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BIOTECHNOLOGY OF PRODIGIOSIN: RECENT DEVELOPMENTS AND TECHNOLOGICAL CHALLENGES

Wani Devita Gunardi[1]*, Margaretha[1], and Kris Herawan Timotius[2]
[1]Department of Microbiology, [2]Department of Biochemistry,
Krida Wacana Christian University (UKRIDA), Jakarta
*Email: wani.gunardi@ukrida.ac.id

ABSTRACT

Background: Prodigiosin is produced by Serratia marcescens. It has several pharmacological benefits, such as anticancer, antimicrobial, and antidaibetic. However, prodigiosin production still faces problems because it cannot be produced effectively, efficiently, and cheaply. Objective: This study aimed to conduct a review that can explain the upstream and the downstream process in prodigiosin production. Methods: Articles were searched from PubMed and ScienceDirect with the keywords prodigiosin and Serratia marcescens from Juny until September 2023 including review and original article. Relevant data and information were then extracted. Results: Prodigiosin has spectrometrical characteristics, which are crucial for evaluating its production, extraction, and purification identification. Submerged or solid-state fermentation is applicable for prodigiosin production, but solid-state fermentation is better. The kind of growing substrates and the cultural condition influence it. The use of oil-based carbon sources is recommended for the high productivity of prodigiosin. In order to have a cheap, effective, and efficient production process, different experiments have been conducted. Standard extraction and purification methods can carry out the downstream process. Conclusion: Prodigiosin can be produced via submerged or solid-state fermentation. Using cheap and readily available substrate are the key to success for the upstream and downstream process. The standard extraction and purification methods are available.This findings can be used as a basis for further research regarding large-scale production of prodigiosin with the cheap, effective, and efficient methode.

Keywords: antibacteria, anticancer, antidiabetes, *Serratia marcescens,* solid state fermentation

ABSTRAK

Latar belakang: Prodigiosin adalah pigmen merah yang dihasilkan oleh Serratia marcencens. Prodigiosin tersebut memiliki beberapa bioaktivitas penting, seperti antikanker, antibakteria, dan antidiabetes. Bioteknologi prodigiosin masih menghadapi kendala karena belum dapat diproduksi secara efektif dan efisien dengan biaya yang murah. Tujuan: Tinjauan pustaka ini bertujuan untuk menjelaskan ciri-ciri utama prodigiosin, perkembangan proses perbanyakan dan proses pengunduhan dalam produksi prodigiosin. Metode: Sejumlah makalah diperoleh dari PubMed dan ScienceDirect dengan kata kunci prodigiosin dan Serratia marcescens. Ekstraksi data dan informasi dilakukan untuk menulis tinjauan pustaka ini. Hasil: Prodigiosin memiliki beberapa ciri spektroskopis yang sangat bermanfaat untuk evaluasi keberadan dan jumlah prodigiosin. Prodigiosin juga memiliki beberapa manfaat farmakologis, sebagai antikanker, antibakteri dan antidiabetes. Proses produksinya tergantung pada strain Serratia marcescens yang digunakan, sistem pertumbuhan, medium pertumbuhan, dan kondisi pertumbuhan. Proses pengunduhan (ekstraksi dan pemurnian) prodigiosin dapat dilakukan dengan berbagai metode standard yang sudah biasa digunakan. Kesimpulan: Perkembangan bioteknologi prodigiosin yang murah, efektif dan efisien dimungkinkan dengan memperhatikan

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sejumlah faktor yang memengaruhi pertumbuhan. Proses pengunduhannya dapat dilakukan dengan cara-cara ekstraksi dan pemurnian yang sudah lazim digunakan. Penemuan ini dapat dijadikan dasar penelitian lebih lanjut mengenai produksi prodigiosin dalam skala besar dengan biaya yang lebih murah, lebih efektif, dan efisien.

Kata Kunci: antibacteria, anticancer, antidiabetes, *Serratia marcescens,* solid state fermentation

INTRODUCTION

Serratia marcescens is a potential biotechnological bacteria because of its capacity to produce a valuable product, prodigiosin, a red pigment. It is a Gram-negative bacteria, facultatively anaerobic (Haddix and Shanks 2018), and a member of Enterobacteriaceae. *S.marcescens* is known as a nosocomial bacteria and opportunistic pathogen. Nevertheless, it is a biotechnological mini-factory in producing prodigiosin (Ferreira, Oliveira et al. 2022, Karczewski, Bäcker et al. 2023, Liébana-Rodríguez, Portillo-Calderón et al. 2023, Moreno, Velandia et al. 2023, Rodríguez, Lobato et al. 2023, Tavares-Carreon, De Anda-Mora et al. 2023).

Prodigiosin has diverse biological activities, particularly its anticancer, antimicrobial, and antidiabetic properties (Choi, Lim et al. 2021, Rodríguez, Lobato et al. 2023). Its anticancer potential has been proven in various cancers (e.g., breast and gastric cancers, lymphoma, colon cancer, and nasopharyngeal carcinoma), lacking harmful side effects on normal cells (Anwar, Albanese et al. 2022). It has an apoptosis-inducing effect in many cancers (Sudhakar, Shobana et al. 2022) and can positively attach to the binding site of caspase-3 firmly with a binding energy score of -17.37 kcal/mol (Sudhakar, Shobana et al. 2022). Therefore, prodigiosin is a potential anticancer drug (Sudhakar, Shobana et al. 2022, Zhao, Gao et al. 2022).

A purified prodigiosin exhibits antibacterial activities (Sudhakar, Shobana et al. 2022). Prodigiosin is stable at different temperatures, pH, and NaCl concentrations and does not show any toxicity for humans (Fürstner 2003, Dos Santos, Rodríguez et al. 2021). Due to its potent antioxidant activity, it is supreme for healing wounds and encouraging the immune system. However, its hydrophobicity limits its bioavailability. Therefore, a prodigiosin carrier is needed to increase its hydrophobicity. A kind of carrier, bionanocomposite, has been developed (Araújo, Zavala et al. 2022). Applying bionanocomposite is a recommended strategy to obtain carriers for prodigiosin transport and release (Li, He et al. 2022).

Prodigiosin has been patented for treating diabetes mellitus without any side effects. Prodigiosin has diabetes-suppressive effect. The prodigiosin's diabetes-suppressive activity is associated with cytokine production regulation. Prodigiosin inhibits cytokine expression, such as IL-2, IL-6, IL-10, IL-12, and IFN-gamma, essential in diabetes mellitus. Therefore, prodigiosin can decrease blood glucose levels (National Center for Biotechnology Information 2023). Prodigiosin is a potent inhibitor of α-glucosidase due to its a very low IC_{50} value of 0.0183 µg/mL, stronger than acarbose, a common antidiabetic drug (Tran, Techato et al. 2021).

The efficiency of the chemical synthesis of prodigiosin is very low. Therefore, the production of prodigiosin by *Serratia marcescens* should be improved to a high level (Han, Xiang et al. 2021). Strategies are needed for prodigiosin production, particularly at a scale-up to large-scale level. Prodigiosin, with its valuable biological activities, can increase its marketability. Therefore, the exploitation of prodigiosin produced by *S. marcencens* is pharmaceutically necessary. Prodigiosin emerges as an exciting agent because of its broad spectrum of bioactivities in biomedical applications, not only anticancer and antibacterial, but also anti-amoebic, anti-Chagas, algicidal, antifungal, antimalarial, antiparasitic, antiviral, and immunosuppressive (Cediel Becerra, Suescún Sepúlveda et al. 2022, Islan, Rodenak-Kladniew et al. 2022). Evaluating the development and challenges in prodigiosin production using minifactory *S.marcescens* is necessary. This review aimed to evaluate the development of upstream and downstream processes of prodigiosin. The various methods of the prodigiosin upstream production and downstream process were evaluated from the previous studies published in scientific literature.

PRODIGIOSIN

Prodigiosin (syn. 2-methyl-3-pentyl-6-methoxyprodiginine) is a promising biomolecule which has many potential biomedical applications. It is an alkaloid produced by bacteria, particularly *Serratia marcescens*. It has a tripyrole chemical structure (Figure 1) (Islan, Rodenak-Kladniew et al. 2022). Besides *Serratia marcescens*, diverse bacteria are also prodigiosin producers, like Hahella, Pseudoalteromonas, Vibrio, Zooshikella, Streptomyces, and Actinomadura. These bacteria produce various prodigiosin isoforms called prodiginine. However, most efforts focus on prodigiosin, while knowledge about other prodiginine members is little (Hu, Withall et al. 2016, Li, He et al. 2022).

UV scan of prodigiosin shows maximum absorbance at 530-538 nm (Balasubramaniam, Alexpandi et al. 2019, Bhagwat and Padalia 2020, Dos Santos, Rodríguez et al. 2021, Liu, Yang et al. 2021). It can be measured quantitatively by a spectrophotometer at its molar extinction coefficient (ε_{535} = 139,800 ± 5,100 $M^{-1}cm^{-1}$) in acidified ethanol. Briefly, the cell material of *S. marcescens* (OD_{650}=1) can be centrifuged, and the obtained pellet can then be extracted with 1 mL acidified ethanol (4% v/v of 1 M HCl). The supernatants can be filtered or again centrifuged till cleared liquid is obtained. Prodigiosin is then measured with a spectrophotometer (Xu, Wang et al. 2021). Analysis of prodigiosin can also be applied to samples from solid-state fermentation. Five grams of the sample can be extracted with ethanol (50 mL) by refluxing at 45°C. The filtered ethanolic solution is then ready for spectrophotometric analysis (Xia, Wang et al. 2016). Production of prodigiosin is calculated and expressed as prodigiosin weight per liter culture (mg/L), as volumetric production time (mg/L/h), or as product per cell dried weight (mg/gDCW) (Domröse, Klein et al. 2015).

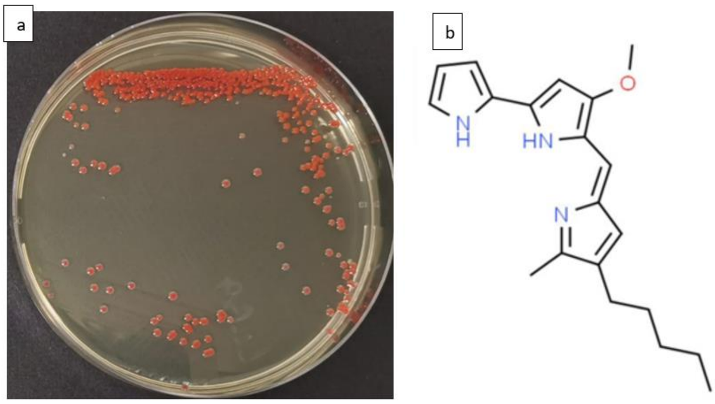

Figure1. Colonies of *Serratia marcencens (a)* and its podigiosin (b) (PubChem)

The molecular weight of purified prodigiosin is 324 Da (de Araújo, Fukushima et al. 2010). FT-IR, UV-Vis spectrometer, GC-MS, and LC-MS/MS, can be used to check the pureness of prodigiosin. LC-MS or GC-MS analysis is helpful to validate the presence of the peak at 323 m/z. (Balasubramaniam, Alexpandi et al. 2019) (Liu, Yang et al. 2021).

The biosynthetic pathway employed by prodigiosin-producing bacteria to produce prodiginine is already known (Hu, Withall et al. 2016). Similar to porphyrins, glycine is a precursor of prodigiosin. Particular genes responsible for prodigiosin biosynthesis can be modified genetically. Therefore, genetic modification techniques can be applied to enhance prodigiosin production. For example, particular strains of *Pseudomonas putida,* which have inserted pig genes from *S.marcescens,* can be used as vehicles for prodigiosin production. The metabolic engineering methods have been used for the bacterial production of prodigiosin and its analogs, focused on the improved production of diverse prodigiosin with more desirable physicochemical properties (Prabowo, Eun et al. 2022).

Generally, prodigiosin has a high hydrophobic character (XLogP3-AA = 4.5), and consequently, it has low bioavailability (Islan, Rodenak-Kladniew et al. 2022). Therefore, to enhance its bioavailability via oral administration, a nanoencapsulated technique has been introduced. Nanoencapsulated is a process encapsulating substances on a nanoscale(Ayala-Fuentes & Chavez-Santoscoy, 2021). Several nanocapsuled that can be used to increase the bioavability such as alginate, chitosan, and casein(Koo dkk., 2023). Although, the recently research encapsulation methode to increase prodigiosin bioavability has been't exiss.

Upstream Process of Prodigiosin

In order to develop a high-throughput and economically realistic prodigiosin-producing process, innovations are undoubtedly needed. Many strategies and methods can be developed for increasing the production yield of prodigiosin. Medium composition, supplementation, cultural conditions, strain improvement, and fermentation methods have been studied (Han, Xiang et al. 2021).

***Serratia marrescens* as prodigiosin producer**

The growth of *S. marcescens* can proceed under various fermentation systems, submerged or solid-state fermentation, batch or continuous/chemostat culture, and planktonic or biofilm growth (Haddix and Shanks 2018).

Small-scale experiments use Erlenmeyer 500 mL flasks, either non- or baffled, with 10% working volume capacity. The most common growing medium used for small scale is LB or TB medium (Table 1). Incubation is at 3O°C with shaking (120 rpm). A cell density of OD_{650}=0.05 can be used as a preculture to inoculate production cultures. Growth parameters can then be evaluated.

For upscale experiments or production scale culture, various production media are introduced. Most media are complex and limited defined media are used (Table 1). Until now, the production cost of commercial prodigiosin is still high, partly caused by the expensive growth medium (Aruldass, Venil et al. 2014). As a result, there is an increase in investigating the use of inexpensive carbon and nitrogen (C/N) sources for large-scale production (Wang, Nguyen et al. 2020).

Carbon sources for *S.marcescens* may be simple carbohydrates, polyol, and oils (Table 1). The cultures can grow in media containing glucose, glycerol, or acetate combined with organic nitrogen. However, pigment production occurs in a nonglucose medium (Andreeva and Ogorodnikova 1999).

The nitrogen source used is usually organic nitrogen. Even though *S.marcescens* can consume inorganic nitrogen sources, it converts it to organic nitrogen. Many studies used various peptones for bacterial growth and prodigiosin production. The growth and production of prodigiosin need peptone combined with a particular carbon source, like mannitol or glycerol (Kurbanoglu, Ozdal et al. 2015, Elkenawy, Yassin et al. 2017).

Various oil sources have recently been considered essential carbon sources for producing prodigiosin. Palm oil exhibits better biomass and prodigiosin yield than peanut and olive oil. The specific growth rate can be improved when it is supplemented with palm oils. Prodigiosin production is favorable in palm oil substrates with high saturated fat content (Abdul Manas, Chong et al. 2020). Different fatty acid-containing oils, peanuts, sesame, and mustard seed cakes are good oil sources. Many oil sources may come from waste and unconventional bioresources. High oil content substrate supports the maximum production of prodigiosin. They are nutrient precursors for producing prodigiosin (Bhagwat and Padalia 2020).

Soybean oil is useful as a carbon source and soy peptone. It provides a carbon source for prodigiosin biosynthesis, relieves feedback inhibition, and upregulates the expression of biosynthetic genes of prodigiosin. A submerged fermentation system in a continuous extraction mode has been created using soybean oil, effectively boosting prodigiosin production while reducing reliance on conventional organic solvents. The soybean oil can undergo recycling after the prodigiosin extraction process (refer to upstream case 7 for details). Under optimal conditions, involving an ideal nitrogen source, such as a C/N ratio of 100/10, temperature at 28°C, and a pH level of 5.0, a substantial prodigiosin yield of 27.65 g/L can be attained (Liu, Yang et al. 2021).

Various industrial agriculture sectors produce a large proportion of waste. Husks, like edible seeds, vegetable peels, and straws of certain crops, are potent materials for prodigiosin production. They are excellent protein, fiber, vitamins, carbohydrates, and mineral sources. Their untreated disposal causes considerable toxic waste. Therefore, to minimize pollution, we need to have environmentally friendly techniques for beneficial and productive activities, including the use as a media for bacterial growth and prodigiosin biosynthesis (Jameel, Umar et al. 2023). For example, as a low-cost substrate, "manipuera" (casava wastewater) has been proven a renewable media for prodigiosin production. In this case, *S. marcescens* can produce prodigiosin at the high level of 49.5 g/L(de Araújo dkk., 2010).

Table 1. Nutrition of *Serratia marcescens*

Nutrient	Compounds
Carbon source	Glucose (Valentina, Alejandra et al. 2019)
	Lactose (Aruldass, Venil et al. 2014, Gondil, Asif et al. 2017)
	Sucrose (Bhagwat and Padalia 2020) (Sudhakar, Shobana et al. 2022)
	Mannose (Jardak, Atoissi et al. 2022)
	Glycerol (Elkenawy, Yassin et al. 2017)
	Mannitol (de Araújo, Fukushima et al. 2010, Kurbanoglu, Ozdal et al. 2015)
	Brown sugar (Aruldass, Venil et al. 2014)
Nitrogen source	Ram horn peptone 0.4% (w/v) (Kurbanoglu, Ozdal et al. 2015)
	Tryptophan (4.0 g/L)(Aruldass, Venil et al. 2014, Sudhakar, Shobana et al. 2022)
	Peptone (Jardak, Atoissi et al. 2022)
	Yeast extract (Gondil, Asif et al. 2017)
	Casein (Valentina, Alejandra et al. 2019)
	Soy peptone (Xia, Wang et al. 2016)
	$(NH_4)_2SO_4$ and $NaNO_3$) (Xia, Wang et al. 2016)
Oil substrate	Palm oil, olive oil, and peanut oil (Abdul Manas, Chong et al. 2020)
	Peanut oil seed cake powder (Bhagwat and Padalia 2020)
	Sesame seed oil (Giri, Anandkumar et al. 2004)
	Soybean oil (Dos Santos, Rodríguez et al. 2021, Liu, Yang et al. 2021)
	Waste frying oil (Bhagwat and Padalia 2020, Asitok, Ekpenyong et al. 2023)
Complex medium	LB (Luria-Bertani): 10 g/L tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L) (Domröse, Klein et al. 2015)
	TB (Terrific-Broth): Casein (2 g/L), enzymatically digested, 24 g/L yeast extract (24 g/L), dipotassium phosphate (9.4 g/L), monopotassium phosphate (2.2 g/L), glycerol (4 mL/L) (Domröse, Klein et al. 2015)
	Nutrient broth (Giri, Anandkumar et al. 2004)
semi-defined medium	Starch (1.6 %), peptone (1.07%), and trace elements [0.3% $CuSO_4$ $5H_2O$, $MgSO_4$ $7H_2O$, $CoSO_47H_2O$, $FeSO_4.7H_2O$, and $MnSO_4.4H_2O$)] (1%) (Chen, Tsai et al. 2018)
Minerals/trace elements	$MgSO_47H_2O$ (1 g/L), NaCl (1 g/L), and K_2HPO_4 (1 g/L) (Valentina, Alejandra et al. 2019)
Renewable resources	"manipueira" (cassava wastewater)(de Araújo, Fukushima et al. 2010)
	squid pen powder (Liang, Chen et al. 2013)

Nutrient	Compounds
Supplement	Farnesol (increases the synthesis of prodigiosin) (Kiziler, Orak et al. 2021)
	Ambigols (increase precursor supply: l-proline and malonyl-Co) (Chilczuk, Monson et al. 2020)
	L- tryptophan (Aruldass, Venil et al. 2014)

Note : carbon and nitrogen source for prodigiosin producing bacteria growth; oil substrate for prodigiosin biosynthesis, relieves feedback inhibition, and upregulates the expression of biosynthetic genes of prodigiosin; trace elements for maximum level prodigiosin production; Suplement for increase the synthesis of prodigiosin

The supplement addition is also crucial for the production of prodigiosin. Farnesol supplementation can increase the synthesis of prodigiosin. Farnesol is a fungal quorum-sensing molecule. It affects the synthesis of prodigiosin. It is non-human toxic and low price. It is an inducer for prodigiosin production at a large scale (Kiziler, Orak et al. 2021).

Ion supplementation, phosphate, and ferrous ions are recommended (Liang, Chen et al. 2013). For achieving maximum level, prodigiosin production needs a suboptimal concentration of inorganic phosphate for growth. More than 0.4 mM, KH_2PO_4, prodigiosin production decreases. The maximum bacterial growth can occur at 1.0 mM. For bacterial growth and prodigiosin production, ferric ion is needed. They occur in the presence of 8 to 16 mg of ferric ion/liter and not at higher concentrations (Williams 1973).

Prodigiosin production

Prodigiosin production by *Serratia marcescens* is influenced by growth conditions. The formation of prodigiosin does not always follow its growth. Both biomass production and prodigiosin production are under the influence of different cultural conditions. As a secondary metabolite, the presence of prodigiosin is not obligatory. Prodigiosin is a signal of a particular condition that exposes the cells (Haddix and Shanks 2018).

A cluster of essential genes is responsible for prodigiosin synthesis. In *S.marcescens*, the synthesis of prodigiosin (Figure 2) is regulated by many genes, around 14 genes, such as pigA, pigB, pigC, pigD, and pigM (Williamson, Fineran et al. 2006, Jia, Liu et al. 2021). In addition, regulatory genes are also discovered, namely genes related to quorum sensing and two-component systems (Xu, Wang et al. 2017, Ravindran, Sunderrajan et al. 2019).

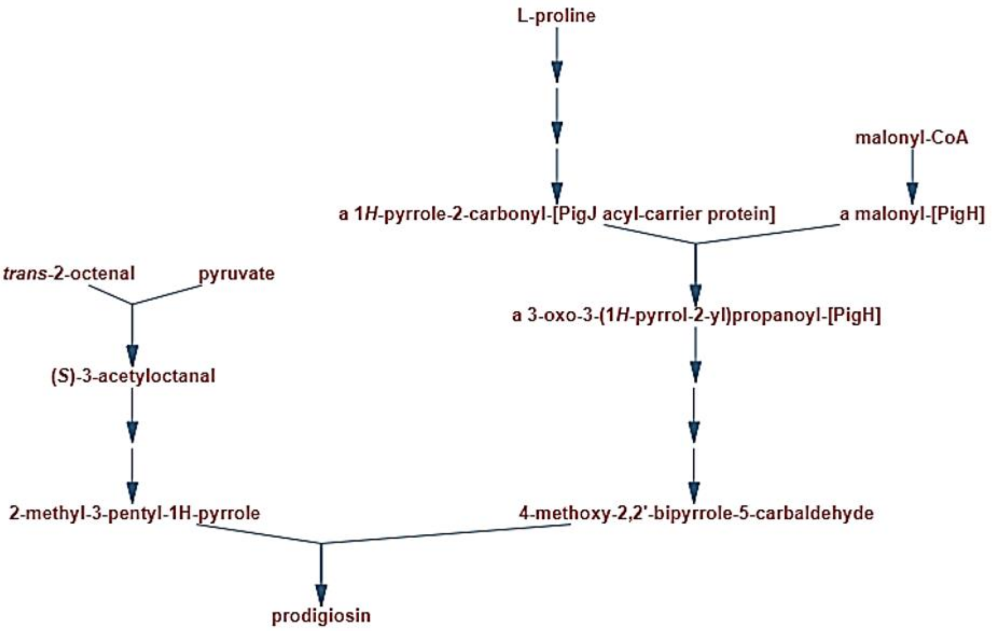

Figure 2. Biosynthesis pathway of prodigiosin (PubChem)

Specific genes play a vital role in governing the synthesis of prodigiosin through quorum-sensing and two-component regulatory systems. Two distinct quorum-sensing mechanisms, SmaI/SmaR (Thomson, Crow et al. 2000, Slater, Crow et al. 2003, Fineran, Slater et al. 2005) and SpnI/SpnR (Horng, Deng et al. 2002), oversee prodigiosin biosynthesis. Quorum sensing (QS) entails the density-dependent regulation of gene expression. In Gram-negative bacteria, a primary QS signaling molecule is N-acyl-l-homoserine lactone (AHL), synthesized by LuxI family proteins. Prodigiosin biosynthesis (see Figure 2) hinges on the pig gene cluster, with AHL-mediated quorum sensing governing the expression of this gene cluster. It's worth noting that QS regulation is not indispensable for the life cycle of S. marcescens (Sakuraoka, Suzuki et al. 2019). The smaI/smaR locus exerts control over Prodigiosin (Pig), where SmaR acts as a repressor of Pig in conditions of low AHL levels produced by SmaI (Fineran, Slater et al. 2005).

Bacteria can detect, sense, and control their environmental condition, especially in various changes in cultural conditions, including temperature, pH, dissolved oxygen, availability of nutrients, and changes in cell density. Therefore, bacteria can form sensors to detect environmental signals and then transform the signals into the regulated target. This system involves two proteins: a specific sensor protein in the cell membrane and a response regulator protein. Both are called two-regulatory systems (TCSs). Specific sensor protein or protein sensor kinase is able to phosphorylate response regulator protein by using phosphate from ATP. Generally, the sensor kinase is histidine protein kinase. The response regulator protein serves as a DNA-binding factor with specificity for the positive or negative regulation of transcription. Typically, the sensor histidine protein kinase detects environmental signals and conveys its phosphate group to the corresponding response regulator protein, which then adjusts the expression of target genes to accommodate the prevailing environmental conditions(Liu, Xu et al. 2023).

At least six types of two-component regulatory systems (TCSs) have been reported for their capacity to regulate the synthesis of prodigiosin are PigQ/PigW (Fineran, Slater et al. 2005), PhoB/PhoR (Gristwood, Fineran et al. 2009), RssB/RssA (Horng, Chang et al. 2010), and EepR/EepS (Stella, Lahr et al. 2015, Jia, Liu et al. 2021), EnvZ/OmpR system (Jia, Zhao et al. 2022), and BarA/UvrY (Liu, Xu et al. 2023). In the case of BarZ/OmpR system (Liu, Xu et al. 2023).

The BarZ/OmpR system can control the expression of more than a hundred under various stress conditions. The function of BarA/UvrY system in Serratia marcescens FS14. The disruption of barA or/and uvrY results in the yield increase of secondary metabolite prodigiosin. BarA/UvrY system represses prodigiosin production by inhibiting the transcription level of pig gene cluster with direct binding to the pigA promoter. BarA/UvrY positively regulates the resistance to H2O2 same as in Escherichia coli highlighting the importance of BarA/UvrY on hydrogen peroxide resistance. The BarA/UvrY system differentially regulates the biosynthesis of the secondary metabolite prodigiosin in S. marcescens FS14 (Liu, Xu et al. 2023).

BarA/UvrY is an important two component regulatory system consisted of the member of subclass of tripartite sensor kinases BarA and its cognate response regulator UvrY classified as FixJ family. It has been suggested that this TCS was stimulated by metabolic end products formate and acetate, even some short-chain fatty acids and can respond to products or conditions of the host organism during infection.

BarA/UvrY is also referred PigQ/PigW Serratia sp. BarA/UvrY were demonstrated to be involved in the expression of noncoding RNAs, including CsrB and CsrC in E. coli. Early studies showed that these small regulatory RNAs tightly control the activity of the global regulatory protein, CsrA. Consequently, the deletion of barA or uvrY gene affects the activity of CsrA protein and then regulates the genes expression involved in carbon metabolism pathways, biofilm formation, motility, and virulence. BarA/UvrY system played important roles in the regulation of cellular survival, virulence, and cellular development in many species of bacteria.

BarA/UvrY system in Serratia marcescens. Strain FS14 is a red-pigmented bacterium producing prodigiosin (2-methyl-3-pentyl-6-methoxyprodiginine) with the pigA-pigN gene cluster. In addition, previous study showed that many environmental factors affected the biosynthesis of prodigiosin, including pH, temperature, oxygen concentration, carbon source and others. Furthermore, it has been reported that some two-component systems in Serratia, such as RssB/A [26], PigQ/W [14] and EepR/S [27] are involved in regulation of the prodigiosin production.

BarA/UvrY two component system negatively regulates the prodigiosin biosynthesis by directly binding to the promoter of pig gene cluster and inhibits the transcription level of genes. It is worth noting that both barA and uvrY mutants exhibit similar sensitivity to hydrogen peroxide as the mutation of BarA-UvrY and the phenotypes were complemented by a plasmid carrying the wild-type barA or uvrY gene. Based on the mutation analyses, this strongly suggests the BarA/UvrY two component system plays differential regulatory roles in prodigiosin biosynthesis and in response to stress and UvrY was solely phosphorylated by BarA in S. marcescens.

Furthermore, prodigiosin synthesis encompasses transcription factors that play crucial roles. These include the affirmative regulators PigP (Fineran, Slater et al. 2005), PigT (Fineran, Everson et al. 2005), PigS (Gristwood, McNeil et al. 2011), PigR, and PigV (Fineran, Slater et al. 2005), as well as the adverse regulators PigX (Fineran, Williamson et al. 2007) and HexS (Tanikawa, Nakagawa et al. 2006).

Many secondary metabolites synthesis, including prodigiosin, is inhibited in a glucose-rich medium. This inhibition involves several genes, such as the GDH (quinoprotein glucose dehydrogenase) gene. GDH activity is related to the glucose inhibition of prodigiosin production. The products of GDH activity have an inhibitory effect. d-glucono-1,5-lactone and d-gluconic acid, but not d-gluconate, can inhibit prodigiosin production. Moreover, the oxidation of d-glucose by GDH can decrease the pH that inhibits prodigiosin production (Kalivoda, Stella et al. 2010).

In the precence of oxygene, S. marcescens will used Fnr to negatively regulate prodigiosin production by binding to the spacer between -10 and -35 region in the promoter prodigiosin biosynthetic gene cluster (Figure 3). The prodigiosin production the prodigiosin yield per cell unit (A_{534}/OD_{600}) for mutant *fnr* was significantly higher than that for wild type(Sun dkk., 2021). Meanwhile, in the high temperature (>30^{0}C) *S. marcescens* will not produce prodigiosin (red pigment) if there is no arabinose (Figure 3). It can be happen because there are HexS as the potent inhibitors of secondary metabolite biosynthesis. Mutation of the *hexS* gene increasing the prodigiosin production whitout arabinose in the high temperature (37^{0}C)(Romanowski dkk., 2019).

Prodigiosin synthesis is associated with the energy condition of the cells. It is related to the ATP production in the cells. Therefore, prodigiosin has an essential role in the bioenergetic process of bacterial cells (Andreeva and Ogorodnikova 1999). Prodigiosin synthesis is correlated with the growth phase of the cell. In batch systems, three phases are recognized: lag phase, exponential or logarithmic phase, and stationary phase. During the lag phase, an increased rate of ATP production occurs. Prodigiosin formation is related to the increased rate of ATP formation (Haddix and Shanks 2018). During the exponential phase, characterized by its high growth rate, prodigiosin declines the ATP levels in the cell. It reduces ATP levels. Prodigiosin biosynthesis is negatively related to ATP levels during high-growth rates. Following the exponential phase, the prodigiosin levels remain high in the early stationary phase and ATP declines. However, at the late stationary phase, the ATP level positively relates to the prodigiosin level per cell (Haddix 2021). The prodigiosin increases ATP production in the lag phase but doubles in the stationary phase. Prodigiosin has both a positive and negative role in ATP biosynthesis. It has a positive role during the lag and stationary phases; meanwhile, during the exponential phase, it has a negative role (Haddix and Shanks 2020, Haddix 2021).

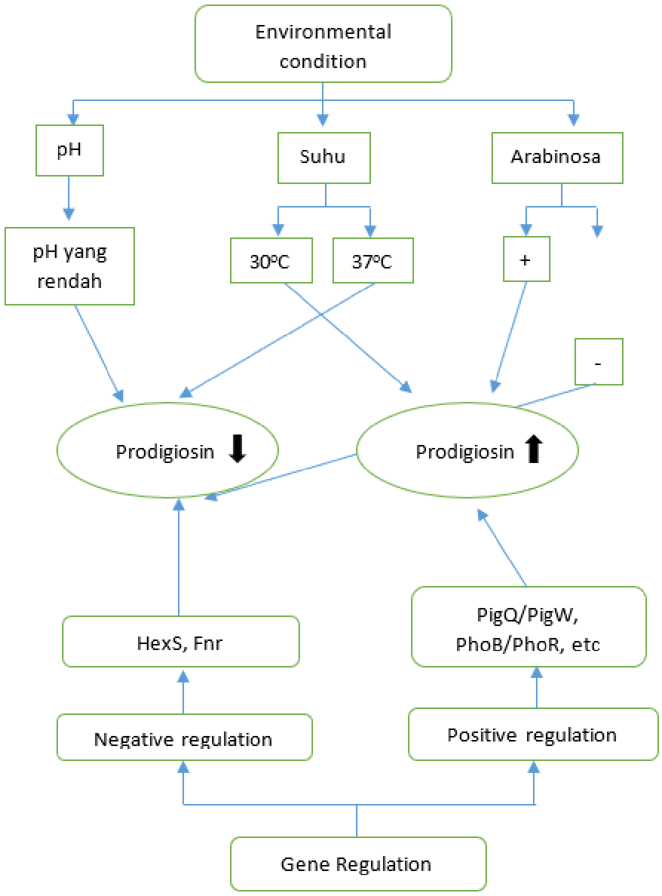

Figure 3. Genetic regulation and environmental impact on prodigiosin production

Cultural conditions

Serratia marcescens that produce prodigiosin have a comprehensive, adaptive response to culture conditions (Cediel Becerra, Suescún Sepúlveda et al. 2022). Several factors crucial for bacterial growth and its ability to produce prodigiosin are pH, temperature, and dissolved oxygen levels (stirring, agitation, aeration, and stirring) (Table 2).

The growth of *S.marcescens* has a broad range of growth pH. However, the optimal pH for growth is 7.0-7.5 (Bhagwat and Padalia 2020, Sudhakar, Shobana et al. 2022). Under certain conditions, *S.marcescens* can grow at pH 9 (Elkenawy, Yassin et al. 2017). Prodigiosin production increases optimally at pH between 6 and 7(Gondil, Asif et al. 2017). The range pH of prodigiosin synthesis by non-proliferating cells is between 5.5 and 9.5, and the optimum pH is 8.0-8.5 (Solé, Rius et al. 1994).

The prodigiosin biosynthesis happens in a narrower span of temperatures than the growth of S.marcenscens (Table 2) (Xu, Wang et al. 2021). Accumulation of prodigiosin occurs in the culture growing at 28ºC, while at 37ºC, no accumulation of the prodigiosin occurs. The maximum production is achieved at 22 °C (Elkenawy, Yassin et al. 2017). Prodigiosin production generally occurs at ambient temperature. In this case, the pigmented culture's biomass yield is much higher than the non-pigmented bacteria (Haddix and Shanks 2018).

The prodigiosin production needs reasonable oxygen transfer rates. In 500-ml Erlenmeyer flasks, the use of internal stainless baffles is suitable for oxygen transfer rates. In a fermentor, either high-speed agitation or low aeration rates are applicable for having optimal oxygen transfer rates. Maintaining a high dissolved oxygen level can proceed with the fermentation process more rapidly. Moreover, changes in pH and cell populations can also be enhanced (Heinemann, Howard et al. 1970, Asitok, Ekpenyong et al. 2023)

The influence of growth rate is also essential for the prodigiosin production. In a dilution rate of 0.057 h, the maximum yield of prodigiosin can be achieved. In the senescent or at a slow growth rate, prodigiosin production increases (Williams 1973, Allen, Reichelt et al. 1983)

Last but not least, NaCl and illumination are environmental factors that influence the growth of the cells and the prodigiosin production. NaCl can inhibit cell growth, but if given at a concentration not exceeding 4-5%, it can increase prodigiosin production (Rjazantseva, Andreeva et al. 1994). Illumination with visible light (< 2,000 lux) can decrease the intensity of prodigiosin but does not change bacterial growth. In addition, light can affect prodigiosin directly (Rjazantseva, Andreeva et al. 1994).

Table 2. Cultural condition of prodigiosin-producing *Serratia marcescens*

Factor	Serratia marcescens growth	Prodigiosin production
Optimal pH	7.0-7.5 (Bhagwat and Padalia 2020, Sudhakar, Shobana et al. 2022)	7.0 (de Araújo, Fukushima et al. 2010)
Optimal Temperature	32 °C (Rodríguez, Lobato et al. 2023)	28 °C (Bhagwat and Padalia 2020) (de Araújo, Fukushima et al. 2010)
Dissolved oxygen levels	low rates of aeration (Heinemann, Howard et al. 1970)	high rate of aeration (Heinemann, Howard et al. 1970)
NaCl 5%	Inhibition (Andreeva and Ogorodnikova 1999, Rodríguez, Lobato et al. 2023)	not exceeding 4-5% (Andreeva and Ogorodnikova 1999)

Upstream cases

Nine upstream cases are discussed in this session. The first six cases are submerged systems. The seventh case is a submerged system with the use of foam cubes. The last two cases are solid-state fermentation. The prodigiosin yields are varied. However, it is higher in solid-state fermentation than in submerged fermentation. Solid-state substrate tends to possess more diverse products than the submerged culture. There is no information on the side products in solid-state fermentation.

***Upstream case 1. Submerged growth* (Heinemann, Howard et al. 1970)**

a. Producing strain: *Serratia marcescens* ATCC 60
b. Production system: Shaken culture and 55-liter stainless-steel fermentor
c. Production medium: 4% autolyzed yeast extract
d. Cultural condition: pH 5.7, aeration, high-speed agitation and low-rate aeration
e. Maximum yield of prodigiosin: 240 µg/mL (at high oxygen rate, pH 8) equivalent to 240 mg/L

***Upstream case 2. Submerged growth* (Tran, Techato et al. 2021)**

a. Production strain: *Serratia marcescens* TNU01
b. Production system: Scaled-up production. 14 L-bioreactor system
c. Production medium: Cassava wastewater, casein (0.25%), $MgSO_4$ (0.05%), K_2HPO_4 (0.1%)
d. Cultural condition: 28 °C
e. Maximum yield of prodigiosin: 6,150 mg/L

***Upstream case 3. Submerged fermentation with brown sugar* (Aruldass, Venil et al. 2014)**

a. Production strain: *Serratia marcescens* UTM1
b. Production system: Shake-flask, 5-l bioreactor
c. Production medium: Brown sugar-based medium with lactose and L-tryptophan supplementation.
d. Production condition: 25ºC, 200 rpm, aeration rate 3 L/min, pH 7.0 (initial)
e. Maximum yield of prodigiosin: ~8,000 mg/mL equivalent to 8 mg/L

Upstream case 4. Submerged fermentation **(Zang, Yeh et al. 2014)**

a. Production strain: *Serratia marcescens* N10612
b. Production system: Batch, 10 mL medium in 100 mL flask
c. Production medium: Sucrose, peptone, yeast extract, NaCl
d. Production condition: 28 ^{o}C, 150 rpm, pH 7.0
e. Maximum yield of prodigiosin: 1,303 mg/L.

Upstream case 5. Submerged fermentation with peptone-mannitol **(Kurbanoglu, Ozdal et al. 2015)**

a. Production strain: *Serratia marcescens* MO-1
b. Production system: Submerged culture
c. Production medium: ram horn peptone+ mannitol
d. Production condition: pH 7, 200 rpm, 28 °C
e. Maximum yield of prodigiosin: 277.74 mg/L

Upstream case 6. Submerged fermentation with UP Foam cubes **(Domröse, Klein et al. 2015)**

a. Production strain: *Pseudomonas putida* with pig gene cluster from *S. marcescens*
b. Production system: 5 L baffled flasks filled with 500 m and PU foam cubes approximately 1 cm^3.
c. Production medium: TB medium
d. Production condition: 20^{o}C
e. Maximum yield of prodigiosin: 2.0 ± 0.1 mg/gDCW and 6.2 ± 0.2 mg/gDCWwere accumulated in *P. putida* strains pig-r1 and pig-r2, respectively

Upstream case 7. Extractive fermentation **(Liu, Yang et al. 2021)**

a. Production strain: *Serratia marcescens* BWL1001
b. Production system: in situ extractive-recycled fermentation, the large-scale batch production.
c. Production medium: Soybean oil as the optimal carbon source (C) and peptone as the optimal nitrogen source (N), with a C/N ratio of 100/10,
d. Production condition: 28 °C, pH 5.0 (initial)
e. Maximum yield of prodigiosin: 27.65 g/L, equivalent to 27,650 mg/L

Upstream case 8. Solid substrate fermentation **(Dos Santos, Rodríguez et al. 2021)**

a. Production strain: *Serratia marcescens* UCP 1549
b. Production system: solid substrate fermentation (SSF)
c. Production medium: 5 g wheat bran, 5% waste soybean oil, saline solution.
d. Production condition: at different temperature, pH, and NaCl concentrations
e. Maximum yield of prodigiosin: 19.8 g/kg dry substrate.

Upstream case 9. Solid substrate fermentation **(Xia, Wang et al. 2016)**

a. Production strain: *Serratia marcescens* Xd-1
b. Production system: Solid substrate fermentation
c. Production medium: Bagasse as an inert carrier, + glycerol+ soy peptone
d. Production condition: 28^{o}C moisture levels 70- 87% (w/w substrate)
e. Maximum yield of prodigiosin: 40.86 g/kg dry solid

Table 3. Prodigiosin yields from various experiments

Ref.	Original data	Yield (mg/L)
(Abdul Manas, Chong et al. 2020)	93 mg/L (with palm oil) 7.8 mg/L (without)	93 (with palm oil) 7.8 (without)
(Chen, Tsai et al. 2018)	103 mg/L	103
(Heinemann, Howard et al. 1970)	240 µg/mL	240
(Kurbanoglu, Ozdal et al. 2015)	277.74 mg/L	277.74
(de Araújo, Fukushima et al. 2010)	0.49 mg/mL	490

Ref.	Original data	Yield (mg/L)
(Bhagwat and Padalia 2020)	0.9 g/L	900
(Zang, Yeh et al. 2014)	1303 mg/L	1,303
(Sudhakar, Shobana et al. 2022)	2,950 mg/L	2,950
(Aruldass, Venil et al. 2014)	~8000 mg/mL	8.00
(Liu, Yang et al. 2021)	27.65 g/L	27,650
(Giri, Anandkumar et al. 2004)	38.75 mg/mL	0.03875
(Xia, Wang et al. 2016)	40.86 g/kg dry solid	
(Dos Santos, Rodríguez et al. 2021)	19.8 g/kg dry substrate	
(Domröse, Klein et al. 2015)	6.2 ± 0.2 mg/gDCW	
(Allen, Reichelt et al. 1983)	3,480 ng/mg protein	

Downstream Process of Prodigiosin

Extraction of prodigiosin

One of the essential factors in extracting any natural product is its efficiency. An efficient method is needed to extract the prodigiosin maximally. Several extraction procedures are applicable for harvesting crude extract of prodigiosin.

The broth from the submerged cultures can be centrifuged to pellet the cells. The pellets collected are then mixed with acidified ethanol or acetone. The supernatant can be separated by centrifugation or filtration. The solvent can be removed by rotatory evaporation. The residue can be partitioned with water and dichloromethane to remove water-soluble impurities. The yielding prodigiosin crude extract is seen as a red solid material. Its purity must be checked by spectrophotometer, HPLC, FTIR, or TLC (Aruldass, Venil et al. 2014, Domröse, Klein et al. 2015).

The pigmented solid substrate or foam cubes can be extracted with a Soxhlet extractor to extract prodigiosin. The round bottom flask should be filled with acidified ethanol, methanol, or diethyl ether (Song, Bae et al. 2006, Domröse, Klein et al. 2015). In addition, the extraction can be conducted by using ultrasonic-assisted reflux extraction (Xia, Wang et al. 2016). In this case, combined ultrasonication can increase the efficiency of the Soxhlet extraction (Khanam and Chandra 2018).

In the coming years, we can anticipate the utilization of environmentally sustainable extraction methods for the retrieval of prodigiosin. These techniques can incorporate the principles of green chemistry to ensure eco-friendly prodigiosin extraction. Examples of such methods encompass ultrasound-assisted extraction, microwave-assisted extraction, pressurized liquid extraction, enzyme-assisted extraction, and, most recently, ionic liquid-assisted extraction, all of which align with eco-friendly practices (Rajendran, Somasundaram et al. 2023).

Khanam and Chandra have been repotrted that ultrasonication extraction methode was observed as the methode with the high yield (98,1±1,76%) compared to the another methode. Thus, they advised that ultrasonication has the potential for commercial application for bioactive pigment extraction. Moreover, they conclude that the present extensively comparative study has revealed the feasibility of ultrasonication as a convenient and cost-effective extraction method for prodigiosin from *S. marcescens*(Khanam & Chandra, 2018).

Purification of prodigiosin

A high amount and good quality prodigiosin is needed. Prodigiosin produced from the fermentation of Serratia marcescnes is probably not yet pure (Khanam and Chandra 2018). Therefore, purification steps are necessary. Various chromatographic techniques are applicable for the purification of the prodigiosin of the crude extract (Hu, Withall et al. 2016). Silica gel column chromatography is applicable for the purification (Song, Bae et al. 2006). Preparative silica gel column chromatography is recommended with dichloromethane as the mobile phase (Domröse, Klein et al. 2015). Preparative HPLC can also be applied for prodigiosin purification (Song, Bae et al. 2006).

CONCLUSION

Serratia marcencens is the most frequently researched for its prodigiosin production. For higher productivity, solid-state fermentation is better than submerged fermentation because the yield product was higher. However both of them, have challenge because the production cost of commercial prodigiosin is still high, partly caused by the expensive growth medium. Therefore, Two fermentation systems, namely using foam cubes and extractive fermentation systems, are promising novel approaches. The use of oil-based carbon sources is recommended for the high productivity of prodigiosin. Extraction and purification methods can be conducted by using Soxhlet and Silica Gel Column Chromatography. The future research in large scale prodigiosin production with the cheap, effective, and efficient methode are interesting.

Authorship contribution statement

Wani Devita Gunadi: Conceptualization and management. **Margretha:** Data curation, writing the draft. **Kris Herawan Timotius:** Review and editing.

Declaration of competing interest

All authors declare that they have no competing financial interests.

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