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Physiological Regulation for Enhancing Biosynthesis of Biofilm-inhibiting Secondary Metabolites in Streptomyces Cellulosae

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ABSTRACT

Although the production of the secondary metabolite is frequently restricted, methods to regulate and optimize their synthesis are extremely beneficial. The current study proposes to enhance the production of antibiofilm metabolite in *Streptomyces cellulosae* (S. cellulosae). It was isolated from soil by growing on Gause's media and identified by colony morphology and genomic sequencing of 16S rDNA. Antibacterial and antibiofilm activities of the isolates were screened against a series of pathogenic bacteria by agar plug diffusion and 96 well microtiter plate methods, respectively. Physiological regulation of the bacterial bioactivity against biofilm formation was monitored under different cultural conditions. The isolated *Streptomyces* sequence analysis of the 16S rDNA was 100% identical to the sequence of S. cellulosae strain NBRC 13027. Physical (temperature and pH) and chemical (carbon, nitrogen, and minerals) culture medium factors have shown variable impacts on the growth and bioactive substances of *S. cellulosae*. Moreover, results of simple linear regression and correlation suggested that most of the physiological regulations with the highest response ($r2= 0.85-0.99$; $p<0.01$) and linearly ($r= 0.88-0.99$; $p<0.01$) were correlated between microbial biomass and crude extract. Lastly, under different culture growth conditions, biofilm inhibition was tested against *Pseudomonas aeruginosa* (*P. aeruginosa*). The physiological regulation results exhibited that 1 µg/mL of the extract was the most efficient concentration against biofilm formation in *P. aeruginosa* while 3 µg/mL is an effective bactericidal dose against *P. aeruginosa*. We concluded that *S. cellulosae* can produce antibacterial and antibiofilm metabolites. Physiological regulation is considered a powerful tool that can be used for increasing the biosynthesis of the active metabolites and biomass.

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Introduction

Natural products are chemical compounds that are spontaneously produced by some biological species that are not specifically involved in the organism's normal growth and development (1). Historically, natural products have played a significant role in drug development and have been the basis of earliest medicines (2). Natural microbial compounds are considered the main and most promising sources of novel medications (3). However, there are a limited number of known bacterial species capable of producing such antimicrobial products. Members of the class Actinobacteria are responsible for producing over 7000 chemicals as natural antibacterial products (4).

In the last century, *Streptomyces* species have been critical in the production of secondary metabolites and drug development. This can be attributed to its wide range of biological activities including the production of a number of potential antibiotics that are used as treatments for infection (5). In addition, some species of *Streptomyces* are able to produce bioactive substances that act as antibiofilm agents (3). The development of microbial resistance against nearly all classes of antibiotics resulted in the loss of their therapeutic functions and their reputation as miracle drugs (6). Now, global healthcare is threatened and facing substantial risks of infectious diseases, potentially becoming one of the major causes of death worldwide (7, 8).

Bacterial signalling pathways control transcription and expression of virulence genes, antibiotic production, and regulation of vital phenotypic characteristics such as biofilm formation (9, 10).

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Biofilms are complex structures containing sessile cells. This trait was developed to enable bacteria to survive and grow in extreme environments (11). Numerous chronic and persistent illnesses are caused by biofilm-forming bacteria, and their proliferation is responsible for more than half of all nosocomial infections (12). Although the temporal aspect of secondary metabolism is undoubtedly a genetic trait, its expression may be considerably affected by environmental adjustments. As a result, secondary metabolism is frequently triggered by nutritional exhaustion, inducer addition, and/or a decline in growth rate (13).

The biosynthesis of biologically active compounds depends on bacterial growth conditions and the utilization of different macro- and micronutrients (14). The local environment determines the biodiversity of microorganisms in ecosystems, and that might contribute to the production of new secondary metabolites (15, 16). Thus, the capacity of bacteria to generate antimicrobial compounds under different environmental conditions can be regulated by enhancing or diminishing the production of secondary metabolites (17). Since the production of secondary metabolites is often limited, methods to regulate and improve their synthesis are highly beneficial (18). Manipulation of nutritional and physical characteristics of culture conditions can be used to improve growth and increase the production of biologically active compounds. Hence, media composition is critical to the productivity and profitability of the overall process. Thus, selecting the appropriate fermentation medium is crucial in the regulation and generation of secondary metabolites (19).

The current study aims to investigate the biosynthetic compounds that potentially have anti-biofilm activity against pathogenic bacteria and to regulate the synthesis of the secondary metabolites through the optimization of bacterial growth factors.

Materials and Methods

Soil sampling and Isolation of Streptomyces

Samples were obtained by removing 10 cm of the upper layer soil surface and collecting samples at 20-40 cm depth in search of *Streptomyces spp.* as previously described (20). Soil were suspended in sterile distilled water and serially dilutions were prepared to inoculate Gause's Synthetic Agar (GSA) plates (soluble starch: 20.0 g; KNO_3: 1.0 g; K_2HPO_4: 0.5 g; $MgSO_4 \cdot 7H_2O$: 0.5 g; NaCl: 0.5 g; $FeSO_4 \cdot 7H_2O$: 0.01 g; agar: 10.0 g; and distilled water: 1,000 mL) (21). The plates were incubated at 28°C for seven days, and cultured spores of Actinomycetes colonies were collected and stored at -80°C until use.

Fermentation (Batch culture method)

100 mL of tryptic soy broth (TSB) was inoculated with 100 µL spore stock ($1x10^8$ spores/mL) to provide homogenous growth and prevent clumping using Erlenmeyer flasks (500 mL) (22). Flasks were incubated at 28°C for 48 h in a shaking incubator at 200 rpm (Multi-Stack Shaking Incubator LSI-5004M, Indonesia). After adjusting the concentration of the pre-culture bacteria to OD_{600} = 0.1, a portion of the inoculum was inoculated (5% v/v) into TSB and incubated for 96 h.

Genomic DNA isolation

The isolated Actinomycetes with a potent antimicrobial activity were selected and aseptically inoculated into 250 mL screw cap bottles containing 30 mL of autoclaved TSB medium. They then were aerobically incubated at 28°C in a shaker incubator at 200 rpm for two days. After harvesting the biomass, genomic DNA was extracted using the Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) following manufacturer protocol. DNA quality and concentration were verified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

PCR amplification of Streptomyces 16S rDNA

PCR primers were designed to amplify the 16S rDNA fragment from the genomic DNA of *Streptomyces spp*. as previously described (23). The sequences and properties of the primers were: forward: 5'-GACAAGCCCTGGAAACGGGGT-3' and reverse 5'-GCTCGTGTCGTGAGATGTTGGG-3', synthesized by Macrogen (Seoul, South Korea). PCR was performed using a DNA thermocycler (Eppendorf, Hamburg, Germany) to amplify the targeted 16S rDNA fragments (~ 900 bp) of the soil isolated Actinomycetes. The reactions were performed in a final volume of 30 µL including 10 µL of Ready-to-use PCR mixture (prepared following manufacturer

protocol EasyTaq® PCR SuperMix (2×)), 1.5 µL of forward primer (10 pmol/µL), 1.5 µL of reverse primer (10 pmol/µL), 3 µL of genomic DNA template, 14 µL of ddH_2O. The program was performed as follows: initial denaturing at 95°C for five min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 40 sec, extension at 72°C for 45 sec, and final extension at 72°C for 3 min.

Gel electrophoresis and sequencing

The amplified PCR products (amplicon ~ 900 bp) were loaded on 1.5 % (W/V) pure agarose (diluted in 100 mL of 1X Tris Borate EDTA (TBE) buffer). 5 µL of the product was run through the gel using electrophoresis at 90 Volts for 1 h. Sequencing was performed by using the Sanger technique (Daejeon, Republic of Korea), and the consensus sequence was obtained using BioEdit version 7.2.5 software program.

Screening for biological activity

Antibacterial activity

Streptomyces was grown on GSA plates and incubated at 28°C for seven days to diffuse microbial metabolites into the agar medium during growth. After incubation, 7mm diameter cores of agar plugs were aseptically removed with a sterile Cork borer and face-to-face deposited on the agar surface (Muller Hinton agar) that previously inoculated the cells of *Micrococcus luteus* (*M. luteus*) ATCC 19404, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 9027, *Escherichia coli* (*E. coli*) ATCC 8739, *Salmonella abony* (*S. abony*) NTCT 6017, *Bacillus subtilis* (*B. subtilis*) ATCC 19659, *Staphylococcus aureus* (*S. aureus*) ATCC 6538, *Acinetobacter baumannii* (*A. baumannii*) ATCC 19606 and *Candida albicans* (*C. albicans*) ATCC 10231. Core deposition was followed by incubation at 30ºC for 24 h for bacteria and 48 h for fungal samples. The antimicrobial activity of the Actinomycetes was measured through the production of the inhibition zone around the agar core plugs (24, 25).

Antibiofilm activity

Screening for antibiofilm activity was performed using a crystal violet staining assay as described (26-28). The analysis was performed on 96-well, flat-bottomed, polystyrene plates (SPL life sciences, Korea).

After incubation at 28ºC and 200 rpm shaking for 96 h, the fermented *Streptomyces* was centrifuged at 25°C and 4000 rpm for 15 min. The cell-free supernatant was filtrated through a sterile syringe filter (pore diameter 0.22 µm). 50 µL of the filtrated culture was then used for antibiofilm screening. Standard bacterial strains were tested (*M. luteus* ATCC 19404, *P. aeruginosa* ATCC 9027, *E. coli* ATCC 8739, *S. abony* NTCT 6017, *B. subtilis* ATCC 19659, *S. aureus* ATCC 6538, *A. baumannii* ATCC 19606 and *C. albicans* ATCC 10231). Subsequent incubation at 37°C for 24 h for bacteria and 48 h for fungi was conducted. The experiments were carried out in five replicates, and the final values are reported as mean ± SD.

The planktonic cells were then washed three times with distilled water (DW) while the sessile cells in the biofilms remained bound to the bottom of the wells. After drying at room temperature, the biofilm cell mass was estimated by adding 200 µL of crystal violet (0.5% V/V) to each well followed by incubation at room temperature for 15 min. The excess unbound crystal violet was then washed and removed with 200 µL DW. The stained biofilm in each well was then resuspended in 200 µL ethanol: acetone (80:20) (v/v). The absorbance of dissolved biofilm was measured at 595 nm using a microplate reader (Biorad spectrophotometer, Beckman Instruments). Biofilm production was expressed as the intensity of the stain that bound to the plates.

Measurement of bacterial viability

Bacterial growth density was determined in both treated (broth medium mixed with extract) and untreated (broth medium alone) samples using the viable plate count method as previously described (29). Bacterial cultures were diluted ten-fold in a 96-well microplate by transferring 20 µL of bacterial culture into 180 µL sterilized DW. Then, from each dilution, 50 µL triplicates were placed on the surface of nutrient agar and left to dry. Plates were inverted and incubated at 37°C for 24 h. Results were estimated as CFU/mL.

Physiological Regulation

The biomass and generation of bioactive metabolites of the *Streptomyces cellulosae* (*S.*

cellulosae) were monitored and regulated physiologically. This was conducted through fermentation under physical (pH and temperature) and chemical (carbon sources, nitrogen sources, NaCl, $MgSO_4 \cdot 7H_2O$, K_2HPO_4, and $FeSO_4 \cdot 7H_2O$) variables of Gause's media.

The bacterial biomass was measured periodically at four-time intervals (24, 48, 72 and 96 h). Five replicates were measured for each time interval. The fermented cultures were extracted twice by using ethyl acetate 1:1 (v/v). The organic layers were separated and dried by a rotary evaporator (Heidolph, GmbH & CO., KG, Germany) under reduced pressure (9, 30). The dried crude product, which was reddish-brown in colour, was suspended in absolute methanol and stored at -20°C. Different concentrations of obtained extract (0.0, 0.5, 1, 2 and 3 µg/mL) were tested against biofilm-forming *P. aeruginosa* ATCC 9027 (previously prepared OD_{600}= 0.1, to prepare a ratio 1:100).

Influence of physical factors

100 mL of sterilized Gause's media was inoculated in triplicate with a stock of pre-cultured *S. cellulosae* in 250 mL Erlenmeyer flasks. A regulation under different pH values (5, 6, 7 and 8.5) was performed, and cultures were grown at 28°C in a shaking incubator (200 rpm) for 96 h. Fermentation under different temperatures (15, 20, 28 and 40°C) was also used as a method of physical regulation with an incubation period of 96 h. and a pH of 7 (31).

Influence of chemical factors

Media starch was substituted with other carbon sources such as sucrose, maltose, glucose and xylose at different concentrations (10, 15, 20 and 25 g/L). The impacts on regulation as a result of the substituted carbon sources were then examined (32). The nitrogen source (KNO_3), sodium nitrate, aspartic acid and peptone were tested at different concentrations (0.5, 1, 2 and 4 g/L).

Other ingredients including NaCl, $MgSO_4$ and K_2HPO_4 were tested at concentrations of 0, 0.5, 1 and 2 g/L while testing for $FeSO_4$ was arranged at a different set of concentrations (0, 5, 10 and 20 mg/L).

Estimation of biomass (dry cell weight)

Culture aliquots of 2 mL were collected at different time intervals (24, 48, 72, and 96 h) and transferred to pre-dried and pre-weighed Eppendorf tubes. They were then centrifuged at 10,000 rpm for 15 min at 4°C. Pellets were then collected and washed in sterile DW, centrifuged at 10,000 rpm for 15 min, and dried at 55°C to obtain biomass weight.

Statistical analysis

GraphPad Prism software version 6 (GraphPad, California, USA) was used for statistical analysis. The experimental results were expressed as the mean ± SD. Groups were compared by analysis of variance using a one-way ANOVA and a Dunnett's multiple comparisons test. A *p*-value of less than 0.01 was regarded as statistically significant. Significance was defined as single star (*) $p < 0.01$, and double stars (**) $p < 0.001$ (grey stars represent significant impact against biofilm and black stars represent significant impact against viable count). Pearson's correlation coefficients (r-value; $p<0.01$) between growth-metabolite data sets has been used as a common approach to identify the direction and strength of correlation between them. Likewise, a simple linear regression (r^2=value; $p<0.01$) was used to determine the response between growth-metabolite data sets.

Results and discussion

Morphology and molecular identification

Rough brown-grey colonies of *Streptomyces* were observed on Gause's agar, and the microscopic examination showed gram-positive, spore-forming, filamentous bacterium with extensively branched aerial mycelia. It showed that the terrestrial isolated bacterium is *Streptomyces* species.

Molecular identification by PCR amplification of 16S rDNA resulted in the generation of ~ 900 bp amplicon of DNA fragments. The sequence analysis of the amplified 16S rDNA was 100% identical to the sequence of *S. cellulosae* strain NBRC 13027. The DNA sequence was deposited in Genbank (NCBI) with an accession number OM978381.

Antimicrobial and Antibiofilm activities

Among the tested microbes, the *S. cellulosae* agar plug showed the highest inhibition activity against the growth of *P. aeruginosa* ATCC 9027, *C. albicans* ATCC 10231 and *A. baumannii* ATCC 19606 (25.0 ± 2.6 mm; 21.0 ± 3.0 mm; 20.33 ± 3.1 mm, respectively). The lowest inhibition zone, however, was recorded against the growth of *E. coli* ATCC

8739 (13.0 ± 2.0 mm) (Table 1). The biofilm inhibition of the filtrated *Streptomyces* culture against *P. aeruginosa* showed the highest impact (p<0.01) among the other tested bacteria (Table 1). Thus, *P. aeruginosa* ATCC 9027 was selected as a biological indicator for quantitative analysis of the biofilm inhibiting bioactive metabolites.

Table 1. The effects of active products of *S. cellulosae* against growth and biofilm of some microbes using agar plugs diffusion assay and crystal violet staining method for biofilm formation. Data represent the mean ± SD of three independent experiments (p<0.01).

Tested strains	Inhibition Zone (mm), (mean ± SD)	Biofilm Inhibition
Micrococcus luteus ATCC 19404	19.2± 2.8	+
Pseudomonas aeruginosa ATCC 9027	25.0± 2.6	++
Escherichia coli ATCC 8739	13.0 ± 2.0	+
Salmonella aboney NTCT 6017	13.8 ± 2.0	+
Acinetobacter baumannii ATCC 19606	20.3 ± 3.1	+
Bacillus subtilis ATCC 19659	13.2 ± 1.3	+
Staphylococcus aureus ATCC 6538	19.2 ± 1.0	-
Candida albicans ATCC 10231	21.0 ±3.0	+

(++): Highly significant effect, (+): significant effect, (-): No significant effect.

Physiological regulation of growth and metabolite production

It has been shown that chemical and physical factors are required for the growth and production of biologically active metabolites. The linear regression provides a suitable model to describe the response between microbial metabolites production and microbial biomass (Table 2).

The highest response (r^2= 0.94 and 0.92; p<0.01) against microbial biomass were recorded for glucose and starch, respectively, while $NaNO_3$, aspartic acid and peptone (as nitrogen sources) exhibited a different response (r^2= 0.98, 0.95 and 0.93; p<0.01, respectively). Addition of inorganic salts to the medium (K_2HPO_4, $MgSO_4$, $FeSO_4$ and NaCl) resulted in higher responses between metabolites and microbial biomass (r^2= 0.99, 0.98, 0.96 and 0.94; p<0.01, respectively).

The results of conducting the test for biomass-metabolites variables under different temperatures and pH values are reported as r^2= 0.94 and 0.85, p<0.01, respectively. Moreover, the results of Pearson's correlation coefficients showed the addition of sucrose and KNO_3 to the medium with negative r= -0.71 and weak r= 0.51 (p<0.01) correlation between biomass and yield of crude extract. Not surprisingly, the correlation of other factors showed that the addition of macronutrients, inorganic salts, physical factors on microbial biomass and the quantity of crude extract exhibited a strong and positive correlation with one another (r= 0.88-0.99; p<0.01). Thus, these results showed that greater microbial growth (biomass) was strongly associated with the production of bioactive metabolites (Table 2).

Table 2. Impacts of applying different chemical and physical factors on the values of simple correlation (r values) and simple linear regression between microbial biomass and dry weight of secondary metabolites (mg/mL). Simple correlation (R-values) to define the direction and strength of the relationship. Simple linear regression (R^2 values) to define the strength response between two variables (p<0.01).

Parameters		
I. Effects of Macronutrients on relationship between biomass (mg/mL) & dry weight metabolites (mg/mL)		
A. Addition of different carbon sources to the medium (g L^{-1})	Simple correlation (R-values)	Simple linear regression (R^2-values)
1. Starch (10, 15, 20, 25 g L^{-1})	0.96	0.92
2. Xylose (10, 15, 20, 25 g L^{-1})	0.88	0.79
3. Maltose (10, 15, 20, 25 g L^{-1})	0.79	0.63
4. Sucrose (10, 15, 20, 25 g L^{-1})	-0.71	0.51
5. Glucose (10, 15, 20, 25 g L^{-1})	0.97	0.94
B. Addition of different nitrogen sources to the medium (g/L)	Simple correlation (R-values)	Simple linear regression (R^2-values)
1. KNO_3 (0.5, 1, 2, 4 g L^{-1})	0.51	0.26
2. Peptone (0.5, 1, 2, 4 g L^{-1})	0.96	0.93
3. $NaNO_3$ (0.5, 1, 2, 4 g L^{-1})	0.99	0.98
4. Aspartic acid (0.5, 1, 2, 4 g L^{-1})	0.97	0.95
II. Effects of Microelements on relationship between biomass (mg/mL) & dry weight metabolites (mg/mL)		
Addition of different mineral sources to the medium (g/L).	Simple correlation (R-values)	Simple linear regression (R^2-values)
1. NaCl (0, 0.5, 1, 2 g L^{-1})	0.97	0.94
2. $MgSO_4$ (0, 0.5, 1, 2 g L^{-1})	0.99	0.98
3. K_2HPO_4 (0, 0.5, 1, 2 g L^{-1})	0.99	0.99
4. $FeSO_4$ (0, 0.5, 1, 2 g L^{-1})	0.98	0.96
III. Effects of physical factors on relationship between biomass (mg/mL) & dry weight metabolites (mg/mL)		
Different pH (8.5, 7.2, 6, 5)	0.92	0.85
Different Temperatures (15, 20, 30,40 °C)	0.97	0.94

In our study, using 1µg/mL of crude extract displayed a significant biofilm inhibition among the tested concentrations (0.0, 0.5, 1 and 2 µg/mL) under different culturing conditions. Different volumes (0.0, 0.5, 1, 1.5, 2 µg/mL) of the extracted metabolites at different pH values (8.5, 7, 6, and 5) and various temperatures (15, 20, 28, and 40°C) exhibited variable inhibitory impacts against the biofilm of *P.*

aeruginosa ATCC 9027. Figure (1 A) illustrates that regulation under pH 8.5 and pH 7 with 1 µg/mL of extracted metabolites extremely reduced the biofilm formation of *P. aeruginosa* (OD_{595nm}= 0.97 ± 0.06 and 1.63 ± 0.39, respectively) when compared to the untreated group (OD_{595nm}= 2.38 ± 0.19). This reduction of biofilm formation at pH 8.5 and pH 7 was accompanied with no significant effect on the bacterial viable count (log_{10}CFU/mL= 11.12 ± 0.06 and 11.13 ± 0.01 respectively, in comparison with the untreated culture (log_{10}CFU/mL= 11.13 ± 0.01). Using 3 µg/mL of the extracted culture at pH 8.5, 7 and 6 was bactericidal against *P. aeruginosa* (log_{10}CFU/mL= 10.88 ± 0.02, 10.87 ± 0.02, and 11.04 ± 0.03, respectively).

In terms of the influence of incubation temperature, it is evident that 28°C provided the most effective biofilm inhibition (Figure 1 B). The extracted metabolites exhibited the most significant biofilm inhibition when 1 µg/mL was used (OD_{595nm}= 2.61 ± 0.08) while it was OD_{595nm}= 3.05 ± 0.02 in the raw bacterial growth. However, the bactericidal effect shown was log_{10}CFU/mL= 11.07 ± 0.01 and 11.07 ± 0.02 respectively, when 3 µg/mL of extracted metabolites under 20°C and 28°C incubation conditions were used.

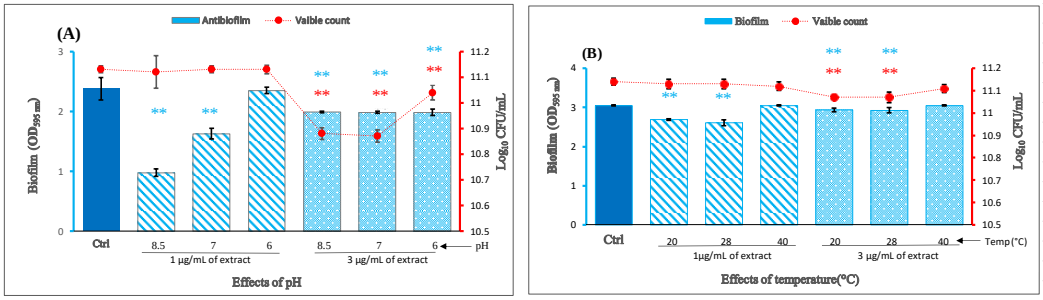

Figure 1. Physiological regulation of extracted metabolites (1 µg/mL and 3 µg/mL) (A): pH values (8.5, 7, 6 and 5). (B): Temperature degrees (15 °C, 20 °C, 28 °C, and 40 °C) for 96 h incubation. Ctrl: untreated culture; red color: represents the effects of crude extract on viable count of *P. aeruginosa*; blue color: represents the effect of the crude extract on biofilm of *P. aeruginosa*.

Different carbon sources (starch, xylose, maltose, sucrose and glucose) at different concentrations (10 g/L, 15 g/L, 20 g/L, 25 g/L) were tested (Figure 2). The minimum amount of carbon that is required to enhance the bioactivity of *S. cellulosae* against biofilm formation of *P. aeruginosa* is 15 g/L or more. Accordingly, the substitution of starch in culture media with glucose yielded the most effective crude extract, in which, the addition of 1 µg/mL significantly inhibited the formation of biofilm (OD_{595nm}= 2.76 ± 0.07 without variation in bacterial viability (log_{10}CFU/mL= 11.12 ± 0.017. Furthermore, using 3 µg/mL of the extract were lethal for *P. aeruginosa* (log_{10}CFU/mL= 10.88 ± 0.04) in comparison to that of control group (log_{10}CFU/mL = 11.14 ± 0.01).

A relatively similar antibiofilm effect was recorded when the starch was replaced with 15 g/L of maltose. Here, a 1 µg/mL of crude extract (OD_{595nm}= 2.10 ± 0.12) exhibited antibiofilm impact with no significant decline in viable count (log_{10}CFU/mL= 11.13 ± 0.02) (Figure 2), while 3 µg/mL of crude extracted culture (starch, sucrose and glucose supplemented groups) displayed a significant bactericidal effect (log_{10}CFU/mL= 10.88 ± 0.04, 10.98 ± 0.03 and 10.88 ± 0.02, respectively) when compared to untreated bacteria (log_{10}CFU/mL= 11.14 ± 0.01).

With regards to the substitution of the nitrogen sources (KNO_3), aspartic acid, $NaNO_3$ and peptone at various concentrations (0.5 g/L, 1 g/L, 2 g/L and 4 g/L) were examined, and it was found that the 1 µg/mL of the culture extract was the most effective biofilm inhibition dose (Figure 3).

Replacing KNO_3 with aspartic acid yielded the most effective crude extract against biofilm formation of *P. aeruginosa* without significant inhibition of growth. It became evident that at least 1 g/L of the nitrogen source (KNO_3, $NaNO_3$ and aspartic acid) was essential for culturing *S. cellulosae* to produce biofilm inhibitory metabolites (OD_{595nm}= 3.01 ± 0.06, 2.97 ± 0.05 and 2.90 ± 0.18, respectively) (Figure 3), whereas the measurements of viable count stayed unchanged (log_{10}CFU/mL= 11.13 ± 0.02, 11.14 ± 0.01 and 11.13 ± 0.02, respectively).

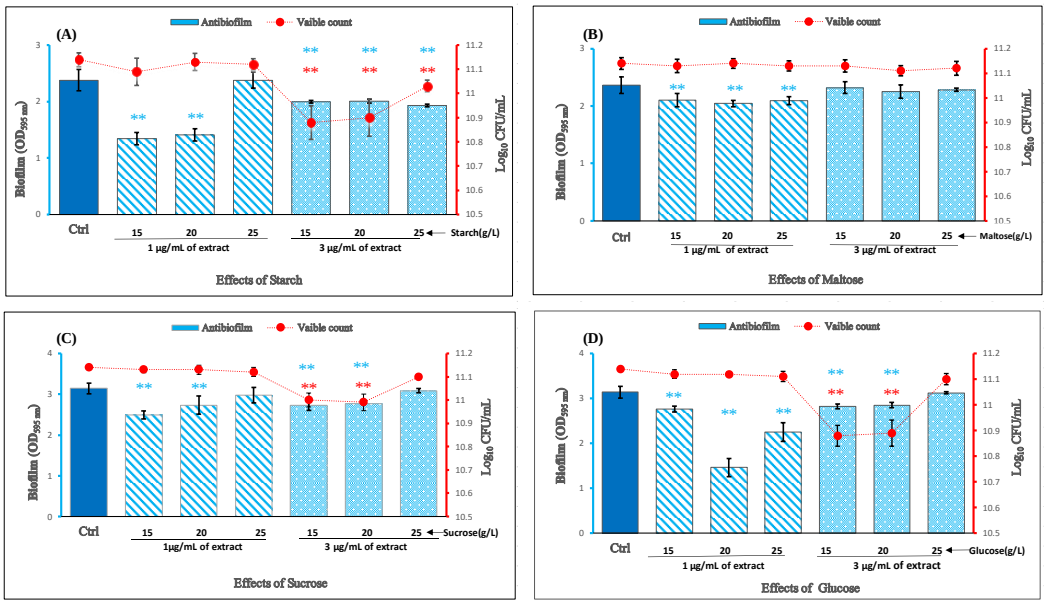

Figure 2. Physiological regulation of the extracted metabolites (1 µg/mL and 3 µg/mL) from S. cellulosae grown under shaker incubator at 28°C for 96 h and pH 7.2 in different carbon sources. Ctrl: untreated culture; red color: represents the effects of crude extract on viable count of *P. aeruginosa*; blue color: represents the effect of the crude extract on biofilm of *P. aeruginosa*.

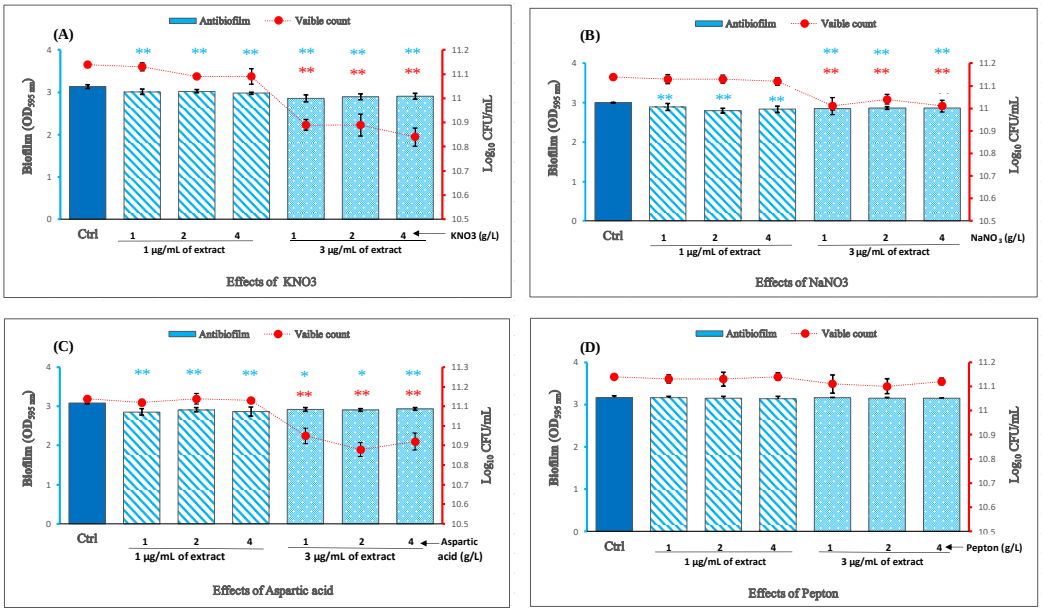

Figure 3. Physiological regulation of the extracted metabolites (1 µg/mL and 3 µg/mL) from S. cellulosae grown under shaker incubator at 28°C for 96 h and pH 7.2 in different nitrogen sources. Ctrl: untreated culture; red color: represents the effects of crude extract on viable count of *P. aeruginosa*; blue color: represents the effect of the crude extract on biofilm of *P. aeruginosa*.

The addition of inorganic minerals to the medium showed that 0.5 g/L of NaCl, $MgSO_4$, K_2HPO_4 and 5 mg/L of $FeSO_4$ were enough to yield effective crude extract in inhibiting biofilm formation (Figure 4). Using 1 µg/mL of the crude extracts significantly reduced the biofilm (OD_{595nm}= 2.90 ± 0.09, 2.87 ± 0.12 and 2.99 ± 0.02, respectively).

Supplementing the media with 5 mg/L of $FeSO_4$ is crucial to enhance production of the biofilm inhibition (OD_{595nm}= 2.87 ± 0.03). The bacterial growth stayed unchanged when testing 1 µg/mL of the metabolite

(log_{10}CFU/mL= 11.13 ± 0.02, 11.12 ± 0.02, 11.12 ± 0.01 and 11.13 ± 0.02, respectively) while 3 µg/mL significantly reduced the value of bacterial viable count (log_{10}CFU/mL= 11.03 ± 0.02, 11.01 ± 0.03, 11.01 ± 0.02 and 11.05 ± 0.02, respectively) (Figure 4).

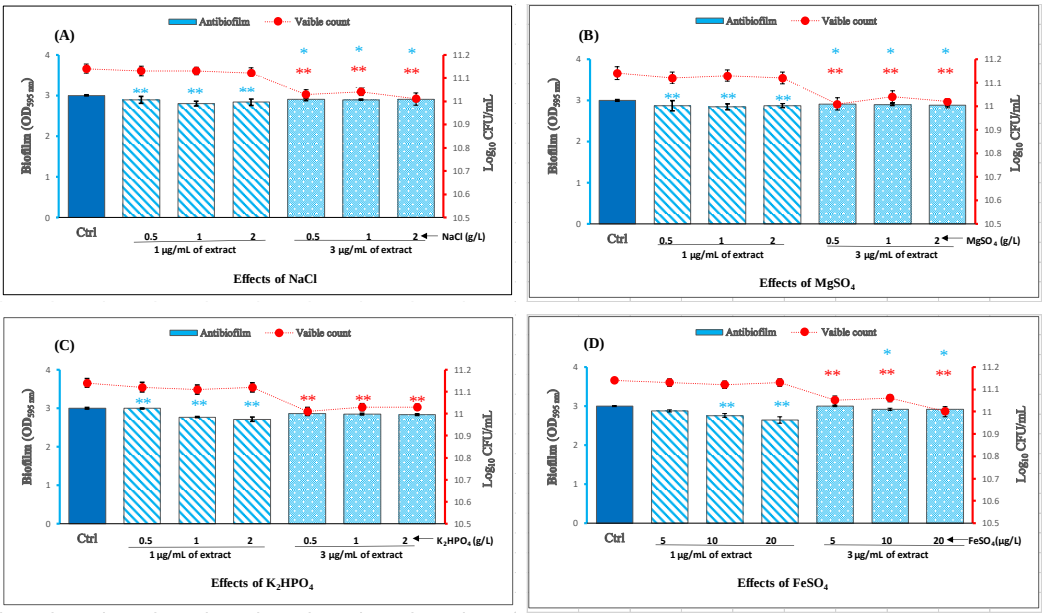

Figure 4. Physiological regulation of the extracted metabolites (1 µg/mL and 3 µg/mL) from S. cellulosae grown under shaker incubator at 28°C for 96 h and pH 7.2. with different minerals (A: NaCl; B: MgSO4; C: K2HPO4 and D: FeSO4). Ctrl: untreated culture; red color: represents the effects of crude extract on viable count of *P. aeruginosa*; blue color: represents the effect of the crude extract on biofilm of *P. aeruginosa*.

Bioactive metabolite production by *Streptomyces spp.* is typically based on bacterial strain, cultural conditions and nutritional requirements in the fermentation medium (33). Biosynthesis of antibiotic compounds is extremely dependent on fermentation conditions which must strike a balance between supplying nutrients for growth, serving as antibiotic precursors, and managing regulatory effects of some of the most potent carbon, nitrogen and phosphate sources (34). It is well articulated that manipulation of the chemical and physical factors of the fermentation medium can be used to improve the growth and production of secondary metabolites (35). Secondary metabolism typically occurs in the overdue growth phase of the producing microorganisms. The temporal nature of secondary metabolism is undeniably genetic; however, expression may be significantly impacted by environmental manipulations (36).

In the current study, agar plug diffusion assays indicated that *S. cellulosae* significantly inhibited the growth of *P. aeruginosa*. Indupalli *et al*. (2015) concluded that *S. cellulosae* crude ethyl acetate extract has potential antimicrobial activities against a wide range of bacteria and fungi (37). The capacity of *Streptomyces* cultures to produce bioactive metabolites is a variable feature that may be considerably enhanced or entirely lost depending on the nutrient availability and growth conditions (38). It has been shown that the addition or depletion of essential nutrients, such as carbon, nitrogen, phosphate and other trace elements, and physical factors have a direct effect on the biosynthesis of antibiotics (39).

Results of the present study showed that alkaline pH (8.5) was the optimum condition for the growth and yield of secondary metabolites. Other studies have shown that the biosynthetic process in productive strains is very sensitive to pH variations (40). Culture media pH has a considerable influence on microorganismal growth kinetics and the production of several enzymes that catalyse metabolite production as well as complex physiological phenomena, including regulation of the secondary metabolite production (40-42). As fermentation progresses, the acidity of the culture medium increases, and thus, the alkaline pH may act as a

buffer to neutralize the acidic conditions and provide optimum pH for bacterial growth and biosynthesis production (43). For reasonably excellent growth and antibacterial metabolite synthesis, *S. cellulosae* displayed a restricted range of tested incubation among four different temperatures. The results emphasized that the maximum production of biomass and bioactive metabolites was obtained at 28°C. Previous studies on *Streptomyces fradiae, Streptomyces kanamyceticus* and *Streptomyces griseolus* demonstrated that incubation temperatures ranging from 25-30°C critically affect growth mass and biosynthesis of neomycin, kanamycin and anisomycin, respectively (44, 45). Consistent with the results reported here, Tawfik *et al.* (1991) reported that incubation above 35°C significantly lowered the cell mass and production of antibiotics by *Streptomyces aureofaciens* and *Streptomyces roseviolaceus* (46).

In a previous study, researchers discovered that different concentrations of carbon sources have different impacts on the generation of biomass and precursors for biosynthesis of secondary metabolites in *Streptomyces spp.* (47). The influence of carbon sources on antibiotic synthesis has been the focus of ongoing research for both industry and academic organizations from both a fermentation standpoint and a biochemical and molecular biology perspective (36). Carbon sources are involved with several regulatory mechanisms for the onset, maintenance and cessation of secondary metabolism (48). In the current work, glucose exhibited the most significant effect on bacterial biomass compared to the other tested carbon sources. Glucose is the simplest monosaccharide that is utilized by most bacterial species. The regulatory effects of glucose on growth and secondary metabolite biosynthesis were previously examined in both *Streptomyces hygroscopicus* and *Streptomyces griseus* (49-51). Another previous study demonstrated that supplementing the culture media with glucose results in a twofold increase in bacterial growth mass (52). Sanchez *et al.* (2010) reported that a carbon source's regulation is contingent on the desired carbon source's consumption, however, with variable impact on responsible genes for the production of secondary metabolites (50).

The current study also investigated the most effective type and quantity of nitrogen sources in the culture medium that efficiently enhanced the production of biomass and bioactive metabolites. Organic nitrogen sources more successfully induce *Streptomyces* in biomass and bioactive metabolite production when compared to inorganic nitrogen sources (35, 53, 54). Some data suggest that not all nitrogen sources support growth equally; bacterial growth in media with rich nitrogen sources is greater than growth in media with poor nitrogen sources (55). It has been reported that regulatory signals from nitrogen metabolism elicit effects at the transcription, translation and post-translational levels, which are necessary in the biosynthesis of antibiotics (56). The flow of nitrogen along the various antibiotic biosynthesis pathways occurs either through nitrogen-containing precursor amino acids (incorporated intact into the antibiotic molecule) or by an antibiotic-specific reaction (the nitrogen atom of the primary metabolite is transferred to a specific intermediate) (55).

Phosphate is considered the most important inorganic component that influences the growth and production of metabolites in a wide range of bacterial strains (38). Previous studies showed that the presence of inorganic phosphate in culture media regulates the synthesis of a wide range of secondary metabolites from various biosynthetic groups including macrolides, tetracyclines, polyether compounds, aminoglycosides and amino acid-derived metabolites (57-60). Similar to the results reported here, another study exhibited that K_2HPO_4 critically impacts the production of neomycin by *S. fradiae* (61). Narayana and Vijayalakshmi (2008) reported that K_2HPO_4 significantly increased the synthesis of antibiotics in *Streptomyces albidoflavus* (53). Additionally, inorganic phosphate is also important for microbial growth enhancement and biosynthesis of various antibiotics (62). Some evidence shows that inorganic phosphate regulates the biosynthesis of secondary metabolites at both the transcriptional and posttranscriptional levels (63, 64).

The overall trend in quantitative biofilm analysis showed inhibitory impacts of extracted metabolites from *S. cellulosae* against *P. aeruginosa.* This might be attributed to the interference of extracted metabolites with the quorum sensing of the biofilm's former bacteria. Chemical signals are more likely to communicate with responsive cells in biofilms with

higher cell densities (65). Inhibition of the biofilm formation may help in the reduction of antimicrobial resistance without inhibiting growth (66). To support this explanation, Park *et al.* (2005) emphasized that *Streptomyces spp*., rather than being an acyl-homoserine lactones (AHL) competitor, can interfere with quorum sensing via an extracellular AHL-degrading enzyme (67). In our study, the inhibitory effects of the extracted metabolite were dose-dependent. Accordingly, using 1µg/mL of the extract reduced the formation of biofilm without killing *P. aeruginosa* while a higher extract volume (2 µg/mL) reduced viable cell count as well as biofilm formation of *P. aeruginosa*. Some reports indicated that certain macrolide antibiotics are capable of repressing *P. aeruginosa* AHL synthesis when applied at subminimal growth-inhibitory concentrations (68, 69).

It seems that *Streptomyces* is an important source of biofilm inhibitors; it produces a variety of types of substances that modulate swarming motility and decreases the availability of iron for biofilm formation in Gram-negative bacteria (26). For instance, Tateda *et al.* (2001) hypothesized that azithromycin may diminish the development of quorum-sensing-dependent virulence factors in *P. aeruginosa* by inhibiting the synthesis of the autoinducer molecules through undefined mechanisms (69). Furthermore, *Streptomyces* can produce a variety of biologically active secondary metabolites simultaneously (70); thus, the extracted metabolites may contain different compounds including biofilm inhibitors and bactericidal compounds with a dose-dependent impact. To this extent, 2 µg/mL of the crude extract was needed to inhibit bacterial viability, while only 1 µg/mL of the extract was required to exhibit antibiofilm activity.

Conclusions

In the present study, the isolated *S. cellulosae* produced a valuable bioactive metabolite with potent antibiofilm and antimicrobial activities against *P. aeruginosa*. The diverse nature of metabolite regulation in *Streptomyces* is one of the most outstanding features. Furthermore, the biomass and metabolite production were monitored and regulated physiologically by both physical and chemical parameters that exhibited variable impacts. Further studies are required to identify and elucidate the structure of the biofilm inhibitor compound and the mechanism of action against biofilm formation in *P. aeruginosa*.

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Authors' Contribution

This research was performed under supervision of Professor Dr. Shwan Kamal Rachid and Dr. Hastyar Najmuldeen, both have equal role in study design, planning and reviewing the manuscript. The sample collection, lab working and manuscript writing was achieved by Kochar Idrees Mahmood.

Interest conflict

The authors declare that they have no conflict of interest.

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References

1. Martín JF, Casqueiro J, Liras P. Secretion systems for secondary metabolites: how producer cells send out messages of intercellular communication. Curr opin microbiol 2005; 8(3): 282-293.
2. Butler MS. Natural products to drugs: natural product-derived compounds in clinical trials. Nat Prod Rep Jun 2008; 25(3): 475-516.
3. Newman DJ, Cragg GMJJonp. Natural products as sources of new drugs over the last 25 years. Journal of natural products 2007; 70(3): 461-477.
4. Berdy J. Bioactive microbial metabolites. J Antibiot Jan 2005; 58(1): 1-26.
5. Dong YH, Wang LH, Xu JL, Zhang HB, Zhang XF, Zhang LH. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. Nature Jun 14 2001; 411(6839): 813-817.
6. Wright GD. Antibiotic resistance in the environment: a link to the clinic? Current opinion in microbiology Oct 2010; 13(5): 589-594.
7. Barbosa T, Levy, SB The impact of antibiotic use on resistance development and persistence. Drug Res upd 2000; 3(5): 303-311.
8. Kazemi E, Kahrizi D. Lack of association between gastric cancer and hopq alleles in Helicobacter pylori. Genetika 2016; 48(3): 893-902.

9. Khattab AI, Babiker EH, Saeed HA. Streptomyces: isolation, optimization of culture conditions and extraction of secondary metabolites. Int Curr Pharm J 2016; 5(3): 27-32.
10. Raghava Rao KV, Mani P, Satyanarayana B, Raghava Rao T. Purification and structural elucidation of three bioactive compounds isolated from Streptomyces coelicoflavus BC 01 and their biological activity. 3 Biotech 2017.
11. Berlanga M, Guerrero R. Living together in biofilms: the microbial cell factory and its biotechnological implications. Microbial cell factories Oct 1 2016; 15(1): 165.
12. Donlan RM. Biofilm formation: a clinically relevant microbiological process. Clin Infect Dis Oct 15 2001; 33(8): 1387-1392.
13. Bibb MJ. Regulation of secondary metabolism in streptomycetes. Current opinion in microbiology Apr 2005; 8(2): 208-215.
14. Yoon YJ, Choi CY. Nutrient effects on FK-506, a new immunosuppressant, production by Streptomyces sp. in a defined medium. J Ferment Bioeng 1997; 83(6): 599-603.
15. Gloer J. 15 Applications of Fungal Ecology in the Search for New Bioactive Natural Products. J Environ Microb relationships 2007; 4: 257.
16. Sharma A. Streptomyces. In: Robinson RK, ed. *Encyclopedia of Food Microbiology*. Oxford: Elsevier; 1999: 2134-2138.
17. Vijayakumar R, Panneerselvam K, Muthukumar C, Thajuddin N, Panneerselvam A, Saravanamuthu R. Optimization of Antimicrobial Production by a Marine Actinomycete Streptomyces afghaniensis VPTS3-1 Isolated from Palk Strait, East Coast of India. Indian J microbiol Jun 2012; 52(2): 230-239.
18. Rachid S, Gerth K, Kochems I, Müller R. Deciphering regulatory mechanisms for secondary metabolite production in the myxobacterium Sorangium cellulosum So ce56. Mol Microbiol 2007; 63(6): 1783-1796.
19. Gao H, Liu M, Liu J et al. Medium optimization for the production of avermectin B1a by Streptomyces avermitilis 14-12A using response surface methodology. Bioresour Technol 2009; 100(17): 4012-4016.
20. Pandey A, Srivastava A, Chandra N, Kumar D, Kumar S. Purification and characterization of bioactive secondary metabolites isolated from soil bacteria. Indian J Microbiol Res 2019; 5(2): 188-193.
21. Wang D-s, Xue Q-h, Ma Y-y, Wei X-l, Chen J, He F. Oligotrophy is helpful for the isolation of bioactive actinomycetes. Indian J microbiol 2014; 54(2): 178-184.
22. Muhamadali H, Xu Y, Ellis DI et al. Metabolomics investigation of recombinant mTNFα production in Streptomyces lividans. Microb cell fact 2015; 14(1): 1-12.
23. Monciardini P, Sosio M, Cavaletti L, Chiocchini C, Donadio S. New PCR primers for the selective amplification of 16S rDNA from different groups of actinomycetes. FEMS Microbiol Ecol Dec 1 2002; 42(3): 419-429.
24. Jiménez-Esquilín A, Roane T. Antifungal activities of actinomycete strains associated with high-altitude sagebrush rhizosphere. J Ind Microbiol Biotech 2005; 32(8): 378-381.
25. Silambarasan S, Kumar EP, Murugan T, Saravanan D, Balagurunathan R. Antibacterial and antifungal activities of Actinobacteria isolated from Rathnagiri hills. J Appl Pharma Sci 2012.
26. Kim YG, Lee JH, Kim CJ et al. Antibiofilm activity of Streptomyces sp. BFI 230 and Kribbella sp. BFI 1562 against Pseudomonas aeruginosa. Appl Microbiol Biotechnol Dec 2012; 96(6): 1607-1617.
27. Najmuldeen H, Alghamdi R, Alghofaili F, Yesilkaya H. Functional assessment of microbial superoxide dismutase isozymes suggests a differential role for each isozyme. Free Radical Biol Med 2019; 134: 215-228.
28. Suo Y, Huang Y, Liu Y, Shi C, Shi X. The expression of superoxide dismutase (SOD) and a putative ABC transporter permease is inversely correlated during biofilm formation in Listeria monocytogenes 4b G. PloS one 2012; 7(10): e48467.
29. Miles AA, Misra SS, Irwin JO. The estimation of the bactericidal power of the blood. Epidemiol Infec Nov 1938; 38(6): 732-749.
30. Elleuch L, Shaaban M, Smaoui S et al. Bioactive secondary metabolites from a new terrestrial Streptomyces sp. TN262. Appl Biochem Biotechnol Sep 2010; 162(2): 579-593.
31. Da Silva I, Martins M, Carvalho C, De Azevedo J, de Lima Procópio R. The effect of varying culture conditions on the production of antibiotics by Streptomyces spp. Isolated from the Amazonian Soil Ferment Technol 2012; 1(3).
32. Jonsbu E, McIntyre M, Nielsen J. The influence of carbon sources and morphology on nystatin production by Streptomyces noursei. J Biotechnol Biomater May 9 2002; 95(2): 133-144.
33. Durante-Mangoni E, Grammatikos A, Utili R, Falagas ME. Do we still need the aminoglycosides? Int J Antimicrb agents 2009; 33(3): 201-205.
34. Coisne S, Bechet M, Blondeau R. Actinorhodin production by Streptomyces coelicolor A3 (2) in iron-restricted media. Lett Appl Microbiol 1999; 28(3): 199-202.
35. Bundale S, Begde D, Nashikkar N, Kadam T, Upadhyay A. Optimization of culture conditions for production of bioactive metabolites by Streptomyces spp. isolated from soil. Adv Microbiol 2015; 5(06): 441.
36. Ruiz B, Chávez A, Forero A et al. Production of microbial secondary metabolites: regulation by the carbon source. Crit Rev Microbiol 2010; 36(2): 146-167.
37. Indupalli MD, Muvva V, Munaganti RK. Streptomyces cellulosae VJDS-1, a promising source for potential bioactive compounds. Int J Pharm Pharm Sci 2015; 7: 57-61.

38. Hassan MA, El-Naggar MY, Said WY. Physiological factors affecting the production of an antimicrobial substance by Streptomyces violatus in batch cultures. Egyptian J Biol 2001; 3(1): 1-10.
39. Abbanat D, Maiese W, Greenstein M. Biosynthesis of the pyrroindomycins by Streptomyces rugosporus LL-42D005; characterization of nutrient requirements. J Antibiot 1999; 52(2): 117-126.
40. Datta A, Kothary MH. Effects of glucose, growth temperature, and pH on listeriolysin O production in Listeria monocytogenes. Appl Environ Microbiol 1993; 59(10): 3495-3497.
41. Guimarães LM, de Araujo Furlan RL, Garrido LM, Ventura A, Padilla G, Facciotti MCR. Effect of pH on the production of the antitumor antibiotic retamycin by Streptomyces olindensis. Biotechnol Appl biochem 2004; 40(1): 107-111.
42. Sole M, Francia A, Rius N, Loren J. The role of pH in the 'glucose effect'on prodigiosin production by non-proliferating cells of Serratia marcescens. Lett Appl Microbiol 1997; 25(2): 81-84.
43. Rao KVR, Rao DB, Satyanarayana B. Optimization of Cultural Conditions for Production of Antibacterial Metabolites from Streptomyces coelicoflavus BC 01. Not Sci Biol 2015; 7(2): 151-159.
44. Abdel-Aal T, Abdelwahed N, Awad G, El Diwany A, Haroun B. Improvement of anisomycin production through mutation and medium optimization for Streptomyces griseolus. Aust J Basic Appl Sci 2011; 5(12): 2637-2648.
45. Vastrad B, Neelagund S. Optimization and production of neomycin from different agro industrial wastes in solid state fermentation. Int J Pharma Sci Drug Res 2011; 3(2): 104-111.
46. Tawfik K, Ramadan E. Factors affecting the biological activity of Streptomyces aureofaciens MY18 and Str. roseviolaceus MR13. Sci 1991; 3(1).
47. Oskay M. Effects of some Environmental Conditions on Biomass and Antimicrobial Metabolite Production by Streptomyces Sp., KGG32. Int J Agric Biol 2011; 13(3).
48. Sanchez S, Demain AL. Metabolic regulation of fermentation processes. Enzyme Microb Technol 2002; 31(7): 895-906.
49. Gesheva V, Ivanova V, Gesheva R. Effects of nutrients on the production of AK-111-81 macrolide antibiotic by Streptomyces hygroscopicus. Microbiol Res 2005; 160(3): 243-248.
50. Sanchez S, Chávez A, Forero A et al. Carbon source regulation of antibiotic production. J Antibiot 2010; 63(8): 442-459.
51. Zhou J, He H, Wang X et al. Optimization of nutrients for dinactin production by a marine Streptomyces sp. from the high latitude Arctic. Biotechnol Bioprocess Eng 2015; 20(4): 725-732.
52. Meidute S, Demoling F, Bååth E. Antagonistic and synergistic effects of fungal and bacterial growth in soil after adding different carbon and nitrogen sources. Soil Biol Biochem 2008; 40(9): 2334-2343.
53. Narayana K, Vijayalakshmi M. Optimization of antimicrobial metabolites production by Streptomyces albidoflavus. Res J Pharmacol 2008; 2(1): 4-7.
54. Wu J-Y, Huang J-W, Shih H-D, Lin W-C, Liu Y-C. Optimization of cultivation conditions for fungichromin production from Streptomyces padanus PMS-702. J chinese Inst Chem Eng 2008; 39(1): 67-73.
55. ter Schure EG, van Riel NA, Verrips CT. The role of ammonia metabolism in nitrogen catabolite repression in Saccharomyces cerevisiae. FEMS Microbiol Rev 2000; 24(1): 67-83.
56. Aharonowitz Y. Nitrogen metabolite regulation of antibiotic biosynthesis. Ann Rev Microbiol 1980; 34(1): 209-233.
57. Doull JL, Vining LC. Nutritional control of actinorhodin production byStreptomyces coelicolor A3 (2): suppressive effects of nitrogen and phosphate. Appl Microbiol Biotechnol 1990; 32(4): 449-454.
58. Hobbs G, Frazer CM, Gardner DC, Flett F, Oliver SG. Pigmented antibiotic production by Streptomyces coelicolor A3 (2): kinetics and the influence of nutrients. Microbiol 1990; 136(11): 2291-2296.
59. Hobbs G, Obanye A, Petty J et al. An integrated approach to studying regulation of production of the antibiotic methylenomycin by Streptomyces coelicolor A3 (2). J Bacteriol 1992; 174(5): 1487-1494.
60. Lounes A, Lebrihi A, Benslimane C, Lefebvre G, Germain P. Regulation of spiramycin synthesis in Streptomyces ambofaciens: effects of glucose and inorganic phosphate. Appl Microbiol Biotechnol 1996; 45(1): 204-211.
61. Majumdar M, Majumdar S. Effects of minerals on neomycin production by Streptomyces fradiae. Appl Microbiol Biotechnol 1965; 13(2): 190-193.
62. Al-ghazali LH, Omran R. Optimization of Medium Composition for Antibacterial Metabolite Production from Streptomyces spp. Asian J Pharma Clin Res 2017: 381-385.
63. Liras P, Asturias JA, Martin JF. Phosphate control sequences involved in transcriptional regulation of antibiotic biosynthesis. Trends Biotechnol 1990; 8: 184-189.
64. Martín JF. Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. J Bacteriol 2004; 186(16): 5197-5201.
65. Webb JS, Givskov M, Kjelleberg S. Bacterial biofilms: prokaryotic adventures in multicellularity. Curr Opin Microbiol 2003; 6(6): 578-585.
66. Hentzer M, Givskov M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. J Clin, Invest 2003; 112(9): 1300-1307.
67. Park S-Y, Kang H-O, Jang H-S, Lee J-K, Koo B-T, Yum D-Y. Identification of extracellular N-acylhomoserine lactone acylase from a Streptomyces sp. and its application to quorum quenching. Appl Environ Microbiol 2005; 71(5): 2632-2641.
68. PechÈre JC. Azithromycin reduces the production of virulence factors in Pseudomonas aeruginosa by inhibiting quorum sensing. Jpn J Antibiot 2001;

54(Suppl C): 87-89.
69. Tateda K, Comte R, Pechere J-C, Köhler T, Yamaguchi K, Van Delden C. Azithromycin inhibits quorum sensing in Pseudomonas aeruginosa. Antimicrob agents Chemother 2001; 45(6): 1930-1933.
70. Niu G, Chater KF, Tian Y, Zhang J, Tan H. Specialised metabolites regulating antibiotic biosynthesis in Streptomyces spp. FEMS Microbiol Rev 2016; 40(4): 554-573.