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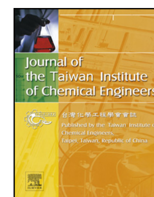

Identification and enhanced production of prodigiosin isoform pigment from *Serratia marcescens* N10612

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ABSTRACT

Serratia marcescens N10612, isolated from soil culture, was found to produce the bio-active prodigiosin isoform pigment (PIP) through nutrition tests. The complex medium consisting of sucrose, peptone and yeast extract was found to be the best composition for PIP production. The optimization of PIP production was carried out by using statistical experiment designs. The optimal concentrations of medium were yeast extract 5.6 g/L, peptone 6.9 g/L, sucrose 31.6 g/L and NaCl 1.0 g/L. Under the optimal conditions, the predicted production of PIP was 1307 mg/L, while the experimental value was 1303 mg/L. Further identification by high resolution mass spectrometry and MNR revealed that the PIP products were prodigiosin and its isoforms. The PIP produced by this strain belongs to shorter carbon chains isoforms (15, 17, 18, 20-C) mentioned in the report. These PIPs are expected to have bioactive functions in medical and clinical applications.

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1. Introduction

Serratia marcescens, a widely distributed bacterium, has been found in air, water, soil and living creatures, including plants and animals [1]. It is capable of secreting different extracellular products, such as chitinase, protease, lipase and nuclease under various conditions [2]. Some secondary metabolites, such as red pigments, can be produced via its metabolism pathways [3,4].

Some natural red pigments were found in the biomass of some species, such as *Pseudomonas*, *Serratia* and *Streptomyces* [5]. One of the red pigments is prodigiosin, which is a cluster of compounds consisting of three pyrrole rings and the pyrrolylpyrromethene core with various alkyl groups [6]. The biosynthesis pathway of prodigiosin has already been conceived, which involved in the synthesis of two pyrrole ring intermediates MBC and MAP [7,8]. Prodigiosin with its isoform pigments, such as: undecylprodigiosin, metacycloprodigiosin, nonylprodigiosin, norprodigiosin and rosephilin, possess multiple medical effects, such as: antibacterial, antitumor, antioxidant and immunosuppressive activities [7,9–12]. Studies on the production of prodigiosin have mainly focused on medium selection, especially the nutrient sources and composition of the medium. Some studies also focused on the effect of process variables, such as temperature and culture aeration control, on prodigiosin production [4,13]. The effects of adding different nutrients, salts and metal ions (ex: ferric acid) for prodigiosin production have been investigated [14–17]. However, few studies have put effort on the prodigiosin isoform pigment (PIP) identification, classification and production.

In this study, the strain, isolated from soil culture and identified as *S. marcescens* N10612, was found incapable of red pigments production when cultured in the traditional nutrient medium. Only under some specified carbon to nitrogen ratio did this strain produce red pigment. This behavior is unusual compared to those of other *Serratia* species in the literature. Therefore, it was used to produce PIP in this study.

In this study, the condition for red pigments production was first studied. The red pigments were then separated and purified to identify their structure and composition. Various carbon and nitrogen sources were investigated. Response surface methodology, such as three-factor Box–Behnken design (BBD), was applied to obtain the optimal conditions for the production of prodigiosin isoform pigments. Box–Behnken design is a statistical method derived from the experiments of three-level factorial designs. Fewer experiments are needed when using BBD to obtain the scientific results [18]. Meanwhile, the structure of the extracted red pigments was identified via high resolution mass spectrometry (HRMS) and NMR detection. The comparison with other studies and discussion on the results are also presented.

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2. Materials and methods

2.1. Chemicals

Beef extract, yeast extract, malt extract, peptone, fructose, lactose, soluble starch, and nutrient agar were obtained from Becton, Dickinson and Company, USA. Glucose, sucrose, ammonium chloride, ammonium sulfate, and sodium nitrate were procured from Showa Chemical Industry Co., Japan. All other reagents and solvents were analytical grade and obtained from Sigma–Aldrich Co., USA.

2.2. Isolation of the strain

The collected soil sample (0.1 g) was inoculated into a 500 mL flask containing 100 mL nutrient broth medium (Difco). The cultivation was conducted at 28 °C, pH 7.0 and 150 rpm for 48 h. The harvested broth was diluted and spread onto the nutrient agar plate to obtain the clusters of single colony. The colony with red color was picked up for the pigments production.

2.3. Determination of 16S rDNA sequence

The method for the isolation of chromosomal DNA was carried out according to the process described by Liu et al. [19]. The 16S rDNA nucleotide sequence was amplified with the primers: p16S-8: 5′-aga gtt gat cct ggc tca g-3′ and p16S-1541: 5′-aag gag gtg atc cag ccg ca-3′ by using the thermal cycler (T100, Bio-Rad, USA). The fragments from PCR were sequenced and identified as *S. marcescens* based on the 16S rDNA sequence (GenBank accession number AJ233431).

2.4. Media and cultivation conditions

The nutrient broth medium (Difco) was used as the enrichment medium and the strain was maintained on nutrient agar. To study the PIP production, this strain was cultivated based on nutrient broth, complete medium and the modification medium of both (compositions shown in Tables 1 and 2), respectively. To study the nutrient effects, modified complete medium 4 (MCM4) in Table 1 was chosen as the central point of Box–Behnken design. To test the carbon and nitrogen sources, different carbon sources, including: sucrose, glucose, fructose, soluble starch and lactose, as well as various nitrogen sources, including: yeast extract, peptone, malt extract, ammonium chloride, ammonium sulfate and sodium nitrate were used.

The seed culture was carried out in a 50 mL flask containing 10 mL MCM4 medium and incubated at 28 °C, pH 7.0 and 150 rpm for 24 h. After that, 1.0% (v/v) of the seed culture was inoculated into 100 mL of main culture medium following the Box–Behnken design and ridge max analysis, and operated under the same conditions as that of the seed culture.

2.5. Isolation and purification of PIP

The harvested culture broth (100 mL) was centrifuged at 1200 × *g* for 10 min to obtain the cells pellet. The pellet was extracted with 300 mL acetone under sonication for 10 min [20]. The supernatant was collected and evaporated to dryness followed by extraction with 50 mL chloroform. The extract was evaporated to dryness followed by purification via a silica gel column chromatography using the mobile phase of toluene/ethyl acetate (9:1 by volume) to obtain the purified red pigment product.

2.6. NMR and mass spectrometry analysis of PIP

The composition of the purified product was characterized by HRMS and NMR. The product was dissolved in D-chloroform ($CDCl_3$) to identify the product by NMR.

2.7. Assay of PIP

A solution of 3 mL of acetone and 1 mL of 0.2 M, citrate buffer (pH 3.0) was added to 1 mL of culture broth, and the mixture was sonicated for 10 min [13,20]. The extraction was centrifuged at 1200 × *g* for 10 min and the supernatant was analyzed by using HPLC (JASCO, Japan) on an RP-18 column (5 μm, 18 mm × 100 mm). The mobile phase was pH 6.0, 10 mM triethylamine in methanol (1/19, v/v) with a flow rate of 1.0 mL/min. The UV–vis detector (JASCO, Japan) at 535 nm was used. The purified PIP was used as the standard to construct the calibration curve [21].

2.8. Assay of biomass

The cell biomass was determined by measuring the absorbance of the sampling broth with a UV–vis photospectrometer at 600 nm (OD_{600}). As for the dry cell weight measurement, the broth was filtered through 0.45 μm filter paper, followed by washing twice and drying at 50 °C. The OD_{600} value was further correlated to the cell dry weight via a calibration curve.

2.9. Optimization of PIP production

Box–Behnken design was employed to screen the medium compositions for PIP production. Yeast extract, peptone and sucrose were selected as the major factors to obtain the mutual influences and the optimal PIP production. The designed levels of the variables are shown in Table 2. The experimental data were analyzed via response surface methodology (RSM) using the SAS program. Ridge max analysis was carried out to estimate the optimal response by increasing the radius from the center of the original design [22].

Table 1
Tests of complex media for PIP production of *Serratia marcescens* N10612.

Medium	Composition						C/N ratio	Biomass (g/L)	PIP (mg/L)
	Beef extract (g/L)	Yeast extract (g/L)	Peptone (g/L)	Glucose (g/L)	Sucrose (g/L)	NaCl (g/L)			
MNB[a]	3	–	5	20	–	0	2.5	0.77 ± 0.01	ND[b]
CM	–	10	10	–	5	5	0.25	12.45 ± 0.15	ND
MCM1	–	5	5	–	10	0	1.0	9.15 ± 0.19	70.28 ± 0.01
MCM2	–	5	5	–	10	5	1.0	7.64 ± 0.23	ND
MCM3	–	1	1	–	10	1	5.0	3.10 ± 0.01	10.95 ± 0.01
MCM4	–	2	2	–	10	1	2.5	5.92 ± 0.04	161.10 ± 0.02

[a] The concentration of every component in medium was modified as indicated. The cultivations were conducted at pH 7.0, 28 °C and 150 rpm for 24 h.
[b] ND means "not detected".

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