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Cultural Conditions for Production of Griseorhodins by a Culture of *Streptomyces californicus* JCM 6910

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The optimum cultural conditions for production of the red pigments, griseorhodin C and dideoxygriseorhodin C, by a culture of *Streptomyces californicus* JCM 6910 were investigated using shake flasks and improved basal medium. Soluble starch as the carbon source, and peptone and yeast extract as the nitrogen sources and the growth factor were excellent for the griseorhodins production. The greatest production of griseorhodins was observed with the cultivation in a medium consisting of 2.5% (w/v) soluble starch, 0.8% (w/v) peptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl (pH 7.2) at 28°C, reciprocal shaking culture (120 osillations/min). Under the optimum conditions, 80 mg/m*l* of griseorhodin C and 63 mg/m*l* of dideoxygriseorhodin C were produced after 3 days cultivation in a 500-m*l* shake flask containing 100 m*l* of the above medium, improving the production of 5 times over initial levels.

Griseorhodin C[1] and dideoxygriseorhodin C[2] are red pigments produced by *Streptomyces californicus* JCM 6910[3]. It might be used as a natural food-coloring agent because of their beautiful color tone and negligible toxicity according to biological test[4]. Its water-solubility is very low and unreliable. When the mixed solution of dideoxygriseorhodin C and peptide were incubated, pigment-peptide complex could be easily produced[5]. Considering the possible industrial applications for this pigment-peptide complex, it is important to increase its yield.

It is well known that the mold, *Monascus sp.*, has been used historically in the fermentative production of red wine. Chin-Fwu Lin[6] reported the optimum cultural conditions for *Monascus*-pigment formation.

This paper describes the results of investigations aimed at establishing the optimum cultural conditions for efficient production of two red pigments.

Materials and Methods

Microorganisms

Streptomyces californicus JCM 6910 was used to obtain the two red pigments (grise-

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orhodin C and dideoxygriseorhodin C). The strain was maintained on the Bennett's[7] agar slants and subcultured every 3-4 weeks.

Medium and Cultivations

The Bennett's medium was composed of 1% (w/v) glucose, 0.1% (w/v) meat extract, 0.1% (w/v) yeast extract and 0.2% (w/v) peptone (Daigo Eiyou Chemical), pH 7.5. When the media was used for agar slant, 2.0% agar was added. Investigations concerning the medium for griseorhodins production were performed by modifying Waksman[8] medium as basal medium, composed of 1.0% (w/v) glucose, 0.5% (w/v) peptone, 0.5% (w/v) meat extract and 0.5% (w/v) NaCl, pH 7.2. The seed culture was prepared as follows. One agar piece from Bennett's agar slant culture inoculated into a 500-m*l* shake flask containing 100 m*l* of the basal medium and incubated at 28°C for 3 days on a reciprocal shaker (120 oscillations/min, 7 cm strokes).

Isolation and Purification of two Red Pigments

The mycelium obtained from the culture broths was extracted with ethylacetate at pH 3. After washing with saturated sodium bicarbonate, the solvent layer was evaporated to dryness, and the residue was dissolved in a small amount of dimethylsulfoxide and subjected to silica gel column chromatography. The benzene-methanol-water (10 : 2 : 1) fraction containing griseorhodins was concentrated to give a dark red crude powder, which was dissolved in a small amount of dimethylformamide and subjected to Sephadex LH-20 column chromatography. Development of the column with dimethylformamide gave two red bands, which were separatelly collected and concentrated to dryness to give amorphous powder of griseorhodin C and dideoxygriseorhodin C, respectively.

Results and Discussion

Effect of Carbon Source

Various mono-, di- and poly-saccharides, sugar alcohols, and organic acids were tested to determine the most favorable carbon sources for griseorhodins production. As shown in Table 1, soluble starch and potato starch were the most suitable. Griseorhodins were produced from other carbon source such as glucose, mannose, galactose, and fructose, but the production levels were relatively low.

In view of the fact that soluble starch and potato starch served as superior carbon sources, the effect of concentration of soluble

Table 1. Effect of carbon sources on griseorhodins production

Carbon source (1%)	Griseorhodin C (mg/m*l*)					Dideoxygriseorhodin C (mg/m*l*)				
	Culture time (day)									
	1	2	3	4	5	1	2	3	4	5
Soluble starch	2.3	12.6	34.5	27.7	10.9	1.1	9.1	26.5	13.3	7.2
Potato starch	1.1	15.9	27.3	21.3	9.7	0.7	6.8	23.7	9.6	2.3
Glucose	—	9.3	16.2	7.0	2.9	—	7.3	12.1	4.5	3.7
Mannose	—	4.2	19.5	5.7	1.5	—	8.2	17.8	7.6	1.3
Galactose	—	7.3	15.9	4.3	2.1	—	5.4	16.5	5.8	0.4
Fructose	—	6.6	20.3	5.2	1.3	—	3.9	15.2	6.1	4.4
Glycerol	—	6.1	21.6	6.9	1.9	2.8	10.9	13.1	8.9	2.0

*Cultivation was carried out as discribed in Materials and Methods except that the test medium substituted soluble starch in the basal medium with the indicated carbon source (1%).

starch on griseorhodins production was investigated in the range from 1.0% to 4.0%. As shown in Fig. 1, griseorhodins production was maximum at a concentration of 2.5%, and 50.2 mg/m*l* of griseorhodin C and 38.2 mg/m*l* of dideoxygriseorhodin C had accumulated after 3 days cultivation.

Effect of Nitrogen Sources

Several kinds of nitrogen sources were examined to find out other suitable substrate than peptone and meat extract for griseorhodins production. The medium were prepared by replacing peptone and meat extract in Waksman medium with other nitrogen sources

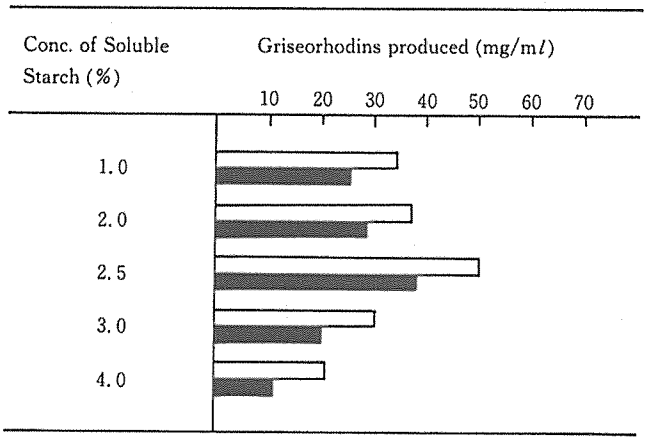

Fig. 1. Effect of concentration of soluble starch on griseorhodins production.
Cultivation was carried out for 3 days as discribed in Materials and Methods except that the concentration of carbon in basal medium was modified as indicated.
□; Griseorhodin C, ■; Dideoxygriseorhodin C.

Table 2. Effect of nitrogen sources on griseorhodins production

Nitrogen source (0.5%)	Griseorhodin C (mg/m*l*)					Dideoxygriseorhodin C (mg/m*l*)				
	Culture time (day)									
	1	2	3	4	5	1	2	3	4	5
$(NH_4)_2HPO_4$	—	2.7	5.3	5.9	0.7	—	—	2.9	0.8	—
$NaNO_3$	—	—	2.7	1.7	0.2	—	—	0.9	1.1	0.3
Monosodium glutamate	0.8	6.6	8.7	2.1	—	—	2.5	6.1	—	—
Peptone	4.4	20.1	24.8	11.2	9.8	2.1	9.9	16.2	5.3	2.2
Urea	—	5.5	7.3	3.1	—	—	2.7	3.4	0.9	—
Yeast ext.	6.1	18.7	20.7	15.9	5.7	3.8	10.7	15.8	11.9	7.8
Meat ext.	1.3	6.5	9.3	3.8	2.7	—	2.3	5.2	4.3	1.9

*Cultivation was carried out as discribed in Materials and Methods except that the test medium substituted peptone and meat extract in the basal medium with indicated nitrogen source (0.5%).

listed in Table 2. The Table shows the results of griseorhodins production from various nitrogen sources examined at the concentration of 0.5%. Among them, organic nitrogen sources such as peptone and yeast extract were found to be the most effective substances promoting griseorhodins accumulation. On the other hand, the meat extract was not good nitrogen sources. In this experiments, it was found that natural nitrogen sources were essential for griseorhodins production, and that peptone and yeast extract were the best of the various nitrogen sources tested.

The effects of concentrations and of the combination of these two nitrogen sources on griseorhodin production were investigated in detail. As shown in Fig. 2, the combination of 0.8% peptone and 0.5% yeast extract led to maximum production of griseorhodins, and 71.2 mg/m*l* of griseorhodin C and 66.7 mg/m*l* of dideoxygriseorhodin C had accumulated after 3 days cultivation.

Effect of Temperature and Medium Volumes in Shake Flask

The effect of temperature on griseorhodins production was investigated within the temperature range from 20℃ to 36℃ using a temperature gradient incubator. The griseorhodins production increased with the rise of temperature and reached a maximum at 28-30℃. The accumulation levels of griseorhodin C with the optimum medium after 3 days cultivation were 69 mg/m*l* at 22-24 ℃, 83 mg/m*l* at 28-30℃, and 42 mg/m*l* at 34-36℃. The accumulation levels of dideoxygriseorhodin C were 43 mg/m*l* at 22-24℃, 63 mg/m*l* at 28-30℃, and 26 mg/m*l* at 34-36℃. At a temperature of 40℃ or above, the griseorhodins production decreased markedly.

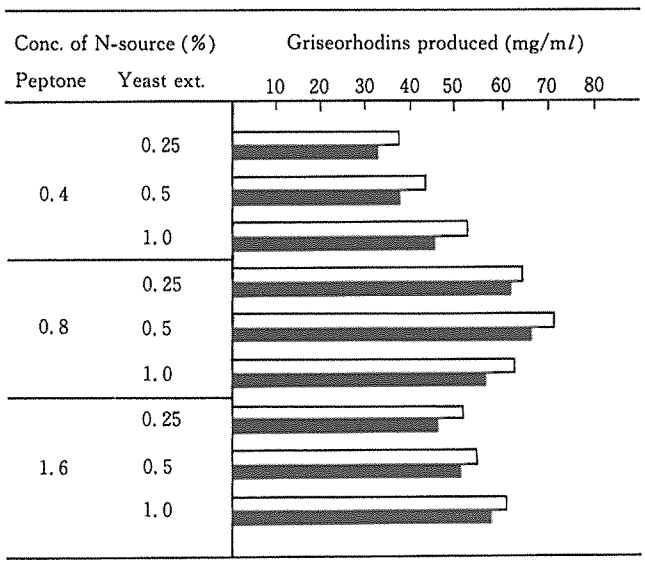

Fig. 2. Effect of concentration of peptone and yeast extract together on griseorhodins production.
Cultivation was carried out for 3 days as described in Materials and Methods except that the concentration of nitrogen in basal medium was modified as indicated.
□; Griseorhodin C, ■; Dideoxygriseorhodin C.

Next, the effect of medium volumes in shake flask was investigated by changing the volume of the optimum medium within the range from 50 m*l* to 200 m*l* per a 500-m*l* shake flask. The highest yield of griseorhodins occured at between 50 m*l* and 100 m*l* per flask. Yield sharply decreased when the volume of the medium was increased more than 125 m*l*. The relationship between the medium volume (m*l*) and the accumulation levels of griseorhodin C (mg/m*l*) with the optimum medium after 3 days cultivation were as follows: 50 m*l*-57 mg/m*l*, 75 m*l*-72 mg/m*l*, 100 m*l*-80 mg/m*l*, 125 m*l*-39 mg/m*l*, 150 m*l*-25 mg/m*l*, 200 m*l*-13 mg/m*l*. The relationship between the medium volume (m*l*) and the accumulation levels of dideoxygriseorhodin C (mg/m*l*) were as follows: 50 m*l*-42 mg/m*l*, 75 m*l*-49 mg/m*l*, 100 m*l*-63 mg/m*l*, 125 m*l*-17 mg/m*l*, 150 m*l*-11 mg/m*l*, 200 m*l*-5 mg/m*l*. It seems high aeration is indispensable for efficient griseorhodins production by the present culture with the reciprocal shaker.

Griseorhodins Production under Optimum Culture Conditions

A typical time course of griseorhodins production under the optimum culture conditions established above in shown Fig. 3. After the beginning of cultivation, griseorhodins production increased with the growth until 3 days cultivation, when both pH and

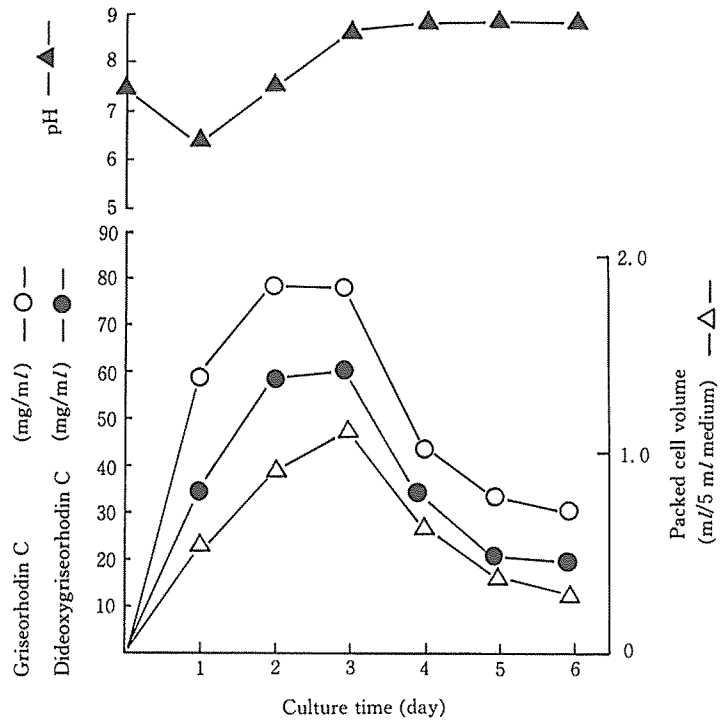

Fig. 3. Time course of griseorhodins production by *Streptomyces Californicus* JCM 6910.
Cultivation was carried out in a 500-m*l* shake flask containing 100 m*l* of the optimum medium consisting of 2.5% soluble starch, 0.8% peptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2, and incubated at 28℃ for 1, 2, 3, 4, 5, or 6 days on a reciprocal shaker (120 oscillations/min, 7 cm strokes).

packed cell volume almost reached the highest level. The levels of griseorhodins production were 80 mg/m*l* of griseorhodin C and 63 mg/m*l* of dideoxygriseorhodin C after 3 days cultivation. Comparing the initial level with the basal medium (16.2 mg/m*l* of griseorhodin C and 12.1 mg/m*l* of dideoxygriseorhodin C), we see that the levels of griseorhodins production was increased almost 5 times by the improved medium.

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放線菌 *Streptomyces californicus* JCM 6910 による グリセオロジン生産の培養条件

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放線菌 *Streptomyces californicus* JCM 6910による2種類の赤色色素（グリセオロジンCとジデオキシグリセオロジンC）生産の最適培養条件を，液体振盪フラスコ培養と基礎培地組成の改良によって検討した。最適培養条件は，培地組成として2.5%(w/v)可溶性澱粉，0.8%(w/v)ペプトン，0.5%(w/v)酵母エキス，0.5%（w/v）食塩（pH 7.2）を用い，培養温度28℃で往復振とう培養することが望ましかった。この条件下で，500 m*l*容振盪フラスコに上記液体培地100 m*l*を入れて振とう培養を3日間行うことにより80 mg/m*l*のグリセオロジンCと63 mg/m*l*のジデオキシグリセオロジンCを生産することができ，初期の基礎培地に比較し5倍量の色素生産を上げることができた。