

An Application of Solid State Fermentation and Elicitation with Some Microbial Cells for the Enhancement of Prodigiosin Production by *Serratia marcescens*

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Abstract

The present study provides an evidence for the successful utilization of interspecies interactions to enhance antibiotic production in solid state fermentation that previously established in liquid cultures. Ground corn, wheat bran, rice husk and soya bean ground were examined in order to choose the substrate that support the prodigiosin production from *Serratia marcescens*. Results revealed that maximum production of prodigiosin was obtained in wheat bran medium, starting from early hours of incubation and reaching its maximum of product yield 47.5 mg.gds⁻¹ (mg per gram of dry substrate) after 48 h of incubation. An enhancement by 2.3-fold in prodigiosin production was obtained as a result of introducing, separately, live and dead prokaryotic and eukaryotic microbial cells as elicitors to *S. marcescens* medium. The highest prodigiosin production (240mg.gds⁻¹) was obtained when *S. marcescens* was cultured in wheat bran medium supplemented with sunflower oil (0.5ml.g⁻¹ substrate) and live cells of *Bacillus subtilis* (0.4ml.g⁻¹ substrate) as elicitor. [DOI: [10.22401/JNUS.21.2.15](https://doi.org/10.22401/JNUS.21.2.15)]

Keywords: Elicitation; Prodigiosin; *Serratia marcescens*; Solid state fermentation.

Introduction

Prodigiosin is a red pigment, cell wall-associated antibiotic that belongs to a group of polypyrrole bioactive compounds called prodiginines [1]. During the past thirty years, researchers have shown an increased interest in prodiginines compounds because of their immunosuppressive and anticancer properties as well as antimicrobial activities [1]. In 1989, the immunosuppressive activity of prodigiosin on cytotoxic T-lymphocytes was discovered [2]. Later, the capability of this compound to inhibit the generation of human B and T lymphocytes was confirmed [3].

Although the common process for the production of secondary metabolites is submerged liquid fermentations, solid state fermentation was widely used for producing these compounds because it has been shown to be more efficient [4]. It is worthy mentioned that solid state fermentation potentially provides superior productivity that require simple technology and low capital costs compared with submerged fermentation. Therefore, it may represent the prospective future technology that can be applied usefully for providing the increasing global demand for secondary metabolites, particularly antibiotics. In this context, high yield of penicillin in a

short time period was achieved by solid state fermentation [5]. The antifungal peptide cyclosporine A was successfully produced by *Tolypocladium inflatum* on wheat bran [6]. In addition, the broad-spectrum antibiotics of tetracycline [7], Cephamycin C [8] and the anti-fungal iturin [9] are other examples of antibiotics that successfully produced by solid state fermentation.

Microbial species in nature live in complex mixtures of populations with diverse interactions and responses that can be existed among them. Researchers believe that many secondary metabolites particularly bioactive compounds may produce in nature as response to these interactions [10]. Thus, an accurate picture of the biosynthetic capacity of a microorganism can be gained in the laboratory from cultivating the microorganism in an environment that mimic its natural setting by co-culturing with other competitor species in mixed cultures. In this context, scientist have focused on utilizing some aspect related to inter-species interactions to stimulate the silent biosynthetic metabolic pathways (elicitation) for novel metabolites in particular bioactive compounds [11] or to develop the productivity of the bioactive compounds-producing strains [12]. Previous studies have reported that

Streptomyces coelicolor increased its antibiotic production as a result of interaction with *Bacillus Subtilis* [12], *E. coli* [13] and heat killed cells of *Bacillus subtilis* and *Staphylococcus aureus* [14]. Although there are some publications in the literature on using solid state fermentation in the production of prodigiosin, this work is the first attempt of using such process along with the exploitation of bacterial inter-species interactions in order to elicit *Serratia marcescens* to increase prodigiosin production.

Materials and Methods

Microbial strains

Soil prodigiosin producing isolate of *Serratia* sp. was used throughout this work provided by Fermentation laboratory, Department of Biotechnology, College of Science, University of Baghdad. This isolate was identified via regular biochemical tests and characterized as *Serratia marcescens* through using primers specifically designed based on the 16s rRNA sequence alignment. The 16s rRNA sequence alignment was compared to other prokaryotic 16S rDNA sequences by using the similarity rank analysis service of NCBI (data not shown).

Bacterial cells of *E. coli* and *Bacillus subtilis* as well as eukaryotic cells of *Saccharomyces cerevisiae* were used in this work as elicitors which already obtained from the Department of Biotechnology in the University of Baghdad.

Cultivation conditions

Luria broth (LB broth) was used for the revival and preculture of *S. marcescens* from a frozen stock for 18h at 30°C and 180rpm which was used as inoculum for the solid state fermentation. A haemocytometer was used to count the cells in the inoculum which was kept in all experiments to be approximately 10^8 cells.ml⁻¹.

250 ml Erlenmeyer flasks contained 5 grams of solid substrate were prepared and 10ml of distilled water was added to obtain moisture level of (2:1) (ml: g). After autoclaving, 1 ml of inoculum was added and incubated at 30°C for two days.

Selection of substrate

Substrates of rice husk, ground corn, soya bean ground and wheat bran were examined for their suitability for growth of *S. marcescens* and prodigiosin production.

Effect of initial moisture ratio

The influence of initial moisture level of the solid substrate on prodigiosin production was investigated at different moisture ratio (0.5:1, 1:1, 1.5:1, 2:1, 2.5: 1, 3:1 ml: g) using distilled water before autoclaving.

Effect of soybean oil on prodigiosin production

An amount of soybean oil was added to the solid substrates in order to determine whether the addition of the vegetable oil could stimulate the production of prodigiosin. The amounts of oil supplemented were 0.25, 0.5 and 1 ml.g⁻¹ substrate.

Elicitation experiments

Inocula of bacterial elicitor cells (*E. coli* and *Bacillus subtilis*) were prepared from an overnight growth culture in LB **broth** which already incubated overnight at 37°C. The number of elicitor cells was adjusted to be approximately 1×10^7 cells/ml using a haemocytometer. Cells of each elicitor were collected by centrifuge at 10000 rpm for 15 min. Boiling water for 30 min was used to prepare heat killed elicitor cells. Elicitor cells were then washed and an equal volume of sterile saline was used to re-suspend the cells. Following the same procedure, elicitor cells of *Saccharomyces cerevisiae* were prepared using sabouraud medium and incubation at 30°C.

In the elicitation experiments, each elicitor (live or heat killed cells) was added to *S. marcescens* culture at zero time at a concentration of 0.1, 0.2, 0.4 ml. g⁻¹ substrate. A pure culture of *S. marcescens* was used as a control culture.

Prodigiosin determination

Fermented medium was extracted with 20 ml of methanol with shaking for at least 3h. The mixture was centrifuged at 11000 and the absorbance of the supernatant was determined at 530nm after removing substrate and cells debris. The concentration of prodigiosin was determined based on using molar extinction coefficient ($E_{530} = 7.07 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$) [15].

Results and Discussion

In general the type and nature of substrate, moisture level/water activity and inoculum size are the important factors that play a crucial role in the growth and activity of the microorganism in a solid state fermentation and consequently production of metabolites. Therefore, these factors were investigated in this study to identify the optimal conditions for prodigiosin production as shown below.

Solid state fermentation

In order to determine the solid substrate that supports the maximal production of prodigiosin, *S. marcescens* was grown indifferent media (wheat bran, soya bean ground, rice husk, starch, peanuts husk). As can be seen in Fig.(1A), wheat bran showed the highest production of prodigiosin compared with other media. The concentration of prodigiosin in this medium after 48 h of incubation was 47.5mg.gds^{-1} . On the other hand, although rice husk and soya bean ground provide a nutritionally good medium for the growth, *S. marcescens* produced less prodigiosin (11.7 and 10.2 mg.gds^{-1} respectively) compared with wheat bran. Furthermore, production of prodigiosin in starch and peanuts husk media were significantly low (2.5 and 3.4 mg.gds^{-1} respectively) albeit the growth of *S. marcescens* was obviously similar to that observed in other media.

In the wheat bran medium, production of prodigiosin started during the first 8 hours of the incubation which then increased dramatically throughout the fermentation period and reached its maximum of 47.5mg.gds^{-1} after approximately 48 h of the incubation Fig.(1B). During the incubation period, the colour of the solid substrate was turned red associated with the accumulation of prodigiosin in the solid substrate.

It is well known that secondary metabolites are synthesized through biosynthetic pathways which usually start with a number of precursors formed from the catabolism of carbon substrates [1]. Several studies have revealed that the biosynthetic pathway of prodigiosin involve incorporation of some amino acids, particularly proline and methionine that serve as essential precursors

for the production of the red pigment. According to the literature, wheat bran is normally contains high level of these amino acids which might be the reason for the increase in the production of prodigiosin observed in the wheat bran medium [9].

Initial moisture level is an important parameter in solid state fermentation which can directly affect the growth and metabolite production. Studies on the effect of moisture have shown that at high moisture level, a reduction in the porosity of the substrate can be occurred as the substrate becomes agglomerated which lower the oxygen diffusion rates leading to decrease the gaseous exchange. Consequently, the rate of substrate degradation is reduced and consequently reduces the growth and metabolite production. On the other hand, if the moisture level is too low, the microbial growth is usually reduced because the substrate is become less accessible as it does not swell. Based on the results presented in Fig.(1C), highest production of prodigiosin in the wheat bran medium was obtained in the culture with an initial moisture level of 1:2.5(ml: gm). Concentration of prodigiosin in this culture was 60.7mg.gds^{-1} after two days of incubation. When the initial moisture level was less or more than 1:2.5(ml:g), production of prodigiosin was consequently decreased.

As mentioned earlier, production of prodigiosin from *S. marcescens* was enhanced in liquid culture as a result of supplementation with plant seed oils. In this context, Y.H Wei and W.C. Chen [16] used soya bean oil, olive oil and sun flower oil for improving prodigiosin production from *S. marcescens* in Modified LB medium. They found that all three oils substrates displayed a stimulatory effect on the prodigiosin production with an increase of 10-14 folds in comparison to the control culture. It was reported that the saturated and unsaturated fatty acid content and the viscosity of the oil are factors that may affect the yield of prodigiosin. The low viscosity oil is generally more accessible to cells, while high saturated fatty acid content may support prodigiosin production [17]. In this work, the inexpensive and easily available soybean oil which contains high level of saturated fatty acid with low viscosity was

selected as a model of oil substrate for enhancing prodigiosin production in the wheat bran medium [18]. As can be seen in Fig.(1D), the oil containing medium displayed 1.6-fold enhancements in prodigiosin production over control. The results revealed that an oil content of 0.5ml.g^{-1} substrate was optimal for soybean oil supplementation in terms of prodigiosin production [100.7mg.gds^{-1}]. Though, based on the literature, this increase in the production of prodigiosin is appreciably low in comparison with those obtained in the liquid medium [16].

The observed correlation between prodigiosin production and oil supplementation might be explained in this way: *S. marcescens* is generally produces lipase which hydrolyses the oil substrates releasing some fatty acids that provide a more accessible carbon and energy sources for the growth and prodigiosin production. However, more research on this topic needs to be undertaken, particularly on the metabolic regulation, before this correlation is more clearly understood.

Elicitation experiments

In the natural habitat, greater competition can be occurred among various types of microorganisms leading to deplete the nutritional resources [10]. For that reason, different strategies might be followed by microorganisms for survive, one of which is the production of antibiotics. *Serratia*

marcescens is ubiquitous that commonly find in the soil, water, plants and animals. Therefore, it is expected that *S. marcescens* has developed an evolutionary recognition mechanism and physiological responses for different species of microorganisms that can be exploited in the lab for making a positive effect in the antibiotic production. Basically, our strategy for eliciting *S. marcescens* is based mainly on mimicking the presence of a second microorganism which acts as a competitor in the fermentation medium. In general, elicitation can motivate the antibiotic producer organism into switching on or activate its secondary metabolite pathways that may yields a novel product of antibiotics [10] or increase the known compounds as obtained in this study. We already tested successfully this strategy in submerged fermentation in *Pseudomonas aeruginosa* culture to enhance phenazine production [19] and in *Streptomyces coelicolor* culture that showed a change in the pigments production pattern, such that the production of the red pigment undecylprodigiosin was significantly increased and the blue pigment actinorhodin reduced [12]. Hence, this work was designed to examine the suitability of this strategy in solid state fermentation for enhancing prodigiosin production.

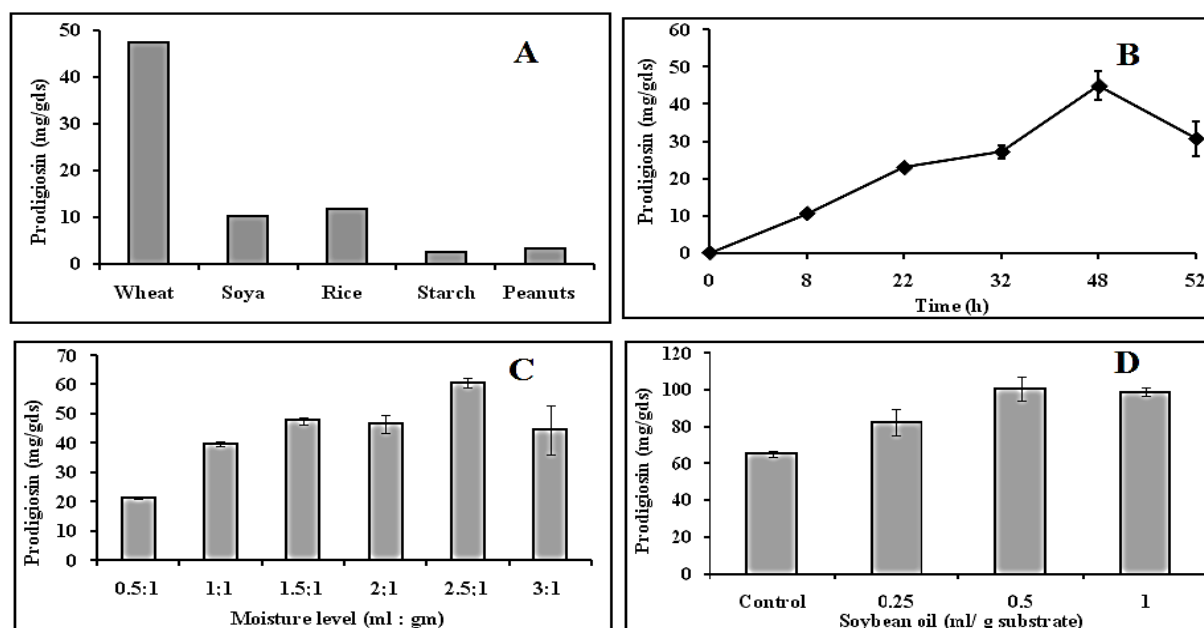


Fig.(1): Production of prodigiosin by *S. marcescens* in solid state fermentation: A, solid state media; B, incubation time; C, Moisture level; D, Wheat bran medium supplemented with soybean oil.

Three microorganisms; the Gram positive *Bacillus subtilis*, Gram negative *E. coli* and the *Saccharomyces cerevisiae*; were selected as competitors (elicitors) in *S. marcescens* culture. Three inoculation levels of live and dead elicitors were tested (0.1, 0.2, 0.4ml.g⁻¹ substrate).

Addition of live cells of elicitors

Our results revealed that the production of prodigiosin was notably enhanced in cultures supplemented with live cells of elicitors compared with the control Fig.(2). The results showed that the maximum increase in prodigiosin production was obtained in the culture supplemented with live cells of *B. subtilis* compared with those elicited with *E. coli* and *S. cerevisiae*. Concentration of prodigiosin after 48h of incubation in cultures elicited with 0.1, 0.2, 0.4ml.g⁻¹ substrate of *B. subtilis* were 184.7, 205.1 and 240.3mg.gds⁻¹ respectively, compared with the control which was 100.47mg.gds⁻¹ Fig.(2). Based on the results presented in Table 1, cultures elicited with *E. coli*, *B. subtilis*, and *S. cerevisiae* displayed approximately 1.8 to 2.3-fold enhancements in prodigiosin production over control.

Addition of heat killed cells of elicitor

Interestingly, the same elicitation effect on the prodigiosin production by *S. marcescens* was observed when dead prokaryotic and eukaryotic microbial cells were used as elicitors. As can be noticed from Fig.(3), production of prodigiosin was noticeably increased in the cultures supplemented with dead elicitor cells compared with pure culture. Similar to the cultures elicited with live cells, an earlier onset of prodigiosin production was observed after 24 hours of incubation reaching its maximum on the second day. Maximum enhancement of prodigiosin was observed in culture supplemented with dead cells of *B. subtilis* compared with those elicited with *E. coli* and *S. cerevisiae*. Concentration of prodigiosin after 48 h of incubation in the cultures elicited with 0.1, 0.2, 0.4ml.g⁻¹ substrate of *B. subtilis* were 169.3, 178.9 and 203.5mg.gds⁻¹ compared with the control which was 104.47mg.gds⁻¹ Fig.(3).

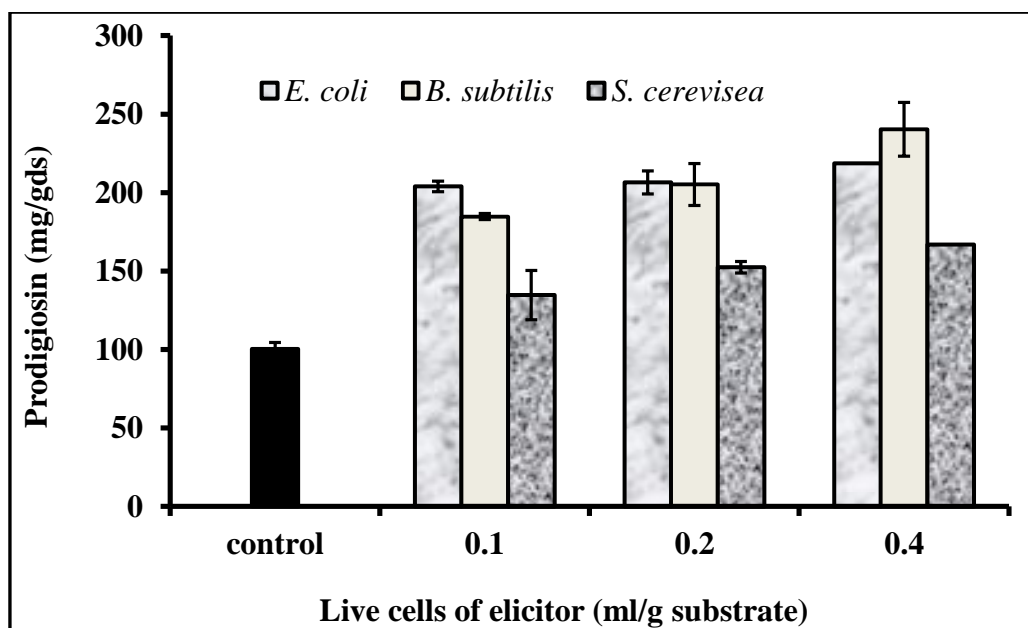


Fig.(2): Prodigiosin production by *S. marcescens* in cultures supplemented with live elicitor cells of *E. coli*, *B. subtilis* and *S. cerevisiae* using wheat bran as a substrate.

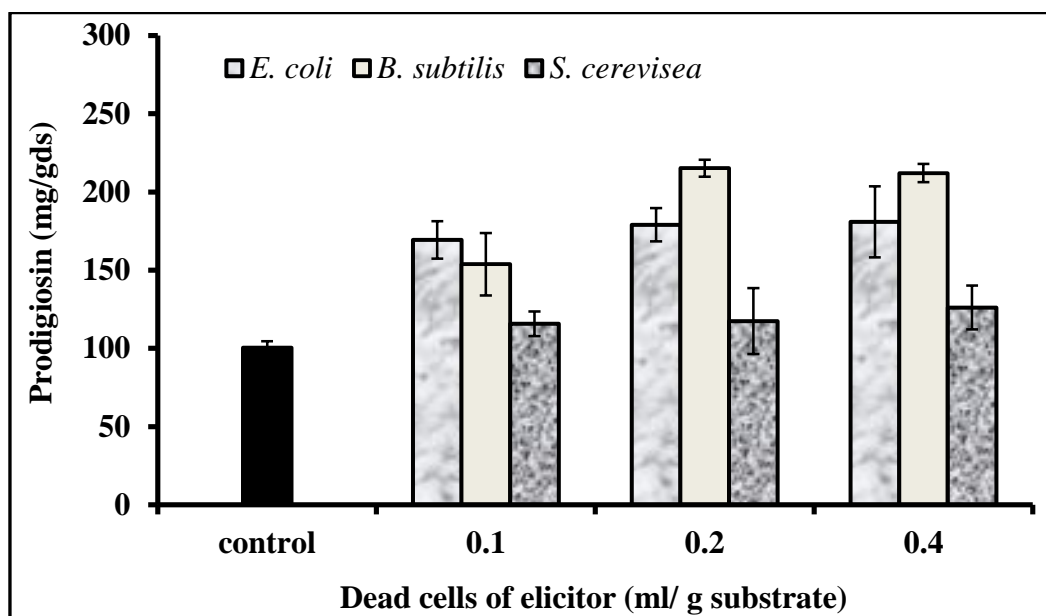


Fig.(3): Prodigiosin production by *S. marcescens* in cultures supplemented with dead elicitor cells of *E. coli*, *B. subtilis* and *S. cerevisiae* using wheat bran as a substrate.

Table (1) summarized the increase in the production of prodigiosin achieved in cultures elicited with both live and dead cells. It can be observed that the highest production of prodigiosin was achieved in the culture supplemented with live cells of *B. subtilis*. Also, a summary of the maximum prodigiosin obtained in this work in solid state fermentation with supplementation of oil and microbial elicitor are presented in Table (2).

Table (1)
Comparison of prodigiosin production achieved in elicited cultures.

Inoculation concentration (ml/gm substrate)	Increased in production of Prodigiosin[fold]					
	<i>E. coli</i>		<i>B. subtilis</i>		<i>S. cerevisiae</i>	
	Live	Heat killed cells	Live	Heat killed cells	Live	Heat killed cells
0.1	2.03	1.69	1.84	1.53	1.38	1.19
0.2	2.06	1.789	2.04	2.14	1.56	1.20
0.4	2.18	1.809	2.39	2.11	1.71	1.290

Table (2)
Production of prodigiosin in solid substrate in comparison with cultures supplemented with oil and microbial elicitor cells.

Solid substrate medium	Production of prodigiosin	
	Mg.gds ⁻¹	Fold-increased
Solid substrate fermentation	60.7	-
Oil supplementation	100.7	1.65
Elicitation with microbial cells	240.3	3.96

One of the most important challenges in the microbiological studies is to elucidate the mechanisms by which microbial cells communicate with each other. The nature of microbial interspecies interactions is known to be due to signaling molecules, detection of cell wall components or even direct cell-to-cell contact. Based on the results, the dead cells of elicitor had the same effect as live cells to elicit *S. marcescens*. Certainly, the occurrence of such microbial cells in the *S. marcescens* fermentation medium did not cause any stress such as nutritional or oxygen stress or even releasing compounds that may cause the elicitation. Furthermore, since live cells of elicitor used in this work (*E. coli*, *B. subtilis* and *S. cerevisiae*) are usually grow poorly in the solid state fermentation, it can be deduced that the mechanism of interaction in this case was not mediated via a compound produced by the elicitors.

Usually, when growth of bacteria ceases due to unsuitable cultivation conditions, cells may lyse because of the autolytic enzymes [20]. Moreover, in order to prepare inocula of dead cells, heat was used to kill the elicitor cells which may damage the bacterial cell wall as well as the outer membrane, enzymes, proteins and nucleic acids. In view of that, one potential explanation of the mechanism of elicitation may be as follows: live and dead cells elicitor may have lysed during *S. marcescens* cultivation in the solid substrate and hence contributed some prodigiosin stimulants or molecules that may work as precursors for the pigment.

Alternatively, direct physical cell-to-cell contact between *S. marcescens* and elicitor cells is another possible elucidation for the elicitation. Although elicitor cells in some cases were used as dead cells, *S. marcescens* may have recognized on the surface of elicitor cells some proteins or receptors that may not have been damaged by the heat. In this context, D.C. Oh *et al.* [11] 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 mediated this interaction; therefore, they suggested that the mechanism of interaction was due to cell-cell direct contact.

Conclusion

In general, the common strategy for producing antibiotics is submerged liquid fermentation, though yields is likely to be low in relation to the cost and energy input. Based on the results obtained in this work, *S. marcescens* produced a considerable amount of prodigiosin in solid state fermentation using wheat bran as a substrate. In addition, the present study provides additional evidence with respect to the strategy of exploiting the interspecies interactions in order to enhance antibiotic production which previously tested effectively in liquid culture. This strategy was successful in solid state fermentation such that the production of this antibiotic was increased approximately by 2.3-fold compared with pure culture.

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