The role of interspecies interactions in the antibiotic production: A potential approach for producing more effective antibiotics

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Antibiotics are complex organic compounds usually synthesized via many separate enzymatic steps through complex pathways which are normally energetically expensive. Why should microorganisms spend their energy producing these compounds unless they play a crucial role in their life cycle or survival in their natural habitat? Scientists believe that antibiotics are synthesized to give the antibiotic producing organism an advantage over any possible competing organisms. This chapter attempts to discuss the following question: is it possible to exploit interspecies interactions to create an effect on the antibiotic production pattern that may form the basis for producing more effective antibiotics. In this field, most research in the literature is focused on exploiting inter-species interactions to induce the unexpressed biosynthetic pathways for new bioactive compounds or to improve the productivity of the antibiotic-producing strains. In the pages that follow, an important aspect of inter-species interactions in the production of antibiotics will be argued that needs to be considered: this is the possibility of inter-species interactions to motivate the producer strains to choose their antibiotic "weapons" that would be more effective against the competitors when the producer strains have the capability to produce one or more than one antibiotic.

Keywords: interspecies-interaction; antibiotics; co-culture; *Actinorhodin*; *Undecylprodigiosin; Prodigiosin; Phenazine*

1. Introduction

In 1990, Davies suggested that ''secondary metabolites are ancient molecules and many of them exert their known biological activities (*e.g.* as antibiotics) through interactions with ancient macromolecules'' [1]. Stone and Williams, [2] argued this opinion and suggested that the producing organisms may have evolved the capability to synthesize antibiotics due to the advantages that may be gained as a result of the functions of these compounds. Wiener [3] studied the ecological role of antibiotics and found that antibiotics production played an important role in preventing the invasion of competitors; however, it did not improve the capability of antibiotic producing organisms to invade the competitor population even if the competitors were sensitive cells. So far, the predominant hypothesis regarding the origin of antibiotics is the competition hypothesis which suggests that antibiotics are synthesized to give the antibiotic producing organism an advantage over any possible competing organisms [4].

Microorganisms in nature exist in complex mixtures of populations that lead to different interactions and responses among them. Scientists believe that these interactions and responses are the driving forces for the production of antibiotics [5]. Therefore, mimicking the natural environmental setting of the microorganisms in the laboratory by co-culturing the microbial species that may interact in nature should give a more accurate picture of the microorganism's biosynthetic potential. In recent years, there has been an increasing interest in investigating and exploiting the interspecies interactions in the production of antibiotics. Most research in this field is focused on exploiting inter-species interactions to induce the unexpressed biosynthetic pathways for new bioactive compounds [6] or to improve the productivity of the antibiotic-producing strains [7; 8; 9; 10]. However, far too little attention has been paid to investigate the role of inter-species interactions in the production of antibiotics in terms of motivating the producer strains to choose their antibiotic "weapons" that would be more effective against the competitors which may be found in their medium when the producer strains have the ability to produce one or more than one antibiotic. This work seeks to study this aspect via investigating the production of four antibiotics in co-cultures using three different antibiotic producers separately: *Streptomyces coelicolor* (produce two antibiotics simultaneously), *Pseudomonas aeruginosa* and *Serratia marcescens* (each produce one antibiotic).

2. Interspecies interactions motivated *Streptomyces coelicolor* to change its production pattern

Streptomyces species are among the most widely used bacteria in industrial microbiology because of their ability to produce a wide range of secondary metabolites. We therefore, chose one of the best characterized members of this species, namely *Streptomyces coelicolor*, as the test microorganism in this study. MT1110 strain of *Streptomyces coelicolor* A3 (2) was used which was SCP1$^-$, SCP2$^-$ and was derived from the wild type strain 1147. It produces two known pigmented antibiotics, undecylprodigiosin (red) and actinorhodin (blue) [11]. It was reported that *Streptomyces coelicolor* changed its antibiotic production pattern when elicited with *Bacillus Subtilis* [7], *E. coli* [8] and *Staphylococcus aureus* [9]. Here, we report the experimental comparison of the growth of pure *E. coli* culture with that in the co-culture of *S. coelicolor* showing the effect of *in-situ* antibiotic production on *E. coli* growth. We also present

our tests on the antimicrobial activities of the produced antibiotics against *E. coli*. These results have led us to present the following question "can interspecies interactions be a strategy towards producing more effective antibiotics?"

2.1 Approach and methodology

In this research, the behaviour of *S. coelicolor* A3 (2) MT1110 was investigated if live cells of *E. coli* C600 were introduced to its culture. We used vegetative inocula of *S. coelicolor* prepared as described in Luti and Mavituna [9]. The vegetative inoculum was initiated using spores at the level of 1×10^9 spores/ml and incubated for two days in an orbital shaker at 28°C and 200 rpm which was then used as vegetative inocula for the subsequent pure and co-culture experiments. The inoculum of *E. coli* was prepared as follows: a few loopfuls of *E. coli* growth from an overnight culture on LB agar was inoculated in to a 250ml Erlenmeyer flask containing 50 ml defined liquid medium and incubated at 37°C for 24 hours. Then, a haemocytometer was used to adjust the number of *E. coli* cells to be approximately at the level of 1×10^7 cells/ml. For the pure cultures of *S. coelicolor*, 5% (v/v) level vegetative inoculum of *S. coelicolor* was used to inoculate 100 ml defined liquid medium with 4 glass beads of 0.4cm diameter in a 500 ml flask. For the co-cultures, *E. coli* was added at 2.5% (v/v) level to the flasks immediately after inoculation with *S. coelicolor*. An orbital shaker was used for incubation at 30°C at 200 rpm for 7 days. Concentrations of actinorhodin and undecylprodigiosin were determined according to the previously described methods [12; 13]. The growth of *S. coelicolor* in the pure and co-cultures was measured as the dry weight of cell material according to the method described in the literature which separates the two species [14]. The growth of *E. coli* was estimated as viable counts in both the pure and the co-culture with *S. coelicolor* using LB agar. All experiments were conducted either in triplicate or duplicate and the results were represented as the arithmetic average.

2.2 Experimental results

The pure culture of *S. coelicolor* produced mainly actinorhodin and only low quantities of undecylprodigiosin (Fig.1). Our results revealed an alteration in the antibiotic production pattern by *S. coelicolor* in the co-culture, such that undecylprodigiosin production was significantly enhanced and actinorhodin decreased. As can be seen in Fig.1, the concentration of undecylprodigiosin in the pure culture was 0.51 mg/L which increased to 2.59 mg/L when the live cells of *E. coli* were added to the *S. coelicolor* culture. Interestingly, actinorhodin concentration decreased significantly from 4.77 mg/L in the pure culture to 0.31 mg/L in the co-culture.

Our results showed that the growth pattern of *S. coelicolor* in the co-culture was not affected; it was similar to that of the pure culture (Fig.2). Furthermore, glucose consumption (data not shown) in both the pure and co-culture was similar and was concomitant with the growth reflecting the changes in the growth trends during the batch culture. The growth of *E. coli* in the defined synthetic medium was investigated both as a pure culture and in co-culture with *S. coelicolor*. For comparison, the pure culture of *E. coli* was prepared using the same cultivation conditions that were used for *S. coelicolor* and inoculated with the same amount of *E. coli* which was added to the *S. coelicolor* culture. It was found that the pure culture of *E. coli* grew well in the defined synthetic medium increasing its concentration of 4×10^2 cells/ml on day zero to 4.8×10^{10} cells/ml on the sixth day of the incubation. On the contrary, *E. coli* growth in the co-culture was inhibited from the second day of the incubation as the concentration of *E. coli* cells decreased continuously for the rest of the incubation compared with that of the pure culture of *E. coli* (Fig.2).

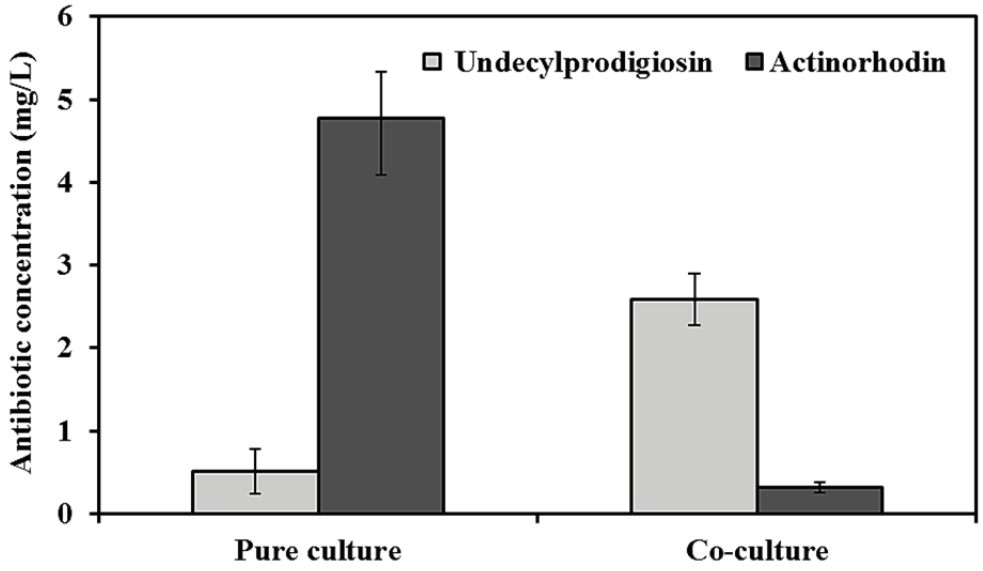

Fig. 1 The concentrations of undecylprodigiosin and actinorhodin produced by *S. coelicolor* on the sixth day of the batch in the pure culture and co-culture with *E. coli*.

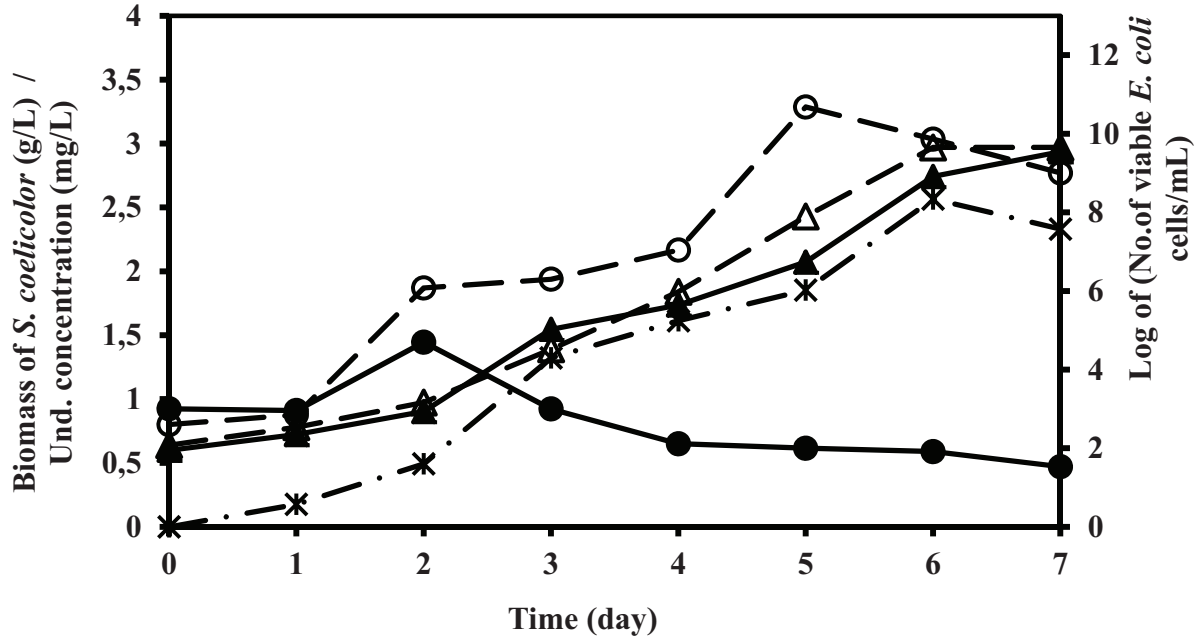

Fig. 2 Growth of *S. coelicolor* and *E. coli* in their pure cultures and in co-culture showing the effect of *in-situ* undecylprodigiosin production on *E. coli* growth: *S. coelicolor* dry weight in the pure culture - -Δ- -, *E. coli* viable cell numbers in the pure culture - -○- - , *S. coelicolor* dry weight in the co-culture —▲—, *E. coli* viable cell numbers in the co-culture —●—, undecylprodigiosin concentration in the co-culture - -×- -.

2.3 Do *S. coelicolor* choose its weapon?

The biosynthesis of antibiotics by *S. coelicolor* shows an interesting example of the molecular regulation of the production of more than one antibiotic. Undecylprodigiosin synthesis is commonly found to precede actinorhodin synthesis [15], however; its production decreases significantly when actinorhodin production begins. The onset and the level of production of undecylprodigiosin and actinorhodin by *S. coelicolor* are significantly affected by the growth rate [13]. It is also reported that actinorhodin production rate increases as the biomass and undecylprodigiosin productions decrease [13]. In our work however, there was no evidence of changed growth rate or nutrient deficiency that might have caused the increase in the production of undecylprodigiosin by *S. coelicolor.*

Antibiotics are synthesized via dedicated biosynthetic pathways that involve several precursors formed from the catabolism of carbon substrates. Undecylprodigiosin and actinorhodin are both derived at least in part, from the same precursor acetyl-CoA (www.KEGG.com). As it is well known, the metabolism in living cells is a highly conserved process in terms of energy usage [16]. Thus, a possible interpretation for the decrease in the production of actinorhodin observed in the co-cultures may be the result of increase in the production of undecylprodigiosin because of the implications on the metabolic costs of producing two antibiotics simultaneously.

We now turn to the important question: why does *S. coelicolor* increase undecylprodigiosin production? A study of the change in the growth of *E. coli* (Fig.2) when co-cultured with *S. coelicolor* provided the first evidence that undecylprodigiosin had an antimicrobial activity against *E. coli*. An inverse relationship was found between the *in-situ* production of the red pigmented undecylprodigiosin by *S. coelicolor* and growth of *E. coli* in the co-culture as shown in Fig. 2. The viability counts of *E. coli* in the co-culture started to decrease at the point when undecylprodigiosin production by *S. coelicolor* began. The decrease in the number of viable *E. coli* cells continued while undecylprodigiosin accumulated in the medium indicating the activity of this antibiotic against *E. coli*.

Then, the next question is; which of the two antibiotics is more effective against *E. coli*? In order to answer this question we compared the antimicrobial activity of the extracted undecylprodigiosin and actinorhodin against *E. coli* using the disk paper diffusion method. Undecylprodigiosin was extracted based on the method described by Wei and Chen [17], which was originally designed for extracting the red pigmented prodigiosin from *Serratia marcescens* because the two compounds are chemically similar and belong to the same family of prodiginines. Actinorhodin was extracted from both mycelia and the supernatant of a pure culture of *S. coelicolor* collected during the stationary phase (day seven) of incubation when the production attained its maximum [18]. For comparison, we used the same number of micro moles from each extracted antibiotic to impregnate the disc paper. We found that undecylprodigiosin was noticeably effective and inhibited the growth of *E. coli* with an inhibition zone of 8mm (Fig.2a) whereas, actinorhodin did not seem to be as effective (Fig.2b). Here, one may speculate that *S. coelicolor* seems to recognise the differences in the effectiveness of its antibiotics and increases undecylprodigiosin production accordingly when challenged with *E. coli*!

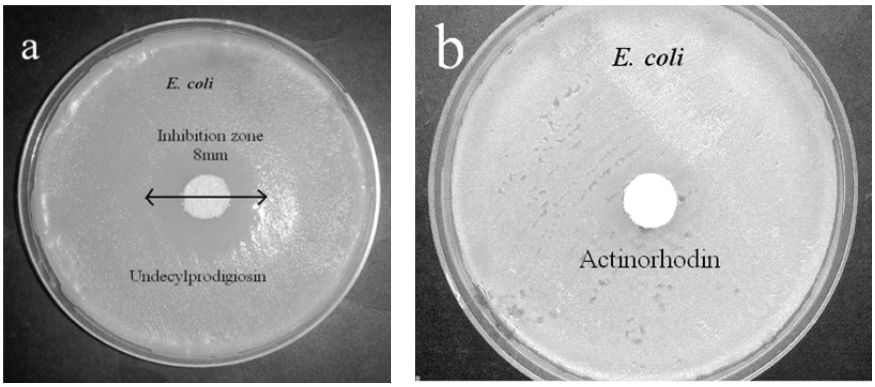

Fig. 3 Antimicrobial activity of the extracted undecylprodigiosin (a) and actinorhodin (b) against *E. coli*.

3. Interspecies interactions increases the production of prodigiosin and phenazine

We investigated the behaviour of *Serratia marcescens* and *Pseudomonas aeruginosa* in terms of antibiotic production if live cells of second microorganism introduced to its culture. Three microorganisms; Gram-negative bacterium *E. coli,* Gram-positive *Bacillus subtilis* and the eukaryotic microorganism *Saccharomyces cerevisiae* were chosen as the likely competitors (elicitors) for *S. marcescens* and *P. aeruginosa.* They were selected as elicitors because of these microorganisms are safe and familiar in the microbial labs that widely used as models in the microbiological studies. Furthermore, they can be found in different environments such as soil, water, air and decomposing plant matter, consequently, evolutionary interaction mechanisms may exist between *S. marcescens* or *P. aeruginosa* with those microorganisms. In addition, none of these microorganisms produce any kind of pigments which may contaminate the measurement and purification of prodigiosin and phenazine. Three levels of elicitor inoculum (1, 2, 3) % were added, separately, to *S. marcescens* and *P. aeruginosa* culture each contain approximately 1.5×10^7 cell/ml. These three levels were chosen as being below, equal and more than the inoculation level of *S. marcescens* and *P. aeruginosa* which was fixed to be 2% contains approximately 2×10^9 cells/ml.

3.1 Approach and Methodology

Prodigiosin producing strain of *S. marcescens* and phenazine producing strain of *P. aeruginosa* were isolated from soil samples which were subjected to regular biochemical tests and molecular identification in order to characterise and confirm their species (Data not shown). Inoculum of *S. marcescens* was prepared as follows: a few loopfuls of *S. marcescens* growth from an overnight culture on nutrient agar was inoculated into a 150 ml Erlenmeyer flask containing 20 ml of chemically defined liquid medium described by Chen and co-workers [19]. This culture was incubated for 24 h in an incubator at 30°C. After the incubation, a haemocytomtere was used to adjust the number of cells to be approximately 2×10^9 cells/ml. Inoculum of *P. aeruginosa* was prepared by following the same procedure using LB broth instead of chemically defined liquid medium and incubation temperature at 37°C.

Inocula of *E. coli* and *Bacillus subtilis* were prepared as follows: a few loopfuls of each bacterium growth from an overnight culture on nutrient agar was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of nutrient broth and incubated at 37°C for 24 h. After the incubation, the number of cells was adjusted to be approximately 1×10^7 cells/ml. Then, the inoculum of each elicitor was centrifuged at 10000 rpm for 10 min and cells were then washed and re-suspended in equal volume of normal saline. Inoculum of *Saccharomyces cerevisiae* was prepared by following the same procedure using sabouraud medium instead of chemically defined liquid medium and incubation temperature at 30°C.

The chemically defined liquid medium [19] and LB medium were used for the production of prodigiosin and phenazine respectively, which were inoculated at a level of 2% (v/v) and then incubated in an orbital shaker at 150 rpm for 48h at 37°C for *P. aeruginosa* and 30°C for *S. marcescens*.

Elicitation of *S. marcescens* was achieved using live cells of *E. coli, B. subtilis and S. cerevisiae* which were available in the Department of Biotechnology, College of Science, University of Baghdad. Three different inoculums sizes of the elicitor (1, 2, 3) % were added, separately, to the *S. marcescens* and *P. aeruginosa* fermentation culture at zero time. The same fermentation conditions were used for all elicitation experiments. For more reliability, all experiments were accompanied with a pure culture of *S. marcescens and P. aeruginosa* which will be referred to as the control and each run was conducted either in triplicate or duplicate and the results were represented as the arithmetic average. After the incubation, samples were taken for the analyses of each antibiotic. Concentration of phenazine was determined using a colorimetric method described by Dietrich and co-workers [20] and concentration of prodigiosin was determined using the colorimetric method described by Venil and Lakshmanaperumalsamy [21] and Williams and

co-workers [22]. The antimicrobial activity of phenazine and prodigiosin against *E. coli*, *B. subtilis* and *S. cerevisiae* was determined.

3.2 Experimental results

As can be seen in Fig. 4, prodigiosin production was significantly enhanced when live cells of *E. coli, B. subtilis* and *S. cerevisiae* were added to the *S. marcescens* fermentation medium. In all co-cultures, prodigiosin production was started after 5 h of incubation which is the normal time of production in the control culture. Similarly, the addition of live cells of *E. coli, B. Subtilis* and *S. cerevisiae* to *P. aeruginosa* culture resulted in a significant increase in the production of phenazine as shown in Fig. 5. Table (1) summarized the enhancement observed in the production of prodigiosin and phenazine as a result of addition of *E. coli, B. subtilis* and *S. cerevisiae* to *S. marcescens* and *P. aeruginosa* fermentation medium.

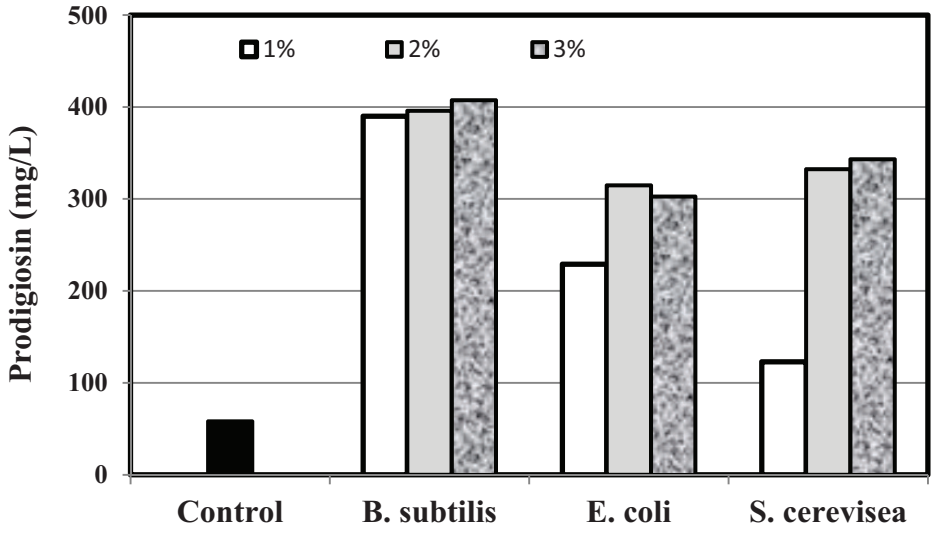

Fig. 4 The concentrations of prodigiosin produced by *S. marcescens* after 48h of the batch in the pure culture and co-culture with *E. coli, B. Subtilis* and *S. cerevisiae*.

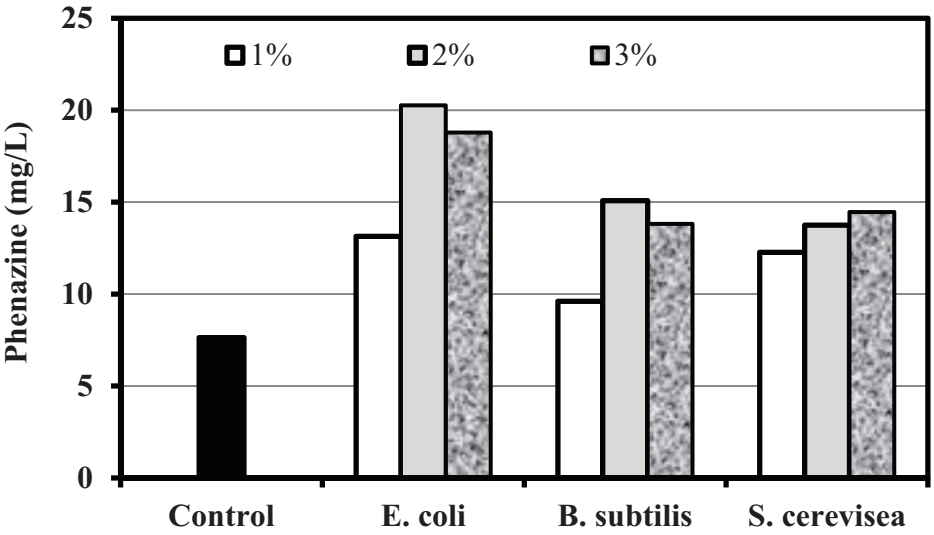

Fig. 5 The concentrations of phenazine produced by *P. aeruginosa* after 48h of the batch in the pure culture and co-culture with *E. coli, B. Subtilis* and *S. cerevisiae*.

Table 1 The enhancement in the production of prodigiosin and phenazine as a result of addition of *E. coli, B. subtilis* and *S. cerevisiae* to *S. marcescens* and *P. aeruginosa* fermentation medium.

Elicitor	Inoculation level %	Production enhancement (fold)	
		Prodigiosin	Phenazine
E. coli	1	3.8	1.8
	2	5	2.7
	3	4.8	2.4
B. subtilis	1	6.7	1.2
	2	6.8	2
	3	7	1.8
S. cerevisiae	1	2.2	1.6
	2	4.8	1.8
	3	5	1.9

3.3 Why do *S. marcescens* and *P. aeruginosa* increased their production of prodigiosin and phenazine

Antibiotics biosynthesis commonly appears in the late phases of the microbial growth culture. Stressful environmental conditions such as reduction of essential nutrients or unexpected change in culture conditions such as pH are factors that generally cause a decrease in the growth rate which normally accompanied with production of antibiotics. In addition, some inducing compounds and signaling molecules may play a crucial role in the production of antibiotics. Though, all these conditions are usually act as factors that trigger the antibiotic production or directing the production during the fermentation period. However, what is important here is the response of *S. marcescens* and *P. aeruginosa* for the presence of second microorganism in their fermentation medium which led to increase in their production of antibiotics more than the normal level in the pure culture (control). It is apparent from the results obtained in all co- cultures experiments; prodigiosin and phenazine production were significantly increased. If the similarity in the culture conditions such as nutrients, pH, temperature and aeration in both pure and co- cultures taken into consideration, it can be said that the environmental conditions may not have been the reason for the increase observed in the prodigiosin and phenazine production levels.

According to the competition hypothesis, antibiotics are synthesized to give the antibiotic producing organism an advantage against others surrounding competing microorganisms for the nutrients and space [23]. Though, at this point, there is a question that needs to be discussed which is why do *S. marcescens* and *P. aeruginosa* increased their production of prodigiosin and phenazine production in the co-cultures? Is there a relation between the antimicrobial activity of these two compounds and the increase observed in the co-culture? In order to answer these questions, we tested the antimicrobial activity of the extracted prodigiosin using the disk paper diffusion method [10] against *E. coli, B. Subtilis* and *S. cerevisiae*. Prodigiosin was extracted based on the method described by Wei and Chen [17]. Based on the results, prodigiosin was noticeably effective and always inhibited the growth of elicitors *E. coli*, *B. subtilis* and *S. cerevisiae* at a concentration of 1 and 0.1%, whereas no inhibition was seen when the concentration was 0.001% (Fig. 6). In addition, the antimicrobial activity of the extracted phenazine was also examined using the well diffusion method [10] against *the elicitors used in the co-cultures*. Our results showed that phenazine was effective and always inhibited the growth of *E. coli*, *B. subtilis* and *S. cerevisiae* as shown in Fig. 7.

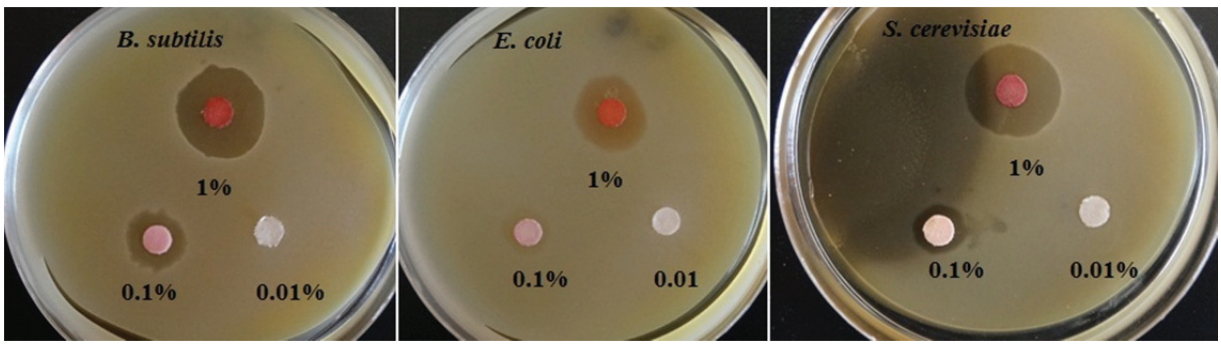

Fig. 6 Antimicrobial activity of the extracted prodigiosin against *E. coli, B. Subtilis* and *S. cerevisiae* using disk paper diffusion method.

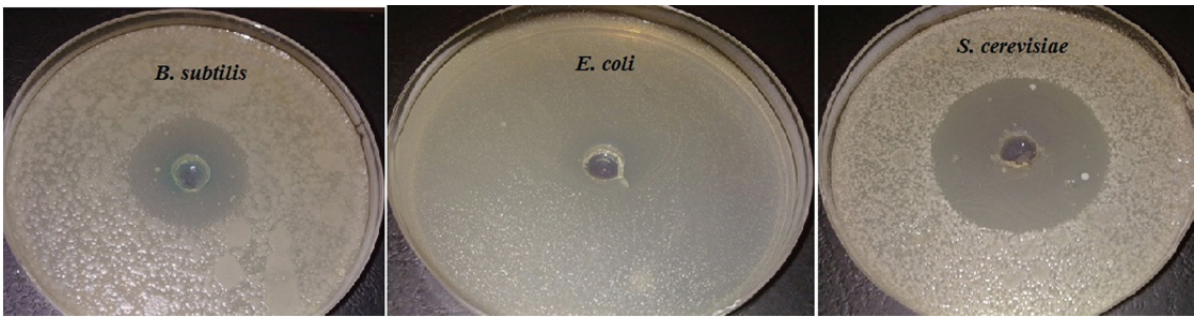

Fig. 7 Antimicrobial activity of the extracted phenazine against *E. coli, B. Subtilis* and *S. cerevisiae* using well diffusion method.

4. Conclusion

Based on the above results, a speculation on the nature of the interspecies interaction that caused the increase in the production of undecylprodigiosin, prodigiosin and phenazine may be as follows: the antibiotic producers may recognise the presence of the second microorganism in the culture through some unknown signalling processes and accordingly modify its antibiotics production pattern as a strategy to defend itself against a 'prospective competitor' by increasing the production of the more effective antibiotic for this competitor. Of course, much more research will need to be performed in order to prove such a speculation. However, the results of this study have gone towards enhancing our understanding of the role of interspecies interactions in the antibiotic production that can be the basis towards producing more effective antibiotics. It is an important issue for future research, if the antibiotic producer organism can indeed recognise its competitor and produce an antibiotic "weapon" accordingly, this may even lead to the production of more effective antibiotics as the antibiotic producer may for instance sense, respond and produce more suitable antibiotics against the pathogenic microbes.

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