CHEMBIOEN-2020 Special Issue

J. Indian Chem. Soc.,
Vol. 97, March 2020, pp. 434-439

Strategies for improved production of prodigiosin by *Serratia mercences*

Tania Paul[a], Tarun Kanti Bandyopadhyay[a], Abhijit Mondal[a] and Biswanath Bhunia*[b]

[a]Department of Chemical Engineering, [b]Department of Bio Engineering,

National Institute of Technology, Agartala-799 046, Tripura, India

E-mail: bbhunia@gmail.com

Manuscript received online 20 December 2019, revised and accepted 28 December 2019

The enhanced production of prodigiosin was performed by two steps approaches. Here in this study, optimization of preservation condition and methodology for seed culturing were applied to attain a higher yield of prodigiosin using *Serratia marcescens* ATCC 14756 in a batch process. Results showed that the yield of prodigiosin was increased while the strain was kept refrigeration for 1 day before subculturing. It was evident that the higher yield of prodigiosin was found with a single time subculturing of previously 24 h preserved strain inside the refrigerator. The maximum yield of prodigiosin is found when the pH of seed media is kept at 6 and the pH of the seed medium was finally shifted after 24 h of subculturing to 7.5. The above final pH (7.5) was considered as a marker for the completion of the seed culturing process. It was evident from results that the production of prodigiosin was found maximum when seed culture was kept inside the refrigerator for 24 h for shocking. The results showed that 1.68 fold higher production of prodigiosin was found by two steps strategies. It was found that enhance the production of prodigiosin was possible by two steps strategies using *Serratia marcescens* ATCC 14756. Therefore, the above strain could be exploited commercially for the production of prodigiosin.

Keywords: Prodigiosin, preservation, seed culturing, *Serratia marcescens*, optimization.

Introduction

Prodigiosin, having molecular formulae $C_{20}H_{25}N_3O$, is a linear tri-pyrrole red pigment. It is considered as a bioactive secondary metabolite that concentrates on cell membranes and also intracellular granules. This bioactive compound is commercially produced from *Serratia marcescens*. The other bacterial sources such as *Serratia plymuthica, Pseudomonas rubra, Hahella chejuensi, Streptomyces coelicolor, Streptomyces lividans, Pseudomonas denitriccans, Vibrio gazogenes, Vibrio psychroerythreus* and *Zooshikella rubidus*[1–4] also able to produce prodigiosin with greater yield. Prodigiosin is commercially exploited for several industrial applications such as anti-bacterial, anti-algal, anti-fungal, anti-malarial, anti-cancer, anti-protozoal, and immunosuppressive, UV protective anti-proliferative activity[3,5–8].

Keeping the demand and usefulness of prodigiosin in the present market, it is urged to develop an inexpensive microbial platform for the production of prodigiosin using biotechnological tools. Prodigiosin is commercially produced using submerged fermentation. The productivity of prodigiosin, therefore, depends on various key parameters involved in this submerged process. Recent reports suggested that optimum requirements of media components along with physical factors involved in the submerged fermentation process are the important factors for the improvement of the yield of prodigiosin. It is evident that the secondary metabolite production process is very complex as several biochemical reactions are involved. Therefore, optimum level of nutrient supplement along with other process parameters during submerged fermentation is challenging task for process engineers[9–14]. Since several environmental factors influence the prodigiosin production process, therefore, these parameters play a significant role in microbial prodigiosin production. Most of the research works, to date, are focused to fulfill the demand for nutrients and physiochemical requirements at an optimum level in the submerged fermentation process. Although several research works are carried out to improve the yield of prodigiosin through optimization of the submerged fermentation process, however, limited research work has been enlightened on alteration of the biochemical pathway

towards higher yield of prodigiosin by artificial development of stress condition on bacteria.

The maintaining of the artificial stress condition from the preservation of microorganisms to cultivation in fermentation media is an important task for a process engineer to achieve a higher yield of prodigiosin. It is evident that inappropriate preservation and regular subculturing have eventually lost the production efficiency of microorganisms for pigment production[15]. Environment condition of seed culture[16] during transfer to production media has played an important role in achieving higher titer of prodigiosin in production media. Optimization of these parameters is required to do judicially before submerged fermentation, so that, a cost-effective platform for the production of prodigiosin can be developed. As per our knowledge concern such a hypothesis, to date, is applied limitedly in submerged fermentation of prodigiosin production. As this hypothesis has potentiality, therefore, an experiment was carried out using *Serratia marcescens* to understand their role in prodigiosin production.

The present work has emphasized the intensification of the prodigiosin production process through optimization of process conditions during the preservation of microorganisms before seed cultivation using *Serratia marcescens.* Additionally, the process condition of seed culture before transferring to production media was critically optimized for higher yield of prodigiosin production.

Materials and methods:

Chemicals and analysis:

Nutrient broth, agar, dextrose, sucrose, lactose, maltose, fructose, and diluent for DNA extraction were analytical grade and purchased from Himedia, Pvt. Ltd. Prodigiosin hydrochloride was purchased from Merck, Germany. The other chemicals used in this study were analytical grade which is commercially available in India. The software package, Graphpad Prism 7 was used for statistical analysis of experimental data.

Microorganism:

Serratia marcescens ATCC 14756 (NCIM-5246) was procured from the National Collection of Industrial Microorganisms (NCIM), Pune, India. The strain was collected in a lyophilized form from the supplier. After collection, the strain was revived by sub-culturing in nutrient agar solid media for 48 h. The incubation temperature for maintaining the growth of bacteria was 30°C. The sub-culturing of bacteria was also carried out on a regular basis in the nutrient broth after an one week interval.

Optimization of preservation time:

The strain grown in petri-plate containing nutrient agar media were kept for 5 days inside the refrigerating condition. One loopful of culture was transferred every day to 250 ml of the conical flask containing nutrient broth media up to 5 days. The petri-plate without keeping inside the refrigerator was considered as control. The nutrient broth media taken for these experiments was 50 ml. Each conical flask containing media was incubated at 30°C for 24 h. 10 percentage (v/v) of seed culture was transferred to the same media and kept at 30°C for 48 h. The sample was withdrawn after 48 h of incubation from liquid media and the production of prodigiosin was measured.

Effects of subculturing:

The effect of subculturing on prodigiosin production was measuring by regular transferring one loopful of a bacterial strain to the conical flask (250 ml) containing 50 ml of nutrient broth media. Each conical flask containing media was incubated at 30°C for 24 h. The transferring of culture was carried out for 5 stages to previously grown media to new liquid media. 10 percentage (v/v) of seed culture was transferred to the same media and kept at 30°C for 48 h. The transferring of culture was also carried out for 5 stages within the solid media containing nutrient agar and kept inside the incubator which is maintained the temperature of 30°C. However, the incubation time was maintained for solid media to 1 day. The sample was withdrawn after 72 h of incubation from liquid media and the production of prodigiosin was measured.

Effects of pH of seed culture:

The effect of pH of seed culture media on the yield of prodigiosin was measuring by transferring one loopful of purified culture having optimized preservation conditions to seed culture media. In these experiments, nutrients broth was used as seed culture media and production media. The experiments were carried out at 250 ml of conical flask having 50 ml of nutrient broth media at 30°C for 24 h and pH of media was maintained at various pH of 5, 6, 7, 7.5, and 8. 10 percentage (v/v) of each seed culture was transferred to same media (pH 7.5) and kept at 30°C for 48 h. The sample

was withdrawn after 72 h of incubation from liquid media and the production of prodigiosin was measured.

Optimization of a shocking period of seed culture:

To understand the effect of a shocking period of seed culture on the production of prodigiosin, the experiments were carried out by keeping the seed culture inside the refrigerator for 12, 24, 36, 48, 60, 72 h. After keeping the seed culture inside the refrigerator for assigned time, 10 percentage (v/v) of each seed culture was transferred to nutrient broth media (pH 7.5) and kept at 30°C for 48 h. The sample was withdrawn after 72 h of incubation from liquid media and the production of prodigiosin was measured. The seed culture without keeping inside the refrigerator was considered as control.

Analytical methods for measurement of prodigiosin:

The UV spectrophotometer was for analyzing the amount of prodigiosin content as per Ref. 17. Briefly, the 10 ml of sample collected from fermentation broth was centrifuged at 10,000 rpm for 10 min. The pellet so obtained after centrifugation was suspended in the equal volume in acidified ethanol (5.0 mL of water (pH 3) and 95.0 mL of ethanol) and kept for 10 min for extraction of prodigiosin from bacteria. The mixture was again centrifuged for 10 min at 15000 rpm to remove the cell debris. The supernatant so obtained after centrifugation was subjected to spectrophotometric analysis at 535 nm and the content of prodigiosin was measured from the standard curve. All experiments were carried out for triplicate.

Results and discussion

Optimization of preservation time:

The effect of preservation time on the production of prodigiosin was illustrated in Fig. 1a. It is evident that preservation time is very much influential for the enhancement of bacterial prodigiosin production. Fig. 1a depicts that the yield of prodigiosin is increased from 72.12 mg/L to 85.5 mg/L while the strain was kept for 1 day inside the refrigerator. However, further increase of preservation time inside the refrigerator is reduced the yield of prodigiosin in production media. It is obvious that sudden change of temperature is created the stress inside the cell which influences the production of secondary metabolites or any kind of pigment production. However, longer refrigeration, cell undergoes spore formation. Hence spore takes longer time for germination under suitable environmental condition which leads to decrease the productivity of pigment[18]. In the present experiment, bacteria grown in solid media is required to keep inside the refrigerator for 1 day to attain the higher yield of prodigiosin in fermentation broth after 48 h of incubation in production media which is considered as optimum preservation time. Interestingly, that 1.19 fold of enhancement of prodigiosin yield was found in comparison with control after one day of preservation inside the refrigerating condition.

Effects of subculturing:

To understand the effect of subculturing on prodigiosin production, experiments were carried out by transferring one loopful of a bacterial strain to the conical flask (250 ml) containing 50 ml of nutrient broth media. The bacterial strain was kept inside the refrigerator for 1 day. The role of subculturing on prodigiosin production has been shown in Fig. 1b. It is evident that prodigiosin production is decreased as a number of subculturing increased. The maximum yield of prodigiosin (85.5 mg/L) is evident when previously 24 h preserved strain inside the refrigerator was subcultured for a single time. The yields of prodigiosin were found as 53.01 mg/L, 32.87 mg/L, 20.38 mg/L and 12.63 mg/L after two,

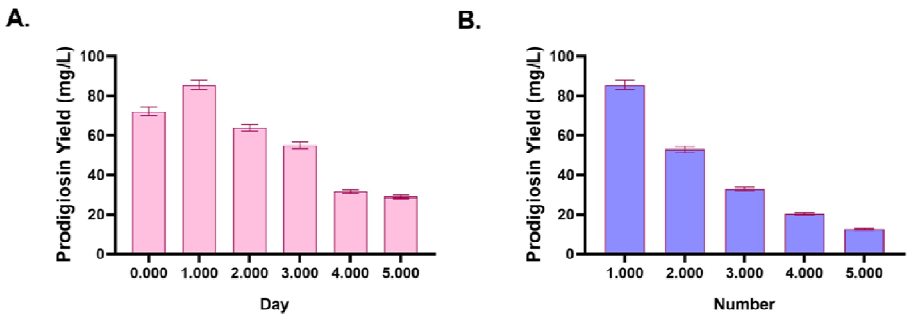

Fig. 1. The effect of (a) preservation time and (b) subculturing on prodigiosin production.

three, four and five times of subculturing respectively. It is obvious that a higher number of subculturing enforces the growth of bacteria through overcoming the stress environment which was previously maintained[19]. The production of prodigiosin after repeated subculturing is exhibited in Fig. 2.

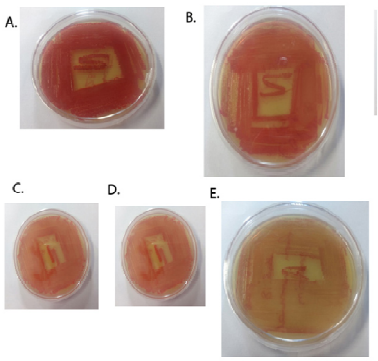

Fig. 2. The prodigiosin production after subculturing in solid media after day (a) 1, (b) 2, (c) 3, (d) 4 and (e) 5.

It is evident that the intensity of colour developed after 24 h incubation in solid media decreases as a number of subculturing increased. It is obvious that when bacteria are kept inside the refrigerator, it has remained as resting cells. In that condition, the cell is under stress condition and consumes minimum energy to survive. However, favorable environmental condition promotes the growth of bacteria[20]. The stress condition is required to maintain for higher yield of prodigiosin production; therefore, the parameters that modulate the stress condition is optimized in this study. Here, the number of subculturing is considered as an important parameter release the stress environment inside the bacteria, therefore, minimum subculturing is found to pay a maximum yield of prodigiosin.

Effects of pH of seed culture:

To understand the role of pH of seed media, the experiments were conducted at various pH of seed media. The variation of productivity of prodigiosin with changing the pH of seed media is illustrated in Fig. 3a. It is evident that pH has a significant role in the enhancement of prodigiosin yield in production media. Fig. 3a depicts that the maximum yield of prodigiosin is found at seed media maintaining pH of 6 and considered as optimum in the present case. In the previous experiment, it is found that bacteria showed a higher yield of prodigiosin when bacteria is kept for 1 day inside the refrigerator as refrigerating condition provides a stress environment. In this study, an attempt was taken to provide constant stress to the seed culture. Therefore, the stress environment is maintained by changing the pH of the media during the development of seed culture. It is evident that the extent of the growth of bacteria is influenced by the pH of the media[21,22]. Since the optimum pH for *Serratia marcescens* is 7.5, therefore, a lower yield of prodigiosin is found within the range of pH from 7 to 7.5. The maximum yield of prodigiosin is found when the pH of seed media is kept at 6. As the pH of media is acidic therefore, it certainly inhibits the growth of bacteria which turns activate the stress environment. Obviously the growth of bacteria is drastically hampered at higher or lower pH, therefore, the yield of prodigiosin is decreased at pH of 5 and 8. It is evident from Fig. 3a that the amount of prodigiosin is affected by the initial level of pH of seed culture. After completion of seed culture, the pH of media is gradually increased and is reached to 7.5 which is due to lowering the acidity of the media[19]. Therefore, in the present study, the pH of 6 is considered a marker to indicate the completion of seed cultivation.

Optimization of a shocking period of seed culture:

In this experiment, seed culture was kept inside the refrigerator for the various time interval for providing the stress environment. The role of a shocking period on the production of prodigiosin is illustrated in Fig. 3b. It is evident from Fig. 3b that the maximum yield of prodigiosin is found as 121.64 mg/L after 48 h of incubation in production media when seed culture was kept 24 h inside the refrigerator. It is evident from Fig. 3b that shocking period is an important parameter for prodigiosin production. Results show that the yield of prodigiosin is gradually increased as shocking time is increased up to 24 h. However, a further increase in the shocking period of seed culture decreases the yield of prodigiosin after 48 h of incubation in production media.

The requirement of customized seed culture is required for the development of the inexpensive fermentative process. The development of appropriate seed culture required for a specific production system depends on the quality of seed culture which depends on the quality of various process parameters of the fermentation process[16]. Obvious, process parameters mainly components of media and physicochemical alter the metabolic activity of the bacteria. Here in this study, an attempt has been taken to evaluate the role of shocking time on the yield of prodigiosin. Results show that

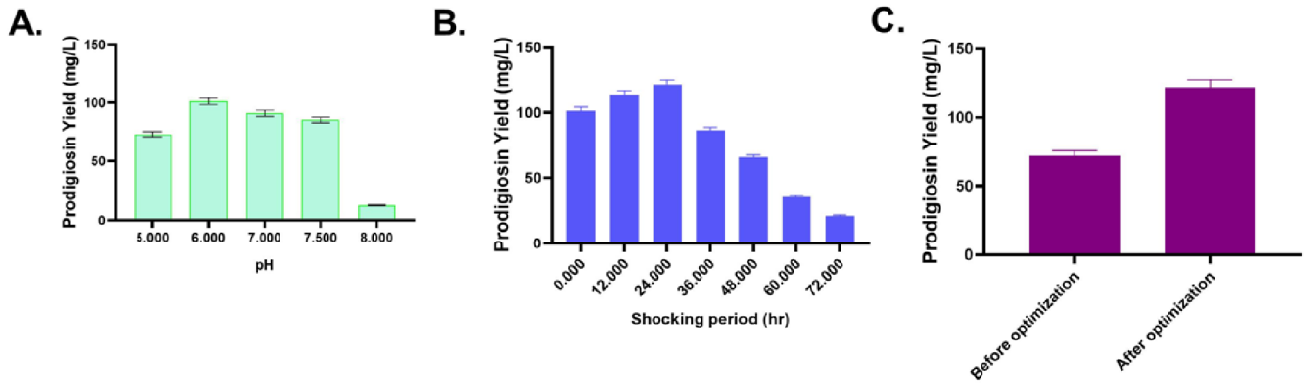

Fig. 3. (a) The effect of pH, (b) shocking period of seed culture on prodigiosin production and (c) the yield of prodigiosin before and after optimization of bioprocess.

the minimum yield of prodigiosin is found when seed culture was kept inside the refrigerator for 72 h as the higher shocking period is decreased the growth of bacteria. It is evident that most of the research publications had extensively studied the optimization of media composition and various process parameters had done to augment the productivity of prodigiosin. Furthermore, extensive research work also carried out to design the reactor and development of the various process. However, limited research works have been enlightened on stress development in preservation and seed culturing. Therefore, the impending implication of stress development during the preservation of bacteria along with seed culturing was convincingly brought to our consideration for seed culture development by an experiment with *Serratia marcescens* ATCC 14756. It is evident from the results that 1.68 fold higher production of prodigiosin has been carried out by two steps strategies (Fig. 3c). The production of prodigiosin is increased from 72.12 mg/L to 85.50 mg/L in the first stage. In this stage, the management of strain was carried out before seed cultivation. However, 121.64 mg/L of prodigiosin is further enhanced upon optimization pH and shocking period of the seed cultivation process.

Conclusion

The present study deeply addresses the role of preservation time, subculturing, environmental condition of seed culturing and the effects of a shocking period on the production of prodigiosin, while experiments have been conducted in a batch process using *Serratia marcescens* ATCC 14756. The potential of these factors has been evaluated based on their role in the yield of prodigiosin. The environmental stress has been provided to the present strain in both stages to understand the role of it in the production of prodigiosin. It is evident that preservation time inside the refrigerator has a significant role in the yield of the product. However, more subculturing of *Serratia marcescens* ATCC decreases the titer of prodigiosin in production media. The initial acidic pH of seed culture has induced the stress to the microorganism and enforces the production of prodigiosin. Before the inoculation of *Serratia marcescens* ATCC in production media, the seed culture is required to provide shock through preservation inside the refrigeration for 1 day. The above hypothesis has successfully implied in the present investigation and 1.68 fold higher production of prodigiosin is achieved.

Acknowledgements

The work provided in this manuscript, is supported by the National Institute of Technology, Agartala, India. All authors acknowledge to honourable Director of NIT Agartala for his constant inspiration.

References

1. J. D'Aoust and N. N. Gerber, *Journal of Bacteriology*, 1974, **118**, 756.
2. D. Kim, J. S. Lee, Y. Park, J. F. Kim, H. Jeong, T. K. Oh, B. S. Kim and C. H. Lee, *Journal of Applied Microbiology*, 2007, **102**, 937.
3. J. S. Lee, Y.-S. Kim, S. Park, J. Kim, S.-J. Kang, M.-H. Lee, S. Ryu, J. M. Choi, T.-K. Oh and J.-H. Yoon, *Appl. Environ. Microbiol.,* 2011, **77**, 4967.
4. A. M. El-Bondkly, M. M. El-Gendy and R. H. Bassyouni, *Antonie Van Leeuwenhoek.*, 2012, **102**, 719.

5. B. Montaner and R. Pérez-Tomás, *Life Sciences*, 2001, **68**, 2025.
6. V. Soto-Cerrato, E. Llagostera, B. Montaner, G. L. Scheffer and R. Perez-Tomas, *Biochemical Pharmacology*, 2004, **68**, 1345.
7. M. Borić, T. Danevčič and D. Stopar, *Microbial Ecology*, 2011, **62**, 528.
8. H. Park, S. G. Lee, T. K. Kim, S. J. Han and J. H. Yim, *Biotechnology and Bioprocess Engineering*, 2012, **17**, 1232.
9. R. P. Williams, C. L. Gott and S. H. Qadri, *Journal of Bacteriology*, 1971, **106**, 444.
10. F. R. Witney, M. L. Failla and E. Weinberg, *Appl. Environ. Microbiol,* 1977, **33**, 1042.
11. M. Sole, A. Francia, N. Rius and J. Loren, *Letters in Applied Microbiology*, 1997, **25**, 81.
12. H. Slater, M. Crow, L. Everson and G. P. Salmond, *Molecular Microbiology*, 2003, **47**, 303.
13. S.-L. Wang, C.-Y. Wang, Y.-H. Yen, T.-W. Liang, S.-Y. Chen and C.-H. Chen, *Process Biochemistry*, 2012, **47**, 1684.
14. I. N. Ryazantseva, V. S. Saakov, I. N. Andreyeva, T. I. Ogorodnikova and Y. F. Zuev, *J. Photochem. Photobiol. B: Biol.,* 2012, **106**, 18.
15. H. S. Tuli, P. Chaudhary, V. Beniwal and A. K. Sharma, *Journal of Food Science and Technology*, 2015, **52**, 4669.
16. P. Dantigny and S. P.-M. Nanguy, *International Journal of Food Microbiology*, 2009, **134**, 16.
17. E. B. Kurbanoglu, M. Ozdal, O. G. Ozdal and O. F. Algur, *Brazilian Journal of Microbiology*, 2015, **46**, 631.
18. S. Shivaji and J. S. Prakash, *Achives of Microbiology*, 2010, **192**, 85.
19. U. S. P. Uday, S. Goswami, K. Gopikrishna, T. K. Bandyopadhyay and B. Bhunia, *3 Biotech*, 2018, **8**, 337.
20. N. Osherov and G. S. May, *FEMS Microbiology Letters*, 2001, **199**, 153.
21. P. Sakthiselvan, B. Naveena and N. Partha, *Brazilian Journal of Microbiology*, 2014, **45**, 1293.
22. V. L. Colin, M. D. Baigorí and L. M. Pera, *AMB Express*, 2013, **3**, 1.