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Continuous Fermentation of a Prodigiosin-Producing *Serratia marcescens* Strain Isolated from Soil

Qi Fu[1], Yujuan Xiao[1*], Xuehui Duan[2], Huabin Huang[1], Zhixia Zhuang[1], Jinhai Shen[1], Yangyue Pei[1], Haojia Xu[1], Meiyu Gan[1]

[1]Department of Science and Technology for Inspection, Xiamen Huaxia University, Xiamen, China
[2]State Key Laboratory of Food Science and Technology, Nanchang, China
Email: fq@hxxy.edu.cn, *xyj@hxxy.edu.cn

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Abstract

A red-pigmented bacterial strain NS-17 was isolated from soil, and physiological and biochemical characteristics and 16S rDNA analysis established this organism as a strain of *Serratia marcescens*. The red pigment isolated from cells of NS-17 was identified as prodigiosin. By analyzing factors affecting the production of prodigiosin, modified medium and culture conditions were set up, and a continuous fermentation method was carried out to take advantage of the mass production of foam during fermentation. Continuous fermentation was achieved in the following conditions: 32˚C, 1:1 ventilation ratio, and medium with a nutrient concentration 5-fold higher than that of the fermentation medium (with half the inorganic salt concentration) supplied as a feed medium at a flow rate of 8 mL/min. For the first time of continuous fermentation of *Serratia marcescens* coupling with foam flotation, a high yield of prodigiosin was obtained. After 56 h of culturing, the total harvest of cells was enhanced 2.33-fold compared to that of batch fermentation, the total amount of prodigiosin was enhanced 2.70-fold compared to that of batch fermentation, and cells and prodigiosin were concentrated in the efflux broth automatically.

Keywords

Serratia marcescens, Prodigiosin, Foam, Continuous Fermentation, Automatic Concentration

1. Introduction

Metabolites of microorganisms represent one of the most important sources of

medicines, especially important for the discovery and development of novel pharmaceuticals. Prodigiosin (PG) is a family of natural red pigments extracted from some species of *Streptomyces*, *Serratia*, *Pseudomonas* and other genera [1] [2]; these pigments are a kind of typical alkaloid compound produced as a secondary metabolite. Some members of this pigment family have shown a variety of drug activities, such as antibacterial [3], antifungal [4], antimalarial [5] [6], antialgal [7] [8], immunomodulating [9] [10] and antitumor [11] [12] activities, displaying important medicinal properties.

Serratia marcescens is the most widely used PG-producing bacterium due to its high production of both biomass and prodigiosin. The traditional production of PG was by batch fermentation, but the yield was not sufficiently high, and because the fermentation process produces considerable foam, operators must add a large amount of defoamer, which is not conducive to downstream extraction and purification. In this study, a red-pigmented bacterium strain NS-17 isolated from soil was identified as *S. marcescens* according to its physiological and biochemical characteristics and 16S rDNA sequence, and the red pigment, PG, was characterized by LC/MS, FT-IR and NMR. Willenbacher [13] and Cui [14] got high yield of product by coupling the fermentation process and foam flotation. To improve the yield of PG and to avoid requiring defoamer, the *S. marcescens* cells were continuously separated from the broth by the foam produced during fermentation, and the efflux broth was collected.

2. Material and Methods

2.1. Bacterial Strain and Media

A red-pigmented bacterium NS-17 was isolated from the soil of the Nanchang area with a nutrient agar plate. NS-17 was incubated in liquid medium containing 4 g of glycerol, 16 g of peptone, 4 g of NaCl and 7 g of KCl at 28˚C and shaken at 160 rpm for 24 h in a shaking incubator.

2.2. Identification of NS-17

Morphological and biochemical tests were done using standard procedures [15]. Ultrastructural studies were performed by scanning electron microscopy.

The 16S rDNA of NS-17 was isolated and sequenced. The sequence was compared with available 16S sequences in GenBank at the National Center for Biotechnology Information (NCBI). A phylogenetic tree was constructed with the Mega program, and 1000 bootstrap resampling was applied to evaluate the robustness of the phylogenetic tree.

2.3. Extraction and Isolation of Pigment

After fermentation, NS-17 cells were collected by centrifugation and washed twice with distilled water. Acidified ethanol (pH 3.0) was added to the cell mass to extract pigment, and the mixture was centrifuged. The pigment in ethanol was isolated using phase separation with water and chloroform. The chloroform

phase containing red pigment was concentrated by rotary evaporation. The red pigment was separated by silica gel column chromatography and eluted with a mixture of hexane: ethyl acetate (2:1, v/v). The concentrated pigment was purified by development in a 1: 8: 1 (v/v) mixture of ligroin: chloroform: methanol using thin-layer chromatography (TLC) and then repurified with further TLC (chloroform: acetidin = 2: 1 [v/v]). The isolated pigment was then collected and dissolved in acidified ethanol. The pigments were dried using a vacuum freeze dryer.

2.4. Characterization of Red Pigments

The purified pigment was dissolved in ethanol, with pH = 5.0 or 9.0 (adjusted by HCl/NaOH) to analyze the absorption spectra under acidic and basic conditions, and the absorbance at wavelengths 300 - 700 nm of the pigment was scanned under those two pH values.

The molecular mass of the pigment was determined using an Agilent LC/MS (HPLC: 1200, eluted with a mixture of methanol: 0.1% formic acid [70:30, v/v]; TOFMS: G6220A, ESI-MS). The ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance III 600 spectrometer (300 MHz), and $CDCl_3$ was used as the solvent. FT-IR spectra of the pigments were recorded with a Nicolet 5700 FT-IR spectrometer.

2.5. Optimization of Flask Culture Conditions

The purified prodigiosin was used to establish a formula between OD_{535} and mass concentration.

The culture broth was mixed with acidic methanol (pH 3.0). The volume of methanol was adjusted according to the OD_{535} of the mixture to ensure the optical density was in the range of 0. 2- 1.0.

Because NS-17 produced minimal pigment in LB broth (the most widely used medium for production of prodigiosin), we tested several media reported for fermentation of prodigiosin [1] [9] [16] [17] [18] and chose the initial medium as follows: glycerol 4 g/L, peptone 16 g/L, NaCl 3 g/L, and KCl 4 g/L; pH 7.0. The initial culture conditions were as follows: inoculum size: 1.0%, 100 mL of medium in flask (250 mL), culture temperature 28˚C, and rotation speed 160 rpm. The production of prodigiosin was measured after 24 h culture.

2.6. Continuous Fermentation

A 50 L bioreactor was used to perform the continuous fermentation under the optimized conditions of the flask culture with no additional defoamer, and the foam was removed as soon as it reached the top of the bioreactor. A medium with a nutrient concentration 5-fold higher than that of the fermentation medium (with half the inorganic salt concentration) was supplied as a feed medium according to the flow rate of efflux foam. The pH of the broth was automatically adjusted with HCl and NH_4·OH.

3. Results and Discussion

3.1. Identification of the Bacterium Strain NS-17

NS-17 was a gram-negative, subspheroidal or rod-shaped bacterium strain (Figure 1). The colonies of NS-17 on nutrient agar were moist, sticky and spreading, with a metallic luster and irregular margins. The organism could liquefy gelatin and use citrate or acetate as the only carbon source; the phenylalanine deaminase, L-arabinose fermentation, pectate decomposition and H_2S tests were negative, while the results were positive for DNAase, V. P, lipase, lysine decarboxylase, ornithine decarboxylase and aesculin hydrolysis tests. Moreover, NS-17 could use trehalose and D-sorbitol but not malonate or alginate. The physiological and biochemical identification tests suggested bacterium NS-17 to be *S. marcescens*.

The 16S rDNA showed the highest similarity to that of *S. marcescens* and 99% similarity to both *S. marcescens* L1 (EF 208031) and *S. marcescens* SA1 (HM 136580). A close evolutionary relationship between NS-17 and some other strains of *S. marcescens* was revealed (Figure 2).

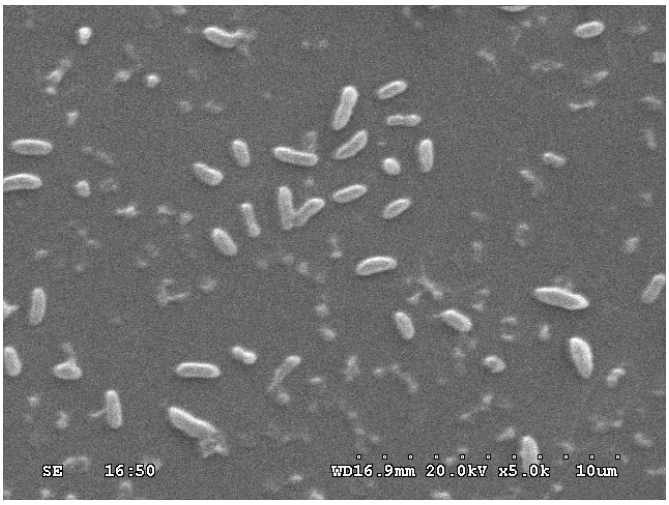

Figure 1. Scanning electron micrograph of cells of strain NS-17 (×5.0 k).

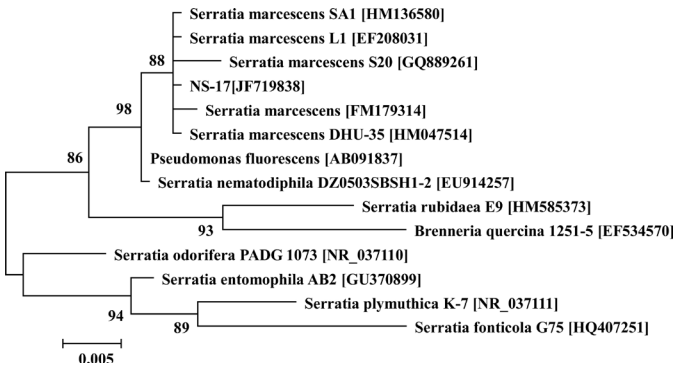

Figure 2. Phylogenetic tree of the NS-17 strain based on 16S rDNA gene sequences. The numbers at the branches represent bootstrap values (1000 bootstrap resampling). The GenBank accession number of each reference species is shown in parentheses.

All the results showed that NS-17 was a member of *S. marcescens*, and the strain was therefore named *S. marcescens* NS-17.

3.2. Characterization of the Pigment

Purified *Serratia marcescens* strain NS-17 was fermented in a shaken flask to produce pigments. After separation with column chromatography and purification by TLC, the red pigment was obtained.

The UV-Vis spectrum of the pigment (**Figure 3**) was similar to the typical spectrum of prodigiosin [2]. The TOFMS analysis result is shown in **Figure 4**. The [M + H] of the pigment was 324.2072, corresponding to $C_{20}H_{25}N_3O$, which is the molecular formula of prodigiosin.

FT-IR absorption of the pigment in KBr was at ν_{max} 3406.91, 2928.02, 2852.17, 1624.86, 1539.83, 1454.07, 1240.98, 1117.75, 1071.29, 960.33 and 772.16. The absorption corresponded to the groups as follows: aromatic NH (3406.91), CH_2 (2928.02 and 2852.17), C = C (1624.86, 960.33 and 772.16), pyrrole skeleton (1539.83 and 1454.07), C-O-C (1240.98, 1117.75 and 1071.29).

The NMR chemical shifts of the pigment were as follows: ^{1}H-NMR ($CDCl_3$, 600 MHz, ppm) δ 0.905 (3H, m), 1.294 (2H, s), 1.311 (2H, m), 1.522 (2H, t), 2.395 (2H, m), 2.565 (3H, s), 3.996 (3H, s), 6.066 (1H, d), 6.355 (1H, brd), 6.688

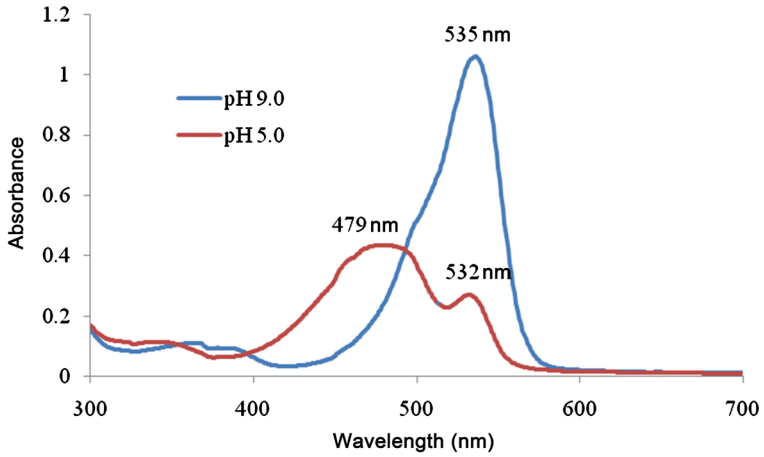

Figure 3. The UV-Vis spectrum of the pigment.

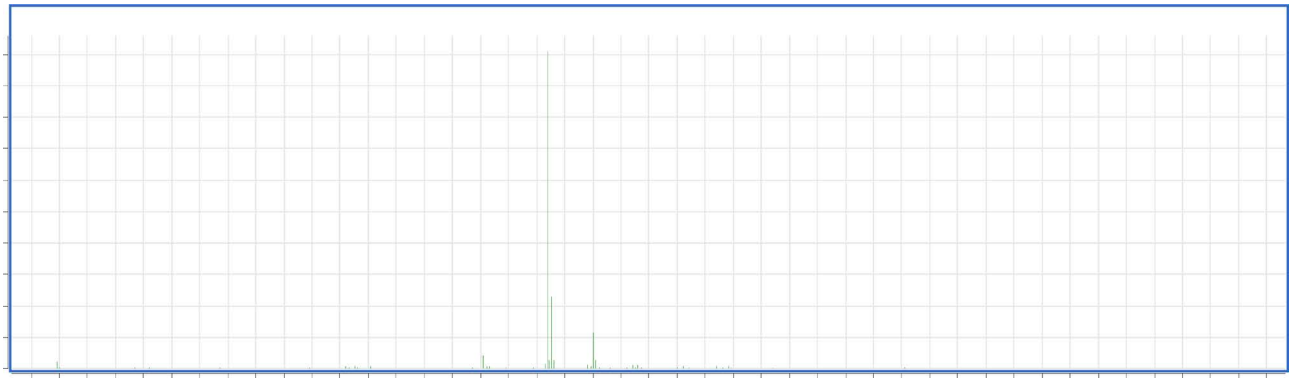

Figure 4. TOFMS of the pigment.

(1H, brd), 6.921 (1H, s), 6.965 (1H, s), 7.091 (1H, d), 12.579 (1H, brs) and 12.731 (1H, brs); and ^{13}C-NMR ($CDCl_3$, 151 MHz, ppm) δ 13.74, 16.96, 22.54, 25.48, 29.71, 31.52, 52.47, 85.86, 108.69, 109.75, 112.32, 120.26, 122.77, 123.62, 124.09, 130.82, 136.22, 138.01, 157.63 and 162.83.

UV-Vis, TOFMS, FT-IR and NMR analyses suggested that the red pigment isolated from NS-17 was PG. The pigment structure is shown in **Figure 5**.

3.3. Flask Culture

The strain NS-17 produced minimal red pigment in LB broth, weakly responded to supplementation with Tween-80 and oil and showed a high production of prodigiosin under the temperature of 30˚C - 32˚C, different from most reported *S. marcescens* strains [2] [3] [19]. The results of single-factor experiments are shown in **Table 1**, with the maximum values presented in bold. Experiments were carried out according to the order in **Table 1** with the factors optimized in the previous steps.

In the investigated factors and concentrations, the optimal conditions for NS-17 to produce prodigiosin were as follows: maltose 6 g/L; peptone 12 g/L; NaCl 1 g/L; KCl 2 g/L; Tween-80 1% (w/v); soybean oil 4% (w/v); pH of medium 8; inoculum size ≥ 1.5%; volume of medium in a 250 mL flask ≤ 50 mL; rotational rate ≥ 190 rpm; and cultivation temperature 32˚C. **Figure 6** shows the yield of prodigiosin according to time for an inoculum size of 1.5%, volume of medium of 50 mL and rotational rate of 190 rpm. The concentration of prodigiosin reached a relatively high value (60.51 mg/L) within 32 h of incubation, and the pH of the culture broth was stable at approximately 8.0, which is supposed to be good for the production of prodigiosin [19] [20] [21].

Oxygen supply significantly affected the concentration of prodigiosin in investigations of the volume of medium and rotational rate. Sufficient oxygen supplementation was the essential condition for prodigiosin fermentation.

3.4. Continuous Fermentation

With a 250 rpm rotor speed and 1:1 ventilation ratio in a bioreactor (GUJS-50L, EASTBIO Ltd., China), a control growth culture was repeated three times under the optimized conditions of the flask culture with 5 g/L of propoxylated glycerin as a defoamer. Group 1 was carried out three times under the same conditions as those of the control group but with no defoamer, and Group 2 was carried out

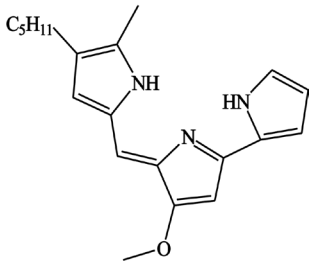

Figure 5. Structure of the PG isolated from NS-17.

Table 1. Single-factor experiments.

1. Carbon sources	Carbon sources	Glycerol	Soybean oil	Amidulin	Sucrose	**Maltose**	Tween-80	Glucose
	PG (mg/L)	12.79	9.49	10.71	12.71	**16.63**	11.37	7.34
2. Maltose	concentration (g/L)	2	4	**6**	8	10	12	
	PG (mg/L)	11.097	16.14	**25.01**	23.09	22.93	23.20	
3. Nitrogen sources	Nitrogen sources	Ammonium sulfate	Ammonium oxalate	**Peptone**	Gelatin	Bean flour		
	PG (mg/L)	0.81	0.95	**23.77**	0.91	1.08		
4. Peptone	concentration (g/L)	4	8	**12**	16	20	24	
	PG (mg/L)	15.51	29.17	**34.83**	26.73	13.71	3.32	
5. NaCl	concentration (g/L)	0	**1**	2	3	4	5	
	PG (mg/L)	23.24	**33.95**	32.82	33.77	33.13	26.65	
6. KCl	concentration (g/L)	0	1	**2**	3	4	5	
	PG (mg/L)	27.61	28.64	**33.59**	33.24	32.76	22.09	
7. Tween-80	concentration (%)	0	**1**	2	3	4	5	
	PG (mg/L)	33.79	**36.74**	35.91	34.09	33.39	31.65	
8. pH of medium	pH value	4	5	6	7	**8**	9	
	PG (mg/L)	0.01	28.13	32.51	38.20	**40.67**	36.45	
9. Inoculum size	Inoculum size (%)	0.50	1.00	**1.50**	**2.00**			
	PG (mg/L)	20.67	39.68	**41.70**	**41.89**			
10. Volume of medium	Volume (mL)	**25**	**50**	75	100	125	150	
	PG (mg/L)	**47.57**	**47.10**	41.89	40.87	30.18	17.87	
11. Rotational rate	Rotational rate (rpm)	100	130	160	**190**	**220**		
	PG (mg/L)	29.43	35.35	47.14	**49.03**	**49.43**		
12. Cultivation temperature	Temperature (˚C)	26	28	30	**32**	34		
	PG (mg/L)	24.14	47.45	52.23	**53.68**	36.77		

three times under the same conditions as those of the control group without a defoamer and with a feed medium that had a 5-fold higher nutrient concentration than that of the fermentation medium (with half the inorganic salt concentration) supplied at a flow rate of 8 mL/min according to the flow rate of the efflux foam. After 56 h of fermentation, the production of cells and PG was measured.

Group	Sample	Dry cell weight (g/L)	PG (mg/L)	Volume (L)	Total dry cell weight (g)	Total amount of PG (mg)
Control	Broth in bioreactor	4.36	65.13	26.40	115.10	1719.43
	Efflux broth	0	0	0		
1	Broth in bioreactor	2.68	17.91	22.70	92.78	1007.53
	Efflux broth	13.42	252.51	2.38		
2	Broth in bioreactor	1.14	1.74	27.20	268.73	4644.63
	Efflux broth	11.54	223.17	20.60		

As the results show, the efflux foam contained a mass of cells and PG, and

continuous fermentation could be successfully achieved with additional supplementation of medium. The efflux foam liquefied automatically in the collector, and the weight of the efflux broth is shown in Figure 6. The PG concentrations in the bioreactor and efflux broth are shown in Figure 7 and Figure 8.

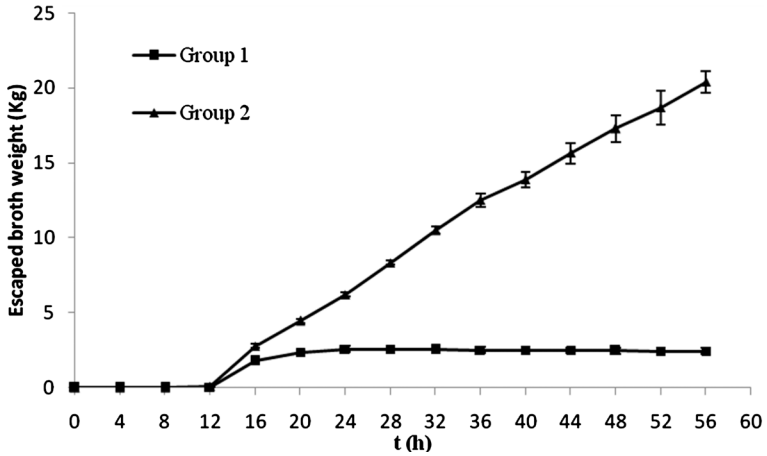

Figure 6. Efflux broth weight in groups without defoamer.

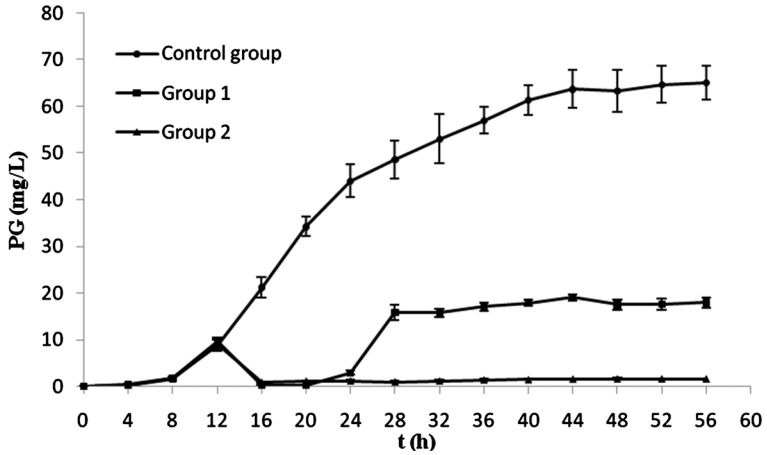

Figure 7. PG concentration in the bioreactor.

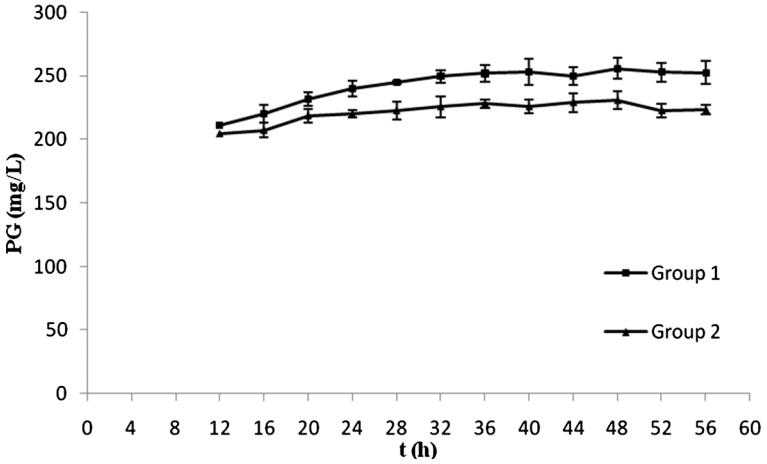

Figure 8. PG concentration in the efflux broth.

Because the process of foam collection was subject to contamination, the continuous fermentation stage of *S. marcescens* was restricted to less than 72 h, and no contamination occurred in 56 h.

4. Conclusion

S. marcescens NS-17 isolated from soil produced a considerable amount of PG in a flask culture under the following conditions: maltose 6 g/L; peptone 12 g/L; NaCl 1 g/L; KCl 2 g/L; Tween-80 1% (w/v); pH 8; inoculum size ≥ 1.5%; and cultivation temperature 32˚C. Based on the foam flotation method, continuous fermentation was performed by removing the foam as soon as it reached the top of the bioreactor; the cells and PG were automatically concentrated in the foam, making the extraction easier and achieving a high production of PG. After 56 h of culture, the total cell harvest was enhanced 2.33-fold compared to that of batch fermentation, and the total amount of prodigiosin was enhanced 2.70-fold compared to that of batch fermentation. However, due to the risk of contamination, long-term culture remains a challenge.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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