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Interspecies Interactions Induced Pigment Production from Non-Producing Isolate of *Streptomyces* MR24 in a Co-culture with *Bacillus subtilis*

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Abstract: In the nature, microorganisms is found in populations of different species in which they interact with each other. It is possible to hypothesise that microorganisms have acquired a diverse of physiological and metabolic responses as a result of inter-species interactions such as production of bioactive compounds. In fact, many of these reponses remain silent in the laboratory and hence many compounds which may be important are not appear. In this work, we utilized microbil interactions to activate silent pathways in order to induce metabolites production. Thirty different isolates of *Streptomyces* were examined for their pigment production in a pure and mixed cultures with *Bacillus subtilis*. In an interestng finding, we recognized a pink pigment produced by the isolate *Streptomyces* MR24 only in the mixed culture. The maximum absorption peak of the extracted pink pigment was approximately at 450 nm. The FT-IR spectra analysis revealed different stretching and bending functional groups at different wave length. The result of FT-IR analysis indicated the presence of O-H, N-H, C=N, C-N, C-C, C-O, C=C and C-H functional groups suggesting that this pigment may possess a good antimicrobial activity which already confirmed as the pink pigment was noticeably effective against *Bacillus subtilis*.

Keywords: *Streptomyces*; pigment; interspecies interaction; co-culture.

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Introduction:

Actinomycetes and in particular the genus streptomyces, continue to be the most vital source of useful natural medical compounds, such as antimicrobial agents, anticancer and antitumor agents (1,2). Most of these bacteria developed a considerable part of their genomes for producing such useful active compounds (3). However, it is clear and scientifically proven that many biosynthetic gene groups encode for the production of these active compounds are remained unexpressed (silent) under the laboratory conditions(4). Scientists have great interest in finding new strategies for activating these gene clusters in order to increase the number and variety of the metabolites produce by actinomycetes.

In the past decade, there has been an increasing amount of literature on finding promising strategies for the activating or triggering the cryptic biosynthetic gene clusters. One of these strategies is the co- cultures approach which is based mainly on exploiting the interspecies interactions among bacteria to activate the silent genes. However, this strategy was focused mostly on improved the expression of known secondary bioactive metabolites in order to increase their production. In this

context, luti and Mavituna (5) reported an increase in the production of undecylprodigiosin production by *Streptomyces coelicolor* when co cultured with dead cells of *Bacillus subtilis* and *Staphylococcus aureus*.Though, far too little attention has been paid to utilizing such strategy for producing noval metabolites from these silent genes (5).

In actinomycetes, the co-culture strategy may offer the basis for increasing the opportunity of discovery of novel compounds which are not produced in pure cultures (6). Interspecies interactions among different species of microorganisms in co-culture results in significant morphological and physiological changes. Ueda and coworkers (7) reported that co-cultivation of actinomycetes with other bacteria stimulated some morphological development and metabolites production.

A number of studies have investigated the interactions between different species in the genus *Streptomyces* with some common soil microorganisms such as *Bacillus* subtilis. These studies have reported a number of important changes occurred as a result of interspecies interaction such as inhibition and modification of growth development (8,9,10), degradation of signaling compounds through increased enzyme production (11), and alteration of production pattern of two antibiotics produce naturally by *Streptomyces coelicolor*(12).

Streptomycetes are very important microbial group in biotechnology due to the large number of bioactive compounds produce by these bacteria. Therefore, stimulating streptomycetes in order to produce novel compounds to meet the commercial requirements represents an important aspect in the industrial biotechnology. Hence, this paper seeks to utilized interspecies interactions in mixed culture in order to induce *Streptomyces* to produce new compounds which are not produced in the pure culture.

Materials and Methods:

Streptomyces isolates:

Soil samples were collected from cultivated and uncultivated area from different locations in Baghdad. The samples were obtained at a depth of 10 cm after removing approximately 3 cm of the soil surface. After dring in an oven, the soil samples were serially diluted using sterile distilled water up to 10^{-7} dilution and one ml from each sample was spreaded on Mannitol soyabean agar (MS agar) and incubated for 10-14 days in 30°C. After incubation, *Streptomyces* isolates were collected based on their morphological appearance.

Next, *Streptomyces* isolates were screened for pigment production in two different media, MS agar (mannitol 20, soya flour 20, agar 20 g/L) and starch medium (starch 20, KNO_3 2, K_2HPO_4 1, Nacl 0.5, $MgSO_4.7H_2O$ 0.5 g/L). Isolates which showed pigmentation under experimental conditions used in this work were excluded. Thirty isolates were exhibited no pigment production and hence were chosen and kept for further investigation.

Preparation of spore inocula of *Streptomyces*:

Streptomyces isolates were first cultured on MS agar slant for 12 days at

30°C and after growth and spore formation, 5ml of sterile distilled water was added. The spores were collected after scrapping the growth with loop and then washed and resuspoended in distilled water. The number of spores was fixed at approximately 10^9 spores/ml using haemocytometer.

Co-cultivation on solid medium:

The effect of interspecies interactions on inducing pigment production was first investigated on solid medium using MS agar and starch medium plates as follow: an amount of spore suspension of the non pigment producing *Streptomyces* isolate was spread in the middle area on the surface of the agar in the form of strip. The plate was allowed to dry and then incubated at 30°C for 24-48 h. Thereafter, a loopful of an overnight *Bacillus subtilis* culture was smeared adjacently only on one side of the *Streptomyces* growth. The other side of the strip was left as a control without *Bacillus subtilis* cells for visual comparison. The plates were then incubated at 30°C for 3-4 days.

Co-cultivation in liquid medium:

Two mL of *Streptomyces* spore onoculum contained approximately 1×10^9 spores/mL was used to inoculate 100 mL of starch liquid medium with 4 glass beads to improve mixing and dispersion of the hyphae. An orbital shaker was used to incubate the culture at 30oC and 200 rpm for 48 hours (13). Next, a fixed inoculation level of 1% of *Bacillus subtilis* was used with three concentrations of *Bacillus subtilis* cells (10^5, 10^6 and 10^7 cell/mL) was used to prepare the co-culture. Thereafter, flasks were incubated again in the orbital shaker at 30°C and 200rpm (5).

Extraction of the pink pigment:

Pink pigment was extracted from 500 mL of co-culture of *Streptomyces* MR24 with *Bacillus subtilis* collected on day 5 of incubation. The culture medium was first centrifuged for 15 min at 5000 rpm. The supernatant was discarded and mycelia was collected to extract the pigment. Four different solvents ethyl acetate, acetone, diethyl ether and methanol were tested for extracting the pigment. 100 mL of solvent was added to the harvested mycelia and mixed vigorously for at least 3 hours at room temperature. The resulting mixture was then centrifuged at 8000 rpm for 10min. The supernatant was collected and filtered through a filter paper (Whatman GF/C). Rotary evaporator was used to concentrate the filtrate and twice the amount of chloroform was added to extract the pink pigment. The two solvents were mixed vigorously in a separatory funnel. Chloroform phase was collected and dried using a rotary evaporator. The resulting pigment was then dissolved in a small amount of water and stored in a dark bottle.

Characterization of the produced pigment:

UV-Visible Spectrophotometry:

The λmax of the pink pigment was dected by UV-Visible spectrum analysis. The λmax is useful to detect the absorbance range of the extracted pigment which was dissolved in ethyl acetate. The UV analysis was performed

in a range level of 200-800 nm in UV-visible spectrometer (Shimadzu).

FT-IR Analysis:

The FT-IR spectra of sample was recorded in order to identify the functional groups in the produced pigment. Two mg of the extracted pigment was added in 200 mg of potassium bromide (KBr- FT-IR grade) and prepared as dry pellet. All measurements were carried out in the range of 400-4000 cm-1 at a resolution of 4.0 cm-1 (Thermo Nicolet – Avatar-330, USA).

Results and discussion:

Thirty isolates of non-pigment producing isolates of *Streptomyces* were collected from soil and already identified based on their morphological appearance. Its appear as non-regular, dry, white to gray in color and tough stony-like colonies. All these isolares were verified that they were unable to produce pigment in two different media MS agar and starch medium under the cultivation conditions used in this study.

As mentioned earlier the aim of this study was to investigate a possible inducing production of bioactive compounds in response to interspecies interaction. For this purpose, *Streptomyces* isolates were cultivated adjacently with *B. subtilis* on the same solid medium as illustrated in fig. 1. In an interestng finding, we recognized a pink pigment produced by the isolate *Streptomyces* MR24 only in the mixed culture in the strarch medium. This pink pigmnent was seen at the interface between the growth of both *Streptomyces* MR24 and *Bacillus subtilis* within four days after inoculation as shown in fig. 1. This pigment was not observed on the growth part of *Sreptomyces* which were not in direct interaction with *Bacillus subtilis*. Moreover, in the pure culture of *Sreptomyces* MR24 on both starch and MS agar, there was no pigment formation even after 7 days of incubation. Certainly, this observation suggested that the formation of the pink pigment was associated to the interaction between the two microorgaisms.

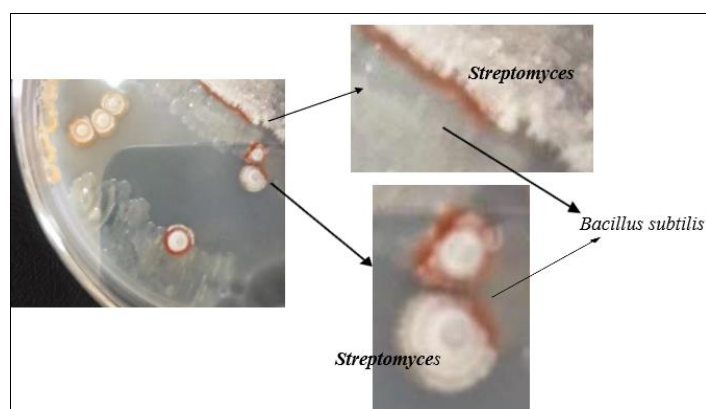

Figure (1): Pink pigment production by *Streptomyces* MR24 in response to interspecies interaction with *Bacillus subtilis* on starch agar medium.

The next step in this research was to test this phenomenon in a liquid medium. Three levels of *B. subtilis* inoculum were added separately to *Streptomyces* MR24 culture at zer time. At first, the ability of *B. subtilis* to grow

in the *Streptomyces* MR24 medium was investigated. Our preliminary observations showed that *Bacillus subtilis* could grew well in the starch medium used to cultivate *Streptomyces* MR24. However, it was necessary to detect the minimum concentration of *B. subtilis* cells that could be added without affecting the growth of *Streptomyces* MR24 due to the faster growth of *B. subtilis* in comparson with *Streptomyces* MR24. Therefore, a fixed inoculation level of 1% of *Bacillus subtilis* was used as being half of *Streptomyces* MR24 inoculation level (2%) with three concentrations of *B. subtilis* cells (10^5, 10^6 and 10^7 cells/mL). Similar to the results observed on solid medium, *Streptomyces* MR24 produced the pink pigment when challenged with *B. subtilis* in all mixed liquid culture. Our results showed that when live cells of *B. subtilis* were added to the *Streptomyces* MR24 culture, the pigenment was again induced (Figure 2). The production of this pigment in the mixed culture was started on the first day of incubation as an pink color particles in the thin smalls hyphae and then continued to accumulate throughout the incubation turning the colour of the culture to pink. The level of pink pigmnet produced was different in the three mixed cultures based on the concentration of *B. subtilis* cells added. It was found that the pigment was increased in a positive relationship with the amount of *B. subtilis*. At the end of incubation, the colour of pure and mixed culture was clearly different; the mixed culture was pink due to the production of the pigment, whereas the pure culture was unpigmented.

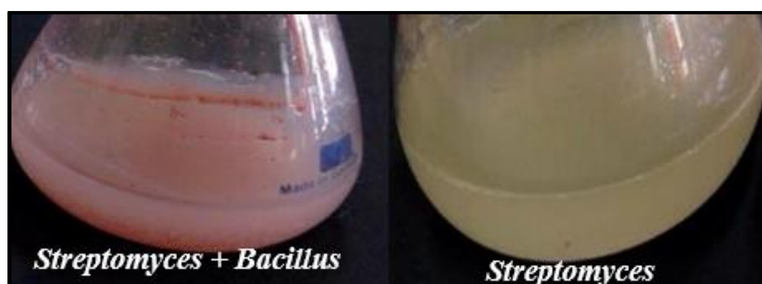

Figure (2): production of pink pigment by *Streptomyces* MR24 in mixed culture with *Bacillus subtilis* after 4 days of incubation compared with pure culture.

Extraction of the pink pigment:

The pink pigment was extracted from a co-culture of *Streptomyces* MR24 culture with *Bacillus subtlis* using foure different solvents. The prilimanry observation revealed that this pigment is an intracellular product therefore, it was extracted from the hyphel pellets only. Results showed that complete pigment extraction was achieved using ethyl acetate which was more efficient compared with other solvents. The solvent was used in an amount untile the hyphae became colorless. Extraction by Liquid-liquid extraction method is mainly based on the movement of molecules from one liquid phase into another immiscible phase according to their solubilities or partition/distribution coefficient. According to the basis of extractive techniques, like dissolves like, solvents tend to dissolve molecules which are similar to their chemical characteristics (14,15). Although both liquids are polar solvents here, water is much more polar than ethyl acetate. Therefore, strong

polar compounds such as sugars and inorganic compounds like salts are expected to remain in the water phase, whereas the organic compounds are expected to move in to the organic solvent phase (ethyl acetate).

Characterization of the pink pigment:

UV/vis analysis scan (200–800)nm of the pink pigment produced was performed and the results showed that the maximum absorption peaks was approximately at 450 nm (fig. 3). On the other hand, the FT-IR spectra analysis presented in fig. 4, revealed different types of bonds and thus different functional groups that absorbed infrared at different wavelength, both stretching or bending. According to the table 1 and fig. 4, the FT-IR spectra spectrum of the pigment showed the presence of peaks at 3394.72 cm-1 and 3224.98 cm-1 indicating the presence of O-H stretching of the hydroxyl group of alcohol. The same peak at same frequency (3224.98 cm-1) was found that may refer to presence of (N-H) bending of amine group. In addition, the peak near 1647.21 cm-1 region attributed to the presence of C=N stretching of aromatic group (oxidized nitrogen). Furthermore, the absorption of the peak in region 1147.65 cm-1 refers to the presence of (C-N) stretching of amine group. The group of C-C stretching as alkane was oserved at frequency 1097.5 cm-1. Though, C-O stretching group of carboxylic acid was found at 1078.06 cm-1. Again, alkane group C=C stretching was seen at wavelength 983.7 cm-1. A band at 754.17 cm-1 corresponding to the presence of N-H bending vibration of amide group was noticed. Finally, the spectra of methane group C-H bending was observed in the regions 630.72 and 453.27 cm-1.

Furthermore, According to IR spectra results, the region that confined between (400 – 1000) cm-1 can be considered as the region of fingerprint reflecting the characteristic features of this pigment (16). Therefore, the fingerprint region of this bacterial pigment included C-H, N-H and C=C which were distincted and non-overlapping peaks. This result can be considered as an evidence for the level of pigment purity (17). In general, the result of FTIR analysis indicated the presence of alcohol, amines, aromatic, alkane, amide and methane compounds. Therefore, it can be said that this bacterial pigment may posses a good antimicrobial and biological activity due to the presence of O-H, N-H, C=N, C-N, C-C, C-O, C=C and C-H functional properties (18,19).

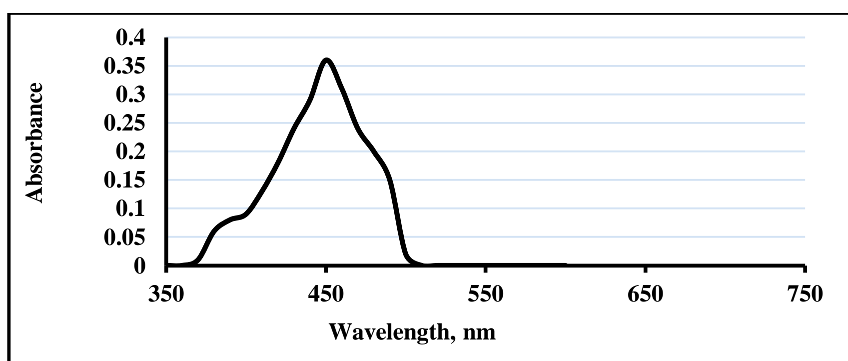

Figure (3): UV/vis spectrum of crude-pink pigment produced by *Streptomyces* in co-culture with *B. subtilis.*

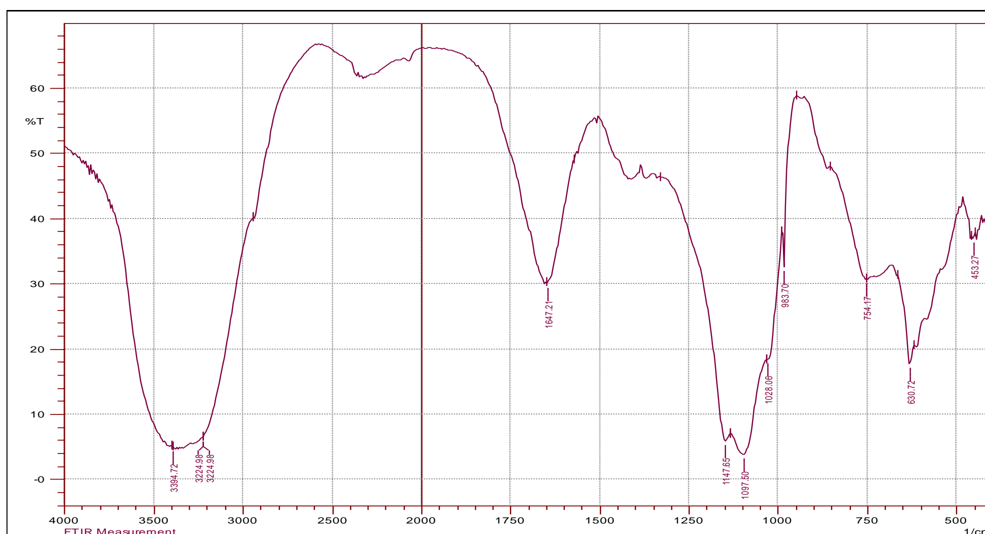

Figure (4): FT-IR spectra (4000 to 400)cm-1 of the pink pigment produced by *Streptomyces* MR24 in mixed culture with *Bacillus subtilis*.

Table (1): FTIR analysis to identify and characterize the functional groups in the pink pigment produced by *Streptomyces* MR24 in mixed culture with *B. subtilis*.

Frequency (Wavelength) cm-1	Functional Group	Name of Group	Vibration Type
453.27	C-H	Methine	Bending
630.72	C-H	Methine	Bending
754.17	N-H	Amide	Bending
983.7	C=C	Alkane	Stretching
1078.06	C-O	Carboxylic	Stretching
1097.5	C-C	Alkane	Stretching
1147.65	C-N	Amine	Stretching
1647.21	C=N	Aromatic (oxidized nitrogen)	Stretching
3224.98	N-H	Amine	Bending
3224.98	O-H	Hydroxylic (alcohol)	Stretching
3394.72	O-H	Hydroxylic (alcohol)	Stretching

***Fingerprint Region: 400 – 1000 cm-1.**

Soil is very rich in several kinds of microorganisms that certainly compate with each other for the poor nutritional resources in such an environment. Hence, soil microorganisms usually produce different types of antimicrobial compounds such as pigments and this is a very important phenomenon that usually soil microorganisms have in order to survive (19). It is widely mentioned in the literature that the driving force for producing antimicrobial compounds is the microbial interspecies interactions (20). Therefore, cultivating the microrganisms alone in pure cultures undoubtedly restrict production of many antimicrobial compounds as the reason for producing them, interspecies interactions, is restricted. Therefore, in order to stimulate the full capability of a microorganism in terms of antimicrobial compounds production, it is necessary to mimic the presence of a potential competitor and this can be achieved in a mixed culture. Actually, this approach can induce the silent metabolic pathway in the pure culture to switing on and consequently yields a potent compounds such as antimicrobial agents. This

certainly what was happened in this work as the pink pigment produced in the mixed culture in response to the presence of *Bacillus subtilis* cells. However, the question now is ‘do the pink pigment has an antimicrobial activity, particulary against *Bacillus subtilis* as this bacterium was the inducer that caus its production?’

In this work, *Bacillus subtilis* was selected as a prospective competitor from the natural environment of *Streptomyces. Bacillus subtilis* is a Gram-positive bacterium which neturally exist in the soil and most possibly, *Streptomyces* has established an interspecies interactions strategy for recognizing and compating this bacterium. The two microorganisms are expected to be found in the same places in the soil for example, plant rhizosphere. In this context, Val and coworkers (21) isolated *Bacillus subtilis* from soil near the plant rhizosphere and as it is well known, *Streptomyces* usually found in the same place for feeding via degrading ligno-cellulosic compounds. In a preliminary experiment, the antimicrobial activity of the extracted pink pigment was performed using the well diffusion method. Based on our results, the pink pigment was noticeably effective and always inhibited the growth of *Bacillus subtilis.* Here, one may assume that: Do *Streptomyces* recognised the presence of *Bacillus subtilis* in its culture and consequently produce this antimicrobial active pigment to defend itself against a potential competitor?

Certainly, more research on this topic needs to be undertaken before such an assumption is established or confirmed. However, it is an important topic for future research, specially considering the possibility of producing new active antibiotics to face the increasing demand as a results of antibiotics resistance.

Finally, genomic analysis studies have showed that the total biosynthetic potential capability of an organism for producing a certain compounds is in fact greater than being exploited or discovered (20). A considerable amount of literature has been published on the silent gene clusters which almost revealed that these genes, if induced to express, may encode for producing novel bioactive compounds4. Since microorganisms in nature found in complex mixtures of populations, interspecies interaction undoubtedly represent the missing factor that cause absence of many compounds to produce in the laboratory.

Conclusion:

Our results presented in this paper support the previous findings of exploiting interspecies interactions to induce the production of different secondary metabolites. Inter-species interactions can stimulate the silent or weak-expressed biosynthetic metabolic pathways for creating new products or increase the production of the known metabolites. Since in nature microorganisms exist in populations involved different types of species that usually interact and response to each other. Therefore, cultivating microorganisms in laboratory may mask thier real potential capability of producing all compounds they are able to form. Hence, it is necessary when cultivating a microorganism in the laboratory, to mimic the interspecies interactions similar to some extent to that occure in the microbial natural setting in order to exhibit all their capibilities.

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