], that are not produced in the monoculture, due to the survival mechanisms. Peak from both co-cultures has a similar configuration and retention time (figure 5). Antimicrobial agent’s mechanism in inhibition against microbes can happen by the inhibition of cell wall and membrane formation, nucleic acid, also inhibition on the ribosomal activity, or important metabolites production [36]. *E. coli* and *S. aureus* have a similar structure, having the cell wall and capsule on its outer cells [37-39]. It is estimated that the inhibition mechanism between both extracts is similar, by inhibition of those structures [36]. The peak at 5 minutes on the monoculture (dashed line) have a wider range than both co-culture extracts. This difference can happen because of the shifting of these compounds to produce other compounds. These other compounds are thought to have a role for the bioactive compounds, due to their absence in the pure culture of DHE5-1. In figure 5, there are peaks that indicate the presence of the compounds therefore more study is needed to purify these compounds and identify their conformation or types with the bioactivity.

4. Conclusion

This research strengthens and complement the other studies about co-cultivation technique potential for the discovery of novel or enhanced activity of current known compounds.
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6. Reference

[1] Jensen Paul R, Mincer Tracy J, Williams Philip G & Fenical William 2005 Antonie Van Leeuwenhoek 87(1) 43-8
[2] Behie Scott W, Bonet Bailey, Zacharia Vineetha M, McClung Dylan J & Traxler Matthew F 2016 Front. Microbiol. 7 2149
[3] Subramani R & Aalbersberg W 2013 Microbiol. Biotechnol. 97(21) 9291-321
[4] Shang Z, Salim A A & Capon R J 2017 J. Nat. Prod. 80 1167-72
[5] Slattery M, Rajbhandari I & Wesson K 2001 Streptomyces tenjimariensis Microb. Ecol. 41(2) 90-6
[6] Abdelmohsen Usama Ramadan, Grkovic Tanja, Balasubrama-nian Srikanth, Kamel Mohamed Salah, Quinn Ronald J & Hentschel Ute 2015 Biotecnol. Adv. 33(6) 798-811
[7] Wu Changseng, Zacchetti Boris, Ram Arthur FJ, Wezel Gilles P Van, Claessen Dennis & Choi Young Hae 2015 Sci. Rep. 5 10868
[8] Dashi Youssef, Grkovic T, Abdelmohsen UR, Hentschel U & Quinn RJ 2014 Mar. Drugs. 22(12) 3046-59
[9] Egan M, Motherway MO, Kilcoyne M, Kane M, Joshi L, Ventura M & Van Sinderen D 2014 Microbiol. 25(14) 282
[10] Huijben S & PaaijmansK P 2018 Putting Evolution in Elimination: Evol. Appl. 11(4) 415-30
[11] Wrede D, Taha M, Miranda AF, Kadali K, Stevenson T, Ball AS & Mouradov A 2014 PLoS One 24(9) e113497
[12] Kitcha S & Cheirsilp B 2014 Appl. Biochem. Biotechnol. 173(2) 522-34
[13] Haque Md Uzzal, Rahman Md Ajijur, Haque Md Anwarul, Sarker Ashish Kumar & Islam Md Anwar Ul 2015 Bangladesh Pharm. J. 18(1) 61-5
[14] Marmann A, Aly AH, Lin W, Wang B & Proksch P 2014 Mar. Drugs. 12(2) 1043-65
[15] Mohseni M, Norouzi H, Hamedi J & Roohi A 2013 Int. J. Mol. Cell. Med. 2(2) 64-71
[16] Kumar S 2012 Textbook of Microbiology(London: JP Medical Ltd)
[17] Pan Hongmiao, Zhang Yongbin, He Gui-Xin, Katagori Namrata & Chen Huizhong 2014 BMC Microbiol. 14 222.
[18] Lu Xin, Yi Lanhua, Dang Jing, Dang Ying & Liu Bianfang 2014 Food Control 46 264-71
[19] Driscoll Amanda J, Bhat Niranjan, Karron Ruth A, O'Brien Katherine L & Murdoch David R 2012 Clin. Infect. Dis. 54(2) S159-64
[20] Shinohara Neide KS, Freitas Fernanda, Pires Edleide, Andrade Samara, Filho Jose Lima & SousaPaulo 2018 Sci. J. Environ. Sciences 4(1) 33-8
[21] Oscar T 2007 Food Microbiol. 24(6) 640-51
[22] Tille P 2013 Bailey & Scott's Diagnostic Microbiology - E-Book(Amsterdam: Elsevier Health Sciences)
[23] Valour F, Senechal A, Dupieux C, Karsenty J, Lustig S, Breton P, Gleizal A, Bousset L, LaurentF, Braun E, Chidiac C, Ader F & Ferry T Infect. Drug Resist. 7 183-97
[24] Pommerville J C 2010 Alcamo's Laboratory Fundamentals of Microbiology(Massachusetts: Jones & Bartlett Publishers)
[25] Lancker J L V 2006 *Apoptosis, Genomic Integrity, and Cancer* (Massachusetts: Jones & Bartlett Learning)

[26] Nystrom T & Osiewacz HD 2004 *Model Systems in Aging* (Berlin: Springer Science & Business Media)

[27] Berg H C 2004 *E. coli in Motion* (Berlin: Springer Science & Business)

[28] Lu Z, Guo W & Liu C 2018 *J. Vet. Med. Sci.* 80(3) 427-33

[29] Vasanthakumari R 2007 *Textbook of Microbiology* (New Delhi: BI Publications Pvt. Ltd)

[30] Andino A & Hanning I 2015 *ScientificWorldJournal* 2015 1-16

[31] Pavia D L, Kriz G S, Lampman G M & Engel R G 2016 *A Microscale Approach to Organic Laboratory Techniques* (Boston: Cengage Learning)

[32] Kovacs V 2016 *Antibiotics: Miracles of Medicine* (Wisconsin: Gareth Stevens Publishing LLLP)

[33] Lorian V 2005 *Antibiotics in Laboratory Medicine* (Philadelphia: Lippincott Williams & Wilkins)

[34] Kaushik Prashant, Andujar Isabel, Vilanova Santiago, Plazas Mariola, Gramazio Pietro, Herraiz Francisco Javier, Brar Navjot Singh & Prohens Jaime 2015 *Molecules* 20(10) 18464-81

[35] Lerman Joshua A, Hyduke Daniel R, Latif Haythem, Portnoy Vasilily A, Lewis Nathan E, Orth Jeffrey D, Schrimpe-Rutledge Alexandra C, Smith Richard D, Adkins Joshua N, Zengler Karsten, Palsson Bernhard O 2012 *Nat. Commun.* 3(1)

[36] Neu H C & Gootz T D 1996 *Medical Microbiology* 4th ed (Galveston: University of Texas)

[37] Gupta S 2012 *The Short Textbook of Medical Microbiology for Dental Students* (London: JP Medical Ltd)

[38] Aneja K R 2003 *Experiments in Microbiology, Plant Pathology, and Biotechnology* (New Delhi: New Age International)

[39] Fetsch A 2017 *Staphylococcus aureus* (Massachusetts: Academic Press)