Kor. J. Appl. Microbiol. Bioeng.
Vol. 17, No. 4, 327-333 (1989)

Production of Purplish-red Pigment in Mixed Culture of *Streptomyces propurpuratus* ATCC 21630 and *Bacillus* sp. R-89

Ryu, Beung-Ho[1]*, Bub-Gyu Park[2], Young-Eh Chi[1] and Ju-Hwa Lee[1]

[1]Department of Food Science and Technology, Kyungsung University, Pusan 608-736, Korea
[2]The Institute of Food Science and Microbiology, Kyungsung University, Pusan 608-736, Korea

Streptomyces propurpuratus ATCC 21630 과 *Bacillus* sp. R-89 의 혼합배양에 의한 적자색 색소의 생산

류병호[1]* · 박법규[2] · 지영애[1] · 이주화[1]

[1]경성대학교 공과대학 식품공학과 [2]경성대학교 종합식품영양소

The purplish-red pigment was formed on agar plate by superimposed streaking of *Streptomyces propurpuratus* ATCC 21630 and strain R-89. The strain No. 89 was ascribable to the genus *Bacillus* and designated as *Bacillus* sp. R-89. Both strain did not produced such pigment when cultivated independently.

For hyperpigment production, we selected the mutant S.P-6 from *Streptomyces propurpuratus* ATCC 21630 by MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) treatment. Maximum purplish-red pigment 1420 mg/m*l* were produced, when the mutant of R-16 and *Bacillus* sp. R-89 were mixed cultured at 30°C for 72 hr.

The artificial synthetic colours are still of great importance to the food industry, finding use in virtually all branches. The most important characteristics by which the quality of food is judged is its appearence and the most important attribute of appearence is colour(1). But the toxicological status of all these colours has been already studied(2-6). Therefore, there has been a continuing trend over the last few years to replace artificially synthetic colours for food with naturally derived or nature equivalent colours. Natural colours from serveral natural sources are used in food among carotenoid, chlorophyll, anthocyanin and beet colours, *etc.* Most of these colours have significant disadvantages in use such as cost, stray characteristic odour, or peppery taste. Their use is hence fairly limited to specialised outlets. For the overcome this problem, hence much work has been produced naturally occuring colours from their sources in nature as well as research in economic synthetic manufacturing routes. Natural colours production of various microorganisms has been reported such as *Monascus* sp.(8, 9), *Streptomyces echinoruber* sp.(10, 11) and *Streptomyces propurpuratus(12, 13).* Furthermore, Nasuno and Asai (14) reported the formation of red pigments by the interaction of molds. Oshima *et al.*(15) also reported that *Streptomyces propurpuratus* produces a purplish-red pigment in mixed culture with other microorganism.

Neopurpuratin (16) is a purplish-red pigment containing ferrous ion, produced by *Streptomyces propurpuratus* ATCC 21630 in cooperation with *Bacillus* sp. under mixed culture. Its productivity, however, is very low and unreliable. Considering the possible industrial applications for this pigment, it is important to increase its yield.

Key words: Purplish-red pigment, hyperpigment production, *Streptomyces propurpuratus, Bacillus* sp. R-89
*Corresponding author

In our present studies, we were isolated and identified the *Bacillus* sp. from soil which produces purplish-red pigment in cooperation with *Streptomyces propurpuratus* ATCC 21630 and investigated the conditions of production by mixed culture of *Streptomyces propurpuratus* ATCC 21630 and *Bacillus* sp. R-89.

Materials and Methods

Microorganisms

Streptomyces propurpuratus ATCC 21630 and *Bacillus* sp. R-89 were used for the studies on the production of purplish-red pigment production by mixed culture. The former was maintained on glycerol-starch-glutamate agar slants and latter on nutrient agar slants.

Media

The glycerol-starch-glutamate (GSG) medium was composed of 1.0% glycerol, 1% soluble starch, 0.1% sodium glutamate, 0.05% $NaNO_3$, 0.025% L-proline, 0.025% K_2HPO_4 , 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ and 0.001% thiamine·HCl.

The nutrient medium was composed of 1.0% meat extract, 1.0% peptone and 0.2% NaCl. For plate and slant cultures, 15g of agar was added to 1*l* of medium.

Production medium of purplish-red pigment by mixed culture was composed of 2.0% glucose, 1.0% peptone, 1.0% meat extract, 0.3% $CaCO_3$ and 0.01% $FeSO_4 \cdot 7H_2O$. Above media pH was initially adjusted at 7.0.

Isolation of induction microorganism for purplish-red pigment produced by mixed culture

Plate culture was carried out as follow ; Preculture of *S. propurpuratus* ATCC 21630 was first streaked on the agar plates of GSG medium and incubated at 28°C for 3 days and then test strains were streaked in parallel with it and the incubation was at 20°C for 4 days. We were selected the strains which produced purplish-red pigment visually.

Identification of *Bacillus* sp. R-89

Microorganisms isolated were identified according to the routine methods recommended by the Society of American Bacteriologist(17). The strain was classified with reference to Bergey's Manual of Determinative Bacteriology, 8th Edition(18).

Selection of hyperpigment-producing mutants of *S. propurpuratus* ATCC 21630

To improve pigment production, *S. propurpuratus* ATCC 21630 were treated by MNNG according to slight modification of Hiroi *et al.*(20).

S. propurpuratus was grown at 28°C for 5 days and then diluted to about 10^7-10^8/m*l* with 0.2M-phosphate buffer, pH 7.0. To 10 m*l* of cells suspension, 10m*l* of MNNG solution which has a concentration of 1 mg/m*l* was added. The mixtures were allowed to stand at 28°C for 90 min without shaking during the treatment periods. The mixture were centrifuged after MNNG treatment. The cells were resuspended in sterile water and then were spread on the production medium plate at 28°C for 10 days. The each colonies were picked out for hyperpigment productivity tests(13).

On the other hand, *Bacillus* sp. R-89 were plated on the nutrient agar plate at 28°C for 2 days. To ascertain whether both isolated strain are concerned in the pigment formation, mutant of *S. propurpuratus* and *Bacillus* sp. R-89 were streaked in parallel together on one plate of GSG agar and incubated at 28°C for 5 days. We selected hyperpigment-producing strains which a purplish-red color appeared around the between *S. propurpuratus* and *Bacillus* sp. R-89 on the GSG agar.

Fermentation of purplish-red pigment by mixed culture

The mixed culture for production of purplish-red pigment was carried out in a 5 *l* jar-fermentor. Before making the mixed culture, the seed culture of *S. propurpuratus* and its partner strain, *Bacillus* sp. R-89 were prepared independently. For *S. propurpuratus* ATCC 21630, a loopful of culture from the GSG agar slant culture was inoculated into a 300 m*l* flask containing 70 m*l* of production medium and incubated at 28°C for 3 days. For *Bacillus* sp. R-89, one loopful of culture from the nutrient agar slant was inoculated into a 300 m*l* flask containing 70 m*l* of nutrient medium and incubated at 28°C for 1 days. Fermentation of the mixed culture was carried out in a 5*l* jar-fermentor containing 1.5*l* of the production medium. The seed culture 2m*l* of *S. propurpuratus* was inoculated to 5 *l* of the production medium and incubated at 28°C, agitation of

Table 1. Pigment formation in mixed culture of *S. propurpuratus* ATCC 21630 with various bacteria.

Test bacteria	Curture time (days) of pigment formation		
	2	3	5
Acetobacter gluconius, IFO 3292	+	+	+
Brevibacterium flavum, ATCC 21528	−	−	−
Pseudomonas aeroginosa, IFO 3446	+	+ +	+ +
Bacillus subtilis, RH-8	+	+ +	+ +
strain, R-12	+	+	+ +
strain, R-30	+	+ +	+ +
strain, R-89	+	+ + +	+ + +

+ ~ + + +; Degree of pigment production, −; None production

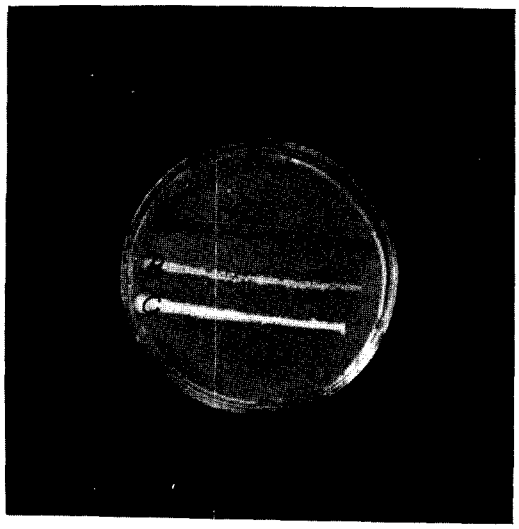

Fig. 1. Purplish-red pigment formation by interaction of *S. propurpuratus* ATCC 21630 and strain R-89 on the plate culture.
A; streak-culture of *S. propurpuratus* ATCC 21630 and strain R-89 (pigmentation),
B; streak-culture of *S. propurpuratus* ATCC 21630 (none pigmentation),
C; streak-culture of *S. propurpuratus* (none pigmentation). Plate culture were carried out according to in Materials and Methods.

250 rpm and aeration of 0.5 vvm. After fermentation of *S. propurpuratus* ATCC 21630 independantly for 8 hr, the seed culture 2 m*l* of *Bacillus* sp. R-89 was added and the mixed culture was incubated for 4 days.

Assay for pigment production of culture broth

Pigment production assay was carried out according to the method of Oshima *et al.*(16) Purplish-red pigment is excreted into culture broth. Ten m*l* of culture broth was tested periodically for analysis of pigment production. After centrifugation at 3,000 rpm for 10 min, pigment in the supernatant was estimated by measuring absorbance at 555 nm. Residual glucose in the supernatant was determined by the Bertrand method(17). Cell growth was measured at 610 nm.

pH measurement of pigment production broth

The pH of pigment production broth during fermentation was measured by pH meter.

Results and Discussion

Pigment formation by mixed-culture of *S. propurpuratus* ATCC 21630 with other microorganisms

To ascertain whether isolated strains are concerned or not in the formation of pigment, we were carried out by plate cultures. *s. propurpuratus* ATCC 21630 and various microorganisms were streaked in parallel together on one plate of GSG agar medium. (Table 1) *S. propurpuratus* ATCC 21630 was first streaked on GSG agar plates and incubated at 28°C for 5 days.

On the other hand, as a control *S. propurpuratus* ATCC 21630 and the test microorganisms were incubated at 28°C for 5 days independently on GSG plate and nutrient medium. They do not formed pigment. But pigment formation was observed on the plate streaking both strains (Fig. 1). Purplish-red color appeared around the streak of *S. propurpuratus* ATCC 21630 with other microorganism (Table 1). *S. propurpuratus* produces a purplish-red pigment

Table 2. Purplish-red pigment formation on plate culture of *S. propurpuratus* ATCC 21630 with various microorganisms.

Test microoganisms	Pigment formation	Remarks
Acetobacter aceti, IFO 3281	±	
Acetobacter gluconicus, IFO 3292	+	
Acetobacter pasturianus, IFO 13751	+	
Aeromonas hydrophila, IFO 3820	−	
Pseudomonas aeroginosa, IFO 3446	+	
Pseudomonas putida, IFO 3738	−	
Bacillus subtillus, RH-8	+	
Brevibacterium lactofermentum, ATCC 21086	−	
Brevibacterium flavum, ATCC 21528	−	
Corynebacterium glutamicum, R-820	−	
Staphylococcus aureus, R-88	−	
Streptomyces albus, IFO 3418	+	
Streptomyces griseus, IFO 3358	±	
Strain, R-12	+ +	isolation from soil
Strain, R-30	+ +	〃
Strain, R-89	+ + +	〃

+ ~ + + +; Degree of pigment production −; None production

Table 3. Morphological characteristic of strain R-89 in comparison with *B. brevis* and *B. cereus*.

	Strain R-89	*B. brevis*	*B. cereus*
Growth in nutrient agar plate	circular, smooth	circular, smooth	circular, smooth
(after 3-5 days at 30°C)	creamed color	creamed color	creamed color
Width of rod (μm)	0.5-0.8	0.5-0.9	0.5-0.9
Length of rod (μm)	1.2-2.0	2.5-3.0	2.0-3.5
Sporangium swollen	−	+	−
Spore shape	ellipsoidal	ellipsoidal	ellipsoidal
Strain gram positive	+	+	+
Vegetative cells	rods, peritrichous, flagella	rods, peritrichous, flagella	rods, peritrichous, flagella

+; positive, −; negative

by interaction with not only strain, R-89 but also several strain of bacteria, although the degree of pigment formation differs with each strain. *S. propurpuratus* ATCC 21630 and strain R-89 isolated from soil produced no pigment independently but they produced a purplish-red pigment in mixed culture. From these data, it were confirmed that *S. propurpuratus* ATCC 21630 produced the purplish-red pigment by interaction with the strain R-89.

As shown in Table 2, the seven strains of bacteria were used as partner of *S. propurpuratus* ATCC 21630 for pigment production in plate culture. We checked the purplish red pigment productivity about the strains described in Table 2. Certainly culture of *S. propurpuratus* ATCC 21630 and strain R-89 formed intensive purplish-red pigment.

Identification of bacteria

Strain R-89 grew easily in nutrient agar plate. The characteristics of the spore slope and vegetative cells in nutrient media were given in Table 3. A comparison of strain R-89 with *Bacillus* species described

Tabl 4. Physiological characteristics of strain R-89.

	Strain R-89	*B. brevis*	*B. cereus*
Anaerobic growth	–	–	–
Hydrolysis of casein	+	+	+
Hydrolysis of urea	–	–	±
Liquefaction of gelatin	+	+	+
Acid from D-glucose	–	+	+
Acid from L-arabinose	–	–	–
Formation of indole	–	–	+
Reduction of nitrate	+	±	+
Degradation of tyrosine	–	±	±
Catalase activity	+	+	+
Oxidase activity	+	±	–
Urease activity	–	–	±
Growth at pH 6.8	+	+	+
Growth at pH 5.7	+	±	+
Growth of temperature	15-40°C	15-40°C	15-40°C

+; positive, –; negative, ±; 11-89% of strain are positive

in Bergey's Manual(19), showed that the species of *Bacillus brevis* and *Bacillus cereus* resembled our organism with respect to the circular, smooth and creamed color and vegetative cells of rods, peritrichous flagella. In general terms, spore shape of strain R-89 are similar with *Bacillus* sp. as references.

Instead, sporangium did not swollen in strain R-89 and *B. cereus*. Physiological characteristics in which strain R-89 differs from two or more of the strains of the *B. brevis* and *B. cereus* as references are the following : urea hydrolysis, nitrate reduction and formation of indole (Table 4). In our opinion, strain R-89 differs from other species of genus, *Bacillus*. Therefore, we can not propose the creation of a new species but these properties taken together with the above morphological characteristics show that the microorganism belong to the geneus *Bacillus* sp. The strain R-89 was designed as the name of *Bacillus* sp. R-89.

Selection of hyperpigment producing mutants of *S. propurpuratus* ATCC 21630

For pigment formation in mixed culture, *S. propurpuratus* ATCC 21630 was precultured at 28°C in a 300 m*l* flask containing 70 m*l* of GSG medium for 3 days. Two m*l* of preculture of *S. propurpuratus*

Table 5. Purplish-red pigment produced by mixed culture between *S. propurpuratus* ATCC 21630 and mutants of *Bcillus* sp. R-89.

	O.D. (610 nm)	Purplish-red pigment (μg/m*l*)
S. propurpuratus ATCC 21630	0.8	960
S.P-4	1.0	1180
S.P-6	1.4	1420
S.P-9	0.9	1030
S.P-10	1.2	1330
S.P-13	1.2	1340
S.P-16	1.1	1250

S.P 4-16; mutant of *S. propurpuratus* ATCC 21630. Mutation of *S. propurpuratus* ATCC 21630 was described in material and methods. Seed culture (2 m*l*) of mutants of *S. propurpuratus* ATCC 21630 was inoculated into 300 m*l* flask containing 70 m*l* of the optimal medium was described in methods and incubated at 28°C. After 8 hr, the seed culture (2 m*l*) of *Bacillus* sp. R-89 was added and the mixed culture was incubated under the same conditions.

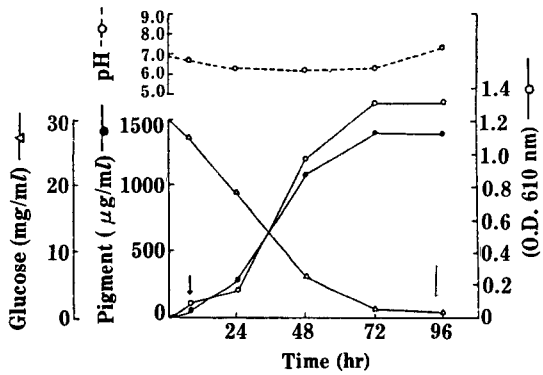

Fig. 2. Time course of purplish-red pigment production in mixed culture. Seed culture (2 m*l*) of mutant S.P-6 was inoculated into a 5 *l* jar-fermentor containing 1.5 *l* of the production medium and incubated at 28°C with agitation of 250 rpm and aeration of 0.5 vvm. After 8 h (arrow), the seed culture(2 m*l*) of *Bacillus* sp. R-89 was added and mixed culture was incubated under the same conditions.

was transferred 70 m*l* of GSG medium in 300 m*l* flask. After incubation at 28°C for 10 hr, 2 m*l* of preculture of a test strain which incubated at 28°C for 1 days with 50 m*l* of nutrient broth was added and mixed culture was incubated at 28°C for 5 days.

By mutation procedures, 6 mutants were selected, and the mutants were allowed to grow with *Bacillus* sp. R-89 in production medium on a shaker for 5 day's at 28°C, and packed cell volume and pigment-production was measured. As shown in Table 5, pigment-productivity of each these mutants which was estimated by absorbance at 555 nm, was improved in comparison with parent organism as follows : 1.48, 1.40 and 1.30 times for the mutants of S.P-6, S.P-13 and S.P-16, respectively. From these results, we decided to use mutant S.P-6 for large scale production of purplish-red pigment by broth culture with the production medium under the optimal conditions.

Fermentation of purplish-red pigment by mixed culture

A typhical time course of pigment production in mixed culture of jar-fermentor established above are given in Fig. 2. When *Bacillus* sp. *R-89* was inoculated at 8 hr after inoculation of *S. propurpuratus* ATCC 21630, pigment production began at 5 hr after the beginning of the mixed culture, and increased until 72 hr of cultivation. At this time, the glucose was almost completely consumed. Purplish-red reached the maxium product of around 1,420μg/m*l* at 72 hr.

Furthermore, the chemical and biological properties of the pigment will be research in detail.

요 약

미생물에 의한 적자색 색소를 생산하기 위하여 *Streptomyces propurpuratus* ATCC 21630과 토양에서 분리한 strain No.89를 한천배지상에서 혼합배양하여 적자색을 형성하였다. 적자색 색소를 형성하는 strain No.89는 *Bacillus* sp. R-89로 명명하였다.

이들 두 균주는 각각 배양하였을 때는 색소를 형성하지 않았다. 적자색 색소를 많이 생산하기 위하여 *Streptomyces propurpuratus* ATCC 21630를 MNNG (N-methyl-N'-nitro-N-nitrosoguanidine)로 처리하여 얻은 변이주 S.P-16과 *Bacillus* sp. R-89를 생산배지에서 혼합하여 발효하였을 때 1420μg/m*l*의 색소를 얻을 수 있다.

Acknowledgment

This paper was supported by the grant from the Korean Traders Scholarship Foundation and Dong Do Industrial Co.

References

1. Walford, J.: Developments in Food colours-1 pp.1-25, Apllied Science publishers, London, (1980).
2. Goldenberg, N.: Why additives-the safety of foods, Chapter 6. Colours-do we need them?, British Nutrition Foundation, London (1977).
3. World Health Organization, Toxicological review of certain food additives with a review of general principles and of specifications, 17th report of the joint FAO/WHO Expert committee on Food Additives, Geneva, (1974).
4. Francis, F.J.: In current aspects of food colorants. Ed. T.E. Furia, 19-27 IRC press, Ohio, (1977).
5. Crampton, R.F.: Colouring agents in Why additives, The safety of foods. The British National Foundation, Forbes publications, London, (1977).
6. Kojima, K.: The toxicological assessment of national food colorants, in chemecal toxicology of food, Elsevier, (1978).
7. Food Additives and Contaminants Committee, International report on the review of the colouring matter in food regulations, p.29 HMSO, London, (1973).
8. Yoshimura, M., S. Yamanaka, K. Mitsugi and Y. Hirose : *Agri. Biol. Chem.*, **39**, 1789 (1975).
9. Hiroi, T., T. Takahashi, T. Sima, T. Suzuki, M. Tsukioka and N. Ogasawara : Production of red-koji in solid culture, Nippon Nageikagaku Kaishi, **55**, 1 (1981).
10. Palleroni, N.J., K.E. Reichelt, D. Mueller, J. Epps, B. Tabenkin, D.N. Bull, W. Schuep, J. Berger : *J. of antibiotics*, 1218 (1978).
11. Schuep, W., J.F. Blount, T.H. Williams, A. Stempel : *J. of antibiotics.*, 1226 (1978).
12. Shinobu, R.: *J. of antibiotics*, **23**, 125, 19 (1985).
13. Oshima, M., N. Ishizaki, A. Handa, Y. Tonooka and N. Kanda : *J. Ferment. Technol.*, **59**, 335 (1981).
14. Nasuno, S., and T. Asai: *J.Gen. Appl. Microbiology*, **8**, 74 (1966).
15. Oshima, M., N. Ishizaki, Y. Tonooka: *J. Ferment. Tech.*, **63**, 131 (1985).
16. Oshima, M., N. Ishizaki, A. Handa: *J. Ferment. Technol.*, **59**, 209 (1981).
17. 이만정 : 식품분석, 동명사, p1110 (1982)
18. Society of American Bacteriologists: Mannual of

microbiological methods, McGraw-Hill Book Co., New York (1957).
19. Buchanan, R.E. and N.E. Gibbons: Bergey's Manual of determination Bacteriology 8th Ed., the Williams and Wilkins Co., Baltimore (1974).
20. Hiroi, T., T. Shima., T. Susuki., M. Tsukioka. and N. Ogasawara: *Agri. Biol. Chem.*, **43**, 1975 (1976).

(Received May 24, 1989)