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Cyclipostins: Novel Hormone-sensitive Lipase Inhibitors from *Streptomyces* sp. DSM 13381

I. Taxonomic Studies of the Producer Microorganism and Fermentation Results

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The cyclipostins are a group of hormone-sensitive lipase inhibitors produced by a *Streptomyces* species. Having verticillate spore chains this strain exhibits significant differences to the known species of the former genus *Streptoverticillium*. Taxonomic studies and fermentation results are presented.

Actinomycetes containing L-diaminopimelic acid (DAP) in their cell walls primarily belong to the family Streptomycetaceae which contain two genera *Streptomyces* and *Kitasatospora*. The latter genus also contain the *meso*-isomer of DAP. The genus *Streptoverticillium*, originally described by BALDACCI in 1958[1], was characterized by the formation of verticils in its aerial mycelium that produce spore chains with smooth spores, and on the basis of studies of 16S rDNA, WITT and STACKEBRANDT (1991)[2] transferred the species of this genus to the genus *Streptomyces*. Most species of the former genus *Streptoverticillium* have been reported to be producers of pharmacologically active compounds[3].

The isolate that produces cyclipostins was found to form verticils containing spore chains with smooth spores. Because based on chemotaxonomic data it belongs to the Streptomycetaceae, and thus it was compared with species formerly assigned to the genus *Streptoverticillium* using the methods of the International Streptomyces Project and biochemical characterization techniques. Differences were obtained to the described verticillate species. The strain has been deposited at the German Culture Collection (DSMZ) under the accession number DSM 13381.

Materials and Methods

Bacterial Strains

The strains used in this study in addition to *Streptomyces* sp. DSM 13381 are shown in Table 1. *Streptomyces* sp. DSM 13381 was isolated on ISP 3 medium from a soil sample collected in Malaysia in 1989.

Morphology and Physiology

The morphological and physiological characteristics of the strains were investigated by growing agar cultures on the various media described by SHIRLING and GOTTLIEB[4]: yeast extract - malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt - starch agar (ISP 4), glycerol - asparagine agar (ISP 5), peptone - yeast extract iron agar (ISP 6), and tyrosine agar (ISP 7). The plates were incubated for 10 days at 28°C. For scanning electron microscopy the strain was grown on ISP 3 agar. The small agar piece was prepared using the method of WINK *et al.*[5].

Utilization of carbohydrates was investigated on ISP 9 medium (SHIRLING and GOTTLIEB[4]) on 12-well microtiter plates. Production of melanoid pigments was observed on ISP 6 and 7 and on Suter medium with or without tyrosine (KUTZNER[6]). Sodium chloride tolerance was likewise tested on microtiter plates (6-well) using a technique based on the

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Table 1. Strains of *Streptomyces* species used for comparative studies.

Species	Strain No.	Source
Streptomyces abikoensis (Umezawa et al. 1951) Witt and Stackebrandt 1991	FH 2491	ATCC 12766^T
Streptomyces albireticuli (Nakazawa 1955) Witt and Stackebrandt 1991	FH 2494	ATCC 19721^T
Streptomyces cinnamoneus subsp. cinnamoneus (Benedict et al. 1952) Witt and Stackebrandt 1991	FH 2573	ATCC 11874^T
Streptomyces ehimense (Shibata et al. 1954) Witt and Stackebrandt 1991	FH 2923	DSM 40253^T
Streptomyces griseocarneus (Benedict et al. 1950)	FH 2575	ATCC 12628^T
Streptomyces griseoverticillatus (Shinobu and Shimada 1962) Witt and Stackebrandt 1991	FH 2101	DSM 40507^T
Streptomyces hachijoensis (Hosoya et al. 1952) Witt and Stackebrandt 1991	FH 2503	ATCC 19769^T
Streptomyces hiroshimensis (Shinobu 1955) Witt and Stackebrandt 1991	FH 2026	IMET 43546^T
Streptomyces kashmirensis (Gupta and Chopra 1963) Witt and Stackebrandt 1991	FH 2504	ATCC 27439^T
Streptomyces kishiwadensis (Shinobu and Kayamura 1964) Witt and Stackebrandt 1991	FH 2505	ATCC 25464^T
Streptomyces lilacinus (Nakazawa et al. 1956) Witt and Stackebrandt 1991	FH 2508	ATCC 23930^T
Streptomyces luteosporeus (Locci et al. 1969) Witt and Stackebrandt 1991	FH 2509	ATCC 33049^T
Streptomyces mashuensis (Sawazaki et al. 1955) Witt and Stackebrandt 1991	FH 2028	IMET 42941^T
Streptomyces mobaraensis (Nagatsu and Suzuki 1963) Witt and Stackebrandt 1991	FH 2513	ATCC 29032^T
Streptomyces morookaensis (Locci and Schonfield 1989) Witt and Stackebrandt 1991	FH 2514	ATCC 14808^T
Streptomyces netropsis (Finlay et al. 1951) Witt and Stackebrandt 1991	FH 2406	LMG 5979^T
Streptomyces olivoreticuli (Locci and Schonfield 1989) Witt and Stackebrandt 1991	FH 1713	ATCC 31159^T
Streptomyces orinoci (Cassinelli et al. 1967) Witt and Stackebrendt 1991	FH 2515	ATCC 23202^T
Streptoverticillium rectiverticillatus (Krassilnikov and Yuan 1965) Witt and Stackebrandt 1991	FH 2516	ATCC 19845^T
Streptomyces roseoverticillatus (Shinobu 1956) Witt and Stackebrandt	FH 2455	DPDU 0819^T
Streptomyces salmonis (Baldacci et al. 1966) Witt and Stackebrandt 1991	FH 2595	NRRL B-1472^T
Streptomyces sapporonensis (Locci and Schofield 1989) Witt and Stackebrandt 1991	FH 2411	ATCC 21532^T
Streptomyces thioluteus (Okami 1952) Witt and Stackebrandt 1991	FH 2487	IFO 13341^T
Streptomyces viridoflavum (Locci and Schofield 1989) Witt and Stackebrandt 1991	FH 2223	DSM 40237^T
Streptomyces sp.	DSM 13381	own isolate

^{T} type strain of the species

method of KUTZNER[6]. Fingerprints of enzymatic activities were obtained with the aid of API 20E test strips[7].

Chemotaxonomic Analysis

Analysis of the whole-cell content of diaminopimelic acid isomers and sugars was carried out by the method of HASEGAWA *et al.*[8]. Phospholipids were analyzed by the method of KÜTZNER *et al.*[6]. For analysis of the whole-cell fatty acid distribution, we used the rapid method based on that of MULLER *et al.*[9] and the information held in our database (WINK *et al.* 2000[10]).

Strain Maintenance and Fermentation

100 ml of nutrient solution (pH 6.0, 2.0% malt extract, 0.2% yeast extract, 1.0% glucose, 0.05% $(NH_4)_2HPO_4$) in a sterile 300 ml Erlenmeyer flask was inoculated with the strain *Streptomyces* sp. DSM 13381 and incubated on a rotating shaker for 7 days at 28°C and 180 rpm. 1.5 ml of this culture was then diluted with 1.5 ml of 99% glycerol and the suspension was stored at −20°C.

For the preparation of a preculture of *Streptomyces* sp. DSM 13381, a sterile 300 ml Erlenmeyer flask containing 100 ml of nutrient solution (15 g/liter glucose, 15 g/liter soy meal, 5 g/liter cornsteep, 2 g/liter $CaCO_3$, and 5 g/liter NaCl) was inoculated with a slant culture (same nutrient solution, with addition of 2% agar) or with 1 ml of a glycerol culture and incubated on a shaker at 28°C and 180 rpm. A 48~96 hours submerse culture (inoculation volume: approx. 10%) of the same nutrient solution is sufficient for the inoculation of 10 and 200 liter fermenters.

Preparation of Cyclipostins A to F

A 200 liter fermenter was charged with 90 liters of nutrient solution (20 g/liter of oat flakes, 2.5 ml/liter of trace element solution, pH before sterilization: 7.8) and the contents were heat-sterilized for 30 minutes. The nutrient solution was then cooled and inoculated with 5% of the volume of *Streptomyces* sp. DSM 13381. Trace element solution: 3 g/liter $CaCl_2 \cdot 2H_2O$; 1 g/liter iron(III) citrate; 0.2 g/liter $MnSO_4 \cdot H_2O$; 0.1 g/liter $ZnCl_2$; 0.025 g/liter $CuSO_4 \cdot 5H_2O$, 0.02 g/liter sodium tetraborate; 0.004 g/liter $CoCl_2 \cdot 6H_2O$, and 0.01 g/liter sodium molybdate. Process time: 72 hours.

Preparation of Cyclipostins N to T2

Use of a medium containing 5 g/liter glucose, 20 g/liter glycerol, 20 g/liter soy meal, 5 g/liter yeast extract, and 3 g/liter NaCl resulted in the formation of cyclipostins N to T2.

Inoculation of the main stage was with 0.15% of a seed culture of *Streptomyces* sp. DSM 13381. Production of these compounds was in a 2 m^3 vessel under the following conditions: temperature 28°C, aeration 60 m^3/hour, pressure 0.5 kg/cm^2, stirrer tip speed 1.2 m/second. The partial oxygen pressure was kept above 30% by stirring, the pH generally remained within a pH 6~6.5 range without external regulation. To avoid product interference during analysis and downstream processing, antifoaming agents were not added. During fermentation, biomass growth was monitored through changes in the packed mass volume (pmv) and glucose and phosphate concentrations.

Results

Characteristics of Strain DSM 13381

Strain DSM 13381 developed beige (RAL 1001) vegetative mycelia on all ISP media tested (Table 3). Aerial mycelia are formed on all ISP media except ISP 6 and these aerial mycelium color is brown-beige (RAL 1011) on media ISP 2, 3, and 4 and white on ISP 5 and 7. Brown exopigments are formed on ISP 2 and 7 and a brown melanoid pigment is formed on Suter medium with added tyrosine.

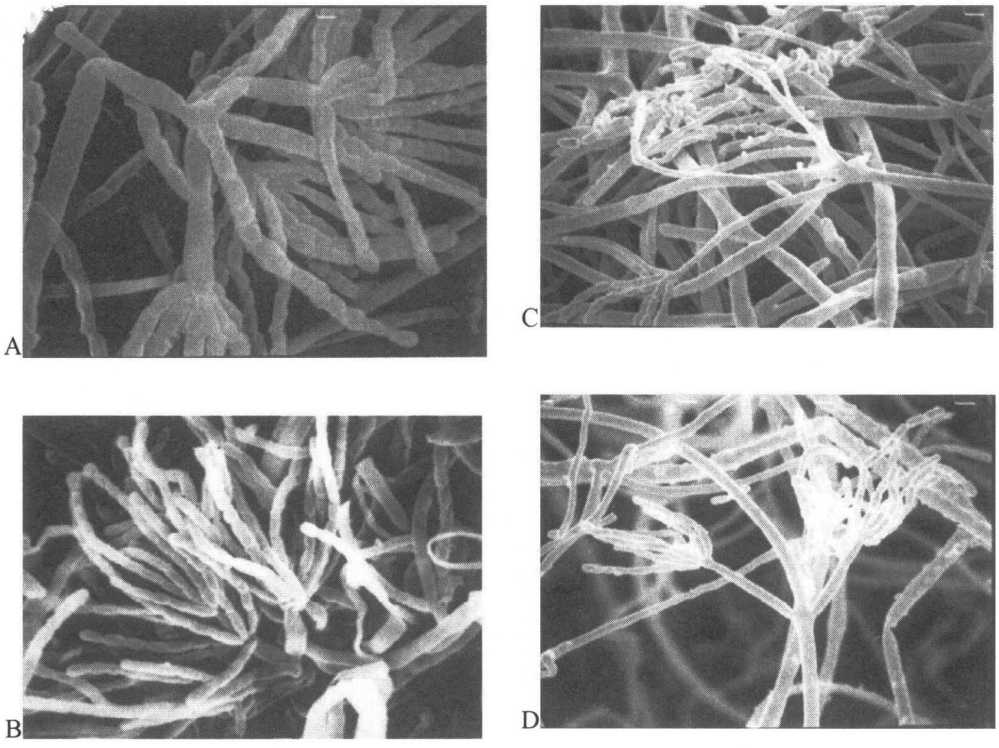
Glucose, arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose, and raffinose are used as the carbohydrate source. With Api 20E test strips, strain DSM 13381 is positive for β-galactosidase and urease and produces H_2S and acetoin.

Comparison of Strain DSM 13381 with *Streptomyces griseoverticillatus* FH 2101 (DSM 40507), *S. kashmirensis* FH 2504 (ATCC 27439), and *S. olivoreticuli* FH 1713 (ATCC 31159)

Comparison of the species of the former verticillated genus *Streptoverticillium* revealed that only *Streptomyces griseoverticillatus*, *kashmirensis*, and *olivoreticuli* exhibited similarity with Strain DSM 13381 in the color of aerial and substrate mycelia. The majority of other species exhibit typical white aerial mycelia and a red substrate mycelium. *S. netropsis* exhibits gray aerial mycelium and *S. thioluteus* an olive-green substrate mycelium while remaining species have rose to pink aerial mycelium. Most of the verticillated species produce soluble pigments, varying from light to dark brown. More than half of the species are melanin-positive.

The substrate mycelium of *S. griseoverticillatus*, *S. kashmirensis*, *S. olivoreticuli* and *S.* sp. DSM 13381 is beige to brown on ISP media. Only *S. griseoverticillatus*

Fig. 1. Scanning electron micrographs of spore chain formation of the different *Streptomyces* species.



A: Strain FH 2101 *Streptomyces griseoverticillatus*, grown on ISP 3 agar for 10 days at 28°C (magnification ×5,000).
B: Strain FH 2504 *Streptomyces kashmirensis*, grown on ISP 3 agar for 12 days at 28°C (magnification ×10,000).
C: Strain FH 1713 *Streptomyces olivoreticuli*, grown on ISP 3 agar for 8 days at 28°C (magnification ×5,000).
D: Strain DSM 13381 *Streptomyces* sp., grown on ISP 3 agar for 7 days at 28°C (magnification ×7,500).

Table 2. Colony characteristics of *Streptomyces griseoverticillatus* FH 2101, *S. kashmirensis* FH 2504, *S. olivoreticuli* FH 1713, and *S.* sp. DSM 13381.

Culture medium	Strain			
	FH 2101	FH 2504	FH 1713	DSM 13381
ISP 2	SM colorless AM beige SP none	SM brown AM beige SP brown	SM beige AM beige SP none	SM brown AM brown-beige SP brown
ISP 3	SM colorless AM rose SP none	SM brown AM beige SP brown	SM beige AM beige SP none	SM beige AM brown-beige SP none
ISP 4	SM colorless AM rose SP none	SM brown AM white SP brown	SM brown-beige AM beige SP none	SM beige AM brown-beige SP none
ISP 5	SM beige AM beige SP brown	SM brown-beige AM none SP brown	SM beige AM beige SP none	SM beige AM white SP none
ISP 6	SM colorless AM rose SP none	SM brown AM none SP brown	SM brown AM none SP brown	SM beige AM none SP beige
ISP 7	SM colorless AM white SP none	SM red-brown AM white SP brown	SM brown AM beige SP brown	SM beige AM white SP none

Note: Formation and color of the substrate mycelium (SM), aerial mycelium (AM), and soluble exopigment (SP).

has colorless substrate mycelium on ISP 2, 3, 4, 6, and 7 except that *S. kashmirensis* has a red-brown substrate mycelium on medium ISP 7. In the case of *S. griseoverticillatus* the aerial mycelium is rose-colored on ISP 3, 4, and 6 and white on ISP 7. *S. kashmirensis* forms white aerial mycelium on ISP 4 and 7 but does not form aerial mycelia on ISP 5 and 6. *S. olivoreticuli* does not form aerial mycelia on ISP 6. Strain DSM 13381 has white aerial mycelium on ISP 5 and 7, but has none on ISP 6. Soluble brown pigment is formed by strain *S. griseoverticillatus* on ISP 5, by *S. kashmirensis* on ISP 2, 3, 4, 5, 6, and 7, by *S. olivoreticuli* on ISP 6 and 7, and by *S.* sp. DSM 13381 on ISP 2 and 6. *S. griseoverticillatus* is melanin-negative, but *S. kashmirensis*, *S. olivoreticuli*, and *S.* sp. DSM 13381 are melanin-positive (see Table 2). The verticillated spore chain morphology of the four strains is shown in Fig. 1.

The strains *S. griseoverticillatus* and *S. olivoreticuli* utilize only glucose as the carbon source. *S. kashmirensis*

Table 3. Carbohydrate utilization by *Streptomyces griseoverticillatus* FH 2101, *S. kashmirensis* FH 2504, *S. olivoreticuli* FH 1713, and *S.* sp. DSM 13381.

Carbo-hydrate	Strain			
	FH 2101	FH 2504	FH 1713	DSM 13381
Arabinose	-	(+)	-	+
Sucrose	-	+	-	+
Xylose	-	(+)	-	+
Inositol	-	+	-	+
Mannitol	-	(+)	-	+
Fructose	-	(+)	-	+
Rhamnose	-	(+)	-	+
Raffinose	-	(+)	-	+

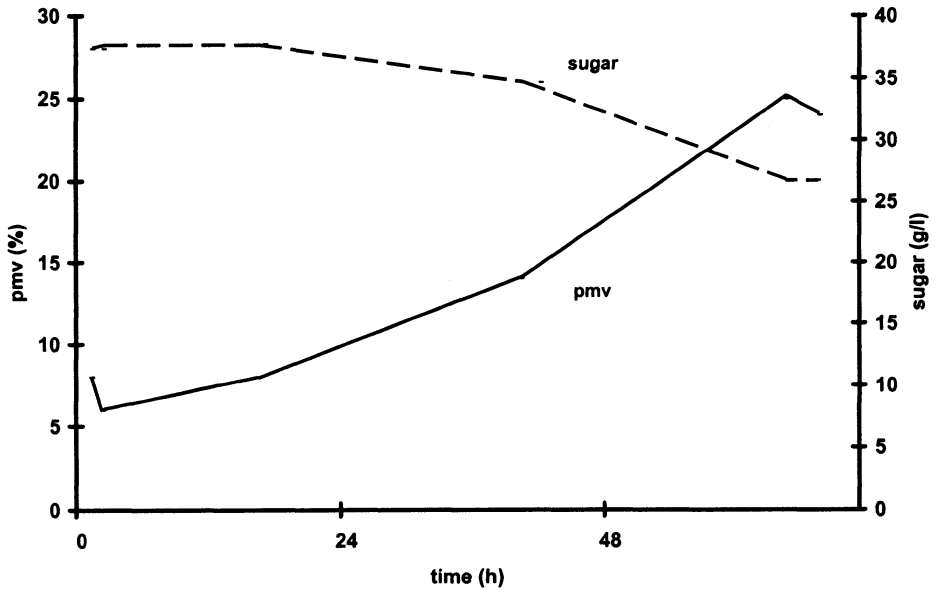
- growth no better than the negative control (basal medium with water)
(+) growth better than the negative control, but not as good as the positive control
\+ growth as good as the positive control (basal medium with glucose)

Table 4. Enzymatic activities of *Streptomyces griseoverticillatus* FH 2101, *S. kashmirensis* FH 2504, *S. olivoreticuli* FH 1713, and *S.* sp. DSM 13381.

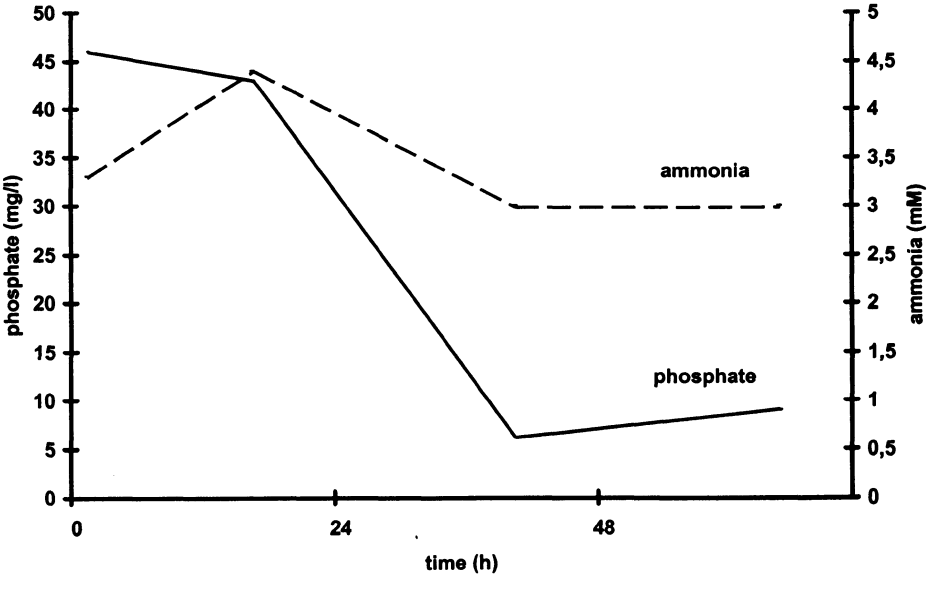
Physiological parameter	Strain			
	FH 2101	FH 2504	FH 1713	DSM 13381
API 20E				
β-Galactosidase	-	-	-	+
Arginine dihydrolase	-	-	+	-
Lysine decarboxylase	-	-	+	-
H_2S production	-	+	+	+
Urease	-	-	-	+
Acetoin production	-	-	-	(+)
Gelatinase	+	-	-	-

Fig. 2. Typical courses of the biomass, sugar (a), phosphate, and ammonia (b) concentrations during cyclipostin fermentation on a 2000 liters scale.

(a)



(b)



Biomass production begins to increase significantly after 20 hours, with a concomitant decrease in substrate concentrations. An initial increase of ammonia concentrations during the first 20 hours was generally observed and may be attributable to enzymatic digestion of the complex protein sources used.

utilizes glucose, sucrose, and inositol well and arabinose, xylose, mannitol, fructose, rhamnose, and raffinose only poorly, whereas the strain *S.* sp. DSM 13381 is able to utilize all the tested carbohydrates (see Table 3). *S. griseoverticillatus* is the only gelatinase-positive strain and is H_2S-negative, whereas *S. kashmirensis*, *S. olivoreticuli*, and *S.* sp. DSM 13381 are H_2S-positive. *S. olivoreticuli* is the only strain positive to arginine dihydrolase and lysine decarboxylase and *S.* sp. DSM 13381 is the only strain positive to β-galactosidase and urease and showing slight acetoin formation (see Table 4).

Fermentation

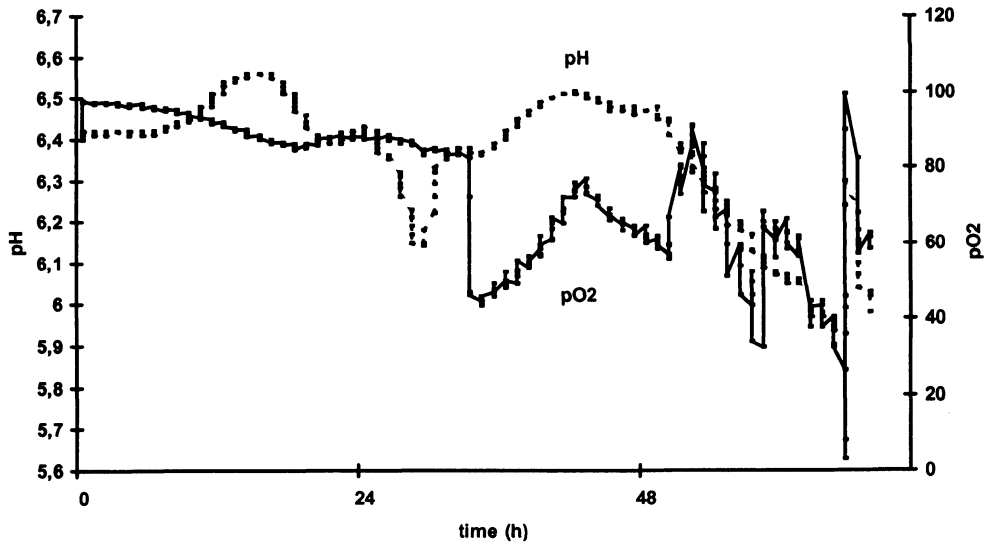
Production of the different cyclipostins can be directed by the choice of nutrient media. Thus, cyclipostins A to F were formed by using medium ISP 3 with oat flakes for fermentation, and cyclipostins N to T2 by using a medium containing glucose (5 g/liter), glycerol (20 g/liter), soy meal (20 g/liter), yeast extract (5 g/liter),. and NaCl (3 g/liter). Regulation of the pH is not necessary. The fermentation time is between 48 and 72 hours with the latter medium and 72 hours with the former. In the glucose/glycerol medium the biomass reached a pmv of 20~25% after about 70 hours. This was accompanied by a decrease in the glucose concentration to about 25~30 g/liter, which was thus far from depleted, whereas the phosphate concentration decreased from 40 to 5 mg/liter. The highest production titers of up to 2 mg cyclipostin P per liter were usually reached after 48 to 72 hours.

The courses of the biomass, sugar, phosphate and ammonia concentrations during a typical large-scale cyclipostin fermentation are shown in Fig. 2, and the courses of the pH and pO_2 concentration shown in Fig. 3.

Discussion

On the basis of its chemotaxonomic and morphological properties, Strain DSM 13381 can be classified as a member of the genus *Streptomyces*[11~13]. Mycolic acids are absent. The cell wall of Strain DSM 13381 was found to contain L-diaminopimelic acid. The phospholipid type is P II with phosphatidylethanolamine as the characteristic lipid. In the fatty acid analysis the dominant acids are 15:0 *iso*, 15:0 *anteiso*, 16:0 *iso*, 17:0 *iso*, and 17:0 *anteiso*. A significant morphological marker is the formation of spore chains in verticillate whorls, which has been described only for the verticillate *Streptomycetes* (former genus *Streptoverticillium*). Among the *Streptomyces* species formerly classified to the genus *Streptoverticillium*, only *S. griseoverticillatus*, *S. kashmirensis*, and *S. olivoreticuli*

Fig. 3. Typical courses of pH and pO_2.



The pH remained within a pH 6~6.5 range without external regulation. The partial oxygen pressure declines continuously to about 30% at which it is controlled *via* stirring. The pronounced fluctuations observed are due to foaming (for easier purification of the cyclipostins, antifoaming agent was not added).

showed some similarities in colony morphology and pigmentation. In its carbohydrate utilization and enzymatic activities and in a complete analysis of colony and pigment formation, DSM 13381 showed numerous differences from *S. griseoverticillatus*, *S. kashmirensis*, and *S. olivoreticuli*. The strain DSM 13381 is the only one utilizing all carbohydrates. It is also the only one with a β galactosidase and a urease activity as well as the production of acetoin.

The strain DSM 13381 is also the producer of a group of secondary metabolites, the cyclipostins, which have not been described for any other *Streptomyces* species. The product spectrum of the various cyclipostins formed is influenced by the composition of the nutrient medium. The structures of the different cyclipostins will be reported in a separate paper (VERTESY *et al.*[14]).

References

1) BALDACCI, E.: Development in the classification of actinomycetes. G. Microbiol. 6: 10~27, 1958
2) WITT, D. & E. STACKEBRANDT: Unification of the genera *Streptoverticillium* and *Streptomyces*, and amendation of *Streptomyces* Waksman and Henrici 1943, 339^{AL}. System. Appl. Microbiol. 13: 361~371, 1990
3) WILLIAMS, S. T.; M. E. SHARPE & J. G. HOLT (Editors): Bergey's Manual of Systematic Bacteriology Volume 4. The Williams and Wilkins Co., Baltimore, 1989
4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int J. Syst. Bacteriology. 16: 313~340, 1966
5) WINK, J.; S. GRABLEY, E. GRANZER, K. HUTTER, D. LUDWIG, M. MAYER, R. THIERICKE & G. TILL: Secondary metabolites by chemical screening. 8. Decarestrictines, a new family of inhibitors of cholesterol biosynthesis from *Penicillium*. J. Antibiotics 45: 56~65, 1992
6) KUTZNER, H. J.; R. M. KROPPENSTED & F. KORN-WENDISCH: Methoden zur Untersuchung von Streptomyceten und einigen anderen Actinomyceten. 4th edition, 1986
7) SMITH, P. B.; K. M. THOMFOHRDE, D. L. RHODEN & A. BALOWS: API System: a multitube micromethod for identification of Enterobacteriaceae. Appl. Microbiol. 24: 449~452, 1972
8) HASEGAWA, T.; M. TAKIZAWA & S. TANIDA: A rapid analysis for chemical grouping of aerobic Actinomycetes. J. Gen. Appl. Microbiol. 29: 319~322, 1983
9) MÜLLER, K. D.; H. HUSMANN & H. P. NALIK: A new and rapid method for the assay of bacterial fatty acids using high resolution capillary gas chromatography and trimethylsulfonium hydroxide. Zbl. Bakt. 274: 174~182, 1990
10) WINK, J.; W. ARETZ, J. MEIWES, G. SEIBERT & G. VOBIS: Friulimicins: Novel lipopeptide antibiotics with peptidoglycan synthesis inhibiting activity from *Actinoplanes friuliensis* sp. nov. I. Taxonomic studies of the producing microorganism and fermentation. J. Antibiotics 53: 807~815, 2000
11) LECHEVALLIER, M. P.; H. A. LECHEVALLIER & N. N. GERBER: Chemical composition as a criterion in the classification of actinomycetes. Adv. Appl. Microbiol. 14: 47~72, 1971
12) LECHEVALLIER, M. P.; A. E. STERN & H. A. LECHEVALLIER: Phospholipids in the taxonomy of actinomycetes. Zbl. Bakt. Mikrobiol. Hyg. I. Abt. Suppl. 11: 335~354, 1981
13) KROPPENSTEDT, R. M.: Fatty acid and menaquinone analysis of actinomycetes and related organisms. *In* GOODFELLOW, M. & D. E. MINNIKIN (*Eds.*). Chemical methods in bacterial systematics. No. 20 SAB Technical Series, Academic Press, pp. 173~199, 1985
14) VERTESY, L.; B. BECK, M. BRÖNSTRUP, K. EHRLICH, M. KURZ, G. MÜLLER, D. SCHUMMER & G. SEIBERT: Cyclipostins, novel hormone-sensitive lipase inhibitors from *Streptomyces* sp. DSM 13381. II. Isolation, structure elucidation and biological properties. J. Antibiotics 55: 480~494, 2002