Production of a High Viscosity Polysaccharide, Methylan, in a Novel Bioreactor

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Received 14 June 1996; accepted 7 September 1996

Abstract: The effect of shear stress on the production of a high viscosity polysaccharide, methylan, from methanol by Methylobacterium organophilum was investigated by using a multidisk mixer. It was observed in the multidisk mixer with defined shear stresses that the specific production rate of methylan increased gradually with increasing shear stress up to 30 Pa, and the production rate was constant beyond 30 Pa. This result suggested that the limited mass transfer from the medium into cells reduced methylan production. A novel bioreactor that provided the large volume of a high shear region was used to increase methylan production. Fed-batch cultures in the novel bioreactor were performed by the dissolved oxygen-stat method of methanol. When 1.13 g/L ammonium ion was added, the concentrations of cells of methylan were 31 and 20.6 g/L, respectively. The productions of cells and methylan in our designed bioreactor were 20 and 50% higher than those obtained in a conventional fermentor. The methylan content reached a maximum of 20.7 g/L in the bioreactor and the viscosity of the fermentation broth was 127 Pa·s, which corresponds to 68 g/L as a xanthan. © 1997 John Wiley & Sons, Inc. Biotechnol Bioeng 54: 115–121, 1997.

Keywords: Methylobacterium organophilum; high viscosity polysaccharide; methylan; multidisk mixer; bioreactor

INTRODUCTION

Methanol has attracted much attention due to its many advantages as a raw material in the field of biotechnology (Faust and Prave, 1986). Methanol would be the most economical and would be expected to be the most useful raw material for the fermentation process that includes the production of organic and amino acids (Ogata et al., 1997; Oki et al., 1973) as well as a single cell protein (Smith, 1981). The advantages of methanol are its low cost, high purity, complete water miscibility, and restricted use by certain microorganisms. Compared with more conventional raw materials, such as glucose or other carbohydrates, its disadvantages are a relatively high heat of fermentation and high oxygen demand. Some studies were made on bacteria that utilized methanol as a carbon source. However, despite the numerous studies on the specific activities of methanol-utilizing bacteria, very little work has been conducted on the biopolymers produced by these microorganisms. Recently, it was found in our laboratory that Methylobacterium organophilum could produce a new viscosity exopolysaccharide from methanol under specific culture conditions (Choi et al., 1991; Lebeault et al., 1991). The high viscosity polysaccharide was named methylan by combining the word methyl from the genus of Methylobacterium and a suffix an.

Microbial polysaccharides show a wide variety of chemical compositions and their physical and biochemical properties are now understood (Bikales, 1993). Polysaccharides are commercially used to produce gels to thicken and stabilize foods, medicines, and industrial products. The production of microbial exopolysaccharides provides a valid alternative, either through the development of products with properties almost identical to currently used polysaccharides, or materials with better rheological characteristics that can be used for new applications.

In polysaccharide fermentation, the viscosity of the culture fluid greatly increases due to the accumulation of extracellular polysaccharides. The increase in viscosity, consequently, leads to oxygen limitation and imperfect mixing (Peters et al., 1989). Another phenomenon is that a slime layer is gradually formed around the cells that acts as a diffusion barrier for nutrients (Funahashi et al., 1987, 1988) and causes a decrease of the production rate of polysaccharides. For the enhanced production of polysaccharides such as xanthan, the stagnant region in a fermentor must be reduced and the slime layer around the cells must be removed by applying high shear (Funahashi et al., 1987, 1988; Peters et al., 1989). To reduce the stagnant region in the fermentor, a bubble column reactor (Pons et al., 1989), air lift reactor (Suh et al., 1990a,b), and fermentor with modified configuration (Lawford et al., 1986) were suggested for polysaccharide production.

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In this article the effect of shear stress on methylan production was investigated by using a multidisk mixer. A high shear novel bioreactor was designed and tested for the enhanced production of the high viscosity polysaccharide, methylan, from methanol (Choi et al., 1991).

**MATERIALS AND METHODS**

**Microorganism and Culture Media**

A facultative methylotrophic bacterium, *M. organophilum* NCIB 11278 KC-1, was used for this study (Choi et al., 1991). Growth medium consisted of 1.0% (v/v) methanol, 1.0 g/L (NH₄)₂SO₄, 1.305 g/L KH₂PO₄, 2.13 g/L Na₂HPO₄·12H₂O, 0.45 g/L (MgSO₄·7H₂O), and 0.2% (v/v) metal solution (which contained 3.3 mg/L CaCl₂·2H₂O, 1.3 mg/L FeSO₄·7H₂O, 130 µg/L MnSO₄·4H₂O, 130 µg/L ZnSO₄·4H₂O, 40 µg/L CuSO₄·4H₂O, 40 µg/L Na₂MoO₄·2H₂O, 40 µg/L CoCl₂·6H₂O, 30 µg/L H₃BO₃). Fermentation medium consisted of methanol [which was initially added at 1.0% (v/v) and then intermittently fed 0.5% (v/v) by the dissolved oxygen (DO) stat method], 0.75 or 1.13 g/L NH₄⁺ (ammonia water was fed for the nitrogen source and pH adjustment), 2.52 g/L KH₂PO₄, 4.24 g/L Na₂HPO₄·12H₂O, 0.9 g/L MgSO₄·7H₂O, and metal solution [which was fed with ratio of 0.1% (v/v) metal solution to 0.075 g/L NH₄⁺].

**Operation of Multidisk Mixer**

A multidisk high share mixer (Kansai Chemical Engineering Co. Ltd., Japan) was used to evaluate shear stress on methylan production. Nine rotation disks and nine fixed disks were alternatively located inside the mixer. Shear stress was generated between the disks and annuli of the fixed disk and rotary disk. Each rotating disk had 12 holes and each fixed disk had 4 holes for air and medium to pass through. The configuration and dimensions are shown in Figure 1 and Table I.

Culture broth (600 mL) with high polysaccharide producing activity was taken from the fermentor and then transferred to the mixer, in which the rotating speed of the disk was varied to generate different shears. The specific production rate of methylan was estimated by measuring the concentrations of cells and methylan in the mixer every 10 min for 60 min. The cell concentration was assumed constant for 60 min. In order to use the cells of the same polysaccharide producing activity in each experiment, culture broth was taken from the fermentor when cell concentration reached about 10 g/L. If necessary, an appropriate amount of concentrated cells and methylan were added to the culture broth to determine the effect of shear on methylan production.

**Culture Conditions in Fermentor and Bioreactor**

A single colony on the agar plate of the growth medium was transferred to a 250-mL flask containing 50 mL of growth medium and was cultivated on a rotary shaker at 30°C at 250 rpm for 20 h, and then 5% (v/v) of culture broth was transferred to new growth medium and cultivated for 12 h under the same conditions. This seed culture was used as the inoculum for the fermentor.

A 5-L fermentor was used for the fed-batch culture of methanol. The working volume of the fermentor was 3 L and temperature and pH were controlled at 30°C and 7.0, respectively. Aeration rate was 1.0vvm and agitation speed was gradually increased from 300 to 1200 rpm for good mixing. Intermittent feeding of methanol was carried out by DO-state fed-batch culture in order to overcome the inhibitory effect of a high concentration of methanol. The DO-stat method is based on the fact that DO level began to rise abruptly when methanol was exhausted (Yano et al., 1979). When the DO level became higher than the setting value (30% saturation), the pump was operated for preset period.

A 10-L novel bioreactor was used for the enhanced production of methylan by the DO-stat fed-batch culture of methanol (Fig. 2). The bioreactor consisted of three impellers: one six blade turbine impeller distributed air bubbles and one large spiral impeller made the upflow stream. Another six turbine impeller, which was attached to the spiral impeller, was set at the middle of the bioreactor and rotated with high speed in the reversed direction to produce the effective mixing. The working volume of the bioreactor was 6 L. Agitation speed was gradually increased to prevent DO limitation and imperfect mixing. The agitation speed of the upper turbine impeller was increased up to 1000 rpm and that of the lower turbine impeller and spiral impeller was increased up to 350 rpm. Other conditions were the same as those of the fermentor.

**Analytical Methods**

Cell growth was estimated by measuring optical density at 570 nm and converting to dry cell weight. The concentration of ammonium ion was determined by the indo-phenol method (Boller et al., 1961) after cells were removed by centrifugation. Methanol concentration was measured by using a gas chromatograph (Shimadzu GC-6A) with a column packed of Chromosorb 101, 80/100 mesh, at 150°C and a flame ionization detector. Methylan concentration was measured by the dry weight and phenol-sulfuric acid methods (Dubois et al., 1956). The dry weight of methylan was determined as follows. Culture broth was diluted to about 10 g/L of methylan with distilled water and centrifuged at 10,000 rpm for 30 min. The supernatant was collected and 2 vol of absolute ethanol were added and centrifuged at 5000 rpm for 15 min and washed twice with ethanol. The washed methylan was dried to a constant weight in an oven at 105°C. The viscosity was measured by a Haake Rotovisco Rheometer (model RV 2) equipped with an MV1 or NV sensor system.

**THEORETICAL DEVELOPMENT**

Shear stress can be calculated by the following assumption and approximation (Whorlow, 1980).
1. Fluid inside a multidisk mixer was homogeneously mixed.
2. The momentum of liquid in the horizontal or vertical direction was ignored compared with that of the rotational direction.
3. The shear effects of disk holes were ignored.
4. The annuli of the fixed and rotary disks were very narrow.

Shear rate could be written for the plate–plate disk viscometer,

\[ \gamma = \Omega r \]  

Shear rate could also be written for a very narrow annulus,

\[ \gamma = \frac{\Omega r_{av}}{r_2 - r_1} \]  

(2)

From Equations (1) and (2), shear rates in the multidisk mixer were calculated.

\[ \gamma_1 = \frac{2\pi N}{\delta_1} (r_2 - r_1), \gamma_2 = \frac{2\pi Nr_i}{\delta_2}, \gamma_3 = \frac{2\pi Nr_2}{\delta_3}. \]
Shear stress in the multidisk mixer could also be calculated (Whorlow, 1980).

\[
\tau_1 = \frac{1}{\pi r_1^2} \int_{r_1}^{r_2} K \left(\frac{2\pi nr}{\delta_1}\right) 2\pi rdr,
\]

\[
\tau_2 = K \left(\frac{2\pi r_2}{\delta_2}\right)^n, \quad \tau_3 = K \left(\frac{2\pi r_1}{\delta_3}\right)^n.
\]

Effective volumes of shear were as follows:

\[
v_1 = 2\pi (r_1^2 - r_1^2)\delta_1,
\]

\[
v_2 = \pi (r_1 + \delta_2)^2 - r_1^2)\alpha_x = 2\pi r_1 \delta_2 \alpha_x,
\]

\[
v_3 = \pi (r_2 + \delta_3)^2 - r_2^2)\alpha_x = 2\pi r_2 \delta_3 \alpha_x,
\]

\[
v_4 = 12\pi r_3^2 \alpha_x + 4\pi r_1^2 \alpha.
\]

The average shear stress was

\[
\tau_{av} = \frac{\tau_1 v_1 + \tau_2 v_2 + \tau_3 v_3}{v_1 + v_2 + v_3} = \frac{K^{n+1}(2\pi)^n}{\delta_1}(n+2) \left[\frac{r_1^{n+2}}{\delta_1^{n+1}} + \frac{r_2^{n+2}}{\delta_2^{n+1}} + \frac{r_3^{n+2}}{\delta_3^{n+1}}\right]. \tag{3}
\]

where

\[
K = A [P]^{n}, \quad \ln K = \ln A + B \ln P, \quad \ln K = C - nD. \tag{4}
\]

A, B, C, and D were constants depending on the properties of polysaccharide. A and B were obtained from viscosity (K) measured at various polysaccharide concentrations (P). Shear rate and shear stress of the polysaccharide solution were measured by the viscometer. The viscosity (K) and flow behavior index (n) were determined from the shear rate and shear stress by using a power law equation. Therefore, C and D could be obtained by Equation (4). In case of methylan production, they were determined to be 0.037, 2.69, 5.364, and 13.148, respectively.

**RESULTS AND DISCUSSION**

**Effect of Shear Stress on Methylan Production**

A multidisk mixer was used to investigate the effect of shear stress on the production of a high viscosity polysaccharide, methylan, from methanol by *M. organophilum*. The changes of cell mass, methylan concentration, and shear stress were determined during 60-min fermentation under the conditions of different rotational speeds, cell concentrations, and methylan concentrations. Figure 3 shows four examples out of 20 experimental results obtained. We assumed that cell concentration was constant during fermentation. Thus, its mean value (X) was used for the calculation of the specific production rate of methylan. Because methylan concentration increased almost linearly during 60-min fermentation, slopes (dP/dt) were used for the calculation of specific production rates of methylan (1/X · dP/dt). Shear stress was calculated from rotational speed, dimensions of the mixer, and methylan concentration. Its mean value was used for data analysis.

The effect of shear stress on the specific production rate of methylan is shown in Figure 4. The specific production rate increased with increasing shear stress up to 30 Pa and remained constant regardless of shear stress beyond 30 Pa. This result indicated that the limited mass transfer from the medium into the cells reduced methylan production due to the polysaccharide layer around the cells. This result was in accordance with a previously reported one for the specific
production rate of xanthan in a rotating drum reactor (Funahashi et al., 1987).

Even though DO levels were almost zero at the high concentration of polysaccharide (>5 g/L), the production of methylan was very effective. This might be due to the nature of *M. organophilum*, a facultative anaerobe (Patt et al., 1976). To verify this idea, we studied the effects of DO levels on the specific production rates of methylan at different aeration rates and agitation speeds on a 5-L fermentor (Fig. 5). These results clearly showed that DO levels tested did not affect the cellular activity expressed as a specific production rate of methylan during the fermentation processes.

**Enhanced Production of Methylan in Novel Bioreactor**

Funahashi et al. (1988) reported that because the specific production rate of polysaccharide was significantly affected by the shear stress generated in the fermentor, the mixing state was one of the crucial factors affecting the productivity of polysaccharide. The whole liquid volume in a conventional fermentor was roughly divided into three regions: the micromixing region around the impeller with high shear stress, the macromixing region dominated by a circulating flow, and the stagnant region. The polysaccharide solution is usually a non-Newtonian fluid with pseudoplastic behavior. The region of high shear is limited to the area near the impeller in a conventional fermentor, which may result in low productivity. A novel bioreactor equipped with a combination of one spiral and two turbine impellers was designed to effectively create a large volume high shear region (Fig. 2).

The methylan production in the bioreactor was carried out by the DO-stat fed-batch mode of methanol. When the total added content of the ammonium ion was 0.75 g/L, methylan was accumulated to 17.6 g/L and cells grew to 23.6 g/L. The production of cells and methylan in our bioreactor was 35 and 40% higher, respectively than those obtained in a conventional fermentor (Fig. 6). The DO level was always over 20% throughout the fermentation process of the bioreactor. When 1.13 g/L ammonium ions were
added, concentrations of cells and methylan reached to 31 and 20.6 g/L, respectively. The production of cells and methylan in our bioreactor was 20 and 50% higher, respectively than those obtained in a conventional fermentor (Fig. 7). However, the maximum specific growth rate in the bioreactor was the same as that in the fermentor. The methylan yield from cells and the methylan productivity were 30 and 60% higher, respectively than those in the fermentor (Table II). The increased cell growth and methylan production rates in the novel bioreactor might be due to the increased volume of the high shear region, resulting in effective mass transfer rate around the cells.

However, it was hardly possible to accumulate over 20 g/L methylan in the new bioreactor even at the high shear stress and unlimited DO levels. The methylan content reached a maximum of 20.7 g/L in the bioreactor and the viscosity of the fermentation broth was 127 Pa·s, which corresponded to xanthan of 68 g/L. No other research has previously reported such a high production of polysaccharides. The present information will be greatly beneficial to the relevant industry for the designing and manufacturing of a bioreactor for the production of polysaccharides in both small experimental and large production scales.

Table II. Comparison of fermentation parameters between conventional fermentor and novel bioreactor.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fermentor</th>
<th>Bioreactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_{\text{max}}$ (h$^{-1}$)</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>$X$ (g/L)</td>
<td>25.32</td>
<td>31.51</td>
</tr>
<tr>
<td>$P$ (g/L)</td>
<td>12.71</td>
<td>20.68</td>
</tr>
<tr>
<td>$P/X$ (g/L)</td>
<td>0.50</td>
<td>0.66</td>
</tr>
<tr>
<td>$Q_{P}$ (g/L h)</td>
<td>0.33</td>
<td>0.52</td>
</tr>
<tr>
<td>$K$ (Pa·s)</td>
<td>24.8</td>
<td>127</td>
</tr>
</tbody>
</table>

Total concentration of supplied ammonium ion was 1.13 g/L.
$r_i$ inner radius of outside in rotary disk (cm)
$r_o$ outer radius of rotary disk (cm)
$r_v$ radius of void fraction disk in rotary disk (cm)
$X$ cell concentration (g/L)

Greek Letters

$\alpha_f$ thickness of fixed disk (cm)
$\alpha_i$ thickness of inside disk in fixed disk (cm)
$\alpha_o$ thickness of outside disk in rotary disk (cm)
$\alpha_r$ thickness of rotary disk (cm)
$\delta_1$ thickness between rotary disk and fixed disk (cm)
$\delta_2$ thickness between inside disk and outside disk in rotary disk (cm)
$\delta_3$ thickness between inside disk and outside disk in fixed disk (cm)
$\mu^{\text{max}}$ maximum specific growth rate (h$^{-1}$)
$\gamma_1$ shear rate between rotary disk and fixed disk (s$^{-1}$)
$\gamma_2$ shear rate between inside disk and outside disk in rotary disk (s$^{-1}$)
$\gamma_3$ shear rate between inside disk and outside disk in fixed disk (s$^{-1}$)
$\tau_1$ shear stress between rotary disk and fixed disk (Pa)
$\tau_2$ shear stress between inside disk and outside disk in rotary disk (Pa)
$\tau_3$ shear stress between inside disk and outside disk in fixed disk (Pa)
$\tau_{av}$ average shear stress (Pa)
$v_1$ volume between rotary disk and fixed disk (cm$^3$)
$v_2$ volume between inside disk and outside disk in rotary disk (cm$^3$)
$v_3$ volume between inside disk and outside disk in fixed disk (cm$^3$)
$v_v$ void volume of rotary disk and fixed disk (cm$^3$)
$\Omega$ angular speed (s$^{-1}$)

Subscripts

1 field between rotary disk and fixed disk
2 field in rotary disk
3 field in fixed disk
av average value
f fixed disk
i inside disk
o outside disk
v void field

References


