Studies on Xylanase from Microorganisms

Part II. Isolation and Selection of Xylanase-producing Microorganisms and the Identification of a New Species of *Streptomyces*

By Hiroshi IIZUKA and Toshiro KAWAMINAMI

The Institute of Applied Microbiology, University of Tokyo

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As xylanase-producing microorganisms, 64 strains belonging to the genus *Streptomyces* were isolated from the barn-yard manures, silages and litters collected in Hokkaido district. Among these isolates the strain 102-1-4, which was found to be a new species under taxonomical studies and named *Streptomyces xylophagus* nov. sp., had the most outstanding ability for the enzyme production. In addition to the isolates, 38 strains of *Streptomyces* and 480 strains of filamentous fungi which have been preserved in our culture collection were also examined on their ability to produce the enzyme. 1) Among the strains of *Streptomyces* tested, only two strains, *St. albogriseolus* IAM 0031 and *St. olivaceus* IAM 0025 were found to have the ability, but their abilities were less than that of *St. xylophagus* nov. sp. 2) Out of 480 strains of fungi tested, *Chaetomium*, *Schyzophyllum*, *Trametes*, *Echinodontium*, *Alternaria*, *Cepharosporium*, *Cercospora*, *Gibberella*, *Glomerella* and *Macrosporium* produced the enzyme. Especially, *Ch. trilaterale* 2264 was the most excellent.

Many studies have been made on industrial uses of amylases and cellulase. However, only a few reports have been published on xylanase which are responsible for degradation of xylan, one of vegetable polysaccharides that exists widely, after starch and cellulose, in natural resources.¹⁾ To date, some strains belonging to *Bacillus*,^{2,3)} *Aspergillus*^{4~6)} and a few other genera^{7,8)} are reported to be able to secrete xylanase into culture media. The authors previously made public⁹⁾ on the purification and some properties of xylanase from a new species of *Streptomyces*. This paper describes the isolation and the identification

of the new species of *Streptomyces*. In addition, some fungi which have been preserved in our culture collection were studied on their ability to produce the enzyme.

EXPERIMENTALS

1. *Isolation of microorganisms.* On the agar plates containing xylan (obtained from Nutritional Biochemicals Corp.) as a sole source of carbon, a few drops of the suspension of each sample which was collected in the vicinity of Obihiro City in Hokkaido were spread and the plates were incubated at 28°C. After certain periods of cultivation, microorganisms which formed clear-cut zones around their colonies were picked up as the strains having the enzyme-secreting ability. The compositions of culture media used in the isolation procedures are as follows:

- 1) Y. Tsuzuki, Saccharides, Iwanami, 1961, p. 187.
- 2) F. J. Simpson, *Can. J. Microbiology*, **2**, 28 (1956).
- 3) M. Takahashi and Y. Hashimoto, Annual meeting of Japanese Agricultural and Chemical Society, 1957.
- 4) R. L. Whistler and E. Masak, *J. Am. Chem. Soc.*, **77**, 1241 (1955).
- 5) S. Fukui and M. Sato, *Bull. Agr. Chem. Soc.*, **21**, 392 (1957).

- 6) S. Fukui, *J. Gen. Appl. Microbiol.*, **4**, 39 (1958).
- 7) H. Sørensen, *Physiol. Plantarum*, **5**, 183 (1952).
- 8) K. Sumizu, M. Yoshikawa and S. Tanaka, *J. Biochemistry*, **50**, 538 (1961).
- 9) H. Iizuka and T. Kawaminami, *Agr. Biol. Chem.*, **29**, 520 (1965).

A. Xylan	10.0 g	B. Xylan	10.0 g
Peptone	10.0	Na-Glutamate	5.0
K ₂ HPO ₄	1.0	MgSO ₄ ·7H ₂ O	0.2
MgSO ₄ ·7H ₂ O	0.5	KCl	0.1
KCl	0.5	FeSO ₄	0.01
Water	1.0 litre	Water	1.0 litre
pH	6.0	pH	7.2
C. Xylan	10.0 g	D. Xylan	10.0 g
Asparagine	3.0	NaNO ₃	2.0
K ₂ HPO ₄	5.0	K ₂ HPO ₄	1.0
MgSO ₄ ·7H ₂ O	2.0	MgSO ₄ ·7H ₂ O	0.5
Water	1.0 litre	KCl	0.5
pH	6.8	FeSO ₄	0.01
		Water	1.0 litre
		pH	6.0

2. *Microorganisms tested.* All strains tested in this paper were subcultured from the culture collection of our laboratory except 64 strains of *Streptomyces* newly isolated from the above-mentioned sources. Table I is a list of genera and number of the strains tested.

TABLE I. LIST OF MICROORGANISMS TESTED FOR XYLANASE PRODUCTION

<i>Streptomyces</i>	Total: 102	
new isolates	64	
stock cultures	38	
Molds	Total: 480	
<i>Chaetomium</i>	35	<i>Alternaria</i> 1
<i>Agaricus</i>	1	<i>Cephalosporium</i> 1
<i>Asterophora</i>	1	<i>Cercospora</i> 13
<i>Collybia</i>	1	<i>Cladosporium</i> 2
<i>Cortinellus</i>	1	<i>Corticium</i> 2
<i>Echinodontium</i>	2	<i>Curvularia</i> 4
<i>Formes</i>	2	<i>Elsinoe</i> 2
<i>Irpex</i>	2	<i>Fusarium</i> 7
<i>Lenzites</i>	4	<i>Gibberella</i> 4
<i>Pholiota</i>	1	<i>Gloeosporium</i> 1
<i>Pleurotus</i>	2	<i>Glomerella</i> 3
<i>Polyporus</i>	3	<i>Helicobasidium</i> 1
<i>Polystictus</i>	4	<i>Helminthosporium</i> 5
<i>Poria</i>	1	<i>Macrosporium</i> 2
<i>Schizophyllum</i>	1	<i>Ophiobolus</i> 1
<i>Stereum</i>	1	<i>Firicularia</i> 2
<i>Trametes</i>	2	<i>Sclerotinia</i> 2
<i>Ustilago</i>	1	<i>Sphaceloma</i> 3
Parasites to Grass	208	<i>Tilachlidium</i> 1
Isolates from		
Rice and Grains ^{10,11)}	150	

3. *Assay of xylanase activity.* *Streptomyces* strains were grown at 30°C in the medium C with shaking. After 3 days cultivation, the broth was filtrated through Toyo No. 2 filter paper to obtain clear filtrate, which was used for enzyme assay. The screening tests for fungi were carried out by almost the same methods as employed in *Streptomyces* strains except for their culture medium. The medium used for fungi was composed of 7% wheat bran and 0.1% yeast extract. One ml of culture filtrate of each test strain was added to 5 ml of 0.5% xylan solution (pH 6.2 for *Streptomyces* and pH 4.0 for fungi). After incubation for 15 min at 55°C, the reaction was stopped by pouring the mixture into 10 ml of Somogyi's reagent A solution, and the amount of reducing sugar produced was determined by Somogyi's method¹²⁾ as xylose. Furthermore, the composition of the reducing sugars was checked by paper chromatographies using *n*-propanol : ethylacetate : water (6 : 1 : 3) and pyrimidine : ethylacetate : water (8 : 2 : 1) as solvents.

RESULTS AND DISCUSSIONS

1. Isolation and selection of xylanase-producing strains

From 101 samples collected in Hokkaido, 64 strains were found to form clear-cut zones around their colonies and all of which belonged to Genus *Streptomyces*. Bacteria and fungi grew also on the agar plates, but they did not show any clear-cut zone. The typical clear-cut zones on the agar plates are shown in Fig. 1. The culture filtrates were checked whether they had actually the xylanase activities by introducing them into the xylan solution. The results are shown in Table II, indicating that No. 102-1-4 was the most potent strain. The reducing sugars in Table II were found to be composed of xylose and xyloligosaccharides by chromatography. In these isolation and selection procedures, *Streptomyces* strains were obtained from all sources as shown in Table III, but most of the isolates

10) H. Iizuka, *J. Gen. Appl. Microbiol.*, **3**, 146 (1957).

11) H. Iizuka, *ibid.*, **4**, 108 (1958).

12) T. Kobayashi and T. Tabuchi, *J. Agr. Chem. Soc. Japan*, **28**, 171 (1954).

FIG. 1. Typical Clear-cut Zones on the Xylan Agar Plates.
Photographed after 3 days cultivation at 28°C.

TABLE II. XYLANASE ACTIVITY OF THE CULTURE FILTRATES OF STREPTOMYCES STRAINS
NEWLY ISOLATED FROM VARIOUS SOURCES

Strain	Source	Reducing sugar (mg)	Strain	Source	Reducing sugar (mg)	Strain	Source	Reducing sugar (mg)	Strain	Source	Reducing sugar (mg)
31-2-1	BM	0.46	44-4-7	BM	0.92	100-4-1	BM	4.01	10-4-2	SG	3.04
34-3-1	BM	5.31	44-5-2	BM	0.43	100-4-2	BM	4.07	11-3-1	SG	0.81
34-3-4	BM	4.31	53-1-1	BM	4.07	102-1-1	BM	2.05	11-3-2	SG	0.75
34-3-5	BM	3.80	53-3-2	BM	3.66	102-1-2	BM	2.61	49-5-1	SG	1.13
34-3-6	BM	3.23	53-4-2	BM	2.32	102-1-3	BM	0.94	49-5-2	SG	3.66
34-5-1	BM	4.46	53-4-3	BM	0.67	102-1-4	BM	6.95	42-3-1	SC	1.56
34-5-2	BM	5.25	53-4-4	BM	1.40	102-3-1	BM	4.45	42-3-2	SC	1.70
36-4-1	BM	1.75	53-5-3	BM	2.18	102-3-2	BM	5.07	72-3-1	SC	3.02
36-5-1	BM	3.91	53-5-4	BM	2.61	102-4-1	BM	4.31	72-3-2	SC	2.86
44-1-1	BM	1.05	53-5-5	BM	1.70	102-4-2	BM	4.18	106-3-1	SP	4.85
44-1-2	BM	0.16	53-5-6	BM	1.27	102-4-4	BM	5.28	106-3-2	SP	2.64
44-3-1	BM	0.67	78-1-2	BM	0.57	102-5-1	BM	3.83	106-3-3	SP	3.02
44-4-1	BM	1.11	78-4-1	BM	0.27	129-3-1	BM	0.57	15-5-1	L	5.31
44-4-4	BM	3.69	83-3-3	BM	0.97	10-3-1	SG	5.42	15-5-2	L	0.84
44-4-5	BM	2.59	83-3-4	BM	2.86	10-3-2	SG	4.15	85-3-1	L	3.10
44-4-6	BM	0.65	100-3-3	BM	1.05	10-4-1	SG	4.10	85-4-1	L	3.83

The xylanase activity was determined as follows: 1 ml of culture filtrate of each test strain was added to 5 ml of 0.5% xylan solution (pH 6.2, adjusted with M/30 phosphate buffer). After incubation for 15 min at 55°C, the reducing sugar was determined by Somogyi's method. The xylanase activity in the table is expressed by the amount of reducing sugar produced.

BM: barn-yard manure, SG: grass silage, SP: potato silage, SC: corn silage, L: litter.

TABLE III. NUMBER OF ISOLATES OF STREPTOMYCES
AND THEIR SOURCES

Source	No. of samples	No. of strains isolated
Barnyard Manure (BM)	24	45
Silage { Grass Silage (SG)	57	8
{ Corn Silage (SC)	8	4
{ Potato Silage (SP)	7	3
Litter (L)	5	4
Total	101	64

came to hand from barn-yard manures. This fact means that *Streptomyces* strains might play an important role in degradation of vegetable fibres and formation of humus of soil.

2. Xylanase production by the stock culture of *Streptomyces*

In accordance with the results of the above

experiments, some *Streptomyces* species in our culture collection were tested on their ability to produce the enzyme. The results are shown in Table IV. In general, the strains had low

TABLE IV. XYLANASE PRODUCTION BY STOCK CULTURE OF STREPTOMYCES

Species	Growth	Enzyme activity (mg)
<i>St. albogriseolus</i> IAM 0031	++	4.6
<i>olivaceus</i> IAM 0025	++	4.1
<i>fradiae</i> IAM 0088	++	±
<i>bobilliae</i> IAM 0002	++	±
<i>coelicolor</i> IAM 0032	++	—
<i>viridochromogenes</i> IAM 0066	++	—
<i>flaveolus</i> IAM 0033	++	—
<i>californicus</i> IAM 0003	+	—
<i>cinamonensis</i> IAM 0020	+	—
<i>flavovirens</i> IAM 0069	+	—
<i>phaeochromogenus</i> IAM 0026	+	—
<i>virginiae</i> IAM 0056	+	—
<i>antibioticus</i> IAM 0076	+	—
<i>griseolus</i> IAM 0021	+	—
<i>griseoluteus</i> IAM 0060	+	—
<i>griseoruber</i> IAM 0110	+	—
<i>flavus</i> IAM 0083	+	—
<i>griseus</i> IAM 0084	+	—
<i>halstedii</i> IAM 0048	+	—
<i>aureofaciens</i> IAM 0087	+	—
<i>albus</i> IAM 0057	—	—
<i>bikiniensis</i> IAM 0019	—	—
<i>hygroscopicus</i> IAM 0072	—	—
<i>lipmanii</i> IAM 0036	—	—
<i>aureus</i> IAM 0092	—	—
<i>flavochromogenes</i> IAM 0059	—	—
<i>lavendulae</i> IAM 0009	—	—
<i>luteovorticillatus</i> IAM 0049	—	—
<i>microflavus</i> IAM 0010	—	—
<i>parvus</i> IAM 0013	—	—
<i>rimosus</i> IAM 0014	—	—
<i>roseochromogenus</i> IAM 0027	—	—
<i>scabies</i> IAM 0015	—	—
<i>venezuelae</i> IAM 0017	—	—
<i>vinaceus</i> IAM 0042	—	—
<i>N. astroides</i> IAM	—	—
<i>N. gardneri</i> IAM 0105	—	—
<i>N. narashinoensis</i> IAM 0113	—	—

The enzymatic activity was determined and expressed as shown in Table II.

xylanase activity, except *St. albogriseolus* IAM 0031 and *St. olivaceus* IAM 0025. However, their abilities were lower than that of the newly isolated strain No. 102-1-4. No clear relationship between the taxonomical position and action to xylan was noted in the experiments.

3. Xylanase production by various kinds of fungi

Out of 480 strains of fungi tested, only 19 strains secreted the enzyme into culture broth as shown in Table V. The enzyme was found more widely among the strains belonging to Genus *Chaetomium* than other genera of fungi tested; 10 out of 35 strains of *Chaetomium* tested showed the enzyme activity and *Ch. trilaterale* 2264 was found to be the most prominent strain. Among *Basidiomycetes*, *Schizophyllum*

TABLE V. XYLANASE PRODUCTION BY VARIOUS KINDS OF FUNGI

Species	Enzyme activity (mg)
<i>Chaetomium atrobrunneum</i> 2101	1.35
<i>Chaetomium aureum</i> 2103	1.24
<i>Chaetomium aureum</i> 2107	1.35
<i>Chaetomium brasiliense</i> 2114	1.21
<i>Chaetomium elatum</i> 2126	0.90
<i>Chaetomium indicum</i> 2222	0.81
<i>Chaetomium indicum</i> 2236	0.62
<i>Chaetomium tomentosum</i> 2260	0.86
<i>Chaetomium trilaterale</i> 2261	1.75
<i>Chaetomium trilaterale</i> 2264	6.08
<i>Alternaria kikuchiana</i> 5005	0.33
<i>Cephalosporium acremonium</i> 5027	1.40
<i>Cercospora melonis</i> 5046	1.40
<i>Gibberella sabinetti</i> 8049	1.37
<i>Glomerella cingulata</i> 8050	1.43
<i>Macrosporium bataticola</i> 5014	1.67
<i>Echinodontium tsugicola</i> 9010	1.37
<i>Schizophyllum commune</i> 9006	3.96
<i>Trametes pini</i> 9007	2.07

One ml of culture filtrate of each test strain was added to 5 ml of 0.5% xylan solution (pH 4.0, adjusted with acetate buffer). After incubation for 15 min at 55°C, the reducing sugar was determined by Somogyi's method. The xylanase activity in the table is expressed by the amount of reducing sugar produced.

commune 9006, *Trametes pini* 9009, and *Echinodontium tsugicola* had the enzyme-producing ability. As long as *Fungi imperfecti* and Plant pathogens are concerned, *Alternaria*, *Cephalosporium*, *Cercospora*, *Gibberella*, *Glomerella* and *Macrosporium* showed the ability. However, their abilities were lower than that of *Chaeto-*

mium trilaterale 2264. Plant parasites and the isolates from rice and grains did not produce the enzyme. Sumizu *et al.*⁸⁾ reported that rice-blight organisms secreted the enzyme, but neither *Piricularia oryzae* 5016 nor 5017 showed the ability in this experiment.

4. Identification of *Streptomyces* sp. No. 102-1-4

This organism shows a number of identifying characteristics which distinguishes it from other allied species of *Streptomyces*. Sporophores are monopodially branched, producing open spirals and the surface of the conidial spore is spiny under electron microscopy as shown in Fig. 2. The growth is usually colourless to yellow and the pigment of the mycelium generally does not diffuse into culture media; aerial mycelium changes to various shades of gray, ranging from light gray to mouse gray according to the media. No coagulation of milk with slow peptonization; the proteolytic action on gelatin is weak and liquefaction starts only after the 15th day of incubation. Melanine formation is negative. Starch is readily hydrolyzed. Nitrate is not reduced to nitrite; hydrogen sulfide formation is positive. Optimum temperature for growth is at 37°C, and good growth is observed at 45°C, too. Judging from the morphological, cultural and physiological characteristics described above, the strain 102-1-4 should be included into Series Cinereus¹³⁾ and closely resembles *St. parvullus*¹⁴⁾ Waksman and Gregory with regard to the colors of the vegetative and aerial mycelia, shape of sporophore, mode of growth in some media, liquefaction of gelatin, peptonization of milk and melanine formation; but it showed marked differences in the reduction of nitrate and soluble pigmentation in some media. Especially, it differs from *St. parvullus* Waksman and Gregory in the surface of conidial spore; that of the strain

Fig. 2. Microscopic Observation of the Sporophore and the Spore of Strain 102-1-4.

A, B: sporophore, C: spore (under electron microscopy)

13) S. A. Waksman, *The Actinomycetes*, Vol. 2, The Williams and Wilkins Co., 1961, p. 117.

14) S. A. Waksman and F. J. Gregory, *Antibiotics and Chemotherapy*, 4, 1050 (1954).

102-1-4 is spiny, while that of *St. parvulus* is smooth. The strain 102-1-4 is distinguished in its good growth at 45°C. Other known species in Series Cinereus such as *St. craterifer*¹⁵⁾ Waksman and *St. cellulosa*¹⁶⁾ Jensen are completely different from the strain 102-1-4 in the morphological characteristics, liquefaction of gelatin, peptonization of milk, hydrogen sulfide formation and reduction of nitrate. Therefore, the strain 102-1-4 is considered to be a new species and it has been named *Streptomyces xylophagus* nov. sp. Iizuka et Kawaminami after its ability to produce xylanase.

DESCRIPTION

Streptomyces xylophagus nov. sp.

xylo Gr. n. xylon wood; *phagus* Gr. v. phagein devour; *xylophagus* M.L. adj. wood-devouring

Type strain: 102-1-4; the strain was deposited to IAM Culture Collection, The University of Tokyo.

Morphology: Sporophores monopodially branched, producing open spirals. Spores spherical to oval, spiny. 0.7 to 1.0 by 0.8 to 1.2 μ .

Sucrose Nitrate agar: Growth thin, colorless. Aerial mycelium abundant, at first white, later becoming brownish gray, with white puffs. No soluble pigment.

Glucose Asparagine agar: Growth pale yellowish brown, later dull orange. Aerial mycelium abundant, powdery, light brownish gray, with white puffs. Soluble pigment faint reddishbrown.

Glycerol Asparagine agar: Growth pale yellowish brown, later dull orange. Aerial mycelium abundant, powdery light brownish gray with white puffs, wrinkled at concentric zone. Soluble pigment none or faint brown.

Nutrient agar: Growth colorless, smooth. Aerial mycelium at first white, becoming brownish gray with age. No soluble pigment. Glycerol Calcium Malate agar: Growth moderate, colorless. Aerial mycelium scant, whitish gray, with white patches. No soluble pigment.

Starch agar: Growth colorless. Aerial mycelium light brownish gray with white patches. No soluble pigment.

Starch Peptone Beef ext. agar: Growth pale yellowish brown, wrinkled. Aerial mycelium light brownish gray with white patches. Soluble pigment none or faint brown.

Potato plug: Growth good, cream-colored. Aerial mycelium abundant, gray. Plug not darkened in 3 days, but later covered with aerial mycelium; so that the tinge of plug can not be observed.

Gelatin: Growth good, cream-colored. Aerial mycelium light brownish gray, powdery, wrinkled. Liquefaction slight in 15 days, moderate in 30 days at 20°C. Soluble pigment grayish red brown.

Milk: Growth cream-colored, no aerial mycelium. Coagulation not observed in 30 days. Peptonization in 21 to 30 days. Soluble pigment pale brown.

Melanine formation: Negative.

Starch hydrolysis: Positive (strong).

Nitrate reduction: Negative.

Hydrogen sulfide formation: Positive.

Cellulose medium: Good growth. Aerial mycelium light brownish gray. Cellulose not decomposed but weakened in 45 days.

Growth on Nutrient agar:

at 20°C; No growth in 24 hr. In 3 days growth pale yellowish brown. Aerial mycelium scant, powdery, white. No soluble pigment.

at 37°C; The best growth. Light brownish gray and powdery aerial mycelium. No soluble pigment.

at 45°C; Good growth, cream-colored, some wrinkled. Aerial mycelium light brownish gray, powdery.

at 55°C; No growth.

15) S. A. Waksman, *Actinomycetes and Their Antibiotics*, Baltimore, 1953, p. 105.

16) H. L. Jensen, *Soil Science*, **30**, 65 (1930).

Carbon utilization: Glucose, Galactose, Mannose, Fructose, Rhamnose, Xylose, L-Arabinose, Ribose, Maltose, Treharose, Cellobiose, Inositol, utilized very well. Sucrose utilized a little in 5 days, but in 7 days utilized as well as Glucose. Raffinose, Dultit, Inuline, not utilized.

Habitat: Barn-yard manure, Hokkaido, Japan; collected in November, 1963.

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