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Growth optimization of mangrove *Streptomyces* for enhancement of actinorhodin production

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ABSTRACT

The family *Streptomycetaceae*, notably species in genus *Streptomyces*, have long been the subject of investigation due to their well-known ability to produce secondary metabolites. Even though thousands of secondary metabolites have been discovered from *Streptomyces*, less attention has been given in understanding the bioprospecting of the secondary metabolites in *Streptomyces* living in mangrove ecosystem. Therefore, the current study was designed to investigate the production of actinorhodin from mangrove *Streptomyces* which was grown in different parameters. Phylogenetic analysis showed that the *Streptomyces* sp. K2-11 belongs to *Streptomyces prasinus* strain NRRL B-12521 with 99.62% similarity. High-performance liquid chromatography analysis has detected the presence of actinorhodin compound in *Streptomyces* sp. k2-11. The compound showed inhibitory activity to *Bacillus subtilis* and no activity against *Streptococcus aureus* based on disc diffusion assay. *Streptomyces* sp. growth in SMMS media was recorded to be the highest (1.49×10^8 cfu/mL) compared with SYE and R2 media. The production of actinorhodin was tested on three different parameters: incubation temperature, pH and glucose levels. The highest production of actinorhodin was recorded at 33°C (1.95×10^{-6} mol/L), pH 5 (7.1×10^{-6} mol/L) and 50% w/v glucose (9.56×10^{-6} mol/L) for 60 h of incubation. The present finding suggested that mangrove *Streptomyces* sp. K2-11 produced high yield of actinorhodin under extreme or stress condition.

Keywords: Actinorhodin; Antibiotic; Mangrove; Secondary metabolites; *Streptomyces*

1. Introduction

Streptomycetes are filamentous, Gram-positive bacteria that exist in a variety of soils, compost, plants and water. These microorganisms also are known as a producer of many secondary metabolites, such as antibiotics. Among the actinomycetes, about 7,600 compounds are produced by *Streptomyces* species. These metabolites are known to have antibacterial, antifungal, antioxidant, neuritogenic, anti-cancer, anti- algal, anti-helmintic, anti-malarial and anti-inflammatory activities [1]. They exhibit a range of life cycles, which are unique among the prokaryotes, and appear to play a major role in the recycling of organic matter in the soil ecosystem [2].

It is commonly known that the production of secondary metabolites from microorganisms, such as antibiotics,

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is the useful mechanism to protect themselves from foreign organisms. The same mechanism was used by humans to produce antibiotics that can kill infection of microorganisms on human body. The uses of antibiotics are increasing worldwide due to population growth, and overuse lead to increasing antibiotic resistance, which raises the price of healthcare products and equipment [3]. The decrease of antibiotic effectiveness contributes to major threat for medical sector. In the United States alone, around 23,000 people die each year as a result of antibiotic resistance infections [4]. Therefore, research on the production of antibiotic is vital to improve the effectiveness of medicine especially those that are related to microbial infection.

The production of secondary metabolites usually coincides with their pathway-specific regulatory genes, which are frequently expressed at low level. The low level effective concentration and remarkable pleiotropic effects usually complicates the regulatory cascades that govern this process. Consequently, the possibility of over-expressing the functional protein (secondary metabolic gene cluster) that is bound to produce the useful compound is predicted to favor the production of the secondary metabolite. Thus, this research was conducted to enhance the production of actinorhodin from *Streptomyces* through growth optimization, which involved various growth parameters and conditions.

2. Methodology

2.1. Bacterial strain

Streptomyces sp. K2-11 was previously isolated from mangrove forest of Tanjung Lumpur, Kuantan, Pahang. Preliminary screening showed antibacterial potential of *Streptomyces* sp. K2-11 against *B. subtilis*. *Streptomyces* sp. K2-11 was maintained on an SYE agar (starch–yeast extract).

2.2. Molecular identification of streptomycetes

Universal primers of 16sF (5′-AGAGTTTGATCCTGGCT CAG-3′) and 16sR (5′-AAG GAG GTGATCCAGCCGCA-3′) were used to target the conserved region of 16S rRNA to amplify an approximately 1.5 kb long DNA fragment. Amplification was performed in a final volume of 50 μL, which consist of 200 ng of template genomic DNA, 0.4 μM of each primer, 25 μL of 2 × MyTaq™ Mix (Bioline, UK) and sterile ultrapure water. The 16S rRNA gene fragment amplification reaction was carried out in an automated thermal cycler (Eppendorf Mastercycler Gradient, Germany) with initial denaturation and denaturation performed at 94°C for 5 min and 30 s, respectively, annealing temperature at 55°C for 60 s, extension at 72°C for 2 min (30 cycles) and final extension at 72°C for 10 min. The PCR products were examined and visualized with 1.0% (w/v) agarose gel electrophoresis, separated at 70 V for 45 min. The product was purified and submitted for sequencing.

2.3. Phylogenetic analysis

The 16S rRNA sequences were manually verified using Sequence Scanner software version 2.0 (Applied Biosystems, ThermoFisher Scientific). The sequences were assembled using the Bioedit Sequence Alignment Editor program. The sequences were matched with the GenBank database using BLASTn (National Center for Biotechnology Information) search tool [5]. The 16S rRNA gene sequences of the isolates and their closest phylogenetic neighbors were aligned and evaluated for their homology using CLUSTALW module. Phylogenetic tree was generated using the Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 [6]. The tree topology was evaluated in a bootstrap analysis based on 1,000 replicates using the neighbor joining algorithm [7].

The nucleotide sequence of the isolate was deposited in the GenBank database via the web-based submission data wizard (http://www.ncbi.nlm.nih.gov/home/submit-wizard.shtml).

2.4. Actinorhodin extraction

Streptomyces K2-11 was grown in SMMS media. After 7 d of incubation, actinorhodin was extracted with 2 L of 3 N KOH from a 2-L sample. The suspension was centrifuged at 4,000 × g for 10 min and the pellet was removed.

2.5. HPLC analysis

Extracted supernatant was subjected to reversed-phase HPLC analysis (PerkinElmer) under the following conditions: column, Phenomenex LunaC18 (2) (4.6 i.d. 150 mm, Waters); column temperature, 40°C; gradient elution with mobile phase comprised of solvent A (acetonitrile, containing 0.5% acetic acid), and solvent B (0.5% acetic acid) (Analytical Laboratory, Kulliyyah of Science, International Islamic University Malaysia). Gradient profile, 0–20 min: 43.5%A, 20–25 min: 43.5%–60%A, 25–28 min: 60%–95%A, 28–32 min: 95%A, 32–35 min: 95%–43.5%A; flow rate, 1.0 mL/min; and detection of 250 and 600 nm using a photo-diode array detector (PD-8020, TOSOH).

2.6. Antimicrobial activity screening

Determination of antimicrobial activity of actinorhodin was performed using a standard antimicrobial susceptibility test. Two test organisms consisted of two Gram-positive bacteria (*Bacillus subtilis* IMR B 144/11 C and *Staphylococcus aureus* ATCC 259233), which were obtained from the laboratory collection.

The antimicrobial activity of the ethyl acetate extracts was assessed using modified Kirby–Bauer disc diffusion method as described by Wanger [8]. The antibiotic extract of the isolates impregnated onto sterile 6 mm in diameter blank antibiotic disc (Oxoid, UK). The concentration of antibiotics was adjusted to 1 mg/mL and 20 μL was injected onto the discs. The disc was then left to dry and placed on the MHA surface seeded with 100 μL test organisms. Standard antibiotic chloramphenicol (10 μg; Oxoid, UK) discs were used as positive control for bacteria, while disc impregnated with methanol was taken as negative control. The plates were incubated at 30°C (48 h). The antimicrobial activity was determined by measuring the diameter of the inhibition zone (expressed in mm), where the colonies are failed to grow. The test was conducted in triplicate and results were expressed as mean ± SD (standard deviation).

2.7. Streptomycetes growth determination on different growth media

Streptomyces sp. K2-11 were grown in all types of broth (SMMS, SYE and R2; 500 mL). As much as 1 mL of culture broth were taken from each conical flask after 5, 10, 20, 30, 40, 50 and 60 h and were transferred into 1.5 mL cuvette. The optical density of *Streptomyces* was determined by using spectrophotometer at wavelength 600 nm. This step was repeated thrice and the results were recorded.

A series of dilution were prepared from 10^{-1} to 10^{-9}-fold and a streak plate technique was performed for dilution (10^{-6}, 10^{-8} and 10^{-9}-fold). All plates were incubated at 30°C and the growth of the colony was observed and recorded.

2.8. Actinorhodin production optimization

Streptomyces sp. K2-11 was cultured in SMMS broth with different conditions and parameters applied in order to optimize the production of actinorhodin. The parameters were temperature (25°C, 27°C, 30°C, 33°C and 37°C), pH (pH 3, pH 5, pH 7, pH 9 and pH 12), and glucose concentration (5% (w/v), 25% (w/v), 50% (w/v), 75% (w/v) and 95% (w/v)).

Fresh *Streptomyces* sp. K2-11 was inoculated into five labelled 500-mL conical flasks containing 200 mL SMMS broth with pH was adjusted to 7 with hydrochloric acid and sodium hydroxide. The broth containing *Streptomyces* was shaken gently. Then, the broth was incubated into five different temperature that at 25°C, 27°C, 30°C, 33°C and 37°C. Actinorhodin was extracted at 5, 10, 20, 30, 40, 50 and 60 h.

Another fresh *Streptomyces* sp. K2-11 was inoculated independently with 200 mL SMMS media pH adjusted to pH 3, pH 5, pH 7, pH 9 and pH 12. The broths were incubated at 30°C with agitation at 150 rpm. The inoculating step was repeated in SMMS broth with customised glucose concentration 5% (w/v), 25% (w/v), 50% (w/v), 75% (w/v) and 95% (w/v). The pH was adjusted to 7 and incubated at 30°C. The antibiotics were then extracted after incubation. The concentration of antibiotics from each parameter was calculated and analysed.

2.9. Actinorhodin quantification

The absorbance of actinorhodin was measured by using spectrophotometer at 640 nm. The actinorhodin concentration was calculated based on an extinction coefficient of 25,320 M^{-1} cm^{-1} [9].

2.10. Statistical analysis

All data were recorded and analysed statistically using Microsoft Excel© 2010 edition and IBM© SPSS Statistics 21.0. In order to understand the relationship among the variables (incubation temperature, pH level and glucose level) or the degree of association between the two, correlation model (Pearson correlation) was adopted. Once the correlation was successfully computed, the probability that observed correlation occurred by chance was determined by conducting significance test. The data were grouped according different parameters (incubation temperature, pH level and glucose level). Each parameter was divided into five different degrees and levels. The concentration of antibiotics from each level was taken at 5, 10, 20, 30, 40, 50 and 60 h of incubation. Thus, the sample size each parameter was n = 40 (including 0 h as zero antibiotic concentration was indicated as a value).

3. Result and discussion

3.1. Phylogenetic analysis

BLAST analysis on 16s ribosomal RNA gene sequence revealed that *Streptomyces* sp. K2-11 is highly similar to *Streptomyces prasinus* strain NRRL B-12521 (Fig. 1) with 99.62% similarity.

Streptomyces prasinus previously known as *Streptomyces bambergiensis*, commonly found as soil inhabitant and was first found by Wallhausser et al. [10] from soil of Bamberg, Germany. *Streptomyces prasinus* was reported to produce secondary metabolites, which were prasinomycin, prasinons A and B [11,12]. Thus far, no study has been reported on the production of actinorhodin by *Streptomyces prasinus*.

3.2. HPLC analysis

The HPLC analysis has successfully detected actinorhodin at retention time 25 min at 420 nm absorbance using combination of gradient acetonitrile and 0.1% acetic acid (Fig. 2). Taguchi et al. [13] reported that in vitro reconstitution systems of the actinorhodin biosynthetic pathway leading to (S)-DNPA had successfully detected and purified actinorhodin at a retention time of 25 min with absorbance at 420 nm.

3.3. Antimicrobial activity screening

The biopotential of *Streptomyces* sp. K2-11 with respect to their antimicrobial properties for actinorhodin was

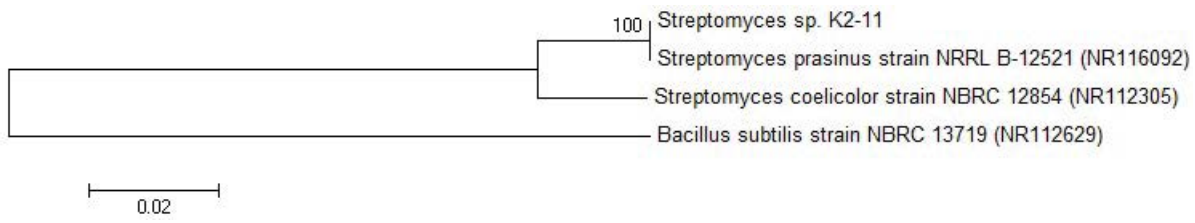

Fig. 1. Phylogenetic tree based on partial 16s rRNA sequence using neighbor–joining algorithm (Tamura-Nei 93 parameter model). *Bacillus subtilis* NBRC 13719 was used as an outgroup.

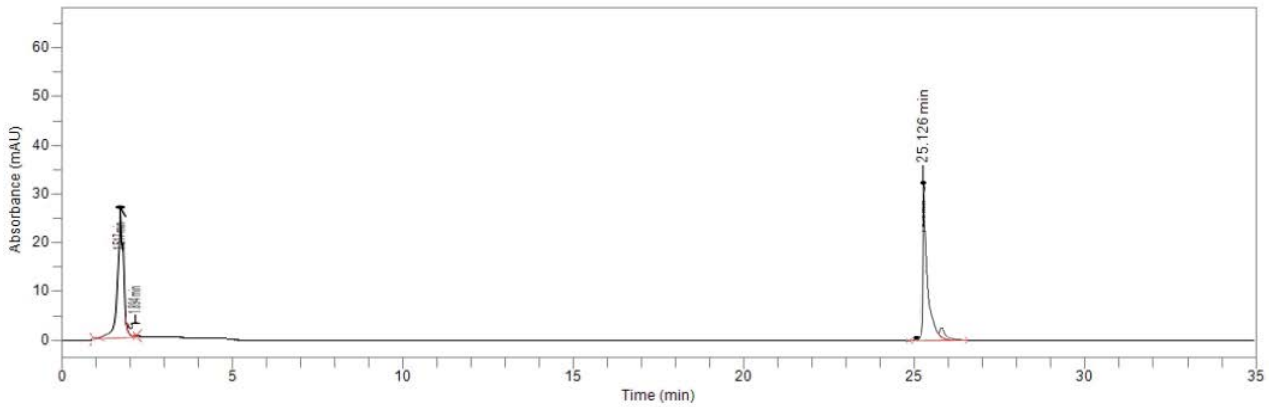

Fig. 2. HPLC profile for actinorhodin from *Streptomyces* sp. K2-11.

further evaluated for its antagonistic potential against two test organisms (two Gram-positive bacteria) using disc diffusion method.

Actinorhodin showed antibacterial activity against *Bacillus subtilis* IMR B 144/11 C (8.0 ± 0.2 mm), but no antibacterial activity was observed against of *S. aureus* ATCC 259233 (Table 1). According to Mak [14], the activity against *S. aureus* ATCC 259233 is intriguing because this strain is reported to be resistant to ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, cephalothin, doxycycline, gentamicin, erythromycin, imipenem, methicillin, penicillin, tetracycline, oxacillin, azithromycin, clindamycin, ceftriaxone, rifampin, amikacin and tobramycin, suggesting that actinorhodin can bypass this resistance. The absence of inhibition zone against of *S. aureus* in this study was probably due to the presence of different structure of actinorhodin [15]. Therefore, further investigation concerning actinorhodin produced by *Streptomyces* sp. K2-11 needs to be conducted in future.

3.4. *Streptomyces sp. K2-11 growth determination on different growth media*

According to Fig. 3, *Streptomyces* sp. K2-11 exhibited different exponential phase in different growth media. In SMMS, the growth reached exponential phase after 5 h of incubation, while in R2 and SYE the exponential phase was between 10 and 20 h, respectively. *Streptomyces* sp. K2-11 recorded the highest growth (9.50×10^6 CFU/mL) in SMMS, followed 3.43×10^6 CFU/mL in R2 and 2.43×10^6 CFU/mL in SYE.

3.5. *Antibiotic production optimization*

Based on Fig. 4, actinorhodin was produced considerably high at 33°C in 60 h of incubation with count of 1.95×10^{-6} mol/L. The lowest concentration of actinorhodin was recorded at 27°C (1.18×10^{-7} mol/L) in 10 h of incubation. Statistical data showed that if one variable change (media and incubation time), it will give significant change to the CFU/mL of the *Streptomyces* sp. K2-11. Analysed data revealed that there was no significant relationship between temperature and actinorhodin production but

Table 1
Antimicrobial activities of actinorhodin from *Streptomyces* sp. K2-11 using disc diffusion method

	Activity against[a] (mm)	
Antibiotic	*Bacillus subtilis*	*Staphylococcus aureus*
Actinorhodin	8.0 ± 0.2	–
Chloramphenicol	25.5 ± 0.6	24.2 ± 0.6

[a]Estimated by measuring the diameter of the clear zone of inhibition produced. Values are recorded as mean of three replications ± standard deviation.

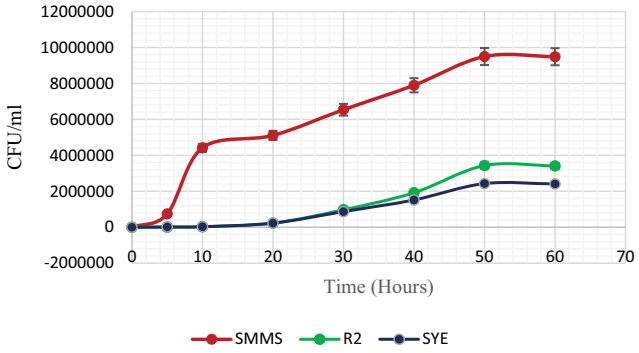

Fig. 3. Colony forming unit over time of *Streptomyces* in different media.

there was significant change of actinorhodin production over incubation time of *Streptomyces* sp. K2-11.

According to Wang et al. [16], the production of actinorhodin was highly depended on pH level. TThe shift of pH level of *Streptomyces* growth environment consequently effect the production of antibiotics [16–18]. Hence, this work has studied the correlation between pH and actinorhodin concentration. The calculation nevertheless has revealed a weak relationship between different pH level and actinorhodin production. Based on Fig. 5, actinorhodin was produced the highest (7.1×10^{-6} mol/L) at pH 5 after 60 h of incubation. The lowest concentration

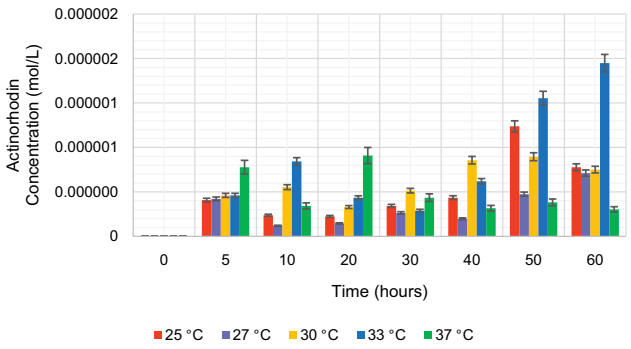

Fig. 4. Actinorhodin concentration production over time in different incubation temperature.

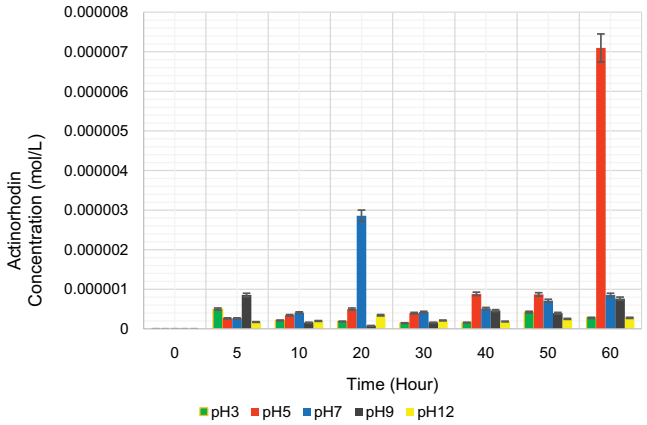

Fig. 5. Actinorhodin concentration production over time in different pH level.

of actinorhodin (7.80×10^{-8} mol/L) was recorded at pH 9 after 20 h of incubation. According to Cihák et al. [19], actinorhodin compounds (actinorhodinic acid and γ-actinorhodin) were detected around 5.5 pH.

Velayudham ans Murugan [20] mentioned that different glucose concentration will affect the production of antibiotics. El-sayed et al. [21] also added that altering glucose concentration in fed batch culture can yield maximum production of natamycin, produced from *Streptomyces natalensis*. However, some antibiotics were produced in scarce in a certain condition of *Streptomyces* growth. In a carbon depleted state, *Streptomyces* secrete secondary metabolite, which is mostly antibiotic, as a response mechanisms [22]. Accordingly, this work examined the effect on actinorhodin concentration as a function of incubation time at various levels of glucose concentrations. The study disclosed that actinorhodin production was the highest when nurtured with 50% w/v glucose concentration at 60 h (9.55×10^{-7} mol/L). Meanwhile in the presence of 75% w/v of glucose, the concentration of actinorhodin was at its lowest count (2.50×10^{-7} mol/L; 10 h of incubation; Fig. 6).

Statistical study shows that the production of actinorhodin had a significant relationship with incubation times in different glucose concentration. Forero et al. [23] reported that actinorhodin production was found to be strongly suppressed by the presence of high glucose concentration

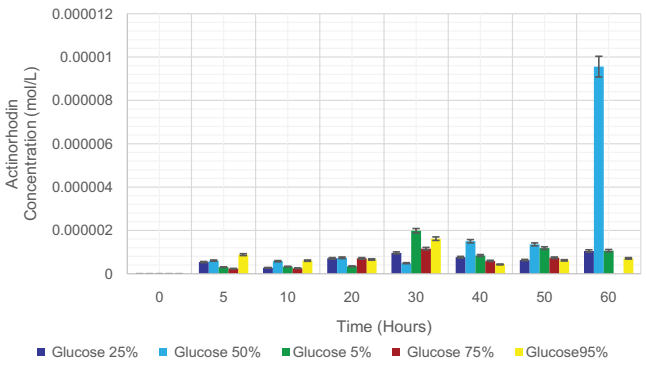

Fig. 6. Actinorhodin concentration production over time in different glucose level.

in *Streptomyces coelicolor*. When the used carbon source glucose was depleted, the *S. coelicolor* produced significant actinorhodin concentration.

4. Conclusion

Streptomyces sp. K2-11 was identified to have 99.62% similarity with *Streptomyces prasinus* strain NRRL-B 12521. Actinorhodin produced by *Streptomyces* sp. K2-11 has successfully detected by HPLC was proven very efficient on the *Bacillus subtilis* growth. In addition, *Streptomyces* sp. K2-11 growth was nourished in SMMS medium rather on SYE and R2 media. Finally and most importantly, manipulation of parameters (pH, temperature, glucose) has remarkably influenced actinorhodin yield in this study.

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