TETRONOTHIODIN, A NOVEL CHOLECYSTOKININ TYPE-B RECEPTOR ANTAGONIST PRODUCED BY *Streptomyces* sp. NR0489

I. TAXONOMY, YIELD IMPROVEMENT AND FERMENTATION

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Streptomyces sp. NR0489 produces tetronothiodin, a novel brain-type cholecystokinin receptor antagonist. This species was differentiated from its related species *S. gelaticus*, *S. griseolus* and *S. hydrogenans* on the basis of their cultural characteristics, such as the utilization of carbohydrates and the presence or absence of various enzymatic activities. We applied the DNA-DNA hybridization method using photobiotin, which proved the genetic difference between the four species mentioned above. The yield improvement effort including single colony isolation, mutation, and protoplast regeneration together with medium optimization resulted in more than an 81-fold increase of the productivity of tetronothiodin as compared to that of the wild type strain.

Using a microbial screen aimed at finding new binding inhibitors of brain-type cholecystokinin (CCK-B) receptors, we discovered a novel antagonist, tetronothiodin, in the fermentation broth of *Streptomyces* sp. NR0489[1]. Since current taxonomic studies on actinomycetes most often require genetic approaches in addition to chemotaxonomy, we applied a DNA-DNA hybridization method using photobiotin which had originally been developed for eubacterial strains. In addition, since yield improvement is often crucial for obtaining a sufficient amount of substances for chemical studies and for biological evaluation, yield improvement and fermentation studies of this strain were also done.

Materials and Methods

Microorganisms

The producing organism, strain NR0489 was isolated from a soil sample collected in Kamakura, Kanagawa Prefecture, Japan.

Several strains of *Streptomyces* (*S. gelaticus* IFO 12866, *S. griseolus* IFO 12777, and *S. hydrogenans* IFO 13475) were used for our comparative taxonomic studies.

Taxonomy

Methods adopted by the International Streptomyces Project (ISP)[2] were used for our taxonomic studies. The cultural characteristics were determined on media recommended by the ISP and WAKSMAN[3]. All cultures were incubated at 27°C for 15 days. The names of colors used in our studies were based on the Color Standard of Nippon Shikisai Co., Ltd.

Activities of 19 enzymes were assayed by the API ZYM system (API System S. A., Montalieu Vercieu, France).

The cell wall preparation was obtained by the method of YAMAGUCHI[4] and the cell wall contents were analyzed by thin layer chromatography[5]. Glycolic acid in the cell wall was analyzed by the method of UCHIDA[6].

DNA was extracted from the cells by the method of MARMUR[7]. The guanine plus cytosine content of the DNA was determined by the method of TAMAOKA and KOMAGATA[8].

The extent of DNA homology between strains was determined by the fluorometric DNA hybridization method using photobiotin in microdilution wells described by EZAKI[9]. Hybridization was carried out for 2 hours at 55°C.

Breeding

We tried a series of breeding experiments to obtain a clone with greater inhibitory activity and more stable productivity. The original clone was mutated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) at pH 9.0 according to DELIC *et al.*[10], protoplasts were regenerated as reported by KOJIMA and OKANISHI[11] and single colonies were isolated in a conventional manner. We altered the composition of the fermentation medium and varied the quantity of each ingredient in order to find an optimum medium for inhibitor production.

Flask Fermentation

The seed and fermentation medium used in the production of tetronothiodin contained the following: 1.5% glucose, 0.75% meat extract, 0.5% Polypepton, 0.1% yeast extract, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% $CaCO_3$, pH 7.0. An antifoam (Nissan Disfoam CA-115) was added when necessary.

A slant culture of the strain was inoculated into 100 ml of the seed medium in a 500-ml baffled Erlenmeyer flask. The inoculated medium was cultured on a rotary shaker (190 rpm) at 27°C for 3 days. Two ml of this culture (seed culture) was added to 100 ml of the fermentation medium in a 500-ml baffled Erlenmeyer flask. Fermentation was carried out for 8 days under the same conditions as described above and the inhibitory activity of CCK-B receptor binding by tetronothiodin was assayed.

Jar Fermentation

The seed culture (12 ml) prepared as described above, was inoculated into 600 ml of the seed medium in each of five 3-liter flasks with baffles. The flasks were shaken at 100 rpm for 3 days at 27°C. The combined cultures (2.8 liters) were transferred to a 200-liter jar fermenter containing 140 liters of the fermentation medium. Jar fermentation was carried out at 27°C with an agitation rate of 250 rpm, air flow rate of 140 liters/minute and internal pressure of 0.5 kg/cm^2. Dissolved oxygen, pH, temperature and agitation speed were automatically monitored every 30 minutes. Inhibitory activity was checked daily. The residual glucose concentration was measured with a glucose analyzer (Beckman, Model 2). Cell growth was expressed as packed cell volume, which was obtained by centrifugation of 10 ml of cultured broth in a conical tube at 3,500 rpm for 5 minutes.

Results and Discussion

Taxonomy

Morphological Characteristics

The spore chains were the *Rectiflexibilis* type and each had 20 to 30 spores per chain.

The spores were cylindrical ($0.33 \sim 0.42 \times 0.7 \sim 1.1\ \mu m$) and their surface was smooth with some wrinkles (Fig. 1). Sclerotic granules, sporangia and flagellated spores were not observed.

Fragmentation of vegetative mycelium was not observed on any of the agar media tested. When the strain was cultured in a liquid medium such as the fermentation medium on a rotary shaker at 27°C for

Fig. 1. Electron micrograph of strain NR0489.

(1/5 Yeast extract-starch agar). Bar represents 1.0 μm.

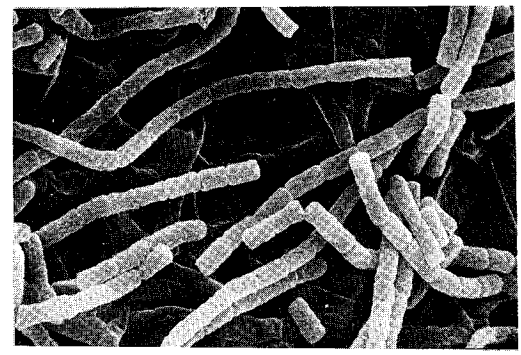


Table 1. Cultural characteristics of strain NR0489, *Streptomyces gelaticus* IFO 12866, *Streptomyces griseolus* IFO 12777, and *Streptomyces hydrogenans* IFO 13475.

Medium		NR0489	*S. gelaticus* IFO 12866	*S. griseolus* IFO 12777	*S. hydrogenans* IFO 13475
Sucrose-nitrate agar	G:	Moderate, colorless	Poor, colorless	Poor, colorless	Poor, colorless
(Waksman medium	AM:	Thin, white	Thin, white	Thin, white	Thin, whitish gray
No. 1)	R:	Colorless	Colorless	Colorless	Colorless
	SP:	None	None	None	None
Yeast extract-malt	G:	Good, colorless	Good, colorless	Good, grayish yellow brown	Good, colorless
extract agar	AM:	Abundant, white~light gray	Scant, brownish white	Abundant, white~gray	Thin, light gray
(ISP medium No. 2)	R:	Dull yellow orange	Dull yellow orange	Brownish gray	Colorless
	SP:	None	None	None	None
Oatmeal agar	G:	Moderate, colorless	Moderate, colorless	Good, brownish gray	Good, colorless
(ISP medium No. 3)	AM:	Thin, white~light gray	Scant, white	Moderate, white~light gray	Thin, light gray
	R:	Colorless	Colorless	Brownish gray	Colorless
	SP:	None	None	Pale yellowish brown	Pale yellowish brown
Inorganic salts-starch	G:	Moderate, colorless	Good, colorless	Good, pale yellowish brown	Good, pale yellowish brown
agar	AM:	Thin, grayish white~light gray	Scant, white	Moderate, white~light gray	Thin, light brownish gray
(ISP medium No. 4)	R:	Colorless	Pale yellowish brown	Light brownish gray	Light brownish gray
	SP:	None	None	None	None
Glycerol-asparagine agar	G:	Poor, colorless	Good, colorless	Good, colorless	Good, colorless
(ISP medium No. 5)	AM:	Thin, brownish white	Thin, brownish white	Abundant, white	Abundant, light brownish gray
	R:	Brownish white	Pale yellowish brown	Pale yellowish brown	Light brownish gray
	SP:	None	Pale yellow orange	Pale yellow orange	None
Glucose-asparagine agar	G:	Good, brownish white	Good, brownish white	Good, light brownish gray	Good, pale yellowish brown
(Waksman medium	AM:	Abundant, white~light gray	Scant, brownish white	Moderate, white~brownish gray	Scant, light gray
No. 2)	R:	Brownish white	Brownish white	Pale yellowish brown	Pale yellowish brown
	SP:	None	None	None	Yellowish brown
Tyrosine agar	G:	Good, pale yellowish brown	Good, pale yellowish brown	Moderate, light brownish gray	Good, pale yellowish brown
(ISP medium No. 7)	AM:	Moderate, white	Scant, white	Moderate, light brownish gray	Thin, light gray
	R:	Pale yellowish brown	Pale yellowish brown	Pale yellowish brown	Pale yellowish brown
	SP:	None	Pale yellowish brown	None	None
Nutrient agar	G:	Good, colorless	Good, colorless	Moderate, colorless	Good, colorless
(Waksman medium	AM:	None	None	Moderate, white	Thin, brownish white
No. 14)	R:	Colorless	Colorless	Colorless	Colorless
	SP:	None	None	None	None

Abbreviations: G, growth; AM, aerial mycelium; R, reverse side color; S, soluble pigment.

3 days, however, it formed submerged spore-like bodies.

Cultural Characteristics

The cultural characteristics of strain NR0489 on various media are summarized in Table 1. Yeast extract - malt extract agar and glucose - asparagine agar supported the best growth and provided abundant formation of aerial mycelia.

Aerial mass color was in the gray color series. Pigmentation of the substrate mycelia was not distinctive. No soluble pigment was observed.

Physiological Characteristics

The physiological characteristics and the utilization of carbohydrate are shown in Tables 2 and 3, respectively. Hydrolysis of gelatin, starch, and milk were negative, nitrate reduction was positive, H_2S production was negative and melanoid pigment was not produced. Strain NR0489 did not grow on yeast extract - malt extract agar supplemented with 4% NaCl. This strain used cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, melibiose, L-rhamnose, salicin, trehalose and D-xylose as sole carbon sources.

This strain grew within a temperature range of 20°C to 37°C, with an optimum temperature range of 27°C to 30°C.

Activities of 19 enzymes by the API ZYM system are shown in Table 4.

Chemotaxonomic Characteristics

Whole cell hydrolysates of strain NR0489 contained LL-diaminopimelic acid but no *meso*-diaminopimelic acid. Glycine was detected in the cell wall. Therefore it was placed in the Type I cell wall group of LECHEVALIER *et al.*

The glycolate test was negative. The DNA from strain NR0489 contained 72.3 mol% guanine plus cytosine.

Table 2. Physiological characteristics of strain NR0489, *Streptomyces gelaticus* IFO 12866, *Streptomyces griseolus* IFO 12777, and *Streptomyces hydrogenans* IFO 13475.

	NR0489	*S. gelaticus* IFO 12866	*S. griseolus* IFO 12777	*S. hydrogenans* IFO 13475
Gelatin liquefaction	−	−	+	−
Starch hydrolysis	−	+	+	+
Milk coagulation at 27°C	−	−	−	−
Milk peptonization at 27°C	−	+	+	+
Nitrate reduction	+	−	+	+
H_2S production	−	−	−	−
Melanin production				
ISP medium No. 1	−	−	−	−
ISP medium No. 6	−	−	−	−
ISP medium No. 7	−	−	−	−
NaCl tolerance	<4%	≤7%	≤7%	<7%
Temperature range for growth	20~37°C	10~37°C	10~37°C	20~37°C
Optimum temperature for growth	27~30°C	27~30°C	20~27°C	27~30°C

+, Positive; −, negative.

Table 3. Carbohydrate utilization of strain NR0489, *Streptomyces gelaticus* IFO 12866, *Streptomyces griseolus* IFO 12777, and *Streptomyces hydrogenans* IFO 13475.

	NR0489	*S. gelaticus* IFO 12866	*S. griseolus* IFO 12777	*S. hydrogenans* IFO 13475
Adonitol	−	+	−	−
L-Arabinose	−	−	+	+
Cellobiose	+	+	+	+
D-Fructose	+	−	+	±
D-Galactose	+	+	+	+
D-Glucose	+	+	+	+
Glycerol	−	+	+	+
Inositol	−	±	−	−
Lactose	+	−	+	+
Maltose	+	+	+	+
D-Mannitol	−	−	−	−
D-Mannose	+	+	+	+
Melezitose	−	±	−	−
Melibiose	+	−	−	−
Raffinose	−	−	−	−
L-Rhamnose	+	+	−	+
Salicin	+	±	+	+
Sucrose	−	±	−	−
Trehalose	+	+	+	+
D-Xylose	+	+	+	+

Table 4. Enzymatic activity in strain NR0489, *Streptomyces gelaticus* IFO 12866, *Streptomyces griseolus* IFO 12777, and *Streptomyces hydrogenans* IFO 13475.

Enzyme assayed for	NR0489	*S. gelaticus* IFO 12866	*S. griseolus* IFO 12777	*S. hydrogenans* IFO 13475
Alkaline phosphatase	+	+	+	+
Esterase (butyrate)	+	−	+	−
Esterase (caprylate)	+	+	+	+
Lipase (myristate)	+	−	+	−
Leucine arylamidase	+	+	+	+
Valine arylamidase	+	+	+	+
Cystine arylamidase	+	+	+	+
Trypsin	+	+	+	−
α-Chymotrypsin	−	+	+	+
Acid phosphatase	+	+	+	+
Phosphoamidase	+	+	+	+
α-D-Galactosidase	−	+	+	−
β-D-Galactosidase	+	+	+	+
β-D-Glucuronidase	−	−	−	−
α-D-Glucosidase	+	+	+	+
β-D-Glucosidase	+	+	+	+
N-Acetyl-β-D-glucosaminidase	+	+	+	+
α-D-Mannosidase	−	+	−	−
α-L-Fucosidase	−	−	−	−

Determination of the Taxonomic Position of Strain NR0489

Based on the taxonomic characteristics described above, we assigned strain NR0489 to the genus *Streptomyces*. Among the known species of this genus, our strain showed some resemblance to *S. gelaticus*

Waksman & Henrici[12], *S. griseolus* Waksman & Henrici[13], and *S. hydrogenans* Lindner, Junk, Nesemann & Schmidt-Thomé[14]. The microbiological characteristics of strain NR0489 were compared with those of the type cultures obtained from IFO and were found to differ clearly from the other species with respect to the properties below. The comparative studies are shown in Tables 1 to 4.

The cultural characteristics of strain NR0489 grown on most media, except for sucrose-nitrate agar and nutrient agar, were different from those of *Streptomyces gelaticus*.

Strain NR0489 grown on yeast extract-malt extract agar and glucose-asparagine agar formed abundant aerial mycelia, whereas *S. gelaticus* grown on any of the media formed poor aerial mycelia.

Starch hydrolysis, milk peptonization, nitrate reduction and NaCl tolerance of strain NR0489 were different from those of *S. gelaticus*. Our strain and *S. gelaticus* also showed different patterns of carbohydrate utilization and enzymatic activity.

Aerial mycelium formation, and pigmentation of the substrate mycelia of strain NR0489 and *Streptomyces griseolus* grown on sucrose-nitrate agar, tyrosine agar and nutrient agar were similar, but when both were grown on all other media they were different.

S. griseolus sporulated more abundantly on most media than did strain NR0489.

The carbohydrate utilization pattern, gelatin liquefaction, starch hydrolysis, milk peptonization and NaCl tolerance of strain NR0489 were different from those of *S. griseolus*. But the enzymatic activity patterns of both were similar.

Aerial mycelium formation and pigmentation of the substrate mycelia of strain NR0489 and *Streptomyces hydrogenans* were similar on sucrose-nitrate agar, tyrosine agar and nutrient agar, but were different on all other media. Moreover, *S. hydrogenans* formed a brown soluble pigment on glucose-asparagine agar while strain NR0489 did not.

The carbohydrate utilization patterns of strain NR0489 and *S. hydrogenans* were similar, but starch hydrolysis, milk peptonization and NaCl tolerance of each were different. The enzymatic activity pattern of strain NR0489 was different from that of *S. hydrogenans*.

S. hydrogenans formed submerged spore-like bodies when grown in fermentation medium on a rotary shaker at 27°C for 3 days as strain NR0489 did, however the other two cultures did not.

The results of the above phenotypic comparison indicated that strain NR0489 was clearly different from the other species.

We carried out DNA-DNA hybridization to confirm this conclusion. EZAKI *et al.*[9] have described the fluorometric DNA-DNA hybridization in microtiter wells using photobiotin instead of radioisotope and nick translation. This method was originally developed for the determination of genetic relatedness

Table 5. DNA relatedness between strain NR0489, *S. gelaticus* IFO 12866, *S. griseolus* IFO 12777, and *S. hydrogenans* IFO 13475.

Strain	% Homology with photobiotin-labeled DNA			
	NR0489	IFO 12866	IFO 12777	IFO 13475
NR0489	100			
S. gelaticus IFO 12866	5.0	100		
S. griseolus IFO 12777	10.5	22.3	100	
S. hydrogenans IFO 13475	5.7	14.3	7.9	100

DNA homology values are expressed as % of labeled DNA reassociated with heterologous DNA compared with that reassociated with homologous DNA (100%).

between eubacterial strains. We applied this method to actinomycete strains of NR0489 and those species mentioned above.

Homology values between strain NR0489 and *S. gelaticus*, *S. griseolus* and *S. hydrogenans* were 5.0%, 10.5% and 5.7%, respectively (shown in Table 5). Therefore strain NR0489 was clearly unrelated to these species from a genetic point of view.

We finally concluded that our strain is a different species from them and assigned it to be *Streptomyces* sp. NR0489.

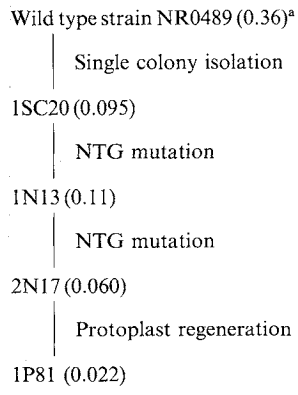
In addition, we also confirmed that *S. gelaticus*, *S. griseolus* and *S. hydrogenans* were genetically unrelated, and that they were distinct species. This supported the results of S. T. WILLIAMS[15].

Breeding

Since the original productivity (IC_{50}: 0.36%) was not enough for further chemical and biological evaluation, yield improvement trials were necessary.

We examined a series of single spore cultures to obtain greater inhibition of CCK-B receptor binding and uniform production. We thus obtained a clone 1SC20 showing an IC_{50} of 0.095% selected from 100 strains. Although this clone showed uniform productivity, it was not enough for purification of the inhibitor. After repeated NTG mutation trials, we obtained clone 2N17 which showed an IC_{50} of 0.060%. The protoplast regeneration method was applied to stabilize productivity, resulting in clone 1P81, which showed a 16-fold greater productivity (IC_{50}: 0.022%) than that of the wild type strain (Fig. 2).

Fig. 2. Breeding of strain NR0489.



[a] IC_{50} values determined by the previously reported method[1].

Table 6. Effect of nitrogen sources on production.

Nitrogen source (concentration: 1%)	% Inhibition at 0.3% broth in the assay mixture	Nitrogen source (concentration: 1%)	% Inhibition at 0.3% broth in the assay mixture
Ajipron E3 (Ajinomoto)	5	Polypepton (Nihon Seiyaku)	70
Medium ingredient S (Yukijirushi)	6	Proteose peptone (Difco)	47
Casamino acids (Difco)	80	Rapeseed cake (Nisshin Seiyu)	24
Casitone (Difco)	90	Skim milk (Difco)	12
Corn meal (Kishimoto Sangyo)	26	Soyaflour FT (Nisshin Seiyu)	24
Corn steep liquor (Hohnen Seiyu)	29	Toast soya (Nisshin Seiyu)	14
Soy peptide hainutoh S (Fuji Seiyu)	15	Totamin S (Kishimoto Sangyo)	60
G powder (Fish meal; Yaizu Suisan)	20	Tryptone (Difco)	67
Kinako	25	Tryptose (Difco)	70
Malt extract (Difco)	26	Yeast extract (Nihon Seiyaku)	84
Meat extract (Kyokuto Seiyaku)	69	Yeast extract (Oriental)	73
Meat extract (Yaizu Suisan)	82	Yeast extract (Difco)	89
Meat extract (Kishimoto Sangyo)	22	Yeast extract (Kishimoto Sangyo)	69
NZ-amine (Sheffield)	83	Fresh yeast (Oriental)	23
Pharmamedia (Traders protein)	13		

Medium Improvement

Although the production of tetronothiodin by the clone in flask culture was high enough, its production in jar fermentation was not always successful because of rather poor growth and delayed production. We thus examined the influence of medium components. The effect of glucose, glycerol, maltose, dextrin, soluble starch, xylose, fructose and rhamnose as carbon sources was examined. Glucose gave the best result. The concentration of glucose and the effect of an additional carbon source were also examined. The addition of 1.5% glucose was optimal for production. A higher concentration of glucose caused delayed production of tetronothiodin.

The production of tetronothiodin by the clone grown on twenty-nine different nitrogen sources was examined. Among them, Casitone, yeast extract, NZ-amine, Polypepton, Casamino acids and meat extract were particularly effective (Table 6). In order to determine the effect of amino acids on production, we

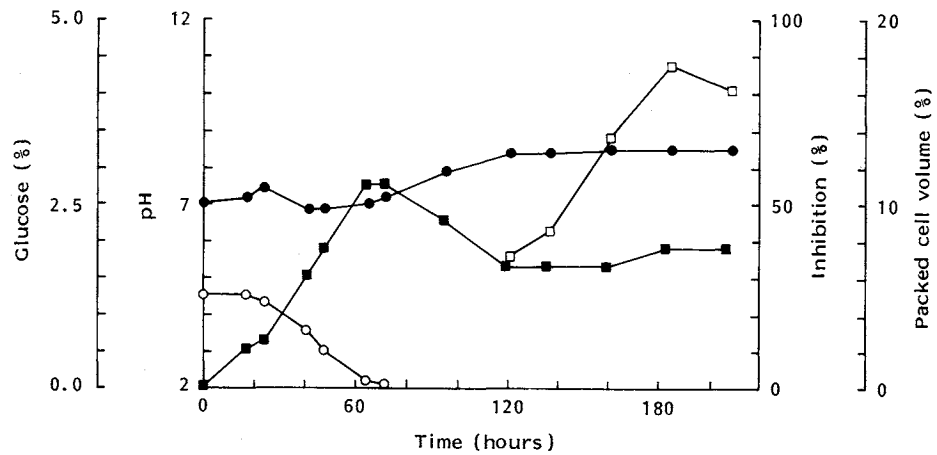
Table 7. Effect of amino acids on production.

Amino acid (concentration: 0.2%)	% Inhibition at 0.2% broth in the assay mixture	Amino acid (concentration: 0.2%)	% Inhibition at 0.2% broth in the assay mixture
L-Alanine	85	L-Lysine	16
	(0.0044)	L-Methionine	20
L-Arginine	2	L-Phenylalanine	49
L-Asparagine	23	L-Proline	79
L-Aspartic acid	40		(0.0072)
L-Cysteine	3	L-Serine	56
L-Glutamine	5	L-Threonine	81
Sodium L-glutamate	76		(0.019)
	(0.010)	L-Tryptophan	74
Glycine	80	L-Tyrosine	59
	(0.014)	L-Valine	84
L-Histidine	36		(0.0061)
L-Isoleucine	70		
L-Leucine	83		
	(0.0044)		

( ): IC_{50} %.

Fig. 3. Time course of tetronothiodin fermentation in a 200-liter fermenter.

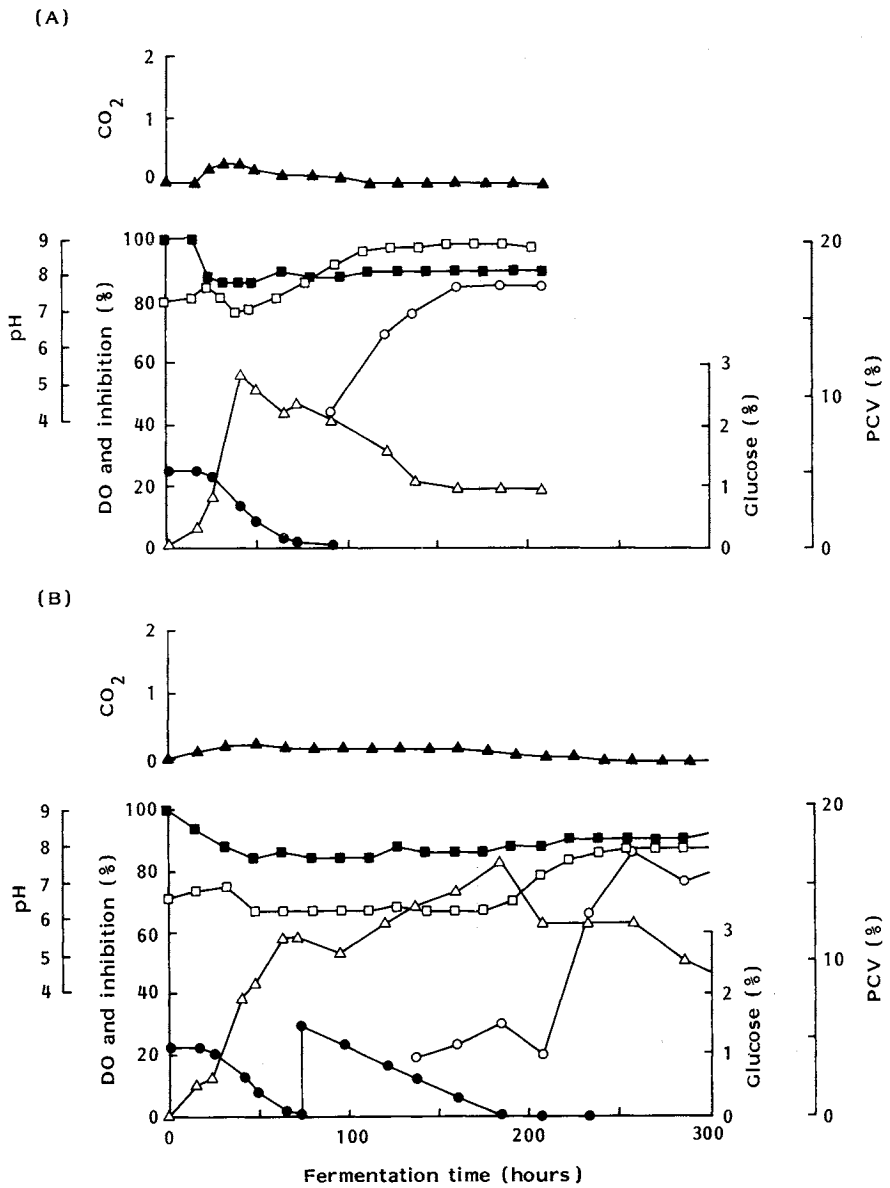
○ Glucose, ● pH, □ inhibition, ■ packed cell volume.



added one of each of 20 amino acids (as the sole nitrogen source) to the production medium. Table 7 indicates that L-alanine, L-glutamic acid, glycine, L-isoleucine, L-leucine, L-proline, L-threonine, L-tryptophan and L-valine were effective, while sulfur-containing or basic amino acids such as L-cysteine, L-arginine and L-methionine were not. Among all the amino acids tested, L-alanine and L-leucine each as the sole nitrogen source resulted in optimal productivity, showing an IC_{50} of 0.0044%. We successfully obtained an 81-fold increase in productivity by clone 1P81 in the optimal medium compared to that of the wild type strain.

Fig. 4. Time course of tetronothiodin fermentation in a 50-liter fermenter.

(A) Batch culture, (B) fed-batch culture, ● glucose, ○ inhibition, △ packed cell volume (PCV), □ pH, ■ dissolved oxygen (DO), ▲ CO_2.



Jar Fermentation

A typical time course of tetronothiodin production in a 200-liter jar fermenter is shown in Fig. 3. The pH was the lowest during the 40~60-hour period and glucose was still being consumed after 60 hours of fermentation. Cells grew exponentially until around 80 hours of fermentation. After the glucose was almost exhausted with the accompanying decrease in cell growth, the production of tetronothiodin began. It reached its maximum after 185 hours of fermentation. This indicated typical production of secondary metabolites.

According to the fed batch culture experiment shown in Fig. 4, the addition of glucose after the depletion of carbon sources resulted in the delayed production (250 hours of fermentation) of tetronothiodin, which was most probably due to catabolite repression.

Acknowledgments

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