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Enhancement of prodigiosin production by *Serratia marcescens* S23 via introducing microbial elicitor cells into culture medium

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Abstract

The present study was designed to investigate the possibility of exploiting the interspecies interaction of microbial cells in order to enhance the production of prodigiosin by local isolate *S. marcescens* S23. Prodigiosin is a promising drug owing to its characteristics of antibacterial, antifungal, immunosuppressive and anticancer activities. *S. marcescens* S23 was isolated from soil sample and already recognized via morphological, biochemical and molecular identification process. The first step was to detect the optimal conditions for maximum prodigiosin production using chemically defined liquid medium. The results revealed that the optimal conditions for prodigiosin production were sucrose as carbon source; peptone as nitrogen source; 60/40% optimum C/N ratio, 2% inoculum size containing 2×10^9 cells/ml, which increased the production of prodigiosin from 1.72 to 416 mg/L. Elicitation experiments were carried out by introducing live and dead cells of *E. coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae*, separately, to the *S. marcescens* culture at zero time. Based on the results obtained in this study, *S. marcescens* increased its production of prodigiosin as a result of interaction with microbial elicitor cells. The maximum enhancement was achieved in the culture elicited with the heat killed cells of *E. coli* at an inoculation level of 3% with an increase of approximately 9-fold, whereas the minimum enhancement was upon elicitation with live cells of *E. coli* and *S. cerevisiae*. Based on the results obtained in this study, elicitation strategy of exploiting interspecies interactions with microbial cells is successful and useful for enhancing the production of antibiotics.

Keywords: *S. marcescens;* Prodigiosin; Elicitation; interspecies interaction.

انتاج المضاد الحيوي برودجيوسين من عزلة محلية لبكتريا *Serratia sp.* ودراسة تأثير استخدام بعض الخلايا الميكروبية كمحفزات للانتاج

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الخلاصة

الدراسة الحالية صممت للتحري عن احتمالية استغلال التفاعل الحاصل من خلال التداخل بين الأنواع وتوظيفيه لزيادة انتاج مادة البرودجيوسين من العزلة المحلية لبكتريا *S. marcescens S23* . البرودجيوسين يعد واحدا من اهم المواد التي تحمل صفات تأهلها ان تكون دواءاً واعداً في المسقبل و من أهم هذه الصفات التي تميز هذه الصبغة الحمراء الميكروبية المنتجة من قبل بكتريا *Serratia marcescens* هي فعاليتها ضد العديد من الميكروبات اي امكانية استعماله كمضاد حيوي بكتيري، مضاد للفطريات بالاضافة الى ان العديد من البحوث التي اشارت الى امكانية استعماله كمثبط مناعي ومضاد للأورام السرطانية.البكتريا *S.*

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S.marcescens S23 عزلت من التربة وشخصت من خلال دراسة الصفات المورفولوجية اعتماداً على (الخصائص المظهرية و المجهرية) و ألاختبارات الكيموحيوية بالاضافة الى استخدام طرق جزيئية. كانت الخطوة الاولى هي الكشف عن الظروف المثلى للوصول الى أقصى قدر من إنتاج مادة برودجيوسين باستخدام وسط انتاجي مرقي (وسط السائل المحدد كيميائيا). وكشفت النتائج أن الظروف المثلى لإنتاج البرودجيوسين كانت كالتالي: السكروز كافضل مصدر للكربون، ببتون افضل مصدر للنتروجين. % ٤٠/٦٠ النسبة المثلى بين الكربون والنتروجين ، الرقم الهيدروجيني 7 و 2% افضل حجم للقاح، حيث تم تحسين انتاجية البرودجيوسين وزيادتها من ١,٧٢ حتى ٤١٦ mg/L. أُجريت تجارب التحفيز من خلال إدخال خلايا حية وميتة من E. coli ، *Bacillus subtilis* و*Saccharomyces cerevisiae* ، ، بشكل منفصل إلى وسط *S.*marcescens في بداية عملية التخمر (وقت الصفر). وبناء على النتائج التي تم الحصول عليها في هذه الدراسة، زادت S.marcescens من إنتاجها من صبغة برودجيوسين نتيجة التفاعل مع الخلايا التحفيز الميكروبية. الحد الأقصى في انتاج صبغة برودجيوسين تم تحقيقه في وسط S.marcescens الحاوي على خلايا E. coli المقتولة حراريا وبمستوى تلقيح ٣% بزيادة قدرها حوالي ٩ أضعاف، بينما كان الحد الأدنى في الانتاجية في تجارب التحفيز مع الخلايا الحية لكل من *S. cerevisiae* و *E.coli*. وبناء على النتائج التي حصلنا عليها في الدراسة الحالية تبين ان استراتيجية التحفيز من خلال استغلال التفاعل بين الأنواع مختلفة من الخلايا الميكروبية ناجحة ومن الممكن استعمالها لزيادة إنتاج المضادات الحيوية.

Introduction

Antibiotics are one of the most important discoveries in the medical science that protect human live against infectious disease. They are secondary metabolites of microbial origin with low molecular weights, which usually at low concentrations inhibit the growth or metabolic activities of other microorganisms [1, 2]. Although the function of antibiotics for the producing organism is still incompletely understood, competition hypothesis suggests that antibiotics give the producing organism an advantage over any possible competitors in a nutritionally poor environment such as soil [3]. Microorganisms in nature exist in complex mixtures of populations that lead to different interactions and responses among them. Scientists believe that these interactions are the driving forces for the production of antibiotics [4]. In biotechnological research, the common procedure for antibiotic production is to use pure culture which, as believed, limiting the real biosynthetic potential of the organism in the laboratory. Therefore, mimicking the natural environmental setting of the microorganisms in the laboratory by co-culturing the microbial species that may interact should give a more accurate picture of the microorganism‘s biosynthetic ability. Interspecies interactions can induce the unexpressed biosynthetic pathways for new antibiotics and other novel products as well as improve the productivity of the antibiotic-producing strains [5, 6].

Prodigiosin is a red pigment, cell wall-associated antibiotic that belongs to a group of polypyrrole bioactive compounds called prodiginines [7]. It produces as a secondary metabolite by many terrestrial (soil) and marine bacterial strains including species of *Serratia*, mainly *S. marcescens* [8]. During the past thirty years, researchers have shown an increased interest in prodiginines compounds due to their immunosuppressive and anticancer properties in addition to antimicrobial activities [7]. In 1989, the immunosuppressive activity of prodigiosin on cytotoxic T-lymphocytes was discovered [9]. Later, the capability of this compound to inhibit the generation of human B and T-lymphocytes was confirmed [10].

The aim of the present work is to study of *S. marcescens* behavior in terms of prodigiosin production in pure culture and culture elicited with three different microbial cells separately. The main goal for this study is the exploitation of bacterial interspecies interactions in order to improve the production of prodigiosin from *S. marcescens***.**

Materials and Methods

Isolation and characterization of soil isolate

Prodigiosin producing strain of *Serratia* sp. was isolated from a soil sample which was subjected to regular biochemical tests in order to characterize the genus. The isolate was further characterized as *Serratia marcescens* through using primers specifically designed based on the 16S rRNA sequence

alignment which was compared to other prokaryotic 16S rDNA sequences by using the similarity rank analysis service of NCBI database by BLAST server. Specific PCR primers used in this experiment are shown below:

16S (F) Forward: 5'TGC CTG ATG GAG GGG GAT AA3'
16S (R) Reverse: 5'CTT CGC CAC CGG TAT TCC TC3'

DNA was isolated from *Serratia* as follow: a single colony was picked from an overnight culture of *Serratia* isolate on MacConkey plates and suspended in 500 µL of lysis sterile buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). Cells were lysed by heating in a heat block at 95 °C for 30 min. The tubes were then cooled quickly on ice and immediately stored at -20 ^{o}C until PCR was performed; aliquots of this lysate were used directly in PCR [11].

DNA amplification was performed using Go *Taq*®Green Master Mix which provided by (Promega-USA). The PCR reaction mixture consisted of 2 µl of each primer, 2 µl of template genomic DNA, 100 ng. PCR was run in a programmable thermocycler having an initial delay at 95 °C for 10 min and final delay at 72 °C for 10 min followed by 30 cycles of denaturation at 95 °C for 1min, annealing at 57 ˚C for 1min followed by extension at 72 °C for 1 min. The PCR product was then electrophoresed in 2% agarose gel and analyzed using gel documentation system. An amount of 25 µL of the PCR ready mix (Go *Taq*®Green Master Mix) was added when the final reaction volume was 50µl to obtain a final concentration of (1X) as recommended by the provider; sterile distilled water was used to achieve a total volume of 50µL after adding each of the primers and a DNA template.

Preparation of bacterial inocula

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. This culture was incubated for 24 h in an incubator at 30˚C. After the incubation, a haemocytomtere (Neubauer improved, Marien field-Germany) was used to adjust the number of cells to be approximately 2×10^9 cells/ml by adding fresh sterile chemically defined liquid medium if necessary.

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 chemically defined liquid medium and incubated at 37˚C for 24 h. After the incubation, a haemocytomter was used to adjust the number of cells to be approximately 1×10^7 cells/ml by adding fresh sterile chemically defined liquid medium if necessary. Then, the inoculum of each elicitor was centrifuged at 10000 rpm for 10 min. If dead cells were required, the culture flask was placed in boiling water for 30 min before separating by centrifugation. Live and heat-killed cells of the elicitor were then washed and re-suspended in equal volume (culture volume before centrifugation) of normal saline. Inoculum of *S. cerevisiae* was prepared by following the same procedure using sabouraud medium instead of chemically defined liquid medium and incubation temperature at 30˚C.

Cultivation methods and media

A chemically defined liquid medium described by Chen and coworkers [14] was used for the cultivation of *Serratia* which contains g/L: (Starch, 10; Peptone, 5; $CaCl_2$.2H2O, 8.82; $FeSO_4.4H_2O$, 0.33; $MgSO_4.7H_2O$, 0.61; $MnSO_4.4H_2O$, 2). The pH was adjusted to 7 prior to autoclaving. The medium was inoculated with *S. marcescens* at level of 2% (v/v) and then incubated in an orbital shaker at 30°C and 200 rpm for 48h. After the incubation, samples were taken for the analyses of prodigiosin. For more reliability, each run was conducted either in triplicate or duplicate and the results were represented as the arithmetic average.

The sources of the carbon and nitrogen significantly affected the production of microbial products. Therefore, several carbon sources (arabinose, cellulose, fructose, glucose, lactose, maltose, mannitol, mannose, methylcellulose, rhaminose, starch, sucrose) and nitrogen sources (peptone, urea, $NH_4.NO_3$, $(NH_4)_2$. SO_4, NH_4.Cl) were tested. In addition, the best concentration ratio between carbon and nitrogen sources that support the maximum production of prodigiosin was investigated. In order to

achieve this goal, six different ratios of the optimized carbon and nitrogen sources were examined (g:g), (13:2, 12:3, 11:4, 10:5, 9:6 and 8:7).

Elicitation experiments

Elicitation of *S. marcesence* was achieved using live or heat-killed cell suspension 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 and 3%) were added, separately, to the *S. marcesence* 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. marcesence* 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.

Determination of growth

The growth of *S. marcesence* was measured as the dry weight of cell material. A known volume of the culture was filtered with vacuum through pre-dried and pre-weighed membrane filter (0.2µm cellulose nitrate membrane filter, Sartorius). The filter paper was thereafter placed in an oven at 60˚C for 24 h, and then weighted. The difference in weights represented the mass of cells in the samples of the culture. This method used with pure culture of *S. marcesence* while in the elicited cultures, this method was not followed because the complexity (difficulty) of the separation between two microorganisms which were growing in the same fermentative liquid.

Determination of prodigiosin concentration

Concentration of prodigiosin was determined using the colorimetric method described by [12, 13]. Prodigiosin was extracted by adding an equal volume of methanol to the cell pellets which were harvested from a known volume of the *S. marcesence* culture by centrifugation at 10000 rpm for 15 min. The mixture was then mixed for at least 3 h. The absorbance at 530 nm of the supernatant was determined after removing cell debris by centrifugation at 10000 rpm for 20 min. The concentration of prodigiosin was calculated by using molar extinction coefficient (E_{530} =7.07 × $10^4 M^{-1}$ cm^{-1}).

$$\text{Prodigiosin g/L} = \frac{\text{O.D}_{530} \times 323.4}{7.07 \times 10^4} \times \text{dillution factor}$$

Where:

O.D 530: Optical density at 530 nm

323.4: Molecular weight of prodigiosin

E 530 =7.07 × 104M-1 cm-1 (Molar extension coefficient of prodigiosin at 530 nm)

Dilution factor = Final volume/ sample volume

Results and discussion

The red pigmented strain isolated from soil taken from Baghdad city, was characterized as belonging to the genus of *Serratia* via various biochemical testes. The major identifier being the morphological and physiological identification by investigating the appearance of the intracellular bright red pigmentation, fishery - urinary culture specific odor, Gram negative, rod shape of cells, oxidase test negative, non lactose fermentation on MacConkey plates, indole test negative and positive for motility test, catalase and voges Proskauer test.

The PCR amplification product for the 16S rRNA based primers gave a sharp band on agarose gel electrophoresis in line (S) corresponding to approximately 550 bp (between 500-600pb) in length compared to the universal DNA molecular ladder as shown in Figure -1.

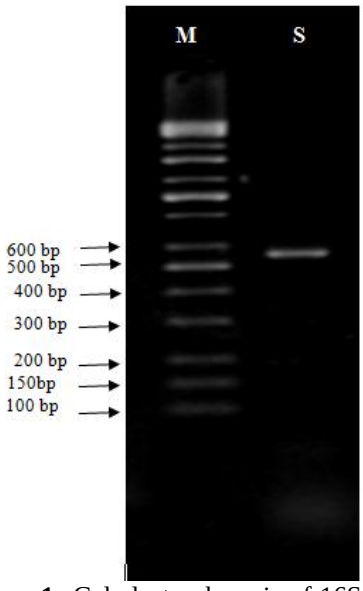

Figure 1- Gel electrophoresis of 16S rRNA gene of wild type of *Serratia* S23migrated on agarose gel (2%) at 120 volts for 1.5 h. Lane M, universal DNA ladder; Lane S, PCR product.

DNA sequencing for the PCR product were commercially performed by Chromas -Pro. Co. Australia as demonstrated below. The sequence was then compared with NCBI database by BLAST server and results reveals that this DNA is belong to *S. marcescens* with 100% similarities.

5'GACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAG
CTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGAC
CAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGT
TGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAGCTTAATACGTTCATCAATTGACGTT
ACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGT
GAAATCCCCGGGCTCAACCTGGG3'

Optimization of medium compositions and culture conditions

The optimized cultural parameters that required for elevating prodigiosin production such as carbon and nitrogen sources, pH and inoculum size were examined. Some physical parameters that were not investigated in this study have been adjusted according to the literature such as temperature (30°C) [14] and agitation rate in the shaker (200 rpm) [15].

Different carbon sources were added to the chemically defined medium in order to select the one that can support the maximum production of prodigiosin. As can be seen in Figure-2a, production of prodigiosin was only promoted in cultures supplemented with fructose, mannitol, sucrose and maltose, whereas it was significantly decreased in the other cultures. Based on the results, the maximum production of prodigiosin was observed in the culture supplemented with sucrose (147.9 mg/L) and therefore it was selected for further experiments. In this context, some literature mentioned that sucrose was found to be the best carbon source in the production of prodigiosin [16, 17]. However, other literature stated that starch was the carbon source that supported the maximum prodigiosin production [14]. In addition, no prodigiosin production was observed in the presence of glucose which agreed with a number of previous studies [18, 19]. Although, it has been recognized in several studies, the basis for the inhibition of prodigiosin by glucose is incompletely understood yet [20-23].The effect of various nitrogen sources on the production of prodigiosin were elucidated using organic nitrogen (peptone and urea) and inorganic nitrogen (NH_4NO_3, $(NH_4)_2SO_4$ and NH_4.Cl). Figure-2b shows that most of the nitrogen sources used, except for peptone, did not support prodigiosin production. The concentration of prodigiosin produced was 149.1 mg/L in the culture supplemented with peptone as a nitrogen source. Therefore, peptone was chosen as a nitrogen source for further experiments. Several studies have revealed that prodigiosin production can be stimulated in culture contains peptone [14,

16]. Based on literature, the biosynthetic pathway of prodigiosin involve incorporation of some amino acids which contain pyrrole-like structures such as proline, histidine and methionine that serve as essential precursors for the production of the red pigment. According to the literature, peptone is normally contains a considerable amounts of these amino acids which might be the reason for the increased observed in the production of prodigiosin [24].

In order to enhance the production of prodigiosin, C:N ratios of 8:7, 9:6, 10:5, 11:7, 12:4 and 13:2 in the chemically defined medium were used to detect the optimal ratio. It was found that microbial growth and production of prodigiosin was significantly affected at high C/N ratio Figure-2c. The optimal C/N concentration ratio of 9:6 (60:40 %) was found to be the best ratio that conferred the maximum prodigiosin production. The maximum concentration of prodigiosin obtained in this culture was 383.6 mg/L. This C:N concentration ratio therefore, corresponds to an increase of 2.5-folds in the maximum prodigiosin concentration compared with C:N ratios that previously used.

In addition, different inoculum sizes ranging from 1 to 5% (v/v) investigated. As shown in Figure-2d, the best inoculums size for the maximum prodigiosin production was 2% yielding approximately 416.0 mg/L under the experimental conditions used in this work.

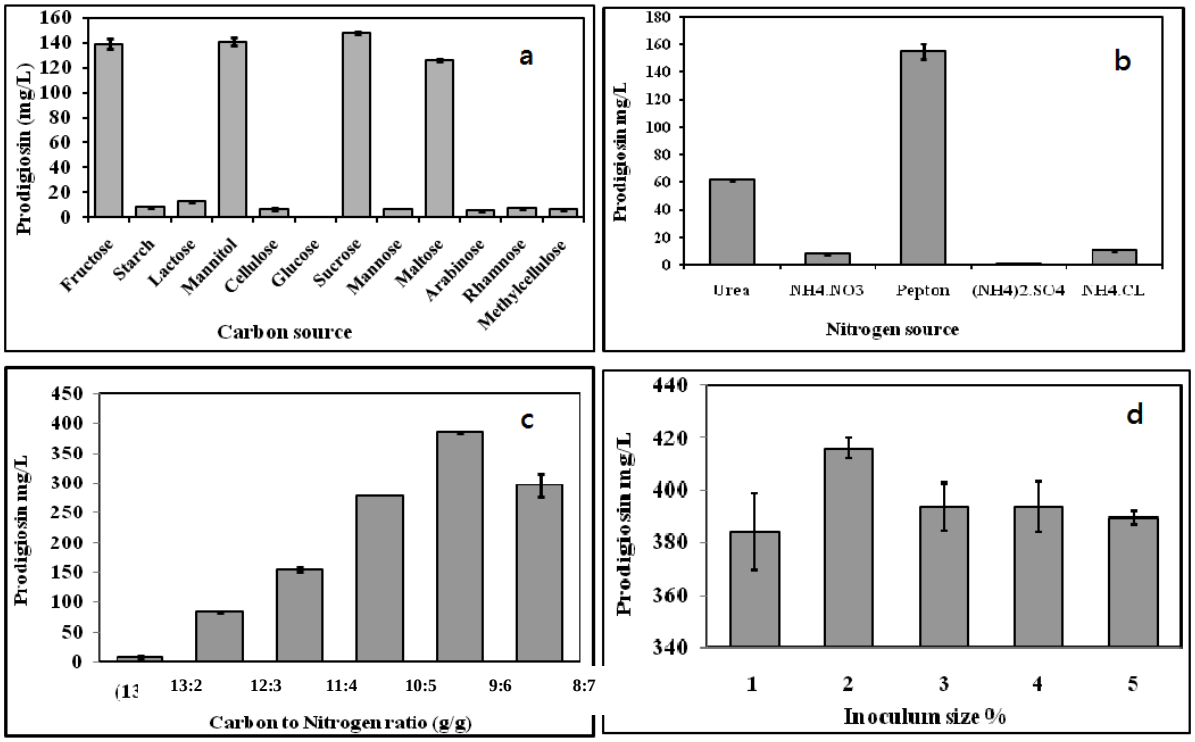

Figure 2- The effect of different carbon sources (a), nitrogen sources (b), C:N ratios (c) and inoculum sizes (d) on the production of prodigiosin by *S. marcesence* S23 in the chemically difened liquid medium at 30˚C.

Under the optimized conditions, the production of prodigiosin started after 12 h of incubation. At the end of the exponential phase (approximately at 30 h of incubation), the concentration of prodigiosin was 250 mg/L and reached its maximum of 414.4 mg/L during the stationary phase after 45 h of incubation Figure-3.

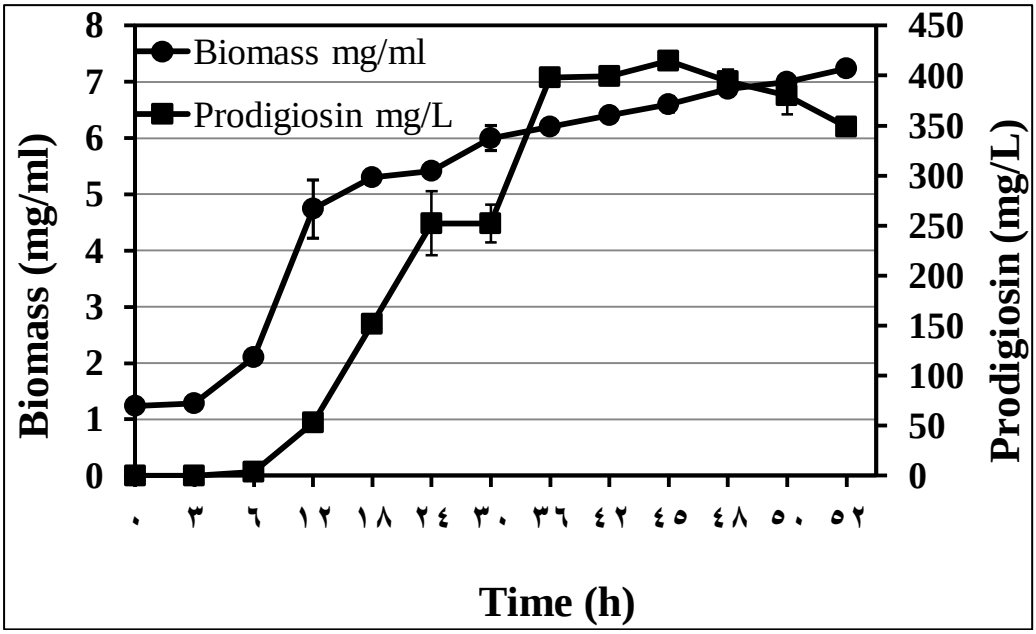

Figure 3- Time course of cell growth and prodigiosin production by *S. marcescens* S23 in a shaker incubator at 200 rpm and 30 °C.

Elicitation experiments

Since microorganisms normally existing with other species in nature, they have developed complex metabolic and physiological responses as a result of such inter-species interactions. Our strategy for the elicitation of *S. marcescens* is based on utilizing some aspects of these interactions by introducing bacterial cells to *S. marcescens* culture. In order to fully exploit any eliciting capability, the bacterial cells (live or heat-killed cells) were added directly into the *S. marcescens* cultures.

One of the challenges that may arise in such a situation is the possibility of the introduced live cells of the second bacterium to become competitors to the main producers. The motivation for our work is to enhance the production of prodigiosin without impacting the growth of *S. marcescens*. Therefore, it is important to keep the elicitor growth under control and this depends on the medium composition and other physico-chemical conditions that may support or reduce the elicitor growth. Furthermore, the concentration of the elicitor cells was fixed to be in a level necessary for elicitation but without overtaking the growth of *S. marcescens*. However, in order to avoid such problem, the eliciting capability of the dead cells of elicitors was examined in comparison with live cells. If the dead cells show an eliciting effect, then any influence on the growth of *S. marcescens* by the competition stress can be avoided.

Three species of microorganisms; Gram-negative bacterium *E. coli*, Gram-positive *B. subtilis* and the eukaryotic microorganism *S.cerevisiae* were chosen as the likely competitors from the natural habitat of *S. marcescens.* They were selected as elicitors because these three microorganisms are safe and familiar in the microbial labs which widely used as a model 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* and those microorganisms. In addition none of these microorganisms produce any kind of pigments which may contaminate the measurement and purification of prodigiosin.

Three different levels of *E. coli* inoculum (1, 2, 3 %) was added to *S. marcescens* culture each contain approximately 1.5×10^7 cell/ml. These three levels were chosen as being below inoculation level of *S. marcescens* which was fixed to be 2% contains approximately 2×10^9 cells/ml.

Addition of E. coli cells

Figure -4 shows that prodigiosin production was significantly enhanced when live cells of *E. coli* were added to the *S. marcescens* fermentation medium. In all elicited cultures, prodigiosin production was started after 5 h of incubation which is the normal time of production in the control culture. Maximum production of prodigiosin was obtained in the culture elicited with 2% level of *E. coli*

inoculums (2.5 g/L) compared with 1.9 and 2.4 g/L attained in cultures elicited with 1 and 3% level of *E. coli* inoculums respectively. Elicitation with live cells of *E. coli* therefore, corresponds to an increase of 3.8, 5 and 4.8-folds in cultures elicited with 1, 2 and 3% inoculation level respectively, in the maximum prodigiosin concentration compared with the control culture.

Interestingly, heat-killed cells of *E. coli* had the same role as live cells in terms of its effect on prodigiosin production by *S. marcescens*. As can be seen in Figure-5, prodigiosin production of *S. marcescens* was enhanced when heat-killed cells of *E. coli* were added to the fermentative medium at zero time. Production of prodigiosin was higher in the cultures elicited with heat killed cells of *E. coli* compared with the control culture. In the elicited culture, no earlier onset of prodigiosin production was observed as the production was started after 5 h of incubation. Maximum production of prodigiosin obtained in the cultures elicited with 1, 2 and 3% level of *E. coli* inoculums was 3.8, 4 and 4.1 g/L respectively, attained after 30 h of incubation. Elicitation with heat killed cells of *E. coli* corresponds to an increase of 8.6, 9 and 9.1 folds in the maximum prodigiosin concentration compared with the control culture.

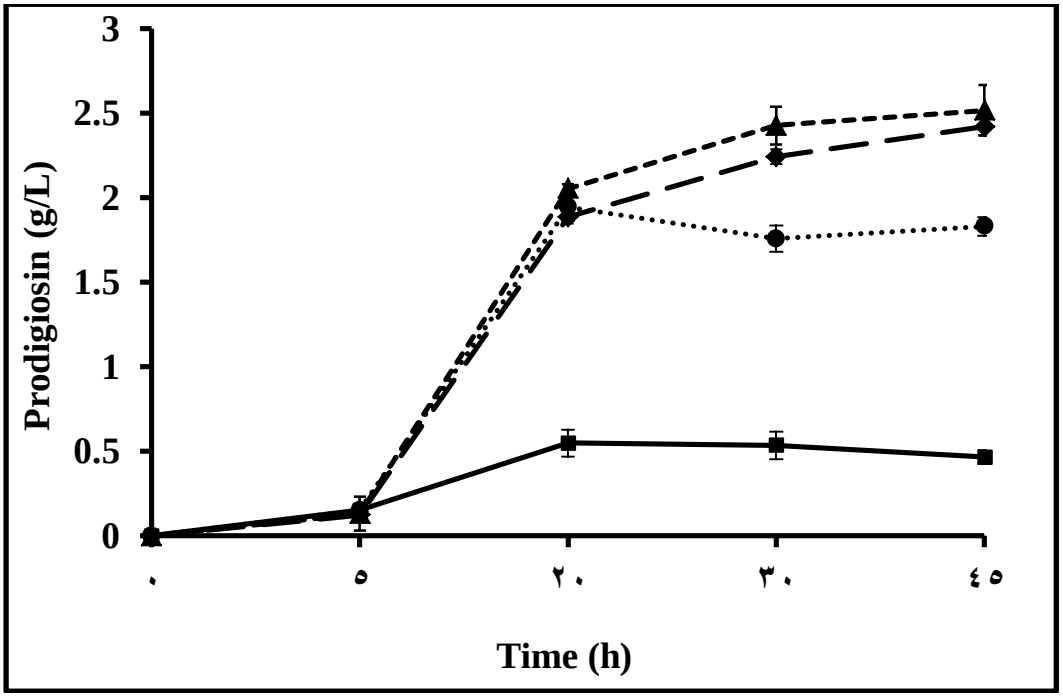

Figure 4- Production of prodigiosin by *S. marcescens* S23 in cultures elicited with three different inoculum levels of live cells of *E. coli*: 1% (···●···), 2% (--▲--), 3% (−♦−) in comparison with control (—■—), in chemically defined medium at 30˚C and 200 rpm.

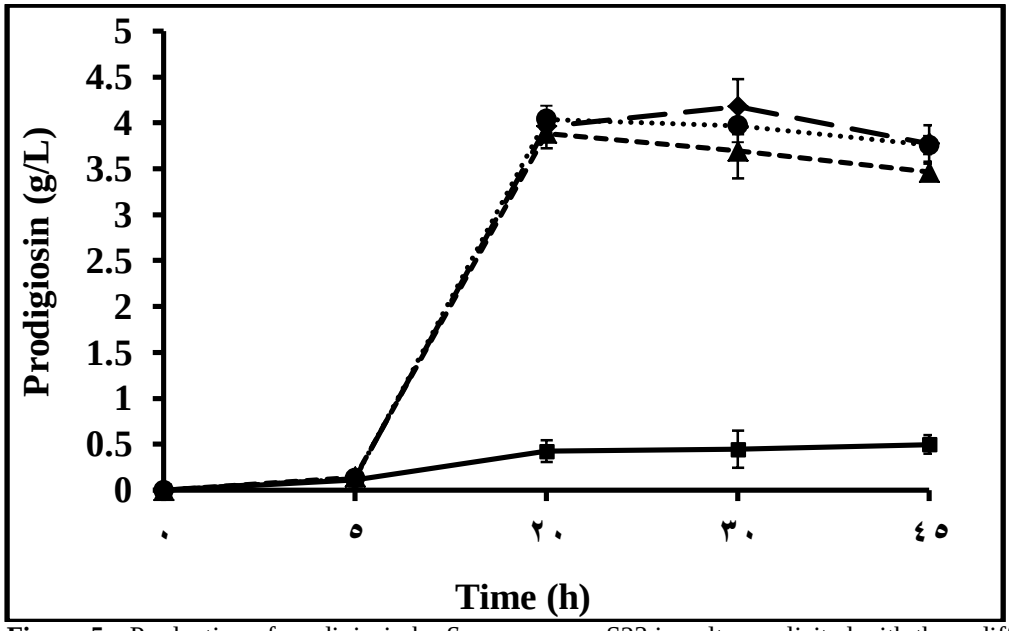

Figure 5- Production of prodigiosin by *S. marcescens* S23 in cultures elicited with three different inoculums of heat-killed cells of *E. coli*: 1% (···●···), 2% (--▲--), 3% (−♦−) in comparison with control (—■—), in chemically defined medium at 30˚C and 200 rpm.

Addition of B. subtilis cells

In addition, results showed that live cells of *B. subtilis* had no considerable effect on prodigiosin production by *S. marcescens* Figure-6. The production pattern of prodigiosin in the control culture and that elicited with live cells of *B. subtilis* were approximately similar live cells of *B.subtilis* were approximately similar. On the other hand, heat-killed cells of *B. subtilis* had the same role as heat-killed cells of *E. coli* to elicit *S. marcescens*. Production of prodigiosin was again stimulated and increased notably in all elicited cultures Figure-7. In the elicited culture, no earlier onset of prodigiosin production was observed as the production was started after 5h of incubation. Maximum production of prodigiosin obtained in the cultures elicited with 1, 2 and 3% level of heat killed cells of *B. subtilis* inoculums was 3.38, 3.44 and 3.5 g/L respectively, attained after 30h of incubation. Elicitation with heat-killed cells of *B. subtilis* therefore, corresponds to an increase of 6.7, 6.88 and 7-folds in the maximum prodigiosin concentration compared with the control culture.

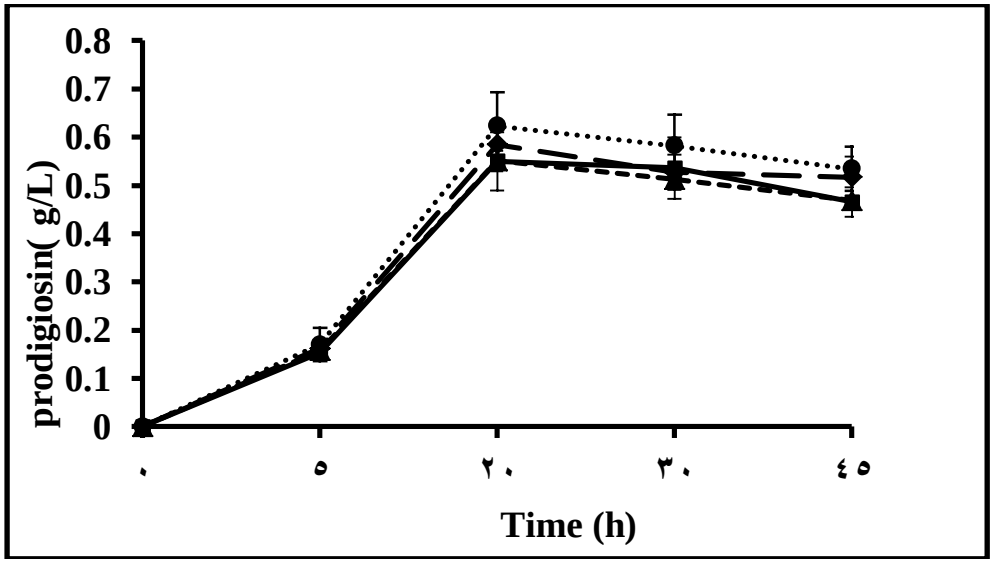

Figure 6- Production of prodigiosin by *S. marcescens* S23 in cultures elicited with three different inoculums of live cells of *B. subtilis*: 1% (···●···), 2% (--▲--), 3% (−♦−) in comparison with control (—■—), in chemically defined medium at 30˚C and 200 rpm.

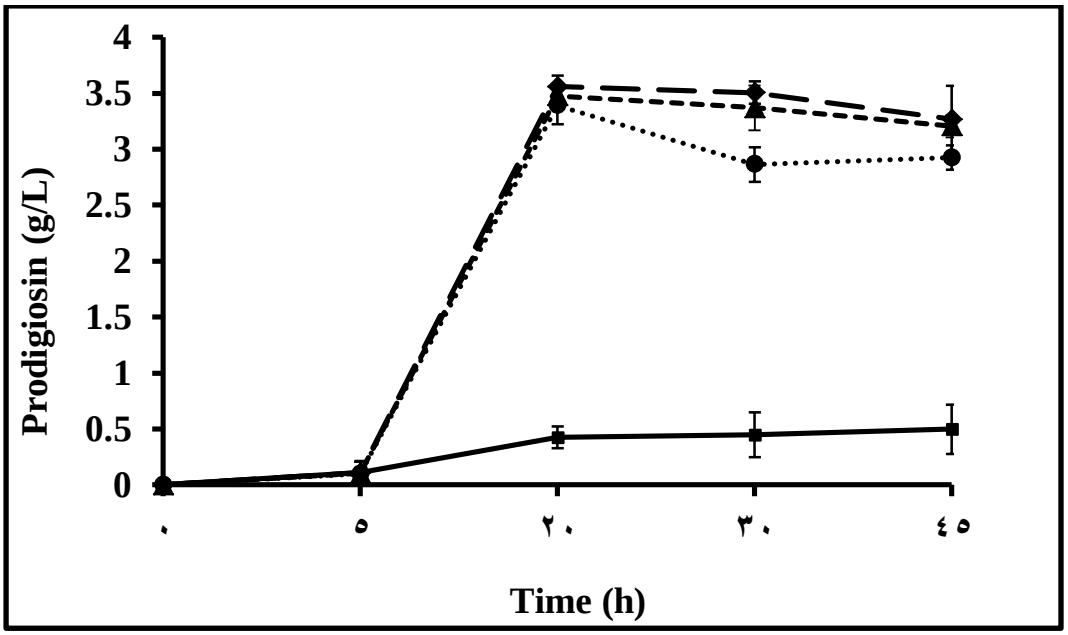

Figure 7- Production of prodigiosin by *S. marcescens* S23 in cultures elicited with three different inoculums of heat-killed cells of *B. subtilis*: 1% (¨●¨), 2% (--▲--), 3% (−♦−) in comparison with control (—■—), in chemically defined medium at 30˚C and 200 rpm.

Addition of *Saccharomyces cerevisiae* cells

Similar to the elicitation with *E. coli* and *B. subtilis*, *S. marcescens* increased its prodigiosin production when challenged with *S. cerevisiae*. Results showed that when live cells of *S. cerevisiae* were added to the *S. marcescens* culture, production of prodigiosin was again stimulated Figure-8. Maximum production of prodigiosin was obtained in the culture elicited with 3% level of *S. cerevisiae* inoculums (2.8 g/L) attained after 30 h of incubation compared with 1.2 and 2.6 g/L attained in cultures elicited with 1 and 2% level of *S. cerevisiae* inoculums respectively. Comparing these values with the maximum concentration achieved in the control culture, the increase in the production of prodigiosin was therefore 2.2, 4.81 and 5.1-folds in the maximum prodigiosin concentration obtained in cultures elicited with 1, 2 and 3% levels of *S. cerevisiae* inoculums respectively.

Heat-killed cells of *S. cerevisiae* had the same role as heat-killed cells of *E. coli* and *B. subtilis* to elicit *S. marcescens*. Figure -9 shows that production of prodigiosin was higher in the cultures elicited with heat-killed cells of *S. cerevisiae* compared with the control culture. Maximum production of prodigiosin obtained in the cultures elicited with 1, 2 and 3% level of *S. cerevisiae* inoculums were 4.1, 3.6 and 3.5 g/L respectively, attained after 45 h of incubation. Elicitation with 1, 2 and 3% levels of heat killed cells of *S. cerevisiae* inoculums therefore, resulted in 8, 7 and 6.8-folds increase in the prodigiosin production compared with the control culture.

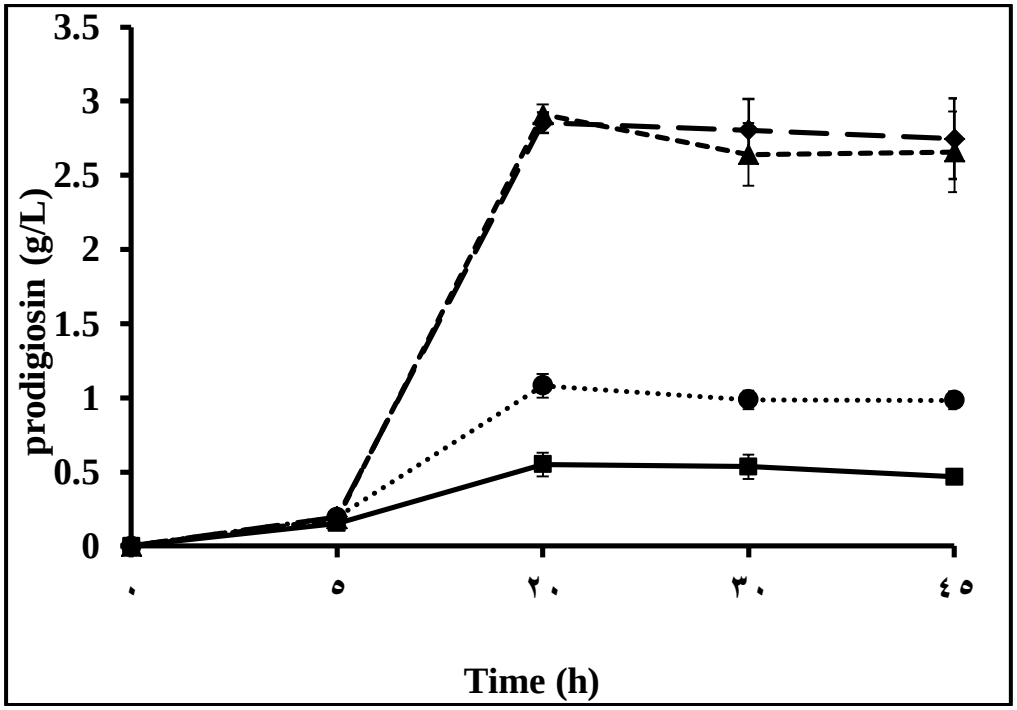

Figure 8- Production of prodigiosin by *S. marcescens* S23 in cultures elicited with three different inoculums of live cells of *S. cerevisiae*: 1% (···●···), 2% (--▲--), 3% (–♦–) in comparison with control (—■—), in chemically defined medium at 30˚C and 200 rpm.

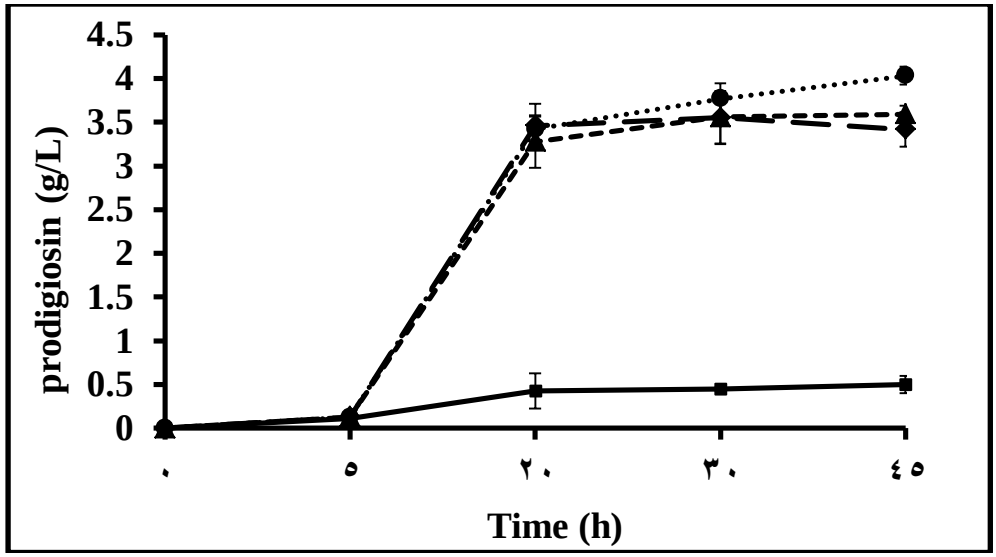

Figure 9- Production of prodigiosin by *S. marcescens* S23 in cultures elicited with of three different inoculums of heat-killed cells of *S. cerevisiae*: 1% (···●···), 2% (--▲--), 3% (–♦–) in comparison with control (—■—), in chemically defined medium at 30˚C and 200 rpm.

Microbial studies have established a fact that, bacteria in nature exist in complex mixtures of populations in which they normally interact and response to each other. Some literatures have mentioned that interspecies interactions importantly affect in the bioactive compounds production, particular antibiotics [5]. According to one of the most accepted hypothesis regarding the origin of antibiotics, antibiotics are synthesized to give the antibiotic producing organism a competitive advantage against others surrounding competing microorganisms for the same nutrients and space [3]. Therefore, the possible results of exploiting the interspecies interactions in the production of antibiotics can be the induction of unexpressed biosynthetic pathways for novel bioactive compounds [26] or the enhancement required in the productivity of the antibiotic-producing strains as obtained in this study. It is apparent from the results achieved in all elicitation experiments; prodigiosin

production was significantly enhanced except of the elicitation with live cells of *B. subtilis* in which no important increased was observed. Therefore, it can be said that elicitation strategy based on exploiting inter-species interaction was useful to enhance prodigiosin production by *S. marcescens* which is a valuable compound due to its antitumor and antimicrobial properties.

One of the interesting findings in this study is that dead cells of elicitors had the same elicitation role as the related live cells in terms of increasing prodigiosin production. This is an important aspect as adding dead cells to the fermentation medium could not affect the growth of the antibiotic producer which certainly, strengthens the case of using such an approach in industrial applications.

Antibiotics biosynthesis commonly appears in the late phases of microbial growth culture. Stressful environmental conditions such as reduction of the essential nutrients usually lead to decrease in the growth rate which normally accompanied with production of antibiotics. In addition, the presences of some inducing compounds are some factor that may affect the production of antibiotics. Molecules of low molecular weight perhaps created at stationary phase to act as factors triggering antibiotics production called signaling molecules [27] Based on results, heat-killed cells of *E. coli B. subtilis* and *S. cerevisiae* gave approximately the same elicitation results as live cells. Obviously, the presence of dead elicitor cells in the *S. marcescens* fermentation medium could not cause any nutritional stress. On the other hand, antimicrobial activity tests revealed that prodigiosin was noticeably active against *E. coli*, *B. subtilis* and *S. cerevisiae* that used as elicitors in this study. This means that growth of *E. coli*, *B. subtilis* and *S. cerevisiae* may inhibited in the *S. marcescens* fermentation medium as results of prodigiosin production. Therefore, it can be suggested that the increase in prodigiosin production as a result of elicitation with live or heat-killed cells may not due to nutrient deficiency cultural stress. In addition, culture conditions such as pH, temperature and aeration in both control and elicited cultures were similar, leading to conclude that the environmental conditions may not have been the reason for the change observed in prodigiosin by *S. marcescens* in elicited cultures.

Alternatively, a possible interpretation for the mechanism of the elicitation may be a physical direct cell-to-cell contact between *S. marcescens* and the live or heat-killed cells of the elicitor bacteria. Although *E. coli*, *Bacillus subtilis* and *S. cerevisiae* in some cases were used as dead cells, *S. marcescens* may have recognized some proteins or receptors on the surface of these cells which may not have been affected by the heat. In this context,. [28] observed an induction of biosynthesis of four novel diterpenoids in a co-culture of a marine bacterium and the marine fungus *Libertella*. The authors found no evidence for signaling molecules; therefore, they suggested that the mechanism of interaction was due to cell-cell direct contact.

At this stage, a speculation on the nature of the elicitation that caused the increase in the production of prodigiosin may be as follows: *S. marcescens* may recognize the presence of the elicitor cells in the culture and increase prodigiosin production as a strategy to defend itself against a prospective competitor. Certainly, more investigations need to be performed before such speculation is confirmed and the reason of elicitation is understood.

Some previous studies have reported the usage of microbial cells as elicitors for enhancing antibiotic production. In this context, [29] used live and dead cells of *B. Subtilis*, *E. coli* and *Staphylococcus aureus* to enhance the production of undecylprodigiosin by *Streptomyces coelicolor.* The pure culture of *S. coelicolor* in a defined medium produce higher concentration of actinorhodin compared with undecylprodigiosin which is more important due to its antitumor activities. In that study, they found that *S. coelicolor* changed its antibiotic production pattern as a result of challenge with elicitor bacteria such that undecylprodigiosin production was significantly enhanced and actinorhodin decreased. Luti and Yonis (2013) reported that production of phenazine from *Pseudomonas aeruginosa* was increased by 2.4, 1.8 and 1.9-folds in cultures elicited with *E. coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* respectively, compared to the pure cultures.

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