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Prodigiosin Production From *Serratia* sp. PDGS[120915] Isolated From Daeyeon Stream Water in Busan

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Prodigiosin is a red pigment characterized by a common pyrrolylpyrromethane skeleton. It is produced by *Serratia marcescens*, *Vibrio psychroerythrus*, *Hahella chejuensis*, etc. Prodigiosin has been reported to possess anticancer, immunosuppressant, antifungal antimalarial, and algicidal activities. However, despite prodigiosin's diverse range of activities, its production rate is significantly low and biosynthesis conditions are difficult. Consequently, the selling price is high, and its usability is limited. This study aimed to increase the efficiency of prodigiosin production according to the culture conditions of *Serratia*. In this study, a bacterial strain PDGS[120915] producing prodigiosin was isolated from lightly contaminated stream water in Busan and identified as a strain of *Serratia* sp. based on 16S rDNA gene sequence analysis and physiological characteristics. The reddish pigment from PDGS[120915] was directly extracted using acidified ethanol, and a characterization analysis confirmed that it was a prodigiosin compound. The optimal conditions for pigment production were 25℃, pH 7, and 0% NaCl concentration for a duration of 14 hr. Furthermore, by treating carbon and nitrogen sources, such as fructose and beef extract, respectively, prodigiosin production increased approximately six-fold and four-fold. Among the minerals tested, 0.1% KCl was found to be the most effective for prodigiosin production. Moreover, casein was identified as the most suitable source for prodigiosin production.

Key words : Optimal culture conditions, prodigiosin, red pigment, *Serratia* sp. PDGS[120915]

Introduction

The genus *Serratia*, a member of the enterobacteriacae, is comprised of a gram negative bacteria. They grow well on ordinary media under anaerobic conditions. Optimum growth of all strains of *Serratia* has been observed at pH 9 and temperatures from 20-37℃. *Serratia* can be distinguished from other genera by its production of three special enzymes DNase, lipase, and gelatinase [6]. *Serratia* also produces a non-diffusible red pigment, prodigiosin, which is only present in a small percentage of isolated cultures under aerobic conditions [14]. Pigmentation is highly variable among species and depends on many factors, such as species type and incubation time.

Prodigiosin is a secondary metabolite formed by the enzymatic condensation of 2-methyl-3amylpyrrole (MAP) and 4-methoxy-2,2'-bipyrrole-5carboxyaldehyde (MBC), leading to a tripyrrole derivative, 2-methyl-3-amyl-6-methoxyprodigiosene [8]. It is isolated from a few species, such as Serratia, Pseudomonas, and Streptomyces [6, 24]. It is sensitive to light and insoluble in water but moderately soluble in alcohol and ether, and soluble in chloroform, methanol acetonitrile, and DMSO [7, 14]. Prodigiosin and its derivatives have been reported to have antimicrobial [6, 28], anticancer [5, 20, 21], immunosuppressive effect [11], antifungal [13], and algicidal activities [12]. Despite these extensive researches, the industrial applications are incomplete because producing prodigiosin is limited [22, 27].

This article was isolated an environmental microorganism (*Serratia* sp. PDGS[120915]), which could produce prodigiosin from slightly contaminated stream water. We have investigate the effects of media compositions, such as carbon, nitrogen sources, and several minerals, on prodigiosin production.

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Materials and Methods

Materials

All reagents used were analytically pure chemicals (commercial grade).

Isolation and identification of bacterial strains

The environmental bacterium PDGS^{120915} was isolated from lightly contaminated stream water in Busan and incubated in LB medium at 25℃ for 14 hr. One strain was selected to produce prodigiosin and identified sequencing of 16S rDNA and pig A (prodigiosin pigmentation gene A) gene. Analysis of 16S rDNA sequence was used to identify the PDGS^{120915} isolated. Bacterial genomic DNA was extracted and purified for the sequence analysis using a Wizard Genomic DNA Prep (Promega Co., Madison, USA). 16S rDNA was amplified by PCR using the universal primer and sequenced. PCR was performed with bacterial 16S rDNA primers 8F (5'- AGAGTTTGATCCATGGC-3') and 1492R (5'-GTTACCTTGTTACGACTT-3'). The PCR thermal profile of 16S rDNA was 94℃ for 3 min, followed by 35 cycles of 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 90 sec. The final cycle included an extension for 7 min at 72℃ to ensure full extension of the products. The pig A region was amplified using two oligonucleotide primers, 5'-ATGGATTTTAA CYTRTCAART-3' and 5'-ACCAGRCCGAGTTCBTGATC-3'. The PCR reaction was run for 30 cycles. The following thermal profile was used for the PCR : pre-denaturation at 94℃ for 5 min, primer annealing at 52℃ for 30 sec, 72℃ for 70 sec, and a final extension step at 72℃ for 7 min. DNA sequencing was performed by a capillary DNA sequencer (Genotech, Korea). The nucleotide sequence of the 16S rDNA from PDGS^{120915} strain has been deposited in the NCBI database under accession number KC007128.

Extraction and estimation of prodigiosin

The isolated PDGS^{120915} was precultured for 24 hr at 30℃ and then re-inoculated in 200 ml LB broth. The cells were harvested by centrifugation at 10,000× g for 20 min. The supernatant was discarded, and the pellet was re-suspended in acidified ethanol (5% HCl and 95% ethanol). The mixture was vigorously shaken until completely suspended, and the suspension was centrifuged at 10,000× g for 20 min. The extract was then dried and used for further analysis.

The absorption spectra were measured using a spectrophotometer in a wavelength range from 400 to 650 nm. The prodigiosin absorption maxima were at 499 nm. Also, the bacterial cell absorption prior to extraction was noted at every step. Isolated prodigiosin was estimated using the following equation [19].

$$\text{Prodigiosin unti/cell} = \frac{[OD_{499} - (1.381 \times OD_{620})]}{OD_{620}} \times 100$$

OD_{499}-Pigment absorbance; OD_{620}-Bacterial cell absorbance; 1.381-Constant

Also, absorption spectra were measured in 535 nm, and pigment concentration was obtained using the purified prodigiosin absorbance standard curve.

Optimization of prodigiosin production

In order to examine the effects of incubation time, initial pH, temperature, NaCl concentration, carbon sources, nitrogen sources, and minerals on the prodigiosin production, 1%(v/v) of the bacterial cell was inoculated in LB broth. The inoculated cell was incubated at different times of incubation 0 to 72 hr. The prodigiosin production was estimated with 2 hr intervals. The bacterial isolate was inoculated in LB broth with various initial pH viz. 4 to 11. The inoculated cells were incubated at 25℃ for 14 hr. The initial pH at which maximum production of prodigiosin was maintained in the following studies. Inoculated cells were incubated at different temperatures viz. 4, 15, 20, 25, 28, 30, 37, and 42℃ for 14 hr. The bacterial isolate was inoculated in LB broth with different NaCl concentrations viz. 0 to 10%. The bacterial cells were inoculated in various culture media such as LB, NB, casein, and soybean oil. Especially casein and soybean oil were used a sole nutrient source in the culture medium test. The bacterial cells were inoculated in LB broth containing various carbon sources. All carbon sources are added at 1% and followed: fructose, galactose, glucose, maltose, mannitol, mannose, sorbitol, sucrose, trehalose, arabinose, cellobiose, lactose, melibiose, raffinose and xylose. The bacterial cells were inoculated in prepared LB broth containing 0.5% nitrogen sources such as peptone, polypeptone, proteose peptone, soytone, tryptone, yeast extract, beef extract, sodium nitrate, ammonium sulfate, and ammonium phosphate. The isolated bacterial cells were inoculated in 0.1% mineral-containing LB broth. Used minerals were $CaCl_2$, $CuSO_4$, $HgCl_2$, KCl, $MgCl_2$, $MnCl_2$, Na_2HPO_4 and $ZnCl_2$. Each mineral was added at 0.1%.

Results and Discussion

Identification of bacteria and pigment

PDGS120915 strain was a mobile, non-spore, anaerobic Gram-negative bacterium forming compact red colonies (Table 1). In 1986, Grimont et al. proposed ribotyping as a general method for molecular bacterial identification [8]. This method can also discriminate between isolates of the same species and has proven to be a useful epidemiological tool in studying various bacteria. Phylogenetic analysis using 16S rDNA sequences and the neighbor-joining method showed that the PDGS120915 strain is a member of *Serratia* close to *S. marcescens* and *S. marcescens* subsp. *sakuensis* KREDT (Fig. 1). The isolated strain showed the highest nucleotide sequence similarity to *S. marcescens* subsp. *sakuensis* KREDT to 98%.

Table 1. Biochemical characteristics of *Serratia* sp. PDGS120915 and other *Serratia* species

Characteristic	1	2†	3‡
Spore	-	-	+
Motility	+	+	+
Anaerobic growth	+	+	+
Utilization of			
Arabinose	-	+	-
Cellobiose	-	-	N/D
Citrate	+	N/D	N/D
Fructose	+	+	N/D
Galactose	+	-	N/D
Glucose	+	+	+
Lactose	-	N/D	-
Maltose	+	+	N/D
Mannitol	+	+	N/D
Mannose	+	+	N/D
Melibiose	-	+	N/D
Raffinose	-	-	-
Sorbitol	+	+	+
Sucrose	+	+	+
Trehalose	+	+	N/D
Xylose	-	-	-
Hydrolysis of			
Gelatin	+	+	N/D
Urea	-	-	N/D
Casein	+	N/D	+
Starch	-	-	-
β-hemolysis	+	N/D	N/D
Production of			
Acetoin	-	+	N/D
H_2S	-	N/D	N/D
Indole	+	-	-
Mixed acid	-	N/D	-
Gas	-	-	N/D

Strains: 1, *Serratia* sp. PDGS120915; 2, *Serratia marcescens*; 3, *Serratia marcescens* subsp. *Sakuensis*
N/D; Not determined
†Grimont and Grimont [7]
‡Ajithkumar B [1]

Prodigiosin, reported by *S. marcescens* [3], had a maximum absorption spectrum at 537-538 nm. As shown in Fig. 2, the maximum absorption spectrum of extracted prodigiosin from *Serratia* sp. PDGS120915 showed similar characteristics (Fig. 2). Also, HPLC profile of purified prodigiosin from *Serratia* sp. PDGS120915 showed similar characteristics (Fig. 3). According to Allen's researches [2], prodigiosin, the pigment produced by *S. marcescens*, was red in acid solution with an absorption maximum at 535-540 nm.

Growthconditions for prodigiosin production

To verify the optimal culture condition for the production of prodigiosin from *Serratia* sp. PDGS120915, the effect of culture conditions on growth rate and amount of pigment production were examined. In 2000, Thomson and co-authors demonstrated that producing prodigiosin was coordinately regulated via a quorum sensing system [26]. In this study, related to growth rate and amount of pigment production, bacterial growth was observed at maximum for 8 hr after incubation, but pigment biosynthesis was produced drastically after 12 hr (Fig. 4A).

From the results of growth and pigment biosynthesis, it was different with other studies. Isolated *Serratia* sp. PDGS120915 was grown broadly at pH 5-12, and optimal growth was observed at pH 5, which is slightly acidic. However, optimal prodigiosin production was observed at pH 7 (Fig. 4B). These results are considered suppressed or non-expressed synthesis of prodigiosin according to inhibited enzyme and membrane transport protein activities.

As shown Fig. 4C, optimal temperature of growth and pigmentation were observed at 25℃, but other prodigiosin-producing bacteria such as *Zooshikella ganghwensis* [17] and *Hahella chejuensis* [16] were observed at 30℃. These results were expected to be caused by habitat differences in marine, soil, and stream water. However, all strains were completely blocked in prodigiosin production at 37℃ [23].

To investigate the effect of NaCl concentration on bacterial growth and secondary metabolites biosynthesis, measured the growth rate and amount of pigment production at the range of 0-10% NaCl. Bacterial growth showed 1.0 or more cell density values in the range of 0-6% NaCl, and optimal condition was observed at 1% NaCl. Meanwhile, the maximum pigment production was 0% NaCl, which was reduced sharply as concentration was increased (Fig. 4D). It was considered that Na^+ was not necessary for the stability of cell membrane and enzyme activity at the growth of *Serratia* sp. PDGS120915.

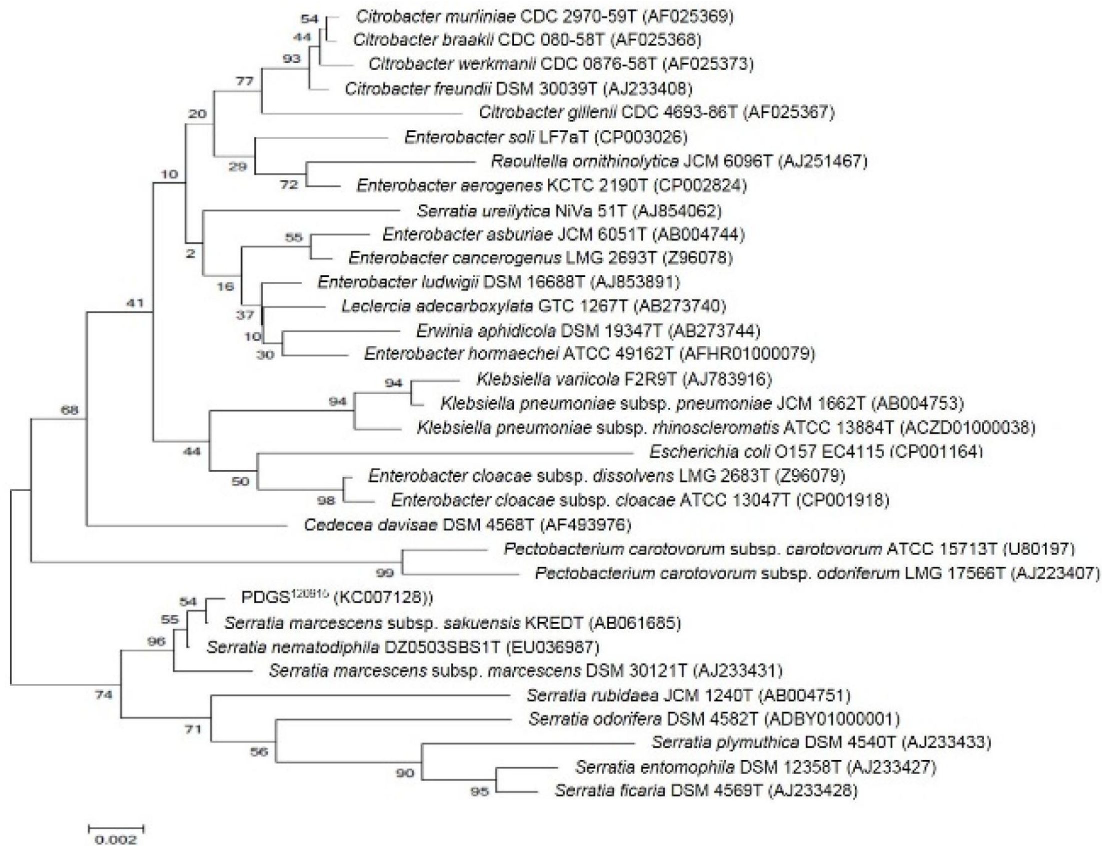

Fig. 1. Neighbour-joining tree based on 16S rDNA sequence, showing relationships between PDGS[120915] and highly homologous group. Number at the nodes are levels of bootstrap support, based on neighbor-joining analyses of 1,000 resample datasets.

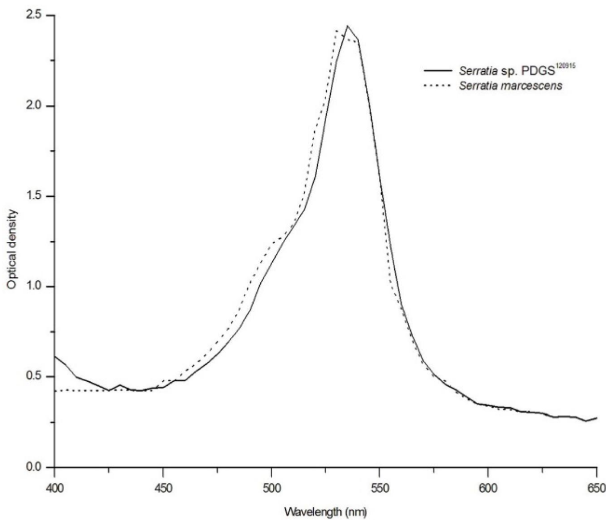

Fig. 2. UV spectrum of prodigiosin isolated from *Serratia* sp. PDGS[120915] and *Serratia marcescens*. Line; *Serratia* sp. PDGS[120915], dotted line; *Serratia marcescens.*

Effects of culture media on the prodigiosin production

Kim and co-authors [15] had demonstrated that soybean oil and casein positively influenced prodigiosin production. However, in our study, soybean oil didn’t have a positive influence. However, casein showed positive influences between 24 hr and 60 hr incubations (Fig. 5). Kim also explained that the easily useful compounds, such as monosaccharides were activated cell growth but inhibited prodigiosin production. In contrast, our results showed that the rapid cell growth led highly prodigiosin production. Casein contains much proline and doesn't have a disulfide bridge. Mentioned as before, the amino acid has an important role in prodigiosin production. So we consider that the prodigiosin production was increased according to the proline oxidase which was produced after the stationary phase.

As summarized in Table 3, when only tyrosin was added to the NB medium, production increased about two-fold. Additionally, there is a study that confirmed an increase of about 2.6 times when methionene and proline were added to 1% peptone water. However, when fatty acids that promote the synthesis of MAP and 2-octanal, such as vegetable oil mixture, are added, production tends to increase. In addition, when hydrophobic polyurethane, which promotes pigment se-

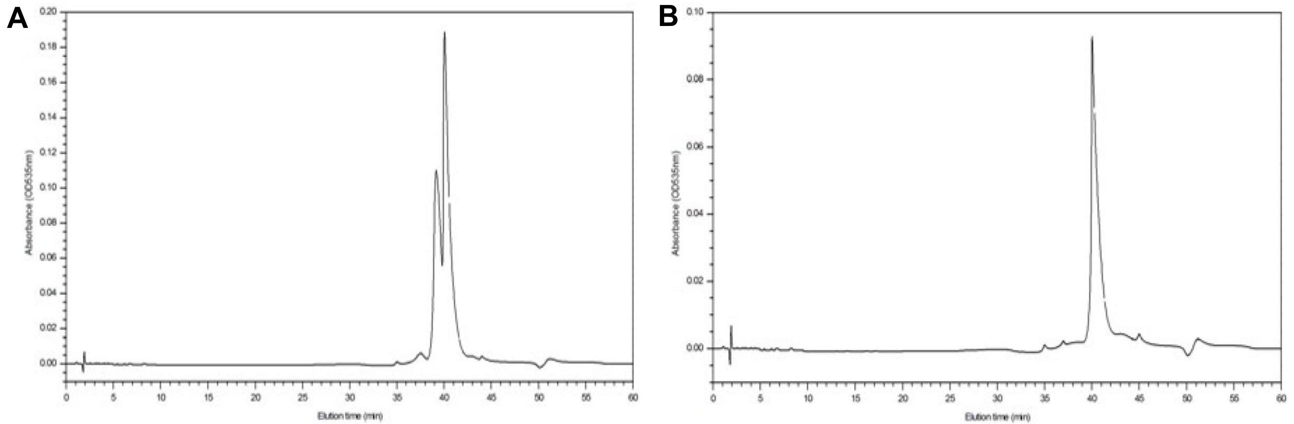

Fig. 3. HPLC profile of prodigiosin from *Serratia* sp. PDGS[120915] (A) and *Serratia marcescens* (B).

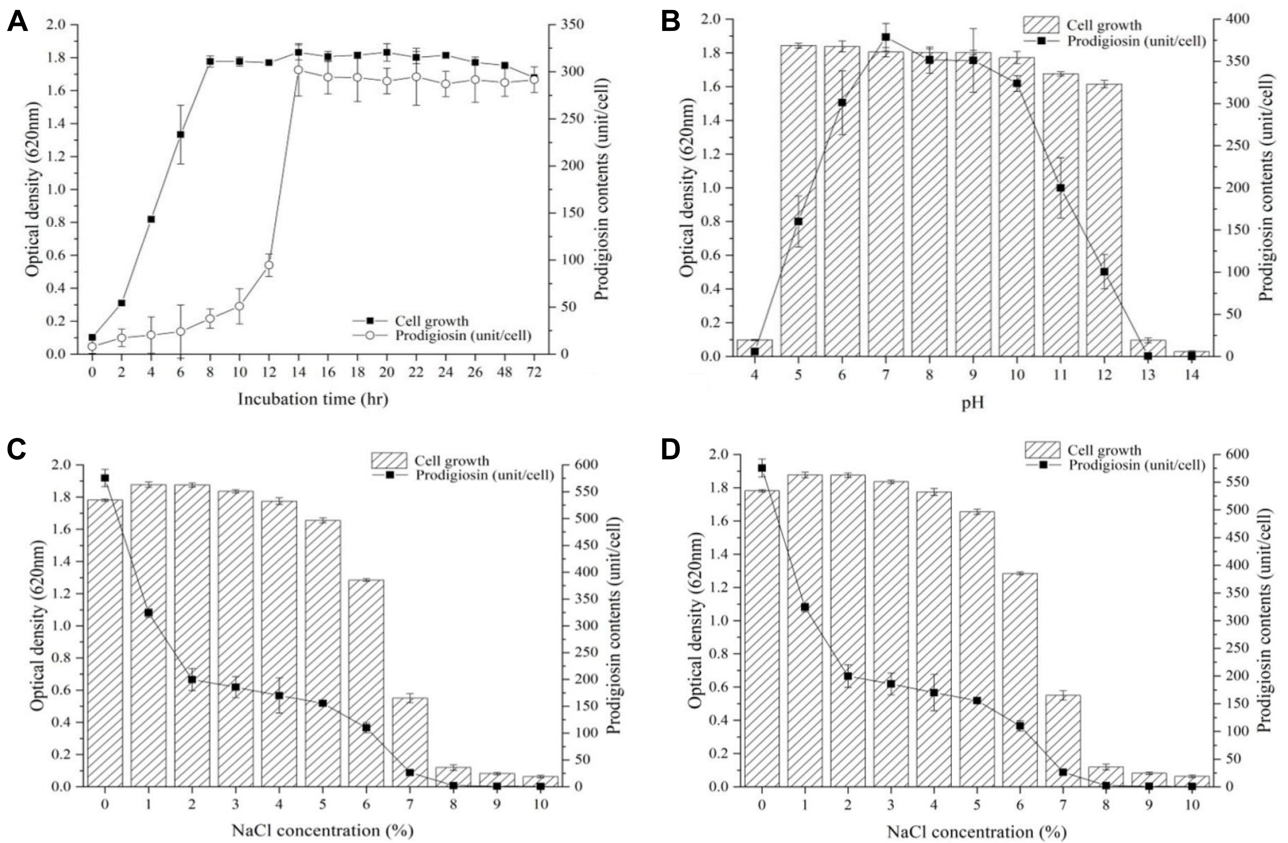

Fig. 4. Effect of culture conditions on the production of prodigiosin. A; Growth curve, B; pH, C; Temperature (℃), D; NaCl concentration (%).

cretion and pigment collection as an adsorbent carrier, was added, an increase of nearly 80 times was confirmed [10].

Absolute comparison is difficult because it is different from the bacterial species used in this study, but since this study also confirmed an increase of about 6 times or more just by adding a carbon source, it is expected that an increase in production can be achieved if additional research is conducted.

Effects of medium composition for the prodigiosin production

To verify the effect of various nutritional sources on growth rate and amount of pigment production, 1% carbon sources, 0.5% nitrogen sources, and 0.1% minerals were add-

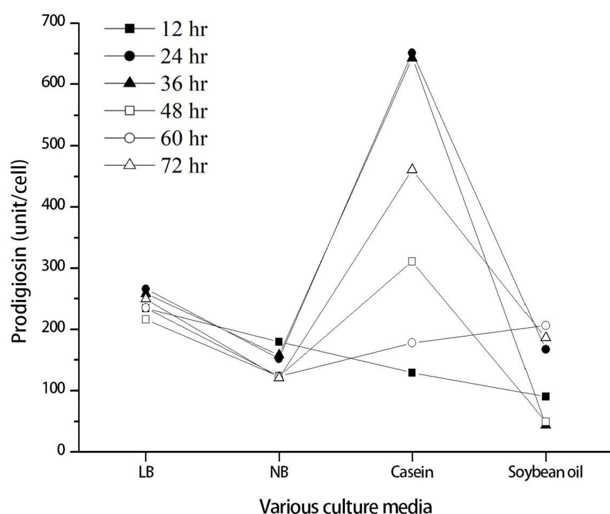

Fig. 5. Effect of various culture media on the production of prodigiosin.

ed in LB medium, respectively. Bacterial growth was promoted in most carbon sources except lactose compared with a control group. An increase of pigment production was observed in most carbon sources. Fructose was the most effective carbon source in bacterial growth and pigment production. It was improved significantly about 613%. Meanwhile, bacterial growth rate was increased in the case of glucose, but pigment biosynthesis was decreased. This inhibition of prodigiosin biosynthesis was caused by glucose, and it showed the same result in other studies [4]. The biosynthesis of prodigiosin is related to proline oxidase, but glucose is inhibition in proline oxidase production [18]. Furthermore, readily available substrates are associated with indirect inhibition effect caused by accumulated pyruvic acid and α-ketoglutaric acid during fermentation [25].

Nitrogen sources are significant components in bacterial metabolism, when organic and inorganic nitrogen sources were added in LB medium, bacterial growth rate and pigment production were increased in most of the nitrogen sources except sodium nitrate compared to a control group. A high increase was observed in beef extracts (about 399%) and

Table 2. Effect of carbon, nitrogen and mineral sources on the cell growth and prodigiosin production

Source	Component	Cell growth (OD_{620nm})	Prodigiosin content (unit/cell)	Prodigiosin content (mg/L)
	Control	1.282	139.91	812.14
Carbon (1%, w/v)	Galactose	1.734	735.73	4270.72
	Glucose	1.417	64.97	377.13
	Maltose	1.686	687.73	3992.09
	Sorbitol	1.613	603.17	3501.24
	Sucorse	1.744	823.39	4779.56
	Fructose	1.789	857.59	4978.08
	Mannitol	1.628	613.25	3559.75
	Arabinose	1.417	297.36	1726.09
	Cellobiose	1.403	244.08	1416.82
	Lactose	1.292	178.25	1034.69
	Xylose	1.429	300.73	1745.66
	Starch	1.538	415.58	2412.33
	Trehalose	1.667	651.18	3779.93
	Melibiose	1.376	238.25	1382.98
	Mannose	1.595	574.27	3333.48
	Raffinose	1.378	239.65	1391.10
Nitrogen (0.5%, w/v)	Peptone	1.347	223.65	1298.23
	Yeast extract	1.620	525.89	3052.65
	Beef extract	1.639	558.74	3243.34
	$NaNO_3$	1.115	23.59	136.93
	$(NH_4)_2SO_4$	1.367	247.01	1433.83
	$(NH_4)_2HPO_4$	1.371	234.76	1362.72
Mineral (0.1%, w/v)	$CaCl_2$	1.406	253.98	1474.29
	KCl	1.409	287.14	1666.77
	$MgCl_2$	1.436	261.54	1518.17
	$MnCl_2$	1.396	255.20	1481.37
	Na_2HPO_4	1.399	262.95	1526.35

Table 3. Medium supplement for prodigiosin production

Microorganism	Supplement	Medium	Prodigiosin yield	Increased fold
S. marcescens WSE	Tyrosin, 0.5%	Nutrient broth	21.28 mg/L	2
P. putida KT2440	Hydrophobic polyurethane	LB medium	94 mg/L	78.33
S. marcescens (SR1)	Vegetative oil mixture, 4%	Casein-enriched medium	765.1 mg/L	ND
S. rubidaea	Methionine, proline	Peptone water 1%	10 mg/L	2.6

ND; data not available

yeast extract (about 376%). Williams and others already reported that prodigiosin biosynthesis is synthesized from various amino acids [18]. Thus, the prodigiosin production were increased the production of nitrogen sources composed of amino acids, minerals and vitamins such as yeast extract and beef extract.

Minerals play an essential role in producing secondary metabolites. Therefore, to verify the effect of various minerals on the growth rate and the amount of pigment production, 0.1% of $CaCl_2$, $CuSO_4$, $HgCl_2$, KCl, $MgCl_2$, $MnCl_2$, Na_2HPO_4, and $ZnCl_2$ were added in LB medium, respectively. Heavy metal minerals such as $CuSO_4$, $HgCl_2$ and $ZnCl_2$ were considered hindrance factors in bacterial growth and pigment production (data not shown). The results are supposed to be related to protein inactivation when the heavy metals combine with a protein with an -SH functional group. $MgCl_2$ and $MnCl_2$ were increased bacterial growth and pigment production (about 187% and 182%). Also, KCl were increased pigment production (about 205%) but bacterial growth did not increase. This result showed that Cl- influenced the pigment synthesis (Table 2).

Acknowledgment

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 하천에서 분리한 *Serratia* sp. PDGS[120915]의 프로디지오신 생산

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프로디지오신은 *Serratia marcescens, Vibrio psychroerythrus, Hahella chejuensis* 등이 생산하는 일반적인 pyrrolylpyrromethane 골격을 특징으로 하는 붉은색 색소이다. 프로디지오신은 항암, 면역억제, 항진균, 항말라리아, 살조 활성을 갖는 것으로 보고되어있다. 프로디지오신은 다양한 활성이 비해 생산율이 현저히 낮고, 생합성 조건이 까다롭다. 이로 인해 판매 가격이 높고, 활용성이 낮다. 본 연구는 Serratia의 배양조건에 따른 생산 효율을 높이기 위한 다양한 연구를 진행하였다. 본 연구에서는 16S rDNA 유전자 서열분석 및 생리학적 특성을 기반으로 prodigiosin을 생성하는 박테리아 균주 PDGS[120915]를 부산의 경미하게 오염된 하천수에서 분리하여 *Serratia* sp.의 균주로 확인하였다. PDGS[120915]의 붉은색 색소를 산성에탄올을 이용하여 직접 추출하고 특성분석을 실시한 결과 프로디지오신 화합물로 확인되었다. 색소 생성은 25℃, pH 7 및 0% NaCl 농도 조건에서 14시간 동안 배양했을 때 최적을 생성을 보였다. 또한 우리는 fructose와 beef extract 같은 탄소 및 질소원을 처리하면 프로디지오신 생산이 각각 약 6배와 4배 증가한다는 것을 발견하였다. 미네랄에서는 KCl이 프로디지오신 생산 증대에 가장 효과적이었다. 카세인은 또한 프로디지오신 생산에 가장 적합한 공급원이었다.