# SAFRACINS, NEW ANTITUMOR ANTIBIOTICS

## I. PRODUCING ORGANISM, FERMENTATION AND ISOLATION

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Safracins, new antibiotics with a novel skeleton, were discovered in a culture broth of *Pseudomonas* sp. The producing organism has been identified as *Pseudomonas fluorescens*. Safracins A and B were isolated by ethyl acetate extraction and chromatography on silica gel.

Safracin A and safracin B were discovered in the course of a continuing search for antibacterial and antitumor antibiotics of bacterial origin. As described in a separate paper[1], the names safracins A and B were chosen on the basis of their structure, similar to that of saframycins produced by Streptomyces[2].

This paper describes taxonomic studies of the producing organism, production by fermentation and purification of antibiotics. Physicochemical properties and structure analysis will be reported in a following paper.

### Taxonomy

The producing microorganism, strain A2-2, was isolated from a soil sample collected in Tagawa-gun, Fukuoka, Japan. The strain has been deposited at the Institute for Fermentation, Osaka, Japan and has been assigned accession number IFO 14128[3].

Strain A2-2 is a non-sporulating Gram-negative rod, 0.6~0.8 by 2.0~3.0 μm in size, and is motile with polar flagella. Poly-hydroxybutylate granules were not observed with a phase contrast microscope. No sheath, stalk or slime is produced. The organism is a non-pleomorphism.

Physiological and biological properties of strain A2-2 are summarized in Tables 1 and 2. A comparison of the above characteristics of strain A2-2 with the descriptions in BERGEY's Manual of Determinative Bacteriology, the 8th edition (1974)[4], suggests that this strain belongs to the genus Pseudomonas. Strain A2-2 is considered to belong to Section 1 of genus Pseudomonas because it requires no growth factors and uses acetate as the sole carbon source, but does not accumulate poly-β-hydroxybutyrate. The results of Tables 1 and 2 show that strain A2-2 closely resembles the species, *P. fluorescens*, but differs from it with respect to the utilization of several carbon sources and phosphatase activity[5,6].

In conclusion, since the characteristics of strain A2-2 are essentially the same as those of *P. fluorescens*, differing in only a few minor points, strain A2-2 was identified as *P. fluorescens*.

### Fermentation

Seed flasks were inoculated with slant cultures and grown for 24 hours at 25°C. The seed medium

Table 1. Physiological reaction of strain A2-2.

| Test | Response | |
|---|---|---|
| | A2-2 | *P. fluorescens* biotype A |
| Nitrate reduction to nitrite | − | − |
| Nitrite reduction to $N_2$ gas | − | − |
| Voges-Proskauer reaction | − | − |
| Indole reaction | − | − |
| Starch hydrolysis | − | − |
| Utilization of citrate | + | + |
| Utilization of ammonium salt | + | + |
| Utilization of nitrate | + | + |
| Pigment production | + | + |
| Urease reaction | − | + |
| Oxidase reaction | + | + |
| Catalase reaction | + | + |
| Growth at 37°C | − | − |
| 20°C | + | + |
| 4°C | + | + |
| Oxidation-Fermentation test | Oxidative | Oxidative |
| 2-Ketogluconate oxidation | − | + |
| Aesculin hydrolysis | − | − |
| Malonate utilization | + | + |
| Arginine dehydrolase | + | + |
| Lysine decarboxylase | − | − |
| Ornithine decarboxylase | − | − |
| Phenylalanine deaminase | − | − |
| Phosphatase test | + | − |
| Utilization of acetate | + | + |

Table 2. Acid production from carbohydrate in OF-basal medium.

| Carbohydrate (1%) | Response | |
|---|---|---|
| | A2-2 | *P. fluorescens* biotype A |
| L-Arabinose | + | + |
| D-Xylose | + | + |
| D-Ribose | + | + |
| Rhamnose | − | + |
| D-Glucose | + | + |
| D-Mannose | + | + |
| D-Fructose | + | + |
| D-Galactose | + | + |
| Maltose | − | + |
| Sucrose | − | + |
| Lactose | + | + |
| Trehalose | + | + |
| Raffinose | − | − |
| Adonitol | + | + |
| Dulcitol | − | − |
| D-Sorbitol | + | + |
| D-Mannitol | + | + |
| Inositol | + | + |
| Ethyl alcohol (3%) | + | + |
| Salicin | − | − |

OF-basal medium (Difco): Tryptone 0.2%, NaCl 0.5%, $K_2HPO_4$ 0.03%, brom thymol blue 0.008%, agar 0.2%.

consisted of 1.5% glucose, 0.5% peptone, 0.2% yeast extract, 0.05% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.4% $CaCO_3$. A 2% of seed culture was used to inoculate the fermentation medium. Using a basal medium of 1% $(NH_4)_2SO_4$, 0.8% KCl, 0.04% $K_2HPO_4$, and 0.8% $CaCO_3$, the effects of carbon and nitrogen sources in the fermentation medium were investigated in 100-ml Erlenmeyer flasks at 25°C for 4 days.

The presence of safracins A and B in culture filtrates was determined as follows: To 5 ml of culture broth adjusted to pH 9.0, an equal amount of ethyl acetate was added and mixed vigorously. After centrifugation at $1,500 \times g$ for 10 minutes, a 5 $\mu$l of upper layer was applied on a column packed with TSK-Gel LS-410 in a high performance liquid chromatograph LC-3A (Shimadzu) and developed with 25% acetonitrile containing PIC-B7 solution at a flow rate of 2 ml/minute. The chromatograph was monitored by a Shimadzu UV spectrophotometric detector SPD-2A at 254 nm. The content of safracin A or B was calculated from the peak height of each standard solution. A typical example of the analysis of the culture broth is shown in Fig. 1.

As shown in Table 3, dried yeast and soybean meal were the most favorable nitrogen sources for safracins production. Dried yeast was chosen as the nitrogen source, as it gave the highest production of safracin B which we wanted. The effect of carbon sources on safracins production was examined using dried yeast as the nitrogen source, and as shown in Table 4 the best results were obtained with mannitol or glucose. Therefore, the effect of combining mannitol and glucose as the carbon source

Fig. 1. High performance liquid chromatography of culture broth of *P. fluorescens* A2-2.

Conditions: TSK-Gel LS-410 column (4×200). Acetonitrile-5 mM PIC-B7 (1:4). Flow rate 2 ml/minute. Monitor at 254 nm.

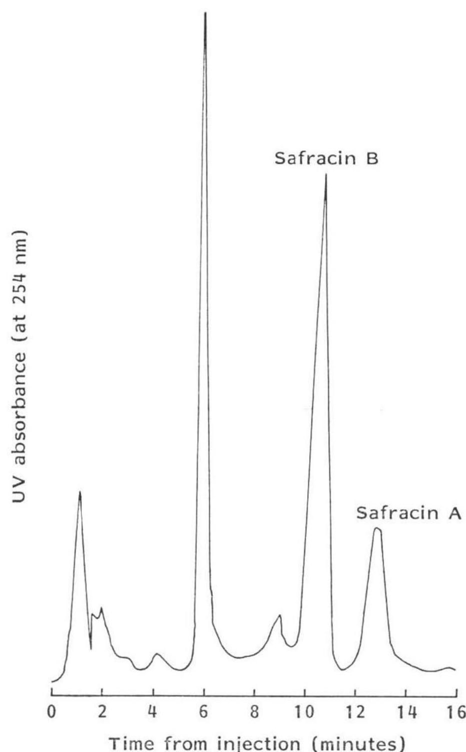


Fig. 2. Time course of fermentation in 30-liter jar fermentor.

Basal medium and cultural conditions: glucose 2%, mannitol 4%, dried yeast 2%, $(NH_4)_2SO_4$ 1%, $K_2HPO_4$ 0.04%, KCl 0.8% and $CaCO_3$ 0.8%.

Agitation rate: 300 rpm. Aeration: 15 liters/minute. Temperature: 25°C.

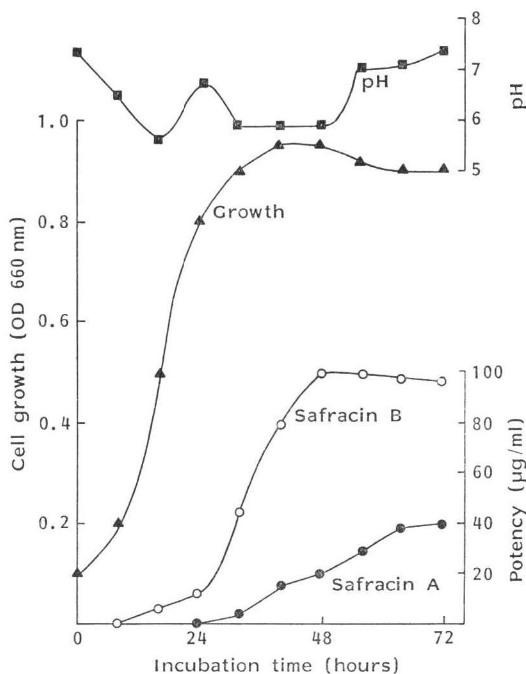


Table 3. Effect of nitrogen sources on safracin A and safracin B production.

| Nitrogen source (%) | | Final pH | Potency (μg/ml) | | |
|---|---|---|---|---|---|
| | | | Safracin A | Safracin B | Total |
| Dried yeast | 0.5 | 7.4 | 10 | 80 | 90 |
| | 1.0 | 7.2 | 12 | 80 | 92 |
| | 2.0 | 7.0 | 16 | 104 | 120 |
| Soybean meal | 0.5 | 7.8 | 32 | 60 | 92 |
| | 1.0 | 7.4 | 30 | 70 | 100 |
| | 2.0 | 7.6 | 24 | 52 | 76 |
| Peptone | 0.5 | 7.1 | 14 | 41 | 55 |
| | 1.0 | 7.1 | 20 | 32 | 52 |
| | 2.0 | 7.2 | 13 | 28 | 41 |
| Yeast extract | 0.5 | 8.0 | 7 | 39 | 46 |
| | 1.0 | 7.7 | 9 | 45 | 54 |
| | 2.0 | 8.0 | 4 | 28 | 32 |
| Meat extract | 0.5 | 8.3 | 12 | 41 | 53 |
| | 1.0 | 8.5 | 18 | 63 | 81 |
| | 2.0 | 8.4 | 16 | 54 | 70 |
| Corn steep liquor | 0.5 | 7.2 | 8 | 47 | 55 |
| | 1.0 | 6.6 | 15 | 59 | 74 |
| | 2.0 | 7.0 | 19 | 63 | 82 |

Basal medium and cultural conditions: glucose 4%, $(NH_4)_2SO_4$ 1%, KCl 0.8%, $K_2HPO_4$ 0.04% and $CaCO_3$ 0.8%.

Initial pH 7.0, cultivated at 25°C.

Table 4. Effect of carbon sources on safracin A and safracin B production.

| Carbon source | Final pH | Potency (μg/ml) | | |
|---|---|---|---|---|
| | | Safracin A | Safracin B | Total |
| Glucose | 6.3 | 14 | 91 | 105 |
| Fructose | 6.3 | 0 | 0 | 0 |
| Lactose | 7.1 | 0 | 0 | 0 |
| Maltose | 7.5 | 0 | 0 | 0 |
| Sucrose | 7.9 | 0 | 0 | 0 |
| Molasses | 8.5 | 0 | 12 | 12 |
| Raffinose | 7.7 | 0 | 0 | 0 |
| Dextrin | 7.7 | 0 | 0 | 0 |
| Soluble starch | 7.7 | 0 | 0 | 0 |
| Glycerol | 7.6 | 7 | 41 | 48 |
| Mannitol | 6.9 | 21 | 106 | 127 |
| Na Citrate | 9.0 | 0 | 0 | 0 |
| Na Acetate | 7.5 | 0 | 0 | 0 |
| Soybean oil | 6.7 | 8 | 53 | 61 |

Basal medium and cultural condition: dried yeast 2%, $(NH_4)_2SO_4$ 1%, KCl 0.8%, $K_2HPO_4$ 0.04%, $CaCO_3$ 0.8%, carbon source 4%.
Initial pH 7.0, cultivated at 25°C.

Table 5. Combination effect of glucose and mannitol as carbon source on safracins A and B production.

| Carbon source (%) | | Potency (μg/ml) | | |
|---|---|---|---|---|
| Glucose | Mannitol | Safracin A | Safracin B | Total |
| 6 | 0 | 21 | 81 | 102 |
| 0 | 6 | 36 | 84 | 120 |
| 5 | 1 | 24 | 90 | 114 |
| 4 | 2 | 27 | 85 | 112 |
| 3 | 3 | 35 | 84 | 119 |
| 2 | 4 | 49 | 96 | 145 |
| 1 | 5 | 38 | 90 | 128 |

Basal medium and cultural condition: dried yeast 2%, $(NH_4)_2SO_4$ 1%, KCl 0.8%, $K_2HPO_4$ 0.04% and $CaCO_3$ 0.8%.
Initial pH 7.0, cultivated at 25°C.

was examined and the combination of 2% glucose and 4% mannitol was most suitable for safracins A and B production (Table 5).

On the base of the above data, a large scale fermentation was carried out and the time course for a typical fermentation in a 30-liter jar fermentor is shown in Fig. 2. Safracin B production began after 16 hours and steadily increased over the next 32 hours, reaching a maximum potency after 48 hours. On the other hand, safracin A production began 12 hours later than safracin B and reached a maximum potency after 72 hours.

## Isolation

High performance liquid chromatography and activity against *Escherichia coli* were used to monitor safracins during their isolation from the culture broth of *P. fluorescens* A2-2.

The whole broth (2.5 liters) was centrifuged in a Sharples centrifugal separator. The supernatant fluid (2 liters) was adjusted to pH 3.0, and then washed with ethyl acetate to remove impurities. The

aqueous layer was adjusted to pH 9.0 and extracted twice with an equal volume of ethyl acetate. After washing twice with 2 liters of water, the ethyl acetate extract was evaporated to dryness *in vacuo*. The dried material was dissolved in a small amount of toluene - chloroform - methanol (2: 6: 1) and put on a column packed with silica gel (50 ml) which had previously been suspended in the same solvent. The column was eluted with the same solvent to obtain safracins A and B. From the above procedures 4 mg of safracin A and 5 mg of safracin B were obtained.

## References

1) IKEDA, Y.; H. MATSUKI, T. OGAWA & T. MUNAKATA: Safracins, new antitumor antibiotics. II. Physicochemical properties and chemical structures. J. Antibiotics 36: 1284~1289, 1983
2) ARAI, T.; K. TAKAHASHI & A. KUBO: New antibiotics, saframycins A, B, C, D and E. J. Antibiotics 30: 1015~1018, 1977
3) Antibiotic safracin A and/or B. Japan Kokai 83-113,192, July 5, 1983
4) BUCHANAN, R. E. & N. E. GIBBONS: BERGEY's Manual of Determinative Bacteriology. 8th ed., p. 217, The Williams & Wilkins Co., Baltimore, 1974
5) COWAN, S. T. & K. J. STEEL: Identification of Medical Bacteria. 2nd ed., Cambridge University Press, London, 1974
6) STANIER, R. Y.; N. J. PALLERONI & M. DOUDOROFF: The aerobic pseudomonads; a taxonomic study. J. Gen. Microb. 43: 150~271, 1966