Acidic Polysaccharide Production from Methane by a New Methane-oxidizing Bacterium H-2

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A thermophilic obligate methane-oxidizing bacterium, H-2, produced two kinds of extracellular acidic polysaccharides (Polymer I and Polymer II) from methane as a single carbon and energy source. The maximum total polysaccharide production was 1.8 g, together with 3.6 g of dry cells, per liter of the culture medium. Polymer I and Polymer II contained 37% and 56% of sugars, respectively, consisting of glucose, mannose, galactosamine and glucuronic acid. In addition, these polysaccharides contained about 38% and 30% of amino acids, respectively.

Methane-oxidizing bacteria have a great advantage in production of single cell protein (SCP) because methane can be obtained in high purity and at relatively low cost. So many methane-oxidizing bacteria have been isolated and investigated.^{1~3)} We have isolated a new thermophilic obligate methane-oxidizing bacterium named H-2⁴⁾ for industrial SCP production. During the study of the culture conditions, we observed the production of extracellular polysaccharide by this strain. Some methylotrophs are known to produce extracellular substances such as polysaccharide from methanol,⁵⁾ and L-serine from glycine.⁶⁾ There are, however, only a few reports on the production of extracellular substances by microorganisms using methane as the carbon source. Söhngen reported that *Pseudomonas methanica* produced slime substances⁷⁾ and recently extracellular polysaccharide production was reported by a mixed methane-oxidizing culture during growth in methane.⁸⁾

In this paper, we dealt with the production of two kinds of polysaccharides by a pure culture of a new methane-oxidizing bacterium from methane as a single carbon and energy source, and with some properties of these polysaccharides.

MATERIALS AND METHODS

The microorganism and its culture. A thermophilic obligate methane-oxidizing bacterium, named H-2 in the previous paper,⁴⁾ was used throughout this study. The culture was carried out as follows: VCR medium⁴⁾ was used for the fundamental culture; the mixed gas consisted of 35% of methane, 10% of oxygen, 5% of carbon dioxide and 50% of air; the temperature was 50°C.

A jar culture was carried out in a mini-type glass jar fermenter (total volume, 2-liters) containing 1 liter of the medium.

Measurements of growth and polysaccharide production. Growth was estimated by measuring optical density at 540 nm (1 OD unit = 400 mg dry cells per liter) with a spectrophotometer.

Polysaccharide was determined by Dubois' method⁹⁾ after the supernatant (crude polysaccharide solution) separated from culture broth by centrifugation at 10,000 × g for 15 min had been dialyzed against deionized water for 6 hr at 4°C.

Purification of polysaccharide. Cetyltrimethylammonium bromide (CTAB) which forms an insoluble complex with anionic polymer was added to the crude polysaccharide solution until no more precipitate developed. The precipitate was washed with deionized water and dissolved in 1 M NaCl. On adding four volumes of acetone to this solution, acidic polysaccharide was precipitated and CTAB remained in the supernatant. The polysaccharide was dissolved and dialyzed against deionized water for 24 hr at 4°C. Fractionation of the

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polysaccharide was carried out by stepwise precipitation with acetone. Purity of the polysaccharide was checked by sedimentation analysis using an analytical centrifuge (UCA-1, Hitachi Co. Ltd., Japan).

Investigation of some physicochemical properties of the polysaccharide. Molecular weight was estimated by sedimentation equilibrium analysis¹⁰⁾ using the above-mentioned analytical centrifuge at 25°C. Viscosity was measured with an Ostwald's viscometer at 20.2°C. Optical rotation was measured with an automatic polarimeter (DIP-4, JASCO, Japan) at 23.3°C.

Investigation of components of the polysaccharide. Sugar components of the polysaccharide were analyzed by thin layer chromatography¹¹⁾ and gas-liquid chromatography.^{12,13)} Especially, 2-aminohexosugar and glucuronic acid were estimated by Elson-Morgan's method¹⁴⁾ and Bitter-Muir's method,¹⁵⁾ respectively.

Amino acid analysis was performed with a Hitachi model 825 amino acid analyzer. Preparation of the hydrolysate for the analysis was carried out by Stein-Moore's method.¹⁶⁾

RESULTS

Polysaccharide production

We determined the optimal culture conditions for polysaccharide production by H-2 on the scale of a 500 ml shaking flask containing 50 ml of medium (Table I). The concentrations of nitrogen sources, sodium nitrate and ammonium chloride, of this medium (VCRP20) were reduced to a half in comparison with those of the VCR medium. Based on these conditions, we carried out a 2-liter jar culture. As shown in Fig. 1, the maximum polysaccharide production was 1.8 g, together with 3.6 g of dry cells, per liter of the culture

broth. The microorganism produced polysaccharides in proportion to the growth.

During the investigation of the optimal phosphate concentration in the medium for polysaccharide production by the microorganism, we observed that polysaccharide productivity of the microorganism was irreversibly decreased when the cultivation was car-

FIG. 1. Time Courses of Polysaccharide Production by H-2 and the Growth.

The fermentation was carried out in a 2-liter glass jar fermentor containing 1 liter of VCRP20 medium as described in the text. The flow rates of methane and oxygen fed into the fermentor were 300 ml/min and 5 ml/min, respectively, as measured with thermal-massflow meters. Symbols: ●, growth; ○, total polysaccharide produced.

FIG. 2. Effect of Phosphate on Polysaccharide Productivity.

The protocol of investigation of the effect of phosphate on polysaccharide productivity of H-2 was as follows: The microorganisms cultured for 20 hours in test media which contained different concentrations of phosphate, 20 to 120 mM, were inoculated into VCRP20 medium. They were further cultured for 20 hours, and measurements of dry cell weight and polysaccharide in the medium were carried out as described in the text.

TABLE I. OPTIMAL CULTURE CONDITIONS FOR POLYSACCHARIDE PRODUCTION

NaNO ₃	1.0 g/liter
NH ₄ Cl	0.25
KH ₂ PO ₄	0.7
K ₂ HPO ₄	2.6
MgSO ₄ ·7H ₂ O	0.4
CaCl ₂ ·2H ₂ O	0.02
FeCl ₃	8.0 mg/liter
CuSO ₄ ·5H ₂ O	0.5
Vitamin B ₁₂	1.0 μg/liter
pH 7.2	Temperature 50°C
Mixed gas CH ₄ -O ₂ -CO ₂ -air	= 7:2:1:10

TABLE II. EFFECT OF CURING AGENTS ON POLYSACCHARIDE PRODUCTIVITY

This test was carried out as follows: The microorganism grown in a 100-ml shaking flask containing 10 ml of VCRP20 medium with an agent for 24 to 72 hr was transferred to a 500-ml shaking flask containing 50 ml of VCRP20 medium. After it had grown to OD 1.5, polysaccharide production was measured as described in MATERIALS AND METHODS.

Agent	(A) Number of isolated colonies	(B) Number of polysaccharide-productive colonies	Ratio of (B)/(A)
Acridine orange (2.0 $\mu\text{g}/\text{ml}^*$)	18	16	0.89
Mitomycin C (0.5 $\mu\text{g}/\text{ml}^*$)	15	2	0.13
Ethidium bromide (0.5 $\mu\text{g}/\text{ml}^*$)	20	16	0.80
Sodium dodecyl sulfate (10 $\mu\text{g}/\text{ml}^*$)	21	3	0.14
Phosphate (80 mM)	20	1	0.05
(40 mM)	20	18	0.90
(20 mM)	42	42	1.00

* Critical concentrations of curing agents for growth of the microorganism.

FIG. 3. Fractionation of Polysaccharide.

Thirty mg of crude acidic polysaccharide was fractionated by stepwise precipitation with acetone. The recovery was 79%.

ried out in a medium containing more than 80 mM phosphate (Fig. 2). The microorganism which had lost the ability of production was cultured in VCRP20 medium for 28 days with 10 successive transfers. But the productivity was not recovered. No irreversible decrease of productivity occurred on changing any other components of the culture medium. In addition, we found that the productivity was lost on treatment of the cells with acridine orange, ethidium bromide, mitomycin C or sodium dodecyl sulfate. In particular, the productivity was easily lost with mitomycin C and sodium dodecyl sulfate (Table II).

Purification of polysaccharide

Crude acidic polysaccharide precipitated

FIG. 4. Profiles of Polymer I and Polymer II on Centrifugal Analysis.

Polymer I is top and Polymer II is bottom. This picture was taken on centrifugation at $1890 \times g$ for 80 min (25°C). The solvent was 10 mM Na-phosphate buffer (pH 7.2).

with CTAB was effectively fractionated by stepwise precipitation with acetone, so that two kinds of acidic polysaccharides were obtained. As shown in Fig. 3, one was precipitated at 50~55% acetone (Polymer I) and the other at

60~65% acetone (Polymer II). Each polysaccharide gave a single peak on analytical centrifugation (Fig. 4). The ratio of Polymer I and Polymer II produced in the crude acidic polysaccharide fraction was about two to one throughout the cultivation period.

Capsular polysaccharides were isolated from the microorganism by treatment with 0.05 N NaOH at 4°C for 30 min, but they were not recovered in the acidic fraction.

Physicochemical properties of the polysaccharide

Some physicochemical properties of Polymer I and Polymer II are shown in Table III. The molecular weight of Polymer II was about three times greater than that of Polymer I, though Polymer I was more viscous than Polymer II. Additionally, both polysaccharides were colloidal in more than 5% trichloroacetic acid or perchloric acid. These polysaccharides were easily decomposed by alkali and irreversibly lost their viscosities on treatment with 0.1 N NaOH at 98°C for 2 min or at room temperature for 24 hr.

Components of the polysaccharide

As shown in Table IV, sugar contents of Polymer I and Polymer II were 36.5% and 56.1%, respectively, and the sugars consisted of glucose, mannose, galactosamine and glucuronic acid in approximate molar ratios of 1:1:5:5 and 1:4:9:6, respectively. The results of elementary analyses revealed that Polymer I and Polymer II contained 6.2% and 5.0% of nitrogen, respectively. In the hydrolysates of these polysaccharides treated with

0.05 N HCl at 80°C for 60 min, no sialic acid was detected by Aminoff's method.¹⁷⁾ Only galactosamine was detected in both polysaccharides by GLC and TLC as a nitrogen containing sugar. So, nitrogen contents of Polymer I and Polymer II due to their sugar components were only 1.3% and 1.8%, respectively.

Then protein contents of these polysaccharides were measured by Lowry's method¹⁸⁾ with bovine serum albumin as standard. Polymer I and Polymer II contained 28.4% and 5.0% of protein, respectively. Amino acid compositions of hydrolysates of both polysac-

TABLE IV. SUGAR COMPONENTS OF POLYMER I AND POLYMER II

Sugar	Polymer I (%)	Polymer II (%)
Glucose	3.2	2.1
Mannose	2.9	10.5
Galactosamine	16.8	23.3
Glucuronic acid	13.6	19.6
Total	36.5	56.1

TABLE V. AMINO ACID COMPOSITIONS OF POLYMER I AND POLYMER II

Amino acid	Polymer I (%)	Polymer II (%)
Aspartic acid	3.33	0.63
Threonine	2.91	0.19
Serine	1.35	0.29
Glutamic acid	3.42	2.97
Glycine	3.86	6.63
Alanine	2.10	0.35
Cysteine	0.40	0.56
Valine	5.10	9.80
Methionine	n.d.	0.22
Isoleucine	1.40	0.26
Leucine	2.66	0.33
Tyrosine	3.80	5.95
Phenylalanine	1.62	0.51
Lysine	1.08	n.d.
Histidine	1.31	n.d.
Arginine	1.74	0.24
Tryptophan	0.46	0.83
Proline	1.19	n.d.
Total	37.7	29.8

n.d., not detected.

TABLE III. PHYSICOCHEMICAL PROPERTIES OF POLYMER I AND POLYMER II

	Polymer I	Polymer II
Sedimentation coefficient (S)	3.9	5.7
Limiting viscosity number (dl/g)	15.5	9.6
Specific rotatory power (degree)	+36	+38
Molecular weight	120,000	340,000

charides are given in Table V. Methionine was not detected in Polymer I. In Polymer II, lysine, histidine and proline were not detected, while four major components, valine, glycine, tyrosine and glutamic acid, occupied 85% of the total amino acids. So, the results of amino acid analyses were quite different from those obtained with Lowry's method. As a result of amino acid analyses, Polymer I and Polymer II were found to contain 37.7% and 29.8% of amino acids, respectively.

DISCUSSION

Accumulation and excretion of polysaccharides has been reported in some methanotrophs.¹⁹⁾ *Methylococcus* NCIB 11083²⁰⁾ grown on methane under nitrogen limitation, accumulates polyglucoses in the cell. Methane-oxidizing bacterium H-2 can produce two kinds of extracellular polysaccharides, Polymer I and Polymer II, without nitrogen limitation. But they cannot function as a reserve carbon and energy source because the microorganism is not able to remetabolize them. We are investigating their roles in the microorganism.

A mixed methane-oxidizing culture is reported to produce heteropolysaccharides containing glucose, galactose, mannose, fucose and rhamnose.⁸⁾ Sugar components of Polymer I and Polymer II produced by H-2 are quite different from those of the latter heteropolysaccharides. Polymer I and Polymer II consist of glucose, mannose, galactosamine and glucuronic acid in approximate proportions of 1:1:5:5 and 1:4:9:6, respectively, and moreover consist of about 38% and 30% of amino acids, respectively. Protein contents of the polysaccharides estimated by Lowry's method are less than those by amino acid analyses. One reason for this is that the polysaccharides have less chromogenic groups²¹⁾ than bovine serum albumin used as a standard for Lowry's method. Both polysaccharides seem to be glycoproteins which involve O-glycosidic linkage to hydroxyamino acid because they are alkali-labile and contain thre-

onine and serine.²²⁾

On growth in media containing more than 80 mM phosphate, H-2 irreversibly loses the ability of polysaccharide productivity. It can be thought that the polysaccharide production depends on a plasmid which is eliminated by phosphate in medium, because the productivity has also been irreversibly lost with agents (mitomycin C, sodium dodecyl sulfate, ethidium bromide and acridine orange) which can remove plasmids from bacteria,²³⁾ though we have not yet been able to detect the plasmid in H-2 by Hansen-Olsen's method.²⁴⁾

Polymer I and Polymer II are the first extracellular substances produced on pure cultivation of a methane-oxidizing bacterium from methane as a single carbon and energy source.

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